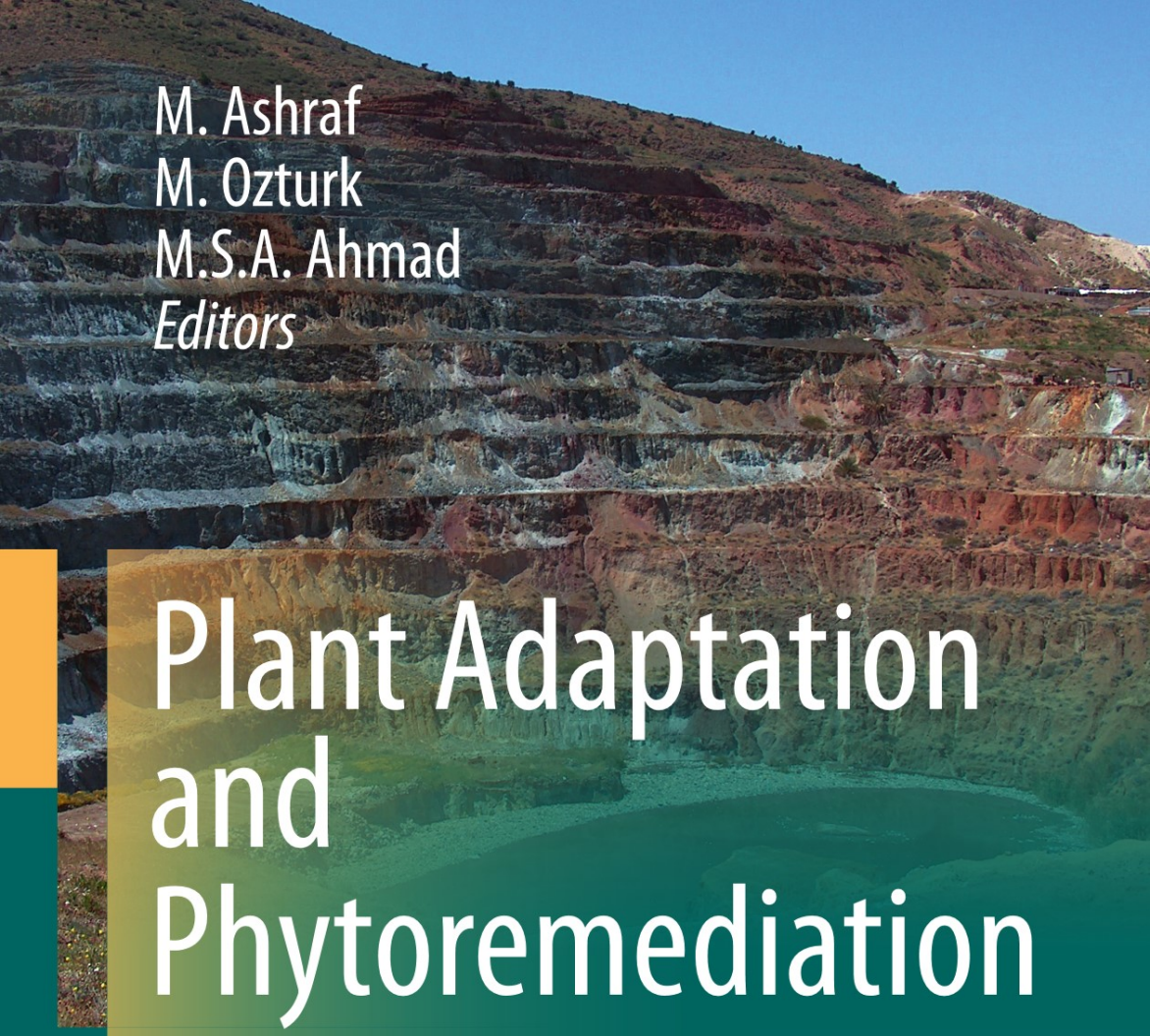

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Part II Phytoremediation

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Chapter 1
Toxins and Their Phytoremediation

Muhammad Ashraf, Munir Ozturk, and Muhammad Sajid Aqeel Ahmad

Abstract The agricultural and industrial revolutions in the last few decades have resulted in increased concentration of toxins in our environment that are now-a-days a major cause of toxicity in plants and animals. Among different toxins, increasing levels of salts, heavy metal, pesticides and other chemicals are posing a threat to agricultural as well as natural ecosystems of the world. These contaminants result in soil, air and water pollution, and loss of arable lands as well as crop productivity. They also cause changes in species composition and loss of biodiversity by bringing about changes in the structure of natural communities and ecosystems. In this situation, different approaches are being adopted to reclaim polluted environments. Among these, *phytoremediation* has a potential in removing these toxins from the environment. This approach is based on the use of natural hyperaccumulator plant species that can tolerate relatively high levels of pollutants in the environment. Pollutants accumulated in stems and leaves of high biomass producing and tolerant plants can be harvested and removed from the site. Therefore, this approach has a potential to remove large amounts of toxins by harvesting the above-ground biomass. However, the effectiveness of *phytoremediation* approach can be increased if we have better knowledge of physiological, biochemical, molecular and genetic bases of plant resistance to natural and anthropogenic induced toxins. All these aspects of toxicity mechanisms and their removal techniques are comprehensively reviewed in this book.

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Contents

1 Introduction

With the increasing human population in the world, the issues related to environmental degradation are becoming more serious (Koptsik et al. 2003; Jarup 2003; Murch et al. 2003). Humans have accelerated the emission of organic and inorganic pollutants such as pesticides, salts, petroleum products, acids, heavy metals etc. Most of the pollutants cannot be easily degraded and hence they accumulate in the environment. Although, some pollutants such as salts and heavy metals naturally occur in soils, industry (Richards et al. 1997; Ortiz-Hernandez et al. 1999; Sharma 2005), and agriculture (Scancar et al. 2000; Yagdi et al. 2000; Delibacak et al. 2002; Suciu et al. 2008) are considered as the major sources of anthropogenic induced pollution in the environment. Accelerated accumulation of toxins in the environment results in soil degradation, deforestation, desertification, loss of species diversity, pollution, acid rain, greenhouse effect and other issues related to environmental degradation.

Toxins or toxic chemicals are the inorganic and organic compounds that have negative effects on plant growth and metabolism. These are emitted into the environment as a result of human activities. For example, salts and heavy metals are released from leakage during extraction by mining, smelting, combustion and industrial effluents (Nriagu and Pacyna 1988; Nriagu 1989). Similarly, extensive use of fertilizers and pesticides in agriculture has resulted in considerable soil contamination.

Other pollutants such as petroleum products, explosives, cyanides etc. also result in considerable toxicity to living organisms.

The toxicity of a particular pollutant is determined in terms of its (i) biological role, (ii) ability to bioaccumulate, (iii) poisonous nature, and, (iv) persistency in the environment (Wildhaber and Schmitt 1996; Barron 2002). However, all these aspects vary greatly for different pollutants depending upon their molecular structure and physical as well as chemical properties (Wildhaber and Schmitt 1996). Unlike organic pollutants which are eventually converted into CO_2 and H_2O, inorganic pollutants such as metals and salts tend to deposit in different environmental components, especially in lakes, and estuarine and marine sediments (Ingersoll et al. 1996; MacDonald et al. 1996). Therefore, their removal is much more difficult as compared to that of organic pollutants and requires a different strategy to adopt for their removal. In addition, metals can easily circulate from one environmental compartment to another. These features make them a highly environmental as well as health hazardous if they accumulate at higher concentrations in the environment (Philp 1995; Hu 2002).

There are various hyperaccumulator species from various groups of bacteria, fungi, lichens, and higher plants that have the ability to uptake, accumulate or detoxify various organic and inorganic pollutants (Verhaar et al. 2000; Gramatica et al. 2002). This process broadly known as *bioremediation* utilizes various mechanisms such as *phytoextraction*, *phytoimmobilization* or *phytostabilization*, *phytotransformation*, *phytodegradation*, *phytostimulation*, *phytovolatilization* and *rhizofiltration* to remove toxic materials from different environmental components especially from soil and water (Schwitzguebel 2000; Cummings 2009). All these strategies are based on different methods and are effective for the removal of specific pollutant. In addition, a particular strategy effective for removal of one pollutant could be entirely useless for the removal of others. For example, *phytoextraction* and *phytoimmobilization* could be remarkably effective for the removal of salts and heavy metals. However, it can be entirely useless for the removal of organic contaminants such as hydrocarbons and explosives where *phytotransformation* or *phytodegradation* could be more effective. Therefore, the selection of a particular plant species to recommend and grow in the contaminated areas depends on the nature of contaminant, mechanism used by that species to remove the Contaminant, tolerance of that plant species to the pollutant and other environmental constraints (Huang and Cunningham 1996; Meagher 2000; Memon et al. 2001).

2 Toxins and Their Types

Toxins are generally classified into biodegradable (organic) and non-biodegradable (inorganic) pollutants (Verhaar et al. 2000; Gramatica et al. 2002). Biodegradable toxins are easily broken down into simpler molecules (CO_2 and water) by the activity of living organisms when they enter in the biogeochemical cycles. Such toxins are generally not harmful as they occur in low quantities in our environment. However, at high concentrations they prove to be highly toxic to all

living organisms. In addition, organic toxins such as petroleum products are toxic even at low concentrations. The examples of biodegradable pollutants include domestic and agricultural residues, petroleum products, urine and fecal matter and sewage water (Cunningham et al. 1996; Kazuya et al. 1999; Aboul-Kassim and Simoneit 2001). In contrast, non-biodegradable toxins cannot be broken down into simple and harmless products by living organisms even over long time period. These include inorganic fertilizers, pesticides and insecticides (DDT), heavy metals (nickel, mercury, copper, lead, aluminum, arsenic etc.), salts (NaCl), oxides of nitrogen and sulphur (NO_2 and SO_2) and cyanides (Van der Werf 1996; Misra and Mani 1991; Sigel et al. 2005). Unfortunately, these toxins persist in the environment for a long period of time and prove harmful to the organisms once they enter in the food chain. Therefore, the removal of these toxins from the environment is much more difficult as compared to bio-degradable one.

Another classification system is based on the environmental components (soil, air or water) in which these toxins accumulate. This classification system reflects the immediate environmental component which is exposed to the degradation by these toxins. Toxins that accumulate in soil include salts, heavy metals, inorganic and organic fertilizers, pesticides, and domestic, agricultural and industrial pollutants etc. Similarly, toxins that are released into air include primary (CO_2, CO, SO_2, NO_2, CH_4, ammonia, volatile organic compounds) and secondary (ozone, peroxyacetylene nitrate) air pollutants. Water pollution is mainly caused by sewage water, residues from food processing units, industrial wastes, petroleum products, fertilizers and pesticides from agricultural runoff etc. Most of the toxins can easily circulate from one environmental component to the other and finally accumulate in soil and water bodies. These pollutants can then be easily taken up by plants and aquatic fauna and flora and transfer to the human body where they cause serious illness and disorders (Philp 1995; Albering et al. 1999; Korte et al. 2000).

2.1 Salts

The excessive amounts of salts in different soil profiles are the largest source of pollutants in the environment causing the problem of salinity world-wide. It is estimated that about 7% of the total earth's land and 20% of the total arable area are affected by high salt contents. In addition, about half of the irrigated area is highly salinized and unfit for cultivation of agricultural crops (Szabolcs 1994; Zhu 2001). The most common salts that create soil salinity problem include NaCl and $MgSO_4$. On the basis of origin, soil salinity can be classified as Primary or natural and secondary or induced soil salinity. Primary or natural soil salinity arises by weathering of minerals derived from highly saline parent rocks (Ashraf 1994). In contrast, secondary salinization results from human interference with natural water regimes. It occurs when native perennial vegetation is replaced by shallow rooted seasonal crops. In addition, other activities such as overgrazing and deforestation greatly reduce plant cover (Ashraf 1994, 2004; Ashraf and Foolad 2007). This results in rise of underground water-table up to 2–3 m and then capillarity brings the salts

dissolved in different soil profiles to the surface causing the problem of secondary soil salinity (Chhabra 1996; Datta and de Jong 2002). In addition, due to reduction in vegetative cover, the amount of water entering underground aquifers (recharge) is increased but water taken up by plants (discharge) is dramatically reduced. This results in rise of water-table bringing the salts stored deep in the soil to the earth surface (Dunin 2002). Sometimes, introduction of exotic crops as well as other plant species and extensive agronomic practices result in altered water-use requirements of the vegetation. If this results in greater recharge of underground aquifers than discharge, the groundwater level will rise, bringing up salts with it and thus causes secondary soil salinity (Srivastava and Jefferies 1996).

Although high level of salt in soil can have a variety of effects on crop plants at biochemical, molecular and physiological levels, the most common effects include inhibition in photosynthesis, nutrient imbalance, changes in metabolic activities, disturbance in solute accumulation, enzyme activities, and hormonal imbalance etc. (Ashraf 1994, 2004; Tester and Davenport 2003; Munns 2005; Munns et al. 2006). It is now widely accepted that salinity inhibits plant growth by four major ways, (i) salt-induced water stress, (ii) specific ion toxicity (ion imbalance or nutritional disorders), (iii) oxidative stress, i.e., production of reactive oxygen species, and (iv) hormonal imbalances (Greenway and Munns 1980; Munns 1993, 2002; Ashraf 2004; Flowers 2004; Munns and Tester 2008). In addition, the degree of growth inhibition due to salt stress depends on the duration of stress, plant growth stage, and type of plant species. However, early growth stages such as germination and seedling stages are contemplated as more susceptible to salt damage as compared to later adult stages (Hamdy et al. 1993).

The salt effects on plant growth and development have been discussed in detail in a number of reviews. Their main focus has been on physiology of salt toxicity and tolerance, intra- and inter-cellular ion transport as well as long distance transport in plants, identification and characterization of traits and/or genes responsible for ion homeostasis, osmotic adjustment, and antioxidants whose expression is regulated by salt stress (Ashraf 1994, 2004; Ingram and Bartels 1996; Tester and Davenport 2003; Flowers 2004; Munns 2005; Munns et al. 2006; Munns and Tester 2008). Of various plant responses to salt stress, accumulation of compatible solutes (organic compounds of low molecular weight) is one of the prominent responses of plants to salt stress, because this phenomenon helps the plant to become acclimated to different stressful environments (Bohnert and Jensen 1996; Ashraf and Harris 2004; Ashraf and Foolad 2007). Various compatible osmolytes such as proline and glycinebetaine are considered as extremely effective in regulating growth under stressful environments and are widely distributed in a wide variety of plants (Rhodes and Hanson 1993). These compatible solutes are of low molecular weight, high solubility, and non-toxic, even if they accumulate at high cellular concentrations. They protect cellular structures from abiotic stress-induced injuries. For example, they promote osmotic adjustment, scavenge reactive oxygen species, stabilize enzymes/proteins, and protect membrane integrity in plants subjected to stressful conditions (Hasegawa et al. 2000; Ashraf and Foolad 2007).

2.2 Heavy Metals

Heavy metals have gained considerable attention as a potential environmental pollutant in recent years (Misra and Mani 1991). This is the result of their excessive use in a number of industrial processes and therefore, their toxicity is more common as compared to deficiency in organisms (Lindberg and Greger 2002). Most metals are commonly used in a multitude of industrial processes, such as manufacture of batteries, alloys, electroplated metal parts, pesticides, textile dyes and steel etc. Consequently, they are emitted to the environment to supplement natural background geochemical sources (Barnes and Rudzinski 2006). The sources of metal pollution in the environment include leakage during extraction by mining and smelting, combustion (particularly during power generation, incineration, smelting and the internal combustion engines) and industrial effluents, (Duce et al. 1991; Galloway et al. 1982; Hutton and Symon 1986; Nriagu 1989; Nriagu and Pacyna 1988).

There are 35 metals that are of a concern to environmental health and 23 of them are called as heavy metals. These include arsenic (As), antimony (Sb), bismuth (Bi), cadmium (Cd), cerium (Ce), copper (Cu), chromium (Cr), cobalt (Co), gallium (Ga), iron (Fe), gold (Au), lead (Pb), nickel (Ni), manganese (Mn), mercury (Hg), platinum (Pt), silver (Ag), thallium (Tl), tellurium (Te), and zinc (Zn) (Philp 1995; Hu 2002). Among these, the most common heavy metals that cause toxicity in plants and animals are arsenic, lead, mercury, cadmium, nickel, iron and aluminum (Hutton and Symon 1986; Chaney and Ryan 1994). Most of the metals are easily absorbed by the plants and bioaccumulate in different organs (Wang et al. 2003). These metals may ultimately enter the human body through ingestion of food, use of metal contaminated water or breathing in air containing toxic metals (Philp 1995; Albering et al. 1999; Jarup 2003).

All metals are not toxic as some of them function as micro-nutrients in less concentration and hence are considered as essential nutrients (Taiz and Zeiger 2006; Timbrell 2005; Pechova and Pavlata 2007). Some of the metals are also called as trace elements (such as iron, copper, manganese, and zinc) due to their extremely low concentrations/requirement in biological systems (Nriagu 1989; Graham and Stangoulis 2003). Since they are found naturally in soil, their adequate amounts are naturally found in our foodstuffs, fruits and vegetables (Ghafoor et al. 1996; Islam et al. 2007). They are also a component of commercially available multivitamin products (Boullata and Armenti 2004). Most of the metals function as a cofactor of a number of metabolic reactions. For example, Fe, Zn, Cu, Ni and Mo are among the common metals that have known biological functions in plants (Westbroek and De Jong 1983; Seiler et al. 1994; Taiz and Zeiger 2006). These metals are mostly required as enzyme activator and some of them are even integral components of a number metaloenzymes. Hence, their deficiency may lead to suppression of growth and development of plants with visible deficiency symptoms reflected as chlorosis and subsequent necrosis of plant tissues (Dixon and Webb 1958; Ghani and Wahid 2007).

Despite the fact that some of the metals function as essential elements in low concentrations, they may become toxic if they accumulate at higher concentrations in

the environment (Verkleij and Prast 1990). Other metals (biologically non-essential) may become toxic to organisms even at very low concentrations (Verkleij and Prast 1990; Islam et al. 2007). The general signs associated with metal toxicity in plants include reduced shoot and root growth, poor development of branching system, deformation of various plant parts and abnormal flower shape, decreased biomass production, leaf spotting, mitotic root tip disturbances, inhibition of germination, and chlorosis that can result in foliar necrosis (Ewais 1997; Madhava Rao and Sresty 2000; Pandey and Sharma 2002; Rahman et al. 2005; Gajewska et al. 2006). Ultimately, all these processes lead to reduction in yield of agricultural crops (Balaguer et al. 1998; Ahmad et al. 2007).

2.3 Herbicides and Pesticides

Herbicides and pesticides have long been used as the most effective means of crop protection by controlling or eliminating the pests and pathogens. They include fungicides, bactericides, insecticides, weedicides, herbicides, rodenticides and algicides (Ellenhorn et al. 1997). These chemical substances are applied to crops at different growth stages e.g., as pre-sowing seed treatments, during crop cultivation and after harvest to protect seeds, grains and cereals from the attack of pests and pathogens and to prolong their storage capacity (Morgan and Mandava 1988; Boesten 2000). These chemicals are applied as liquid sprays, powder and dusts, seed-treatments, oil-based solutions and aerosols. Different examples include dichlorodiphenyltrichloroethane (DDT), benzene hexochloride, lindane, malathion, and 2,4-dichlorophenoxy acetic acid etc. (Morgan and Mandava 1988; Laws and Hayes 1991). Most of the pesticides can effectively control pests and pathogens and therefore, they are the most popular, economical and effective technology for crop protection among farmers of different regions of the world (Mandava et al. 1985).

Although application of these chemical compounds is regarded as an effective mean to control pest and pathogens, their application can have adverse effects on plants and animals including invertebrate and vertebrate species (Schluz 2004). These pesticides and herbicides can enter the atmosphere and ecosystems during their preparation and processing procedures, application methods, post-application evaporation and volatilization and water runoff (Van der Werf 1996; Shreiver and Liess 2007). In addition, disposal of expired chemicals into soil and water bodies is also a major source of their pollution in the environment (Bacci 1994). Among different classes of these chemicals, insecticides are the most important in damaging environment and causing toxicity to living organisms. This is followed by fungicides and bactericides and herbicides (Marer 2000; Goel and Aggarwal 2007).

The toxic/damaging effects of pesticides and herbicides on organisms and environment are determined by a number of features. These include (i) their chemical nature (systemic or non-systemic), (ii) active ingredients (formulation), (iii) organism exposed, (iv) persistency in the environment, and (v) concentration used for application (Van der Werf 1996). Besides these facts, some other factors such as personnel skill of the applicator (farmer), time of application and weather condition also contribute significantly towards the pesticide's actual toxicity and can make

them extremely hazardous. These chemicals accumulate in soil and water bodies and prove extremely toxic to the non-target organisms including plants and animals as well as humans (Jeyaratnam 1990).

2.4 Cyanides

Cyanides are organic compounds that comprise the cyano group (C≡N) in their structure. Cyanide toxicity is also known as prussic acid poisoning (Vogel et al. 1987). Different forms of cyanides include hydrogen cyanide (HCN), potassium cyanide (KCN) and sodium cyanide (NaCN). Among these, HCN is a colorless gas with odor just like a bitter-almond while NaCN and KCN are white powders with a similar odor as that of HCN. Both NaCN and KCN are converted into HCN when they get mixed in water and cause toxicity to living organisms (Curry and LoVecchio 2001). Cyanogenic compounds occur naturally in certain bacteria, fungi, algae and higher plants. Therefore, they occur in a variety of food and plant products. Cyanogenic compounds naturally occur in a number of plant families including Poaceae, Papilionaceae, Sambucaceae, Euphorbiaceae and Rosaceae. They are found in small amounts in various plant fruits such as apple seeds, citrus seeds, plums, mango stones, peach stones and bitter almonds (Poulton 1990; Wong-Chong et al. 2006).

In plants, cyanides are generally found in bound forms as cyanogenic glycosides and play an important role in plant defense against herbivory. For example, cassava roots have been reported to contain excessive amount of cyanogenic glycosides (Emmanuel and Emmanuel 1981). Among different cyanogenic glycosides found in plants, amygdalin is the best characterized one, which is present in a number of plant species especially in the leaves and seeds of cherry, almond and peach, etc. (Santamour Jr 1998; Sánchez-Pérez et al. 2008). For example, cherry kernels may yield up to 170 mg while bitter almond pulps up to 250 mg 100 g^{-1} dry weight. Overall, cyanogenic glycosides have been reported to occur in more than 3000 plant species (ca. from 130 families) and thus these species have a potential to produce HCN toxicity if ingested by animals and humans. However, actual incidence of cyanide poisoning is low, because these plants are not frequently eaten up by animals or humans (Curry and LoVecchio 2001).

In addition to natural sources, cyanides are also released by various industrial sources. For example, thiocyanate is discharged in a variety of industrial wastewater discharges, while cyanogen halides are released upon chlorination or bromination of water containing free cyanides (Zheng et al. 2004). Cyanides are also used as a raw material during the production of chemicals (nylon and plastic), adhesives, cosmetics, dyes, computer electronics, pharmaceuticals, and road salts, pesticides, rodenticides, wine, anticaking agents, fire retardents, pharmaceuticals, painting inks, and other materials (Kjeldsen 1998). In addition, they are also directly used in a variety of processes, including electroplating and hydrometallurgical based gold and silver extraction (Kavanaugh 2004). Current industries that produce cyanide as a by-product include chemical manufacturing, iron and steel making, petroleum

refining, and aluminum smelting (Wong-Chong et al. 2006). Overall, the approximate production of cyanides is 1.4 million tons per annum (Mudder and Botz 2001) which means over 10,000 tons of cyanide are being released into the environment each year (Mudder and Botz 2001; Korte et al. 2000).

Cyanogenic compounds, if accidentally ingested by animals or hydrolyzed by plants, prove extremely toxic (Schnepp 2006; Barillo 2009). This is mainly due to their ability to uncouple cytochrome C oxidase in mitochondria. HCN can readily bind to Fe in cytochrome in a stable and irreversible bonding (Cooper and Brown 2008). These result in disruption of electron transport chain thus blocking aerobic respiratory pathway that contributes to 95% of the energy produced in the cells in the form of ATP (Taiz and Zeiger 2006). In animals, tissues which are primarily dependent on aerobic respiration for source of energy, e.g., heart and central nervous system are markedly affected (Schnepp 2006; Barillo 2009). Thus, due to the blockage of ATP synthesis, plants or animals die quickly as no energy will be available to perform routine activities.

2.5 Toxic Explosives

Immense industrial and military activities are the main causes of substantial contamination of the environment with toxic explosives. Worldwide, a number of explosive-manufacturing, testing and storage facilities and military bases are contaminated with these chemicals. In addition, inappropriate disposal of explosive wastes and old and non-functioning weapons also contribute considerably towards environmental pollution (Pennington and Brannon 2002). The most common examples of explosives at hazardous waste sites are nitroglycerine (NG), 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (Royal Demolition Explosive - RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7- tetrazine (Rosenblatt 1980; Best et al. 1999). Among these, the most toxic materials used in military activities include TNT and RDX (Jenkins et al. 2006). Despite the threat of explosion upon exposure to large quantities of these explosives, exposure to these explosives such as TNT can cause severe health hazardous effects such as abnormal liver function, anemia, skin irritation, and cataracts. Similarly, RDX cause severe spasm when inhaled or eaten in large quantity. TNT and RDX also cause long-term health effects such as failure of nervous system and heart, which could lead to death of affected individuals (Lynch et al. 2002). In some cases, these toxic wastes may leach down to groundwater causing toxicity far away from the contaminated sites (Best et al. 1999).

There is only a little work on the effect of explosive materials on plants. However, the available literature suggests that these chemicals including nitroglycerine, TNT, RDX have a variety of effects on plants growing in contaminated areas (Harvey et al. 1991; Just and Schnoor 2004; Vila et al. 2007a; Rao et al. 2009). These effects include retardation of seed germination, growth (fresh and dry biomass) and development, and induction of leaf chlorosis and necrosis of plant tissues (Peterson et al. 1996; Robidoux et al. 1996; Vila et al. 2007b). Since the chemicals are mutagenic, they can also cause lethal mutations in animals as well as plants (French et al. 1999; Podlipna et al. 2008).

3 Plant Resistance to Toxins

3.1 Salts

The extent of the adverse effects of salt stress on crops or other naturally growing plants greatly differs and it depends on the type of species or cultivar, growth stage and interaction with other environmental constraints (Ashraf 1994; Ashraf et al. 2008; Munns and Tester 2008). Therefore, a variety of information is available in the literature depicting genetic variation for salt tolerance in crop plants. For example, while appraising the relative salinity tolerance in field pea, canola, dry bean, and durum wheat, Steppuhn et al. (2001) ranked these crops in an ascending order as dry bean < field pea < durum wheat < canola. Of different *Brassica* species, *B. napus* was found as the most salt tolerant, while *B. campestris* and *B. nigra* the most salt-susceptible (Kumar 1995). Some other studies entailing the exploration of mechanism of salt tolerance in canola have shown that cv. Dunkeld has high salt tolerance due to having higher photosynthetic, antioxidant, ion exclusion and osmotic adjustment capacities which make it highly salt tolerant (Ali et al. 2006; Ulfat et al. 2007; Ashraf and Ali 2008).

Plants use different mechanisms to overcome high salt concentration in soil. These include osmoregulation, compartmentalization of toxic ions, ion excretion, scavenging of reactive oxygen species and accumulation of compatible solutes etc. Salt tolerance in plants can be achieved by avoiding high ion concentration, i.e., delayed germination or maturity until favorable conditions, salt exclusion at root level or preferential root growth in non-saline areas, compartmentation of salts in vacuole or specialized cells such as salt glands and salt hairs or storage in older leaves, and selective discrimination of Na^+ against K^+ or Ca^{2+} (Marschner 1995; Hasegawa et al. 2000; Munns 2002, 2005; Tester and Davenport 2003; Flowers 2004). The antioxidant defense system includes antioxidant compounds (tocopherols and carotenoids) and enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and many others. Plants differ in their ability to scavenge ROS. For example, SOD in plants can catalyze the dismutation of superoxide to dioxgyen and hydrogen peroxide. Peroxidase or catalases can counteract H_2O_2 (Shalata and Tal 1998; Garratt et al. 2002).

Accumulation of compatible solutes such as polyols, sugars, glycinebetaine, proline, and other free amino acids is considered as one of the most vital components of salt tolerance in plants. Under saline conditions, these solutes not only allow the cells to adjust the osmotic potential to a level in the cytoplasm so as to maintain a sufficient amount of water content (Bohnert and Jensen 1996; Subbarao et al. 2001; Yokoi et al. 2002), but also safeguards proteins from the salt-induced dissociation of their respective subunits (Incharoensakdi et al. 1986). Moreover, in photosynthetic organisms, these organic solutes play a vital role in maintaining integrity of photosystem II at high levels of salt (Murata et al. 1992; Papageorgiou and Murata 1995), as well as the activity of enzymes involved in the mechanism of photosynthesis (Yokoi et al. 2002; Bohnert and Jensen 1996) such as ribulose 1,5- bisphosphate carboxylase/oxygenase (Nomura et al. 1998). Among the compatible solutes,

accumulation of proline and glycinebetaine plays a crucial role in osmoregulation and osmotolerance in plants (Rhodes and Hanson 1993; Hasegawa et al. 2000). They also protect membranes and proteins against the destabilizing effects of abiotic stresses such as salt stress and water stress. In addition, their ability to scavenge free radicals generated under stress conditions renders them as an important marker of salt tolerance (Kavi Kishore et al. 2005; Ashraf and Foolad 2007).

3.2 Heavy Metals

Although some of the metals function as essential elements such as copper and zinc in low concentrations, they may become toxic if they accumulate at higher concentrations in the environment (Verkleij and Prast 1990). Other metals (non-essential) may become toxic to organisms even at very low concentrations (Verkleij and Prast 1990; Loska et al. 2000; Islam et al. 2007). The concentration of essential elements in organisms is generally controlled homeostatically i.e., they are taken up from the environment according to the nutritional demand of a plant (Sigel et al. 2005; Mueller-Roeber and Dreyer 2007; Alloway 2008), except for some elements like selenium, iodine and technetium (Wolterbeek 2001; Windisch 2002). If this regulatory mechanism breaks down either due to insufficient supply (deficiency) or excess (toxicity) of metal, its effects on growth are manifest as deficiency or toxicity symptoms in organisms (Grusak et al. 1999; van Wuytswinkel et al. 1999; Grusak 2002; Welch 2002).

The differential variability of uptake of different metals depends on various aspects such as the metal itself, the absorbing organism, the physico-chemical properties of the soil environment and the levels of other important metals and complex chemicals present in waters from different sources (Cataldo and Wildung 1978; Battarbee et al. 1988; Antosiewicz 1992). For example, free ions are largely bioavailable forms of a metal, and the free ion concentration is usually a potential indicator of toxicity (Seiler et al. 1994). However, in some other cases the situation is different. For example, in case of mercury, the organic form (methylmercury) is more toxic than the inorganic mercury ion (Wright and Welbourn 2002). In addition, the valency of a particular metal ion also has great influence on its bioavailability and mobility in soil and plants (Deoraj 2003; Deoraj et al. 2003).

A great deal of controversy exists in the literature on the prospective mechanisms of metal tolerance. This is likely due to a lack of knowledge on issues related to metal toxicity or due to the complexity of plant responses to metal toxicity. Furthermore, a variety of mechanisms may have been evolved in different species to tolerate high amounts of metals and even within the same plant species more than one mechanism may be operational (Memon et al. 2001; Meharg 2005; Gao et al. 2007). In most studies, plant species are tested for tolerance ability by using only one or a combination of a few metals. However, under natural conditions, most of the sites are polluted with more than one type of pollutants (organics and in-organics) having varying degrees of toxicity. In addition, other environmental and geophysical features also contribute considerably for their availability and uptake. Therefore, it

becomes extremely difficult to distinguish their toxicity and mechanism operative for their tolerance in pants (Cataldo and Wildung 1978; Antosiewicz 1992; Deoraj et al. 2003).

Plants can employ numerous strategies to counteract excess external metal levels. These can be categorized into two main types, i.e., limiting the uptake or transport of metals, and internal metal tolerance mechanisms (Taylor 1987; Clemens 2006). In the first strategy, the toxic effects of metals are reduced by preventing the entry of excess metals in the plant by reduced uptake. This is brought either by complexing or precipitating metals in the root zone. Plants have the ability to precipitate metals by elevating the pH of the rhizosphere or by excreting them in the form of anions (Taylor 1991). However, a great deal of work has been done with limited number of metals such as Al and extensive work for other metals is essential to appraise the extensive validity of this mechanism.

True metal tolerance in plants could be, however, realized if metals are sequestered/compartmentalized within the cell of different tissues so that metals are unable to react with metabolically active cellular substances (Volesky 1990; Barley et al. 2005; Rajamani et al. 2007). In many studies, a significant increase in the level of organic molecules and amino acids (such as histidine) has been reported to occur in roots of metal stressed plants (Hall 2002). These results suggest that the complexation of metals with these organic molecules and amino acids might be involved in reduced delivery of metals from roots to shoots and hence reduced toxicity in aerial parts. However, once metals are transported to the aerial parts, there must be an effective mechanism to reduce their toxicity. As a first strategy, compartmentation of metal ions in the vacuole is the most plausible method of cellular sequestration (Rajamani et al. 2007). In addition, most of the metals lead to the production of reactive oxygen species. Therefore, most of the plants have evolved an effective scavenging system consisting of enzymatic (superoxide dismutase, peroxidase, catalase, glutathione reductase and ascorbate reductase) and non-enzymatic (proline, ascorbic acid, tocopherols, glutathione, carotenoids and phenolics) antioxidants. These antioxidants scavenge reactive oxygen species and protect micro- and macro-molecules and other cellular structures from oxidative damage (Luna et al. 1994).

3.3 Herbicides and Pesticides

Herbicides and pesticides have different effects on animals and plants. A few of these chemicals are selective in nature while others are broad spectrum in action. Therefore, broad spectrum pesticides are more hazardous to environment and organisms as compared to selective one (Laws and Hayes 1991; Marer 2000). Most of these chemicals persist in the environment which ultimately proves extremely toxic to non-target plants and animals. In addition to the toxic effects of these chemicals to plants and animals, these chemicals also contribute to soil degradation and affect soil microorganisms (Arthur Coats 1998; Andreu and Pico 2004).

Pesticide pollution causes considerable threats to a wide variety of non-target organisms including useful soil microbes, crops, livestock and other aquatic species.

Avoiding or minimizing the use of toxic chemicals is essential to improve continued existence of these non-target organisms (Calderbank 1989; Goel and Aggarwal 2007). It is now well known that soils have diverse composition and mainly consist of mineral particles and organic matter. Different types of pesticides may interact with the soil and form toxic residues in soils with minerals and organic matter, which may not be recovered from the soil even through extensive extraction (Gevao et al. 2000). The bioavailability of these bound residues is of great significance that determines toxicity to microorganisms and plants (Khan 1982; Calderbank 1989). Although, it has been documented that the activities of soil microorganisms primarily depend on the release of bound residues from the soil, but other factors like agronomic practices and application of some other chemicals that may change the chemical nature of soil may cause the release of soil bound residues (Khan 1982; Calderbank 1989; Goel and Aggarwal 2007). This might result in recycling of the compounds into the soil solution that could be ultimately absorbed by the plants and causes sever toxicity in plants (Andreu and Pico 2004).

Excessive use of pesticides and herbicides has been shown to produce a variety of toxicity symptoms in plants. However, there is great variation in toxicity symptoms depending upon type of chemical, active ingredient and concentration in the growing environment (Morgan and Mandava 1988; Boesten 2000; Hendersona et al. 2006). The most common toxicity symptoms in non-target plants are inhibition of seed germination, growth retardation, loss of photosynthetic pigments, damages to the photosynthetic machinery, fruit drop, reduced yield and a variety of other symptoms. These defects could result in chlorosis and necrosis of plant tissues eventually leading to the death of whole plants (Nair et al. 1993; Hendersona et al. 2006; Shreiver and Liess 2007).

3.4 Cyanides

The concentrations of cyanogenic glycosides greatly vary with phenology, growth stage, infection by pathogens, herbivory and environmental conditions (Gebrehiwot and Beuselinck 2001; Dzombak et al. 2006; Ballhorn et al. 2007). In plants, cyanogenic glycosides are usually compartmentalized in cell vacuoles and thus cells are prevented from their toxicity (Gruhnert et al. 1994; White et al. 1994; Gleadow and Woodrow 2002). Therefore, cyanogenic glycosides in plant tissues are not toxic unless they are hydrolyzed by plant enzymes (or rumen microorganisms) to form free HCN (White et al. 1998). This hydrolysis is usually carried out by the enzyme β-glucosidase that is found in plant cytoplasm. This conversion is also enhanced when the plant cells are injured (crushing, insect attack, herbivory) or when the plants are subjected to sever environmental stresses such as wilting or freezing stress (Ballhorn et al. 2009).

Some plants species contain an enzyme system that is able to detoxify cyanide by converting certain amino acids such as alanine and asparagine to cyanogenic glycosides in which a simple sugar is bonded to a cyanide molecule (Miller and Conn 1980; Galoian et al. 1982). In some plant species, β-cyanoalanine synthase

(CAS) was found to be able to catalyze the conversion of cyanide plus cysteine to β-cyanoalanine and sulfide (Miller and Conn 1980; Maruyama et al. 2001). This enzyme occurs in a number of higher plants and plays a vital role in the metabolism of cyanides (Maruyama et al. 2001). Since mitochondria are potential sites of cyanide toxicity and this enzyme is exclusively localized in this organelle, its principal physiological role has been attributed to its detoxification capability of cyanides (Manning 1988). In another study conducted on both cyanogenic as well as non-cyanogenic plants, asparagine was the only metabolic product found when they were exposed to labeled ^{14}CN (Manning 1988). In an experiment by Yu et al. (2004) 28 plants belonging to 23 families were appraised for their performance for removal of cyanide. These authors found that most of the plant species were capable of readily metabolizing cyanide to non-toxic chemical. This evidence shows that the mechanism of cyanide detoxification in plants needs to be fully explored.

3.5 Toxic Explosives

The toxicity of explosives containing nitro groups is usually attributed to the number of nitro groups. It has been suggested that different plants can take up and degrade toxic explosives such as nitroglycerine into simpler non-toxic compounds. In this regard, Podlipna et al. (2008) showed that the toxicity of nitroglycerine decreased with the decreasing number of nitro groups during *phytodegradation* of these chemicals by mustard (*Sinapis alba*), *Juncus inflexus*, *Phragmites australis* and flax (*Linum usitatissimum*). Most recently, genetically engineered plants have been shown to have greater ability to detoxify these compounds. In these plants, toxic explosives such as TNT are converted to different compounds that are used by the plant enzymes for further processing (Rylott and Bruce 2008). In response to the explosive presence several genes are up-regulated, including transferases, which by transferring a particular residue to the acceptor molecule, alter its bioactivity, solubility and/or transport properties (Ekman et al. 2003; Mezzari et al. 2005). A full characterization of the activity of the most promising enzymes such as transferases should be performed so that new concepts are added to the biochemical scheme of transformation of toxic explosives.

4 Phytoremediation of Toxins

Phytoremediation, a subcategory of *bioremediation*, is generally defined as removal of toxins from the environment by the use of hyperaccumulator plants. This word has been derived from the Greek “Phyto” meaning plant, and Latin “Remedium” meaning refurbishing balance, removal, or remediation. Thus, in the process of *phytoremediation*, pollutant/toxins from contaminated soils, water or air are mitigated/removed by using plants which are able to hold, breakdown or remove metals, salts, insecticides, pesticides, organic solvents, toxic explosives, crude oil

and its derivatives, and a variety of other contaminants from different environmental components. *Phytoremediation* is generally considered as efficient, inexpensive and environment-friendly technique, as compared to other mechanical or chemical methods of remediation that involves excavation of soil from contaminated site and ex-situ treatment for the removal of contaminants (Cunningham and Ow 1996).

Phytoremediation of contaminated soils can be achieved through various processes. These include *phytoextraction*, *phytoimmobilization* or *phytostabilization*, *phytotransformation*, *phytodegradation*, *phytostimulation*, *phytovolatilization* and *rhizofiltration* (Schwitzguebel 2000; Cummings 2009). Of these strategies, *phytoextraction* or *phytoaccumulation* consists of natural or induced (enhancement through use of chelating agents) potential of plants, algae and lichens to uptake and remove pollutants from soil, water environment by accumulating them into harvestable biomass. This method is traditionally used for the removal of heavy metals and salts from the contaminated soils. *Phytostabilization* is stabilization of the toxic pollutants over a long-term. Some plants have natural ability to immobilize pollutants by providing a region around the roots where these pollutants can be precipitated and stabilized. Unlike *phytoextraction*, *phytostabilization* involves sequestering of toxins into the *rhizosphere*, thereby preventing metal uptake by plant tissues. Therefore, pollutants turn out to be less mobile and bioavailable to plants, wildlife, livestock, and humans. *Phytotransformation* is the conversion of different types of organic pollutants by certain plant species to non-toxic substances. In addition, microorganisms living in soil and water and those associated with plant roots may metabolize these substances to non-toxic ones. However, it is imperative to note that these tenacious and complex compounds cannot be degraded to simple molecules such as water, carbon dioxide etc. by plant metabolism. However, in this process, a change in their chemical structure is brought about that reduces their toxicity to living organisms. *Phytostimulation* involves the enhancement of uptake of pollutants by increasing the activity of soil microorganisms to degrade the contaminants. This involves normally the activity of those organisms that live in association with the roots of higher plants. *Phytovolatilization* is the removal of substances from soil or water and hence, their release into the atmosphere. *Rhizofiltration* is the filtration of contaminated water through a mass of roots so as to remove toxic substances or surplus nutrients (Raskin and Ensley 2000).

The use of *phytoremediation* approach for the removal of environmental toxins has been greatly appreciated due to its environmental friendliness. In comparison to the conventional methods being used for cleaning up contaminated soil that damage soil structure and hamper soil fertility, *phytoextraction* can clean up the soil without causing any major change in soil quality and fertility. Another potential benefit of *phytoextraction* is that it is comparatively cost-effective as compared to any other traditional clean up method in vogue. In addition, the effectiveness of plants in the process of *phytoremediation* can be easily monitored by their growth potential under contaminated soils (Salt et al. 1995, 1997; McIntyre and Lewis 1997; Sadowsky 1999; Raskin and Ensley 2000; Schwitzguebel 2000). Despite all these advantages, the process of *phytoremediation* is criticized due to its certain limitations. For example, it can reclaim only surface soils as well as up to the depth

occupied by the plant roots. As this process depends on the ability of plants to uptake and degrade/metabolize, so more time is required as compared to traditional but highly efficient methods used for cleaning of contaminated soils. In addition, with plant-based remediation systems, preventing leaching of pollutants to groundwater aquifers is not easy without the complete removal of the pollutants from the soil. The survival of the plants growing in the contaminated land is determined by the extent of toxicity of pollutants. Finally, there is always a risk of bio-accumulated contaminants in plants to enter into the food chain, from primary producers to primary consumers and upwards, and finally to humans (McIntyre and Lewis 1997; Chaudhry et al. 2002; Prasad 2004a, b; Lupino et al. 2005).

Remediation of saline soils by using highly salt tolerant plants (halophytes) has been suggested as an economical approach. Some halophytic species (e.g., those of *Atriplex*, *Suaeda, Salsola*, *Chenopodium* and *Portulaca*) could uptake salt ions through roots and metabolize or store them in the leaves through the process of *phytoextraction* (McKell 1994; Grieve and Suarez 1997). The salt uptake and accumulation by these halophytes can reduce the salt level at least at rhizospheric level, and make the soil suitable for growth of the agricultural crops with better yield (Zuccarini 2008). This approach seems to be effective because many halophytic and highly salt tolerant plant species naturally grow on highly saline soils and hence can be employed to reclaim saline soils. This approach appears to be less expensive when conventional soil reclamation and advanced biochemical and genetical modification approaches are costly. However, it should be clear that the salt tolerance ability varies greatly within species as well as within populations of the same species. In addition, it also depends on interaction of salinity stress with other environmental adversaries that limit plant growth under that set of environments (Ashraf 2004). Therefore, the successes of a particular halophyte may differ greatly under different environments that need to be explored by proper experimentation. In addition, if the *phytoremediation* potential of halophytes is aided by other conventional techniques, the amelioration processes would be more fast, effective, reliable and sustainable (Ashraf et al. 2008).

Heavy metals from contaminated soils can best be removed by *phytoextraction* or *phytoaccumulation* techniques without destroying the soil structure and fertility. In this approach, toxic metals are absorbed and accumulated into the biomass that can be easily harvested and removed from the contaminated areas (Huang and Cunningham 1996; Chaney et al. 2000; Lasat 2000). *Phytoextraction* can be achieved using natural or chelate assisted extraction of heavy metals from the contaminated soils. Continuous or natural *phytoextraction* involves the removal of metals depending on the natural ability of a particular plant species to accumulate metal contaminants without showing any significant symptoms of toxicity (Salt et al. 1995, 1997). In contrast, in chelate assisted or induced *phytoextraction*, the *phytoremediation* potential of different species is enhanced by synthetic chelates such as ethylenediaminetetraacetic acid (EDTA), *S,S*-ethylenediaminedisuccinic acid (EDDS), trisodium nitrilotriacetate (Na_3NTA), *N*-hydroxyethyl-ethylenediamine-triacetic acid (HEDTA), ethylenediamine di-(*o*-hyroxyphenylacetic acid) (EDDHA), trans-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA), ethylene glycol-bis(β-aminoethyl ether),

N,*N*,*N′*,*N*-tetraacetic acid (EGTA), and diethylenetriaminepentaacetic acid (DTPA) (Blaylock et al. 1997; Kulli et al. 1999; Kayser et al. 2000; Grcman et al. 2003; Kos and Lestan 2003). These chelates generally increase the mobility and uptake of metal contaminants by plants many-folds as compared to natural conditions. However, it must be understood that the success of *phytoextraction* technique mainly depends on the ability of a plant species to (i) extract large quantities of heavy metals into their roots, (ii) translocate the heavy metals to above-ground parts, and (ii) produce a large quantity of plant biomass (Grcman et al. 2003; Kos and Lestan 2003; Luo et al. 2004). Other factors such as growth rate, element selectivity, resistance to disease, methods of harvesting, are also important in determining the success of this technique (Baker et al. 1994; Cunningham and Ow 1996). Therefore, slow growth, shallow root system and small biomass production limit the potential of hyperaccumulator species (Brooks 1994). This technique has successfully been used for the removal of almost all known metal contaminants by various plant species.

Phytovolatilization involves the uptake of contaminants from polluted soil and their transformation into volatile compounds and their extraction into the atmosphere by transpiration. This technique is relatively less useful for removal of heavy metals as the pollutant must (i) be taken up by plants through roots, (ii) pass through the xylem to the leaves (iii) be converted into some volatilable compounds, and (iv) volatilize to the atmosphere (Mueller et al. 1999). Despite these limitations, this technique has been reported to be useful for the removal of mercury from the polluted soils by transgenic tobacco plants carrying bacterial mercury detoxification genes *merA* and *merB* (Rugh et al. 1996, 1998; Bizily et al. 1999, 2000). The genes (*merA*) encodes the enzyme mercuric ion reductase that reduces ionic mercury (Hg^{+}) to the less toxic volatile $Hg^{(0)}$ using NADPH reducing equivalents. In this process, the mercuric ion is transformed into methylmercury (CH_3Hg^{+}) and phenylmercuric acetate (PMA), that are fat-soluble and finally to metallic elemental mercury $Hg^{(0)}$ that is volatile at room temperature (Langford and Ferner 1999). In another study, plants growing on high selenium media have been shown to produce volatile selenium in the form of dimethylselenide and dimethyldiselenide (Chaney et al. 2000). However, this technique has the biggest disadvantage that most of the pollutants evaporated into the atmosphere are likely to return back to the ecosystems by precipitation (Hussein et al. 2007). Additionally, the success of this technique has a been test only for a limited scale under controlled conditions and a lot of work has to be done for determining its effectiveness for other metals as well as under field conditions.

Rhizofiltration i.e., removal of metals by passing through a mass of roots, can be used for the removal of lead, cadmium, copper, nickel, zinc and chromium, which are primarily retained with in the roots (Chaudhuri et al. 2002; United States Environmental Protection Agency Reports 2000). This technique has been tested using different crop plants such as sunflower, Indian mustard, tobacco, rye, spinach and corn, as well as tree plants such as poplar (Chaney et al. 1997; Eapen et al. 2003; Pulford and Watson 2003; Biró and Takács 2007; Lee and Yang 2009). Among these, sunflower and poplar have the greatest ability to remove metals from the contaminated environment (Prasad 2007; Zacchini et al. 2009). The greatest

benefit of the *rhizofiltration* method is that it may be conducted in-situ, with plants being grown directly in the contaminated soil and water bodies. It does not involve removal and ex-situ treatment of contaminants. Therefore, it is considered as a relatively cheep procedure with low capital costs. Operational costs are also low but it depends on the type of contaminant as well as selection of plant species. Additionally, crop may be converted to biofuel, used as a substitute for fossil fuel or used in other domestic and agricultural purposes (Chaudhry et al. 2002; Rugh 2004). Despite this, the applicability of this method is very limited. First of all, the plants species selected may grow well in moderately contaminated areas but might show poor performance in highly contaminated sites. Secondly, contaminants that lie in deep soil below the rooting depth will not extracted by this method. Therefore, plants with shallow root system will not be much effective as the deep-rooted plants. Thirdly, it normally takes many years to reduce the concentration of the contaminant to regulatory levels. Fourthly, most sites are contaminated with a variety of contaminants including metals, inorganics and organics. In this case, the use of plants for removing the pollutant through *rhizofiltration* will not be sufficient and would require support of some other methods. Plants grown on polluted water and soils may become a threat to animal and human health. Therefore, a careful attention should be taken while harvesting and only non-fodder crops should be chosen for the remediation of soil and water through the *rhizofiltration* method (Cunningham and Ow 1996; Chaudhry et al. 2002).

In *bioremediation* of herbicides and pesticides, plant metabolism contributes to their removal by transformation, break down, stabilization or volatilization after uptake from soil and groundwater. Biodegradation of these chemicals is mainly carried out by both bacteria and plants. However, bacterial degradation of these chemicals is more efficient as compared to plants (Roberts et al. 1993; Allison et al. 1995; Hall et al. 2000; Hendersona et al. 2006; Liao and Xie 2008). *Bioremediation* by microbes is mostly active in the upper layer of the soil surface, where the organic matter is the source of nutrients for their activity (Navarro et al. 2004). The degradation process consists of formation of metabolites and their decomposition to inorganic and simple products that are generally harmless to living organisms (Sassman et al. 2004, Sparks 2003, Kale et al. 2001). Some fungal species such as *Phanerochaete chrysosporium* and *Phanerochaete sordida* have also been shown to actively degrade pesticides such as DDT from the contaminated soils. This extremely toxic chemical was transformed into comparatively less toxic products such as DDD and DDE (Bumpus and Aust 1987; Safferman et al. 1995). Although both these chemicals are less toxic to micro-organisms, which have the ability to metabolize and detoxify them into more simple products and their high concentration can prove extremely toxic to these organisms (Bumpus and Aust 1987; Safferman et al. 1995; Osano et al. 1999).

In addition to the role of bacteria in biodegradation of herbicides and pesticides, many plants contain certain enzymes that can break down and convert ammunition wastes, chlorinated solvents such as trichloroethylene and other herbicides to simpler and harmless molecules. The enzymes include oxygenases, dehalogenases and reductases (Black 1995). In some studies, it has bee reported that some

grass species such as big bluestem, switchgrass, and yellow Indian-grass have a potential to remove pesticide residues from the contaminated soils. These species can develop a region around rhizosphere with microflora that can readily detoxify pesticide residues (Hoagland RE, Zablotowicz 1995; Marchand et al. 2002; Hendersona et al. 2006). Specific strains of atrazine-degrading bacteria have been shown to have atrazine chlorohydrolase that can enhance the rate of biotransformation of atrazine in soil. In addition, these prairie grasses were also found to reduce the rates of leaching of pesticides from soil to ground water (Hendersona et al. 2006). In another study by Coats and Anderson (1997) some members of *Kochia* sp. were found to be effective in degradation and detoxification of various chemicals such as atrazine and trifluralin. In this case, most of the degradation occurred in the rooting zone (rhizosphere), suggesting that micro-organisms residing in the rhizosphere of these plant were involved in enhanced degradation of these pesticides. Additional experimentation on members of *Kochia* sp. by the same authors have shown to be promising for the removal of pesticide from soils and groundwater (Arthur and Coats 1998). In laboratory experiments, poplar tree with fast growth potential and deep root system were found to be very successful in the removal of atrazine and arochlor from soil and groundwater. In this case, poplar plantations absorbed and metabolized these harmful compounds to less toxic chemicals (Burken and Schnoor 1996; Burken and Schnoor 1997; Nair et al. 1993).

Various plant species have the potential to remove cyanides from the polluted environments. These include hybrid willows (*Salix matssudana* Koidz x *Salix alba* L.), weeping willows (*Salix babylonica* L.), basket willows (*Salix viminalis*), poplar (*Populus deltoides*), upright hedge-parsley (*Torilis japonica*), Chinese elder (*Sambucus chinensis*), snow-pine tree (*Cedrus deodara* (Roxb.) Loud), water hyacinth (*Eichhornia crassipes*) and many other plant species (Ebbs et al. 2003; Yu et al. 2004 2005; Larsen et al. 2004; Taebi et al. 2008). However, their remediation ability varies greatly and differs with plant species, age and level of toxin in the environment. Hence, the decision whether to use a particular species for *phytoremediation* of cyanides should be carefully evaluated before any sound recommendation. In addition, it has also been shown that the removal of cyanide may also be carried out by certain species of micro-organisms through the process of biodegradation (Dubey and Holmes 1995).

As mentioned earlier, some plant species have the ability to uptake, transport and detoxify the cyanogenic compounds. The basic detoxification mechanism in tolerant species is *phytodegradation* in which the conversion of cyanides to cyanogenic glycosides is carried out by specific enzymes. This helps these plants to reduce the level of cyanide to non-toxic levels and maintain growth under cyanide polluted environment. In view of a report a small amount of cyanides can also be evaporated through *phytovolatilization* (Trapp and Christiansen 2003). This postulation was confirmed by the work of Yu et al. (2004) in which it was found that 1.5% of total cyanide fraction could be evaporated through leaves. However, they suggested that this small fraction is not sufficient enough to confirm whether the process of *phytovolatilization* is involved in the removal of cyanides from contaminated soils. Later, Larsen et al. (2004) did not find a significant relationship between

evaporation and removal of cyanides by basket willows. However, they confirmed the involvement of two potential enzymes beta-cyanoalanine synthase and beta-cyanoalanine hydrolase in the ability of willow to detoxify cyanides. This evidence, although insufficient, shows that *bioremediation* of cyanides from the environments polluted can be carried out mainly by biodegradation and on a limited scale through *phytovolatilization*.

The primary solution for the remediation of soils affected with explosive chemicals is soil evacuation and ex-situ treatment by incineration or secured land-filling. However, this method is extremely cost-intensive, destructive to the environment, and not practicable by any means. In this situation, *bioremediation* is an affordable and environment-friendly method and has been evaluated using a number of bacterial strains and a few plant species. A number of fungi, yeast, bacteria and other microorganisms present in the root zone (rhizosphere) of higher plants have been shown to break down organics such as explosives, fuels and solvents (French et al. 1998; Bhadra et al. 1999; Burken et al. 2000; Hawari et al. 2000). Among plants, willow and poplar have been extensively used in the cleaning-up of soils contaminated with toxic explosives. It has been reported that hybrid poplar (*Populus deltoids* x *P. nigra*) is very effective in removal of TNT when it was grown in hydroponic solution, but it translocated only 10% of total TNT to the foliar parts (Thompson et al. 1998). In another study, clones of hybrid willow (*Salix clone* EW-20) and Norway Spruce (*Picea abies*), were found to be very effective in readily metabolizing TNT to non-toxic intermediates (Schoenmuth and Pestemer 2004).

A limiting factor for using *phytoremediation* approach of explosives is that it is a very slow and in most of the cases an incomplete process. This leads to accumulation of a variety of intermediate metabolites that can be further incorporated into the food chain and may ultimately reach humans (Dietz and Schnoor 2001; Aken 2009). Recently, a number of bacterial genes have been introduced into plants to enhance inherent limitations of plant detoxification capacities. For example, various bacterial genes encoding enzymes involved in the detoxification of explosives have been successfully introduced in plants. In this regard, the genes encoding nitroreductase and cytochrome P_{450}, have been successfully engineered in a number of plants. This has resulted in a considerable improvement in uptake, detoxification and tolerance to toxic explosives by these plant species (Cherian and Oliveira 2005; Park 2007; Aken 2009).

5 Conclusion

Although *phytoremediation* is very helpful in removing contaminants from polluted soil and water, it is absolutely not the complete answer to all contamination problems. It is a fact that once pollutants are added to the environment, they cannot be completely removed due to their ability to circulate among different environmental components and food chains. Therefore, as a first strategy, we must try to avoid

or reduce the addition of pollutants to the environment. Secondly, if soil or water environment has been polluted, we must adopt in-situ and environment-friendly approach such as *bioremediation* to overcome this problem rather than ex-situ and destructive remediation methods.

The use of *phytoremediation* approach to remove contaminants has been greatly appreciated due to its environment friendliness. Perhaps, the greatest benefit of this approach is that plants are directly planted in the contaminated soils and it does not involve massive soil evacuation and ex-situ treatment for removal of contaminates. This feature greatly reduces the operational as well as capital costs incurred and renders this method less expensive than any other in-situ and ex-situ clean-up methods. In comparison to the traditional methods used for removing contaminants from contaminated soil that degrade structure of soil and reduce fertility, *phytoremediation* can clean-up the soil exclusive of bringing about any major change in soil quality and fertility. In addition, the effectiveness of plants in the process of *phytoremediation* can be easily monitored by examining their growth potential when grown in contaminated soils. Some crop products may be converted to biofuel, used as a substitute for fossil fuel or employed in other domestic and agricultural purposes.

Despite the attractiveness of *bioremediation* as environment-friendly, economical and feasible approach, it has certain limitations as its full potential is still being discovered. First of all, most plants have shallow root system and can generally grow and remediate in only top soil up to 3–4 feet. Even if we use deep-rooted plants, it can effectively remediate up to a depth of only 10 feet and thus may not be effective for the remediation of groundwater. Secondly, it requires a considerable time-period to effectively remediate a contaminated site and bring the level of contaminants to acceptable levels. It also requires a continuous monitoring of the effectiveness during this process that increases capital cost. Thirdly, in most of the *bioremediation* techniques such as *phytoextraction* and *phytostabilization*, plants uptake pollutants from soil and then transport and accumulate them to their above-ground parts such as stems or leaves. In this case, pollutants are not completely biodegraded to non-toxic compounds, but accumulate in plant tissues. This can be extremely harmful to primary (herbivores) and secondary (human) consumers. Fourthly, sometimes, it is impossible to predict the byproducts of transformation process and in this case degradation of some pollutants, such as DDT leads to accumulation of byproducts such as DDE and DDD that proved extremely toxic in most organisms. Although some microorganisms have the capacity to detoxify or metabolize them (DDE and DDD) to more simple and harmless products, their high concentrations can be toxic to them. Finally, some pollutants are extremely resistant to biodegradation and some are recalcitrant in nature. Therefore, the removal of these compounds requires superior and efficient organisms or alternative methods.

An extensive research work is required to fully understand the mechanism of bioremediation. It could be achieved through immense work in the fields of physiology, molecular biology, and biochemistry. Different species of plants and microorganisms need to be identified and carefully evaluated for their *bioremediation* potential. In addition, different genes found in micro-organisms with a potential

of *bioremediation* can be identified and introduced into crop plants and trees. This would enhance the efficiency of natural hyperaccumulator species for the effective removal of environmental pollutants. Since most of the soils and water bodies are polluted with more than one type of pollutants, an integrated approach should be used to get the maximum benefits of *bioremediation*.

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Part I
Toxins and Resistance Mechanisms

Chapter 2
Molecular Mechanisms and Genetic Basis of Heavy Metal Toxicity and Tolerance in Plants

Nand Lal

Abstract Heavy metal pollutants are mainly derived from growing number of anthropogenic sources. As the environmental pollution with heavy metals increases, some new technologies are being developed, one of these being phytoremediation. Hyperaccumulator plant varieties can be achieved by using methods of genetic engineering. An uptake of excessive amounts of heavy metals by plants from soil solution leads to range of interactions at cellular level which produce toxic effects on cell metabolism in terms of enzyme activity, protein structure, mineral nutrition, water balance, respiration and ATP content, photosynthesis, growth and morphogenesis and formation of reactive oxygen species (ROS).On the basis of accumulation of heavy metals plants are divided into three main types; (i) the accumulator plants, (ii) the indicator plants, and (iii) the excluder plants. Generally, the accumulation of heavy metals in plant organ is in series root > leaves > stem > inflorescence > seed. Most of plants belong to excluder group and accumulate heavy metals in their underground parts. When roots absorb heavy metals, they accumulate primarily in rhizodermis and cortex. In intracellular parts, highest concentration of heavy metals is found in cell wall. Tolerance of plants against heavy metals is due to reduced uptake of heavy metals and increased plant internal sequestration. In the increased plant internal sequestration mechanism, plant is manifested by interaction between a genotype and its environment. There are biochemical machineries in plants that work for tolerance and accumulation of heavy metals. Metal transporters are involved in metal ion homeostasis and transportation. Some amino acids and organic acids are ligands for heavy metals and these amino acids and organic acids play an important role in tolerance and detoxification. Phytochelatins (PCs) are produced in plants under stress of heavy metals and play role in binding heavy metals to complexes and salts and sequestering the compounds inside the cell so that heavy metals can not disturb the cell metabolism. The genes for phytochelatin synthesis have been isolated and characterized. Another low molecular weight (6–7 KDa) cysteine-rich compounds known as metallothioneins (MTs) also play an important

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role in detoxification of metals. In the plants growing under unoptimal temperature, there is high expression of heat shock proteins (HSPs), which normally act as molecular chaperones in protein folding, but may also function in the protection and repair of protein under metal-stress. Genes for heavy metal resistance have been isolated, manipulated and used to produce transgenic plants. Introduction of above genes and heterologous metallothionein genes to raise novel transgenic crop plants is under progress and holds promise to develop superior metal tolerant/hyperaccumulator crop plants.

Keywords Heavy metals · Metal toxicity · Phytoremediation · Metal binding proteins · Metal transporters · Phytochelatins · Metallothioneins · Heat shock proteins · Transgenic plants

Contents

1 Introduction

It is well known that plants require some nutrients for their proper growth and metabolic processes. Among these essential nutrients, some are required in relatively very high value (range above 10 mmol kg^{-1} of dry wt.) and are called as macronutrients. Some nutrients are required in very trace quantity (range below 3.0 mmol kg^{-1} of dry wt) and are known as micronutrients. Among these required micronutrients, Cu, Zn, Mn, Fe are heavy metals, required in very small quality. If these are found in soil above required level, they become toxic to plants. Besides these essential nutrient heavy metals, non-nutrient heavy metals are also found among which Cd and Pb are most widespread. Fifty-three of ninety naturally occurring metals are considered as heavy metals. These are characterized by specific density above 5 g cm^{-3} and relative atomic mass above 40. Environmental pollution with such heavy metals is a subject of great concern. These pollutants are derived from growing number of diverse anthropogenic sources such as; industrial effluents

and wastes, burning liquid and solid fuel, smelting and foundry work, urban run-off, sewage treatment plants, boating activities, agricultural fungicide run-off, domestic garbage dump and mining activities.

Plants often accumulate heavy metals to concentrations exceeding their levels in soil by several folds, wherefrom they enter the food chain. The capacity of plants to accumulate such metals and tolerate their high concentrations is species-specific trait. Plants ideal for phytoremediation should grow fast, have high biomass and tolerate or accumulate a range of heavy metals in their harvestable parts. More than 400 plant species have been reported to hyperaccumulate heavy metals (Brooks 1998). Most of these species fall short of biomass, only recently some plants have been reported to be ideal such as Chinese brake fern (*Pteris vittata* L.), an arsenic hyperaccumulator with a considerable biomass, fast growing, easy to propagate and perennial in nature (Ma et al. 2001; Chen et al. 2002).

When bound on the cell surface and within cells, heavy metal ions interact with the functional groups of proteins, nucleic acids, polysaccharides, and substitute for other metal ions already bound to these functional groups. Various metabolic disorders arise, and it is usually difficult to tell, which is primary and which one is secondary. Many heavy metals manifest high affinity for sulphur containing ligands and strongly bind to latter. When these enter the cell, they interact with protein and change their native conformational structure. During interaction with enzymes, they mask the active site of enzyme and disturb the enzyme activity.

As for as the environment is contaminated with heavy metals, there is need of technology to clean the environment with suitable techniques, which must be easy to handle, cost-effective and feasible. For this purpose there are some techniques such as; soil replacement, solidification, washing strategies and hytoremediation. Among these technologies, phytoremediation has gained most attention because it is cost effective, feasible and easy to handle. The high accumulation of heavy metals in plants was first reported in 1865 in *Thlaspi caerulescens* (Sachs 1865), but the term "hyperaccumulator" was coined by Brooks et al. (1977). They defined this term during the study of Ni concentrations in a plant and concluded that plants having concentrations higher than 1000 $\mu g\ g^{-1}$ of dry weight (0.1%) should be called hyperaccumulators. A hyperaccumulation of Ni, Zn, Cd, Pb, Cu, As, Co and Mn have been reported. It is not common in all terrestrial higher plants. Only less than 0.2% of all angiosperms have been identified as metal hyperaccumulators. The cruciferae family is well represented among these, *Brassica juncea* is a heavy metal accumulator plant with a high biomass and is well applicable for phytoremediation strategy. Recently, transgenic plants have also been developed for hyperaccumulation of heavy metals (Zhu et al. 1999a, b). Genetic engineering can be applied to this technique to get more remarkable results.

2 Heavy Metal Toxicity

In soil solution, the chemical form of heavy metal is dependent on other ions present in the vicinity of heavy metal ions and soil pH. Differences in solubility, absorbability, transport and chemical reactivity in these metals will lead to specific

differences in toxicity within the body of living organisms (Stohs and Bagchi 1995). Plants are organisms exposed to different kinds of stresses, such as air pollution, drought, temperature, light, heavy metals, salinity, freezing, UV radiation and nutritional limitation. Hall (2002) reported that the toxicity symptoms observed in plants in the presence of excessive amounts of heavy metals may be due to range of interactions at cellular level. The toxic effects may be direct or indirect and appear as metal-induced toxic effect on cell metabolism in terms of; enzyme activity, protein structure, mineral nutrition, water balance, respiration and ATP content, photosynthesis, growth and morphogenesis, and formation of reactive oxygen species.

Inhibition of plant growth is often used in the environmental tests for toxic heavy metals. Growth inhibition by heavy metals results from metabolic disorders and direct effects on growth, e.g., due to the interactions with cell wall polysaccharides decreasing cell wall plasticity. In the plant species like *Phaseolus vulgaris* and *Pisum sativum*, the seed coat is readily permeable to Pb^{2+} and seeds do not germinate in the presence of Pb salts. Root growth is more sensitive to heavy metals than shoot growth (Obroucheva et al. 1998; Seregin and Ivanov 1997; Titov et al. 1995; 1996; Nesterova 1989). This evidence correlates with the data that heavy metals accumulate predominantly in roots (Seregin and Ivanov 2001). To assess the ecological impacts of heavy metals, it is important to determine the lowest concentration that inhibits root growth. Further studies will show in detail whether the mixed salts of various metals produce additive synergistic or antagonistic effects. They notably affected root morphology. At moderate concentrations, the number of lateral roots decreases to a lesser extent than the primary root length, and the root system acquires a denser pattern. The initiation of lateral roots is very tolerant to heavy metals, probably due to the endodermal barrier and the specific structure of the cells in the central cylinder. Denser root systems develop when heavy metals decrease the final size of elongated cells, and therefore the distances between lateral root initials.

Majority of heavy metals have strong affinity toward SH group of enzymes and usually inhibit their activities during this interaction by blocking the SH group or masking the active site of enzyme. There are about hundred known enzymes, whose activity is affected by SH group interaction with heavy metal ions (Seregin and Ivanov 2001). Table 2.1 presents effects of two common heavy metals, Cd and Pb on certain enzyme activities. The resistance of one and the same enzyme to heavy metals varies with plant species. The decline in enzymatic activity by exposure of heavy metals is crucial for understanding the multidirectional effects of these metals on diverse aspects of cell metabolism. In some cases these ions even promote enzyme activity. The direct stimulation of catalase, peroxidase and superoxide dismutase has not been proved unambiguously because these activities decrease following short exposure to heavy metal ions. Apparently it is the oxidative stress that enhanced the activities of the stress-related enzymes by increasing the levels of free radicals and peroxides in the cytoplasm. The tolerance of particular enzymes, activation of particular enzyme system and maintaining the metabolisms in the stress-affected cells are possible causes for plant tolerance to an excess of heavy metals.

Table 2.1 Effect of Cd and Pb on some plant enzyme activities

Enzyme	Metabolic process	Metal	Enzyme activity	Type of interaction	Plant species
δ-Aminolaevulinate dehydrogenase	Chlorophyll synthesis	Pb	↓	Interaction with SH groups; Pb-induced zinc deficiency	*Pennisetum typhoideum*
Protochlorophyllide reductase	Chlorophyll synthesis	Cd	↓	Interaction with SH groups	*Hordeum vulgare*
The enzyme system of photolysis	Water photo-oxidation	Cd	↓	Interaction with SH groups	*Lycopersicon esculentum*
Ribulose-1, 5-bisphosphate carboxylase/oxygenase	CO_2 fixation	Cd Pb	↓	Interaction with SH groups Cys173 and Cys458	*Hordeum vulgare*, *Cajanus cajan*, *Avena sativa*
Phosphoenolpyruvate carboxylase	CO_2 fixation	Cd Pb	↓	–	*Zea mays* *Cajanus cajan*
Glyceraldehyde-3-phosphate Dehydrogenase	Calvin cycle	Cd	↓	Interaction with SH-groups	*Valerianella locusta* *Cajanus cajan*
Ribulose-5-phosphate kinase	Calvin cycle	Cd	↓	Interaction with SH-groups	*Valerianella locusta*
Nitrogenase	N_2 reduction	Cd Pb	↓	–	*Glycine max* *Azolla filiculoides*
Nitrate reductase	NO_3^- reduction	Cd Pb	↓	1. Interaction with SH-groups 2. Decrease in NO_3^- uptake	*Pisum sativum* *Phaseolus vulgaris* *Lycopersicon esculentum*
Root H^+-ATPase	Ion transport	Cd	↓	Conformational changes	*Zea mays* *Helianthus annuus* *Triticum aestivum*
Hexokinase	Glycolysis	Cd	↓	–	*Pisum sativum (seeds)*
Glucose 6-phosphate dehydrogenase	Pentose phosphate pathway	Cd	↓	–	*Pisum sativum (seeds)*
Carboanhydrase	Reversible CO_2 hydration	Cd Pb	↓ ↓ ↑	Zinc deficiency Molecular modification	*Glycine max* *Melica nutans (sensitive)* *Melica nutans (tolerant)*

Table 2.1 (continued)

Enzyme	Metabolic process	Metal	Enzyme activity	Type of interaction	Plant species
Cu-Zn superoxide dismutase	Destruction of superoxide ions	Cd Pb	↓ ↑	Zinc deficiency	*Phaseolus vulgaris* *Lupinus luteus*
Mn-superoxide dismutase	Destruction of superoxide ions	Cd	↑	–	*Phaseolus vulgaris*
Peroxidase	Polyphenol oxidation with H_2O_2	Cd	↑	–	*Phaseolus vulgaris* *Lemna sp.,* *Oryza sativa* *Hordeum vulgare*
Protease	Protein hydrolysis	Cd	↑	–	*Lemna sp., Oryza sativa*
Catalase	Destruction of H_2O_2	Pb	↑	–	*Zea mays*

Note: ↓ Indicate decrease in activity; ↑ indicate increase in activity

Similarly if the heavy metal ions interact with native proteins, it may denaturate and change their structures. Since heavy metals change the protein structure hence it does not function properly and may cause toxicity to that particular cell.

They also check uptake mechanism of both cations (k^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Fe^{3+}) and anions (NO_3^-) by affecting the absorption of other ions via diverse mechanisms.Their relative inputs differ in various cases, therefore we observe variations within different plant species. The two well-known mechanisms involved in the decrease of macro- and micronutrient uptake by heavy metals are; physical and chemical mechanism depending on the size of metal ion radii such as competition between Cd^{2+} and Zn^{2+} and Cd^{2+} and Ca^{2+}, and metal-induced disorder in the cell metabolism leading to the changes in the membrane enzyme activity and membrane structure. For example, Cd^{2+} drastically changes the lipid composition of membranes and increases the contents of palmitic as well as linoleic and linolenic acids, but all classes of lipids decrease (Ouariti et al. 1997a). The overall changes in membrane permeability and inhibition of membrane enzyme could shift the ionic balance in cytoplasm.In the same way uptake of nitrate declines, when exposed to the heavy metals, resulting in lower nitrate reductase activity and disturbed nitrogen metabolism (Burzynski and Grabowski 1984; Hernandez et al. 1996; Ouariti et al. 1997b). Notable changes in ionic balances are observed in various plant species and their tissues.

It has also been reported that under heavy metal stress conditions transpiration rate and water content in treated plants declines. This process involves various mechanisms (Fig. 2.1) such as; reduction in the area of leaves due to growth retardation, smaller guard cells, decrease in the contents of the compounds maintaining cell turgor and cell wall plasticity thus leading to growth inhibition, increase in the

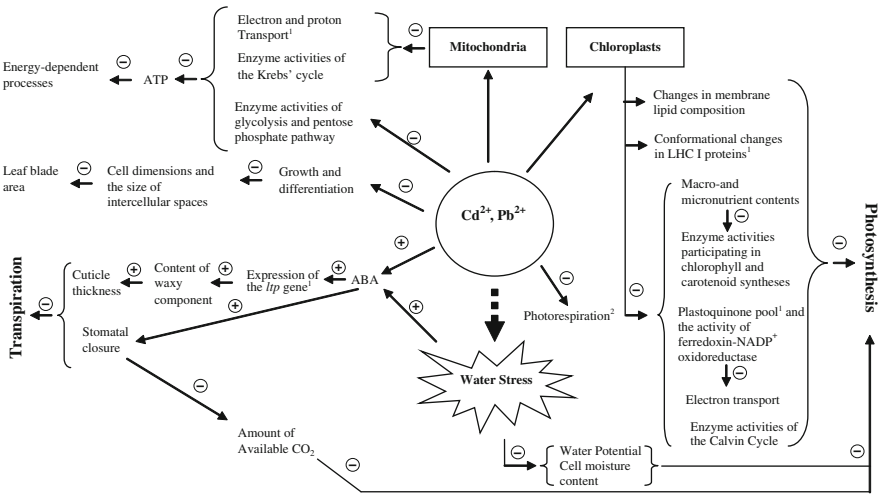

Fig. 2.1 Effects of Cd and Pb on photosynthesis, respiration and water uptake. Regime 1-concern only Cd; 2-concern only Pb; (−) Decrease, Inhibition; (+) Increase, Activation

Abscisic acid (ABA) content thus inducing stomatal closure, disordered respiration and oxidative phosphorylation which cause a disarray in the plant water regime. During the effects on the ABA metabolism, Cd^{2+} promotes the expression of *ltp* gene in the epidermis encoding the proteins for nonspecific lipid transfer. The latter effect leads to the accumulation of monomers arriving at the site of cutin synthesis and increase in the cuticle thickness, thus hindering transpiration (Hollenbach et al. 1997). Moreover, the water stress induced by heavy metals promotes superproduction of proline, an osmoregulating antioxidant and stress-protecting substance (Kuznetsov and Shevyakova 1999).

At a concentration of about 1 m*M*, Cd^{2+} reduces oxygen consumption by roots and tobacco cell-suspension culture. Dithiothreitol, a SH-agent, alleviated Cd^{2+} exerted inhibition of mitochondrial respiration and restrained their swelling. Presumably this heavy metal inhibits the transport of electrons and protons in the mitochondria and thus disorganizes the electron transport chain and remarkably affecting ATP formation. Using the labeled glucose, Reese and Roberts (1985) have demonstrated that heavy metals do not notably affect the glycolysis and the pentose phosphate pathway but considerably inhibit succinate oxidation via the Krebs cycle.

The distorted chloroplast ultrastructure generally leads to a decline of the photosynthetic rates due to restrained synthesis of chlorophyll, plastoquinone, and carotenoids; the obstructed electron transport; an inhibition in the enzyme activities of the Calvin cycle; and CO_2 deficiency due to stomatal closure (Fig. 2.1). Heavy metal ions change the lipid composition of thylakoid membranes. Lower chlorophyll content is a typical effect of Cd^{2+} and Pb^{2+}; in particular, chlorophyll *b* is more affected than chlorophyll *a*, apparently due to the inhibition of chlorophyll-synthesizing enzymes and the lack of Mg and Fe. The effect of one and the same metal concentration on chlorophyll content varies with the plant species. The inhibition of chlorophyll synthesis by heavy metals is often manifested as chlorosis. Cd^{2+} also restricts the PSII-related electron transport, probably as a result of the structural and functional changes in thylakoid membranes, the reduced ferredoxin-$NADP^+$ oxido-reductase activity, and arrested plastoquinone synthesis.

Heavy metals produce chromosomal aberrations as well as mitotic disarrays, such as C-mitoses, resulting in a higher metaphase percentage, just like the weak effect of colchicine. When Wierzbicka (1994) followed C-mitoses in onion roots, the maximum percentage of C-metaphases was observed between 6 and 10.5 h of exposure, in the interval corresponding to the minimum mitotic index (MI), then the percentage of C-metaphases decreased. Thus, the highest level of C-metaphases is correlated with the drop in MI. The lower numbers of prophases and telophases and higher number of metaphase can be correlated with the longer mitosis.

The inhibition of cell division by heavy metals may involve different mechanisms. It is not yet clear whether or not the latter include the direct metal-DNA interactions. Though the possibility of direct interaction between metal ion and DNA has been demonstrated experimentally (Alex and Dupois 1989), it is not clear whether such ions at low concentrations can reach the nucleus. Moreover, mitoses may be affected by interactions of metals with SH-group of proteins, disruption of cell metabolism and GA functions, etc.

Diverse mechanisms are involved in a decline in the rates of cell division and elongation in the roots affected by heavy metals. These mechanisms include direct binding to DNA, metal-induced aberrations, expansion of the mitotic cycle, inhibition of microtubule development, decrease in cell wall plasticity, and reduction of the glutathione pool (Fig. 2.2). Many substances inhibit cell division and elongation, and, in this case, the two processes do not considerably differ in their sensitivity towards the inhibitory agent. The toxic effects of Cd and Pb on cell division and elongation are typical of other metals, while the alternative stress factors produce other mitotic disorders. The specific responses to heavy metals in diverse plant tissues and species depend on the extent of disorder and the capacity to synthesize metal-binding chemicals and in this way to eliminate the absorbed heavy metals from the active metabolism.

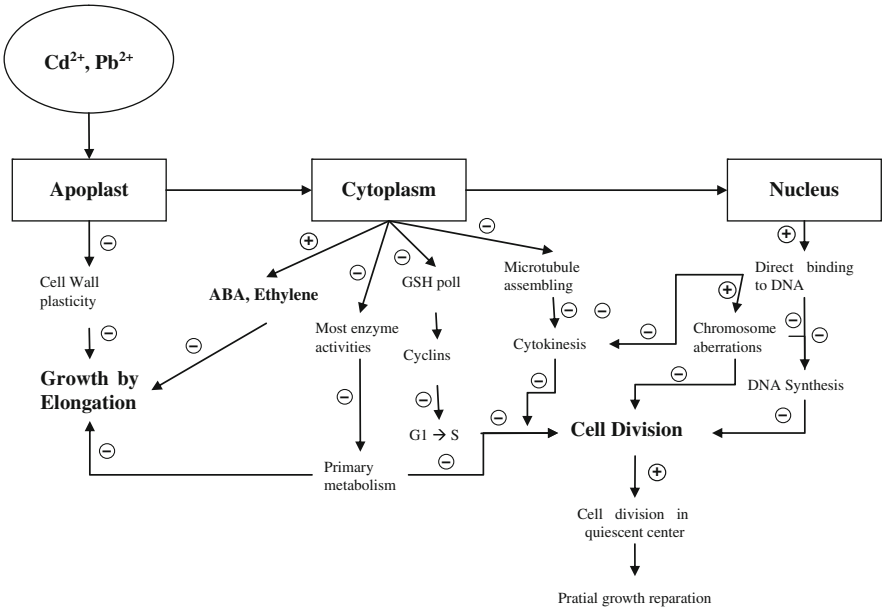

Fig. 2.2 Distribution of Cd and Pb on cell division and elongation

The intoxication with pollutant metals induces oxidative stress because they are involved in several different types of ROS-generating mechanisms. ROS intermediates are partially reduced form of atmospheric oxygen (O_2). Superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) or a hydroxyl radical ($HO^{\bullet}$). These radicals occur transiently in aerobic organisms because they are also generated in plant cells during normal metabolic processes, such as respiration and photosynthesis. Although some of them may function as important signaling molecules that alter gene expression and modulate the activity of specific defense proteins, they can be extremely harmful to organisms at high concentrations. These can oxidize

proteins, lipids, and nucleic acids, often leading to alterations in cell structure and mutagenesis. There are many potential sources of ROS in plants, in addition to those that come from reactions involved in normal metabolism, such as photosynthesis and respiration. The balance between the steady-state levels of different ROS are determined by the interplay between different ROS-producing and ROS-scavenging mechanisms. A variety of proteins function as scavengers of superoxide and hydrogen peroxide. These include, among others, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX), glutathione reductase (GR), thioredoxin, and the peroxiredoxin family of protein. These protein antioxidants are supplemented with a host of non-protein scavengers, including, but not limited to, intracellular ascorbate and glutathione. The intoxication with some heavy metals induces oxidative stress because they are involved in several different types of ROS-generating mechanisms.

3 Heavy Metal Tolerance

The tolerance to high levels of heavy metals depends on two mechanisms; the reduced uptake of heavy metals and increased plant internal sequestration.

Primary heavy metal ions enter plants from soil via the root system. At the root surface, heavy metal ions bind with the carboxyl group of mucilage uronic acid, which is found at the covering of root system, but the ability of mucilage to bind heavy metals differs for different metals.

The entrance through leaves is little and is related to the leaf morphology e.g., downy leaves absorb the heavy metals better from atmosphere. The uptake rate depends on the pH of soil solution, organic matter content and concentrations of other ions in the soil. At higher pH value, the solubility of many metal salts in soil solution declines due to the formation of less soluble compounds, as a result their biological availability in the soil decreases. Adding synthetic chelating agents, such as EGTA and EDTA to the soil polluted with heavy metals enhances the uptake and this characteristic can be used for cleansing the soils polluted with heavy metals. In addition to this other ions present in the soil solution considerably affect the uptake of heavy metals by various plant tissues. There is no particular mechanism known, probably other ions present in the soil solution interact and compete with each other thus leading to less biological availability of metal ions and reduction in their uptake. In the increased plant internal sequestration mechanism plant is manifested by interaction between a genotype and its environment (Hall 2002). Because some plants possess a range of potential mechanisms that may be involved in the detoxification of heavy metals, they are tolerant to metal stress. These mechanisms involve; binding to cell wall, reduced uptake or efflux pumping of metals at the plasma membrane, chelation of metals in the cytosol by various ligands such as phytochelatins, metallothioneins, and metal-binding proteins, repair of stress damaged proteins and compartmentation of metals in the vacuole by tonoplast-located transporters.

4 Localization and Distribution of Heavy Metals and Their Transport in the Plants

On the basis of accumulation of heavy metals, plants are divided into three main types;

(i) *accumulator plants* which accumulate amass metals primarily in shoots;
(ii) *indicator plants* which accumulate metal concentrations in different plant tissues corresponding to high or low concentrations in the environment and
(iii) *excluder plants* which maintain low metal concentrations in their shoots even if the external metal concentration in the environment is high.

Generally the heavy metal content in various plant organs decreases in the following sequence; root → leaves → stems → inflorescence → seed. However, this order sometimes varies with plant species. Roots usually manifest the maximum content of heavy metals. Leaves vary with age in their ability to accumulate heavy metals, some heavy metals accumulated preferable in the youngest leaves of plant. Whereas in other maximum content is found in senescing leaves.

The seed coat presents the first barrier for heavy metal absorption by germinating seeds. Obroucheva et al. (1998) has reported that some heavy metals enter the seed coat and are mainly found in the cell wall of seed coat. Heavy metals did not enter the embryos, even at lethal concentrations. When roots absorb heavy metals, they accumulate primarily in rhizodermis and cortex (Table 2.2) with few exceptions, where accumulation occurs in the endodermis cell wall (Lane and Martin 1977). Notable amount of heavy metals has been found in the root hair, however, it is

Table 2.2 Distribution of Pb in different tissues/zones of root

	Zea mays (CCD)	
Tissue	LD_{50}	LD
Seed coat	+	+
Rhizodermis	+	+
Root hairs	+	+
Cortex	+	+
Endodermis	−	+
Stelar parenchyma	−	+
Xylum parenchyma	+	+
Xylum	−	+
Quiescent centre	−	+
Root cap	+	+

Note: Distribution of Cd in root tissue of *Zea mays* plant. LD-Lethal dose (10^{-2} m*M* $Cd(NO_3)_2$), LD_{50} − the concentration producing 50% root growth inhibition following 48 h incubation (10^{-3} m*M* $Cd(NO_3)_2$. (+) indicates the high metal concentrations and (−) low metal concentrations

uncertain if this accumulation is important for uptake. The multilayer cortex seems to reduce the toxic effects of metal ions on other tissues by binding most of these in the cell wall thereby serving as the second barrier and defending plants from the toxic effects of heavy metals. In most cases (except radish) the heavy metal content in the endodermis are lower than in the cortex, and sometimes they are absent from cortex. There are differing reports in this connection because endodermal ultrastructure varies with plant species, and different concentrations of heavy metals are used for root incubation. At high external concentrations, the heavy metal content is practically the same in the endodermis and cortex. In the site where the lateral roots break through the endodermis heavy metals enter the stele more rapidly. At sub-lethal concentrations heavy metals are not found in the stellar parenchyma. However, in some plants these have been reported from apoplast of basal root region and sometimes even in vacuoles of root stellar cells. At the levels approaching the lethal concentrations (particularly Cd) they pass over through the cortex and endodermis and are found in considerable amounts in the cells of vascular cylinder.

The penetration of heavy metals in root meristem has not been studied sufficiently. However, detailed evidence comes from Wierzbicka (1987), who investigated the Pb uptake in onion roots. Pb has been observed in the external layer of root cap cells within several minutes and penetrated the two cell layer of root cap and two layers of the protodermis cells in the following 5 to 10 min. It reached the root cortex within 1 h and was evenly distributed in the cell walls of six external cell layers, and in the seventh to tenth layer, Pb was found only in the anticlinal cell walls. The total amount in the root constantly increased and following 70–85 min incubation the label was found in all cell layers including procambium though the levels in the procambium were the lowest. It was almost totally absent from the quiescent center region, due to some peculiar characteristics of cell wall and plasmalemma in these cells.

Very little is known about heavy metal distribution in stem and leaf tissues in the plants grown in metal solutions. Most of the metals are localized in the rhizodermis and cortex and do not cross the endodermal barrier at sub-lethal concentrations. At lethal concentrations endodermal barrier is broken, and a flux of heavy metals enters the stellar tissues. Root surface tissues are the barriers preventing the uptake into the root. The study of the endodermal barrier restricting the transport helps us to understand the mechanism of plant resistance. Heavy metals use several routes to reach the shoot. Their uneven distribution in plant tissues depends on transport mechanism.

Various methodologies such as X-ray microanalysis, electron microscopy, histochemistry and autoradiography have been used to study the intracellular localization. In many plants species, decreasing series of heavy metal concentrations in a cell follows the pattern as; cell wall → vacuoles → golgi apparatus → endoplasmic reticulum → nucleus. Cell wall of monocot and dicot plants is made of pectin and hemicelluloses; with varying contents in different plant species and tissues; which affect the cation-binding capacity. The strength of bonds between metals and the particular component of cell wall varies with the values of stability constant, which is measured in terms of log k. Cell wall works as barrier and prevents the transport

of heavy metals into the cytoplasm. In the sensitive plants exposed to heavy metals, the cytoplasm gets disorganized since heavy metals entry the cytoplasm.

A considerable portion of metals stays as globular aggregates at the external plasmalemma surface and thus is excluded from cytoplasm. However, some ions enter the cytoplasm by penetrating the plasmalemma. The mechanism of this penetration has not been studied at length. Apparently some ions enter the cell via passive transport, the active transport also may be employed by the uptake system. Most metal ions accumulate in the vacuoles within the cell and together with the cell wall and vacuole comprise up to 96% of the absorbed metals. They are deposited in vacuole in the form of complexes and salts, a large-scale elimination from cytoplasm occurs which works as a mechanism of metal detoxification.

The elucidation of mechanism of transporting the absorbed heavy metal to the vacuole is very important for understanding mechanism of plant tolerance. The steps of mechanism are as follows:

- heavy metals ions may enter from the external solution to ER immediately connected to apoplast,
- accumulation of the compounds of high affinity for heavy metals; such as organic acids and compounds that form low soluble complexes with heavy metals; in the vacuole results in their deposition in form of complexes and salts,
- metal sequestration may depend on synthesis of phytochelatins in the cytoplasm, which bind these into lasting compounds.

The fact that heavy metals are found in golgi apparatus and endoplasmic reticulum is possibly related with the metal secretion through the cell surface and into vacuole. A small quantity of heavy metals is reported to reach nuclei, chloroplast and mitochondria.

The binding of metals to apoplastic proteins has not been studied sufficiently. It has been reported that Cd enhances the protein content in barley apoplast (Blinda et al. 1997), but the role of these proteins is not known. Probably they promote callose and suberin deposition that prevents the uptake of heavy metals.

4.1 Amino Acids and Organic Acids

Plants produce a range of ligands for Cd, Cu, Ni, and Zn. Carboxylic acids and amino acids, such as citric acid, malic acid, and histidine (His), are potential ligands for heavy metals and, so, could play a role in tolerance and detoxification (Rauser 1999; Clemens 2001; Hall 2002).The Cd-and Zn-citrate complexes are prevalent in leaves, even though malate is more abundant. In the xylem sap moving from roots to leaves, citrate, and His are the principal ligands for Cu, Ni, and Zn. Recently, Salt et al. (1999) identified putative Zn-His complexes in the root of the closely related Zn hyperaccumulator *T. caerulescens*. Kramer et al. (1996) observed a 36-fold increase in the concentration of free His in the xylem exudates of the

Ni-hyperaccumulator *Alyssum montanum* and a significant linear correlation in the xylem exudate concentrations of free His and Ni in several Ni-hyperaccumulators in the genus *Alyssum*. The addition of equimolar concentrations of exogenous L-His to a Ni-amended hydroponic rooting medium enhances Ni flux into the xylem in the non-accumulator *A. montanum* and *Brassica juncea* cv. *vitasso*. In *B. juncea*, reducing the entry of L-His into the root by supplying D-His instead of L-His or L-His in the presence of a 10-fold excess of L-alanine did not affect root Ni uptake, but reduced Ni release into the xylem., The root His concentrations are constitutively 4.4-fold higher in the hyperaccumulator *A. lesbiacum* as compared to *B. juncea* and did not increase within 9 h of exposure to Ni (Kerkeb and Kramer 2003). However, no increase has been observed in the concentration of free His in root, shoot, or xylem sap in *Thlaspi goesingense* in response to Ni exposure (Persans et al. 1999).

4.2 Phytochelatins (PCs)

Murasugi and his group in 1981 first discovered the peptides in Cd-binding complexes produced in fission yeast, *Schizosaccharomyces pombe* exposed to Cd^{2+} ions and named these as cadystins (Murasugi et al. 1981). Cadystins are of two type cadystin A and B. These cadystins were latter named as phytochelatins. Phytochelatins are capable of binding various metals including Cd, Cu, Zn, As, Cd or metalloid As. The structure of PCs is (γ Glu-Cys)nX in which X is Gly, γ-Ala, Ser or Glu and n = 2−11 depending on the organism. Many plants cope with the higher levels of heavy metals by binding them in complexes with PCs and sequestering the complexes inside their cells. Biosynthesis of PCs from their common precursors; glutamine (Glu, E), cysteine (Cys, C) and glycine (Gly, E) is presented in Fig. 2.3. The pathway is completely overlapped with that of GSH biosynthesis because PCs are synthesized from GSH as a direct substrate. The biosynthesis of GSH consists of two sequential reactions mediated by γ-glutamyl-cyst synthatase (γECS) and both reactions require ATP as substrate. The γEC synthetase activity is induced by many metals, including Cd, Hg, Cu, Ni, Pb, As and Zn, however, Cd is by for the strongest inducer. Also, the activity of γEC synthase is inhibited by treatment with buthionine sulfoximine (BSO). PC synthase mediates the synthesis of PCs from GSH. This enzyme is constitutively expressed but requires metal inducers. Cd^{2+} ions are most efficient activators among the metal ions. There

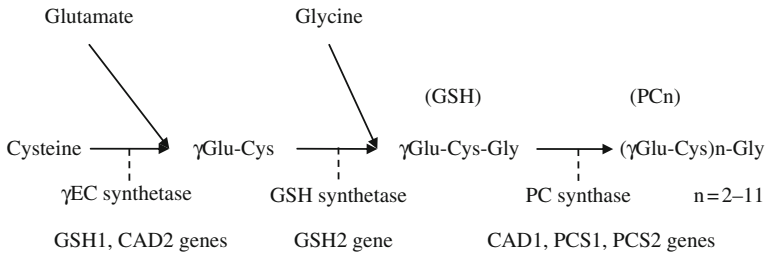

Fig. 2.3 Biosynthesis of PCs in higher plants

are genes encoding the key enzymes for PC biosynthesis (Fig. 2.3). More recently, PC synthase gene (*PCS1*, *CAD1*) has been isolated from *A. thaliana* (Ha et al. 1999). This gene may be more widespread and have more general functions. PCs are also reported to be involved in the homeostasis of Zn^{2+} and Cu^{+}/Cu^{2+} by providing a transient storage form for the ions (Grill et al. 1988; Thumann et al. 1991). The induction of PCs by the anion arsenate has been observed in a survey for peptide-inducing metal ions (Grill et al. 1987) and suggests a unique mode of PC synthase activation. However, Maitani et al. (1996) failed to demonstrate an As-PC complex. This result indicates that PCs do not fulfill a detoxifying function during As poisoning. Raab et al. (2004) have developed a method to ascertain the nature of As-PC complexes in extracts of the As-tolerant grass *Holcus lonatus* and the As hyperaccumulator *Pteris cretica* using parallel metal (loid)-specific (inductively coupled plasma-mass spectrometry) and organic-specfic (electrospray ionization-mass spectrometry) detection systems. In *H. lanatus*, the As(III)-PC3 complex was the dominant complex, although GSH, PC2, and PC3 were found in the extract. *P. cretica* only synthesizes PC2 and forms dominantly the GSH-AS(III)-PC2 complex. In both plant species, As is dominantly in non-bound inorganic forms, with 13% being present in PC complexes for *H. lanatus* and 1% in *P. cretica* (Raab et al. 2004).

4.3 Metallothioneins (MTs)

Detoxification of metals by the formation of complexes is used by most of the eukaryotes. Metallothioneins (MTs) are low molecular weight (6–7 kDa), cysteine,-rich proteins found in animals, higher plants, eukaryotic microorganisms, and some prokaryotes (Kagi 1991). The biosynthesis of MTs is regulated at the transcriptional level and is induced by several factors, such as hormones, cytotoxic agents, and metals, including Cd, Zn, Hg, Cu, Au, Ag, Co, Ni, and Bi (Kagi 1991).They are divided into Class I,Class II and Class III MTs on the basis of their cystein content and structure. Class I contain 20 highly conserved Cys residues based on mammalian MTs and are widespread in vertebrates, whereas Class II are without this strict arrangement of cysteines and are mainly found in plants and fungi. Class III are found in a few higher plants and are also low molecular weight proteins with high cysteine content, but the cysteine distribution is different than mammalian MTs. Although it is believed that MTs could play a role in metal metabolism, their role in plants remains to be determined owing to a lack of information, and their precise function is not clear (Hall 2002).

4.4 Heat Shock Proteins (HSPs)

HSPs characteristically show increased expression in response to the growth of a variety of organisms at temperatures above their optimal growth temperature. They are found in all groups of living organisms and are classified according to their

molecular size. HSPs are now known to be expressed in response to a variety of stress conditions, including heavy metal stresses (Vierling 1991; Lewis et al. 1999). They act as molecular chaperones in normal protein folding and assembly, but may also function in the protection and repair of proteins under stress conditions. Presently only a couple of reports of increased HSP expression in plants in response to heavy metal stress are available. Neumann et al. (1995) observed that HSP17 is expressed in the roots of *Armeria maritima* plants grown on Cu-rich soils. It was also reported that a short heat stress given prior to heavy metal stress induces a tolerance effect by preventing membrane damage. Clearly, more molecular evidence is required to support such an important repair or protective role.

4.5 Other Metal-Binding Proteins

Metal-binding proteins and peptides in plants can enhance metal tolerance or accumulation.These metal-binding peptides or proteins should be preferentially metal specific so that only toxic metals like Cd, Hg, and Pb are sequestered rather than essential ones like Zn and Cu. Ryu et al. (2003) isolated and characterized a novel Cu-binding protein (BP) in the Asian periwinkle *Littorina brevicula*, which is highly resistant to a wide range of heavy metal concentrations and has its metal-binding protein(s) induced in the presence of Cd. They found that Cu-BP contained an equal amount of Zn in non-exposed physiological conditions following purification by Sephacryl S-100 chromatography. However, Zn is replaced by Cu at the binding site upon the addition of excess Cu (100 μmol L^{-1} $CuCl_2$) to the cytosol or after a long period (60 d) of exposure of plants to the metal ion (150 μg L^{-1} $CuCl_2$). The molecular weight of the purified protein was determined as 11.38 kDa using MALDI-TOF MS analyses. This Cu-BP is distinct from common mollusc MT in that it contains a significantly lower number of Cys (eight residues) and high levels of the aromatic amino acids (Tyr and Phe). In addition, the protein contains His and Met, which are absent in the MT-like Cd-BP of *L. brevicula*. The Cu-BP of *L. brevicula* functions in the regulation of Zn as well as Cu, which is an essential component of hemocyanin under physiological conditions. This protein is possibly involved in the detoxification mechanism against a heavy burden of Cu (Table 2.3).

Table 2.3 Peptides/proteins contributing to heavy metal tolerance and accumulation

Peptides and proteins	Related heavy metals
Phytochelatins	Cd, Zn, Hg, Cu, Ag, Ni, Au, Pb, As
Metallothioneins	Cd, Zn, Hg, Cu, Ag, Ni, Au, Pb, As
Heat shock proteins	Cu
Cpx-type heavy metal ATPases	Cu, Zn, Cd, Pb
Nramp	Cd
CDF family proteins	Zn, C, Cd
ZIP family	Cd, Zn, Mn
Metal-binding protein	Zn, Cu, Cd

5 Molecular Mechanism of Heavy Metal Accumulation in Plants

Metal cation homeostasis is essential for plant nutrition and resistance to toxic heavy metals. Therefore, heavy metal transport is a very exciting and developing field in plant biology. Although there is no direct evidence for a role for plasma membrane efflux transporters in heavy metal tolerance in plants, recent investigations have revealed that plants possess several classes of metal transporters that may be involved in metal uptake and homeostasis in general and, thus, could play a key role in tolerance (Table 2.4). These include heavy metal (or CPx-type) ATPases, the natural resistance-associated macrophage (Nramp) family of proteins, cation diffusion facilitator (CDF) family of proteins and the zinc-iron permease (ZIP) family. Of course, many plant metal transporters remain to be identified at the molecular level.

Table 2.4 Genes isolated and introduced into plants with increased heavy metal resistance and uptake

Genes	Plants	Related heavy metal	References
AtNramps	*Arabidopsis*	Cd	Thomine et al. (2000)
A library enriched in Cd-induced DNAs	*Datura innoxia*	Cd	Louie et al. (2003)
At Pcrs	*Arabidopsis*	Cd	Song et al. (2004)
CAD1	*Arabidopsis*	Cd	Ha et al. (1999)
gshI and *gshII*	*Brassica juncea*	Cd	Zhu et al. (1999a)
PCS cDNA clone	*B. juncea*	Cd	Heiss et al. (2003)

The CPx-type heavy metal ATPases have been identified in a wide range of organisms and have been implicated in the transport of essential, as well as potentially toxic metals like Cu, Zn, Cd, and Pb across cell membranes. Responsive-to-antagonist 1 (RNA1), a functional Cpx-ATPase, plays a key role in the operation of the ethylene signaling pathway in plants. Hirayama et al. (1999) identified an *Arabidopsis* mutant RNA1 that shows ethylene phenotypes in response to treatment with trans-cyclooctene, a potent receptor antagonist. Genetic epistasis studies revealed an early requirement for RNA1 in the ethylene pathway. Functional evidence from yeast complementation studies suggestes that RNA1 transports copper and this CPx-ATPase may have a role in delivering copper to the secretory system, which is required in the production of functional hormone receptors. The Cpx-ATPases are thought to be important not only in obtaining sufficient amounts of heavy metal ions for essential cell functions, but also preventing their accumulation at toxic levels.

The Nramp family defines a novel family of related proteins that have been implicated in the transport of divalent metal ions. Thomine et al. (2000) reported that Nramp proteins play a role in Fe and Cd uptake; interestingly, disruption of an *AtNramps 3* gene slightly increases Cd resistance, whereas over-expression results in Cd hypersensitivity in *Arabidopsis*.

The CDF proteins are a family of heavy metal transporters implicated in the transport of Zn, Cd, and Co. Certain members of the CD family are thought to function in heavy metal uptake, whereas other catalyse efflux, and some are found in the plasma membranes and others are located in the intracellular membranes. A recent study by van der Zaal et al. (1999) suggests that the protein zinc transporter of *Arabidopsis thaliana* (ZAT1) may have a role in zinc sequestration. Enhanced zinc resistance was observed in transgenic plants over-expressing ZAT1 and these plants showed an increase in the zinc content of the root under conditions of exposure to high concentrations of zinc. However, this transporter is not confined to root tissue; northern blotting analysis indicated that ZAT1 was constitutively expressed throughout the plant body and was not induced by exposure to increasing concentrations of zinc.

Up till now 15 members of the *ZIP* gene family have been identified in the *A. thaliana* genome. Various members of the Zip family are known to be able to transport iron, zinc, manganese, and cadmium. Pence et al. (2000) cloned the transporter *ZNT1*, a *ZIP* gene homolog, in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. They found that *ZNT1* mediates high-affinity Zn uptake as well as low-affinity Cd uptake. Nothern blot analysis indicated that enhanced Zn transported in *T. caerulescens* results from a constitutively high expression of *ZNT1* in the root and shoots. Sequence analysis of *ZNT1* revealed that it is a member of recently discovered micronutrient transport gene family, which includes the *Arabidopsis* Fe transporter *IRT1* and the *ZIP* Zn transporters (Pence et al. 2000). Assuncao et al. (2001) have cloned two *ZIP* cDNA (*ZNT1* and *ZNT2*) while working on the populations of *T. caerulescens* from different sources. They found them to be highly expressed in root tissue. The fact that down-regulation of transcript levels was not observed in response to high concentrations of zinc suggests that a constitutively high level of expression of these transporters may be a distinctive feature of hyperaccumulator plants. Lombi et al. (2002) have also cloned an ortholog of the *A. thaliana* iron transporter IRT1 from *T. caerulescens*, which also belongs to the *ZIP* gene family. Many plant metal transporters remain to be identified at the molecular level and the transport function, specificity, and cellular location of most of these proteins in plants remains unknown.

The two primary strategies used to isolate and identify genes contributing towards heavy metal resistance in plants have been functional complementation of yeast mutants defective in metal ion transport with plant cDNA expression libraries and the identification of putative transporters by virtue of sequence similarities with databases of plant cDNA and genomic sequences that have determined.

Up till now, a few genes that contribute to Cd resistance in plants have been identified. Thomine et al. (2000) isolated *AtNramp* cDNAs from *Arabidopsis* and observed that these genes complement the phenotype of the metal uptake-deficient yeast strain *smfl*. The *AtNramps* show homology to the *Nramp* gene family in bacteria, yeast, plants, and animals. Expression of *AtNramp* cDNAs increases Cd^{2+} sensitivity and Cd^{2+} accumulation in yeast. In *Arabidopsis*, *AtNramps* are expressed in both roots and aerial parts under metal replete conditions. The results of Thomine et al. (2000) show that *Nramp* genes in plants encode metal transporters

and that *AtNramps* transport both the nutrient metal Fe and the toxic metal Cd. Two differential screening steps have been used to screen the Cd-induced library, resulting in eight putative Cd-specific cDNAs of a pool of 94 clones. Reverse transcription-polymerase chain reaction (RT-PCR) was used to confirm that four of these eight clones were Cd specific. One of the four Cd-specific cDNAs had homology to a sulfur transferase family protein in *A. thaliana*. Song et al. (2004) screened an *Arabidopsis* cDNA library using yeast (*Saccharomyces cerevisiae*) expression system using the Cd(II)-sensitive yeast mutant *ycf 1* and then yielded a small Cys-rich membrane protein (*Arabidopsis* plant cadmium resistance; *AtPcrs*). Database searches revealed that there are nine close homologs in *Arabidopsis* and the homologs have also been found in other plants. Four of the five homologs tested also increased resistance to Cd(II) when expressed in *ycf 1*. It has been found that AtPcrl localizes at the plasma membrane in both yeast and *Arabidopsis*. *Arabidopsis* plants over-expressing *AtPcrl* exhibited increased Cd(II) resistance, whereas antisense plants that showed reduced *AtPcrl* expression were more sensitive to Cd(II). The over-expression of *AtPcrl* reduced Cd uptake by yeast cells and also reduced the Cd content of both yeast and *Arabidopsis* protoplasts treated with Cd. Thus, it appears that the Pcr family members may play an important role in the Cd resistance of plants (Moffat 1999).

Several investigators have isolated genes for the PC synthases, which make the metal-binding peptides when the cell is exposed to toxic metals (Moffat 1999). Ha et al. (1999) isolated the *CAD1* gene, using a positional cloning strategy, which was proposed to encode PC synthase in *Arabidopsis* and their experiments showed that expression of the CAD1 mRNA is not influenced by the presence of Cd. The position of the gene was mapped using molecular markers and a candidate gene identified from the *Arabidopsis* genome initiative genomic sequence. Zhu et al. (1999a) over-expressed the *Escherichia coli* counterparts of ECS (*gsh1*) and glutathione synthetase (*gshII*) in *Brassica juncea*, resulting in transgenic plants that accumulate more Cd than wild-type plants. Over-expression of *E. coil gshII* in the cytosol increased Cd concentrations in the shoot up to 25% and total Cd accumulation per shoot up to three-fold compared with the wild type. Moreover, Cd accumulation and tolerance was correlated with the level of *gshII* expression and Cd-treated GM plants had higher concentrations of glutathione, PC, thiolsulfur and Ca than wild-type plants. Over-expression of *E. coli gshI* in the plastids resulted in transgenic plants that, in a hydroponic system, grew better than the wild-type plants even though shoot Cd concentrations were 40–90% higher than in the wild-type plants. The over-expression of *E. coli gshI* increased the biosynthesis of glutathione (1.5- to 2.5-fold) and PCs in transgenic plants. Oven et al. (2002) isolated and functionally expressed a cDNA *GmhPCS1* encoding homophytochelatin synthase from *Glycine max*, a plant known to accumulate homophytochelatins rather than PCs upon exposure to heavy metals. The catalytic properties of *GmhPCSI* were compared with the PC synthase AtPCSl from *A. thaliana*. When assayed only in the presence of glutathione, both enzymes catalysed PC formation; *GmhPCSl* accepted homoglutathione as the sole substrate for the synthesis of homophytochelatins, whereas *AtPCS1* did not. Heiss et al. (2003) isolated a PCs cDNA clone from

B. juncea cv. *vitasso*, a candidate species for phytoremediation, and revealed a close relationship of *BjPCS1* with PCs proteins from *A. thaliana* and *T. caerulescens*.

Plant MT-like genes have been isolated from several plant species, including maize, soybean, rice, wheat, tobacco, and *Brassica napus*, but their role in metal detoxification has not yet been established. Type I MT like genes are expressed predominantly in the roots, whereas type II MT-like genes are expressed primarily in the leaves (Mejare and Bulow 2001). Transgenic plants that express MTs have been scored for enhanced Cd tolerance and Cd accumulation or modified Cd distribution. A human MT-II gene was introduced into tobacco and oilseed rape and it was found that the growth of these transgenic seedlings was unaffected up to Cd concentrations of 100 μmol L^{-1} (Misra and Gedamu 1989). The human MT-II gene and MT-II fused to the β-glucuronidase gene were expressed in tobacco under the control of the CaMV 35S promoter with a double enhancer (35S2). *In vitro* grown transgenic seedlings expressing the fusion protein accumulated 60–70% less Cd in their shoots than did control plants (Elmayan and Tepfer 1994).

Most of the work on hyperaccumulators has focused on the physiological mechanisms of metal uptake, transport, and sequestration, but relatively little is known about its genetic basis. Persistent exposure of natural populations to inadequate or toxic micronutrient availability would be expected to provoke evolutionary adaptation, providing that the appropriate genetic variation is available in the populations in question. The plant species occurring on metal-enriched soils provide striking examples of microevolutionary adaptation to toxic heavy metal availability. Most of these species are "facultative" metallophytes: they occur on both normal as well as metalliferous soil types. Well-known examples are *Festuca ovina*, *F. rubra, Agrostis capillaries, A. gigantean, A. stolonifera, A. canina, Deschampsia cespitosa, D. flexuosa, Minuartia verna, T. caerulescens, and Silene vulgaris* (Schat 1999). All these species have been shown to exhibit a very pronounced inter-population variation in the degree of heavy metal tolerance. Plants from metalliferous sites are often 5- to 50-fold more tolerant to particular heavy metals than plants from non-metalliferous sites (Schat and Ten Bookum 1992).

Genetic variations among plants in their ability to accumulate metals is of great theoretical importance because it is the raw material on which natural selection acts to influence the evolution of hyperaccumulation. Although some degree of hyperaccumulation occurs in all members of the species that can hyperaccumulate, there is evidence of quantitative genetic variation in the ability to hyperaccumulate, both between and within populations. Such variation does not appear to correlate positively with either the metal concentration in the soil or the degree of metal tolerance in the plants. The genotypic differences between populations described above are of great interest to researchers trying to understand and manipulate the genetics of hyperaccumulation. Relatively few studies have been designed to test the magnitude and genetics of within population variability. Pollard et al. (2002) have conducted a study on *T. caerulescens* from five populations representing a variety of soil types in Britain and Spain, including Zn/Pb mine soil, serpentine soils high in Ni/Co/Cr, and non-metalliferous soils. Plants grown from seeds, collected as sib families, were cultivated hydroponically in solutions of uniform metal concentration (either Zn or Ni).

Populations varied in their metal hypemccumulation when grown in the uniform hydroponic solution. An analysis of variance revealed these differences between populations to be statistically significant.

Studies using controlled crosses, inter-specific hybrids, and molecular markers are beginning to shed light on the genetic control of this variation. Macnair et al. (1999, 2000) has proved that it is possible to generate F1, hybrids between *A. halleri* and the non-accumulator *A. petraea* (L.) Lam., which can then be back-crossed with the parental species to make an F2 array. The F2 population is highly variable, including individuals that accumulated as little Zn as *A. petrae,* individuals that accumulated as much as *A. halleri*, and a range of intermediates. The segregation of tolerance to Cu, Zn, and Cd in these crosses appeared to be governed largely by either one major gene or two additive genes, depending on the level of tolerance of the tolerant parent (Schat 1999). In general, the inheritance of adaptive high-level metal tolerance appears to be governed by a single major gene in other metallophyte species as well. The F2 crosses between equally tolerant plants from different geographically isolated mines do not segregate. No more than two loci for Cu tolerance, two for Zn tolerance, and one or two for Cd tolerance have been found among plants from a total of four Cu-tolerant, five Zn-tolerant, and three Cd-tolerant isolated *Silene vulgaris* mine populations (Schat et al. 1996; Schat 1999).

6 Conclusion

As molecular physiology provides greater insights into the specific genes that control metal accumulation, we may learn more about the genetic and regulatory factors that influence variable expression of the hyperaccumulating phenotype.

The application of powerful genetic and molecular techniques may surely identify a range of gene families that are likely to be involved in transition metal transport. Considerable progress has been made recently in identifying plant genes encoding metal ion transporters and their homologs in hyperaccumulator plants. Therefore, it is hoped that genetic engineering may offer a powerful new means by which to improve the capacity of plants to remediate environmental pollutants.

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Chapter 3
Biomonitoring of Heavy Metal Pollution Using Lichen (*Pseudevernia furfuracea* (L.) Zopf.) Exposed in Bags in a Semi-arid Region, Turkey

Ahmet Aksoy, Zeliha Leblebici, and M. Gökhan Halici

Abstract In this study, the lichen *Pseudevernia furfuracea* (L.) Zopf. samples were collected from Çat Forests near the village of Sızır in Sivas province and exposed in bags in 29 different sites of Kayseri city, Turkey. The elements Pb, Cd, Cu, Zn, Cr, and Co were analysed by ICP-OES in the lichen samples. Lichen bags were exposed for two periods (dry and wet) starting from the beginning of July 2005. In the wet period, ıt was observed that the lichen accumulated a larger quantity of metals. The contents of heavy metals in lichen samples were found to be in range of 0.16–0.31 $\mu g\ g^{-1}$, 9.50–18.89 $\mu g\ g^{-1}$, 23.50–68.24 $\mu g\ g^{-1}$, 3.10–30.81 $\mu g\ g^{-1}$, 0.07–2.54 $\mu g\ g^{-1}$, and 3.33–5.63 $\mu g\ g^{-1}$ for Cd, Cu, Zn, Pb, Cr, and Co, respectively. *Pseudevernia furfuracea* has been found to be a useful biomonitor of the six heavy metals studied because of greater lichen resistance to the dry and stressing conditions of urban environments.

Keywords Accumulation · Lichen · Heavy metal · ICP-OES · Kayseri

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1 Introduction

Monitoring trace metal deposition using lichen bags is inexpensive, independent of power supply, and can provide information on the bioavailability of persistent atmospheric pollutants and their biological effects (Bargagli 1998; Brown 1984; Carreras and Pignata 2002; Castello 1996; Figueira et al. 2002). In the last 30 years, plant leaves, lichens, and mosses have been increasingly used for assessing the atmospheric deposition of trace elements and/or biological effects of airborne pollutants (Bargagli 1998; Figueira et al. 2002; Aksoy and Öztürk 1996; Aksoy and Öztürk 1997; Aksoy et al. 1999).

Knowledge of the uptake and accumulation processes of airborne pollutants, their persistence in moss and lichen bags, and possible synergistic and/or antagonistic effects of climatic and environmental factors is scant. The relationship between concentrations in atmospheric deposition and those in lichen and moss bags is also poorly investigated.

In fact, a purely instrumental approach to pollution monitoring has several weak points: despite the precision of measurement, recording gauges do not give information either on the bioavailability of pollutants or on their biological effects, and pollutants occurring at very low concentrations, such as trace elements, are often neglected. This can lead to gross underestimation of possible health effects, as some metals have synergistic toxicity and a hazard may exist even under low-dose exposure conditions. In urban areas, where lichens are often scarce or even absent, the "bags technique" has been set up and developed in order to monitor city air pollution. Bags consist of a mesh or grid, generally made of nylon, containing water-washed lichens. This technique has the following advantages: uniformity of entrapment surface and exposure period, flexibility both in site selection and in the number of stations that can be chosen, known original concentrations of contaminants in the biomonitors and greater collection efficiency for most elements. In addition, bags eliminate the possibility of contamination via root uptake and, in comparison with dust fall jars or bulk samplers, offer lower cost and higher efficiency. The major limitation of the method is in the unknown collection efficiency for different contaminants. Thus, the measured metal concentrations might reflect relative rates of deposition and not the total atmospheric load of contaminants. The duration of exposure is another critical aspect of biomonitoring by bags. Biomonitors may reach a saturation point for the uptake of an element and biomonitoring performance may also be altered by climatic and environmental conditions (Bargagli 1998). Compared with instrumental monitoring, concentrations of trace elements in the thallus are easily quantifiable with common analytical procedures and are related to those in wet and dry atmospheric depositions. The use of biomonitors is found to provide a high density of sampling points, which

is indispensable for drawing reliable maps of pollutant depositions, and for giving information on long-term pollution effects (Bargagli et al. 2002).

Plasma Optical Emission Spectrometer (Inductively Coupled Plasma Optical Emmision Spectrometry = ICP-OES) is suitable for heavy metal determination and it is preferred by many research centres (Lara et al. 2001; D'angelo 2001).

In 2005, a bioaccumulation study of trace elements was carried out in the Kayseri urban area in Turkey using the lichen *Pseudevernia furfuracea* (L.) Zopf., transplanted in 29 city sites. The sites were selected near automatic air pollution and where meteorological monitoring devices were already fixed. In this study; Pb, Cd, Cu, Zn, Cr, and Co contents in exposed bags of *P. furfuracea* were measured.

2 Material and Methods

2.1 Study Area

Kayseri is a densely populated city (1,560,432 people in 2000). In the city, there is a definite boundary which distinguishes between urban and suburban sites. Urban sites were chosen at least 10 m away from a main road, and urban roadside sites were selected mainly near the city center along main roads. All urban roadside samples were chosen between 0 and 5 m, usually not more than 2 m away from the main road. Urban park sites were chosen from five large parks in Kayseri, mainly near the roads where the traffic density is not so high. Industry sites were chosen from the industrial area of the city. Shanty sites were chosen from five shanty zones around the city and control sites were chosen south of the Kayseri and more than 10 km away from any source of pollution. The city is crossed every day by an average of 162,000 vehicles driving through the city (Anonymous 2003). According to measurements from these stations, the study area has a Mediterranean climate characterised by dry summers and warm temperatures. In Kayseri, the annual rainfall is 368.4 mm and a mean annual temperature 10.6°C (Fig. 3.1). The urban area of Kayseri is affected by contamination from SO_2 and particle matter (PM) in the atmosphere (Fig. 3.2).

2.2 Lichen Sampling and Bag Preparation

Pseudevernia furfuracea was collected from Çat forests on the bark of pine trees in the rural area of Sızır in the Sivas Province (39º 24.665′ N, 35º 51.369′ E, Turkey), at nearly 1582 m above sea level, far from large urban and industrial settlements. Homogeneous specimens were made by carefully mixing the collected materials. In the laboratory, lichen samples were cleaned from soil particles and submitted to seven consecutive washings with distilled water. Spherical bags 3–4 cm in diameter were assembled using nylon mesh (10 × 10 cm wide with 1 mm 2 meshes) and closed by nylon wire. Lichen thalli (400–450 mg) were placed in each bag. This amount exceeded 100–200 mg suggested as optimal by Gailey and Lloyd (1986) in order to assure enough material for chemical analysis.

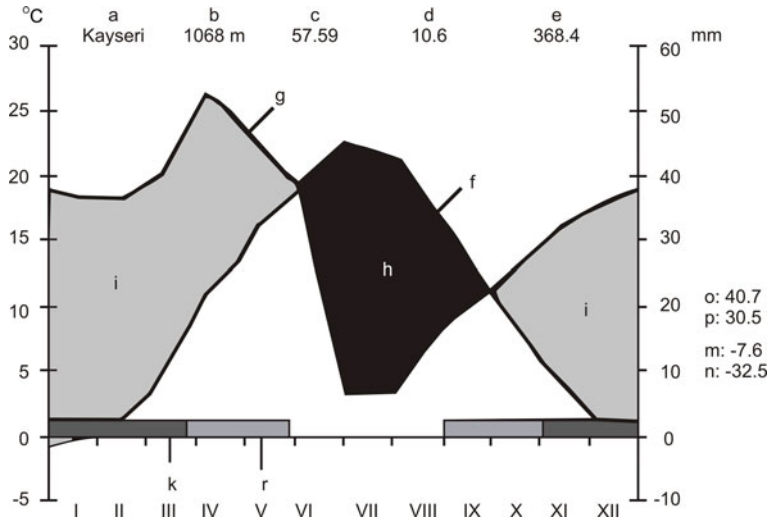

Fig. 3.1 Ombrothermic diagrams for Kayseri (Halici et al. 2005). *a* meteorological station, *b* altitude, *c* observation (years), *d* average annual temperature (°C), *e* average annual precipitation (mm), *f* temperature, *g* precipitation, ***h*** dry season, *i* precipitation season, *k* frost months, *m* average minimum temperature (°C), *n* minimum temperature (°C), *o* maximum temperature (°C), *p* average maximum temperature (°C), *r* probable frost months

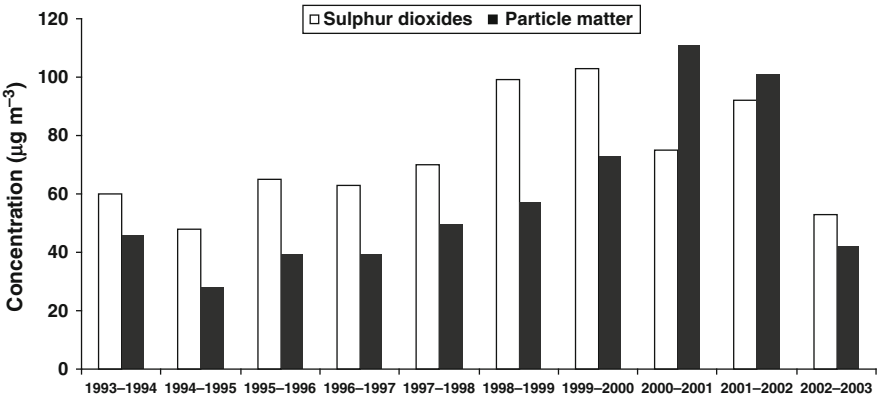

Fig. 3.2 Sulphur dioxides (SO_2) and particle matter (PM) in the atmosphere of the city of Kayseri (Anonymous 2003)

2.3 Sample Collection

At the beginnig of July 2005, lichen bags were placed in 29 urban exposure sites (Table 3.1). In order to evaulate lichen element accumulation in two different time periods, dry and wet, the bags were gathered in two moments; two bags at the end

Table 3.1 The localities where the lichen licen bags were placed

Study area of kayseri	Stations
Urban ($n = 6$); Urban sites were chosen at least 10 m away from a main road	Sivas Main Road
	Belsin Main Road
	Fevzi Çakmak Main Road
	Talas Main Road
	Osman Kavuncu Main Road
	Çevreyol Main Road
Urban park ($n = 5$); Urban park sites were chosen from five large parks in Kayseri	Kumalı Park
	İnönü Park
	Fuar Park
	Gültepe Park
	Erciyes Üniversity Park
Urban roadside ($n = 6$); Urban roadside sites were selected mainly near the city center along main roads	Ziya Gökalp
	Yeni District
	Beyazşehir
	Anayurt
	Yenişehir
	Fuzuli
Industry ($n = 5$); Industry Sites were chosen at least 5 m away from a industrial area	Organize Erkoton Industry
	Organize Yurtkan Furniture
	Organize Günka Industry
	Organize Mahya Industry
	Organize Esen Furniture
Shanty ($n = 5$);Shanty sites were chosen from five shanty zones around the city	Argıncık
	Yeşil District
	Eskişehir Vineyard
	Yıldırım Beyazıt
	Erkilet
Control ($n = 2$); More then 10 km away from any source of pollution	Hisarcık
	Ali Forest
Original Samples	Çat Forest

of the dry season (after four weeks of exposure) and the other two during the wet season (after four weeks of exposure).

2.4 Sample Preparation and Chemical Analyses

An aliquou of lichen was dried at 105°C to determine the dry weight. For the measurement of metal concentrations, 0.5 g of each homogenised sample was mineralised in a microwave oven (CEM Marsh Microwave) in Teflon vessels with 10 ml of concentrated (65%) nitric acid. The digests were diluted in double distilled water and analysed by Inductively Coupled Plasma (ICP-OES). The contents of Zn, Pb, Cd, Cu, Cr, and Co were determined. An SPSS statistical program was used to calculate all statistical analysis (ANOVA).

2.5 Results and Discussion

The mean levels of Pb, Cd, Zn, Cu, Cr and Co found in dry and wet season *Pseudevernia furfuracea* in different sites are presented in Tables 3.2 and 3.3. By comparing the Pb concentrations of lichens from studied sites with a control site, significant variations were observed (Table 3.2). The urban roadside with the highest human activities, together with high vehicular density congestion, shows the highest Pb level (30.81 $\mu g\ g^{-1}$)which is significantly higher than that of the control sites (3.10 $\mu g\ g^{-1}$). Similar kinds of observations were made by Loppi, while studying *Flavoparmelia caperata* thalli as indicators of temporal variations of air pollution in the town of Montecatini Terme, Central Italy (Loppi et al. 2004).

The significant correlations found among most of the elements considered may reflect for the related elements as common sources of emissions. The correlation between Fe, Zn, Pb, Cr, Ni, and Cu which are considered as indicative of vehicle emissions (Garty et al. 1985; Ward 1989) and partly associated with tyre and brake abrasion, is consistent with the fact that in Kayseri, urban traffic is the main form of pollution.

The mean Cd concentration in urban roadside (0.31 $\mu g\ g^{-1}$) and industry sites (0.29 $\mu g\ g^{-1}$) are slightly higher than those of the urban sites (0.17 $\mu g\ g^{-1}$), urban parks (0.21 $\mu g\ g^{-1}$), shanty sites (0.25 $\mu g\ g^{-1}$) and significantly higher than the control sites (0.09 $\mu g\ g^{-1}$) in wet season (Table 3.2). The concentrations of Cd in both wet and dry seasons are significantly higher from the urban roadside, industry site, and urban park than from the control site, probably indicating an accumulation of motor vehicles, dust raised by metal businesses, and other human activies. The most important sources that cause cadmium pollution are fossil fuels of vehicles, metal businesses, plastics, house tools construction and sewers (Markert 1993). All of the study sites are polluted by Cd except rural sites. According to Allen (1989), plants from unpolluted natural environments contain 0.01–0.3 $\mu g\ g^{-1}$ cadmium (Allen 1989).

Zinc is an essential element in plant growth and plays an important role in the biosynthesis of enzymes. Normal concentrations of Zn in plants are in the range of 10- 100 $\mu g\ g^{-1}$ (Allen 1989). The highest levels of Zn were found in industry sites (68.24 $\mu g\ g^{-1}$) and lowest at the control site, (23.50 $\mu g\ g^{-1}$) in wet season (Table 3.2). Zinc concentration in the lichen samples was linearly related to the traffic. It is reported that the most important sources that cause Zn pollution are fuels, fossil, fertilizers and metal alloys (Markert 1993). Elevated Zn levels in industry, urban roadsides, urban sites, urban parks, and shanty sites show the effect of traffic volume and tyre wear from vehicles. According to Adamo et al. (2002), concentration of Zn in lichens greater than 100 $\mu g\ g^{-1}$ (65 to 243) indicates that the environment is polluted with Zn. By following their criteria, we can say that the province of Kayseri is not polluted by Zn as its amount is far below 100 $\mu g\ g^{-1}$.

The mean Cu concentrations in industry sites (18.89 $\mu g\ g^{-1}$) and urban roadside sites (14.55 $\mu g\ g^{-1}$) are slightly higher than urban parks (13.49 $\mu g\ g^{-1}$), urban (11.24 $\mu g\ g^{-1}$), and shanty sites (11.04 $\mu g\ g^{-1}$) which in turn are higher than the control site (4.50 $\mu g\ g^{-1}$) in wet season determined using *P. furfuracea*

Table 3.2 Pb, Cd, and Zn concentrations in *Pseudevernia furfuracea* (μg g^{-1} dry weight) after dry (DS) and wet season (WS) in the survey area of Kayseri city, together with standard deviations (SD)

Elements	Pb		Cd		Zn	
Season	DS	WS	DS	WS	DS	WS
Urban ($n = 6$)	18.81 ± 1.94	22.39 ± 2.94	0.13±0.10	0.17±0.08	53.96±2.85	55.27±2.28
Urban park ($n = 5$)	19.88 ± 0.63	22.00 ± 1.37	0.12±0.04	0.21±0.06	51.34±3.49	55.09±2.74
Urban roadside ($n = 6$)	28.34 ± 4.57	30.81 ± 1.94	0.24±0.13	0.31±0.22	55.45±5.19	58.30±5.70
Industry ($n = 5$)	21.60 ± 1.81	23.87 ± 2.74	0.26±0.29	0.29 ±0.10	61.11±7.02	68.24±6.52
Shanty ($n = 5$)	19.76 ± 1.07	22.65 ± 2.62	0.09±0.05	0.25±0.08	47.18±4.69	48.76±7.97
Control ($n = 2$)	2.92 ± 1.00	3.10 ± 1.03	0.06±0.09	0.09±0.10	20.13±3.14	23.50±0.51
Original Sample ($n = 2$)	1.79 ± 0.98		0.04±0.02		17.12±0.42	

Table 3.3 Cr, Cu and Co concentrations in *Pseudevernia furfuracea* ($\mu g\ g^{-1}$ dry weight) after dry (DS) and wet season (WS) in the survey area of Kayseri city, together with standard deviations (SD)

Elements	Cr		Cu		Co	
Season	DS	WS	DS	WS	DS	WS
Urban ($n = 6$)	1.10±0.59	1.09±0.20	10.97 ± 0.96	11.24 ± 2.14	4.49±0.34	4.64±0.37
Urban park ($n = 5$)	1.24±0.34	1.35±0.23	12.58 ± 2.10	13.49 ± 0.54	4.64±0.02	4.93±0.10
Urban roadside ($n = 6$)	1.83±0.35	1.95±0.09	12.38 ± 1.90	14.55 ± 2.44	4.77±0.10	5.34±0.49
Industry ($n = 5$)	2.34±1.02	2.54±0.48	16.53 ± 8.40	18.89 ± 5.82	4.91±0.45	5.63±0.10
Shanty ($n = 5$)	1.19±0.83	1.21±0.15	10.46 ± 1.15	11.04 ± 1.35	4.38±0.04	4.49±0.32
Control ($n = 2$)	0.04±0.11	0.07±0.03	3.08 ± 1.17	4.50 ± 0.86	3.04±0.01	3.33±0.72
Original sample ($n = 2$)	0.03±0.01		1.92 ± 0.45		2.01±0.08	

(Table 3.3). It is known that, the most important sources of Cu pollution are indicated as animal fertilizers, pesticides, sewage, ashes, metal businesses, iron and steel industry (Markert 1993). High concentrations of Cu come from industry and exhausts of vehicles in the industry, urban roadside, urban park, urban and shanty sites. According to Loppi et al. (2004), the high levels of Cu contamination in *Flavoparmelia caperata* in Italy occurred mainly in study areas where traffic is high.

The higher Cr concentrations in industry sites (2.54 $\mu g\ g^{-1}$) and urban roadsides (1.95 $\mu g\ g^{-1}$) were slightly higher than the urban park (1.35 $\mu g\ g^{-1}$), urban (1.09 $\mu g\ g^{-1}$) and significantly higher than the control site (0.07 $\mu g\ g^{-1}$) in *P. furfuracea* during wet season (Table 3.3). The most important sources of Cr pollution are known to be sewage, plastics, metal business, and iron and steel industry (Markert 1993). Bennett and Wetmore (1997) investigated Cr content in four lichens in national parks which showed variation from 0.58 to 1.85 $\mu g\ g^{-1}$.

When Table 3.3 is examined for Co, the highest value is seen in industry sites (5.63 $\mu g\ g^{-1}$) in lichen samples in wet season (Table 3.3). It is reported that the most important sources that cause Co pollution is plastics (Markert 1993). High concentrations of Co come from the industry, urban roadside, urban park, urban, and shanty sites.

Analysis of variance (ANOVA) was used to determine if significant differences were present among means of wet season (WS) samples of *Pseudevernia furfuracea* (Table 3.4). According to the results there are no statistical differences of Cd contents in lichen samples.

The statistical analysis of the results show that, the highest Zn content was observed in industry sites and the lowest Zn content was observed in control sites. This stuation was normal, because the industry sites are near the industrial areas and the residues of processed mines accumulated around the factory are the source of pollution. There are no statistical significance for urban and shanty sites, and the highest Cr concentration was determined in industry sites. Furthermore, the highest Co concentrations were obtained from the industry sites and the lowest Co concentrations obtained from the control sites. Furthermore, it is observed that the differences of Co concentration for urban sites, urban parks and urban roadsides are not statistically important. Differences in Cu concentrations of the samples collected from all sites in the study area are statistically important. According to the statistical analysis of the results, the highest Pb concentration was observed in urban roadside sites, and the lowest Pb concentration in the control sites. We presume that the highest Pb concentration in urban roadside sites due to the fact that these sites are close to residential areas with high traffic activity.

Analysis of Paired-Samples T test was used to determine if significant differences were present among the means of the wet and dry seasons (Table 3.2 and 3.3). Differences of heavy metal concentrations of samples collected from industry sites which in the study area are not statisically important ($T = -1.956$; $SD = 5$; $p = 0.108$; $p > 0.05^{ns}$). In contrast, the urban roadside stations are statisically important ($T = -2.674$; $SD = 5$; $p = 0.044$; $p < 0.05^{ns}$).

Overall, the present study confirms that lichens are efficient metal accumulators and they can be effectively used in biomonitoring studies. The concentrations of six

Table 3.4 Average Pb, Cd, Zn, Cr, Cu and Co concentrations in *Pseudevernia furfuracea* (μg g^{-1} dry weight) wet season (WS) in the survey area of Kayseri city, together with standard deviations (SD)

Elements	Pb	Cd	Zn	Cr	Cu	Co
Urban ($n = 6$)	22.39 ± 2.94^{b}	0.17 ± 0.08^{a}	55.27 ± 2.28^{bc}	1.09 ± 0.20^{ab}	11.24 ± 2.14^{ab}	4.64 ± 0.37^{bc}
Urban park ($n = 5$)	22.00 ± 1.37^{b}	0.21 ± 0.06^{a}	55.09 ± 2.74^{bc}	1.35 ± 0.23^{b}	13.49 ± 0.54^{b}	4.93 ± 0.10^{bc}
Urban roadside ($n = 6$)	30.81 ± 1.94^{c}	0.31 ± 0.22^{a}	58.30 ± 5.70^{c}	1.95 ± 0.09^{c}	14.55 ± 2.44^{b}	5.34 ± 0.49^{bc}
İndustry ($n = 5$)	23.87 ± 2.79^{b}	0.29 ± 0.10^{a}	68.24 ± 6.52^{d}	2.54 ± 0.48^{d}	18.89 ± 5.82^{c}	5.63 ± 0.10^{c}
Shanty ($n = 5$)	22.65 ± 2.62^{b}	0.25 ± 0.08^{a}	48.76 ± 7.97^{b}	1.21 ± 0.15^{ab}	11.04 ± 1.35^{ab}	4.49 ± 0.32^{b}
Control ($n = 2$)	3.10 ± 1.03^{a}	0.09 ± 0.10^{a}	23.50 ± 0.51^{a}	0.07 ± 0.03^{a}	9.50 ± 0.86^{a}	3.33 ± 0.72^{a}

Different letters in the same column indicate significant differences at $P < 0.05$ (ANOVA)

elements detected in *Pseudevernia furfuracea*, after exposure in bags in the urban area of Kayseri, compared with the element concentration in the orginal and in the extraurban control site samples give a clear indication of urban air contamination by trace elements. Lichen accumulation capacity increases with wet conditions. The correlation between Pb, Zn, Cr, Cu, Cd, and Co confirm that vehicular traffic plays a prominent role in terms of air pollution in the Kayseri province.

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Chapter 4
Heavy Metal Toxicity in Plants

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Abstract Although many metal elements are essential for the growth of plants in low concentrations, their excessive amounts in soil above threshold values can result in toxicity. This detrimental effect varies with the nature of an element as well as plant species. Heavy metal toxicity in plants depends on the bioavailability of these elements in soil solution, which is a function of pH, organic matter and cation exchange capacity of the soil. Nonessential metals/metalloids such as Hg, Cd, Cr, Pb, As, and Sb are toxic both in their chemically combined or elemental forms, and plants responses to these elements vary across a broad spectrum from tolerance to toxicity. For example, the bioaccumulation of heavy metals in excessive concentrations may replace essential metals in pigments or enzymes disrupting their function and causing oxidative stress. Heavy metal toxicity hinders the growth process of the underground and aboveground plant parts and the activity of the photosynthetic apparatus, which is often correlated with progress in senescence. To avoid the toxicity, plants have developed specific mechanisms by which toxic elements are excluded, retained at root level, or transformed into physiologically tolerant forms. In this chapter, we have discussed the toxic effects of heavy metals

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on plant growth and their detoxification mechanisms that enable them to tolerate high levels of metals in the soil environment.

Keywords Heavy metal · Cadmium · Chromium · Photosynthesis · Tolerance

Contents

1 Introduction

Heavy metals are defined as the elements having density greater than 5 g cm^{-3} (Adriano 2001). Some heavy metals namely, cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni) and zinc (Zn) are considered to be essential for plants, whereas chromium (Cr), and antimony (Sb) are found essential for animals (Misra and Mani 1991; Markert 1993). These metal elements can directly influence growth, senescence and energy generating processes due to their high reactivity. Their concentration in soil beyond permissible limits is toxic to plants either causing oxidative stress through free radicals and/or disrupting the functions of enzymes by replacing essential metals and nutrients (Henry 2000; Prasad 2008). Although changes in cell metabolism permit plant to cope with, yet the reduction in plant growth is the primary symptom of metal toxicity. However, response of plants to excess of metals depends on their growth stage (Skórzyńska-Polit and Baszynski 1997). For example, Maksymiec and Baszyński (1996) reported that beans (dicotyledonous plants) and alfalfa (Peralta-Videa et al. 2004) were more resistant to heavy metals at the early growth stage. Conversely, in older plants

exposed to heavy metals the adaptation mechanisms in older plants exposed to heavy metals are not so flexible and efficient. Therefore, the toxic effects of heavy metals on the plant physiology and metabolism are much more pronounced.

Among the heavy metals, chromium and cadmium are of special concern due to their potential toxicity to both animals and plants even at low concentrations (Sharma et al. 1995; Das et al. 1997; Shukla et al. 2007). The chromium toxicity in plants varies from the inhibition of enzymatic activity to mutagenesis (Barcelo et al. 1993). The visible symptoms include leaf chlorosis, stunting, and yield reduction (Das et al. 1997; Boonyapookana et al. 2002). Cadmium (Cd) is particularly dangerous pollutant due to its high toxicity and great solubility in water (Pinto et al. 2004). Reports indicate that in some plant species Cd interacts with the absorption of metal nutrients such as Fe, Zn, Cu and Mn (Zhang et al. 2002; Wu and Zhang 2002), in addition to inducing lipid peroxidation and chlorophyll breakdown in plants, resulting in an enhanced production of reactive oxygen species (ROS) (Hegedüs et al. 2004). Cadmium also inhibits the uptake of elements such as K, Ca, Mg, Fe because it uses the same transmembrane carriers (Rivetta et al. 1997). Its accumulation in plants may also pose a serious health hazard to human beings through food chain; however, it poses an additional risk to children by direct ingestion of Cd-contaminated soil (Nordberg 2003).

2 Origin and Occurrence

Heavy metals exist in colloidal, ionic, particulate and dissolved phases. The soluble forms of metal elements are generally ions or unionized organometallic chelates. In soil, the concentrations of metals range from traces to as high as 100,000 mg kg^{-1} which depends on the location and the type of metal (Blaylock and Huang 2000). Amongst chemical elements, Cr is considered to be the seventh most abundant element on earth and constitutes 0.1 to 0.3 mg kg^{-1} of the crystal rocks (Cervantes et al. 2001). About 60–70% of its total world production is used in alloys and 15% in chemical industrial processes, mainly leather tanning, pigments, electroplating and wood preservation (McGrath 1995). Chromium has several oxidation states ranging from Cr^{2-} to Cr^{6+}; however, valences of I, II, IV and V have also been shown to exist in a number of compounds (Krishnamurthy and Wilkens 1994). Additionally, Cr(VI) is considered to be the most toxic form of chromium and is usually associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxyanions. Cr(III) is less mobile, less toxic and is mainly found bound to organic matter in soil and aquatic environments (Becquer et al. 2003). Cr occurs mostly in the form of Cr(III) in soil, and within the mineral structures in the form of mixed Cr(III) and Fe(III) oxides (Adriano 1986). Cr and $Fe(OH)_3$ is a solid phase of Cr(III) having even lower solubility than $Cr(OH)_3$ (Rai et al. 1987). Hence, in the environment total soluble Cr(III) remains within the permissible limits for drinking water for a wide range of pH (4–12) due to precipitation of (Cr, Fe) $(OH)_3$ (Rai et al. 1989; Zayed and Terry 2003). Similarly, major source of Cd is the parental material, but the anthropogenic activities have also enhanced the amount of Cd in soil (Kabata-Pendias and Pendias 2001). Heavy metals are normally present at low concentrations

in freshwaters (Le Faucheur et al. 2006), but the discharge of effluents from a wide variety of industries such as electroplating, metal finishing, leather tanning, chrome preparation, production of batteries, phosphate fertilizers, pigments, stabilizers, and alloys has impacted aquatic environments (El-Nady and Atta 1996; Booth 2005; Stephens and Calder 2005). In addition, large areas of cultivated land have also been reported to be contaminated by As and Cd due to agricultural and industrial practices (McGrath et al. 2001; Verma et al. 2007). Cadmium pollution is also given off from rubber when car tires run over streets, and after a rain, the Cd is washed into sewage systems and collected in the sludge, which could be an additional source of Cd contamination. Reports indicate that the composted sludge from Topeka, Kansas contains 4.2 mg kg^{-1} Cd (Liphadzi and Kirkham 2006).

3 Mobility, Uptake and Accumulation of Heavy Metals

Heavy metals entering our environment are transported by water and air and deposited in soil and sediments where they could be immobilized (Ozturk et al. 2008). However, the bonding process may take considerably long period of time. It has been noted that at the beginning of the binding process the bioavailable fraction of metal elements in soil is high, but decreases gradually in due course of time (Martin and Kaplan 1998).

Metal solubility and bioavailability to plant is mainly influenced by the chemical properties of soil such as, soil pH, loading rate, cation exchange capacity, redox potential, soil texture, clay content and organic matter (Williams et al. 1980; Logan and Chaney 1983; Verloo and Eeckhout 1990). Generally, higher the clay and/or organic matter and soil pH, the metals will be firmly bound to soil with longer residence time and will be less bioavailable to plants. Soil temperature as well is an important factor accounting for variations in metal accumulation by crops (Chang et al. 1987).

The bioavailability of metals is increased in soil through several means, the most indigenous being the secretion of phytosiderophores into the rhizosphere to chelate and solubilise metals that are soil bound (Kinnersely 1993). Acidification of the rhizosphere and exudation of carboxylates are considered potential means to enhancing metal accumulation. Heavy metals are captured by root cells of the plants after their mobilization in the soil, and their movement in the soil depends mainly upon: (i) diffusion of metal elements along the concentration gradient which is formed due to uptake of elements and thereby depletion of the element in the root vicinity; (ii) interception by roots, where soil volume is displaced by root volume after growing, and (iii) flow of metal elements from bulk soil solution down the water potential gradient (Marschner 1995). Cell wall behaves as an ion exchanger of comparatively low affinity and low selectivity where metals are first bound. From the cell wall, the transport systems and intracellular high-affinity binding sites mediate and drive the uptake of these metals across the plasma membrane. A strong driving force for the uptake of metal elements through secondary transporters is created due to the membrane potential, which is negative on the inside of the plasma membrane and may exceed −200 mV in root epidermal cells (Hirsch et al. 1998). However, the

uptake of some heavy metals has been reported to be passive, metabolic or partially metabolic and partially passive (Cataldo et al. 1983; Bowen 1987).

The uptake of metals, both by roots and leaves, increases with increasing metal concentration in the external medium. Nevertheless, the uptake has no linear relation with increasing concentration. This is mainly because the metals bound in the tissue cause saturation that is governed by the rate at which the metal is taken up. The uptake efficiency of metals by the plants (or accumulation factor) is highest at their low concentrations in the external medium. This is examined both in solution culture and in soil for Cd which may probably be due to low concentration of metal per unit of absorption area, resulting in low competition between ions at the uptake sites while the situation is otherwise at high concentrations (Greger et al. 1991; Greger 1997). Both essential and non-essential metals can be taken up by leaves. In the form of gases, they enter the leaves through the stomata, whereas in ionic form metals mainly enter through the leaf cuticle (Lindberg et al. 1992; Marschner 1995). Hg^o in gaseous form is taken up via stomata (Cavallini et al. 1999) and its uptake is reported to be higher in C_3 than C_4 plants (Du and Fang 1982). The uptake occurs to a high degree through ectodesmata, non-plasmatic "channels" (which are less dense parts of the cuticular layer) that are situated foremost in the epidermal cell wall/cuticular membrane system between guard cells and subsidiary cells. Furthermore, the cuticle covering guard cells are often different to that covering normal epidermal cells (Marschner 1995).

Most of the metal elements are insoluble in the vascular system of plants and unable to move freely, thus usually form sulphate, phosphate or carbonate precipitates immobilizing them in apoplastic (extracellular) and symplastic (intracellular) compartments (Raskin et al. 1997). High cation exchange capacity of cell walls further limits the apoplastic transport of metal ions unless the metal ion is transported as a non-cationic metal chelate (Raskin et al. 1997). The apoplast continuum of the root epidermis and cortex is permeable for movement of solutes. In the apoplastic pathway the water and solute particles can flow and diffuse without any cross membrane, hence the pathway remains relatively unregulated. The cell wall of the endodermal cell layer acts as a barrier for apoplastic diffusion into the vascular system.

Generally, prior to the entry of metal ions in the xylem, solutes are to be taken up by root symplasm (Tester and Leigh 2001). Metals once taken up by the root symplasm, their further movement from root to the xylem is mainly governed by three processes, including: (i) metal sequestration into the root symplasm, (ii) symplastic transport into the stele, and (iii) release of metals into the xylem. The ion transport into the xylem is generally mediated by membrane transport proteins. Metal elements which are not needed by the plants effectively compete the essential heavy metals for their transport using the same transmembrane carriers.

Cr(III) uptake by the plant is mainly a passive process, while Cr(VI) transport is mediated by sulphate carrier. However, its affinity is low (Skeffington et al. 1976). Due to this reason inhibitors like, sodium azide and dinitrophenol inhibits the uptake of Cr(VI) by barley seedlings but this does not happen in case of Cr(III) (Skeffington et al. 1976). Group VI anions (e.g., SO_4^{2-}) also inhibit the uptake of chromates whereas Ca^{2+} stimulates its transport (Shewry and Peterson 1974). This inhibition of chromate transport is due to the competitive inhibition because of the chemical similarity, while stimulated transport of Cr(VI) due to Ca is attributed to its essential

role in plants for the uptake and transport of metal elements. (Zayed and Terry 2003; Montes-Holguin et al. 2006).

There exists no correlation between Cr concentrations in plant tissues and that in soils. However, some plants like *Brassica* species show an unusual ability to take up heavy metals from root substrates and accumulation of these metals in their parts (Kumar et al. 1995). Even though it seems a common tendency of all plant species to retain Cr in their roots, but with quantitative differences (Zayed and Terry 2003). It is observed that leafy vegetables (e.g., spinach, turnip leaves) that tend to accumulate Fe appear to be the most effective for the translocation of Cr to the plant top (Cary et al. 1977). While those leafy vegetables (e.g., lettuce, cabbage) that accumulate relatively low concentrations of Fe in their leaves were considerably less effective for the translocation of Cr to their leaves. Some plant species are reported to attain substantially higher shoot/root concentration ratios than other species (Zayed and Terry 2003). However, reports show that a 'Soil–Plant Barrier' well protects the food chain from toxicity of heavy metals which implies that levels of heavy metals in edible plant tissues are reduced to levels safe for animals and humans due to one or more of the following processes: (i) prevention of uptake of metal element(s) due to its insolubility in soil, (ii) prevention of translocation of metal element(s) by making them immobile in roots or (iii) lowering the phytotoxicity of the metal element(s) to permissible level both for animals and human beings (Chaney 1980).

Some elements (e.g., B, Cd, Mn, Mo, Se, Zn) are easily absorbed and translocated within plant tissues, while others (e.g., Al, Ag, Cr, Fe, Hg, Pb) are less mobile due to their strong binding to soil components or root cell walls (Chaney 1983a, b). However, beyond certain concentrations, all of these elements are mobilized within the transport system of the plant, even against a concentration gradient. For example, kinetic data demonstrate that essential Cu^{2+}, Ni^{2+} and Zn^{2+} and nonessential Cd^{2+} compete for the same transmembrane carrier for their transport (Crowley et al. 1991). Metal chelate complexes may also be transported via specialized carriers across the plasma membrane as is the case for Fe–phytosiderophore transport in graminaceous species (Cunningham and Berti 1993).

Amongst the factors influencing the metal accumulation in plants, soil pH is usually the most important parameter (Ramos et al. 2002; Piechalak et al. 2003; Kirkham 2006; Deng et al. 2006). At higher soil pH, metal elements in soil solution form low soluble compounds and decrease their bioavailability, while metal bioavailability to plants increases at lower soil pH (Seregin and Ivanov 2001). However, Cr is reported to enhance Cd accumulation in plants such as *H. verticillata* and *Chara corallina* (Rai and Chandra 1992; Rai et al. 1995), but the accumulation of Cr is found to be greater in comparison to Cd when applied separately (Shukla et al. 2005; Singh et al. 2006). It is probably due to the fact that the properties of Cr make this element more available for plant uptake.

4 Mechanism of Metal Tolerance

Plants use complex processes to adapt their metabolism to rapidly changing environment. These processes include perception, transduction, and transmission of stress

stimuli (Turner et al. 2002; Xiong et al. 2002; Kopyra and Gwóźdź 2004). The adaptation to stressing conditions includes mechanisms of resistance and tolerance, later involves the immobilization of a metal in roots and in cell walls (Garbisu and Alkorta 2001). Tolerance deals with the internal sequestration of the toxic element. The plants develop a series of mechanisms to avoid heavy metal toxicity which include: (i) production of reactive oxygen species by auto oxidation and Fenton reaction, (ii) main functional group blocking, and (iii) displacement of metal ions from biomolecules (Clemens 2006). All these mechanisms operate as strategies to grow on contaminated soil. It has been determined that plants are able to grow in contaminated soils because; (i) they prevent the metal uptake through aerial parts or maintain low and constant metal concentration over a broad range of metal concentration in soil by holding metals in their roots (metal excluders) (Cunningham 1995), (ii) they actively accumulate metals in their aerial tissues due to the production of metal binding compounds (chelators) or alter metal compartmentalisation pattern by storing metals in non-sensitive parts (metal indicators), and (iii) they can concentrate metals in their aerial parts to levels far exceeding than soil (hyperaccumulators) (Raskin et al. 1994; Baker et al. 1994). The mechanisms used for hyperaccumulation are still unknown. The criteria to classify plants as hyperaccumulators are: (i) plants that can accumulate either As, Cu, Cr, Ni, Pb, or Co >1000 mg kg^{-1} or zinc >10 000 mg kg^{-1} in their shoot dry matter (Baker et al. 1994; Brown et al. 1994 Ma et al. 2001; Brooks 1998; Reeves and Baker 2000) or Mo>1500 mg kg^{-1} (Lombi et al. 2001), (ii) plants which accumulate metals in shoots 10–500 times more than normal levels (Shen and Liu 1998), (iii) plants accumulating more of an element in shoots than in roots (Baker et al. 1994), and (iv) when an enrichment coefficient (element in shoot/element in soil) >1 is observed (Wei et al. 2002). Very few higher plant taxa have adaptations that enable them to survive and to reproduce in soils heavily contaminated with Zn, Cu, Pb, Cd, Ni, and As (Dahmani-Muller et al. 2000; Pulford and Watson 2003). Tree roots of these plants can actively forage towards less contaminated zones of soil (Turner and Dickinson 1993) and, even with highly reduced growth, they can "sit and wait" for favorable growth conditions (Watmough 1994). Such species are divided into two main groups: the so-called (i) pseudometallophytes that grow on both contaminated and non contaminated soils and the (ii) absolute metallophytes that grow only on metal contaminated and naturally metal-rich soils.

5 Effect on Growth and Development

Heavy metals either retard the growth of the whole plant or plant parts (Shafiq and Iqbal 2005; Shanker et al. 2005). The plant parts which have the direct contact with the contaminated soils normally the roots exhibit rapid and sensitive changes in their growth pattern (Baker and Walker 1989). The significant effects of a number of metals (Cu, Ni, Pb, Cd, Zn, Al, Hg, Cr, Fe) on the growth of above ground plant parts is well documented (Wong and Bradshaw 1982). Heavy metals mainly affect plant growth through the generation of free radicals and reactive oxygen species (ROS), which pose constant oxidative damage by degenerating important

cellular components (Pandey et al. 2005, Qureshi et al. 2005). For example, in cucumber plants, Cu limits K uptake by leaf and inhibits the photosynthesis via sugar accumulation resulting into the retardation of cell expansion (Alaoui-Sosse et al. (2004). Similarly, rice seedlings exposed to Cd or Ni (Moya et al. 1993) and runner bean plants treated with Cd (Skórzyńska-Polit et al. 1998) and Cu (Maksymiec and Baszyński 1998) have shown an increase in carbohydrate content and a decrease in photosynthesis, resulting in growth inhibition. The typical symptoms of Cd toxicity of rice plants are wilted leaves, growth inhibition, progressive chlorosis in certain leaves and leaf sheaths, and browned root systems, especially the root tips (Das et al. 1997; Chugh and Sawhney 1999). In addition, in maize (*Zea mays*) Cd also reduces plant growth (Talanova et al. 2001; Liu et al. 2003/2004). Tomato plants irrigated with polluted water also show some phenotypic deformities like stunted growth, fewer branching and less fruiting. However, accumulation of heavy metals in fruits appears to be extremely low as compared to the stems, roots, and leaves (Gupta et al. 2008).

5.1 Germination

Seed germination and early seedling growth are quite sensitive towards changing environmental conditions (Seregin and Ivanov 2001). The germination performance and growth rate of seedings are therefore often used to assess the abilities of plant tolerance to metal elements (Peralta et al. 2001). The higher concentrations (1, 5 and 10 μM) of heavy metals (Cu, Zn, Mg and Na) inhibit seed germination and early growth of barley, rice and wheat seedlings significantly compared to control (Mahmood et al. 2007). Since seed germination is the first physiological process affected by toxic elements, the ability of a seed to germinate in a medium containing any metal element (i.e., Cr) would be a direct indicative of its level of tolerance to this metal (Peralta et al. 2001). The seed germination of *Echinochloa colona* is reduced to 25% at 200 μM Cr treatment (Rout et al. 2000), and high levels (500 ppm) of Cr(VI) in soil reduce germination of *Phaseolus vulgaris* up to 48% (Parr and Taylor 1982). Jain et al. (2000) observed reductions upto 32 and 57% in sugarcane bud germination at 20 and 80 ppm Cr, application respectively. In another study by Peralta et al. (2001) lucerne (*Medicago sativa* cv. Malone) germination was reduced to 23% at 40 ppm Cr(VI) treatment. The reduced germination of seeds under Cr stress could either be a depressive effect of Cr on the activity of amylases or transport of sugars to the embryo axes, or an increase in protease activity (Zeid 2001).

5.2 Root

In plants, roots are the first organs to come into contact with toxic elements and they usually accumulate more metals than shoots (Salt et al. 1995; Wójcik and Tukiendorf 1999; Rout et al. 2001). The inhibition of root elongation appears to be

the first visible effect of metal toxicity. Root elongation can be reduced by either the inhibition of root cell division and/or the decrease of cell expansion in the elongation zone (Fiskesjo 1997). Since inhibition of root elongation appears to be the first visible effect of metal toxicity, the root length can be used as an important tolerance index (Piechalak et al. 2002; Belimov et al. 2003; Odjegba and Fasidi 2004; Han et al. 2007).

It is reported (Han et al. 2004) that Cr(III) precipitates in the roots of *Brassica juncea* avoiding translocation. In accordance with another study (Peralta et al. 2001), alfalfa plants grown in solid media watered with 20 mg L^{-1} of Cr(VI), the ratio of Cr in shoots to Cr in roots was approximately 43%. This is an indication that most of the 50% of the absorbed Cr is kept in roots.

The response of roots to heavy metals has been extensively studied in both herbaceous plant species and trees (Khale 1993; Punz and Sieghardt 1993; Hagemeyer and Breckle 1996, 2002). After the work of numerous researchers (Barcelo and Poschenrieder 1990; Punz and Sieghardt 1993; Hagemeyer and Breckle 1996; 2002) the main morphological and structural effects caused by metal toxicity in roots can be summarized as: (i) decrease in root elongation, biomass and vessel diameter, (ii) tip damage, (iii) root hair collapse or decrease in number of roots, (iv) increase or decrease in lateral root formation, (v) enhancement in suberification and lignifications, and (vi) alterations in the structure of hypodermis and endodermis.

The metal toxicity varies with the type of metal elements. Chromium severely affects the root length as compared to the other heavy metals (Prasad et al. 2001). Mokgalaka-Matlala et al. (2008) observed that the root elongation decreased significantly with increasing concentrations of As(V) and As(III) in *Prosopis juliflora*. It has been reported that the root length in *Salix viminalis* is affected more by Cr than by Cd and Pb (Prasad et al. 2001). According to Fargaŝvá (1994; 1998) the inhibition effect of Cr on *S. alba* root growth is in fact similar to that of Hg, and stronger than that of Cd and Pb, while Ni reduced root length less than Cr. The order of metal toxicity to new root primordia in *S. viminalis* is reported to be Cd>Cr> Pb (Prasad et al. 2001).

5.3 Stem

The metal elements adversely affect the plant height and shoot growth as well (Rout et al. 1997). The reduction in plant height might be mainly due to reduced root growth and regulation of lesser nutrients and water transport to the aerial parts of the plant. Cr transport to the aerial part of the plant can have a direct impact on cellular metabolism of shoots contributing to the reduction in plant height (Shanker et al. 2005). Anderson et al. (1972) observed reduction of 11, 22 and 41% respectively compared to control in oat plants at 2, 10 and 25 ppm of Cr content in nutrient solutions in sand cultures. A similar reduction in height of *Cucumis sativus, Lactuca sativa and Panicum miliaceum* due to Cr(VI) was observed by Joseph et al. (1995). Cr(III) inhibits shoot growth in lucerne cultures (Barton et al. 2000). Sharma and

Sharma (1993) observed a significant reduction in height of wheat (cv. UP 2003) when sown in sand with 0.5 μM sodium dichromate in a glasshouse experiment after 32 and 96 days. A significant reduction in height of *Sinapis alba* at a level of 200 or 400 mg kg^{-1} of Cr in soil along with N, P, K and S fertilizers was reported by Hanus and Tomas (1993). Very recently, a reduction in stem height at various concentrations (10, 20, 40 and 80 ppm) of Cd and Cr have been reported in *Dalbergia sissoo* seedlings compared to the control (Shah et al. 2008).

5.4 Leaf

A healthy leaf growth, area development and total leaf number contribute to crop yield (Shanker et al. 2005). Metal elements like Cd, however, induce morphological changes such as drying of older leaves, and chlorosis and necrosis of younger leaves. *Datura innoxia* plants grown in an environment contaminated with Cr(VI) exhibited toxic symptoms at 0.2 m*M* of Cr(VI) in the form of leaf fall and wilting of leaves at 0.5 m*M* Cr(VI) in soil (Vernay et al. 2008). None of these symptoms were, however, visible in the medium with excessive Cr(III). Sharma and Sharma (1993) and Tripathi et al. (1999) found that a high concentration (200 ppm) of Cr(VI) severely affected the leaf area and biomass of *Albizia lebbeck* seedlings. These authors used higher contents of Cr(VI) in leaf growth traits as bio-indicators of heavy metal pollution and in the selection of resistant species. An addition of 100 ppm of Cr(VI) to soil showed up to 45% decrease of dry leaf yield in bush bean plants (Wallace et al. 1976). There appears a reduction in leaf area and leaf dry weight in *Oryza sativa, Acacia holosericea* and *Leucaena leucocephala* treated with tannery effluent of varied concentration (Karunyal et al. 1994). In a study on the effect of Cr(III) and Cr(VI) on spinach, Singh (2001) reported that Cr applied to soil at the rate of 60 mg kg^{-1} and higher levels reduced the leaf size causing burning of leaf tips or margins and slowed leaf growth rate. According to Pedreno et al. (1997) heavy metal contamination, especially Cr, preferably affected the young leaves in tomato plants.

5.5 Dry Biomass

Plant biomass is an indicator of crop productivity in terms of dry matter yield. Increased photosynthetic process is considered as the basis for the building up of organic substances which accounts for 80–90% of the total dry matter of plant (Bishnoi et al. 1993a; b). However, heavy metals like Cr and Cd showed reduced biomass production in *Bacopa monnieri* (Tokalioglu and Kartal 2006). According to another study, in an environment with varying contents of Cr, fronds of *Azolla* species showed toxicity symptoms in terms of increased fragmentation, change in color, development of necrosis and an overall decrease in biomass production as compared to controls (Aora et al. 2006). A Cr(VI) concentration above 2.5 μg mL^{-1} severely effects the dry matter production in *Vallisneria spiralis*

(Vajpayee et al. 2001). According to Zurayk et al. (2001), combined effect of salinity and Cr(VI) caused a significant decrease in the dry biomass accumulation of *Portulaca oleracea*. Cauliflower (cv. Maghi) when cultivated at 0.5 m*M* Cr(VI) showed restricted dry biomass production (Chatterjee and Chatterjee 2000). The effect of Cr(VI) on biomass production (Kocik and Ilavsky 1994) in sunflower, maize and *Vicia faba* grown in soil with Cr(VI) concentration of 200 mg kg^{-1} Cr(VI) was negligible but uptake of Cr into plant tissue was positively correlated with their contents in the soil. A distinct reduction in dry biomass at flowering stage of *S. alba* was noted when Cr(VI) was given at the rates of 200 or 400 mg kg^{-1} soil along with N, P, K and S fertilizers (Hanus and Tomas 1993). In pot trials in soil duly amended with Cr at the levels of 100 or 300 mg kg^{-1}, a reduction in yield of barley and maize has also been reported (Golovatyj et al. 1999). Dry matter production decreased dramatically in tomato and corn plants with increasing concentrations of Cd, decrease in yield of both crops was observed at 0.1 mg L^{-1} Cd and reached to acute toxicity at 2 mg L^{-1} (Yildiz 2005).

6 Effect on Plant Physiology

Plants exhibit morphological and metabolic changes in response to metal stress that are believed to be adaptive responses (Singh and Sinha 2004). For instance, Cd not only inhibits growth (Lunáčková et al. 2003, Dong et al. 2005), but also brings about changes in various physiological and biochemical characteristics such as water balance, nutrient uptake (Vassilev et al. 1997, Dražić et al. 2006, Scebba et al. 2006) and photosynthetic electron transport around photosystems PS I and PS II (Siedlecka and Baszynski 1993, Skórzyńska-Polit and Baszynski 1995, Vassilev et al. 2004). Similarly, Cr inhibits electron transport, reduces CO_2 fixation, chloroplast disorganization (Zeid 2001; Davies et al. 2002; Shanker 2003), decreases water potential, increases transpiration rate, reduces diffusive resistance, and causes a reduction in tracheary vessel diameter (Vazques et al. 1987).

6.1 Photosynthesis

The photosynthetic apparatus appears to be very sensitive to the toxicity of heavy metals, which invariably affect the photosynthetic functions either directly or indirectly by inhibiting the enzyme activities of the Calvin cycle and CO_2 deficiency due to stomatal closure (Seregin and Ivanov 2001; Linger et al. 2005; Bertrand and Poirier 2005).

Negative impacts of Cr on photosynthesis in terrestrial plants are well cited in the literature. According to a study by Bishnoi et al. (1993a) the effect of Cr was rather more pronounced on the PS I than on the PS II activity in isolated chloroplasts of pea plant. Vernay et al. (2007) observed photoinhibition in the leaves of *Lolium perenne* due to the effect of 250 μM Cr on the primary photochemistry of PSII and noted a

decrease in the maximal photochemical efficiency of PSII of plants at 500 μM Cr. Shanker et al. (2005) argued that Cr caused oxidative stress in the plants because Cr might enhance alternative sinks for the electrons due to the reduction of molecular oxygen (part of Mehler reaction). According to Rocchetta et al. (2006), the overall effect of Cr ions on photosynthesis and excitation energy transfer could be due to Cr induced abnormalities (widening of thylakoid and decrease in number of grana) in the chloroplast ultrastructure.

Though the effect of Cr on photosynthesis in higher plants is extensively studied (Foy et al. 1978; Van Assche and Clijsters 1983), it is not well understood to what extent Cr induces inhibition of photosynthesis either due to disarray of chloroplast ultrastructure and inhibition of electron transport or the influence of Cr on the enzymes of the Calvin cycle (Vazques et al. 1987). Krupa and Baszynski (1995) explained some hypotheses concerning the possible mechanisms of heavy-metals toxicity on photosynthesis and presented a list of key enzymes of photosynthetic carbon reduction, which were inhibited in heavy-metal treated plants (mainly cereal and legume crops). It has been noticed that the 40% inhibition of whole plant photosynthesis in 52-day-old pea plant (*Pisum sativum*) seedlings at 0.1 m*M* Cr(VI) was further enhanced to 65 and 95% after 76 and 89 days of growth respectively (Bishnoi et al. 1993a). Disorganization of the chloroplast ultrastructure and inhibition of electron transport processes due to Cr and a diversion of electrons from the electron-donating side of PS I to Cr(VI) is a possible explanation for Cr-induced decrease in photosynthetic rate. It is possible that electrons produced by the photochemical process are not necessarily used for carbon fixation as evidenced by low photosynthetic rate of the Cr stressed plants. Bioaccumulation of Cr and its toxicity to photosynthetic pigments in various crops and trees has been investigated extensively (Barcelo et al. 1986; Sharma and Sharma 1996; Vajpayee et al. 1999). Bera et al. (1999) studied the effect of Cr present in tannery effluent on chloroplast pigment content in mung bean and reported that irrespective of Cr concentration, chlorophyll *a*, chlorophyll *b* and total chlorophyll decreased in 6-day-old seedlings as compared to control. Chatterjee and Chatterjee (2000) reported that in cauliflower (cv. Maghi) grown in refined sand with complete nutrition (control) and Co, Cr and Cu at 0.5 m*M* each, a drastic decrease in chlorophylls a and b in leaves was recorded. The order of stress was Co > Cu > Cr. Conversely, a study on the Cr and Ni tolerance in *E. colona* showed that the chlorophyll content was high in tolerant calluses in terms of survival under high Cr concentration (Samantaray et al. 2001). Chromium(VI) at 1 and 2 mg L^{-1} significantly decreased chlorophyll *a* and *b* and carotenoid concentrations in *Salvinia minima* (Nichols et al. 2000). The decrease in the chlorophyll *a*/*b* ratio (Shanker 2003) brought about by Cr indicates that Cr toxicity possibly reduces the size of the peripheral part of the antenna complex. It has also been hypothesized that the decrease in chlorophyll *b* due to Cr could be due to the destabilization and degradation of the proteins of the peripheral part (Shanker et al. 2005). A significant decrease in contents of chlorophyll and carotenoid was established under the influence of Cd at both growth stages. This effect was dependent on Cd concentration in nutrient solution (Šimonova et al. 2007). PS II is inactivated by heavy metals such as Cd (Siedlecka and Baszynski 1993). This effect is related

to disorders in chlorophyll biosynthesis or chlorophyll destruction. Moreover, PS II reaction centers and PS II electron transport are affected by an interaction of Cd impairing enzyme activity and protein structure. The interaction of heavy metals with the functional SH-groups of proteins according to Van Assche and Clijsters (1990) is a possible mechanism of action for heavy metals. However, an earlier study by Haghiri (1974) reported that high Cd content in the growing medium suppressed the Fe uptake by plants, while Root et al. (1975) stated that Cd-induced chlorosis in corn leaves could possibly be due to changes in Fe:Zn ratios. In others plant species Cd toxicity appeared to induce phosphorus deficiency or reduced transport of manganese (Goldbold and Huttermann 1985).

6.2 Water Relation

Water can be considered as a major factor in the plant growth regulation since it affects directly or indirectly all growth process (Kramer and Boyer 1995). Plants raised in metal contaminated soils often suffer drought stress mainly due to poor physicochemical properties of soil and shallow root system, therefore, researchers are interested in investigations on plant water relation under heavy metal stress. Selection of drought resistance species can be considered to be an important trait in phytoremediation of soils polluted with heavy metals (Barcelo et al. 2001).

Heavy metals can induce stress in plants through a series of events leading to decreased water loss, (i.e., enhanced water conservation), decrease in number and size of leaves, stomatal size, number and diameter of xylem vessels, increased stomatal resistance, enhancement of leaf rolling and leaf abscission, higher degree of root suberization (Barcelo and Poschenrieder 1990).

It has been suggested that heavy metals can affect root hydraulic conductivity by multiple mechanisms operating on the apoplastic and/or the symplastic pathway. A reduced cell expansion may occur at their relatively low concentrations in the growth medium without any damage to cell integrity. For example, in bean plants leaf expansion growth in bean plants exposed to 3 μM Cd was inhibited after 48 h. The bulk leaf turgor remained unaffected, however, there was a decline in relative water contents (Poschenrieder et al. 1989). The data suggested that a Cd induced decrease of cell wall extensibility might have resulted in the decline of hydraulic conductivity of the leaf system in bean plants.

Chatterjee and Chatterjee (2000) concluded that excess Cr decreased the water potential and transpiration rates, and increased diffusive resistance and relative water content in cauliflower leaves. Barcelo et al. (1985) also observed a decrease in leaf water potential in bean plants treated with Cr. Bush bean plants when treated with Cr exhibited toxicity symptoms such as decreased turgor and plasmolysis in the epidermal and cortical cells and decrease in tracheary vessel diameter, which ultimately resulted in reduction of longitudinal water movement (Vazques et al. 1987).

Turner and Rust (1971) reported the wilting of various crops and plant species due to Cr toxicity, but little information is available on the exact effect of Cr on water

relations of higher plants. Impaired spatial distribution and reduced root surface of Cr-stressed plants can lower the capacity of plants to explore the soil surface for water. A significantly higher toxic effect of Cr(VI) in declining the stomatal conductance could be instrumental in damaging the cells and membrane of stomatal guard cells. This could affect the water relationship in all plant species.

6.3 Essential Nutrients

Heavy metals as micronutrients are essential for biological and physiological functions of plants. These functions include biosynthesis of proteins, nucleic acids, growth substances, chlorophyll and secondary metabolities such as metabolism of carbohydrates and lipids, stress tolerance, structural and functional integrity of various membranes and other cellular compounds (Päivöke and Simola 2001; Tu and Ma 2005). However, heavy metals like Cr and Cd interfere with the proper functioning of micronutrients. Reports indicated that higher concentrations of Cr in soil reduced the N content and increased the P concentration in oat plant tissues, slashed the micronutrient (Cu, Zn, Mn, and Ni) uptake in plants, decreased the levels of Fe and Zn with an increase of Mn contents in bush bean, interfered with the uptake of Ca, Cu, B, K, Pb and Mg in soybeans, diminished uptake of Fe, Zn and Mn in maize and reduced the uptake of Fe, Ca, Cu, Mg, Mn and Zn in sugar beat (Zayed and Terry 2003 and references therein). Since Cr is a toxic and non essential element, plants may lack any specific mechanism for its transport. Moreover, being structurally similar and having competitive binding abilities to common carriers to that of essential elements, can affect uptake and transport of mineral nutrients in plants in a complex way. For instance, Cr may reduce S and Fe uptake. Similarly, P and Cr are competitive for surface sites and Fe, S and Mn are competing Cr for transport binding. Thus, the competitive ability of Cr makes its swift entry into plant system.

Numerous studies on the effect of Cr on different plants are available in the literature. For example, Sujatha and Gupta (1996) observed that irrigation with tannery effluents with higher Cr contents resulted in micronutrient deficiencies in several agricultural crops. Khan et al. (2001) noted a decrease in N, P and K contents in dried rice plants treated with water having 0.5 ppm Cr. According to Barcelo et al. (1985), a strong correlation exists between chlorophyll pigments and Fe and Zn uptake in Cr-stressed plants. Cr hinders the availability of nutrients like Fe, Mn, Cu and Zn in plant parts like roots, leaves and stem (Sharma and Pant 1994). The N, P, K, Na, Ca and Mg contents in stems and branches of tomato plants treated with Cr at 50 and 100 mg L^{-1} were significantly reduced (Moral et al. 1995). Likewise, Moral et al. (1996) also reported negative effect of Cr on Fe absorption in *Lycopersicon esculentum* M. plants. Shanker (2003), however, explains that impediment of nutrient transport in heavy metal stressed plants is due to the inhibition of the activity of plasma membrane H^+ATPase.

Cadmium also influences the uptake and transport of essential elements in plants either reducing their availability in soil or lowering the microbes in soil

(Moreno et al. 1999). Cd toxicity causes the nutritional deficiency in plants (Das et al. 1997), inhibition of chlorophyll synthesis and disorganization of chloroplast structure (Clarkson and Luttage 1989; Rivetta et al. 1997). Reports show that a reduction in the uptake of Fe by maize plants and the Cd concentration was increased in soil coupled with an accumulation of Cd in the tissues of roots and shoots of plants (Liu et al. 2006).

7 Effect on Enzymes and Other Compounds

Enzymatic activity is indispensable in enhancing stress reaction response in plants through biosynthesis of signaling molecules. It is reported that heavy metals impede the enzymes associated with photosynthetic carbon reduction cycle and all of three phases of the Calvin cycle such as, carboxylation, reduction and regeneration, especially carboxylation phase, in plants (Krupa and Baszynski 1995: Prasad 1995; 1997).

According to Sheoran et al. (1990), Cd and Ni reduce photosynthetic activity in plants by inhibiting various enzymes (Rubisco, 3-PGA kinase, NADP, NAD glyceraldehydes 3-P dehydrogenase, aldolase and FDPase) of the photosynthetic carbon reduction cycle. The toxicity of cadmium also damages cell membrane and inactivates enzymes possibly through reacting with SH-group of proteins (Mathys 1975: Fuhrer 1988), which reflects the inhibitory effects of Pb^{2+}. Cd^{2+}, Zn^{2+} and Cu^{2+} on the activity of the chloroplast enzymes (Stiborova et al. 1986; Assche and Clijsters 1990; Guliev et al. 1992). However, many of the metal sensitive plant enzymes (rubisco, nitrate reductase, alcohol dehyrogenase, glycerol-3-phophate dehydrogenase and urease) are reported to be Cd tolerant in the form of a Cd-PC complex (Kneer and Zenk 1992). In an investigation involving *Zea mays* seedlings exposed to 50 μM Cd for 5 days, Cd enhanced enzymatic activity involved in sulfate reduction by acquiring more label from $^{35}SO_4{}^{2-}$ (Nussbaum et al. 1988).

Several investigations are available on the hyperactivity of antioxidative enzymes in various plants under Cu, Pb, Zn stress (Ali et al. 2003; Assche and Clijsters 1990). Nevertheless, fewer reports are available on the role of enzymatic antioxidant system in protecting plants from the toxic effects of reactive oxygen species (ROS) under Cr stress environment. This demonstrates the hypothesis that the antioxidant system, besides its function in detoxification, may also be a sensitive target of Cr toxicity in plants. Inside the cell, a reduction of Cr(VI) to Cr(III) owes to the formation of free radicals due to strong oxidative ability of Cr. (McGrath 1982; Cervantes et al. 2001). Thus, plants growing in a Cr(VI) stressed environment are prone to potential risk induced by ROS. Therefore, in response to Cr stress antioxidative defense systems, consisting of several non enzymatic and enzymatic mechanisms, are activated in the cell. One of the protective mechanisms is the enzymatic antioxidant system, which involves the sequential and simultaneous action of a number of enzymes including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) (Clijsters et al. 1999). Samantaray et al. (2001) and Poschenrieder et al. (1991) observed that Cr toxicity increased the CAT activity in bean plants.

However, Cr depressed the enzyme activity in *Zea mays*, *Triticum* spp., and *Brassica chinensis* (Sharma et al. 2003). Montes-Holguin et al. (2006) suggested that iron–porphyrin biomolecules (CAT) are able to interact with Cr through their iron center, affecting the availability of the active form of iron resultantly suppressing the CAT activity.

7.1 Root Fe(III) Reductase

Heavy metal toxicity hinders the Fe mobility and uptake by plants, and restrains reduction of Fe(III) to Fe(II) and its availability to plants. Consequently, Fe deficiency causes chlorosis in plants (Shanker and Pathmanabhan 2004). Under Fe-deficient conditions, an enhancement of the root Fe(III) reductase activity thereby increases the capacity to reduce Fe(III) to Fe(II)-a form in which roots absorb Fe (Alcantara et al. 1994). Similarly Cr application to iron-deficient *Plantago lanceolata* roots enhanced the activity of root-associated Fe(III) reductase. The examination by Wolfgang (1996) in a split root experiment applying Cr and iron-free treatments to root medium exhibited intermediate FeEDTA reductase activity as compared to non-split control plants. Under iron deficient conditions, addition of Cr(III) at 2 μM restricted ferric chelate reductase in roots of alfalfa plants, whilst at 10 μM it tended to stimulate ferric chelate reductase in media containing cobalt, nickel, chromium, and copper (Barton et al. 2000).

7.2 Nitrate Reductase

Various tree species are affected by higher contents of heavy metals. In Cr(VI) stressed *Albizia lebbeck* plants, nitrate reductase (NR) activity of leaves has been observed to be substantially enhanced as compared to control. However, the activity is negatively correlated with other parts i.e., root and shoot length, leaf area and biomass of the plants (Tripathi et al. 1999). Similarly, Cr concentration up to 200 μM significantly restrained the NR activity in *Nelumbo nucifera* (Vajpayee et al. 1999) and *Nymphaea alba* plants (Vajpayee et al. 2000). Although low concentrations of Cr (1 μM) enhance the NR activity, higher concentrations render it toxic, by significantly inhibiting the enzymatic activity (Panda and Patra 2000). Heavy metal like Cd is also instrumental in reducing nitrate reductase activity at higher concentrations and the absorption and transport of nitrate from roots to shoots of plants (Hernández et al. 1996). Similar reduction in the enzymatic activity due to Cd was also exhibited in *Silene cucubalus* plants (Mathys 1975).

7.3 Antioxidant Enzymes

Oxygen affect the cell metabolism in two ways, either by providing the energy for enzymatic combustion of organic compounds, or by causing a damage to aerobic cells due to the formation of reactive oxygen intermediates (Bartisz 1997), which

could excessively be produced in various compartments or organelles even under normal conditions. However, living organisms possess highly efficient defense systems called antioxidative or antioxidant systems against the toxicity of reactive oxygen intermediates (ROIs). These defense systems are comprised of both non-enzymatic and enzymatic constituents.

Heavy metals, at low concentrations, promote the antioxidant activity of enzymes. However, at higher metal contents catalase activity is reduced and SOD activity remains unaffected (Gwozdz et al. 1997). A study on the Cr(VI) effect on SOD activity of root mitochondria in pea plants revealed that SOD activity increased by 20% at 20 μM Cr content, whereas higher Cr levels (200 μM) substantially reduced SOD activity (Dixit et al. 2002). The specific activity of catalase in sugarcane is inhibited at Cr dose ranging between 20–80 ppm (Jain et al. 2000). According to Chatterjee and Chatterjee (2000), an excess of Cr (0.5 m*M*) restricted the activity of catalase in leaves of cauliflower. The activity of peroxidase and catalase was reportedly increased in tolerant calluses than in non-tolerant ones in *Echinochloa colona* (L) plants at Cr treatment of 1.5 mg L^{-1} (Samantaray et al. 2001). The application of Cr at a concentration of 15 μM showed an increase in the catalase and peroxidase activities in calli derived from *Leucaena leucocephala* (K8) growing on Cr treated as compared to untreated soil (Rout et al. 1999). Similarly, cadmium adversely intervenes the antioxidant enzymes.

8 Conclusion

Several heavy metal elements are essential for biological and physiological functions of plants, including biosynthesis of proteins, nucleic acids, growth substances, synthesis of chlorophyll and secondary metabolities, stress tolerance, structural and functional integrity of various membranes and other cellular compounds. However, beyond permissible limits, these metal elements become toxic depending upon the nature and species of metal and plants. Metal toxicity may inhibit electron transport, reduce CO_2 fixation, and cause chloroplast disorganization. It may also affect plant growth through the generation of free radicals and ROS, which pose a threat for constant oxidative damage by degenerating important cellular components. Visible symptoms of metal toxicity include drying of older leaves, chlorosis, necrosis of young leaves, stunting, wilting, and yield reduction. In addition, heavy metal stress can induce a series of events in plants leading to decrease in number and size of leaves, enhancement of leaf rolling and leaf abscission changes in stomatal size and resistance, and higher degree of root suberization. However, plants use complex processes (perception, transduction, and transmission of stress stimuli) and several non enzymatic and enzymatic mechanisms such as, SOD, POD, CAT and APX which activate the cell to adapt their metabolism to metal stress.

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Chapter 5
Mechanism of Free Radical Scavenging and Role of Phytohormones in Plants Under Abiotic Stresses

Parvaiz Ahmad, Shahid Umar, and Satyawati Sharma

Abstract Environmental stresses result in the generation of reactive oxygen species (ROS) in plants. ROS accumulate in cells and lead to the oxidation of proteins, chlorophyll, lipids, nucleic acids, carbohydrates etc. Cells have evolved intricate defense systems including enzymatic (superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductases (GR), monodehydroascorbate reductases (MSHAR), dehydroascorbate reductases (DHAR), glutathione peroxidase (GPX), guaicol peroxidase (GOPX) and glutathione-*S*- transferase (GST) and non-enzymatic systems such as ascorbic acid (ASH), glutathione (GSH), phenolic compounds, alkaloids, non-protein amino acids and α-tocopherol, which can scavenge the indigenously generated ROS. Plant stress tolerance mediated by antioxidants has been shown by many workers. Antioxidant resistance mechanisms may provide a strategy to enhance plant stress tolerance. Various enzymes involved in ROS-scavenging have been manipulated, over-expressed or down-regulated to add to the present knowledge and understanding of the role of antioxidant system. ROS induce the synthesis of several plant hormones, such as ethylene, salicylic acid (SA), jasmonic acid, brassinosteroids, abscisic acid (ABA) etc. These Phytohormones are required for growth and development of plants and defense responses during environmental stresses. The present review throws light on the enzymatic and non-enzymatic antioxidants in plants to enhance stress tolerance in plants and also in particular the role of brassinosteroids and ethylene during abiotic stress tolerance in plants.

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Contents

1 Introduction

Environmental stresses like temperature, drought, alkalinity, salinity, UV radiation are dangerous to plant life (Van Breusegem et al. 2001). According to FAO (2004) approximately, 22% of the world agricultural land is saline and the land under drought stress is expanding at an alarming rate (Burke et al. 2006).

Abiotic stress environment can induce a wide number of responses in plants ranging from readjustments of transport and metabolic processes leading to growth inhibition (Jaleel et al. 2007b, 2008; Ahmad et al. 2008a). During the exposure of plants to stress, a number of genes and gene products are expressed including proteins and they may be responsible for tolerance to these stresses (Mathur et al. 2008).

The primary effect of abiotic stress is ion imbalance and hyper-osmotic stress. During stress molecular oxygen receives electrons from high energy level to produce reactive oxygen species (ROS) (Mittler 2002) that are harmful to plant cells at high concentrations. ROS such as hydrogen peroxide, superoxide ions, singlet oxygen, peroxides etc. are toxic molecules for plant metabolism (Apel and Hirt 2004). All cellular macromolecules including DNA are damaged due to the deleterious effects of ROS (Tuteja et al. 2009) (Table 5.1).

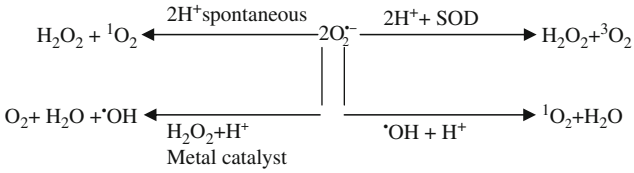
Table 5.1 Reactive oxygen species and oxidative stress

Name	Basic sources
Singlet oxygen (1st excited singlet state) $^1O_2$	Photoinhibition; UV irradiation; PS II e- transfer reactions (chloroplasts)
Superoxide anion $O_2^{\bullet -}$	Formed in many photooxidation reactions (flavoprotein, redox cycling); Mehler reaction in chloroplasts; mitochondrial e- transfer reactions; glyoxysomal photorespiration; peroxisomal activity; nitrogen fixation; reactions of O_3 and $OH^{\bullet}$ in apoplastic space; defense against pathogens; oxidation of xenobiotics
Hydrogen peroxide H_2O_2	Formed from $O_2^{\bullet -}$ by dismutation; photorespiration; ß-oxidation; proton-induced decomposition of $O_2^{\bullet -}$; defense against pathogens
Hydroxyl radical $OH^{\bullet}$	Decomposition of O3 in apoplastic space; defense against pathogens; reactions of H_2O_2 with $O_2^{\bullet -}$ (Haber-Weiss); reactions of H_2O_2 with Fe^{2+} (Fenton); highly reactive with all macromolecules
Perhydroxyl radical $O_2H^{\bullet}$	Protonated form of $O_2^{\bullet -}$; reactions of O_3 and $OH^{\bullet}$ in apoplastic space
Ozone O_3	UV radiation or electrical discharge in stratosphere; reactions involving combustion products of fossil fuels and UV radiation in troposphere

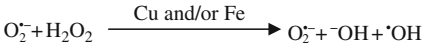
2 ROS Production

The main site of ROS production in plants through photorespiration during light is chloroplast and peroxisomes (Foyer and Noctor 2003) and mitochondria during darkness (Moller 2001). Chloroplast is a major producer of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in plants. Asada (2006) has demonstrated that the sites of ROS production in chloroplast thylakoids are PSI and PSII.

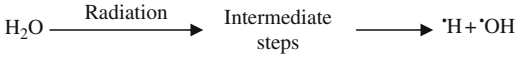
Superoxide ($O_2^{\bullet -}$) is produced as byproduct at complexes I and III of mitochondria. Superoxides ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are produced during metabolism. The former is produced by NADPH oxidase in plasma membrane and has an important role in several metabolic processes (Torres and Dangl 2005). The most important reactive oxygen species are oxygen derivatives (Tuteja et al. 2001; 2009) that are produced through the complete reduction of O_2, as shown below:



Hydroxyl radicals are produced from hydrogen peroxide which is an oxidizing agent. It can affect biomolecules of the cell. Hydroxyl radicals are produced through Harber-Weiss reaction (1934).



Radiations are also known to generate hydroxyl radicals in plants. The high energy of radiations (X-rays or gamma-rays) in the cell sap splits the covalent bonds of water.



The life span of hydroxyl radicals is very short (micro-seconds) but they are highly reactive among radicals studied so far.

Plant systems are equipped with enzymatic and non-enzymatic antioxidants such as: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione redutase (GR), ascorbic acid (AsA), glutathione etc. They minimize the deleterious effects of ROS. Every compartment of the cell contains one or more antioxidants that act on a particular ROS and detoxifies it (Nobuhiro and Mittler 2006). Introduction or over-expression of selected genes is the promising way to generate stress tolerant plants (Mathur et al. 2008).

3 Enzymatic Antioxidants

3.1 Superoxide Dismutase (SOD; EC 1.15.1.1)

Superoxide dismutase is a metalloenzyme, which coverts $O_2^{-\bullet}$ to H_2O_2. It was first found in maize (Scandalios 1993). SOD is classified on the basis of metal ions attached to their active site, as Cu/Zn-SOD, Mn-SOD, Fe-SOD, and Ni-SOD. Cu/Zn-SOD is found in the cytosol and chloroplast of the plant cell, whereas Mn-SOD in the mitochondrial matrix and peroxisomes. SOD regulates the concentration of superoxide anionic radical, and it has received great attention because of its protective effect against oxygen toxicity (Nordberg and Arner 2001). Hence, SOD has gained considerable interest in the pharmaceutical and food industries (Meyer et al. 2005). The over-expression of SODs combats the negative effects

of oxidative stress and has a significant role in tolerance and survival of plants. Experimental results showed that during salt stress, SOD activity increases in pea, maize, tea, mustard and mulberry (Ahmad et al. 2008b; Tuna et al. 2008; Upadhyaya et al. 2008; Ahmad 2010; Ahmad et al. 2010). Arbona et al. (2008) also showed the strong induction of SOD (up to 1.4 fold) in Carrizo citrange as compared to that in Cleoptra mandarin in response to flooding. In other studies, SOD activities were found to be low in salt sensitive cultivars and high in salt tolerant cultivars of tomato and *Plantago* under salt stress (Shalata et al. 2001; Sekmen et al. 2007). Total SOD activity showed marked enhancement under salinity in *Morus alba* (Harinasut et al. 2003). *Picea asperata* has been shown to have increased SOD activity during high light and drought stress (Yang et al. 2008). Qiu-Fang et al. (2005) demonstrated that under high NaCl concentration, chloroplast SOD, thylakoid bound SOD and stroma SOD were enhanced, and the increase being more in chloroplast SOD. Zhang et al. (2008) observed over-expression of 9-*cis*-epoxycarotenoid dioxygenase (NCED) gene *SgNCED1* in transgenic tobacco plants which induced the activity of SOD thereby resulting in improved growth in transgenic tobacco under drought and NaCl stresses. SOD activity increased under drought stress in *Euphorbia esula* (Davis and Swanson, 2001), maize (Pastori *et al*. 2000; Jiang and Zhang 2002), wheat (Singh and Usha 2003; Shao et al. 2005), rice (Wang et al. 2005), *Phaseolus acutifolius* (Turkan et al. 2005), and the SOD activity was higher under salinity stress in *Catharanthus roseus* (Jaleel et al. 2007a). Expression of Cu/Zn-SOD and APX genes in transgenic fescue plants showed tolerance to methyl viologen (MV), and heavy metal stress (Lee et al. 2007). Expression of Fe-SODs in *Lycopersicon esculentum* seedlings may help plants in the development of heat-shock tolerance (Camejo et al. 2007). Constitutive over-expression of Cu/Zn-SOD in the transgenic tobacco cytosol, reduced the ozone-induced necrosis (Pitcher and Zilinskas 1996).

3.2 Catalases (EC 1.11.1.6)

Catalases, mainly localized in the peroxisomes, are responsible for the conversion of $2H_2O_2$ to $O_2 + 2H_2O$ (Srivalli et al. 2003; Ben-Amor et al. 2005). They are present in all aerobic eukaryotes and are important in the detoxification of H_2O_2 generated in peroxisomes (microbodies), involved in β-oxidation of fatty acids, the glyoxylate cycle (photorespiration) and purine catabolism. Multiple isozyme forms of catalase have been found in plants. Castor bean and *Arabidopsis* contain two and six isozyme forms, respectively (Frugoli et al. 1996). They can direct dismutation of H_2O_2. Plants have been shown to contain catalase in multiple forms, e.g., maize contains three isoforms, *CAT 1*, *CAT 2* and *CAT 3*, which are located on separate chromosomes and are differentially expressed and independently regulated (Scandalios 1990). Peroxisomes and cytosol contains *CAT 1* and *CAT 2,* and *CAT 3* is located in mitochondria. Plants contain multiple CAT isozymes, e.g., two in *Hordeum vulgare* (Azevedo et al. 1998), and as many as 12 isozymes in mustard (Frugoli et al. 1996). CAT isozymes have been shown to be regulated temporally and spatially and may respond differently to light (Skadsen et al. 1995). Catalases

are the principal scavenging enzymes which directly dismutate H_2O_2 into H_2O and O_2 during stress (Van Breusegem et al. 2001). There are reports which show that increasing catalase activity helps the plant to adapt the harsh conditions and maintains the metabolic processes by minimizing the toxic level of H_2O_2 (Sekmen et al. 2007; Vital et al. 2008). Abiotic stress leads to the up-regulation of the genes responsible for the expression of catalase in alfalfa nodule, tea, cotton and tobacco (Sekmen et al. 2007; Upadhyaya et al. 2008; Vital et al. 2008; Zhang et al. 2008). Sekmen et al. (2007) demonstrated that increase in catalase activity was more in salt tolerant *Plantago maritima* than that in salt-sensitive *Plantago media*. Continuous waterlogging in *Citrus melo CPB 4475* and *Carrizo citrange* showed that CAT activity increased 1.7 fold and 3.0 fold, respectively as compared to that in control plants (Arbona et al. 2008). Yang et al. (2008) observed that CAT activity significantly increased in dragon spruce (*Picea asperata* Mast.) seedlings subjected to the combined effect of drought and high light.

Catalase activity increased in maize (Pastori et al. 2000; Jiang and Zhang 2002); *Allium schoenoprasum* (Egert and Tevini 2002), and wheat (Dalmia and Sawhney 2004; Shao et al. 2005); *Phaseolus acutifolius* (Turkan et al. 2005) under drought stress. An increase in catalase activity was reported in many higher plants under drought stress (Reddy et al. 2004). Similar results were found in *Lotus corniculatus* (Borsani et al. 2001) and rice (Wang et al. 2005). However, Harinasut et al. (2003) showed that CAT activity did not respond to increasing salt concentration in salt tolerant mulberry cultivar, Pei. Decrease in CAT activity in leaves of *Bruguiera parviflora* under NaCl stress was also observed by Parida et al. (2004). The decreasing CAT activity in some plants reflects the importance of peroxidase as well as SOD/ascorbate-glutathione cycle as oxygen reactive scavenging systems (Harinasut et al. 2003).

3.3 Ascorbate Peroxidase (APX, EC 1.11.1.1)

Ascorbate peroxidase is an essential antioxidant enzyme, which has a leading role in detoxification or scavenging of H_2O_2 in water-water and ascorbate-glutathione cycles. The excess of H_2O_2 is reduced to H_2O and O_2 in the presence of APXs (Kangasjärvi et al. 2008). Five different isoforms of APX family have been found in different compartments of the cell (Noctor and Foyer 1998).

Ascorbate peroxide activity increased under drought stress in *Euphorbia esula* (Davis and Swanson 2001), *Zea mays* (Jiang and Zhang 2002), wheat (Dalmia and Sawhney 2004), *Phaseolus acutifolius* (Turkan et al. 2005) and soybean (Van Heerden and Kruger 2002). Increased APX activity was observed under drought stress in *Vigna* (Manivannan et al. 2007) and *Catharanthus* plants under salt stress (Jaleel et al. 2007a). Zhang et al. (2008) reported that transgenic tobacco over-expressing 9-cis-epoxycarotenoid dioxygenase (NCED) gene *SgNCED1* showed increased activity of APX and improved growth under mannitol-induced drought stress. The mRNA of cytosolic ascorbate peroxidase showed up-regulation during drought stress in alfalfa nodules (Naya et al. 2007). Different abiotic stress increases APX activity in different plants, e.g., waterlogging in citrus (Arbona et al. 2008),

NaCl and paraquat stress in cotton calli (Vital et al. 2008), salt stress in *Arabidopsis* (Lu et al. 2007). Giacomelli et al. (2007) observed that *Arabidopsis thaliana* deficient in two chloroplast ascorbate peroxidases (stromal APX and thylakoid APX) showed accelerated necrosis induced by light at lower levels of AsA in the cell. Simultaneous over-expression of Cu/Zn-SOD and APX genes in chloroplasts of transgenic fescue plants showed resistance to abiotic stresses (Lee et al. 2007).

3.4 Glutathione Reductase (GR, EC 1.6.4.2)

GR is a flavo-protein oxidoreductase, found in both prokaryotes and eukaryotes that catalyses the NADPH-dependent reduction of oxidized glutathione (GSSG) to its reduced form (GSH). In the cell, GR is located in the chloroplast stroma, mitochondria, cytosol and peroxisomes. Plants have multiple forms of this enzyme, eight in pea (Edwards et al. 1990) and two in wheat (Dalal and Khanna-Chopra 2001). There are reports which showed that different environmental stresses induce GR activity. For example, GR activity significantly increased with chilling stress in cucumber (*Cucumis sativus* L.), and wheat (*Triticum aestivum*) with high temperature (Keles and Oncel 2002), and in alfalfa nodules during water stress (Naya et al. 2007), and in cotton calli during NaCl and paraquat stress (Vital et al. 2008). Semane et al. (2007) also demonstrated that a significant increase in the messenger RNA level of genes involved in GSH synthesis (*gsh1 and gsh2*) during Cd stress in *Arabidopsis* (Semane et al. 2007).

Interestingly, higher glutathione levels were also observed in transgenic tobacco, which over-expressed human DHAR gene (dehydroascorbate gene) and the increased GR levels protected these plants from membrane damage when subjected to MV and NaCl (Lee et al. 2007). DHAR over-expressing plants also had improved tolerance for other abiotic stresses like low temperature and high salinity levels. However, with increasing NaCl concentration, a decrease in GR activity has been reported in roots of salt sensitive genotypes of wheat (BR5001) by Azevedo-Neto et al. (2006). More decrease in GR activity was observed in salt-sensitive *Plantago media* than that in salt tolerant *Plantago maritima* (Sekmen et al. 2007). Upadhyaya et al. (2008) observed that some clones of *Camellia sinensis* showed increased GR activity under water stress and rehydration treatments decreased the GR activities in all the tested clones. Ding et al. (2007) also reported increased GR activity in mango fruit after exogenous oxalic acid or salicylic acid treatment under chilling stress.

4 Non-enzymatic Antioxidants

4.1 Ascorbic Acid (Vitamin C)

Among the non-enzymatic antioxidants AsA is the most extensively studied molecule and is found in various plant cell types (Horemans et al. 2000; Smirnoff 2000). Although the precursor of L-ascorbic acid is D-glucose, its biosynthetic pathway is still unclear (Foyer and Noctor 2005). Normally, ascorbate occurs in the

reduced form (AsA). (90% of the ascorbate pool) and its intracellular concentration ranges from 20 m*M* in the cytosol to 300 mM in the chloroplast (Noctor and Foyer 1998). The synthesis of ascorbate takes place in mitochondria and is transported to other cell components through a proton-electrochemical gradient or through facilitated diffusion (Horemans et al. 2000). AsA has effects on different physiological processes including growth regulation, differentiation and metabolism of plants. The basic role of AsA is to protect plants from the deleterious effects of H_2O_2 and other toxic derivatives of oxygen. AsA acts essentially as a reductant and it scavenges many types of free radicals. In the ascorbate–glutathione cycle, APX utilizes ascorbic acid and reduces H_2O_2 to water and generates monodehydroascorbate (MDA). MDA can also be reduced directly to AsA. The electron donor is usually NADPH and catalyzed by monodehydroascorbate reductase (MDAR). AsA can directly scavenge $^1O_2$, $O_2^{\bullet -}$ and $^{\bullet}OH$ radicals produced in the cell. AsA helps to regenerate tocopherol from tocopheroxyl radical which in turn provides protection to the membranes against oxidative stress. The synergistic action of AsA with other antioxidants plays a significant role in reducing the damaging effect of oxidative stress and gives tolerance to plants against environmental stresses (Foyer and Noctor 2005). Over-expression of *A. thaliana* MDAR gene (*AtMDAR1*) in tobacco plants showed enhanced tolerance to ozone, salt and PEG (Eltayeb et al. 2007). This tolerance may be due to the increased levels of AsA which mainly resulted from the enhanced activity of MDAR (Eltayeb et al. 2007). AsA reacts non-enzymatically with superoxide, hydrogen peroxide, and singlet oxygen.

4.2 Vitamin E (α-Tocopherols)

Tocopherols, a lipid soluble antioxidant found in all plant parts and are potential scavengers of ROS and lipid radicals (Kruk et al. 2005). Kagan (1989) has reported that tocopherols are important part of membranes in biological systems, where they play both antioxidant and non-antioxidant functions. Out of four isomers of tocopherols (α-, β-, γ-, δ-) found in plants (Kamal-Eldin and Appelqvist 1996), α-tocopherol is extensively studied. The molecular stricture of α-tocopherol has three methyl groups that give the molecule highest antioxidant property. Tocopherols are shown to be scavengers of oxygen radicals, especially $^1O_2$ and during chain propagation step, lipid auto-oxidation is prevented by tocopherols and this makes them effective free radical traps (Serbinova and Packer 1994). Munne-Bosch (2005) demonstrated that one molecule of α-tocopherol can scavenge up to 120 $^1O_2$ molecules by resonance energy transfer. It is well established that oxidative stress up-regulates the genes for tocopherol synthesis in plants (Wu et al. 2007). Antioxidants including α-tocopherol and AsA contributes to chilling tolerance in tomato plants and plays a protective role in oxidative stress induced damages to membranes. Many workers have reported that water stress is accompanied by increasing levels of tocopherols (Wu et al. 2007; Shao et al. 2007). α-tocopherol is synthesized from γ-tocopherol in chloroplasts by γ-tocopherolmethyltransferase (γ-TMT; VTE4). Leaves of many plant species including *Arabidopsis* contain high

levels of α-tocopherol, but are low in γ-tocopherol. It has been suggested that γ-tocopherol or its respective derivative 5-nitro-γ-tocopherol (5-NγT), may prolong early development by reducing the level of NOx. The germinating seeds of mustard, tobacco and *Arabidopsis* have been found to contain 5-NγT (Desel et al. 2007). Bergmüller et al. (2003) reported that during oxidative stress (high light, high temperature, cold treatment) the amounts of α-tocopherol and γ-tocopherol increased in wild type, and γ-tocopherol in *Arabidopsis* mutant line (*vte4-1*). However, chlorophyll content and photosynthetic quantum yield were very similar in wild type and *vte4-1*, suggesting that α-tocopherol can be replaced by γ-tocopherol in *vte4-1* to protect the photosynthetic apparatus against oxidative stress. Giacomelli et al. (2007) found that the concentrations of α-tocopherol, ascorbate and glutathione showed increase in response to high light in different genotypes of *Arabidopsis,* and the four ascorbate deficient *vtc2* genotypes accumulated more glutathione under control light than the others. Tocopherol cyclase (VTE1) encoded by *VTE1* gene acts as a catalyst in the synthesis of tocopherol (Liu et al. 2008). Over-expressing *VTE1* from *Arabidopsis* in transgenic lines of tobacco showed decreased lipid per-oxidation, electrolyte leakage and H_2O_2 content in comparison with the wild type. Thus, they concluded that increase in vitamin E is due to expression of *VTE1* in plants and this also leads to enhanced tolerance to environmental stresses (Siefermann-Harms 1987).

4.3 Glutathione (GSH)

GSH may be the most important intracellular defense against damage by ROS. The tripeptide (γ-GluCysGly) glutathione is one of the crucial metabolites in plants. Plant tissues contain GSH in reduced form which is abundantly found in all compartments of the cell (Jimenez et al. 1998). It plays a central role in several physiological processes, including regulation of sulfate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics (Xiang et al. 2001) and the expression of stress-responsive genes (Mullineaux and Rausch 2005). GSH has also been associated with several growth and development related events in plants (Rausch and Wachter 2005); its role is to maintain the reduced state of cells and is an important scavenger of $^1O_2$, H_2O_2 and $OH^\bullet$ (Larson 1988; Smirnoff 1993; Noctor and Foyer, 1998). In an anti-oxidative defense system, GSH has an important role as it regenerates ascorbic acid (another antioxidant) *via* the Ascorbate–Glutathione cycle (Foyer and Halliwell 1976; Foyer et al. 1997). It also plays an indirect role in protecting membranes by maintaining α-tocopherol and zeaxanthin in the reduced state. Increase in stress levels showed a gradual decrease in glutathione concentrations and the redoxed forms were changed in to oxidized forms, leading to metabolic system failure (Tausz et al. 2004). GSH is a precursor of PCs (Phytochelatins), which are able to control heavy metal concentrations in the cell. The role of GSH in the antioxidant defense system provides a strong basis for its use as a stress marker. Freeman et al (2004) have demonstrated that increasing concentration of GSH is correlated with oxidative stress tolerance in plants during metal stress. *Arabidopsis* plants with

low concentrations of glutathione were susceptible to even low concentrations of Cd (Xiang et al. 2001).

Manipulation of GSH biosynthesis increases resistance to oxidative stress (Sirko et al. 2004). It has been observed that upon Cd exposure, one of the main responses observed was the up-regulation of genes involved in sulfur assimilation–reduction and glutathione metabolism in the roots of *Arabidopsis* (Herbette et al. 2006). Feedback inhibition of γ-glutamylcysteine synthase (γ-ECS) by GSH is a basic central point for GSH synthesis (Noctor and Foyer 1998). Oxidation of GSH to GSSG decreases GSH levels and allows increased γ-ECS activity under stressed conditions (Noctor and Foyer 1998). Environmental stresses trigger an increase in ROS levels in plants and the response of glutathione can be crucial for adaptive responses. Antioxidant activity in leaves and chloroplast of *Phragmites australis* was associated with a large pool of GSH, protecting the activity of many photosynthetic enzymes against the thiophilic bursting of Cd exerting a direct important protective role in the presence of Cd (Pietrini et al. 2003). Increased concentration of GSH has been observed with increasing Cd concentration in *Brassica juncea* (Qadir et al. 2004), *Pisum sativum* (Metwally et al. 2005), and *Sedum alfredii* (Sun et al. 2007). However, decay in GSH content in *Pinus sylvestris* roots (Schutzendubel et al. 2001), *Populus* × *Canescens* roots (Schutzendubel and Polle 2002) and *Oryza sativa* leaves (Hsu and Kao 2004) has been reported under Cd stress. Cadmium-induced depletion of GSH has been mainly attributed to phytochelatin synthesis (Grill et al. 1985). Vacuoles of tobacco leaves and *Avena sativa* have been shown to accumulate PC-heavy metal complexes (Vogelli-Lange and Wagner 1990) and these complexes were reported to transport through the tonoplast (Vogelli-Lange and Wagner 1990). The decline in the levels of GSH might also be attributed to an increased utilization for ascorbate synthesis or for a direct interaction with Cd (Pietrini et al. 2003). The variety of responses to oxidative stress induced by heavy metals like Cd, is not only due to the Cd levels but it also depends on the plant parameters like species, age of the plant and duration of the treatment.

Srivastava et al. (2005) reported an appreciable decline in GR activity and GSH pool under copper stress, but a significant increase under NaCl stress. ROS scavenging enzymes and GSH concentration have been found to be in higher concentrations in the leaves of cultivar Pusa Bold than in CO 4 cultivar of *Vigna radiata*, and the higher concentrations of oxidized glutathione (GSSG) were detected in cultivar CO 4 as compared to that in Pusa Bold (Sumithra et al. 2006). Hence, it was concluded that Pusa Bold has an efficient antioxidative system that is responsible for its protection against oxidative damage than cultivar CO 4.

5 Phytohormones

5.1 Brassinosteroids (BRs)

Brassinosteroids (BRs) are potent plant growth regulators of steroidal nature that are synthesized by plants affecting many aspects of plant growth. The most abundant one is brassinolide. It was first isolated from the pollen of *Brassica napus*. It plays an

important role in growth and development of plants and is involved in different plant physiological responses (Sasse 2003). It is suggested that BRs have high biological activity and they regulate several morpho-physiological processes in plants, such as growth, germination, flowering, senescence, proton pump activation, stress tolerance, xylem differentiation and gene expression (Clouse 1996; Clouse and Sasse 1998; Li and Chory 1999). So far 42 BRs and four brassinosteroids conjugates have been characterized (Fujioka 1999). Li et al. (1998) observed that application of brassinolide to water stressed maize seedlings increased the activities of enzymatic and non-enzymatic antioxidants, whereas Vardhini and Rao (2003) showed that during osmotic stress BRs increase the activity of CAT and decrease the peroxidase and AsA oxidase activities in sorghum. Increase in anti-oxidative enzymes by BRs has also been reported in salt stressed rice seedlings (Núñez et al. 2003) and cadmium stressed chickpea (Hasan et al. 2008). Hayat et al. (2007) have also reported that BRs increase anti-oxidative activities and photosynthesis in mustard plants under cadmium stress. The foliar application of 24-epiBL or 28-homoBL improved growth and increased anti-oxidative enzymes in *Vigna radiata* under aluminum stress (Ali et al. 2008a) and in *Brassica juncea* under salt and nickel stresses (Alam et al. 2007; Ali et al. 2008b). Increases in photosynthesis and relative water content have also been observed in the above-mentioned plant species. Positive correlations have been seen between BR levels and tolerance to cold stress and photo-oxidation in cucumber plants (Xia et al. 2009). BR treatment induced the expression of genes *MAPK1, MAPK3* and *RBOH* and those related to anti-oxidative defense (Xia et al. 2009). Fariduddin et al. (2009) showed that treatment of *Brassica juncea* seedlings raised from the seeds treated with 28-homobrassinolide (HBL) improved growth, photosynthetic parameters and antioxidant enzymes under copper stress. The elevated antioxidant enzyme and proline might be responsible to overcome the toxic effects of copper in *B. juncea*.

5.2 Ethylene (C_2H_4)

Ethylene (Eth) is produced in most living plant cells and is considered as a plant hormone. Ethylene has many roles in various physiological processes, such as germination, growth, development, senescence and abscission as well as in defense and resistance (Wang et al. 2002). Environmental stress induces the production of ethylene in large amounts (Wang et al. 2006). Induction of ethylene biosynthesis has been shown in spring wheat during osmotic stress (Li et al. 2004) and in maize under UV-B radiation (Wang et al. 2006).

The biosynthesis of ethylene has two main steps: (i) Conversion of S-adenosyl L- methionine to ACC (1-aminocyclopropane-1-carboxylic acid) in the presence of catalyzing enzyme ACS (ACC synthase) and (ii) Cleavage of ACC to ethylene in the presence of ACO (ACC oxidase) (Fig. 5.1) (Zarembinski and Theologis 1994). Eth production in the tissues is very less as the activity of ACS enzyme is very low. During stress, the ACS activity is increased which in turn increases the production of ethylene. Tomato exposed to ozone stress induces ACS expression like *LE-ACS1A*, *LE-ACS2*, and *LE-ACS6*, and potato also shows the expression of *ST-ACS4* and

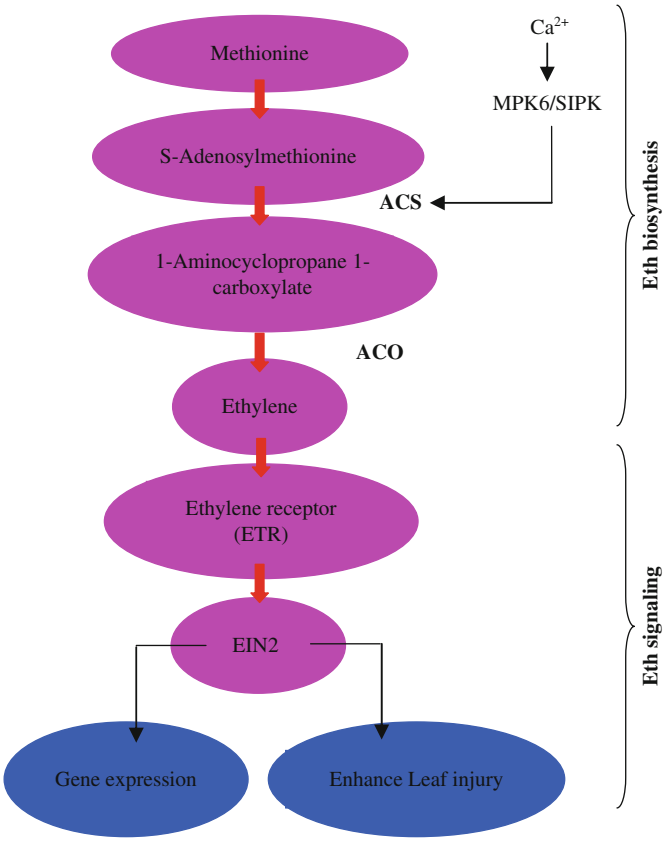


Fig. 5.1 Ethylene Biosynthesis pathway and signaling in stressed plants. In MPK 6 in Arabidopsis and SIPK in tobacco regulates ACC synthase (ACS) whose activity is controlled with cytosolic free Ca^{2+}. Ethylene gets attached to ethylene receptots (ETR) and signaling is transmitted through EIN2

ST-ACS5 (Tuomainen et al. 1997; Schlagnhaufer et al. 1997). Liu and Zhang (2004) observed that ACS accumulation is due to MPK6 induced phosphorylation in ACS2 and *ACS6* (Fig. 5.1) and thus leads to elevated levels of cellular ACS activity, indicating that ozone-induced ethylene evolution might be regulated not only by the transcription level of *ACS6*, but also post-transcriptionally through the MAPK signaling pathway. There is a strong correlation between ROS and ethylene levels in plant physiological responses. For example, it was found that this phytohormone and active oxygen species are responsible for the initiation of root nodules and it also acts as a transducer of downstream of the *Nod* factor response in the tropical, semi-aquatic legume *Sesbania rostrata* (D'Haeze et al. 2003). Tanaka et al. (2005) showed that ABA induced stomatal closure is inhibited by ethylene in *Arabidopsis*. H_2O_2

induced stomatal closure results in loss of function in *Arabidopsis* mutants, which suggests an important role of ethylene in guard cell ROS signaling and stomatal closure (Desikan et al. 2005). Pretreatment with ethylene increases ozone tolerance in pea (*Pisum sativum*) and mung bean (*Vigna radiata*) (Mehlhorn 1990). A dual role for ethylene in ozone tolerance has also been observed in different genotypes of silver birch (*Betula pendula* Roth). An ozone-tolerant silver birch clone produced little ethylene in response to ozone treatment, and ethylene production occurred temporally (Vahala et al. 2003).

6 Conclusion

Abiotic stress disturbs the balance between the production and removal of ROS which are in equilibrium at normal metabolic conditions. ROS induce oxidative damages to many biomolecules like membrane lipids, proteins, nucleic acids, chlorophyll etc. The $OH^{\bullet}$ is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone. The polyunsaturated fatty acids (PUFAs) linoleic acid and linolenic acid are particularly susceptible to attack to $^1O_2$ and $HO^{\bullet}$, giving rise to complex mixtures of lipid hydroperoxides. Extensive PUFA peroxidation decreases the fluidity of the membrane, increases leakiness and causes secondary damage to membrane proteins. ROS also leads to oxidations of proteins and are essentially irreversible, whereas, a few involving sulfur-containing amino acids are reversible. Protein oxidation is widespread and often used as a diagnostic marker for oxidative stress. Mounting evidence links oxidants and oxidative stress to senescence, impaired photosynthesis and necrosis in plants.

To control the level of ROS and to protect the cells under unfavourable environmental conditions, plants posses the ability to scavenge/detoxify ROS by producing different types of ROS Scavenging antioxidants. The components of antioxidant defense system are enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include SOD, CAT, APX, MDHAR, DHAR and GR and non-enzymatic antioxidants are GSH, AA (both water soluble), carotenoids and tocopherols (lipid soluble). Interestingly, higher plants also developed specific ROS-scavenging systems in different organelles to efficiently remove the ROS produced in these cellular parts; and, in particular under environmental stress such as salt stress, they coordinately work to provide plant cells with a highly efficient machinery for detoxifying ROS. A great deal of research has established that the induction of the cellular antioxidant machinery is important for protection against salt stress, metal stress, drought etc. various organelles have their own ROS scavenging system so that the organelles remove ROS more efficiently. They coordinately work to protect plant cells from ROS induced oxidative damage, e.g., Cytosolic APX1 can protect chloroplasts during light stress, which is a cross-compartment protection of thylakoid and stromal/mitochondrial APXs by cytosolic APX1.

7 Future Perspective

Plant biotechnologists are with the aim to increase the resistance of plants through genetic engineering. Up-regulation of certain anti-oxidative genes resulting in detoxification of ROS has been successful to some extent. This has added to the current knowledge in this area, but many reports are ambiguous at the same time. Improving the metabolic activities intricately involving superoxide scavenging, needs to be considerably taken care of, rather than enhancing the activity of antioxidant enzymes alone. Also the antioxidant mechanism of plants can be fortified by manipulating the antioxidant enzymes. Multiple genes which are affected under abiotic stresses indicate that there could not be a single marker for stress tolerance. Plant hormones are also responsible for the development of the plant and have a role in defense during environmental stresses. Much effort is still required to uncover in detail each product of genes induced by abiotic stress and signal transduction pathways. Researchers should look forward for defined set of markers to predict tolerance towards a particular type of stress.

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Chapter 6
The Role of Arbuscualr Mycorrhizae in Inducing Resistance to Drought and Salinity Stress in Crops

Ghazala Nasim

Abstract Arbuscular mycorrhizal (AM) fungi are commonly occurring soil microbes whose association with roots can have wide ranging effects on growth of the host plants. These fungi are frequent root colonizers of trees, shrubs, terrestrial orchids and a broad range of plants in temperate and tropical habitats. During the establishment of AM symbiosis, a range of chemical and biological parameters are affected in plants. These fungi are considered instrumental in promoting plant establishment and growth in these environments by enhancing plant nutrient and water uptake, protecting plants from root herbivores and pathogens and improving soil structure. This symbiosis is alleged to improve plant resistance to drought and nutrient stress. There are several reports which show that AM induce physiological drought tolerance, involving both increased dehydration avoidance and dehydration tolerance. Majority of the experiments have shown that when the symbiosis improves host drought resistance it does so by aiding drought avoidance.

AM symbiosis has frequently increased resilience of host plants to salinity stress. The AM plants in the saline soils had increased phosphate and decreased Na concentrations in shoots compared to non-AM ones. Salt resistance has been shown to improve by AM colonization in a number of crops like maize, mungbean, clover, cucumber, lettuce, tomato, and many more. A correlation has been established between AM colonization and improved osmoregulation or proline accumulation. AM colonization has also been documented to improve NaCl resistance in tomato, with the extent of improvement related to salt sensitivity of a cultivar. AM improvement of salt resistance has usually been associated with AM-induced increases in P acquisition and plant growth. However, there are scanty reports of AM induced effects on host plants being more pronounced when plants were exposed to osmotic stress in salinized soils.

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Contents

1 Introduction

Only in the last few decades, botanists and mycologists have realized that most terrestrial plants live in symbiosis with soil fungi (Krishna 2005). The term mycorrhiza, coined to reflect this reality, comes to us, moreover, from the combination of two words, one Greek "mikes" (fungus) and the other Latin "rhiza" (roots). It therefore basically designates the symbiotic association between fungi and plant roots. Among the types of mycorrhizae observed in nature, one is found on the vast majority of cultivated plants. It is the arbuscular mycorrhiza, which lives in association with approximately 85% of herbaceous plants. This means therefore that in the plant world, mycorrhizal symbiosis is the rule rather than the exception.

Arbuscular mycorrhizal fungi (AMF), which are microscopic soil fungi, colonize the roots and their rhizosphere simultaneously and spread out over several centimeters in the form of ramified filaments. This filamentous network dispersed inside as well as outside the roots allows the plant to have access to a greater quantity of water and soil minerals required for its nutrition. In return, the plant provides the fungus with sugars, amino acids and vitamins essential to its growth (Harley and Smith 1983).

The colonized plant is better nourished and better adapted to its environment. It obtains increased protection against environmental stresses (Sylvia and Williams 1992), including drought (Augé et al. 2007, 2008), cold (Charest et al. 1993), salinity, and pollution (Leyval et al. 1997). In addition, symbiosis tends to reduce the incidence of root diseases and minimizes the harmful effects of certain pathogenic agents (Dehne 1982; St-Arnaud et al. 1995). By and large, the growth and health of colonized plants is improved. At the same time, they obtain increased protection against environmental conditions detrimental to their survival.

Given that the majority of cultivated plants used for human and animal food purposes are colonized by mycorrhizae, we can consider utilizing this symbiosis for the benefit of agriculture, by selecting the best plant-fungus combinations (Abbott and Robson 1991). It is then possible to promote healthier cropping systems and to reduce the use of chemical inputs (pesticides, fertilizers), while ensuring crop profitability and environmental quality.

2 Arbuscular Mycorrhiza and Environmental Stresses

Recent evidence suggests that colonization of root systems by VA mycorrhizal fungi affords host plants greater resistance to environmental stresses like drought stress (Sanchez-Diaz and Honorubia 1994; Allen and Bosalis 1983; Nelson and Safir 1982; Augé 2000, 2001). Mycorrhizal plants may avoid drought to some extent through enhanced water uptake at low soil moisture levels. In onion, the effects appear to be conferred through improved phosphorus nutrition (Nelson and Safir 1982). While in Bromus and rose, some other mechanism prevails (Bildusan et al. 1986). An influence on host osmotic potential has been observed in wheat (Allen and Bosalis 1983).

These fungi also play a vital role in alleviating the effects of salinity (Al-Karaki et al. 2001). By improved nutrient acquisition, AM fungi compensate for the nutritional imbalances imposed by salinization, (Sylvia and Williams 1992). Some other environmental stresses such as micronutrient imbalances, industrial effluents (Oliveira et al. 2001), heavy metal toxicity (Chaudhry et al. 1999; Leyval et al. 1997), biocide treatment, (Heggo et al. 1990), slurry application (Chistie and Kilpatrich 1992), sulfur dioxide fumigation (Clappert et al. 1990) and wild fire recovery (Puppi and Tartnlini 1991), involves the use of AM fungi, (Barea et al. 1993). Some AM fungi are adapted to adverse conditions so they can benefit plants under a variety of environmental stresses, (Mosse et al. 1981). AM can also reduce the toxicity of certain metals for plants, while at non-toxic or such optimal level, their acquisition is enhanced by symbioses, (Bethlenfalvay 1992; Sylvia and Williams 1992; Barea et al. 1993; Khan et al. 2000). AM also plays positive role in protecting plants from pH extremes, (Sylvia and Williams 1992).

Mycorrhizal fungi interact with a wide assortment of organisms in the rhizosphere. The results can either be positive, neutral, or negative on the mycorrhizal association or a particular component of the rhizosphere. For example, specific bacteria stimulate EM formation in conifer nurseries and are called mycorrhization helper bacteria. In certain cases, these bacteria eliminate the need for soil fumigation (Azcón-Aguilar and Barea 1992; Garbaye 1994; Gryndler 2000).

The interaction between *Rhizobia* and AM fungi has received considerable attention because of the relatively high phosphorus demand of N_2 fixation. The two symbioses typically act synergistically, resulting in greater nitrogen and phosphorus content in combination than when each is inoculated onto the legume alone.

Legumes are typically coarse-rooted and therefore inefficient in extracting phosphorus from the soil. The AM fungi associated with legumes are an essential link for adequate phosphorus nutrition, leading to enhanced nitrogenase activity that in turn promotes root and mycorrhizal growth.

Mycorrhizal fungi colonize feeder roots and thereby interact with root pathogens that parasitize the same tissue. In a natural ecosystem where the uptake of phosphorus is low, a major role of mycorrhizal fungi may be protection of the root system from endemic pathogens such as *Fusarium* spp. Mycorrhizae may stimulate root colonization by selected biocontrol agents, but our understanding of these interactions is meager. Much more research has been conducted on the potential effects of mycorrhizal colonization on root pathogens. Mycorrhizal fungi may reduce the incidence and severity of root diseases (Linderman 2000, 1994; Hooker et al. 1994). The mechanisms proposed to explain this protective effect include: (i) development of a mechanical barrier-especially the mantle of the EM to infection by pathogens, (ii) production of antibiotic compounds that suppress the pathogen, (iii) competition for nutrients with the pathogen, including production of siderophores, and (iv) induction of generalized host defense mechanisms (Duchesne 1994). Role of arbuscular mycorrhizae as biological control agents soil-born plant pathogens have been elucidated by Azcón-Aguilar and Barea, (1996) and Singh and Singh (1996).

3 Arbuscular Mycorrhiza and Abiotic Stresses

The concept of an arbuscular mycorrhiza (AM) has been intensively advocated as a temporally and spatially complex symbiosis representing a suite of hosts and fungi, as against the more traditional "dual organism" view. These associations are important on natural and managed ecosystems due to their nutritional and non-nutritional benefits to their symbiotic partners. They can alter plant productivity, because AMF can act as biofertilizers, bioprotectants, or biodegraders (Xavier and Boyetchko 2002). AMF are known to improve plant growth and health by improving mineral nutrition or increasing resistance to tolerance to biotic and abiotic stresses (Clark and Zeto 2000; Turnau and Haselwandter 2002; Takeda et al. 2007).

Reports are mounting concerning the role of AM in responses to elevated atmospheric CO_2. Measurements of the contributions of AM fungi at various levels require the use of different response variables. For example, hyphal nutrient translocation rates or percent AM root infection may be important measures at the individual plant level, but hyphal biomass or glomalin production and turnover are more relevant at the ecosystem level. There is a discrepancy between our knowledge of the multifaceted role of AM fungi in plant and ecosystem ecology and most of the current research is aimed at elucidating the importance of this symbiosis in global-change scenarios. A framework for more integrated and multifactorial research on mycorrhizal involvement in regulating CO_2 responses may also serve to enhance communication between researchers working at different scales on large global-change ecosystem projects. A series of investigations have summarized the role of

anthropogenic pollution in general and CO_2, SO_2, O_3 pollution in particular, affecting mycorrhizal fungal communities (Cairney and Meharg 1999; Rillig and Allen 1999; Staddon and Fitter 1998).

One major line of work entailed studying the responses of the mycorrhizal communities to pollution stress, since under field conditions these fungi are crucial for the transfer of minerals from the soil solution to tree roots (Harley and Smith 1983). Effects of acid precipitation and gaseous pollutants have been shown to reduce root growth and mycorrhizal development. Indirect effects of pollutants in reducing photosynthesis and hence carbon allocation to the root system, may also inhibit mycorrhizal developments. Effect of elevated CO_2 (Rillig and Allen 1999) and other gaseous pollutants have recently been reviewed by Dighton and Jansen (1991), but most of the work was based on researches with ectomycorrhizae. Shaw et al. (1992a, b) studied the effects of SO_2 and O_3 on the mycorrhizae of Scots pine These were fumigation experiment based on collecting data of fruit bodies above-ground and taking root harvests below ground. Toermorshuizen and Shaffers (1987) observed that under Scots pine in the Netherlands mycorrhizal fruit bodies were not depressed by air pollution in young stands than they were in mature stands. Studies by Brown and Roberts (1988) discussed the effects of ozone on foliar leaching in Norway spruce *Picea abies* confounding effects due to N_2O_5 production during ozone generation in fumigation experiments. Shafer and Schoeneberger (1994) have indicated the mycorrhizal connection in the relationship of air pollution and ecosystem health.

The term "mycobioindication" was first of all coined by Kraigher et al. (1996). In their discussion of mycobioindication of forest site pollution, they employed a supposedly pollution sensitive (*Hydnum rufescens*) and supposedly insensitive (*Paxillus involutus*) fungal species of ectomycorrhizae. However, they emphasized that further screening of comparable forest sites differently influenced by pollution was needed to confirm the choice of species. The literature is wanting as regards the role of VA mycorrhizal fungal species as indicators of air pollution (Nasim et al. 2007). There are, however, sporadic reports of some fumigation studies employing AM. During a pioneer study, McCool et al. (1979) investigated the effects of ozone and HCl gases on the development of mycorrhizal fungus, *Glomus fasciculatum* and growth of Citrus sp. He noticed that higher concentration than the normal ones inhibited the growth and spread of *G. fasciculatum*. In subsequent studies, and Heath et al. (1982) concluded that the air containing higher concentration of ozone affects the rate of photosynthesis by reducing the photosynthetic capacity of the chloroplast and inhibits mycorrhiza formation in return. In another preliminary study, the effect of ozone exposure on mycorrhiza formation and growth of a forage grass, *Festuca arundinacea*, were studied (Ho and Trappe 1984). This grass usually forms abundant mycorrhizae (Ambler and Young 1977) but when exposed to 0.1 ppm ozone for three months a significant decrease in root weight and intensity of mycorrhizae formation was observed. In 1983, Brewer and Heagle, exposed soybean plants to ozone in open-top chambers in sterilized and unsterilized soil inoculated with AM and Rhizobium. Their results indicated that soybean infected with *Glomus geosporum* was less sensitive to adverse growth and yield effects of ozone.

3.1 Drought Stress

Stress is defined an external factor that exerts a disadvantageous influence on the plant. In most, cases stress is measured in relation to growth or to the primary assimilation processes (CO_2 and mineral uptake) which are related to overall growth. Under both natural and agricultural conditions, plants are constantly exposed to stress. Some environmental factors (such as air temperature) can become stressful in just a few minutes, whereas others may take days to weeks (soil water) or even months (some mineral nutrients) to become stressful (Taiz and Zeiger 2006). In this section we would focus our discussion on drought stress and role of arbuscular mycorrhiza in alleviating this stress (Table 6.1).

Table 6.1 Impacts caused by drought on plants

Drought resistant strategies vary with climatic or soil conditions: Water stress has several effects on growth. Of particular importance is a specific limitation to leaf expansion or otherwise plants have to complete their life cycles to avoid the onset of drought and rapid depletion of water through much expanded leaves.
Decreased leaf area is an early response to water stress: As water content of the plant decreases the cell shrinks and the cell walls relax. This decrease in cell volume results in lower hydrostatic pressure. The plasma membrane becomes thicker and compresses as it occupies a smaller area than before. Inhibition of cell expansion results in a slowing of leaf expansion.
Water deficit stimulates leaf abscission: In response to water stressed conditions the leaves will undergo senescence and will fall off. This leaf area adjustment is an important long-term change that improves the fitness for water-limited environment.
Water deficit enhances root extension into deeper, moist soil: Inhibition of leaf expansion reduces the consumption of carbon and energy during photosynthesis, and a greater proportion of the plant's assimilates can be distributed to the root system, where they can support further growth.
Mid-noon Stomatal closure due to stress induced ABA synthesis: Stomata may close during the peak hours of the day in response to severe water stress. This is also facilitated by the production of excess amount of ABA and translocation of the same in the transpiration stream initiating the closure of stomata.
Water stress limits the photosynthesis within the chloroplast: Rate of photosynthesis is less sensitive to turgor as compared to leaf expansion. However, Mg^{2+} concentration in chloroplast may influence photosynthesis during water stress.
Osmotic adjustment of cells helps maintain plant water balance: As soil dries up, its matric potential becomes more negative. Plants are able to take up water as long as their water potential is more negative than the water potential of the soil. Osmotic adjustment, or accumulation of solutes by cells is a process by which water potential can be decreased without an accompanying decrease in turgor. These solutes which are generally accumulated are called compatible solutes or osmolytes.
Water deficit alters energy dissipation from leaves: The evaporative cooling lowers leaf temperature and is highly effective for the survival of plants in arid environments. When transpiration slows down, the leaf temperature increases. Under these circumstances reduced leaf surface area, oreientation of leaves away from sunlight, wilting, leaf rolling, presence of hair or pubescence on the leaf surface, by layer of reflective epicuticular wax, or grey-white appearance are effective strategies adopted by the plants.

Table 6.1 (continued)

Water deficit increases resistance to liquid-phase water flow: With and increasing development of water stress the resistance to the flow of water in the plant increases sharply (Blizzard and Boyer 1980). As plant cells lose water, they shrink. When root shrinkage during the day is pronounced, the root surface moves away from the soil particles that hold the water, as a results the delicate root hairs are damaged. Another reason may be the deposition of suberin, a water impermeable lipid increasing the resistance to water flow. Another reason may be cavitation, or breakage of water column under tension.
Water deficit increases wax deposition on leaves: During water stress, production of a thicker cuticle that reduces water loss from the cuticle (cuticular transpiration) is a common observation.
Water deficit may induce Crassulacean Acid metabolism: Crassulacean Acid Metabolism (CAM) is a plant adaptation in which stomata open at night and remain closed during the day. Therefore the water use efficiencies of CAM plants are among the highest measured in all higher plants. The phenomenon of CAM is characteristic of succulent plants such as *Bryophyllum* or cacti. A few succulent species display facultative CAM, switching to CAM when subjected to water deficit or saline conditions (Hanson and Ting 1978).
Drought as a cause of Dieback and decline of trees: The decline is a general loss of vitality throughout the entire tree caused by a systemic disease or by a sequence of stressing events that causes, the tree to deplete its energy reserves. Twig and branch dieback is initiated in the tree as a response to poor growing conditions, physical injury to the tree and/or pest attack. Usually a combination of physical, climatic and pest problems lead to decline and dieback of trees (Clatterbuck 2001, 2006). Drought is a primary contributing factor to tree decline. Extended drought can influence the health of shade trees by the loss of absorbing roots. Most of the roots occur in the top 6–12 inches of the soil. Once the upper soil becomes dry, many absorbing roots dry out and die. Leaves and stems can also be damaged by drought conditions, especially when there is little water available for evaporative cooling and for photosynthesis and food production. Trees that occur on these soils or convex surfaces (ridges and ridge crests) where soil does not have much water holding capacity are more susceptible to drought that others. Some species of trees are more drought tolerant than others (Clatterbuck 2001). Trees may not readily show initial drought symptoms (curling of leaves, gradual loss of leaves, thinning of the crown) because of stored food reserves that reside in the woody tissues. However, as these stored food is depleted, drought symptoms become more prevalent. Drought symptoms can be delayed for two or more years as food reserves slowly deplete and imbalance between the aboveground and belowground tissue occur, making it difficult for many to believe that drought was actually the problem.

Drought is an evocative term. It comes with connotation of severe financial hardship among farmers in rich countries, to malnutrition, even famine, among farmers in poor countries. If prolonged it can lead to major social upheaval, mass migration, and desertification, not only in the sense that the affected region is deserted by its former inhabitants but also because over-farmed land may become so degraded that it can no longer support human habitatition even when the prolonged drought is over (Passioura 2007).

'Drought' has many meanings in relation to crop production. These range from: statistical, to a meteorological; through yield being limited by too little water to an agronomist; to sudden severe water deficits to many molecular biologists (Passioura 2007). Laboratory scientists typically work at short time scales. One area that has

attracted much attention is desiccation tolerance, the ability of plants to survive severe water deficit. Work with transgenic involving CBF/DREB transcription factors is proceeding apace. This is covered by 300 patents that also refer to drought tolerance (Passioura 2007).

3.1.1 Morphological and Anatomical Effects

Plants facing the problem of drought or continued water stress may respond in terms of exhibiting certain changes in morphological or anatomical features. Of particular importance in this connection are surface area, shape and arrangement of leaves and their internal structure. Small changes in leaf water status can have relatively large effects on critical physiological processes such as photosynthesis and water transport (Franks 2006; Taiz and Zeiger 2006). Because of this, leaves appear to be designed to maintain a certain degree of hydraulic homeostasis, both across species and across environments (Cowan and Farquhar 1977; Farquhar et al. 2002; Franks 2006).

3.1.2 Metabolic Effects

Continued episodes of water stress lead to inhibition of plant growth and photosynthesis, as well as to other effects. The process that is most affected by water deficit is cell growth. More severe water stress leads to inhibition of wall and protein synthesis, accumulation of solutes, closing of stomata and inhibition of photosynthesis.

3.1.3 Drought Resistance

Drought resistance mechanisms have been divided into several types. At the first level the phenomenon may be distinguished into desiccation postponement (ability to maintain tissue hydration) and desiccation tolerance (ability to function when dehydrated) which are sometimes referred to as drought tolerance at high and low water potentials respectively. A third category is drought escape which comprises plants that complete their lifecycles during the wet season, before the onset of drought. These are the only true drought avoiders. Among the desiccation postponers are water savers and water spenders. The water savers use water conservatively saving some in the soil for use late in the life cycle, whereas the water spenders aggressively absorb water, often using prodigious quantities (MacMahon and Schimpf 1981; Levitt 1972) (Table 6.2).

3.1.4 Mycorrhiza and Plant Water Relations

Water is one of the major global problems facing humankind at the moment and that is likely to be ever increasing in the future. Furthermore, there would be an increased competition for water resources available for agriculture in the future, despite the fact that there will be an ever increasing demand for water resources

Table 6.2 List of impacts caused by arbuscular mycorrhiza on plants exposed to drought

An immense magnitude of work has been published in the form of 200 peer reviewed papers on the influence of AM fungi on water relations, photosynthetic rates and drought responses of 90 host species representing 69 genera.

Stomatal conductance and transpiration:
AM and Non-AM plants often display different transpiration rate and stomatal conductances to water vapour being higher in the case of AM plants. AM effects on stomatal conductance have been observed with similar frequency under amply watered and drought conditions. AM symbiosis has also affected stomatal sensitivity to atmospheric water status (humidity). AM induced increases in transpiration and stomatal conductance in non-stressed plants are often stable but have been found to be three times that of P-limited NM controls. Stomatal conductance and leaf Ψ are linked functionally: changes in one usually derive changes in other. Thus when AM symbiosis hastens or postpones leaf dehydration, this would naturally be associated with altered stomatal behaviour. The extent of this alteration, however, may vary with different combinations of host plants and AM fungi (Augé et al. 2008).
Photosynthesis:
AM plants often show higher photosynthetic rates than their experimental non-AM counterparts, which is consistent with AM effects on stomatal conductance. Like stomatal conductance and transpiration, photosynthesis is stimulated by AM symbiosis about as frequently under non-stresses as under drought conditions. As with stomatal conductance, different AM fungi have different effects on photosynthesis during drought (Dixon et al. 1994).
Leaf hydration:
Tissue hydration or water status is typically quantified by measuring Ψ or its components or water content. Leaf Ψ of non-stressesd plants has usually not been affected by AM symbiosis. However, leaf osmotic potential may differ in AM and Non-AM plants during drought. Osmotic potential tends to be higher in leaves of AM plants than non-AM plants which means that AM plants are not as strained by the water stress as non-AM plants.
Root hydration:
Root Ψ components and water contents are more difficult to measure than corresponding leaf parameters and root water relations of AM and NM plants have seldom been compared. Nodule water content was higher in AM than in Non-AM alfalfa plants. Symplastic water fractions were increased by AM symbiosis in droughted rose roots.
Hydraulic conductivity and hyphal water transport:
Root hydraulic conductivity is generally not improved by AM symbiosis in the absence of AM-induced growth or P effects. In fact it was lower in AM plants. The hyphae of various AM fungi differ in their influence on water uptake, despite similar intra- and extra-radical hyphal extensions.
Soil drying rates and moisture relations:
AM root systems can dry soil more quickly and thoroughly than NM root systems, signified by larger declines in soil water contents or soil Ψ over time. This is probably because the shoots of the AM plants were larger (more evaporative leaf surface area) or the root systems of AM plants were larger or more finely divided (more water absorptive surface area) than those of non-AM plants (Okon et al. 1996).
Growth and nutrient uptake during drought:
AM symbiosis usually increases host growth rates during drought by affecting nutrient acquisition and possibly hydration. It has also typically increases water use efficiency and colonization by different fungi affects water use efficiency differently. As soil first begins drying, shoot growth can be inhibited before any leaf dehydration occurs through a root-to shoot non-hydraulic signaling mechanism. AM effects on host growth during drought are often related to improved P acquisition, as the available P in the soil is reduced by soil drying. It has been observed that copper and zinc concentration were higher in leaves of droughted AM than non-AM plants. While manganese and boron concentration was lower in leaves of AM than non-AM plants. Shoot concentration of nitrogen, potassium, calcium, magnesium, iron, sodium and molybdenum appear to be affected little by AM symbiosis in drought conditions.

Table 6.2 (continued)

Metabolic effects during drought: AM plants respond more quickly to the onset of drought than non-AM plants. This is also reflected in their metabolism. A plant more strained by water stress would be expected to be more metabolically perturbed. AM plants of tobacco accumulated less glucose and fructose in leaves and roots than non-AM ones in drought conditions. While a fungal disaccharide trehalose greatly increased in AM plants during drought. Concentration of amino acids in drought stressed AM plants have been reported to increase along with an increase in the activities of several enzymes. While during drought the concentration of ABA in xylem sap is reported to be low in AM plants. Chlorophyll concentration is high in leaves of AM than non-AM plants.
Morphological effects during drought: AM effects on plant water relations and metabolism during drought have been associated with morphological and phenological effects. In some plants early and enhanced leaf abscission were recorded during drought in AM plants, while in some the leaf drop decreased in AM plants under stress. AM soybean had less drought-induced pod abortion. Leaf movements were greater in AM plants under stress. AM rose leaves had less epicuticular wax and lower cutical weight than non-AM plants. AM plants show reduced wilting under water stress and recover more quickly from wilting when provided with ample water. However, stomatal density is not significantly affected in AM plants during drought.
Mechanisms: The best understood mechanism of AM mediated responses under water stress conditions involves AM effects on plant size. The size of a plant can affect its water relations and drought responses. Enhanced P uptake is the most dramatic means by which AM fungi affect overall plant biomass, but AM effects on carbon and nitrogen relations can also influence host size. Both overall plant size and within-pant relationship, such as root-to-shoot ratios, can influence plant behaviour, particularly when soil water becomes limiting.

Modified from Augé (2001)

available for agriculture to meet the needs of the increasing world population. A range of strategies have been proposed to cope with global water scarcity which include desalinization, virtual water and food trade, increasing agriculture yields, and improving the efficiency of water use in agriculture.

Biotechnology can play a significant role to address the last two possibilities. Through a number of investigations innumerable attempts have been made to genetically modify the plants so that they are able to withstand water stress conditions either through drought tolerance or drought avoidance. Here the specific application of symbiotic soil fungi has been discussed in relevance to water use in agriculture. This is regarding the inoculation of crops with arbuscular mycorrhizal fungi.

The extensive amount of research literature on the subject indicates that mycorrhizae often have a substantive impact on water movement into, through and out of host plants, with consequent effects on plant tissue hydration and leaf physiology. They usually increase host growth rates during drought, by affecting nutrient acquisition and possibly hydration, and typically water use efficiency, which are influenced by the kind of fungi involved (Augé 2001)

Mycorrhizal fungi can therefore be applied as biofertilizer with the aim of increasing growth potential and reducing water and fertilizer use and are used in

crop production, horticulture, habitat restoration, bioremediation and forestry. The mycorrhizal fungal innoculum may be applied in a number of ways e.g., by simply applying soils known to contain the desirable mycorrhizal fungi to areas lacking the fungi or using one of the many commercially available products available worldwide (Schwartz et al. 2006). Benefits, however, are not granted and a number of factors have to be considered when assessing their potential application, such as competition with other soil microorganisms as well as the dependence of the plant species on mycorrhizae, the nutrient status of the soil and the inoculum potential of the mycorrhizal fungi already present in the soil (Sylvia et al. 2005).

3.1.5 Mycorrhiza and Soil Water Relations

The contributions of AM fungal hyphae in terms of improving soil structure and its water holding capacity are substantial (Miller and Jastrow 2000). Not only can mycorrhizal fungi influence plant growth overall (and hence soil water regimes), but mycorrhizal plants exhibit different water relations from their non-mycorrhizal counterparts (Augé 2001, 2004). AM symbiosis has been reported to result in altered rates of water movement into, through and out of host plant, with consequent effects of tissue hydration and leaf physiology. For example, higher stomatal conductance and transpiration can occur in the mycorrhizal situations (Ebel et al. 1997; Augé et al. 2004). More efficient exploration of water by mycorrhizal fungi may lead to more extreme wet/dry cycles, which could have very strong consequences for soil aggregation (Six et al. 2004). Additionally, because the symbiosis can allow leaves to fix more carbon during water stress (Duan et al. 1996), carbon inputs into the soil would be expected to be increased, which might be especially important in more arid environment. Hyphae and roots can be viewed as a "sticky string bag" from a mechanistic point of view. Basically, the hyphae of AM fungi contribute to the entanglement and enmeshment of soil particles to form aggregates, the basic building blocks of soil structure. Furthermore, the glycoprotein, glomalin, deposited on the cell wall of the AM fungus is rather stable hydrophobic glue that might enable the fungus to cope with gas-water interfaces during aerial growth. In addition, the hydrophobicity of the deposited glomalin may reduce macro-aggregate disruption during wetting and drying events (Miller and Jastrow 2000).

3.1.6 Molecular Basis for Drought Resistance

Of all the abiotic stresses that curtail crop productivity, drought is the most devastating one and the most recalcitrant to breeder's efforts. In the past, breeding efforts to improve drought tolerance have been hindered by its quantitative genetic basis and our poor understanding of the physiological basis of yield in water limited conditions (Passioura 2002; Blum 1988). Further complexity derives from the occurrence of other abiotic stresses that often amplify the negative impact of drought on growth and metabolism (Mittler 2002, 2005). From an application point of view, it is crucially important to select genotypes able to optimize water harvest and water

use efficiency while maximizing yield in relation to the dynamics of the drought episodes prevailing in each target environment (Bacon 2004).

The genetic basis of the molecular, cellular and developmental responses to drought involves many gene functions regulated by water availability. Genomics based approaches provide access to agronomically desirable alleles present at quantitative trait loci (QTLs), that affect such responses, thus enabling us to improve the drought tolerance and yield of crops under water limited conditions more effectively. Marker-assisted selection is already helping breeders to improve drought related traits. Analysis of sequence data and gene products should facilitate the identification and cloning of genes at target QTLs. Based on such ideas, we envision a quick broadening of our understanding of the genetic and functional basis of drought tolerance. Novel opportunities will be generated for tailoring new genotypes "by design". Harnessing the full potential of genomics-assisted breeding will require a multidisciplinary approach and an integrated knowledge of the molecular and physiological processes influencing tolerance to drought (Tuberosa and Salvi 2006).

Among a seemingly endless list of morpho-physiological characters, the roots traits seem to be of much significance due to the crucial role of roots in harvesting water from the soil. Roots show a high degree of plasticity as regards water and nutrient uptake. Although this plasticity is under genetic control to a varying degree and several QTLs have been identified for in rice and maize, most recently, QTLs for the response of leaf elongation rate to soil moisture, temperature and evaporative demand have been identified. Remarkably, a model based on the combined effects of the major QTLs was able to predict 74% of the variability for leaf elongation rate measured among recombinant inbred lines of the mapping populations. Applying this modeling approach to root elongation rate sould provide valuable insight onto the role of root plasticity in the 'Genotype x Environment' (GxE) interaction under different water regimes and allow MAS to be used more effectively to tailor drought-tolerant genotypes by improving the root architecture.

During the past decade, an increase in QTL studies for drought-related traits and the first encouraging results in QTL cloning (Salvi and Tuberosa 2005) has lead us to a better understanding and to be able to effectively manipulate the traits influencing drought tolerance. This molecular assisted breeding will help us to face the challenges posed by the decreasing availability and escalating price of irrigation water. The successful exploitation of genomics to enhance drought tolerance will only be possible within a coherent, interdisciplinary context able to provide a thorough understanding of the factors limiting crop yield in drought-prone environment.

3.2 Nutrient Stress

Arbuscular mycorrhiza (AM) having a great influence on overall plant physiology contributes to improved plant health and growth, particularly under suboptimal conditions (Peuss 1958; Hirrel and Gerdemann 1980; Sharma et al. 1992). AM can

improve the uptake of water (Augé 2001) and nutrients (George 2000). Carbon assimilation and export from leaves may also be increased in mycorrhizal plants (Douds et al. 2000; Gernns et al. 2001).

3.3 Salinity Stress

Soil Salinization is an ever-present threat to crop yield. It is a widespread problem. Approximately, 7% of the global land surface is covered with saline soils (Ruiz-Lazano et al. 1996). Out of 1.5 billion ha cultivated land, about 77 million ha (5%) are affected by excess salt content mainly induced by irrigation with ground water of high salt content (Munns et al. 1999). It is well known that crop production is low in saline soil, mainly due to salt toxicity to plants leading to a decrease in plant water holding capacity, the imbalance of nutrient uptake, and toxicity of ions towards plant photosynthesis (Katerji et al. 1998; van Hoorn et al. 2001). The responses to salt stress comprise an array of changes at the molecular, biochemical and physiological levels (Garg and Manchanda 2008).

Mycorrhizal symbiosis is a key component in helping plants survive under adverse environmental conditions (Augé et al. 1992). Arbuscular mycorrhizal fungi widely occur in salt stressed environment (Wang and Liu 2001). Recently, many researchers reported that AM fungi could enhance the ability of the plants to cope with salt stress (Yano-melo et al. 2003; Rabie 2005; Jahromi et al. 2008) by improving plant nutrient uptake (Canterall and Linderman 2001; Asghari et al. 2005), and ion balance (Zandavalli et al. 2004; Giri et al. 2007), protecting enzyme activity (Rabie and Almadini 2005; Giri and Mukerji 2004, and facilitating water uptake (Berta et al. 1990; Ruiz-Lazano and Azcon 1995). Shi et al. (2002) and Shi and Guo (2006) found that salt stress could decrease photosynthetic ability and induce physiological drought in plants which leads to a decrease in crop production. There are few reports which indicate that AM colonization could enhance relative water content in *Zuchhini* leaves Colla et al. (2008), water potential of maize plants (Feng et al. 2000a; b) and chlorophyll concentration in the leaves of several plant species like *Sesbania aegyptica*, *S. grandiflora*, and *Lotus glaber* (Giri and Mukerji 2004; Sannazzaro et al. 2006; Colla et al. 2008). Sheng et al. (2008) evaluated the influence of arbuscular mycorrhizal fungus *Glomus mosseae* on characteristics of the growth, water status, chlorophyll concentration, gas exchange, and chlorophyll fluorescence of maize plants under salt stress. Maize plants were subjected to five levels of NaCl for 55 days. The results of this experiment by Sheng et al. (2008) that mycorrhizal maize plants had higher shoot and root dry weights than non-mycorrhizal plants when being exposed to salt stress, which means that mycorrhizal plants grow better than non-mycorrhizal plants under saline conditions. This is in line with many greenhouse studies on tomato (Al-Karaki and Hammad 2001), cotton (Feng and Zhang 2003), barley (Mohammed et al. 2003), and maize (Feng et al. 2000a, b).

In an experiment while evaluating the effect of AM inoculation on salt-induced nodule senescence in *Cajanus cajan* (Pigeon pea) it was reported that many of the

physiological and biochemical plant processes were affected by salt stress as a result of triggering premature nodule senescence along with a reduction in N-fixing ability of the nodules. In an experiment of 80 days, the plants were exposed to fairly high salinity regimes of 4, 6, 8 dS m^{-1} with and without mycorrhizal inoculation. Various parameters linked to nodule senescence were assessed like nodulation, leghemoglobin content, and nitrogenase enzyme activity measured as acetylene reduction activity (ARA). Two groups of antioxidant enzymes were studied: (1) enzyme involved in detoxification of O^{2-} radicals and H_2O_2 namely, superoxide dismutase, catalase and peroxidase, and (2) enzymes that are important components of the ascorbate glutathione pathway responsible for the removal of H_2O_2, namely, glutathione reductase and ascorbate peroxidase. The results of the experiment showed that AM significantly improved nodulation, leghemoglobin content and nitrogenase activity under salt stress. Activities of the rest of the enzymes mentioned above increased markedly in mycorrhizal-stressed plants. In some of the previous studies by Alguacil and others (2003) it was reported that increased antioxidative enzyme activities could be involved in the beneficial effects of mycorrhizal colonization on the performance of plants grown under semiarid conditions. Similar observation as those of Garg and Manchanda (2008) were noticed by Ruiz-Lozano and others (2001) and Porcel and others (2003) in soybean under drought stress (Table 6.3).

Arbuscualr mycorrhizal symbiosis is often alleged to improve plant resistance to drought stress (Cho et al. 2006) and AM plants often far batter during drought than their non –AM counterparts (Augé and Moore 2005; Augé et al. 2007). The intensity of mycorrhizal effect can increase with the intensity of drought (Subramanian and Charest 1998).

AM symbiosis has also been reported to increase resilience of host plants to salinity stress, perhaps with greater consistency than to drought stress (Cho et al. 2006). Growth in saline soils was increased by inoculation with *Glomus* spp. with AM plants having increased phosphate and decreased Na^+ concentrations in shoots compared to uninoculated controls (Giri and Mukerji 2004). Salt resistance was improved by AM colonization in maize (Feng et al. 2002), mung bean (Jindal et al. 1993) and clover (Ben Khaled et al. 2003), with the AM effect correlated with improved osmoregulation or proline accumulation. AM colonization also improved NaCl resistance in tomato, with extent of improvement related to salt sensitivity of the cultivar (Al-Karaki 2000; Al-Karaki et al. 2001). AM improvement of salt resistance has usually been associated with AM-induced increase in P acquisition and plant growth, although two of three AM fungi tested were able to protect cucumber plants from NaCl stress compared to similarly sized non-AM plants (Rosendahl and Rosendahl 1991). Alfalfa was also more effectively protected against salinity stress by AM symbiosis than by P-supplementation (Azcon and El-Atrash 1997), and the improvement of NaCl resistance in lettuce induced by several AM fungi was not attributed to nutrition (Ruiz-Lozano et al. 1996).

Since solutes can concentrate in the soil solution just outside roots as soil dries (Stirzaker and Passioura 1996), and since AM symbiosis often increases plant resistance to salinity stress, one can speculate that the amount of salts in drying soil may

Table 6.3 Impacts of salinity on plants

A highly complex and extensive problem in agriculture is the accumulation of salts from irrigation water. Evaporation and transpiration remove pure water (as vapor) from the soil and this water loss concentrates solutes in the soil. When the quality of irrigation water is poor and when there is no opportunity to flush out accumulated salts to a drainage system with an occasional excess irrigation, salts can quickly reach levels that are injurious to salt sensitive species (Taiz and Zeiger 2006).

Soil Salinization impairs plant function and soil structure:
High concentration of Na^+ results into sodicity while the increase in total salt concentration is called as salinity. The high Na^+ concentration of the sodic soils cannot only injure plants directly but also degrade the soil structure, decreasing porosity and water permeability. A sodic clay soil is very hard and impermeable.
Salinity depresses growth and photosynthesis in sensitive species:
Plants are divided into two broad groups on the basis of their response to high concentration of salts. *Halophytes* are native to saline soils and complete their life cycle in that environment. *Glycophytes* (sweet plants), or non-halophytes are not able to resist salts to the same degree as halophytes. Usually there is a threshold concentration of salts above which glycophytes begin to show signs of growth inhibition, leaf discolouration, and loss of dry weight. Among crops, date palm and sugar beet are highly tolerant crops (Greenway and Munns 1980). Species like *Suaeda maritima* and *Atriplex nummularia*, which are highly tolerant to salt show growth stimulation at Cl^- concentration many times greater than the lethal level of sensitive species.
Salt injury involves both osmotic effects and specific ion effects:
The increase in salt concentration is just similar to that of sol water deficit. Some plants can adjust osmotically when growing in saline soil and in this way prevent loss of turgor, which would slow extension growth of cells while generating a lower (more negative) water potential. Specific ion effect occurs when injurious concentration of Na^+, Cl^-, or SO_4^{2-} accumulates in the cells. A high Na^+ to K^+ ratio and high ratio of total salts inactivate enzymes and inhibit protein synthesis resulting into an inhibition of photosynthesis.
Plants use different strategies to avoid salt injury:
Plants avoid salt injury by exclusion of ions from the leaves or by compartmentation of ions in vacuoles. The salts may be excluded at the level of roots or may be secreted through salt glands and crystallize in the form of harmless crystals.
Salt stress induces synthesis of new proteins:
Exposure to NaCl induces synthesis of proteins associated with improved tolerance to NaCl. In tissue culture, cells of *Citrus* species or tobacco (*Nicotiana tobaccum*) have been acclimated to tolerate unusually high concentration of salts.

be one experimental factor that can explain why AM fungi increased drought resistance in some studies but not in others i.e., perhaps AM effects on drought resistance are linked to AM effects on salt resistance; in those reports where AM symbiosis did improve drought resistance, AM fungi may have helped to overcome plant susceptibility to an osmotic or NaCl stress that developed as soil dried (Cho et al. 2006). Cho et al. (2006) found that in *Sorghum bicolor* plants, salinity stress tended to nullify an AM-induced change in drought response. In another experiment, Augé at al. (2008) observed that in the case of squash leaves, across all AM and NaCl treatments, the leaf hydraulic conductance change in synchrony with stomatal conductance corroborating leaf tendency towards hydraulic homeostasis under varying rates of transpirational water loss.

3.4 Heavy Metal Stress

An immense load of heavy metals such as Pb, Cr, As, Cu, Cd and Hg is being added to our soils through industrial, agricultural and domestic effluents. These elements can either be absorbed in soil particles or leached into ground water. Problems associated with the contamination of soil and water such as animal welfare, health, fatalities and disruption of natural ecosystems is well documented (He et al. 2005). Human exposure to these metals through ingestion of contaminated food or uptake of drinking water can lead to their accumulation in humans, plants and animals. Lead, copper, zinc and cadmium are also found naturally on soils and can cause significant damage to the environment and human health as a result of their mobility and solubilities (Shuman 1985; Khan 2006).

Heavy metals in the soil are associated with a number of soil components which determine their behavior in the soil and influence their bioavailability (Boruvka and Drabek 2004). The cell wall components such as free amino acids, hydroxyl, carboxyl and other groups of soil fungi can bind to potentially toxic elements such as Cu, Pb, Cd, etc., (Kapoor and Viraraghavan 1995). Many filamentous fungi can sorb these trace elements and are used in their commercial biosorbants (Morley and Gadd 1995). The proteins in the cell walls of AM fungi appear to have similar ability to sorb potentially toxic elements by sequestering them. There is evidence that AMF can withstand potentially toxic elements. Gonzalez-Chavez et al., (2004) pointed out that glomalin produced on hyphae of AMF can sequester them. AMF plays a significant ecological role in the phytostabilization of potentially toxic trace element polluted soils by sequesteration and, in turn, help mycorrhizal plants survive in polluted soils (Khan 2005). Glomalin, an iron-containing glycoprotein produced by the hyphae of AMF fungi (Wright and Upadhyaya 1998), is released into soil by AMF hyphae (Driver et al. 2005). These authors have shown in the case of Glomus intraradices that glomalin is tightly bound in AMF hyphae and spore walls. Small amounts were found to be adhered to soil via release into liquid medium from the hyphae and not through secretion. It has been hypothesized that glomalin has a role in the immobilization ('filtering') of heavy metals at the soil-hypha interface, i.e. before entry into fungal-plant system.

There has been few analytical studies of AM in polluted soils during which some workers observed that the external mycelium of AMF was the main site for trace element localization (Kaldorf et al. 1999; Turnau 1998), while others reported selective exclusion of toxic and non-toxic elements by adsorption onto chitinous cell walls (Zhou 1999), or onto glomalin, the extracellular glycoprotein (Wright and Upadhyaya 1998), or intracellular precipitation. All these mechanisms have implications in reducing a plant's exposure to potentially toxic elements. Gonzalez-Chavez et al. (2002) studied the form and localization of Cu accumulation on the extraradical mycelium of three AM fungi isolated form the Cu and As polluted soil. The authors reported differential capacity of AMF to absorb and accumulate Cu as determined by TEM and SEM. However, an insight into the nature of accumulation and mechanisms involved require further research (Khan 2006).

The AMF can be screened for their ability to produce maximum level of extraradical mycelium in polluted soils (Joner et al. 2000), and to utilize adapted AM fungi to help accumulate heavy metals both within the plant roots (phytoaccumulation) and the extrametrical fungal mycelium (Khan 2006).

4 Conclusion

Mycorrhizae are symbiotic associations that form between the roots of most plant species and fungi. These symbioses are characterized by bi-directional movement of nutrients where carbon flows to the fungus and inorganic nutrients move to the plant, thereby providing a critical linkage between the plant root and soil. As a result, mycorrhizal plants are often more competitive and better able to tolerate environmental stresses than are nonmycorrhizal plants.

Mycorrhizal associations vary widely in form and function. Arbuscular mycorrhizal fungi belong to the order Glomales and form highly branched structures called arbuscules, within root cortical cells of many herbaceous and woody plant species. These structures are meant for bilateral transfer of growth factors.

Plant responses to colonization by mycorrhizal fungi can range from dramatic growth promotion to growth depression. Factors affecting this response include the mycorrhizal dependency of the host crop, the nutrient status of the soil, and the inoculum potential of the mycorrhizal fungi. Arbuscualr mycorrhiza confers resistance to water and salinity/nutrient stress in plants.

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Chapter 7
Predicting Growth, Carbon Sequestration and Salinity Impacts of Forestry Plantations

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Abstract Farm forestry is an increasingly important form of diversifying farm income and helping to deal with environmental issues including dryland salinity, global warming and climate variability. Here we briefly describe the development, use and spatial application of improved versions of the plantation growth model, 3-PG, to provide estimates of productivity and carbon sequestration as well as salinity impacts. Several forestry scenarios using eucalypt species and *Pinus radiata* were tested with application to the Corangamite Catchment in south western Victoria, Australia.

Keywords Corangamite catchment · Carbon sequestration · Salinity stress · Forests

Contents

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1 Introduction

Amongst important issues in Australia at present are dryland salinity, climate variability and the need for water conservation. Rainfall is predicted to decrease in many parts of southern Australia over the next few decades. In south-eastern Australia, occurrence of dryland salinity is typically scattered and patchy, with stream salinity often a greater concern than land salinisation. Growing trees on farms for commercial or semi-commercial benefit (farm forestry) is an increasingly important and recognised form of diversifying farm income and providing environmental services in Australia. Tree planting may help reduce in-stream and end-of-catchment salinity, provide habitat to enhance biodiversity, produce timber and sequester carbon to offset greenhouse gas emissions and address global warming and climate variability.

The Corangamite catchment (13,350 km^2) located in south-western Victoria, Australia (Fig. 7.1) provided a test region for applying predictive modeling as part of the Commercial Environmental Forestry (CEF) project (Polglase et al. 2006). Dryland salinity is estimated to affect 17,250 ha of land in this catchment (Nicholson et al. 2006) with stream salinisation an important issue in the northern part of the catchment. Land use is predominantly agriculture, including dairy in the higher rainfall areas to the south, broad-acre cropping in the north and mixed cropping and grazing throughout the extensive volcanic plains in the centre. There are more than 45,000 ha of plantation forestry in the catchment, mostly *Pinus radiata* (radiata pine) and *Eucalyptus globulus* (blue gum) mainly in higher rainfall (>700 mm) areas, with smaller farm forestry plantings (including *E. cladocalyx* – sugar gum) where rainfall is lower (450–700 mm).

Fig. 7.1 Location of the Corangamite catchment in south-western Victoria, Australia

The plantation growth model, 3-PG (Physiological Principles in Predicting Growth), originally developed by Landsberg and Waring (1997) and variously modified since then (e.g., Sands and Landsberg 2002), is a process-based forest growth model widely tested and applied (Sands and Landsberg 2002, Almeida et al. 2004,

Dye et al. 2004, Stape et al. 2004). In its simplest form, the model requires monthly climate inputs (total short wave incoming radiation, mean temperature and vapour pressure deficit, and total rainfall), knowledge of soil texture, soil water holding capacity, an indication of soil fertility, initial number of trees per hectare, and initial values for stem (including bark and branches), foliage and root mass per hectare to initialise the model at a selected age. The model incorporates simplifications of some well-known relationships, with the aim of describing complex physiological processes so that they can be applied to plantations or even-aged, relatively homogeneous forests. Many of the parameters used in 3-PG need to be calibrated for individual species or different genotypes within a species, however there are parameter sets for several species available in the literature (e.g., Almeida et al. 2004, Paul et al. 2007, Morris 2003).

Here we briefly describe the development, use and spatial application of two recent versions of the plantation growth model, 3-PG, for various forestry scenarios to provide predictions of growth, carbon sequestration, water use and salinity impacts at catchment and farm scales.

2 Materials and Methods

Two versions of 3-PG were developed and applied. 3-PG2 was improved to include the ability to model over- and under-storey, different planting configurations, responses to environmental factors such as soil water stress and salinity (termed 'growth modifiers'), and the water balance is now calculated in a more detailed way (Polglase et al. 2006; Almeida et al. 2004). 3-PG2 was used to spatially model (as 3-PG2 Spatial or 3-PG2S; 100 × 100 m grid resolution) growth, carbon sequestration and water use for the entire Corangamite catchment region. 3-PG+ (Morris 2003) was further modified to improve water balance prediction capability (using daily time-step climate inputs to better estimate run off and infiltration) to predict growth, carbon and water use, and it was also used within a hydrological modelling framework, the Catchment Analysis Tool (CAT, Beverly et al. 2006), as CAT_3-PG+ (20 × 20 m grid resolution), to predict impacts of stream salinity and flows, for salinity-prone, northern areas of the Corangamite catchment. CAT includes a suite of one dimensional farming system models linked to a distributed surface hydrology model and a groundwater model. Both 3-PG2S and 3-PG+_CAT used spatial input data layers including soil depth, soil texture, fertility index, road networks, hydrology and digital elevation.

3-PG2S was run for 21 forestry scenarios (combinations of species, silvicultural management and site fertility rating), and 3-PG+_CAT was run for five scenarios. The species of interest - *Eucalyptus globulus*, *E. cladocalyx*, *Corymbia maculata* (spotted gum) and *P. radiata* – were deemed to have suitable commercial prospects for regions of low to moderate annual rainfall (500–800 mm). The scenarios were developed in consultation with Department of Primary Industries Victoria and several private forestry companies as a compromise between reasonable practices for species being considered and constraints of modelling. Models were run for the

entire plantable area (i.e., areas not occupied by roads, buildings, parks, existing native forest and plantations).

In order to calibrate both models for different species, trees at representative sites were destructively sampled into biomass components to compare with model predictions of biomass, which is later converted to stem diameter and volume in 3-PG. In order to test growth and carbon sequestration predictions using 3-PG, site, soil (texture, structure, depth) and tree growth (height, stem diameter at breast height, leaf area index, calculated stand stem volumes) data were collected for each species from existing plantations within the Corangamite and other catchments in Victoria. Soils data were subsequently used to check spatially-predicted soil depths and estimate soil water holding capacity and site fertility/quality for input into 3-PG. Analysed tree growth data were compared with predictions.

3 Results and Discussion

Initial testing of 3-PG+ and 3-PG2 suggested that these models capture the effect of major environmental gradients on growth for the four species considered. Based on regression and model efficiency analysis, there was generally good agreement between observed and predicted growth (the model explained between 61% and 84% of the observed growth) and carbon sequestration, and in the case of CAT_3-PG+, for stream flows and salt loading.

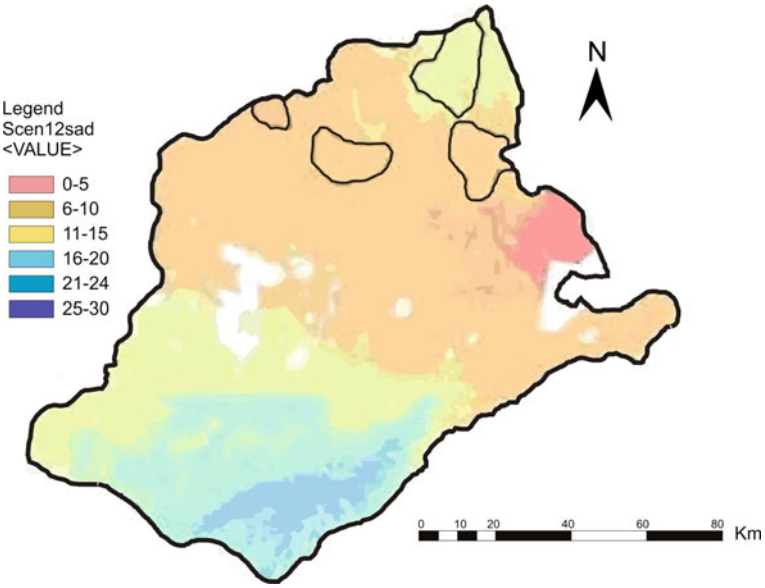

Fig. 7.2 Spatial output layer of the Corangamite catchment for mean annual increment in stem volume (MAI, m^3 ha^{-1} y^{-1}) of *P. radiata* (30 years, sawlog, medium fertility) from 3-PG_2S

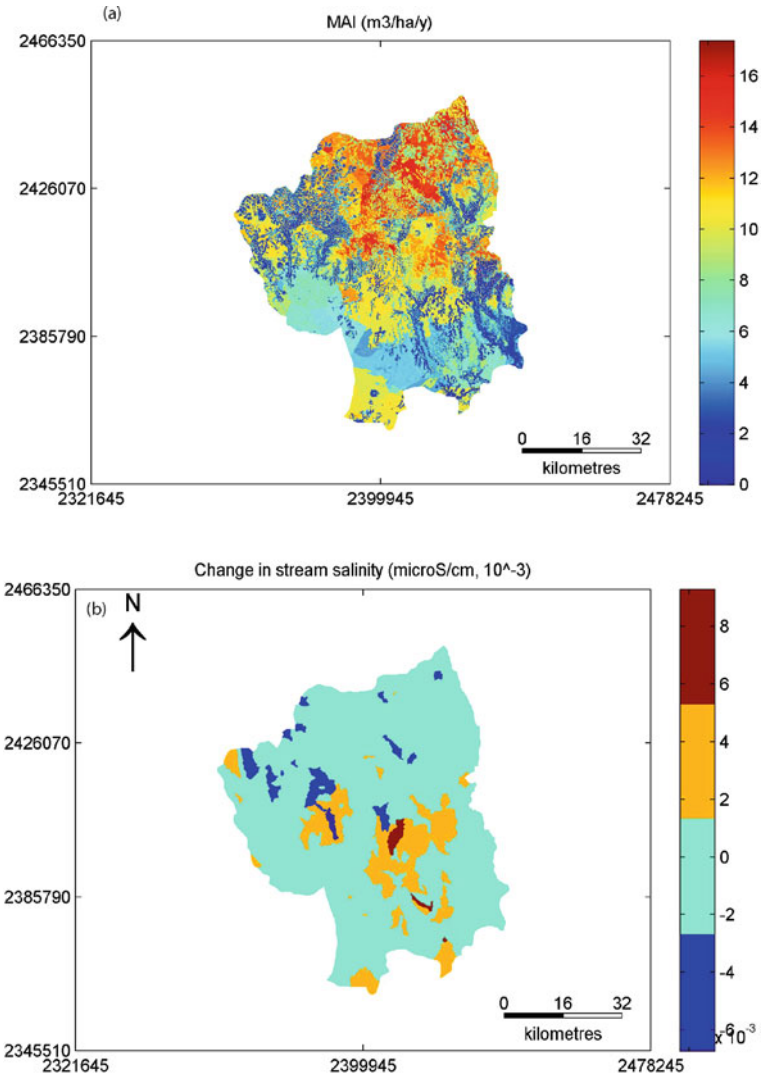

Fig. 7.3 Spatial output layer for the northern part of the Corangamite catchment from CAT_3-PG+ modelling for (**a**) mean annual increment in stem volume (MAI, $m^3\ ha^{-1}\ y^{-1}$) and (**b**) change (positive number means an increase and negative number means a decrease) in stream salinity ($\mu S\ cm^{-1} \times 10^{-3}$) for *P. radiata* (30 year sawlog rotation, initial stocking of 1500 stems per ha, final stocking of 250 stems per ha)

Effects of forestry scenarios on stream flow and salt load varied with species, scenario and sub-catchment. This means that there will be trade-offs between the reduction in stream flow and the salinity of these streams for different parts of the catchments depending on which species is planted and whether the system is a long (e.g., sawlog) or short (e.g., pulpwood) rotation. Generally, by planting trees in those

parts of the catchment where water moves more freely and salts are more prevalent, there will be a tendency for greater reduction in movement of salts and water to streams. However, stream salinity will vary with the relative impact of stream flow and salt load. Modelling results would also be expected to differ if only certain parts of landscape within the catchment were targeted for forestry.

For all the scenarios that were run it was predicted that plantations reduced stream salinity but also stream flow, the extent dependent on which species and scenario was tested. Example spatial outputs are presented here for *P. radiata* (30 year rotation for sawlog production) for (i) estimated stem volume growth (using 3-PG2S) over the entire Corangamite sub-catchment (Fig. 7.2), and (ii) estimated growth and change in stream salinity[1] (using CAT_3-PG+) for the northern part of the Corangamite catchment (Fig. 7.3a, b). Shortcomings in 3-PG+ include an inability to include more than one thinning as a management option (3-PG2 overcomes this), and effects of soil salinity and groundwater were not accounted for.

4 Conclusions

The plantation growth model, 3-PG, which was modified, calibrated and extensively verified, has been applied at catchment and farm scales to provide spatial estimates of productivity, carbon sequestration and salinity impacts of tree planting on farms. Within scenarios, stream salinity was predicted to decrease in some parts of the region and increase in others. Information obtained from modeling approaches coupled with further field studies provides land managers and government agencies with increased confidence and flexibility in making land use decisions, especially with respect to forestry.

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[1] 'End-of-valley' stream salinity (EC in $\mu S\ cm^{-1}$) was calculated by dividing salt load by stream flow.

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Chapter 8
Structural and Functional Adaptations in Plants for Salinity Tolerance

Mansoor Hameed, Muhammad Ashraf, Muhammad Sajid Aqeel Ahmad, and Nargis Naz

Abstract Salt tolerance in plants is a multifarious phenomenon involving a variety of changes at molecular, organelle, cellular, tissue as well as whole plant level. In addition, salt tolerant plants show a range of adaptations not only in morphological or structural features but also in metabolic and physiological processes that enable them to survive under extreme saline environments. Morpho–anatomical adaptations include xeromorphic characteristics like thick epidermis and sclerenchyma, well developed bulliform cells, increased density of trichomes and increased moisture retaining capacity by increasing cell size and vacuolar volume. Development of excretory structures like vesicular hairs and salt glands is another major structural adaptation and very crucial for salt tolerance. Physiological adaptations include restricted toxic ion uptake, increased succulence, osmotic adjustment and exclusion of toxic Na^+ and Cl^-.

Keywords Succulence · Osmotic adjustment · Salt exclusion · Ion uptake

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Contents

1 Introduction

Soil salinity is among the major abiotic stresses that limits crop productivity worldwide (Hu et al. 2005) since most crops are sensitive to soil salinization (Munns 2002). There are two major processes of soil salinization; geo–historical processes and man–made. Most of the worldwide salt–affected lands are the result of natural causes, i.e., from accumulation of salts over long time period, and this occurs mainly in arid and semiarid zones (Rengasamy 2002). One way of soil salinization is weathering of the rocks that releases soluble salts, which is mainly in the form of sodium chloride and calcium chloride (Szabolcs 1989), other being salt accumulation due to the deposition of salts from oceans by wind or rain (Munns and Tester 2008). Man–made saline soils are mostly found in (semi) arid lands as a result of over-irrigated agriculture, and hence in the rise of water tables. This is the main factor of increasing salinity in agricultural lands (Munns et al. 2002).

Soil salinity is an ever–increasing problem worldwide and it is estimated that the saline soils approach 930 million ha, about 7 percent of the total land worldwide (Szabolcs 1994). Nearly, one third of the total 230 million ha under irrigation is uncultivable due to soil salinity (Oldeman et al. 1991; Ghassemi et al. 1995). Of this total, 15.57% is located in Africa, 5.07% in Australia, 0.57% in Mexico and Central America, 1.80% in North America, 20.21% in South America, 26.70% in North and Central Asia, 24.25% in Southern Asia, and 5.82% in Southeast Asia (Massoud 1974). A large number of plants are found to grow on these areas but tolerance varies greatly not only among species but also within species. Among monocotyledonous crop plants, rice is the most sensitive, bread wheat moderately tolerant and barley the most tolerant. The halophytic tall wheatgrass, a relative of wheat is one of the most salt tolerant of all monocots (Munns and Tester 2008). In dicots, salinity tolerance is even more diverse. For example, some legumes are even more sensitive than rice (Läuchli 1984). Alfalfa is relatively tolerant to salt, and halophytes for example some *Atriplex* spp. grow well at extremely high salinities (Flowers et al. 1977).

There is a wider range of salt tolerance in natural populations, which is reported to be evolved naturally in numerous grass species like *Agrostis*, *Festuca*, *Lolium*, and *Poa* (Humphreys et al. 1986; Acharya et al. 1992). Such plants provide outstanding materials for studying the mechanisms of adaptations they use to tolerate

high concentrations of salt (Ashraf 2003). Such adaptations have been evaluated in several grass populations from quite diverse habitats such as estuaries and coastal areas, marine and fresh water salt marshes, and dry–land salinities. Examples are *Sporobolus virginicus* (Naidoo and Mundree 1993), *Cynodon dactylon* (Pasternak et al. 1993; Hameed and Ashraf, 2008), *Spartina patens* (Ashour et al. 1997), *Urochondra setulosa* (Gulzar et al. 2003), *Ochthochloa compressa* and *Aeluropus lagopoides* (Naz et al. 2009), and *Imperata cylindrica* (Hameed et al. 2009).

The main objectives of this chapter are to present the physio–biochemical aspects of salinity tolerance in naturally adapted salt tolerant plants and to correlate them to the structural adaptations found in different plants to cope with highly saline adverse environments.

2 Adaptive Components of Salt Tolerance

Salt tolerance is a complex phenomenon involving a variety of mechanisms. It can be defined as the ability of the plants to complete their growth cycle with an acceptable growth and yield (Flowers et al. 1986; Colmer and Flowers 2008). Three major factors affect the plant growth under salinity, water stress, ion toxicity, and nutrient uptake and translocation, and as a result, disturbance of ionic balances such as K^+ and Ca^{2+}. Physiological drought may play a crucial role, which restricts the water uptake by plants (Table 8.1). On contrary, excess salt uptake by plants interrupts the cellular functions and this damages vital physiological processes, i.e., photosynthesis and respiration (Marschner 1995). Furthermore, mechanisms like increased leaf resistance (fewer stomata, increased cuticle and epidermis thickness, and mesophyll resistance) could prevent turgor loss from leaf and root surface, and hence better water efficiency.

Plant tolerance to saline environments is of broad spectrum ranging from glycophytes (that are sensitive to salt) to halophytes (that tolerate high concentrations of salt). The acquired salt tolerance may be of hereditary nature in some species (Niknam and McComb 2000), i.e., passed along to offspring. Halophytic or salt tolerant species can adopt multiple strategies to survive under high salinities by controlling the levels of ions their shoots or particularly in leaves. The mechanisms involved are restricting or excluding the ion uptake at root level, and hence minimizing the translocation of salts to the shoot (Flowers and Colmer 2008).

Genkel (1954) divided the halophytes into three groups: euhalophytes, crinohalophytes, and glycohalophytes, but this classification has been modified by Nagalevskii (2001) and Zhao et al. (2002). Salt tolerance in euhalophytes is based on accumulation, as they accumulate salts in their tissues, crinohalophytes depend on excretion of toxic ions like Na^+ and Cl^- as they are capable of excreting salts out of the plant body, and glycohalophytes rely on avoiding mechanism by preventing the accumulation of excess salts (Voronkova et al. 2008). The growth rate can be linked to the accumulation of salts in the plant leaves that plant takes up from the roots, so the continuation of growth under saline environments is an indication of high degree of salt tolerance.

Table 8.1 Physiological and biochemical mechanisms of salt tolerance in some highly salt tolerant or halophytic plant species

Plant species	Ion uptake and transport	Osmotic adjustment	Ion exclusion
Monocots			
Aeluropus lagopoides	Restricted uptake of Na^+ and Cl^- (Naz et al. 2009), and increased uptake of K^+ and Ca^{2+}	Accumulation of Na^+ and Cl^- in shoot, in addition to retention of K^+ and Ca^{2+} (Naz et al. 2009)	Excretion of only Na^+ and Cl^- ions (Naz et al. 2009)
Cymbopogon jwarancusa	Increased uptake of Ca^{2+}, and increased K^+ in shoots	Accumulation of total free amino acids and soluble proteins	
Cynodon dactylon	Restricted uptake of Na^+ accompanied by high uptake of K^+ and Ca^{2+} (Hameed and Ashraf 2008)	Accumulation of soluble sugars, proline and total free amino acids (Hameed and Ashraf 2008)	Excretion of Na^+ and Cl^-
Imperata cylindrica	Increased uptake of Ca^{2+}	Accumulation of total free amino acids and proline	
Lasiurus scindicus	Increased uptake of Ca^{2+}		
Ochthochloa compressa			Excretion of Na^+ and Cl^-
Panicum antidotale		High water use efficiency (Hameed and Ashraf 2009)	
Sporobolus arabicus	Restricted uptake of Na^+ and Cl^-	Accumulation of free amino acids and proline	Excretion of Na^+ and Cl^-
Sporobolus ioclados	Restricted uptake of Na^+ and Cl^-	Accumulation of free amino acids, soluble proteins and soluble sugars	Excretion of Na^+ and Cl^-
Dicots			
Cressa cretica	Restricted uptake of Na^+ and Cl^-		Excretion of Na^+
Fagonia indica		Accumulation of Na^+ and Cl^- in shoot, in addition to retention of K^+ and Ca^{2+}	
Haloxylon recurvum	Increased uptake of Na^+ and Cl^-	Dumping off Na^+	Excretion of Cl^-
Haloxulon salicornicum		Dumping off Na^+ and Cl^-	
Salsola baryosma		Dumping off Na^+ and Cl^-	
Suaeda fruticosa		Dumping off Na^+ and Cl^-	

Morphological features of the plant roots can prevent salts in large quantities. At cellular level, physiological and metabolic features can counteract salts if salts do enter the roots (Winicov 1998). Plants generally use two mechanisms to tolerate high salt concentrations. Firstly, the avoidance, i.e., keeping the salts away from the metabolically active tissues (Munns and Tester 2008). This is through passive exclusion of ions (by a permeable membrane), active expelling of ions (by ion pumps), or by dilution of ions in plant tissues (Allen et al. 1994). Secondly, compartmentalization of accumulated salts in the vacuoles of plant cells (Munns 2002). These two methods are vital for preventing toxic ions to accumulate or causing damage to the plant tissues, and therefore, they could be employed for identifying markers for genetic manipulation of salinity tolerance in plants.

Salt tolerant or halophytic plants can minimize the detrimental effects of salts (i.e., ion toxicity, nutritional disorder, osmotic stress) by modifying morphological, anatomical and physiological mechanisms of salt tolerance (Poljakoff-Mayber 1975; Hameed et al. 2009). Extensive root system (root length and proliferation) and the presence of salt secreting structures (e.g., salt glands) on the leaf surface may prove vital in plants (Marcum et al. 1998; Naz et al. 2009). The salt tolerance of plants may involve: (a) restricted or controlled uptake of salts, (b) tissue tolerance, (c) accumulation of salt in inert areas (e.g., vacuoles), (d) ion discrimination (e.g., uptake and translocation of ions like K^+, Na^+, Cl^- and SO_4^{2-}), (e) production of low molecular weight protective osmolytes like enzymes, hormones, antioxidants, etc. (Gorham and Jones 1990; Munns and Tester 2008). These mechanisms may be responsible for variations in the salt tolerance within plant genotypes or species (Table 8.1).

Soil reclamation is a very expensive and physically difficult process to practice. However, cultivation of salt tolerant species/varieties is the most practical solution, particularly when salinity is relatively low. When a plant is exposed to increased soil salinity, a primary response is decreased plant water potential, and this is due to a decrease in both osmotic and water potentials of the soil. Accumulation of osmotically compatible cellular solutes (e.g., sugars, proteins, free amino acids) is one of the well–characterized responses of plants to such low water potential. In salt tolerant species, accumulation of osmotically compatible solutes directly correlates with Na gradients in soil and thereby reduces the detrimental effect of salt stress (Briens and Larher 1982; Lee et al. 2007). Mechanisms involved in salinity tolerance or adaptations crucial for the plant survival are still not well understood. Therefore, there is a need to identify appropriate morpho-anatomical or physio-biochemical indicators of salinity tolerance in halophytic and other salt tolerant plants (Ashraf and Harris 2004).

2.1 Morphological Traits

Salinity–induced changes in root morphology, anatomy, and ultrastructure as well as some physiological implications of the altered growth patterns have been reviewed earlier at length. Excess salinity has been reported to inhibit both root cell division

and cell expansion (Zidan et al. 1990). Generally, in glycophytes, root growth is less affected by salinity than either vegetative shoot growth or fruit and seed production (Maas and Nieman 1978). Depending on the type of plant species, the level of salinity stress and the composition of the external solution, root growth may be stimulated, inhibited or unaffected (Delane et al. 1982, Waisel 1985).

Soil salinity directly affects plant growth and development, especially crop species (Chinnusamy et al. 2005; Ashraf 2009). In general, dicotyledonous halophytes show optimal growth up to 250 m*M* NaCl (Flowers et al. 1986). However, in monocotyledonous halophytes growth is generally not simulated by salts or if so, then it is at 50 m*M* NaCl or less (Glenn 1987; Glenn et al. 1999). Rooting parameters (depth, proliferation and weight) are reported to be associated with salinity tolerance. Root weights increase under salinity in the grasses (Marcum et al. 1998). Shoot biomass production in highly salt tolerant species like *Leptochloa fusca* and *Puccinellia distans* is not affected by salinity. On the other hand, *Pennisetum divisum* has the lowest fresh and dry biomass of both shoots and roots and is very sensitive to salinity stress (Ashraf and Yasmin 1997).

2.2 Anatomical Traits

Both halophytes and non–halophytes exhibit remarkable anatomical changes when exposed to elevated levels of salinity (Maas and Nieman 1978). However, most conspicuous changes are notable in leaf. Longstreth and Nobel (1979) reported a smaller increase in the mesophyll area/leaf area in *Atriplex patula* (halophyte) than that in *Phaseolus vulgaris* and *Gossypium hirsutum* (both glycophytes). This reveals a greater tendency of *Atriplex* to maintain constant mesophyll area, and is an adaptive feature which reflects greater degree of shielding to photosynthetic mechanisms from harmful effects of salts. Zoysiagrass (*Zoysia* spp.) does not show any change in the density of salt glands when grown under salinity (Marcum and Murdoch 1990). Enhanced salt tolerance of *Zoysia* spp. is proportional to a greater density of salt glands in different species (Figs. 8.1 and 8.2) followed by exclusion of shoot ions through leaf glands (Marcum et al. 1998).

Many salt tolerant plants, particularly dicotyledonous halophytes are characterized by xeromorphic characteristics (Table 8.2) such as thick succulent leaves (Fig. 8.3), which apparently aid sufficient water supply (Vakhrusheva 1989). Smaller reduced leaves with dense covering of pubescence are also a characteristic of xerophytes, which accounts for a successful survival of halophytes under dryland salinities (Mokronosov and Shmakova 1978).

Salt secretion by micro-hairs has been detected only in certain Chloridoideae, all having ‘chloridoid type’ micro-hairs with basal cell partitioning membranes. It has not been detected in many species with micro-hairs lacking basal cell partitioning membranes. For example, the ‘chloridoid type’ micro-hairs of *Sporobolus elongatus* and *Eleusine indica* do not secrete salt, despite their possession of partitioning membranes (Amarasinghe and Watson 1988). At leaf level, there are certain appendages which help the plant to secrete excess salts from the main body. Most important

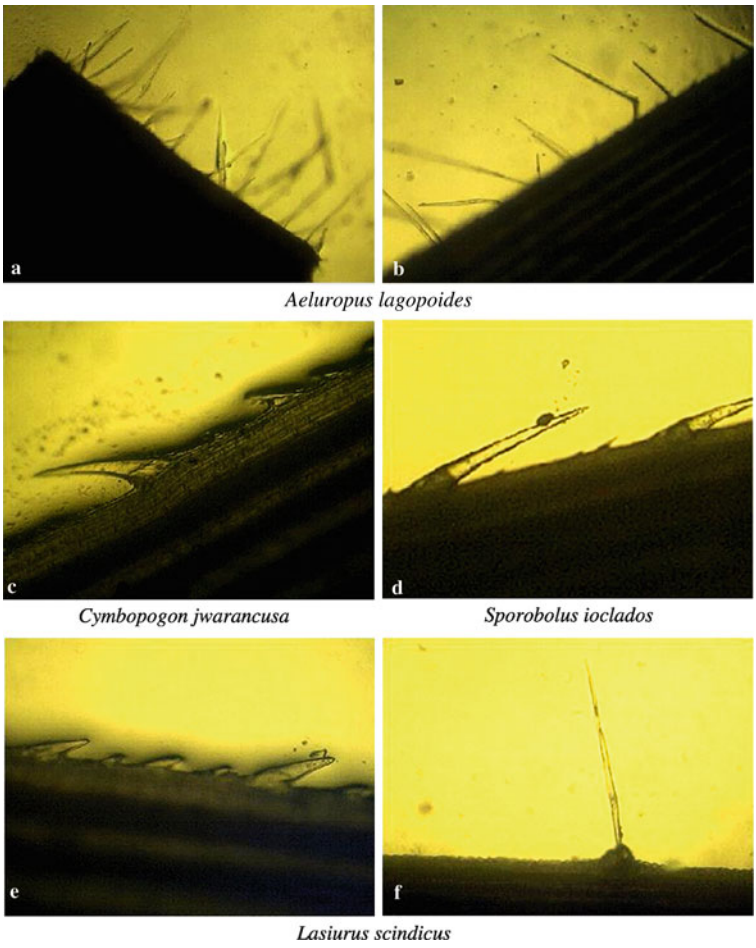

Fig. 8.1 **a** Dense hairiness in *Aeluropus lagopoides* on leaf surface. **b** Glandular and simple hairs on leaf margins in *A. lagopoides*. **c** Marginal hairs in leaf of *Cymbopogon jwarancusa.* **d** Salt secretory hairs on leaf margins. **e** Marginal hairs in leaf of *Lasiurus scindicus*, and **f** Glandular hairs on leaf surface in *L. scindicus*

among these are salt secretory trichomes (e.g., *Atriplex* spp.), second type is multicellular salt glands which occur in many desert and coastal habitat flowering plants, and are confined to the members of families including Poaceae, Aveceniaceae, Acanthaceae, Frankeniaceae, Plumbaginaceae and Tamaricaceae (Mauseth 1988; Thomson et al. 1988; Marcum and Murdoch 1994). In contrast, the stem of halophyte *Salicornia fruiticosa* has a simple cortex and single layered epidermis which is thin–walled and the photosynthetic tissue has palisade and parenchymatous cells for storage of water (Fahn 1990).

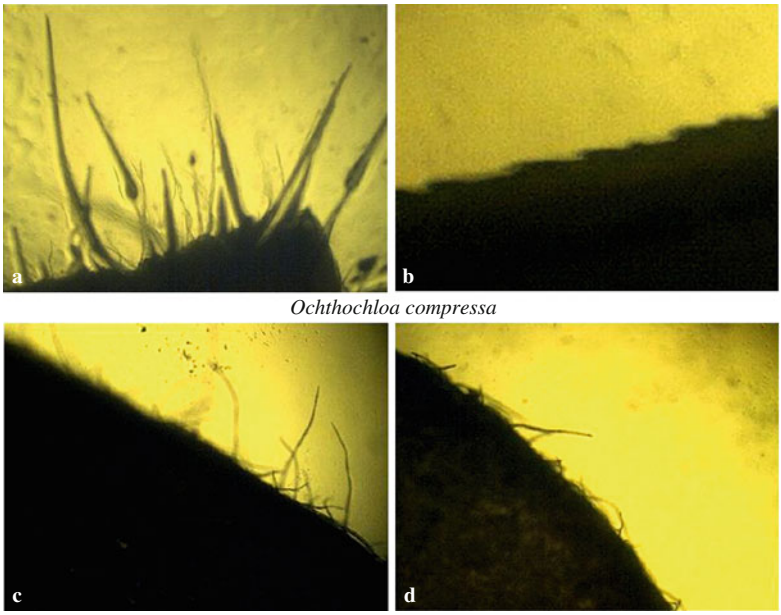

Fig. 8.2 **a** Dense hairiness in *Ochthochloa compressa* on leaf surface with a mixture of glandular and simple hairs and trichomes. **b** Marginal hairs on leaf in *O. compressa*. **c** Dense hairiness in *Cressa cretica* on leaf surface, and **d** Leaf margins

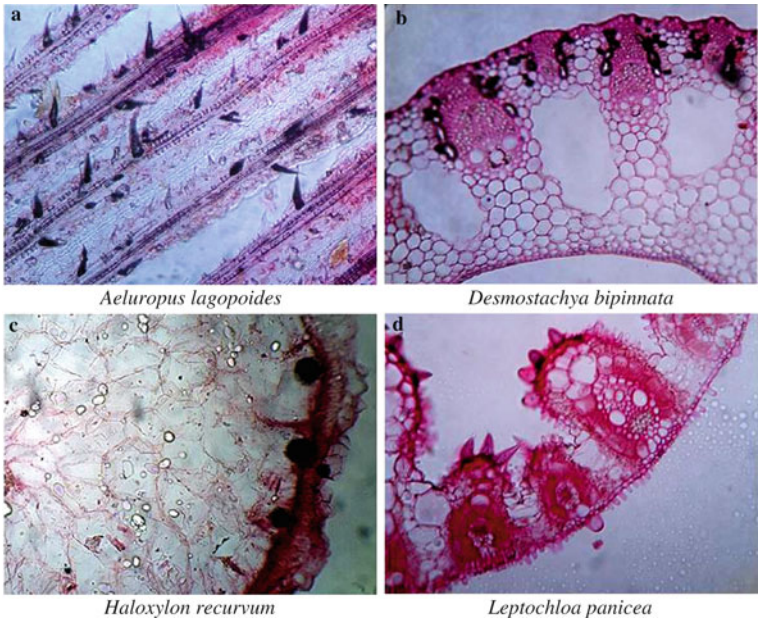

Fig. 8.3 **a**, **e** Dense cover of leaf trichomes in *Aeluropus lagopoides.* **b** Aerenchyma in leaf sheath in *Desmostachya bipinnata*. **c** Leaf succulence in *Haloxylon recurvus*, and **d** Dense cover of microhairs on both leaf surfaces and trichomes on adaxial surface in *Leptochloa panicea*

Table 8.2 Anatomical mechanisms of salt tolerance in some highly salt tolerant or halophytic plant species

Plant species	Development of xeromorphic characteristics	Structural modifications to salt stress	Salt excretory structures
Monocots			
Aeluropus lagopoides	Dense hairiness on both leaf surfaces as well as leaf margins, and increased sclerification in stems	Increased sclerification in root outside endodermis	Ion exclusion through micro hairs
Cymbopogon jwarancusa	Increased sclerification in stem and leaf and increased trichome density		
Cynodon dactylon	Increased hairiness (trichomes)		Ion exclusion through micro hairs
Imperata cylindrica	Succulence in leas midrib, highly developed bulliform cells, increased sclerification in leaf and root, and reduced stomatal density and pore area (Hameed et al. 2009)	Formation of aerenchyma in leaf sheath, increased area of vascular tissue, and enlarged bulliform cells (Hameed et al. 2009)	
Lasiurus scindicus	Increased sclerification in stem and development of bulliform cells	Increased sclerification in roots	
Ochthochloa compressa	Dense hairiness on adaxial leaf surface and leaf margins		Ion excretion through salt glands and micro hairs
Panicum antidotale	Development of bulliform cells and extensive leaf rolling, and reduction in stomatal density and size (Hameed and Ashraf 2009)		

Table 8.2 (continued)

Plant species	Development of xeromorphic characteristics	Structural modifications to salt stress	Salt excretory structures
Sporobolus arabicus	Increased succulence and sclerification in stem	Development of aerenchyma in leaf sheath	Ion exclusion through micro hairs and leaf sheath
Sporobolus ioclados	Increased sclerification below exodermis and increased leaf hairiness	Increased endodermis thickness	Ion exclusion through micro hairs
Dicots			
Cressa cretica	Increased sclerification in stem and increased cuticle and epidermis thickness in leaves		
Fagonia indica	Increased succulence in leaves		
Haloxylon recurvum	Increased succulence and sclerification in stem, and increased succulence in leaves		
Haloxulon salicornicum	Increased sclerification in stem		
Salsola baryosma	Increased succulence in stem		
Suaeda fruticosa	Increased succulence in leaves		

Stomatal features like density and size are critical for controlling transpirational loss from leaf surface and even more critical under physiological droughts (Hameed et al. 2009). The importance of stomatal characteristics in avoiding water loss through leaf surface has been reported several species like *Distichlis spicata* (Kemp and Cunningham 1981), barley (Gill and Dutt 1982), and wheat (Akram et al. 2002).

The roots of saline desert plants have reduced cortex to shorten the distance between epidermis and stele. The casparian strip is much wider in the highly dry and salt marsh habitat plants, as compared to mesophytes. In saline habitat plants, the endodermis and exodermis (hypodermis with casparian band) represent barriers (Fig. 8.4) of variable resistance to the radial flow of water and ions from cortex

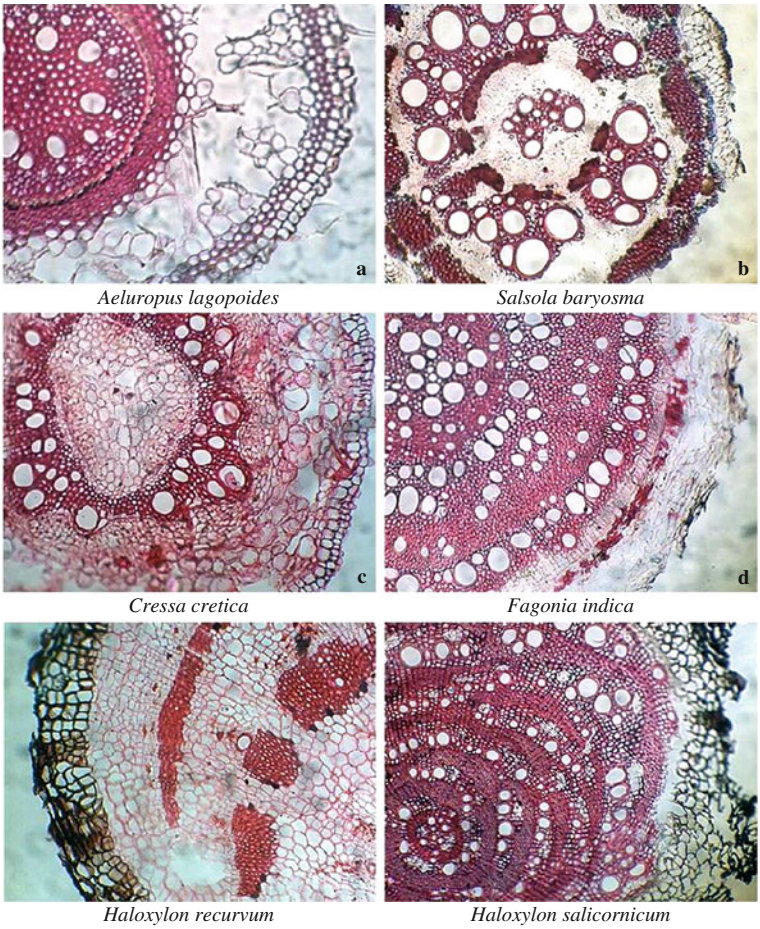

Fig. 8.4 Roots **a** Sclerification surrounding vascular region above endodermis and in vascular region in *Aeluropus lagopoides,* **b** Sclerification in patches in vascular regions with unusually large metaxylum vessels in *Salsola baryosma* and **e** *Haloxylon recurvum*, **c** Sclerification of vascular region with highly developed storage parenchyma in pith and cortical regions in *Cressa cretica,* Highly sclerified central region in **d** *Fagonia indica* and **f** *Haloxylon recurvum*

to the stele under prevailing conditions (Hose et al. 2001; Taiz and Zeiger 2002). Such adaptation is advantageous for efficient functioning of endodermis, when the protoplasts are attached to the large portions of the radial and transverse walls of endodermal cells (Fahn 1990).

Drought avoidance is a vital adaptive strategy against salt stress. Modifications like highly developed bulliform cells (important for leaf rolling) can play an important role in avoiding water loss during physiological drought caused by salinity (Abernethy et al. 1998; Alvarez et al. 2008). Thick epidermis is a characteristic feature of many salt tolerant terrestrial species (Ristic and Jenks 2002) and this is one of the most valuable mechanisms relating to xeric adaptation to prevent water loss (Jenks and Ashworth 1999; Zhao et al. 2000).

Root aerenchyma is reported to be a distinctive attribute of waterlogged plants. Colmer and Flowers (2008) summarized characteristics of aerenchyma in halophytic species, but this is exclusively under waterlogged conditions. Aerenchyma formation in halophytes may aid in efficient solute transport in addition to oxygen (Hameed et al. 2009). Increased sclerenchyma under salinity stress not only provides rigidity to the tissues or organs, but also vital for reducing water loss through plant surface. Increased sclerification has been reported by several researchers in salt tolerant or halophytic plants, e.g., *Spartina alterniflora* (Walsh 1990), *Puccinellia tenuiflora* (Zhao et al. 2000), and *Prosopis strombulifera* (Reinoso et al. 2004).

2.2.1 Succulence

Succulence (both leaf and stem) is one of the most noticeable features in halophytes, which provides not only more space for dumping off toxic ions in the plant body, but also increasing the total plant water content (Waisel 1972; Drennan and Pammenter 1982), and this is crucial for balancing out ion toxicity. Leaf succulence is very rare in monocots (Hameed et al. 2009), but relatively common in dicots, such as *Kandelia candel* (Hwang and Chen 1995) and many other halophytes (Flowers and Colmer 2008). It is not very much clear as succulence is simply a response to salinity or is the response of adaptive value of halophytic plants (Waisel 1972).

Increased succulence in halophytes in response to increasing salinity is presumed to be of adaptive nature (Waisel 1972). Succulence is very much greater in halophytic dicotyledonous species than in monocotyledonous ones (Flowers et al. 1986). There is also evidence of a rapid increase in vacuolar volume and in the concentration of Na^+ (Mimura et al. 2003) in the cells of mangrove *Bruguiera sexangula*, which is a potential mechanism to cope with a rapid increase in external salt concentration.

2.2.2 Salt Excretion

Halophytes utilize salts in osmotic adjustment, which lowers water potentials of their tissues. Accumulation of toxic ions in large quantities in leaves, while avoiding their toxic effects seems to be an important strategy for growth and survival

under harsh climates (Greenway and Munns 1980). Balancing of growth and ion accumulation is the major phenomenon of salt tolerance in some species, while in others excess of toxic ions is secreted via secretory structures like salt glands and micro-hairs (Drennan and Pammenter 1982; Flowers and Yeo 1986). *Spartina* spp. are the example where shoot mineral content is regulated by the ionic secretion through specialized salt glands. Salts are also released by the leaf surface through cuticle or in guttation fluid; but they also become concentrated in salt hairs (Stenlid 1956).

Many species exude Na salts onto the leaf surface (Drennan and Pammenter 1982; Marcum et al. 1998; Naidoo and Naidoo 1998), which is effective in reducing Na concentration in plant tissues, i.e., *Sporobulus* spp. (Lipschitz and Waisel 1974; Marcum and Murdoch 1992). Salt secretory trichomes, characteristic of *Atriplex* spp., are bladder–like hairs projecting out of leaf surface. They consist of a large secretory or bladder cells on the top and a stalk consisting of one or sometimes a few cells (Samoui 1971; Dickison 2000). All these cells contain mitochondria, dictyosomes, ribosomes, endoplasmic reticulum and a large flattened nucleus. The chloroplasts are rudimentary or partially developed. The only difference lies in that a single large vacuole is present in bladder cell and many small vacuoles in the stalk cell (Osmond et al. 1969). A symplastic continuum exists from the mesophyll cells to the bladder cells for the movement of ions. The external walls of bladder and stalk cells are cutinized, while inner primary walls are not (Thomson and Platt-Aloia 1979).

In grasses, the glands are generally bi–celled, i.e., an outer cap cell and a subtending basal cell. They may be sunken, subsunken, extending out of epidermis (Lipschitz and Waisel 1974; Marcum and Murdoch 1994) or lie in bands or ridges (Marcum et al. 1998). In dicotyledonous species, the salt glands are multi–cellular, consisting of basal and secretory cells. The number of cells may vary from 6 up to 40 in different genera (Fahn 1990). For example, in *Tamarix* spp. the salt glands consist of two basal collecting cells and outer six highly cytoplasmic secretory cells (Mauseth 1988). However, the glands of *Avicennia* and *Glaux* comprise several secretory cells positioned above a single disc–shaped basal cell (Rozema et al. 1977). The position of the epidermal glands may be lateral (*Tamarix*), present in epidermal depression (*Glaux*) or projecting out of abaxial surface of leaf–like trichomes in *Avicennia* (Thomson et al. 1988).

2.3 Physiological/Biochemical Traits

Salinity causes many adverse effects on plant growth which may be at physiological or biochemical levels (Munns 2002; Munns and James 2003), or at the molecular level (Mansour 2000; Tester and Davenport 2003). In order to assess the tolerance of plants to salinity stress, growth or survival of the plant is measured because it integrates up– or down–regulation of a variety of physiological mechanisms (Niknam and McComb 2000). Cell growth rate depends on cell wall extensibility as well as turgor (Lockhart 1965).

2.3.1 Osmotic Adjustment

Accumulation of exceptionally high concentrations of inorganic ions as well as organic solutes is an important physiological adaptation in both halophytic and salt tolerant species (Pitman 1984). In salt excretory plants, salt is kept away from photosynthesizing or meristematic cells. In these plants, osmotic balance is generally achieved via extensive accumulation of organic solutes and/or inorganic ions. However, in plants where salt inclusion is the prime mechanism, accumulation of some inorganic ions (predominantly Na^+ and Cl^-) regulates the osmotic adjustment (Wyn Jones and Gorham 2002; Ashraf 2004). Both organic and inorganic solutes are essential for osmoregulation in plants, especially under saline environments. However, their relative contribution to osmotic adjustment varies from plant to plant or species to species, or even within different tissue of the same plant (Ashraf 1994; Ashraf and Bashir 2003; Hameed and Ashraf 2008).

There is a variety of compatible osmolytes in higher plants. Important among these are soluble sugars, organic acids, and soluble proteins. The important amino acids that accumulate in the plants are alanine, arginine, glycine, leucine, serine, and valine, along with the imino acid proline, citrulline and ornithine (Rabe 1990; Mansour 2000; Ashraf 2004). Osmoregulation via accumulation of free amino acids and in particular, glycinebetaine is the principal strategy in many plant species to tolerate salt stress (Martino et al. 2003). Amides such as glutamine and asparagine (Dubey 1997; Mansour 2000), and proline (Ashraf 1994; Abraham et al. 2003) have also been reported to accumulate in large amounts in higher plants in response to salt stress.

2.3.2 Ion Selectivity

A major feature of the solute transport by plants in saline conditions is the degree of selectivity, particularly between potassium and sodium (Ashraf et al. 2005). One of the most important physiological mechanisms of salt tolerance is the selective absorption of K^+ by plants from the saline media (Ashraf et al. 2006). Halophytic or salt tolerant species differ from salt–sensitive ones in having restricted uptake or transport of Na^+ and Cl^- to the leaves despite an effective compartmentalization of these ions. This is critical in preventing the build–up of toxic ions in cytoplasm (Munns 2002; Ashraf 2004). Ion imbalance, particularly that caused by Ca^{2+} and K^+ is the most important and widely studied phenomenon affected by salt stress, which is directly influenced by the uptake of Na^+ and Cl^- ions (Munns 2002 Munns et al. 2006). Maintaining better concentrations of K^+ and Ca^{2+} and limiting the Na^+ uptake are vital for the salt stress tolerance in plants (Karmoker et al. 2008). Higher K^+/Na^+ or Ca^{2+}/Na^+ ratios are characteristic to the tissue salt tolerance, and are often used as a screening criteria for the salt tolerance (Munns and James 2003, Ashraf 2004; Song et al. 2006).

2.3.3 Salt Exclusion

Halophytes or highly salt tolerant plants have both types of mechanisms that enable them to survive and grow for long times in saline soils. They exclude salts efficiently in addition to effective compartmentalization of the salts in vacuoles. Glycophytes, on the other hand, exclude the salts but they are unable to compartmentalize them. The mechanism of salt exclusion involves transport of salts to the leaves and subsequently excreted out of the plant body thereby reducing salt concentration in plant tissues. Salts translocated in the transpiration stream are deposited and their concentration increases with time. This results in much higher salt concentrations in older leaves than those in younger leaves. Mechanisms conferring salt exclusion (both at cellular and whole plant levels) have been reviewed by many authors (Greenway and Munns 1980; Storey and Walker 1999; Jeschke 1984). Salt exclusion is the most important adaptive strategy regulating the internal salt load of halophytes. As an example, about 98% of salt was reported to be excluded in the mangrove species *Avicennia marina* growing in 500 m*M* NaCl (Ball 1988). In perennials, exclusion is particularly important and it is more vital to regulate the incoming salt load in the plant body (Amtmann and Sanders 1999; Hasegawa et al. 2000).

2.3.4 Intracellular Ion Compartmentation

Sequestering of Na^+ and Cl^- in the vacuoles of the plant cells is ideal situation for plants under salt stress. Exceptionally, high concentrations of salts are found in leaves, which still function normally. Concentrations well over 200 m*M* are common in halophytic or highly salt tolerant species, and such concentrations will severely inhibit the activity of several enzymes in vivo (Munns and Tester 2008).

2.3.5 Stomatal Responses

Although there are few data available on stomatal responses of different plant species, it is possible to identify two types of stomatal adaptations to increasing salinity (Flowers et al. 1997): the guard cells can utilize sodium instead of potassium to achieve their normal regulation of turgor (Ashraf 1994), or the ionic selectivity of the guard cells that use potassium and are capable of limiting the sodium intake (Robinson et al. 1997). This mechanism may be very important in non-secretory halophytes that lack secretion mechanisms, and it may therefore be of particular interest as a potential contributor to the development of salt tolerance in crops. Sodium can substitute for potassium in the stomatal mechanism (Flowers and Colmer 2008). In *Suaeda maritima*, sodium is the major cation under salinity in the guard cells of closed stomata (Flowers et al. 1989). Stomatal regulation by sodium provides a vital regulatory mechanism for the control of excessive salt translocation in the shoot, when a plant capacity to compartmentalize increases. In glycophytes,

accumulation of sodium ions damages the stomatal function, and this disruption supports their lack of survival under saline conditions (Robinson et al. 1997).

3 Conclusion

Salt tolerant plants adapt specific structural and physiological modifications to cope with high salinities. Morpho–anatomical adaptations include the prevention of undue water loss from the plant by the development of thick epidermis and sclerenchyma, well developed bulliform cells for extensive leaf rolling, and increased density of trichomes, and this is vital in water limiting environment under high salinities. Increased moisture retaining capacity is the other adaptive feature which is critical under physiological drought due to salinity stress. Development of excretory structures like vesicular hairs and salt glands is a major structural adaptation and very crucial for salt tolerance. Physiological adaptations include restricted toxic ion uptake at root level. At cell level, succulence is crucial for dumping off toxic ions in relatively inert areas like vacuoles. Toxic ions like Na^+ and Cl^- are important for osmotic adjustment in highly salt tolerant species. Lastly, the most important point is that ion exclusion which is one of the most vital phenomena for high salt tolerance in plants.

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Part II
Phytoremediation

Chapter 9
Plant Resistance to Anthropogenic Toxicants: Approaches to Phytoremediation

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Abstract The problem of soil preservation and restoration has became more intense due to continued deterioration of the ecological systems of the world. This problem is especially important for Azerbaijan, where environmental pollution by heavy metals and oil products is increasing. Though the nature of toxicity of these two factors is different, they both affect plant productivity, including agricultural crops and human health. This review is devoted to the analysis of modern conceptions on fundamental physiological mechanisms of plant resistance to toxic levels of heavy metals and organic pollutants in soils, also of their uptake and translocation in plants. Different aspects of the nature of toxicity of metals and petroleum hydrocarbons and genetic basis of plant resistance to them, hyperaccumulation mechanisms of heavy metals by some plant species and approaches to phytoremediation of both inorganic and organic pollutants are discussed.

Keywords Contamination · Heavy metals · Petroleum hydrocarbons · Toxicity · Resistance · Phytoremediation

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Contents

1 Introduction

Natural and anthropogenic pollution caused by various chemical contaminants to date is at the cutting edge of ecological problems requiring an active special interference of humanity for their solution. Environmental contamination is a critical factor potentially affecting plant productivity and causing a large risk not only to plants, but also to human and animal health. Every year, owing to over population in a number of countries, as well as an intensification of anthropogenic activities such as industrial and urban growth and increasing of oil–and–gas production, thermal power plants and vehicles use, chemicalixation of agriculture; also due to the natural phenomena as subterranean waters, rocks, mud volcanoes and loss of vegetation and wildlife, environmental pollution becomes a more and more intense global problem. Among of the contaminants of soils, water and air heavy metals (HM) and petroleum hydrocarbons (PH) are widespread and pose a serious threat to ecosystems.

Azerbaijan is also one of the most polluted territories in the world with the relevant critical ecological problems. Man-caused pollution is a main source of the contaminations of all the ecosystem components and the most common contaminants here are the waste products of petroleum, chemical and metallurgical industries.

Interactions between plants and an environment are of a complicated nature. Plants as a functional part of ecosystems are the complex organisms that respond rapidly to any changes in their surroundings. Even slight changes in environmental conditions may influence plant physiological–biochemical processes provoking their alteration and corresponding response reactions (Marschner 1983; Ernst 1998; Fitter and Hay 2002). Plants being sensitive or resistant to the phytotoxicity of various pollutants differ in their response and tolerance mechanisms to increasing doses of contaminants. Some lower and higher plant species are capable to survive and assimilate high levels of certain environmental contaminants when growing on polluted areas. In the last decades, based on plants tolerant mechanisms and their accumulation capacity, an environmental-friendly green technology has been developed for remediation of soils, water and air (Baker 1981; McCutcheon 1998; Lasat 2000; Ernst 2006).

This chapter mainly aims to discuss the nature of metal and petroleum hydrocarbon phytoxicity in contaminated soils. An emphasis on plant resistance mechanisms to these toxicants, mechanisms of uptake and translocation of contaminants in plants and the approaches to phytoremediation – the use of plants for the decontamination of polluted soils will be discussed. Results of the authors' own research carried out so far concerning the tolerance mechanisms of the indigenous plant species to heavy metals are also discussed.

2 Environmental Contaminants

A global environmental pollution by various chemicals becomes a growing central ecological problem disturbing the human communities at all levels and requires its detailed investigations for an effective solution. Due to increase of the many-sided fields of anthropogenic activities the numerous types of contaminants of a different nature are the main initiators of serious problems for the terrestrial, water and atmospheric ecosystems. HM and PH among of these pollutants are the most common, representing the more negative effects to living organisms.

In many cases, organic pollutants are noticed to be accompanied by heavy metals and even vice versa. The petroleum contamination is considered to promote the changes in the structure and some physico-chemical properties of soils and as a consequence increase the concentrations of mobile forms of HM, mainly in the recently polluted soils. For instance, the concentrations of Mn increase for four times, and those of Co, Mo and Cu two times (Bakhshiyeva and Akimova 2001). Moreover, some heavy metals such as Ni, V, Fe, Zn, Co and Cu are known to be associated with some groups of petroleum compounds (Chicarelli et al. 1990).

Age duration of heavy metal contamination is estimated for hundreds of years, while pollution of environment by organic materials is more recent (Adriano et al. 2005).

2.1 Toxic Heavy Metals

For the basic metabolism of plants, 19 elements have been selected, of which there are metals and non-metals. Chemical elements with the metallic properties (plasticity, electro- and heat conduction, specificity of ligands etc.) on their density are classified as light and heavy metals, but on their necessity for living organisms they are considered as macroelements, micronutrients/trace metals and toxic metals.

Metals with a density <3.5 g cm^{-3} are accepted to be light metals, while with a relatively high density (>5–6 g cm^{-3}) are heavy metals with atomic number >20, atomic mass >40. Number of HM is 23. Among light and heavy metals, both macro- and micro-elements, and toxic elements occur.

Heavy metals play various functions in living organisms. Depending on concentrations they can be beneficial or harmful for plant development, animal and human health. Micronutrients such as iron (Fe), copper (Cu), zinc (Zn), and

manganese (Mn) required in only low concentrations and are essential for plant basic metabolism; in addition, cobalt (Co), chromium (Cr), nickel (Ni), and tin (Sn) are necessary for human nutrition; besides all these metals, arsenic (As) and vanadium (V) are also essential for animal health (Adriano et al. 2005; Ernst 2006). At the same time, many essential heavy metals at any excess level can be toxic for creatures and result in undesirable modifications of biological systems (Marschner 1983; Adriano et al. 2005; Ernst 2006). While some heavy metals being non essential have no biological roles. As these metals are not necessary for living organisms, they are poisonous even in low concentrations and hence are considered as toxic metals. Highly toxic heavy metals are cadmium (Cd), lead (Pb), mercury (Hg), and thallium (Th).

Under normal conditions, soils, water and air contain a low background of heavy metals. But close to industrial units, mining sites, and along intensive roads, the high levels of heavy metals are noticed as a result of ever-increasing anthropogenic activities (smelting of metalliferous ores and steel-smelting, gas exhaust, energy production, transport exploitation, defense industry etc.). Municipal waste products and agricultural chemical applications are also among the pollution sources of ecosystems.

The man-caused HM emissions are deposited on the soil surface and remain not degraded. They remain for a long-time in different soil compartments, thereby they offer a potential source of long-term pollution of ecosystems. Atmospheric deposition can be moved away on wide distances from their release sources in dependence on a wind direction or washed by rain into the soil. A strong correlation is revealed to be between the distance and soil HM total and extractable levels, i.e., soil concentrations of HM decrease with the distance from polluting source (Senthilkumar et al. 2005; Alirzayeva et al. 2006).

HM contamination embraces the increasingly more territories and sites all over the world and their areas are stretched every day. For instance, approximately 20,000 ha of arable land are contaminated by HM only in Bulgaria (Andonov 2005). About 180,000 ha of soil in some regions around metallurgical units of Romania are affected by HM (Vrinceanu et al. 2005). Over 80,000 sites in the urban environment or related to former mining sites in Australia are subjected to HM (Naidu et al. 2003). About 50,000 ha private vegetable gardens in Switzerland are also often polluted by HM (Martin et al. 2005). About 840,000 ha of area in Azerbaijan suffer from excess of various chemicals, including HM. The annual contaminant emissions from only a vehicle use here are over 750,000 tons (Babayev 2003; Mamedov 2003).

Metals were shown to be associated with several soil fractions as: free ions and soluble metal complexes in soil solution; absorbed to inorganic soil constituents at ion-exchange sites; bounded to soil organic matter; precipitated as oxides, hydroxides and carbonates; embedded in structure of the silicate minerals (Lasat 2000).

HM in metalliferous soils are known to differ on their bioavailability for plants and the levels of their available fractions are noticed to be significantly less than the total HM contents. A bioavailability depends on metal solubility in soil solution. A mobility and accessibility of metals can be affected by a number of soil factors such as pH, clay and organic matter contents, redox potential, root exudates

etc. (Hesse 1971; Harter 1983; Kaschl et al. 2002a–c; Naidu et al. 2003). Due to the plant–soil interaction, presence of microorganisms and release of root exudates, an alteration in the chemical availability of HM mainly occurs in the rhizosphere, particularly, where pH values endure to significant changes. In particular, metal solubility increases with the decrease in of pH values (Aijen 2004). Correspondingly, the metal desorption from soil binding sites and release of metal ions into solution is usually facilitated by low pH due to H^+-competition for binding sites (Lasat 2000; Fitter and Hay 2002). The metal bioavailability depending on soil types and nature of pollution decreases also in the presence of other metals and chelators.

Metal mobility can be affected also by the various types of organic matters (insoluble, dissolved and colloidal) (Tyler and McBride 1982; Kaschl et al. 2002c). The soluble metal-organic matter complexes increase the bioavailability and mobility of metals in the soil, while the insoluble organic complexes effectively remove metals from the solution (Kaschl et al. 2002a). Metals display specificity in this composting. In particular, cadmium, as opposed to Cu and Zn, is shown to demonstrate a tendency to preferably associate with larger, humified and less soluble organic matters (Kaschl et al. 2002a; 2002b).

The plants when grown on contaminated soils with a higher metal bioavailability directly are faced with phytotoxicity of HM. Phytotoxicity is shown as a variety of symptoms during plant growth and development. However, these symptoms may differ depending on type of metal, degree of metal toxicity, plant species and their accumulation capacity. Mechanisms of phytotoxicity are very different (Ernst et al. 1992; Ernst 1998; Seregin and Ivanov 2001). Interacting with different functional groups, in particular, SH-groups of proteins, nucleic acids, polysaccharides and a number of low molecular weight compounds HM affect various developmental and biochemical processes and have a toxic action on plant metabolism (Balsberg-Pablsson 1989; Ernst 1998; Khudsar et al. 2004; Seregin and Kozhevnikova 2006; Liu et al. 2008).

2.2 Petroleum Hydrocarbons

Some widespread xenobiotics, namely, the petroleum products play a significant role in modern life as a result of the industrial revolution of the past century. Hazardous crude oil or its derivative fuels in process of production, exploration, refining, transport and storage are the main sources of environmental contamination by PH.

Because the PH are ubiquitous in various environmental compartments, almost insoluble in water, recalcitrant and difficult to compose, they pose a serious threat to ecosystems. A special danger is caused by unforeseeable consequence and state of emergence at transportation, transfer and storage processes.

According to the US Environmental Protection Agency in the United States about 35% leaks from storage tanks were revealed in only 1986 (Onwurah et al. 2007). Similar spills were also noticed in the other regions of US, i.e., Texas, Rhode Island and the Delaware Bay (Anonymous 1989). More than 200,000 barrels of crude oil from the tanker Exxon Valdez were spilled in Prince William Sound, Alaska (Hagar

1989). The toxic effect of spilled crude oil was found to remain here even after more than ten years (Short et al. 2002). There are the other examples such as Nigeria where only during 4 years (1976–1980) about 784 incidences of oil spills took place resulting in the release of 56.1 million barrels of crude oil into aquatic and terrestrial ecosystems (Awobajo 1981). The Gulf War in 1991 led to the destroying of numerous oil installations causing extended oil pollution in the ecosystem of Kuwait and at least 25% of the desert was exposed to the serious problems (Pilcher and Sexton 1993; Brown and Porembski 2000).

Azerbaijan is considered as the oldest oil-producing country of the world and oil production and other industrial fields connected with it have almost 160 years of history. The Absheron peninsula of Azerbaijan, including the large cities such as Baku and Sumgayit, and the Caspian Sea are among the most ecologically devastated areas in the world, because of severe air, soil and water pollution as a result of highly developed petroleum producing, refining and transporting branches of industries in this area. More than 30,000 ha of soils of the Republic are contaminated by oil and oil products, and more than 10,000 ha of this soil area in Absheron are heavily polluted (Mamedov 2004).

Crude oil is a complex of different kinds of hydrocarbon components (Reis 1996) that are produced during various refining processes. The main hydrocarbon categories are aliphatics, aromatics, asphaltenes and resins. Aliphatic hydrocarbons, consist of alkanes, alkenes, alkynes and cycloalkanes, but aromatic hydrocarbons are monoaromatics and polycyclic aromatic hydrocarbons. The asphaltenes are phenols, fatty acids, ketones, esters and porphyrins, and the resins are pyridines, quinolines, carbazoles, sulfoxides and amides (Colwell and Walker 1977).

Hydrocarbons differ in their physical–chemical properties. The normal alkanes are rapidly degraded. Volatility and solubility of hydrocarbons decline, but their degradation time period increases with an increasing of their molecular weight and a number of aromatic rings in the molecular structure. The small aromatics are fairly soluble in water, rapidly evaporated and degraded. Generally, the aliphatics are more volatile than aromatics (Association for Environmental Health and Sciences 1998).

A bioavailability of PH, just as one of heavy metals, depends on organic matter contents in soils. Organic matter binding to lipophylic PH can reduce their bioavailability. Similarly, a toxicity of PH for the living organisms mainly depends on their molecular weight. It increases with the decrease in molecular weight, since a low molecular weight hydrocarbons can easily enter into the plant cells and tissues. Hence, light crude oils containing mainly low molecular weight hydrocarbons are considered to be more toxic than heavy crude oils having a higher molecular weight compounds (Reis 1996).

Petroleum toxic compounds that change the soil chemical properties, can have a negative effect on soil microorganisms and plants. PH can significantly reduce the availability of the plant nutrients in soil as a result of rapid growth of populations of oil degrading bacteria which use up or immobilize the available nitrogen and phosphorus (Xu and Johnson 1997). They also induce a drought stress due to the hydrophobicity of petroleum-polluted soils and can limit the availability of soil water in which the nutrients are dissolved and thereby reduce the accessibility of

nutrients to plants and microorganisms (Schwendinger 1968; Li et al. 1997). In that way PH create the negative conditions for good plant growth and development and their phytotoxic effect is increased more.

Phytotoxicity depends on PH types, their compositions and concentrations, plant species and soil types. Some plant species were shown to exhibit the visual symptoms of PH toxicity such as chlorosis and yellowing through reduction of the photosynthetic pigments; a growth reduction as the shortening of under- and aboveground organs, and the perturbations in developmental parameters (Chaineau et al. 1997; Malallah et al. 1998; Adam and Duncan 1999; Pena-Castro et al. 2006; Meudec et al. 2007). The reduction of transpiration of willows and poplars growing in soils with diesel and gasoline was found to be by 10% at 810 mg kg^{-1} total hydrocarbon concentrations and by 50% at 3,910 mg kg^{-1}. However, at this site gasoline was more toxic to the tree species than diesel (Van Epps 2006). While contamination with 13.6% of diesel also inhibited the germination of perennial ryegrass (Siddiqui et al. 2001) and 50 g kg^{-1} diesel led to the reduction of root biomass to 20% in oil seed rape cultivar Martina (Adam and Duncan 1999). On the other hand, willows and poplars, as well as grass and legume species were able to grow in hydrocarbon contaminated soils at 40,000 mg kg^{-1} total petroleum concentrations (Van Epps 2006). Besides, it was shown that the different types of hydrocarbons (diesel oil, gasoline and crude petroleum) inhibited hydrolase activity in the sandy soils, while in the clayey soils, diesel oil stimulated the enzyme activity. Gasoline had the highest inhibitory effect on hydrolase activity in both soils. At the same time, a phytotoxic effect of diesel or petroleum on barley and ryegrass was observed in both soil types (Labud et al. 2007).

3 Mechanisms of Plant Resistance to Toxicants

Resistance of various plants to inorganic and organic pollutants depends on their genetic peculiarities and the physiological–biochemical mechanisms of different nature. At that, plant resistance varies greatly between plant species as well as their ecotypes and in dependence on the types and nature of contaminants.

Plants when grown on metal-enriched soils are obliged to modify their physiological processes to adapt to the strained environment, because their primary metabolism cannot guarantee plant survival. Most likely, the only option for plant survival on metal enriched soils is evolution of resistance mechanism (Ernst 2006). At the same time, it is supposed that specific mechanism determines the plant resistance to metal surplus, e.g., to Ni is not revealed (Seregin and Kozhevnikova 2006). Mechanisms of plant constitutive and adaptive tolerances to HM excluding some of them (Cu, Cd, Zn etc.) are also incompletely studied.

The roots are the main plant organs which are earliest exposed to soil pollutants and play a major role in plant response reactions. The root apex is generally accepted to be the primary site responded by blocking or accumulating various soil chemicals than the mature root tissues (Delhaize et al. 1993; Delhaize and Ryan 1995; Horst 1995). A nature of metal distribution in the meristematic tissues depends mostly

on anatomical and physiological peculiarities of apex, and to a lesser degree on physico–chemical properties of their ions (Seregin and Kozhevnikova 2008). At cadmium accumulation, the roots of oil rape and sunflower were shown to be the major organs (Herrero et al. 2003). A compartmentation of Cd in the root vacuoles of tobacco plants is considered to be one of the premier physiological mechanisms of Cd tolerance (Vogeli-Lange and Wagner 1990).

An interaction between different metals and their competition in the rooting medium can cause the differences in root absorption and translocation capacities of plants and/or result in a low overall metal toxicity (Keltjens and Beusichem 1998; Herrero et al. 2003) as well as a deficiency or surplus of their entry into plants (Seregin and Kozhevnikova 2006). Synergistic and antagonistic effects of metal interactions on metal uptake have been shown. A decrease in shoot concentration of Cd was observed in the presence of other metals (Herrero et al. 2003), for example Cu (Keltjens and Beusichem 1998), and Zn (Cataldo et al. 1983).

The roots of plants grown on petroleum-polluted soils were found to behave differently than the ones on metal-contaminated soils. The initial observations indicate that the plant roots have a tendency to avoid oil contaminated areas completely, if a surrounding uncontaminated soil is present (Adam and Duncan 1999). In this case, they do not grow into lower soil layers and distribute near the soil surface. Due to depletion in oxygen reserves caused by petroleum hydrocarbons in contaminated soils (Bossert and Bartha 1984) the roots showed a tendency to improve their respiration by this way. If there are no available uncontaminated soils, the roots will grow through contaminated regions until they find more suitable surviving conditions (Adam and Duncan 1999). Thus, some plant species have developed the specific tolerance to PH stress. It is shown that even wide used edible plant *Zea mays* in comparison to the many other crops, displayed a high tolerance level and could be grown on soils contaminated by 21% of crude oil and it still produced a fresh yield of about 60% than on normal soils (Ayotamuno and Kogbara 2007).

One of the resistance mechanisms of plants to various contaminants is the release of root exudates containing mostly the organic acids, amino acids, phenolic compounds, and sugars (Marschner and Romheld 1996; Dakora and Phillips 2002). They are carbon and nitrogen sources for improving the chemical status of petroleum contaminated soils with a low bioavailability of nutrients, and play a certain role as chelators of HM for their detoxification in metal-contaminated soils.

Chelator compounds of various natures can act both in soils and in plants for the blocking and detoxification of pollutants in plants and thereby playing a role in tolerance, sequestration and transport of inorganics and organics (Ross 1994).

Compounds such as some organic acids, amino acids etc. forming not soluble complexes with metals in soils make them not available for plants and prevent their uptake by roots. But these compounds, along with the oligo- and poly-peptides such as glutathione (Xiang et al. 2001; Blum et al. 2006), phytochelatins (Keltjens and Beusichem 1998; Kolodyazhnaya et al. 2006; Wunschmann et al. 2007), and

metallothioneins (Burdin and Polyakova 1987; Robinson et al. 1993; Seregin and Ivanov 2001) which are the initial chelators of HM in plants, form complexes and isolate them into metabolically less active cell structure, such as vacuoles and prevent their transport on plants thereby weakening the HM ion toxicity on plant cells.

Phytochelatins (PCs) and metallothioneins (MTs) are two classes of a cysteine-rich low molecular weight peptides that bind to HM by thiolate coordination, and maintain a metal ion homeostasis in cytosol and mediate a heavy metal tolerance in plants (Clemens 2001; Cobbett and Goldsbrough 2002; Wunschmann et al. 2007). Unlike phytochelatins, metallothioneins of higher plants are initial gene products (Grill et al. 1987).

In particular, PCs synthesis is considered to be induced under excess of heavy metals, e.g., Cd, Cu, Zn, Ni, Pb, Hg etc. (Kahle 1993; Keltjents and Beusichem 1998; Inouhe 2005), while Fe, Mo, Cr etc. do not result in their synthesis (Kolodyazhnaya et al. 2006). In particular, Cd being a potentially toxic metal can be sequestered and detoxified by PCs due to their intracellular complexation in its innocuous forms (Steffens 1990). Formation of complex with a low molecular weight compounds (<10 kD), e.g., with organic acids is shown to play a certain role in plant resistance to Ni (Seregin and Kozhevnikova 2006).

Some enzymes such as proteases, phosphatases, peroxidases, dehydrogenases, hydrolases, dehalogenases and others are thought to be involved in a range of important processes, including the defensive responses of plants to various external effects both of abiotic and biotic stress factors (Ali-zade et al. 2001; Segarra et al. 2002). Plant resistance to pollutants also can be associated with an enhanced induction of activities of some stress antioxidant enzymes – peroxidase, superoxiddismutase, catalase etc. (Schickler and Caspi 1999; Seregin and Ivanov 2001). In case of soil organic pollution, the intra- and extra-cellular enzymes (dehalogenases, mono- and dioxygenases, peroxidases, phosphotases etc.) of plants and microorganisms are considered to play an important role in degradation of organic pollutants both in the soils and the shoot/root tissues (Dixit and Pant 2000; Susarla et al. 2002; Vasileva-Tonkova and Galabova 2003; Wolfe and Hoehamer 2003; Pilon-Smits 2005; Muratova et al. 2007). Plants contain a set of specific metabolic isoenzymes and the corresponding genes, some enzymes are involved in oxidations of xenobiotics, while others are associated with xenobiotic metabolism in plant cells, transport of intermediates and compartmentation processes (Macek et al. 2000; Pena-Castro et al. 2006). From the assigned identities of the isolated cDNAs, an induction of complex and multifactorial molecular response of plants by petroleum hydrocarbon stress was shown (Pena-Castro et al. 2006).

An immobilization of the metal in the cellular wall (Cosio et al. 2005; Seregin and Kozhevnikova 2008) and induction of synthesis of heat-shock proteins (Sanita di Toppi and Gabrielli 1999; Heckathorn et al. 2004) have been also proposed to be resistance mechanisms of plants.

A more attractive peculiarity of plants to tolerate to strained soil conditions is thought to be their individual capacities of uptake and translocation of pollutants.

4 Mechanisms of Uptake and Translocation of Contaminants in Plants

Growing environmental pollution by HM has stimulated a study of mechanisms of metal uptake from soils and their distribution in plants (Baker 1981; Hall 2002; Seregin and Kozhevnikova 2008).

Plants are known to differ on their mechanisms of uptake of various pollutants from the environment (Marschner 1983; McCutcheon 1998; Siciliano and Germida 1998; Lasat 2000; Schat et al. 2000; Pilon-Smits 2005; Ernst 2006). Capacity of plants to accumulate and store them in different organs is unequal, too. This is obviously caused by two factors: genetic differences in uptake, translocation and resisting or storing of contaminants by plants, and environmental factors.

An uptake of pollutants by plant roots is also different for organic and inorganic compounds (Pilon-Smits 2005). An uptake of inorganics like nutrient elements is known to be realized both by passive diffusion and mainly active transport. An active transport of Ni^{2+} was shown to play an important role in its uptake from medium with the low concentrations of Ni, whereas the mechanism of passive transport dominates at higher concentrations of Ni (Temp 1991). Metals usually pass the root membranes with an aid of membrane transporter proteins (Pilon-Smits 2005) belonging to the family of CDF (cation transport) proteins (Yoshihiro et al. 2004). It is remarkable, that a binding domain of proteins recognizes only specific ions and is responsible for transporter's specificity (Lasat 2000). Metal-phytosiderophores which increase the bioavailability of soil metals also are important in their uptake from soils (Marschner and Romheld 1996; Schaaf et al. 2004; Pilon-Smits 2005).

However, there are no transporters for organic compounds in plant membranes. Depending on their hydrophobicity, the organic pollutants have a tendency to be taken up and also to be moved into and translocated between root symplast and xylem apoplast as well as to enter the leaf by simple diffusion (Pilon-Smits 2005).

HM uptake and accumulation by some plant species in large amounts is defined by their morphological/physiological features (Seregin and Kozhevnikova 2006). Many plants can accumulate heavy metals in high levels, while some are significantly distinguished by their sensitivity to excess of HM. This difference can be due to various mechanisms, including a preparation to HM uptake and transport; binding of HM to cell walls and vacuoles or cytoplasm; changes in rate of HM transport from roots to shoots and their store in different root tissues; synthesis of enzymes, increase in plant resistance to HM; activation of mechanisms of their removal from cells (Van Steveninck et al. 1990; Brooks 1998; Seregin and Ivanov 2001; Guo et al. 2004; Ernst 2006).

Solubility of metals in soil solution is an essential factor for their uptake. Several plants can change metal availability directly (uptake) and indirectly by different mechanisms. It was shown that the metal accumulating plants with a high potential to extract HM from soil, *Thlaspi caerulescens* L. and to a lesser extent *Salix viminalis* L. making a change in the rhizosphere can alter the HM distribution in different soil pools (Hammer and Keller 2002).

An uptake of both organic and inorganic pollutants is also connected with the influence of soil rhizosphere microorganisms, which are in symbiosis with roots.

The considerable changes in their community and population size are revealed in soil rhizosphere in comparison with the bulk soils (Anderson, et al. 1993; Siciliano et al. 2003). A role of soil microorganisms is diverse, including exudation by them of organic compounds to soil, which increases a bioavailability and metal uptake by roots (Fe, Mn, Cd etc.), can also directly influence metal chemical properties, making them innocuous and immobile (e.g., Cr, Hg, Pb and Cd) (Lasat 2000).

Plants, due to on their differential HM accumulating abilities are classified into 3 groups: *accumulators*, which accumulate high levels of metals in aboveground easily harvestable organs independence of metal concentrations in soils; *indicators*, which reflect the levels of metal concentrations in rhizospheric soil; *excluders* have the restricted uptake of metals into roots and their limited translocation to shoots even under high contamination in the growth medium (Baker 1981; Antosiewicz 1992).

A majority of plants belong to the excluder group. For example, a low transport to shoots of Pb accumulated in roots in many plants is explained by its strong retention in the cell walls of root cortex; by a weak mobility of metal ions (Seregin and Ivanov 2001), and/or long distance between roots/shoots (Blaylock and Huang 1999). A rate of metal uptake and transport depending on plant species is one of the important factors of plant resistance (Yang et al. 1995). In excluder plants, polysaccharides of mucilage covering roots play an important role in HM uptake processes and its rate depends on metal nature. The binding of mucilage to HM significantly limits the metal intake into the roots and can be an important barrier of the root systems to metals (Morel et al. 1986; Seregin and Ivanov 2001).

Due to the ability to accumulate one or another level of HM, plants are identified as accumulators and hyperaccumulators (Brooks et al. 1979). The latter can accumulate both the high levels of essential microelements and also the significant amounts of non-functional metals, such as Cd, Pb, and Ni and they have additional mechanisms of their detoxification. Hyperaccumulator plants differ by high concentrations of HM in their shoots, about 100-fold excessive than in the non-accumulator ones (Brooks 1998; Lasat 2000). For the first time Brooks et al. (1979) used this term to describe plants with Ni-concentrations >1000 $\mu g\ g^{-1}$ (0.1%) in their dried leaves. An important parameter characterizing the plant abilities to accumulate and transport ions from roots to shoots is bioaccumulation factor (BF–HM concentration ratio of shoot/soil), that is considered to be greater than 1 in metal hyperaccumulator plants (Baker 1981).

To date, about 400 plant species have been identified as metal–hyperaccumulators with high genetic capacity to accumulate huge amounts of HM in their shoots (Baker et al. 2000). In particular, to date about 300 species of Ni hyperaccumulators have been found (Seregin and Kozhevnikova 2006). Hyperaccumulator terrestrial vascular plant species endemic to metalliferous soils for other metals (e.g., Zn, Cu, Co, Pb) have also been revealed, but their amount is much less.

The major proportion of metals is located in plant rhizodermis and cortex during their uptake by roots (Obroucheva et al. 1998; Tung and Temple 1996; Vodnik et al. 1999; Seregin and Ivanov 2001). Seregin and Kozhevnikova (2008) have reviewed the role of various tissues of roots and shoots in HM transport and accumulation in the two plant groups (excluders and hyperaccumulators) and classified

these tissues on their participation in transfer and distribution of some HM (Cd, Pb, Ni, Sr) in plants as: (i) absorbents (rhizodermis), (ii) with barrier functions (endodermis and exodermis), iii) accumulators and presentators (epidermis and cortex), (iv) collectors (pericycle), v) inter-organic transporters (xylem and phloem), (vi) storage (root apex).

Metal distribution in hyperaccumulator plants is very likely to be regulated by an efficiency of a number of detoxification mechanisms and defined on features of metal transport. For example, Ni was shown to be easily transported to tissues of stele, while distribution of Cd and Pb to central cylinder is restricted by endodermis. It can define the specificity of Ni toxic action and be of one of the reasons of influx of this ion to aboveground organs of accumulator plants (see Seregin and Kozhevnikova 2006).

Accumulation and distribution of organic compounds in plants are somewhat different. Organic pollutants can be degraded both in plant root and shoot tissues. Thus, unlike inorganics, their accumulation in plants comprises three phases: enzymatic modification and enzymatic degradation, conjugation and sequestration in cell walls (Pilon-Smits 2005).

To date the detail knowledge of the metabolic and genetic processes regulating a metal tolerance gives a possibility to enhance a plant resistance and accumulation of HM using some biotechnological approaches and genetic engineering. Plants with ability to survive at the high levels of contamination can be developed by introducing various genes, which provide a binding or removing HM ability and also influence on the synthesis of enzymes alleviating the toxic effect of HM (Pilon-Smits 2005; Kolodyazhnaya et al. 2006).

The plants possessing genetic potentials for uptake, extraction, degradation, metabolization and immobilization of pollutants are good tools for cleaning up of contaminated soils in phytoremediation process.

5 Phytoremediation of Polluted Soils

To date, due to colossal and extensive contamination world-over and to provide a safety to ecosystem, the detoxification/remediation of soils polluted by organics and inorganics is of great importance.

Among of existing cleanup options of pollutants, *in situ* by the biological treatment systems, in particular phytoremediation is more practicable and includes phytostimulation, phytostabilization, rhizofiltration, phytoextraction, phytovolatilization, phytodegradation etc. (Baker 1981; Cunningham et al. 1996; McCutcheon 1998; Lasat 2000; Pilon-Smits 2005).

Phytoremediation is a natural inexpensive technology to remediate the environment. Phytoremediation can be used for decontamination of both organic and inorganic pollutants in soil, water and air. About 64% of the polluted sites are revealed to contain mixtures of organics and inorganics (Ensley 2000), where organics tend to be less reactive and are not accumulated readily as HM. Thus HM are most likely to cause toxicity, limit plant growth and phytoremediation (Pilon-Smits 2005).

During phytoremediation, plants participate both directly and through symbiotic relations with their associated microbes in the processes of uptake, transport and detoxification of pollutants, especially in case of petroleum contamination. Plant and microorganism symbiotic relations are considered to be as mutually beneficial actions. Rhizospheric effect enhances the plant survival and its abilities of degradation/detoxification of petroleum hydrodcarbons at their toxic or low levels (reduced bioavailability), decreases a time, and increases a rate of degradation. Penetrating and breaking–up the soil, plant roots create the macropores that provide microorganisms by air and water resources thereby increasing microbial activities and biodegradation rates of pollutants. The accelerated degradation of organic pollutants by microorganisms in the planted soils in comparison with unplanted ones has been demonstrated (Huang et al. 2005; Juhanson et al. 2007; Olson et al. 2007).

A potential of some grasses and legumes, as well as some trees – poplar, willow etc. (see Susarla et al. 2002; Merkl 2005) have been shown to play a significant role in degradation, containment, and transfer of petroleum hydrocarbons. It is well known that the root exudates promote the colonization of microbes, increase the microbial biomass, and enhance microbial metabolic activity in root zone for accelerated degradation of organic pollutants (Schnoor et al. 1995; Yoshitomi and Shann 2001; Alkorta and Garbisu 2001; Ryan and Firestone 2001; Hinsinger et al. 2006; Juhanson et al. 2007).

Hyperaccumulator plants with high production of biomass, a deep root system, fast growth and high tolerance to metals are good tools in the environmental biotechnology for decontamination of soils contaminated by HM (Baker 1981; Lasat 2000; Pilon-Smits 2005; Almeida et al. 2007).

In recent years, all over the world, an increasing attention is being paid to discover the new plant species with high HM accumulating capacity and the list of these species is expanding every day. In particular, a study of potentials of resistance to various pollutants of some *Artemisia* L. species belonging to *Asteraceae* (Compositae) family widespread on different contaminated regions of the world revealed their high HM accumulation capacity (Morishita and Boratynski 1992; Samkaeva et al. 2001; Bashmakov and Lukatkin 2002; Toderich et al. 2002; Kim et al. 2003; Li et al. 2003; Takeda et al. 2005). Thus, based on these investigations, some *Artemisia* species are identified as accumulators of HM.

The ones from indigenous flora of Azerbaijan are remarkable for their easy reproduction and high introduction in highly contaminated areas by HM and petroleum hydrocarbons were investigated in this respect by the authors. All 5 *Artemisia* species tested (*A. fragrans*, *A. scoparia*, *A. szovitsiana*, *A. caucasica* and *A. arenaria*) were found to be dominant and widespread on contaminated areas of Azerbaijan and they grow vigorously on polluted soils by organics and inorganics without showing any symptoms of toxicity (Alirzayeva et al. 2006). Research carried out by the Canadian scientists on assessment of the abilities of some plant species to survive in crude oil-contaminated soils also revealed the hydrocarbon tolerance possibilities of *Artemisia frigida* (Robson et al. 2003). At the same time, the 5 *Artemisia* L. tested species from Azerbaijan flora displayed a significant accumulation capacity of HM in their different parts, mainly in shoots. Data on BF for some

HM, mainly for Zn, Cu and Cd were much higher than one, which proposed that these species can be considered as potential tools for phytoextraction. Especially, *A. scoparia* with its large biomass and high adaptation ability was revealed to have more potential for a phytoremediation approach on polluted soils, mainly by Zn (Alirzayeva et al. 2006).

6 Conclusion

A more detailed study and definitive elucidation of mechanisms of resistance of plants to heavy metals and petroleum hydrocarbons, including their uptake, transport and translocation in shoot/root tissues, which are determined by plant genetic peculiarities will give possibilities to manipulate and choose the appropriate biotechnological approaches to develop the plants with enhanced tolerance to various soil contaminations and their HM/PH accumulation/degradation capacities. For a successful solution of these issues primarily a monitoring of indigenous flora widespread on contaminated sites and subsequent selection of the more suitable plant species with large biomass production, deep root system, high growth rate and capacity to reproduce under these severe adverse conditions, and accumulate of pollutants in their aboveground parts are of great importance for phytotechnologies of cleaning up and remediation of soils.

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Chapter 10
Biochemical and Molecular Aspects in Phytoremediation of Selenium

L.F. De Filippis

Abstract The element selenium (Se) is considered a finite and non-renewable resource on earth, and has been found to be an essential element in humans, animals, micro-organisms and some other eukaryotes; but as yet its essentiality to plants is in dispute. There is no doubt that adequate levels of selenium are important to animal and human health, and some selenium compounds have been found to be active against cancers. A limited number of plants growing on selenium rich soils can accumulate very high levels of selenium (i.e., hyperaccumulate selenium), and are classified as selenium tolerant, however, many more plants do not accumulate selenium to any great extent, and are selenium sensitive. Plants vary considerably in their physiological and biochemical response to selenium, and a revision of the physiological responses of plants to selenium is presented; especially growth, uptake, transport and interaction of selenium with other minerals. The review also details the biochemical responses of plants to selenium, the assimilation of selenium in plants and possible incorporation into proteins. Molecular approaches to understanding selenium toxicity and tolerance have increased the knowledge of mechanisms of action, and the molecular biology of selenium in transgenic plants is detailed; with special reference to the similarity with sulphur metabolism, sulphur/selenium transporters and important assimilation enzymes. Phytovolatilisation of selenium will be summarised, which is a unique method for plants to metabolise selenium to more volatile forms in order to eliminate selenium from tissues, and eventually from the soil and water. Finally, the application of phytoremediation in selenium rich environments is reviewed in light of the possible use of plants to decontaminate selenium from soil and water environments, and perhaps also produce a product which could be used in mineral supplementation of foods, and even fighting cancers.

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Abbreviations

APS	adenosine 5'-phosphosulphate
APSe	adenosine 5'-phosphoselenate
Cys	cysteine
Cysth	cystathione
DMS	dimethylsulphide
DMSP	dimethylproprionate
DMSe	dimethylselenide
DMDSe	dimethyldiselenide
DMSeP	dimethylselenioproprionate
GPX	glutathione peroxidase
GSH	glutathione
GSSeSG	selenodiglutathione
HAST	high affinity sulphate transporter
LAST	low affinity sulphate transporter
MeCys	*S*-methylcysteine
MeSeCys	*S*-methylselenocysteine
MeSeCysSeO	methylselenocysteine seleno-oxide
Met	methionine
S	sulphur
Se	selenium
SeCys	selenocysteine
Secysth	selenocystathione
SeGSH	selenoglutathione
Sehocys	selenohomocysteine
SEM	SeCys + MeSeCys
SeMet	selenomethionine
SeMMet	selenomethylmethionine

Contents

1 Introduction

The element selenium (Se) was discovered in 1817 by the Swedish chemist Berzelius, Jons Jakob and named after the Greek moon goddess ‘selene’. Selenium belongs to the Periodic Table Group VIA; the group that also contains sulphur (S) and tellurium (Te). However Se compounds, minerals and seleniferous soils have a long history. In 1295 Marco Polo reported that during his famous journey from Venice through Asia Minor to China, his horses suffered from a typical necrotic hoof disease when the horses ate poisonous plants; the symptoms are now known to be due to Se toxicity from animals ingesting high levels of Se present in accumulator plants (Birringer et al. 2002). As early as 1842 evidence became available for the toxicity of Se to animals, but the first recorded written evidence of Se poisoning in livestock was reported in 1856 by the US Army surgeon, Madison (Whanger 2002). In 1884 a television system was developed using Se pohotocell technology in imaging (Chasteen and Bentley 2002). Therefore Se played a fundamental role in xerography, or in other words early versions of televisions and photocopiers. The photoconductivity of Se compounds has had a profound influence on humanity, and Se compounds have found many roles in the electrical, electronic and semiconductor industries. As well, Se is often used in agriculture, paint and pigment production, volcanisation, oil refinery, glass manufacturing, coal and electricity generation, metallurgy and lately medicine (Lemly 1997; 2004).

The toxicity of Se and Se compounds in domestic animals had been identified and described for many years, however it was not until the Kesterson Reservoir

controversy in the USA in the 1980s that scientists and health regulators were made aware of Se as an environmental contaminant. The reservoir contamination was traced back to Se loaded agricultural drainage water, which had been allowed to flow into the reservoir from adjoining farms (Ohlendorf et al. 1986; Saiki and Lowe 1987). Interest in the environmental impact of Se has increased since this incident 25 years ago. However in nature, Se toxicity is more often found in arid and semi-arid regions of the world that have seleniferous, alkaline soils derived from weathering of Se rich rocks and shales. Contamination of land and water by Se is inevitable due to the geochemical balance of sulphur versus selenium (i.e., ratio of S:Se) being roughly 3000:1 in rocks while the same balance in waters are closer to 8,000,000:1. Seleniferous soils exist in China's Great plains, Canada, a belt in Mexico, pockets in Latin America, parts of New Zealand and Australia, North-West and Great plains regions of the USA, parts of Ireland, in Russia and the Punjab in India (Baker and Brooks 1989; Baker et al. 2000; Dhillon and Dhillon 2003; Haug et al. 2007; Sharma et al. 2009).

In trace amounts, Se is an essential micronutrient and has important beneficial roles in microorganisms, animals, a number of other eukaryotes and humans. However Se has not been shown to be an essential microelement to vascular plants (Pilon-Smits et al. 2009). Nutrition and health benefits of Se include combating heart disease, thyroid disease (hypothyroidism) and strengthening the immune system (Hartikainan 2005). Numerous studies have also demonstrated the anti-carcinogenic role that some organic forms of Se have, especially lung, colon and prostrate cancer, with the most responsive cancers being prostrate and lung cancers (Ellis and Salt 2003; El-Bayoumy and Sinha 2005). It is also true that Se and Se resources could be described as non-renewable and in many cases compounds in short supply around the world, and there is a strong case not only to protect Se resources and minerals, but also to find better ways of extracting Se resources for nutritional and health reasons. Haug et al. (2007) have provided a world Se budget which clearly demonstrates how vulnerable and in short supply Se is around the world, and we should begin to address this problem and how we use this scarce resource.

Environmental pollution of Se can have an impact on human health, agricultural productivity and the stability of natural ecosystems. Even low-level contamination if present on a large enough scale can represent large economic and logistical barriers to effective and timely treatment. At present, aggressive engineering based technologies and/or excavation and entombment of Se contaminated sites may not be cost effective, and therefore not easily justified; and at any rate it may have marginal impacts (Berken et al. 2002; Rugh 2004). Therefore *in situ* biological remediation could be the most appropriate corrective option for treatment of a wide range of low impact contamination due to Se. In many situations, and because of the low toxicity of Se contamination the economic value placed on remediating this type of pollution is often not considered a high priority. However if decontamination is coupled to an economic positive outcome from the extracted material, as could be achieved in the case of Se, then the economics could well be different; especially if a Se rich bi-product could be manufactured for a world-wide scarce resource. Bioremediation typically refers to microbial mediated processes which attempt to clean a site, while

phytoremediation refers to plant mediated clean-up procedures. Part of this review will deal with biological aspects of phytoremediation of Se contaminated areas, but for a more general review of phytoremediation see Pilon-Smits (2005) and Banuelos (2006).

The chemistry of Se has been reviewed extensively by a number of authors (Birringer et al. 2002; Pilon-Smits et al. 2009) and this review will only basically cover areas of need. Se important in human health and cancer treatment has also been well reviewed recently (Combs 2005; El-Bayoumy and Sinha 2005), and we will not deal with these topics. Excellent reviews on Se in higher plants were published by Terry et al. (2000) and Sors et al. (2005b), and we intend to concentrate on more recent developments, and focus on bioremediation implications, although the physiology, biochemistry and molecular biology of Se must at times refer back to these reviews.

2 Physiology

2.1 Types of Se Accumulator Plants

Most plants contain naturally low tissue concentrations of Se, typically less than 5 mg Se kg^{-1} dry weight; and rarely does Se content exceed 15 mg Se kg^{-1} dry weight in plants. This is true even if plants have been grown in high Se containing soils, although compared to controls in soils low in Se they do take-up more Se; these plants are called Se non-accumulators (Ernst 1982; Baker and Brooks 1989; Mayland et al. 1989; Bell et al. 1992). A limited number of plants, especially from the Fabaceae and Brassicaceae can accumulate considerably higher levels of Se in leaves, and are often found on soils that are naturally enriched with Se (i.e., seleniferous soils). These accumulator plants can be further sub-divided into two groups (Dhillon and Dhillon 2003; White et al. 2007):

(a) Primary accumulators (hyperaccumulators) – which have concentrations of Se in leaves in the range of 70–300 mg Se kg^{-1} dry weight, and discrimination coefficients (DC) between Se and S (Se/S) of more than 2.5 in solution culture. DC = [Se/S] plant / [Se/S] solution. Examples include various species of *Astragalus, Stanleya pinnata, Melilotus officinalis, Grindelia squarrosa, Neptunia amplexicaulis, Bertholletia excelsa*, and species of *Lecythis, Morinda, Happlopappus* and *Machaerantha* (Marschner 1995, White et al. 2004).

(b) Secondary accumulators – which take-up Se in proportion to the amount of Se available in the soil and have a DC of less than 2.5. Tissue concentrations of Se are in the range of 5–30 mg Se kg^{-1} dry weight. Plants in this group include species of *Aster*, *Attriplex, Brassica juncea* and *Brassica napus* (canola), species of *Comondra, Grayia, Gutierrezia, Siderenthus* and *Castileja* (Huang and Wu 1991; White et al. 2004).

A list of tested primary and secondary accumulator plant species is given in Table 10.1, although only about 185 plant species were tested by White et al. (2004)

Table 10.1 Plant family and species grown in hydroponic solution containing 1 m*M* sulphate and 0.5 μM selenate with leaf Se concentrations and DC (Se/S ratio) values of greater than 1.4 shown. Species with DC values of less than 1.4 are not shown, and the list is modified from data compiled by White et al. (2004, 2007)

Family	Species	Leaf Se (mg kg^{-1} dw)	DC (Se / S)
Fabaceae	*Astragalus racemosus*	282.8	14.14
	Trifolium subterraneum	14.6	1.76
	Astragalus sinicus	6.9	1.68
	Medicago lupina	5.7	1.64
	Trifolium repens	7.5	1.62
	Trifolium pratense	6.4	1.53
	Medicago sativa	6.0	1.41
Brassicaceae	*Stanleya pinnata*	68.6	3.27
	Brassica nigra	17.9	2.37
	Raphanus sativa	22.2	1.93
	Brassica arvense	24.4	1.75
	Brassica carinata	17.0	1.72
	Sinapis alba	21.9	1.70
	Brassica juncea	21.0	1.63
	Brassica oleracea	33.0	1.51
Solanaceae	*Solanum tuberosum*	9.8	2.02
	Solanum melongena	5.8	1.80
	Lycopersicon pennellii	21.2	1.77
Poaceae	*Panicum miliaceum*	11.9	2.21
	Oryza sativa	11.3	2.12
	Cynodon dactylon	14.1	1.97
	Bromopsis inermis	12.7	1.91
	Agrostis stolonifera	13.8	1.90
	Boutelouga gracilis	6.9	1.87
	Dactylis glomerata	7.1	1.80
	Hordeum vulgare	12.3	1.73
	Holcus lanatus	8.7	1.70
	Sorghum bicolor	7.1	1.67
	Lolium multiflorum	7.5	1.65
	Sporobolus airoides	8.8	1.60
Asteraceae	*Machaeranthera tanacetifolia*	15.2	1.81
	Helianthus annuus	7.6	1.61
	Machaeranthera bigelovii	5.7	1.51
Caryophyllaceae	*Atriplex hortensis*	6.5	1.41
	Beta vulgaris	5.6	1.36
Malpighiaceae	*Linum usitatissimum*	13.4	1.90
Cucurbitaceae	*Cucumis sativa*	10.4	1.62

and White et al. (2007). It is worth noting that although there is a relationship between higher Se accumulation and a higher DC ratio, this is not always true. For example, *B. arvense, B. juncea* and *B. oleracea* have moderate DC ratios of 1.50–1.75 yet contain high leaf Se content (21–33 mg Se kg^{-1} dry weight), but in contrast *B. gracilis, D. glomerata* and *S. melongena* have high DC ratios of 1.80–1.87 yet contain low leaf Se content (5.8–7.1 mg Se kg^{-1} dry weight). Se accumulators certainly can grow on seleniferous soils, but not all plant species growing there may accumulate Se. For example, the genera *Astragalus* contains both Se accumulator species and Se non-accumulator species, and these different types of plants can grow next to one another on the same soil. Most forage and crop plants, as well as grasses contain less than 5 mg Se kg^{-1} dry weight in their tissues, and therefore are classified as non-accumulators (Ernst 1982; Baker et al. 2000; Freeman et al. 2006).

Chemical forms of Se accumulated in crops and other important dietary products to humans are summarised in Table 10.2. It is apparent from this table that most crop plants accumulate Se as SEM (SeCys + SeMCys), and the problem with this is not so much the chemical form of Se found, but that levels in most of these crop plants is too low for dietary needs. On the other hand phytoplankton mostly have a very low Se concentration and Se is mostly as selenite. Fish, dairy products, meat and milk have Se mostly in the form of selenate and selenite, and this is also not satisfactory. Fortified crop plants tested so far accumulate Se mainly in the form of SeMCys, but wether this is the desired chemical form or not required for human nutrition has not been thoroughly tested. It is assumed from very few reports on experimental animals like the rat values in Table 10.2, that the chemical form of Se important in human diets and even cancer prevention is an organic form of Se (El-Bayoumy and Sinha 2005; Haug et al. 2007; White and Broadley 2009). The conclusion from Table 10.2 is that young sprouting seedlings of fortified crops best achieves the beneficial and dietary needs for humans.

2.2 Se Toxicity and Tolerance

When Se sensitive plants are exposed to elevated levels of Se in the soil root medium they may exhibit varying symptoms such as stunted growth, chlorosis, withering, drying of leaves and premature death of the whole plant (Mengel and Kirkby 1987; Mikkelson et al. 1989). There are differences between Se accumulator and Se non-accumulator plants in the threshold values of Se that determine toxicity:

(a) Primary accumulator plants – Se toxicity is shown at values between 2000 and 4000 mg Se kg^{-1} dry weight shoots. Plants in this group include *Astragalus, Stanleya, Neptunia* and *Brassica* (Broyer et al. 1972; Galeas et al. 2007).

(b) Secondary accumulator plants – Se toxicity shown at values between 75 and 900 mg Se kg^{-1} dry weight shoots. Plants tested in this group include clover, strawberry clover, bent grass, ryegrass, rice, buffalo grass, alfalfa and tall fescue (Wu et al. 1988; Sharma et al. 2009).

(c) Non-accumulator plants – Se toxicity shown at values between 2 and 25 mg Se kg^{-1} dry weight shoots. Plants tested in this group include wheat, rice, pea, mustard, kidney beans and alfalfa (Zayed et al. 1998; Sharmasarkar and Vance 2002).

Table 10.2 Distribution and percentage of different selenocompounds identified in various biological and food materials. Modified from Whanger (2002) and Hartikainan (2005). SEM is the sum of SeCys plus MeSeCys

Plant species (type)	Selenate	Selenite	SEM	SeCys	MeSeCys	Others
Wheat grains	12–19		56–83	24–32	11–24	4–26
Wheat straw	97					3
Corn			61–64	15–16		20–24
Rice	1–3	5–13	68–81	6–10		19–31
Soybean			>80			
Lucerne	5–5		70–81			
Ryegrass	10–15		66–78			
Red clover	5–8		72–81			
Grassland legumes			51–70	19–39	10–13	
Vegetables (20 types)	1–50		40–50			
Lettuce	10–12		35–40			
Tomato	15–20		55–65			
Oil seeds and nuts	10	25	40	15	25	
Phytoplankton	1	83	3.2		12.8	
Astragalus prelongus	1.4	9	37		52	
Arabidopsis thaliana	25	15	40	5	10	
Rats (selenite injected)			16–30	24–40		20–34
Rats (SEM injected)			14–23	22–57		15–40
Enriched yeast	0–4	0–27	23–59	0–21	6–20	5–51
Enriched garlic	2–5	8		1–13	47–87	4–36
Enriched onion				7–38	42–55	21–35
Enriched broccoli (sprouts)	10		25	30	25	15
Enriched broccoli (florets)	5		25	21	23	21
Enriched leeks (bulbs)	12–25				35–50	1–3
Enriched potatoe				15–20	50–60	5–10
Fish (17 different types)	15–36	5–30				
Dairy products, milk (low and normal fat) and eggs	5.4	25	30			
Meat products	10–20	25–50	10–20			
Commercial Se feed supplement for livestock	0.6	98.7	0.7			

The threshold range in non-accumulator plants generally vary with plant age and sulphur supply. Younger plants can be more susceptible to toxicity, and tolerance to Se toxicity increases with increasing sulphate supply (Brown and Shrift 1981). The threshold toxic value in non-accumulator plants also depends on the form of Se applied; with selenate and selenite being the main toxic forms to plants. This may be linked to both these forms of Se being readily absorbed and translocated in plants and assimilated in the inorganic forms (Eustice et al. 1980). In most studies selenate is more toxic to plants than selenite (Sors et al. 2005b).

The predominant mechanism involved in Se toxicity is almost certainly due to the incorporation of SeCys and SeMet into proteins in place of Cys and Met (Anderson and Scarf 1983). Additionally, Se may diminish the actual rate and efficiency of

protein synthesis because the substitution of Se amino acids into proteins may mean a less effective or slower rate of protein synthesis during translation (Eustice et al. 1981). But there may be other mechanisms involved such as effects on chlorophyll biosynthesis, as demonstrated by the symptoms of chlorosis. Interference with the reduction of nitrate in leaves and the inhibition of glutathione accumulation are other possible effects. Glutathione levels are critical in anti-oxidative reactions and stress, and evidence suggests Se decrease these reactions, but may also diminish plant defence mechanisms against disease organisms (Aslam et al. 1990; Mugesh et al. 2002; Sharma et al. 2007). It is worth noting however that high levels of Se, especially in hyperaccumulating plants have been shown to protect the plant from leaf chewing insects and other herbivorous animals eating the plants (Boyd 2007; Freeman et al. 2007; Freeman et al. 2009).

A number of possible modes of tolerance to toxic compounds have been described by Pilon-Smits (2005) and may involve any of six mechanisms; these include differences in adsorption, conjugation, sequestration, enzymatic modification, enzymatic degradation and volatilisation. Tolerance in Se accumulator plants appears to be due to a number of mechanisms under the categories above (Neuhierl et al. 1999; Wang et al. 1999; Ellis and Salt 2003):

(a) Decrease in excessively high concentrations of Se being transported into cells of leaves (adsorption/transportation).
(b) Accumulation of Se in Se amino acids, but these seleno-amino acids are not incorporated into normal protein synthesis (sequestration)(enzymatic modification).
(c) Compartmentation of Se as selenate in the vacuole and away from more sensitive cytoplasmic reactions (sequestration).
(d) Increase ATP sulphurylase and SeCys methyltransferase activities to reduce inorganic Se to organic forms of Se, although other enzymes and reactions are also required (enzymatic modification).
(e) Conjugation with glutathione (GSH) and an increase in anti-oxidation protective reactions (conjugation).
(f) Conjugation with Se binding proteins and polypeptides, decreasing inorganic Se content (conjugation).
(g) Increase volatilisation of mainly organic forms of Se out of plant cells and tissues (volatilisation).

In tolerance mechanisms, the key role of the two enzymes ATP sulphurylase and SeCys methyltransferase are of prime importance, and these enzymes have been the main focus of more recent studies in Se tolerance, including transformation and use of transgenic plants with increased tolerance to Se. However, recently the role of Se specific and non-specific binding proteins and polypeptides are being increasingly recognised as having additional effects in increasing Se tolerance (see Table 10.3 for a summary).

Table 10.3 Summary of molecular genetic studies and transgenic reports on plants associated with effects on selenium tolerance and selenium accumulation, including the origin of the genes

Transgene	Gene origin (plant species)	Transgenic plant species	Effects on Se tolerance and accumulation	Reference
SMT Selenocysteine methyltransferase	*A. thaliana*	*B. oleracea*	Upregulation caused higher Se levels, but little effect on Se toxicity; complex interactions with S	Lyi et al. (1995)
APS2 isoform of ATP sulphurylase	*A. thaliana*	*N. tabacum*	No significant effects on Se accumulation and Se tolerance	Hatzfeld et al. (1998)
APS1 isoform of ATP sulphurylase	*A. thaliana*	*B. juncea*	Increase in Se accumulation and an increase in Se tolerance	Pilon-Smits et al. (1999)
CyS crystathionine -γ-synthase	*A. thaliana*	*B. juncea*	Lower Se levels in shoots and increased Se tolerance	Van Huysen et al. (2003)
SMT selenocysteine methyltransferase	*A. bisulcatus*	*A. thaliana*	Increase in foliar Se levels and increase in tolerance to selenite, but not selenate	Ellis et al. (2004)
SMT selenocysteine methyltransferase	*A. bisulcatus*	*A. thaliana*	Increase in total Se levels and increase in tolerance to selenite, but not selenate	Le Duc et al. (2004)
SMT selenocysteine methyltransferase	*A. bisulcatus*	*B. juncea*	Increase in total Se levels and increase in tolerance to selenite, but not selenate	LeDuc et al. (2004)
APS isoform of ATP sulphurylase	*A. thaliana*	*B. juncea*	Increase in Se accumulation and an increase in Se tolerance	Van Huysen et al. (2004)
CyS Crystathionine -γ-synthase	*A. thaliana*	*B. juncea*	Lower Se levels in shoots and increased Se tolerance	Van Huysen et al. (2004)

Table 10.3 (continued)

Transgene	Gene origin (plant species)	Transgenic plant species	Effects on Se tolerance and accumulation	Reference
APS1 isoform of ATP sulphurylase	*A. thaliana*	*A. thaliana*	Decreased Se accumulation and Se tolerance	Sors et al. (2005)
PaAPR APS reductase	*A. thaliana*	*A. thaliana*	Decrease in foliar Se and increase selenate tolerance	Sors et al. (2005)
SATm Mitochondria serine acetytransferase	*T. goesingense*	*A. thaliana*	No significant effects on Se accumulation and tolerance	Sors et al. (2005)
Selenium binding polypeptides/proteins (SBP)	*A. thaliana*	*A. thaliana*	Resistance to Se achieved due to overexpression of Se binding proteins	Agalou et al. (2005)
AtCpNifS chloroplast protein like Se Cys lyase	*A. thaliana*	*A. thaliana*	Enhanced selenate tolerance by reducing Se incorporation into protein	Van Hoewyk et al. (2005)
A number of Se associated genes and gene families	*A.thaliana*	*A. thaliana (RT-PCR used to detect upregulation)*	Se tolerance was linked to upregulation (higher) activity of *APS*, *SULTR* and *SMT*	Zhang et al. (2006)
Selenocysteine lyase (SeCys lyase)	*A. thaliana*	*B. juncea*	Higher selenate tolerance probably by reducing Se incorporation into protein	Banuelos et al. (2007)

Table 10.3 (continued)

Transgene	Gene origin (plant species)	Transgenic plant species	Effects on Se tolerance and accumulation	Reference
SMT Selenocysteine methyltransferase	*A. thaliana*	*B. juncea*	Increase in total Se levels and increase in tolerance to selenite, but not selenate	Banuelos et al. (2007)
SULTR 1,2,3 Sulphate proton transporters	*A. thaliana*	*A. thaliana (knock-down gene technology)*	Selenate accumulation reduced by HAST transport, little effect on selenite	Lydiate et al. (2007)
AtCpNifS chloroplast protein like Se Cys lyase	*A. thaliana*	*A. thaliana*	Confirm higher selenate tolerance by reducing Se incorporation into protein	Van Hoewyk et al. (2008)
SBP 1,2,3 Se binding protein gene family	*A. thaliana*	*A. thaliana*	Elevated tolerance to heavy metal cadmium (Cd) by Se protein also binding Cd	Dutilleul et al. (2008)

2.3 Se Uptake and Transport

Selenate is accumulated in plant cells against an electrochemical potential (or gradient) by active transport driven by ATP (ATPase). Selenate readily competes with the uptake of sulphate, and both anions appear to be taken-up by a number of sulphate transporters in the root plasma membrane (Abrams et al. 1990). The sulphate transporters modulate Se uptake in bacteria and yeasts, and at least two types of these transporters are also present in plants. The S/Se transporters described belong to two main classes (Fig. 10.1):

(a) Transporters that have high affinity for sulphate (HAST). This is likely to be the primary transporter involved in sulphate uptake from the soil, and is expressed mainly in roots with a K_m for sulphate of 7–10 μM. HAST is also considered to be involved in selenate uptake; and

(b) Transporters with a low affinity for sulphate (LAST). This secondary transporter is more likely to be involved in intercellular transport of sulphate, expressed in both the roots and shoots with a K_m for sulphate of 100 μM. LAST is also considered to be involved in selenate uptake (Clarkson and Luttge 1991; Smith et al. 1995; Cherest et al. 1997).

Selenite uptake on the other hand may not be mediated by membrane transporters, as hydroxylamine a respiratory inhibitor inhibits selenite uptake by only about 20%, however hydroxylamine inhibited selenate uptake by 80% (Arvy 1997). Abrams et al. (1990) showed that SeMet uptake by wheat seedlings was coupled to metabolism as evident by the inhibition of uptake by the metabolic inhibitor

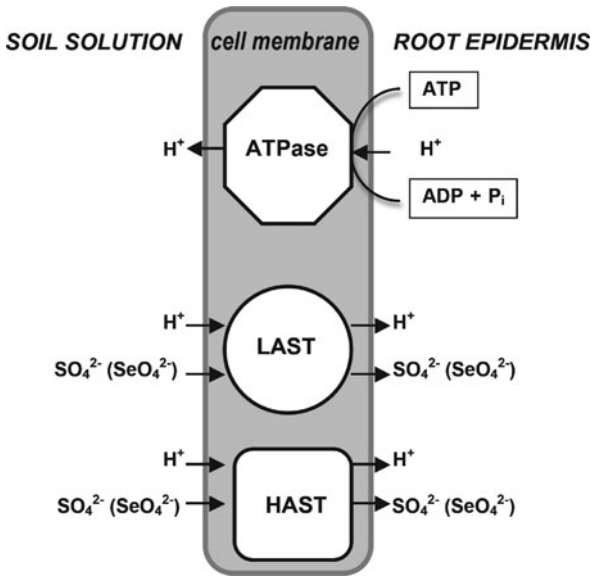

Fig. 10.1 Selenate and sulphate uptake across the root cell membrane driven by ATP (ATPase). LAST is the low affinity sulphate transporter and HAST is the high affinity sulphate transporter (Modified from Sors et al. 2005b)

dinitrophenol and anaerobic conditions. Se concentrations in xylem exudate in roots exceeded that in the external medium by 6–13 times when selenate was added. However when selenite was added Se concentrations in the xylem were always lower than the outside solution, and tends to confirm that membrane transporters may not be involved in selenite uptake (Smith et al. 1997).

Translocation of Se from the roots to the shoots is highly dependent on the form of Se supplied. Selenate is transported more readily than selenite or organic Se compounds. For example, more than 50% of Se was transported from the roots to the shoots within 3 hours when selenate was added. Whilst less than 10% Se was transported from the roots to the shoots when selenite or organic Se was added (Shrift and Ulrich 1976). The reason may be that selenite is more easily converted to organic Se than selenate, and selenate is more strongly retained in the roots after transportation from the soil to the root by HAST. As well, the other conclusion could be that only selenate is readily available in the roots for transportation to the leaves by LAST. The distribution of Se in plants also differs with the type of Se accumulating plant species under investigation:

(a) Se accumulators – Se is accumulated most in young leaves, early vegetative growth, during reproductive stages and seeds; while Se content in mature leaves is reduced greatly (Broyer et al. 1972; Sors et al. 2005a).

(b) Se non-accumulators – Se is often similar in seeds and grains, and in the roots; with lower amounts in the stem and leaves (Arvy 1997; Asher et al. 1977).

Apart from the form and concentration of Se being important, the concentration of sulphur present is important (see Sect. 2.4 below). Plants can also absorb volatile forms of Se from the atmosphere, via the leaf surface and stomata. The Se can quickly be translocated down, probably in the phloem and accumulates in the roots as inorganic selenite, selenoglutathione (SeGSH) and protein bound seleno-methionione (SeMet) (Terry et al. 2000).

2.4 Se Interaction with Other Salts

Sulphates compete with selenate for uptake. Sulphate salinity (i.e., Na_2SO_4) therefore drastically inhibits plant selenate uptake. However, not all Se type plant species are affected in the same way:

(a) Se accumulator plants – selenate is preferentially taken up over sulphate, and so plants can take up high amounts of Se despite the high sulphate salinity present; and

(b) Se non-accumulator plants – have high discrimination for sulphate, and selenate uptake can be significantly inhibited by increasing sulphate supply (Banuelos et al. 1995; Zayed et al. 1998).

On the other hand, chloride salinity (i.e., NaCl) has a much reduced effect on Se uptake, but generally there can be a small decrease in shoot accumulation of Se with

increasing NaCl levels (Wu and Huang 1991; Bell et al. 1992); but this may well be more of an indirect effect of NaCl generally decreasing plant metabolism.

Se is often associated with minerals also containing heavy metals, especially Cu, Ag, Hg and U (Broadley et al. 2007) therefore it is not surprising to find interactions between Se and heavy metals. For example De Filippis (1979) demonstrated that selenite and cysteine decreased the sub-lethal effects of zinc and mercury, including organic mercury to the freshwater alga *Chlorella*. In a recent study there appeared to be an association between Se binding proteins and a decrease in cadmium (Cd) toxicity, these binding proteins are usually rich in sulphydryl groups which may well explain the observations in *Chlorella* (Dutilleul et al. 2008). In reclamation of uranium mines there was present a growing risk of toxic levels of Se being released as a secondary problem to uranium toxicity (Sharmasarkar and Vance 2002). Finally, in phytoremediation of sites from mercury and organomercurials, Bizily et al. (1999) demonstrated that volatilisation of Hg was important and was a process similar to Se volatilisation. The genes for Hg volatilisation have been cloned and transgenic plants have been successfully used in phytoremediation; this appears to be a system in many ways similar to what is being proposed for Se phytoremediation (Rugh 2001).

3 Biochemistry

3.1 Se as an Essential Element

There is some evidence that Se may be required for growth and development in algae, but the question of Se being an essential element (micronutrient) in higher plants remains unresolved (Yokata et al. 1988; Whanger 2002; Pilon-Smits et al. 2009). In Se accumulating plants, indications are that Se may be required for maximum growth potential, especially those endemic to seleniferous soils (Broyer et al. 1966; Broyer et al. 1972). Even in the best studied Se accumulating plant *Astragalus pectinatus* the results of additional Se application in experiments have had differing results (Shrift 1969; Stadtman 1990). It is fair to point out that other nutrients can complex the situation such as phosphates and sulphates, however the experiments so far have not used controls where residual Se is not present at all; and indeed such experiments may be near impossible to perform (Forshhammer and Boek 1991; Stadtman 1996). This is simply because there will always be trace amounts of Se in plants, coming from impurities in the nutrients used or even coming from the atmosphere.

An alternative approach to try to resolve essentiality was to try to detect Se incorporation into Se dependent enzymes, with an integral SeCys residue as present in animals and bacteria (see Sect. 3.3) (Axley et al. 1991). To conclude, the evidence so far from molecular studies available is quite strong that there is no clear evidence for essential selenoproteins in higher plants, but part of the machinery for the synthesis of selenoproteins may be present in plants (see Sect. 4.2) (Berry et al. 1991; Berry et al. 2001).

3.2 Se Assimilation

Higher plants metabolise Se via the sulphur assimilation pathway. Most of the sulphur assimilation pathway is well characterised and described for Se non-accumulator plants. The various biochemical steps in this pathway are described below (Zayed et al. 1999; Sors et al. 2005b).

(a) ATP sulphurylase – Selenate is absorbed by roots via the sulphate transporters (Fig. 10.1) and is usually transported through the xylem without modification to the leaves. Once selenate is inside leaves it enters the chloroplasts where it is metabolised by the enzymes of sulphate assimilation. The first, most critical and rate limiting step is the reduction of selenate to APSe by ATP sulphurylase (Burnell 1981), which is accumulated in the chloroplasts. However, if the same plants are supplied with selenite, organo-Se compounds similar to SeMet are assimilated. De-topped plants supplied with selenate accumulated only selenate in the roots; strongly supporting that the chloroplasts are the sites for ATP sulphurylase activity and selenate reduction (Shaw and Anderson 1972; Pilon-Smits et al. 1999).

(b) Reduction of adenosine 5'-phosphoselenate (APSe) to selenide (Se^{2-}) – The next series of metabolic steps where evidence is available is that APSe can further be reduced to selenide (Se^{2-}) via two pathways; one enzymatically and the other non-enzymatically (Fig. 10.2a):

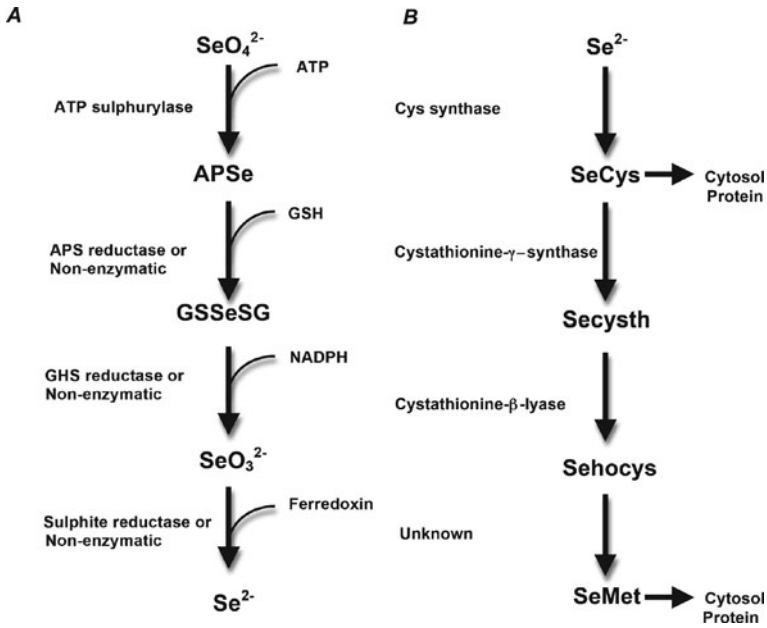

Fig. 10.2 **a** Pathway for selenate activation and reduction to selenide, which can be either enzymatic or non-enzymatic. **b** Pathway of selenide conversion to selenocystein (SeCys) and/or selenomethionine (SeMet) and incorporation of both into proteins

1. *Non-enzymatically* – with the aid of GSH, NADPH and FADH; however GSH reductase (i.e., glutathione reductase) may be necessary as a side reaction (Anderson 1993; Ng and Anderson 1979); and
2. *Enzymatically* – via APS reductase and sulphite reductase; although one non-enzymatic step may also be required (Arvy 1997; Terry et al. 2000).

The intermediate compound selenite (SeO_3^{2-}) can also undergo other transformations besides its final assimilation and reduction to selenide, and enter alternate pathways. This is achieved non-enzymatically by reduction to GS-Se-SG, which is reduced to the selenol (SeGSH). SeGSH is glutathione conjugated selenide. For example plants supplied with selenite can oxidise Se to selenate (Ng and Anderson 1979); a sort of reverse reaction to normal Se assimilation.

3.3 Incorporation of Se into Protein

It is proposed that plants like bacteria incorporate and assimilate SeCys specifically into protein, or after it is metabolised to SeMet. It is likely that this process also occurs in the chloroplasts. In both cases Cys synthase converts Se^{2-} to SeCys, which can be a reverse reaction if the enzyme SeCys lyase is present. SeCys is converted to Secysth by the enzyme cystathionine-γ-synthase, then to Sehocys by another enzyme cystathionine-β-lyase, and finally to SeMet by what is as yet an unknown mechanism (Fig. 10.2b). Finally, either a direct or an indirect pathway of incorporation into proteins takes place for both SeCys and/or SeMet (Foyer and Halliwell 1976; Goutierrey-Marcos et al. 1996):

(a) Direct – SeCys is incorporated via a specific SeCys t-RNA into the selenoproteins.

(b) Indirect – SeCys is converted to SeMet as above (Fig. 10.2b), and a specific SeMet t-RNA incorporates SeMet into selenoproteins.

3.4 Localisation of Se Pathways

A summary of the cellular and sub-cellular localisation of the enzymes and metabolites in the selenium assimilation pathway are given below:

(a) Chloroplasts – for the selenate reduction pathway all enzymes and metabolites have been localised in chloroplasts, wether the reactions are enzymatic or non-enzymatic. Cys synthase and maybe also cystathionine-γ-synthase and cystathionine-β-lyase are localised in the chloroplast. At least until the synthesis of Sehocys most reactions occur in chloroplasts (Kim and Leustek 1996; Setya et al. 1996; Ravanel et al. 1998; Turner et al. 1998).

(b) Cytoplasm – SeMet production from Sehocys and methylation of SeMet to SeMMet, DMSeP and DMSe are thought to occur within the cytoplasm (Fig. 10.3a) (James et al. 1995; Terry et al. 2000).

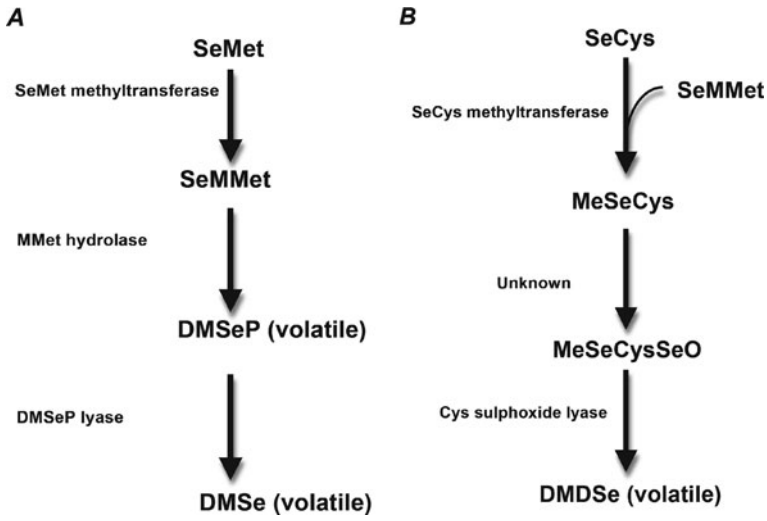

Fig. 10.3 **a** Pathway for the production of volatile forms of Se, DMSeP and DMSe from selenomethionine (SeMet). **b** Additional pathway of production of the volatile DMDSe from selenocysteine (SeCys)

(c) Selenium accumulator plants – the pathway for assimilation of inorganic Se is thought to be mostly the same as described above for Se non-accumulator plants. However Se accumulators differ in that they metabolise the SeCys primarily into various seleno amino acids which are not incorporated into essential proteins. The pathway by which these Se amino acids are synthesised is probably similar to sulphur amino acids (Nigam et al. 1969; Peterson and Robinson 1972).

In the Se hyperaccumulating plants *Astragalus bisulcatus* and *Stanleya pinnata*, elemental Se was localised ultrastructurally by Freeman et al. (2006) and its distribution and chemical forms differed considerably. In *A. bisulcatus* Se was predominantly accumulated in the trichomes of young leaves, and the Se was mostly in the organic form of MeSeCys and γ-glutanyl-MeSeCys. In young leaves only 30% maximum was in the form of inorganic Se (i.e., selenate or selenite). In *S. pinnata* the Se was mostly accumulated near the leaf edges and surface globular structures in epidermal cells; most of the Se was in the form of MeSeCys (Fig. 10.3b). This was in contrast to non-accumulator plants like *A. thaliana* where most of the Se was present in the inorganic form in the vascular tissues and mesophyll cells. In hyperaccumulating plants the Se is mobile in both the xylem and phloem of young leaves, and compartmentation into organoselenium in specific organs and tissues appears to be a unique property of Se hyperaccumulator plants (Freeman et al. 2006).

4 Molecular Biology

4.1 Sulphate Transporters

Initial research on yeast enabled the first sulphate transporter genes to be cloned in plants. These were identified as important in conferring resistance to high concentrations of selenate. Using first strand complementation between yeast and plants three genes (*SHST1, SHST2* and *SHST3*) encoding sulphate transporters were isolated in a legume (*Stylosanthes amata*) (Breton and Surdin-Kerjan 1977; Smith et al. 1995), and another gene (*HUST1*) was isolated from barley (Smith et al. 1995; 1997). Amino acid sequence and protein structural analysis suggested that the transporters contained multiple (up to 12) membrane spanning domains. Using highly conserved cDNA regions, cDNA homologous to the sulphate transporters have been isolated in *Arabidopsis*, Indian mustard, soybean and corn (Davidian et al. 2000). Consistent with the two main classes of transporters in plants and other organisms, gene families for these have also been identified:

***SHST3* gene family for low affinity transporter (LAST)** – which is expressed in both the roots and shoots, and this appears to be the main transporter gene for intracellular transport from the apoplast to the symplast. This transporter gene is modulated strongly by the sulphur status of plants and elevated GSH down-regulate transcription of the genes.

***SHST1* and *SHST2* gene families for high affinity transporter (HAST)** – which is expressed primarily in the roots, and is primarily responsible for the accumulation of sulphate from the soil to the root.

SHST1/2 – over-expression of these genes increased selenate accumulation by at least two fold in Indian mustard, however most of the Se was accumulated and retained in the roots.

SHST3 – over-expression of this gene did not significantly lead to an accumulation of selenate in plant roots, but rather allowed Se to be translocated throughout the plant.

Selenite uptake appears not to be modulated by transporters in the membranes of plants. However selection which resulted in an *A. thaliana* '*sel*' selenite mutant were found to contain less of the sulphate transporter gene *Sultin 1* in the root cortex (Shrift and Ulrich 1976; Abrams et al. 1990). This gene was found to be similar to the *SHST1* gene involved in transporting of both sulphate and selenate from the soil to the root. There are also other sulphate transporter genes (e.g., *Sultin 2* and *Sultin 3*) reported but their role in Se transportation and Se tolerance is not as well described (Table 10.3). From the few studies so far it is highly likely that in Se hyperaccumulating plants the inducible high affinity transporter (HAST) is perhaps simply more selective for selenate rather than for sulphate (Terry et al. 2000).

4.2 Genetic Code and Se Proteins

The incorporation of the active seleno amino acid SeCys into essential selenoproteins is a co-regulation process directed by a UGA codon. UGA normally functions as a universal terminating codon (one of three) present in higher plants (Boek et al. 1991). In order for the process to occur and for integration into proteins to proceed, both specific secondary structural elements in the mRNA and a unique SeCys-charged tRNA that contains the UGA anti codon are required (Stadtman 1996). A key reaction is the activation of selenide to form selenophosphate by the enzyme selenophosphate synthase. Selenophosphate is the Se donor for the conversion of the serine binding tRNA to the SeCys binding tRNA (Stadtman 1996).

Attempts at definitively ascertaining if selenoproteins are present in plants have yielded differing and inconclusive results. Sabeh et al. (1993) found a 6 KDa tetrameric protein in *Aloe vera* which they claim is the selenoprotein GSH peroxidase (GPX). Molecular evidence also suggests that although GPX like enzymes are present in higher plants they appear not to be selenoproteins (Anderson 1993). Peptide sequencing of purified proteins have confirmed that Cys and not SeCys is present in the active site for most of these plant GPX like enzymes. However there appears to be part of the machinery for the synthesis of selenoproteins in plants in that the UGA decoding tRNA has been demonstrated in beet and algae (Hatfield et al. 1992; Eshdat et al. 1997).

4.3 Key Enzyme Genes

ATP sulphurylase – there is experimental evidence supporting selenate is transported into the chloroplast upon uptake, where sulphate and probably selenate assimilation takes place. Mutation studies suggest that increasing expression of genes encoding ATP sulphurylase can increase selenate tolerance of plants up to ten-fold (Pilon-Smits et al. 1999). In addition, with the overexpression of ATP sulphurylase the biosynthesis of organoselenium compounds is maximised, allowing cells to tolerate increased levels of Se because levels of selenate have been reduced (Leustek et al. 1994). Overexpression of an ATP sulphurylase gene (*APS1*) in Indian mustard produced a two-fold higher accumulation of glutathione, and a 2-3 fold increase in total Se content of leaves. Almost the same effects were found in *A. thaliana* of increase Se content with overexpression of an isoform of the gene *APS2*, however in tobacco overexpression of this gene had no significant effects (Saito et al. 2000). Sors et al. (2005a) demonstrated in *A. thaliana* that overexpression of *APS1* decreased Se levels and Se tolerance. A number of subsequent studies detailed in Table 10.3 have confirmed the important role of ATP sulphurylase for increasing tolerance to Se in a number of transgenic plants.

Selenocystein methyltransferase – selenocystein methyltransferase (*SMT* genes) is an important enzyme in Se hyperaccumulating plants, in that large amounts of Se methyl protein are produced, and the enzyme selenocysteine methyltransferase catalyses the methylation of SeCys to MeSeCys. One of the earliest molecular

transformation reports by Lyi et al. (1995) was using this gene, where the *SMT* gene from *A. thaliana* was transferred to *B. oleracea* and affected Se levels in transformed plants. The enzyme has also been cloned in *Astragalus bisulcatus* and overexpression of this enzyme in *Astragalus* leads to both MeSeCys and MeCys synthesis, suggesting the enzyme can methylate both (Van Huysen et al. 2003). Overexpression of *SMT* in *A. thaliana* and *B. juncea* increased foliar and plant tissue Se levels, and increased tolerance to selenite, however *SMT* expression had no significant effect on selenate tolerance (summary in Table 10.3). The *SMT* protein has been characterised and is 65–70% structurally similar to the enzyme homocysteine methyltransferase (*HMT*) from *A. thaliana* and rice (*O. sativa*) (Ellis et al. 2004). Together the evidence suggests that *SMT* and *HMT* have similar structure and function; as well as their Se homologues. This may be an effective sink for both Se and S in plants, however it cannot explain the preference of *Astragalus* hyperaccumulating plants for Se over S.

APS reductase – the constituative expression of APS reductase (*PaAPR*) was investigated and isolated from the bacterium *P. auruginosa* and expressed in *A. thaliana*. There was increased sulphate reductive capacity and accumulation of reduced inorganic and organic forms of sulphur (Bruhl et al. 1996). When treated with selenate, plants increase selenate reduction (65–80%) suggesting it had the capacity to reduce APSe. This was accompanied by a decrease in foliar Se and increased selenate tolerance (Table 10.3). In *Astragalus*, APS reductase activity was similar in non-accumilating and hyperaccumulation species.

Serine acetyltransferase – serine acetyltransferase (*SATm*) is a key enzyme leading to Cys biosynthesis, and this enzyme which in many reports is localised in the mitochondria plays an important regulatory role. In transgenic tobacco where *SAT* overproduction was present, results indicated a drastic increase in o-acetyl serine (OAS) and Cys, and glutathione levels six times higher were recorded (Losi and Frankenberger 1997). However the plants showed no difference in Se accumulation or tolerance (Table 10.3). As well, the hyperaccumulator *Astragalus* was not correlated to higher expression of *SAT*, and it appears that Cys synthesis does not limit selenate accumulation.

Selenocysteine lyase – this enzyme in Se assimilation has been cloned and expression of this gene in *B. juncea* originally sourced from *A. thaliana* appeared to reduce selenate toxicity, and Banuelos et al. (2007) attributed this to a reduction in incorporation of Se into proteins (see Table 10.3). The gene used in this study may well be similar to the *AtCpNifS* chloroplast gene used below by Van Hoewyk et al. (2005).

Selenocysteine transferase – this enzyme was also cloned and expression of this gene in *B. juncea* sourced from *A. thaliana* appeared to have little effect on selenate toxicity but had a small effect on selenite toxicity (Banuelos et al. 2007). The gene describe here may well be similar to the *SMT* gene family used above (Table 10.3), but its full name was not used in the report.

Cystathionine-γ-synthase – another important enzyme in Se assimilation has been cloned and overexpression of *CyS* genes in *B. juncea* lowered Se levels in shoots and increased Se tolerance (Table 10.3).

Chloroplast selenocysteine lyase (*AtCpNifS*) – genes for a chloroplast protein-like SeCys lyase enzyme have been cloned. When this gene was overexpressed in *A. thaliana* it enhanced selenate tolerance by reducing Se uptake into proteins (Van Hoewyk et al. 2005).

Selenium binding proteins (*SBP123*) – a more distant related family of genes that induce higher levels of binding polypeptides and proteins, well studied in *A. thaliana*. It was recently found by Dutilleul et al. (2008) that expression of what was considered specific binding proteins for Se also conveyed tolerance to the heavy metal cadmium (Cd); most likely also by binding this heavy metal (Table 10.3).

Sulphate proton transporter genes – The *Sultr 123* family of genes regulates sulphate transporters, and by association may also regulate Se transportation. Lydiate et al. (2007) using 'knock-down' technology in *A. thaliana* of *Sultr 123* genes reduced HAST transportation of Se, but had little effect on selenite transportation (Table 10.3). The *Sultr* family of genes are likely to be similar to the *SHST* family of genes described before.

For such advancement in molecular and genetic studies as outlined above, it must be pointed out the very important contribution of research by Zeibur and Schrift (1971) where they successfully initiated in tissue and callus culture various species of *Astragalus*. Without the aid of tissue culture, mutagenic and genetic studies on critical enzymes of Se assimilation in different species of *Astragalus* would have been difficult. Another important molecular study was that of Wang et al. (1999) where they clearly demonstrated Se tolerance could be increased via simple selection methods. Analytical methods such as the use of radioactive Se, enzymatic detection assays, immunoblotting and two-dimentional (2-D) electrophoresis separation were also used in this study, which have become standard techniques in later research.

In the future, molecular investigations on Se will need to follow the lead of three other important investigations, which have laid the foundation for more detailed research:

(a) Mapping of quantitative trait loci (QTL) associated with Se tolerance, like the study of Zhang et al. (2006) and Zhang et al. (2007) where selenate tolerance was linked to root growth and epistatic to other important traits, and these genes could be mapped on different chromosomes of *A. thaliana*.

(b) Microarray analysis to compare many up-regulated and down-regulated genes and metabolites between different Se performing clones, like in the study of Tamaoki et al. (2008), where it was found that reactive oxygen radicals and plant hormones were important in Se tolerance.

(c) Proteomic analysis to confirm and detail molecular differences in polypeptide and protein fragments where up-regulated and down-regulated genes and metabolites are involved, and even if the proteins identified contain seleno amino acids or not; like the clinical studies reviewed by El-Bayoumy and Sinha (2005).

4.4 Methylation and Volatilisation

After SeMet is synthesised it can be methylated and converted to dimethylselenide (DMSe) which is the major volatile Se compound in non-Se accumulating plants. The enzymatic steps are well known (Giovanelli et al. 1980; Bourgis et al. 1999) (Fig. 10.3a), however no detail knowledge of the enzymes, except SeMet hydrolase at the molecular level have been investigated. Plants can also volatilise Se as dimethyldiselenide (DMDSe) via oxidative and subsequent methylation with an intermediate DMSeP which is also volatile. The enzymatic and biochemical steps are also well known but no molecular biology knowledge is available (Fig. 10.3b).

5 Phytovolatilisation

5.1 Se Volatilisation

In summary, SeMet may be methylated to Se-methyl-Met (SeMMet) by a series of enzymatic steps which eventually can produce DMSe, or indirectly via the intermediate phosphorylated DMSeP. In either case DMSe (Fig. 10.3a) is produced, and it can be volatilised with the aid of the enzyme DMSeP lyase thought to exist in plants (Hanson et al. 1994; Hanson et al. 1997). By analogy with the production of DMS (dimethyl sulphide) in plants DMSP occurs in chloroplasts. However since roots volatilise more DMSe than shoots or leaves it must be assume that all the enzymes necessary, and especially SMMet hydrolase and DMSeP lyase are also present in roots. The synthesis of SeMet appears to be rate limiting for Se volatilisation (Hanson et al. 1997) and the conversion of SeMet to DMSeP is also rate limiting in plants (Hanson et al. 1997). In accumulator plants in particular methylation to DMSe is abundant before it is volatilised. Similarly, all of the enzymes and steps for production and volatilisation of DMDSe from SeCys are known, except the enzyme that converts MeSeCys to MeSe CysSeO, or this step may be a non-enzymatic step (Fig. 10.3b).

5.2 Variation Amongst Plants

The rate of Se volatilisation varies widely amongst plant species. Rates can be from a high of 200–300 mg Se m^{-2} leaf area day^{-1} in rice, broccoli, cabbage and *Astragalus* to less than 15 mg Se m^{-2} leaf area day^{-1} in sugar beet, bean, lettuce, tomato, alfalfa and tall fescue. In trials, wetland plants showed a 50-fold variation in Se volatilisation, with a low rate of 1 mg Se kg^{-1} dry weight d^{-1} attained for selenate, to a higher rate of 4 mg Se kg^{-1} dry weight d^{-1} for selenite in *Azzola*. The plant *Salicornia bigelovii* had a high rate of Se volatilisation of 420 μg Se m^{-2} soil d^{-1}, and was between 10 and 100 times greater than other species tested; including

salt grass, cord grass, cotton, *Eucalyptus* and canola (Duckart et al. 1992; Terry and Lin 1999).

5.3 Plant/Microbe Interactions

Bacteria, fungi and algae can assimilate and volatilise Se independently of plants; and the rates achieved can be considerably higher than in plants. The question therefore arises in Se volatilisation is how independent are plants in volatilising Se by the presence of microbes in the rhizosphere. An early indication of some dependence by plants on microbes was obtained when de-topped roots were treated with antibiotics (Terry et al. 1992; Brady et al. 1996; De Souza and Terry 1997; Pilon-Smits et al. 1999). The rate of Se volatilisation was reduced by antibiotics by as much as 95% for selenate supplied broccoli. Subsequent research was done to try to resolve this question with sterile and non-sterile tissue culture plants. Using Indian mustard it was shown that Se volatilisation did require a rhizosphere to volatilise substantial Se from selenate and selenite; but this was not the case when SeMet was added (Rael and Frankenberger 1996; Fan et al. 1997).

The role of the rhizosphere microbes appeared to be somewhat specific for selenate and its uptake, by producing heat labile compound(s) that were proteinaceous in nature; possibly the amino acid derivative o-acetylserine (OAS) and the amino acid serine which can stimulate the uptake of selenate by the sulphate transporters. There was no such stimulation with selenite supplied plants, and indications were that the rhizosphere organisms aided in the production of organic Se compounds like SeMet, which can be converted to DMSeP and DMSe, and both of these compounds are more readily volatilised (Thompson-Eagle et al. 1989; Zayed et al. 1998).

5.4 Environmental Factors

The ability of plants to volatilise Se is influenced by the concentration of Se around the roots and the chemical form of Se supplied. There was a direct linear relationship between an external Se concentration and internal plant tissue concentration of Se in Indian mustard supplied with selenate or selenite (De Souza et al. 1999). Se volatilisation was also correlated to plant tissue concentrations, and selenite treated plants released 10–15 times more Se than plants supplied with selenate. However plants supplied with SeMet volatilised Se at an even higher rate; but plants supplied with DMSeP volatilised Se at the highest rate recorded (Terry et al. 1992). These findings were consistent with studies described before for aquatic plants in constructed wetlands (Terry 1998).

An important environmental factor in volatilisation of Se is the concentration of sulphate compared to selenate in the soil. Se volatilisation can be inhibited strongly by the presence of sulphate in the range of 0.25–10 m*M*. Rates of volatilisation decreased from 97 to 14 μg Se m^{-2} leaf area day^{-1} with the higher sulphate supply

(Zayed et al. 1998). The rate of inhibition generally decreases with an increase in the S:Se ratio in plant tissue. The inhibition of volatilisation suggests that sulphur compounds out compete Se compounds for the active sites of the enzymes responsible for Se volatilisation. In the field, rates of volatilisation vary enormously, and also vary with the time of the year (Martens and Suarez 1997). Se volatilisation is at its highest rate in spring and early summer. In wetlands, Se volatilisation is dependent on many parameters, like Se concentration, water sediment, the plant used, microbial biomass in sediment, pH, salinity, dissolved oxygen, depth and temperature. However the most important factors appear to be water temperature, Se concentration in roots and microbial biomass in the sediment (Hanson et al. 1997; Terry and Lin 1999).

6 Phytoremediation

6.1 Process

Low level large scale contamination presents monumental economic and logistical barriers to effective, timely treatment. A number of technologies have been successfully applied, and all fall into the two broad categories below:

Engineering based technologies – which can be aggressive and are usually applied to cleanup more acute polluted point sources. These can be not cost effective or even environmentally justified for marginally affected sites. The methods can be diverse but usually include excavation and entombment or variations of these methods Lynch and Moffat (2005). The methods are not likely to diminish or alleviate the hazardous material, and more importantly they cannot reduce landfill capacity. Engineering based approaches are usually applied to where more rapid responses are required but can cause secondary problems in the long term (Pilon-Smits 2005; Banuelos 2006). These engineering methods and their possible application to Se remediation will not be covered in this review.

***In situ* biological remediation** – could be a cost effective and more appropriate corrective option for treatment of wide-spread, low impact contamination (Banuelos 2001). The methods fall into two sub-categories of:

Bioremediation – a microbial induced process, and
Phytoremediation – which refers to a plant based clean-up processes.

6.2 Plant Species

A variety of plant taxa possess a remarkable natural ability to accumulate metals (phytoextraction) or even degrade organic compounds (phytodegradation). Superior Se phytoaccumulating species of plants have been characterised, identified and

studied at the physiological, biochemical and molecular level. Even more, a selected few of these important plants have been well described at the molecular and genetic level, and a very small number have been genetically manipulated. For example, Banuelos et al. (2002) have identified and transformed the functional trait (actually a key enzyme) from a Se hyperaccumulating species (*A. bisulcatus*) to the non-accumulator *A. thaliana*; conferring increased Se tolerance and some increase accumulation of Se (see Table 10.3). Metal hyperaccumulating plants and their identification have been recognised for a relatively long time (Berken et al. 2002), and have been used in different ways by researchers and ecologists. Some of the ways metal and metalloid hyperaccumulating plants have been used include:

Phytomining – historically metal hyperaccumulating plants were only recognised for their ability to identify sites or areas useful as possible mining sites, mostly of sought after deposits of metals (phytoprospecting) and recovery of the metals (Baker et al. 2000).

Revegetation – more recently plants that can survive high metal content have been used increasingly in revegetation projects, some necessary by legislation, and yet others done for aesthetic purposes, as for example barren, eroding mining or industrial impacted soils. Recovery of metals was not a primary objective (i.e., as in phytomining) as it was deemed that recovery was too expensive and uneconomic (Sors et al. 2005a). However these practices and other technologies have lead to the 'invention' of more refined phytoremediation techniques.

Metal recovery – plant based recovery of soil based metals and their reuse has been described only for nickel (Ni) and thallium (Th); which have high economic value. Other toxic metals for example like mercury (Hg), lead (Pb), arsenic (As), cadmium (Cd) and caesium (Cs) have little economic value and are also extremely toxic; these must be processed as hazardous waste and so far have not been proposed to be used in conjunction with accumulation in plants (Freeman et al. 2004).

Biological beneficial minerals – essential minerals could be good candidates for combined phytoextraction and use in for example dietary supplements. These include zinc (Zn), iron (Fe) and selenium (Se), which have been used in crop fortification for increased essential mineral enrichment of edible crops (Finley 2005). Indian mustard (*B. juncea*), *Astragalus* species and a number of other crops and vegetable species have been fortified for Se for many years now (a list is presented in Table 10.2) (Mayland et al. 1989; Parker et al. 1991).

6.3 Para-Phytoremediation

Such mixed-benefit strategies as described just before should be considered to be 'para-phytoremediation', which combines and identifies the useful part of the remediation method in plants with their ability to detoxify the environment in which plants are grown (Wu et al. 1988; Wu 2004). There may be other products that could be obtained from plants loaded with potentially toxic and valueless metals and metalloids, apart from nutritional enhancement for essential micronutrients

and environmental detoxification. One such benefit proposed is energy production which accompanies incineration, and is a procedure required to process and dispose of hyperaccumulating plant biomass. Another possible product in the case of Se could be in the extraction of biopharmaceutical compounds used in cancer treatment (Banuelos 2006). The attraction and benefits of these proposals are that these so called 'crops' could be grown on otherwise non-productive lands for profit; and these could be strong incentives to cost-effective treatment of toxic area for not only energy, but paper, fibre, building materials and health supplement/treatment.

6.4 Problems

An obvious concern over phytoremediation techniques, especially in using genetically modified plants is the possible transfer of undesirable traits to elite plants and crop cultivars for agriculture (Hanson et al. 1997; Terry et al. 2000). The concern over hyperaccumulation and high levels of for example Se during uptake into plants may limit the use of phyto-crops for food or animal consumption. However technology exists to identify the fate of most of these toxic compounds, and their toxicity; as demonstrated with the development of chemo preventitive enriched Se accumulating (fortified) edible crop plants like potato, radish and other vegetables in Australia, UK, USA and other parts of the world (Table 10.2) (Broadley et al. 2004; Lefsrud et al. 2006; Pedrero et al. 2006; Haug et al. 2007; Zhao et al. 2007).

A major environmental problem is how to clean-up Se from constructed wetlands and their waters. An affective solution appears to be to use 'artificially constructed wetlands'. Up to 90% of Se from oil refinery effluent has been shown to be removed by wetlands, and Se was substantially contained in the sediment. But a considerable amount was present in plant tissue, and a reasonable amount also volatilised into the atmosphere (10–30%). Wetland efficiency for removal of Se depends on the most suitable plant species planted and some species like cattail grass (by size of its biomass) and widgeon grass (by amounts hyperaccummulated) removed the most Se in trials so far (Banuelos 2006; Nyberg 1991). A full review of Se removal by constructed wetlands is presented by Wu (2004), and it will not be dealt with further in this review.

7 Conclusions/Future Directions

World selenium (Se) resources need to be managed so that this non-renewable vulnerable resource is not squandered. Se uptake, mobilisation and assimilation are quite well understood and are similar to sulphur, however there are some steps not well understood, especially enzymatic and non-enzymatic steps leading to the reduction to selenide. Se hyperaccumulating plants do have differences in uptake and sequestration of Se which require more investigations, and essentiality of Se to

higher plants also needs to be resolved. Growth potential of Se plants as agricultural crops for biomass production and identification of the chemical species of Se present and their quantification in plants is necessary for any use in health supplementation. Seleniferous soils are potentially useful in their use, but the soils need to be better identified and field testing needs to be done before they may be considered potentially usable for an intense agricultural system of farming. It is also clear that just simple biofortification of crops needs to be considered carefully for value and effects. Perhaps a new method combining the use of Se-enriched sprouts (i.e., young tender shoots) provided through the germination of seeds of selected plants in Se rich soils is an interesting new concept worth considering and trialling.

Molecular studies and overexpression of genes encoding proteins involved in Se uptake, transport and assimilation have been reported, and we can still expand on these types of experiments and observations. In this way further strategies for genetic engineering of Se accumulation, transformation and toxicity will become evident, and the use of transgenic plants for use in a variety of ways could be evaluated. Phytoremediation offers a cost effective and environmentally friendly alternative or complementary technology to conventional bioremediation techniques. However the underlining biological processes of phytoremediation are still largely unknown in many cases, and important areas which need more detail investigations are plant-microbe interactions, mechanisms of degradation and transformation, volatilisation, chelation, binding and detoxification. The feasibility of mixed-use strategies for phytoremediation is worth considering with the use of genetically improved phytocrops in Se enriched soils. In this regard there is value in enhancement of traits in plants useful in phytoremediation such as high biomass and growth potential in seleniferous soils, which might otherwise be considered agriculturally non-productive land. Se-hyperaccumulating plants (wether naturally occurring or transgenic plants) have possibilities in that they combine pollutant decontamination with production of a product with beneficial properties to humans and animals.

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Chapter 11
Perspective on Phytoremediation for Improving Heavy Metal-Contaminated Soils

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Abstract Heavy metal pollution of soil is a significant environmental problem and has its negative potential impact on human health and agriculture. Phytoremediation strategies with appropriate heavy metal-adapted rhizobacteria (for example, mycorrhizae) have received more and more attention. Some plants possess a range of potential mechanisms that may be involved in the detoxification of heavy metals, and they manage to survive under metal stresses. High tolerance to heavy metal toxicity could rely either on reduced uptake or increased plant internal sequestration, which is manifested by an interaction between a genotype and its environment. A coordinated network of molecular processes provides plants with multiple metal-detoxifying mechanisms and repair capabilities, which allow plants to survive under metal-containing soil environments. The growing application of

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molecular genetic technologies has led to an increased understanding of mechanisms of heavy metal tolerance/accumulation in plants and, subsequently, many transgenic plants with increased heavy metal resistance, as well as increased uptake of heavy metals, have been developed for the purpose of phytoremediation. This article reviews advantages, disadvantages, possible mechanisms, current status and future directions of phytoremediation for heavy metal contaminated soils and environments.

Keywords Phytoremediation · Heavy metals · Soil · Mechanisms · Signal transduction · Phytohormones · Transcription factors · Biotechnology · Hyperaccumulator · Gene expression

Contents

1 Introduction

Phytoremediation of metals is being developed as an effectiveand environment-friendly solution for heavy-metal–contaminatedsoils (Barceló and Poschenrieder 2003; Banuelos et al. 2007; Aina et al. 2007). In recent years, major scientific strides have been takenin understanding the soil chemical and plant molecular-geneticmechanisms that drive metal hyperaccumulation in plants. Becausehyperaccumulators are mostly low biomass and slow-growing plants,current research is focused mainly on designing transgenic plantsthat can overcome this deficiency. The complexity of plant-metal interactions and influences of the environment, andspecific matrix factors that control the chemical speciationof the metal, and interactions of other toxicants that may bepresent at the site all add to the strategy of phytoremediation (Bassirirad 2000; Bauer and Bereczky 2003). Extensive progress has been made in characterizingand modifying the soil chemistry of the contaminated sites topromote/accelerate metal phytoremediation. However, extensivefield deployment of this technique on a large scale is stillbeing hampered by a lack of specific understanding of the complex interactions between metal, soil, and plant systems that are instrumental in metal uptake, translocation, and storage in plants. A multidisciplinary research effort that integrates the work

of plant biologists, soil chemists, microbiologists, and environmental engineers is essential for the success of phytoremediation as a viable soil cleanup technique in metal-contaminated sites (Brewer et al. 1999; Bennett et al. 2003).

Phytoremediation is the use of a plant's natural ability to contain, degrade, or remove toxic chemicals and pollutants from soil or water. It can be used to clean up metals, pesticides, solvents, explosives, crude oil, and contaminants that may leak from landfill sites. The term phytoremediation is a combination of two words – phyto, which means plants, and remediation, which means to remedy (Clemens 2006; Denton 2007; Shao et al. 2008a, b, c, d, e).

Researchers are investigating phytoremediation potential by using plants such as sunflower, ragweed, cabbage, geranium, *Thlaspi caerulescens*, *Arabidopsis thaliana*, *Lycopersicon esculentum*, *Zea mays*, *Hordeum vulgare*, *Oryza sativa*, *Pisum sativum*, *Lotus japonicas*, *Brassica*, *Sedum alfredii*, *Cannabis sativa*, as well as other less known species. The plants are often used in combination with other traditional technologies for cleaning up contaminated sites because of the phytoremediation limitations (Cobbett 2002; Curie and Briat 2003; Citterio et al. 2003; Czako et al. 2006) There are many advantages of phytoremediation for heavy metal-contaminated soils (Table 11.1).

Table 11.1 Advantages of phytoremediation

Advantages	Disadvantages
1. Environment friendly, cost-effective, and aesthetically pleasing 2. Metals absorbed by the plants may be extracted from harvested plant biomass and then recycled 3. Phytoremediation can be used to clean up a large variety of contaminants; 4. May reduce the entry of contaminants into the environment by preventing their leakage into the groundwater systems	1. Relies on natural cycle of plants and therefore takes time 2. Phytoremediation works best when the contamination is within reach of the plant roots, typically three to six feet underground for herbaceous plants and 10 to 15 feet for trees 3. Some plants absorb a lot of poisonous metals, making them a potential risk to the food chain if animals feed upon them

2 Understanding Mechanisms of Phytoremediation for Improving Heavy Metal Contaminated Soils

2.1 Heavy Metal Accumulation in Plants

Heavy metals can be accumulated in various plant organs, which belong to the long-term effects of heavy metal action (Cunningham et al. 1995; Datta and Sarkar 2004). Their presence was detected in roots, stems, leaves, seeds and fruits. The cell wall is

suggested to be the main accumulation site of Cd and other heavy metals. A similar accumulation site was found in vacuoles, especially in the case of Zn. In stems, Zn accumulated along the walls of vascular bundles, and in roots along cell walls. Its deposition occurred either in the form of simple Zn salts or proteins and carbohydrates complexes with Zn. Irons of heavy metals are detoxificated in the cytosol by high-affinity ligands like amino acids, organic acids and two types of peptides: PCs (phytochelins) and MTs (metallothioneins) (Deckert 2008; Doty 2008). It is generally assumed that the major sites of metal sequestration are vacuoles of root cells. PC-Cd complexes are transported into the vacuole, where heavy metal complexes are formed. Accumulation of heavy metals in chloroplasts is still controversial (Eide et al. 1996; Dhankher et al. 2002).

Ni was found to accumulate in seeds of *Raphanus sativus*, its level being maximal after 10 h of treatment (Elizabeth 2005). In wheat leaves, most of Ni accumulated up to the 3rd day after the application because of a fast and long distance transport of this metal (Fox and Guerinot 1998; Fayiga et al. 2004). Roots and shoots of *Pisum sativum* showed different metal accumulation capabilities. Ni amount in roots increased as a function of metal supply and was markedly higher than in shoots. In maize, Ni accumulated in chloroplasts of the bundle sheath cells and in the root apex. In chloroplasts, Ni was found to be more associated with their lamellar fraction than with the stroma and envelope (Gleba et al. 1999; Ghosh and Singh 2005; Huang and Cunningham 1996).

The content of Hg in tomato seedlings increased concurrently with Hg concentration and exposure time. More Hg was accumulated in roots than in above ground plant parts. Mature tomato leaves contained the greatest, whereas younger ones the smallest Hg content (Savenstrad and Strid 2004).

In rice seedlings growing at increasing lead concentration, Pb was distributed in an organ-dependent specific manner, which was greater in roots than in shoots. Pb was unevenly distributed in roots, where different tissues act as barriers to apoplastic and symplastic Pb transport, restricting its transport to shoots (Rugh et al. 1998; Hartley-Whitaker et al. 2001, 2002; Kramer 2005; Haydon and Cobbett 2007).

2.2 Genes Involved in Heavy Metal Perception and Signal Transduction

2.2.1 Heavy Metal Sensors

There are limited data on metal perception and signal transduction pathways in plants. The perception of extracellular signals is thought to be mediated by receptor-like protein kinases. The receptor-like kinase involved in heavy metal stress in plants has been reported very recently. The gene coding for lysine motif receptor-like kinase in barley was shown to be induced by Cr, Cd, Cu during leaf senescence (Fusco et al. 2006). The proteomic study on Cd-treated rice roots indicated the induction of putative receptor protein kinase. However, more detailed study on the function of this putative receptor has not been published so far.

2.2.2 Signaling Involved in Calcium, Reactive Oxygen Species (ROS) and Mitogen-Activated Protein Kinases (MAPK)

The heavy metal stress signaling in plants involves calcium changes, MAPK cascades and transcriptional activation of the stress-responsive genes (Gasic and Korban 2007; Li et al. 2005; 2006). The expression of metal-induced barley receptor-like kinase is also mediated by Ca level. It was suggested that certain metals (Cd, Ni, Co) may cause perturbation in intracellular calcium level and interfere with calcium signaling by substituting Ca in calmodulin regulation (Kim et al. 2007). By using calcium indicator, it was recently proved that metals such as Cd and Cu induce calcium accumulation in rice roots (Yeh et al. 2007). The treatment of tobacco cells and Scots pine roots with Cd and lupine roots with Pb caused the generation of H_2O_2(Meda et al. 2007). The Cd-producing oxidative burst in tobacco is mediated by calmodulin and/or calmodulin-dependent proteins. Thus, available data suggest the involvement of Ca/calmodulin pathway in signaling of metal response in plants (Sunkar and Zhu 2004).

MAPK pathway is involved in the transduction of extracellular signals to intracellular targets in all eukaryotes (McCully 1999; Pence et al. 2000; Shao et al. 2008). It was recently indicated that Cd and Cu activate four different MAPKs (SIMK, MMK2, MMK3 and SAMK) in alfalfa, whereas Cd induces one such kinase (ATMEKK1) in *Arabidopsis* and one (OsMAPK2) in rice (Persans et al. 2001; Sasaki et al. 2006; Kassis et al. 2007). However, it is not clear if activation of MAPKs occurs by direct action of these metals or through ROS, which also activates MAPK cascade in *Arabidopsis* or it occurs via action of other mediators (Wawrzynski et al. 2006). Recent information shows that Cd- and Cu-induced MAPK activation requires the involvement of calcium-dependent protein kinase (CDPK) and phosphatidyl-inositol 3-kinase (PI3 kinase) (Yazaki et al. 2006). Therefore, the current model for Cd and Cu signal transduction pathway states that both metals induce ROS production and calcium accumulation. The CDPK and PI13 kinase may be involved in metal-induced MAPK activities. However, both of these metals induce MAPK activation via distinct ROS-generating systems, therefore the MAP responsiveness may differ depending on the type of metals and ROS involved. MAPKs usually link the cytoplasmic signal to nucleus, where they activate other protein kinases, specific transcription factors and regulatory proteins (Sunkar et al. 2006; Shao et al. 2008).

2.2.3 Phytohormone Signaling

The signaling pathways involving abscisic acid (ABA), salicylic acid (SA) and auxin (IAA) also participate in the response to heavy metals, as respective *cis*-DNA regulatory elements were detected in heavy metal-induced genes. The auxin-responsive mRNA was detected in Cd-treated *Brassica juncea* plants (Lindblom et al. 2006). Proteomic analysis of Cd-treated *Arabidopsis thaliana* showed the induction of nitrilase protein, which is involved in auxin biosythesis (Roth et al. 2006). The transcription activation of the gene (*SAMT)* involved in biosynthesis of SA was detected

in pea treated with Hg. It is known that Cd induces the biosynthesis of ABA and ethylene, which in turn evoke various stress responses. All these data confirm that phytohormones play a role in plant responses to heavy metals. However, it is not clear if they play the signaling role in activation of heavy metal-responsive genes, or serve as effectors of certain heavy metal-imposed reactions to participate in both processes.

2.2.4 Heavy Metal – Induced Transcription Factors and Heavy Metal Responsive Elements

Little is known about transcriptional processes in plants in response to heavy metals as well as functional link between signaling pathways and responses at transcription level. The transcriptional profiling of plants treated with various heavy metals indicated that they can induce into heavy metal-induced transcription factors (LeDuc et al. 2006). The Cd-induction of transcripts for basic region leucine zipper (bZIP) and zinc finger transcription factors has been detected in *Arabidopsis thanliana* and *Brassica juncea* (Ramos et al. 2007). Screening of Cd-responsive genes in *Arabidopsis thanliana* indicated that *DREB2A* gene is up-regulated by Cd. The DREB proteins bind to dehydration response element and in Cd-treated *Arabidopsis thaliana*, *DREB2A* preferentially activates the *rd29A* gene, which is thought to play an important role under cold, high-salt and dehydration (Rosen 2002; Srivastava et al. 2005; Shao et al. 2008). On the other hand, one of the Cd-induced bZIP transcription factor (OBF5) in *Arabidopsis thaliana* binds to promoter region of glutathione transferase gene (*GST6*), which is known to be induced by auxin, SA and oxidative stress (Qi et al. 2007). The Zn treatment of *Arabidopsis thaliana* caused the induction of one type of transcription factor (bHLH), whereas the expression of two others (WRKY and zinc-finger, GATA-type) was decreased in the presence of excess of Zn (Ouelhadj et al. 2007). Despite existing data on the heavy metal-induction of different transcription factors, it is still not clear if these activations are specific to particular heavy metal ,common to most of the metals, related to oxidative stress (caused directly or indirectly by most of the heavy metals), mediated by phytohormones or connected with the general plant stress response (Sun and Zhou 2005). The process of ROS-mediated transcription activation of factors is thought to be a common link in different stress responses in plants. Therefore, among all possible pathways, ROS seems to play a key, but not the only one, role in activation of heavy metal-induced transcription factors in plants. Other organisms, such as yeast and animals, contain specific heavy metal-induced transcription factors which bind to heavy metal responsive element present in promoters of heavy metal-responsive genes (Cobbett 2002). The *cis*-acting elements related to heavy metal responsive elements have been found within promoters of a few plant genes, including metallothionein-like genes, however there is no evidence that these sequences confer heavy metal responsiveness of these genes. So far only two types of *cis*-DNA elements, which may be functional in heavy metal response, have been described in plants (Deckert 2008). One type is iron-dependent regulatory sequences (IDRS), which are responsible for the iron-regulated transcription of genes involved

in Fe acquisition. The second one has been recently identified within the promoter region of *PvSR2* gene from *Phaseolus vulgaris*. *PvSR2* gene encodes a heavy metal stress related protein, whose expression is strongly stimulated by Hg, Cd, As and Cu, but not by other environmental stresses such as UV radiation, high temperature or pathogens. The heavy metal-responsive elements were localized within two regions of *PvSR2* gene promoter. Region I contains a motif similar to the consensus metal-regulatory element of the animal metallothionein genes, whereas the region II represents a novel heavy metal-responsive element in plants and has no similarity to previously identified *cis*-acting DNA elements involved in heavy metal induction.

According to the above concerning the activation of various transcription factors, which also confer the response to other stimuli, the lack of specific heavy metal-induced transcription factors and very limited data on the function of *cis*-acting and metal-specific DNA elements indicate that plants employ a wide array of mechanisms to activate the genes required to cope with the excess of heavy metals in their environment (Rocovich and West 1975; Ma et al. 2001; Rupali and Sarkar 2004). Possible molecular mechanisms of phytoremediation for heavy metal-contaminated soils, in combination with signaling pathways and transcription regulation, has been summarized in Fig. 11.1.

2.2.5 Phospholipid Signaling

Phospholipid signaling plays a crucial role in serving as a second messenger in plant responses to heavy metal stress (Shao et al. 2008). Phospholipds are rapidly produced in response to a variety of stimuli by the activation of lipid kinases or phosphatases. The expression of phospholipase D was shown to be induced by ABA, cold, drought, high salinity, wound and pathogen interactions (Bergmann and Munnik 2006). Some results indicate that this pathway may also be involved in plant response to heavy metals as the increased level of phospholipases transcripts were observed in cadmium-treated plants and phosphatidyl-inositol 3-kinase was shown to take part in cadmium and copper activation of MAPKs in rice roots (Yeh et al. 2007). The growing evidence suggests that plant signaling consists of network of pathways operating during various stress situations and that the crosstalk exists among stress responses, phytohormones and ROS signaling (see Fig. 11.1) (Sunkar and Zhu 2004; Sunkar et al. 2006; Fujita et al. 2006; Shao et al. 2008).

2.2.6 Posttranscriptional Regulation of Heavy Metal-Dependent Genes By MicoRNAs

MicroRNAs (miRNA) and short interfering RNAs (siRNAs) are small noncoding RNAs that have recently come out as a global important regulator of mRNA degradation, translational repression and chromatin modification (Sunkar and Zhu 2004). MicroRNAs are small, 21–22 nucleotides long, RNA molecules that can contribute to the regulation of gene expression in plants by directing an endoribonuclease complex to degrade the target mRNAs.The involvement of miRNAs in regulation of gene expression is mostly known for various developmental processes

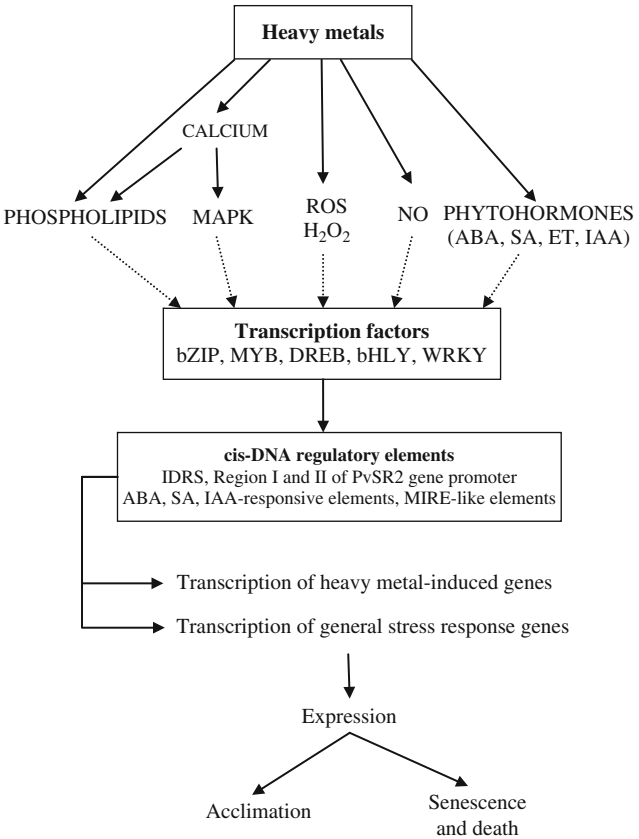

Fig. 11.1 Possible molecular mechanisms of phytoremediation for heavy metal-contaminated soils, in combination with signaling pathways and transcription regulation

(Dugas and Bartel 2004; Shao et al. 2008), but recently their participation in stress responses has been paid more attention (Sunkar and Zhu 2004; Shao et al. 2008). The predicted targets of number of *Arabidopsis thaliana* microRNA families, designated as miR398, are the mRNAs coding for cytoplasmic and chloroplast Cu-Zn-superoxide dismutase (Cu,Zn-SOD:CSD1 and CSD2) and a subunit of mitochondrial cytochrome C oxidase (COX5b-1). It was shown that miR398 expression is down-regulated transcriptionally by heavy metals, light and other oxidative stresses. This down-regulation of miR398 is important for up-regulation of mRNAs coding for Cu-Zu-SOD and oxidative stress response (Sunkar et al. 2006). Further studies indicated that the same microRNA (mir398) regulated copper homeostasis and mediated this regulation by controlling the degradation of Cu-Zn-SOD mRNA when Cu was limited (Yamasaki et al. 2007). It is clear that posttranscriptional processes involving microRNAs play important roles in regulating plant heavy metal dependent genes, which is a fine performance of acclimating mechanisms of higher

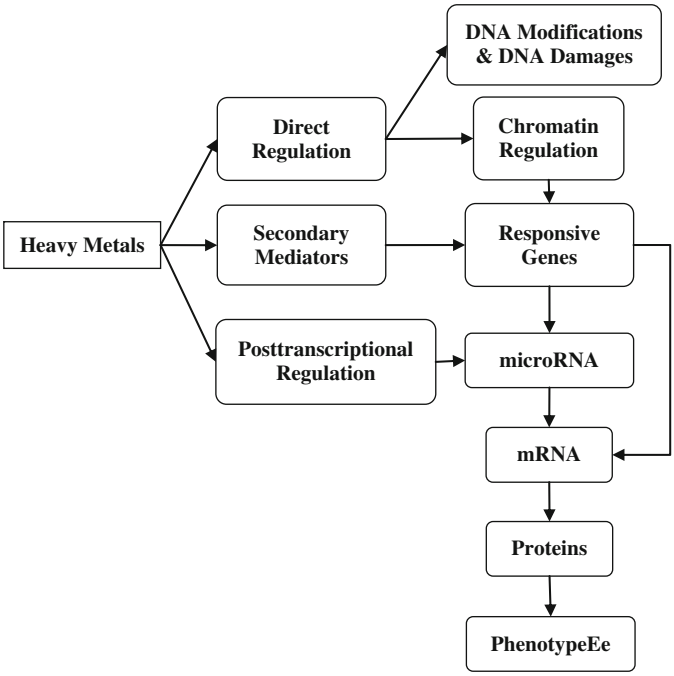

Fig. 11.2 A framework for the gene expression and regulation when plants are exposed to heavy metals

plants under the changing environment. A possible framework for the gene expression and regulation when plants are exposed to heavy metals is summarized in Fig. 11.2.

3 Important Standards for Heavy Metal Hyperaccumulator Plants

How do heavy metal hyperaccumulator plants achieve this remarkable bioaccumulation of soil heavy metals? Researchers have identified several characteristics that are important:

1. The plant must be able to tolerate high levels of the element in root and shoot cells; hypertolerance is the key property which makes hyperaccumulation possible. Such hypertolerance is believed to result from vacuolar compartmentalization and chelation. The most direct demonstration used isolated vacuoles from protoplasts of tobacco cells which had accumulated high levels of Cd and Zn. Whether hypertolerance in the known hyperaccumulators is due to an enhancement of these mechanisms is not yet known. However, electron

microprobe analysis supports vacuolar compartmentation for Zn in the leaves of the hyperaccumulator *Thlaspi caerulescens*.

2. A plant must have the ability to translocate an element from roots to shoots at high rates. Normally root Zn, Cd or Ni concentrations are 10 or more times higher than shoot concentrations, but in hyperaccumulators, shoot metal concentrations can exceed root levels. Researchers recently found that although the chemical forms of Ni found in extracts of leaves of Alyssum hyperaccumulators are the chelates with malate and citrate, in the xylem exudate histidine chelates about 40% of the total Ni present; nearly all of the histidine in exudate is chelated with Ni. Whether Ni^{2+} or a mixed chelate such as Ni (histidine, malate) is pumped into the xylem by a membrane transporter remains unknown. Additions of histidine to nutrient solution increased Ni tolerance and transport to shoots by *Alyssum montanum*, a non-hyperaccumulator species.
3. There must be a rapid uptake rate for the element at levels which occur in soil solution. Here quite different patterns have been observed in different groups of hyperaccumulators. Studies showed that *T. caerulescens* accumulated Zn and Cd from nutrient solution only about as well as tomato and *Silene vulgaris* did, but tomato was severely injured at 30 μM Zn, *S. vulgaris* at 320 μM Zn, and *T. caerulescens* only at 10,000 μM Zn. Because this species can keep tolerating and accumulating Zn and Cd at high soil solution levels, it is found in nature with 1–4% Zn while surrounding plants are <0.05% Zn (Zn excluders). Further, studies have shown that Zn hypertolerant genotypes of *T. caerulescens* require much higher solution Zn^{2+} (104-fold) and leaf Zn concentrations (100–300 mg kg^{-1} vs. 10−12 mg kg^{-1} in normal plants) to grow normally than do related non-hyperaccumulator species. By implication, the highly effective compartmentalization to reduce the toxicity of Zn and Cd appears to require the plant to accumulate much more Zn to have adequate supply. In contrast, the Ni-hyperaccumulator *Alyssum* species accumulate remarkably higher shoot Ni levels compared to other species grown at the same Ni^{2+} activity in solution. The Se-hyperaccumulating species similarly accumulate higher shoot Se levels and many can volatilize Se at high rates growing beside plants with more normal levels and slow volatilization.

4 Biotechnology and Phytoremediation of Heavy Metal Contaminated Soils

Biotechnology approaches to develop phytoremediation plants have been examined. Traditional plant breeding can only use available genetic diversity within a species to combine the characteristics needed for successful phytoremediation. Researchers expected that increasing the concentrations of metal binding proteins or peptides in plant cells would increase metal binding capacity and tolerance. Although plant cell cultures expressing mammalian metallothioneins (MTs) or phytochelatins (PCs) are more tolerant of acute Cd toxicity, the transfer of mammalian metallothionein

genes to higher plants appears to provide no benefit for phytoremediation. Further, when natural metal hypertolerant plants were examined, the concentration of PCs showed no difference, suggesting that hypertolerance to Cd and Zn in these plants was not due to the hyperaccumulation of PC peptides. The evidence for the role of PCs is that their presence does correlate with normal levels of metal tolerance, since mutations that abolished PC production in *Arabidopsis* and fission yeast resulted in hypersensitivity to Cd. Cd-sensitive (hypotolerant) single gene mutants *cad1* and *cad2* of *Arabidopsis thaliana* have been identified and studied (e.g. PC synthesis). For a plant species with normal tolerance (*A. thaliana*), PCs were essential for the normal level of tolerance (Cunningham et al. 1995; Wu et al. 2006; Doty 2008; Shao et al. 2008).

Although these studies have allowed cloning of genes involved in acute Cd tolerance, and characterization or confirmation of metabolic pathways, the environmental relevance of findings from such acute Cd exposure has not been established. An alternative view of Cd-catalyzed PC biosynthesis is that chelation of PCs with Cd alleviates the feedback inhibition of the PC-synthase; as long as Cd activity in the cytoplasm is high, an enzyme supports more transfer to form more PCs and longer PCs. Because the level of Zn present in nearly all environments is 100 times higher than that of Cd, if an acute toxic Cd dose is provided, the plants would be killed by Zn. Even the formation of the sulfide-stabilized high molecular weight Cd-PC complex in vacuoles may result from the acute toxic Cd supply without Zn. Further, the finding that the hmt1 vacuolar membrane pump protein (which restored Cd hypertolerance to mutant fission yeast) transported both Cd-PCs and PCs without Cd, raises questions about how the pump works to induce Cd hypertolerance *in vivo*. Cadmium (Cd) phytotoxicity in soil is a recent anthropogenic effect, whereas Zn phytotoxicity and co-accumulation of trace levels of Cd are normal biogeochemical phenomena. It seems increasingly likely that the Cd hypertolerance mechanisms are incidental biochemical phenomena. Although Cd-PCs can be found at low levels in plants in the environment, they account for only a small fraction of the tissue Cd (Suzuki et al. 2001; Jonak et al. 2004).

Another goal of developing transgenic plants with increased metal binding capacity was to use these metal-binding factors to keep Cd in plant roots, thus reducing Cd movement to the food chain or into tobacco. Vacuolar compartmentation of Cd only in roots may reduce Cd translocation to shoots; expression in plants of the hmt1 vacuolar pump for Cd-PCs from fission yeast has not yet been successful, and modification of gene sequences may be required before its effectiveness can be tested (similar to the mercury reductase gene sequence changes). The expression of MT as the whole protein, the Cd binding '-domain' part of the protein, or a fusion protein with -glucuronidase, under several promoters increased Cd tolerance of tobacco and other plants, but had little effect on Cd transport to shoots(Pence et al. 2000). Recently use of the improved 35S2 promoter may have increased the ability of MT to keep Cd in roots, however, tests have not yet progressed to soil studies which must be the important measure of success. Some promising genes that are involved in phytoremediation of heavy metal–contaminated soils in plant roots are listed in Table 11.2.

Table 11.2 Some promising genes involved in phytoremediation of heavy metal–contaminated soils in plant roots

Gene	Functions of gene products	Plant species	Roles of gene products	Gene regulation
Atfro2	Ferric chelate reductase	*Arabidopsis thaliana*	iron reductase	Induction
Psfro1		*Pisum sativum*	Iron reductase	Induction
Atirt1	ZIP transporter	*Arabidopsis thaliana*	Fe II transport in root epidermis	Up-regulation
Atirt2		*Arabidopsis thaliana*	Iron (metal) transporter	Up-regulation
Leirt1		*Lycopersicon esculentum*	Iron (metal) transporter	Up-regulation
Leirt2		*Lycopersicon esculentum*	Iron (metal) transporter	Similar expression
Psrit1		*Pisum sativum*	Iron (metal) transporter	Induction
Osrit1		*Oryza sativa*	Iron (metal) transporter	Induction
Atnramp1	NRAMP	*Arabidopsis thaliana*	Iron (metal) transporter	Up-regulation
Atnramp3		*Arabidopsis thaliana*	Iron (metal) transporter	Up-regulation
Atnramp4		*Arabidopsis thaliana*	Iron (metal) transporter	Up-regulation
Lenramp1		*Lycopersicon esculentum*	Putative iron(metal)transporter	Up-regulation
Lenramp3		*Lycopersicon esculentum*	Putative iron(metal)transporter	Similar expression
Osnramp1		*Oryza sativa*	Iron (metal) transporter	Not analysed

Table 11.2 (continued)

Gene	Functions of gene products	Plant species	Roles of gene products	Gene regulation
Osnramp3		*Oryza sativa*	Putative iron(metal)transporter	Not analysed
Atfrd3	Transporter MATE	*Arabidopsis thaliana*	Putative transporter	Weak up-regulation
Lechln	Nicotianamine sysnthase	*Lycopersicon esculentum*	Nicotianamine synthase	Similar expression
Hvnas		*Hordeum vulgare*	nicotianamine synthase	Induction
Osnas		*Oryza sativa*	Nicotianamine synthase	Induction
Hvnaata	Phytosiderophore enzyme	*Hordeum vulgare*	nicotianamine aminotransferase	Induction
Hvnaatb				
Hvids2		*Hordeum vulgare*	Putative dioxygenase	Induction
Hvids3				
Lefer	Regulator	*Lycopersicon esculentum*	regulator, putative transcription factor	Similar expression

5 Conclusion

Extensive progress has been made in characterizing soil chemistry management needed for phytoremediation, and physiology of plants which hyperaccumulate and hypertolerate metals. It is increasingly clear that hypertolerance is fundamental to hyperaccumulation, and high rates of uptake and translocation are observed in hyperaccumulator plants. Fundamental characterization of mechanisms and cloning of genes required for phytoremediation has begun with the mercuric ion reductase, and *hmt1* expression in higher plants is expected soon. Improved hyperaccumulator plants and agronomic technology to improve the annual rate of phytoextraction and to allow recycling of soil toxic metals accumulated in plant biomass is important to support commercial environmental remediation, which society can afford in contrast with present practices. Although most phytoremediation systems are still in development, or in plant breeding to improve the cultivars for field use, application for Se phytovolatilization has already begun. Many opportunities have been identified for research and development to improve the efficiency of phytoremediation. Progress had been hindered by limited funds for research and development for 15 years since the first report of the model for phytoremediation. New commercial firms are moving into this field and phytoremediation technologies will be increasingly applied commercially in the near future.

At the present time, phytoremediation is an emerging technology and there is still a significant need to pursue both fundamental and applied research to fully exploit the metabolic and growth habits of higher plants. It is precisely the purpose of the European COST Action 837 to stimulate the development and evaluate the potential of plant biotechnology for the removal of organic pollutants and toxic heavy metals from wastewater and contaminated soils.

Heavy metals affect plant gene expression at different scales. They can influence DNA directly and may act via modification of chromatin structure. The activation of heavy metal stress-responsive genes occurs by a complex array of signaling pathways, which is a dimensional network. The various secondary mediators participate in the activation of regulatory proteins that bind to promoter regions of target genes. Some of these processes constitute a general plant stress response and are not solely specific to the heavy metal stress. The regulation of plant genes by heavy metals also occurs post-translationally by microRNA silencing. The framework for the mechanism is referred to Figs. 11.1 and 11.2, although there are more details remained to be known.

Overall, the main limitations of heavy metal phytoextraction technology for soil remediation are related to low-deep penetrating roots, low yields of hyperaccumulator plants and the disposal of their metal-enriched biomass and the little knowledge about the detoxifying process in plants and soil. So, phytoremediation is very much dependent on plant and soil factors, such as soil suitability for plant growth, depth of the contamination, depth of the plant root system, level of contamination, and urgency in cleaning up. Furthermore, there is also need for a full understanding of the physiology, biochemistry, molecular biology, and uptake process of the plants

employed. In combination with biotechnology, selection of new hyperaccumulators (including ferns) is also a challenge.

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Chapter 12
The Structural and Functional Characteristics of Asiatic Desert Halophytes for Phytostabilization of Polluted Sites

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Abstract Phytoremediation, the use of plants to extract, sequester, and/or detoxify pollutants through biological processes is an effective, in situ, non-intrusive, low-cost, ecologically friendly, socially accepted technology to remediate polluted soils. Crystalline to fibrillar wax formations, appressed to surfaces of guard cells appear to originate from guard cells in the vicinity of the stomatal aperture. Formations may arise from evaporation of plant water at the interface between stomatal antechambers and substomatal cavities, leaving salt ions behind to precipitate. Many questions remain unanswered regarding their ecological and physiological significance as well as their occurrence and prevalence in both time and space. Such functions would be of considerable adaptive value in the light of their possible relationships to the impact of pollutants. An attempt has been made here to address these questions by analysing the morphology of salt glands and intracellular salt crystals using SEM micrographs of *Salsola, Eremopyrum, Aeluropus litoralis, Tamarix* and other desert plants.

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Contents

1 Introduction

Salinization is one of the major ecological and production problems currently facing the agricultural and pastoral sectors in Central Asian countries. Overuse of major rivers of Central Asia (Amudarya, Zerafshan, Syrdarya) for the production of cotton and other crops has resulted in the rise of water tables, waterlogging, and ecological disasters like salinisation around the receding Aral Sea. On the other hand contamination by heavy metals and chemicals released from agricultural activities as well as from gold mining, uranium and oil-gas industries have been frequently reported from Kyzylkum sandy Desert (Walter and Box 1983; Black et al. 2003; Gintzburger et al. 2003; Toderich et al. 2005a, b; Aparin et al. 2006; Toderich et al. 2009). Technogenic industrial establishments randomly distributed on the areas with sandy-loam/clay soils and on large sand dunes are to a large extent responsible for the degradation of fertile lands in Kyzylkum desert and Priaralie regions from Uzbekistan, Tajikistan and Kazakistan. Large-scale industrial developments in the southeast and central Kyzylkum for the last 15–40 years have aggravated land degradation of these territories. The mine tailings of radioactive waste deposits (as well as the dumps of uranium ores below industrial grade and the mining of underground leaching areas) situated on the left bank of Zerafshan River near Navoi city is also dangerous for the ecology of the region (Tsukatani et al. 2008). The mobility of toxic pollutants can be highly facilitated by both chemical characteristics of soils and the diversity of desert plant communities. Plants under such environments face multiple stresses caused by high soil salinity, heavy metals, organic pollutants and long-term water shortages. The wild arid plants play a significant role in the phytoremediation of the contaminated soils by heavy metals, in which, microbial populations are known to affect heavy metal mobility and availability to the plant (Ottenhof et al. 2007).

Initial studies on the plant cellular mechanisms affecting the bioremediation of elemental and/or organic pollutants suggests that there is a great promise for the use of desert plants in large-scale environmental clean-up efforts, but very little information is available on the accumulation of toxic ions and/or heavy metals derived from soils and water and passing through plants to the food chain. Factors related to the uptake of chemical compounds, such as tannins, nitrates, metals, and oxalates (some of which may be toxic for plant development) too have not been adequately studied. The native desert plants have the advantage of being highly adapted to the hyper-arid and contaminated conditions. There is a limited number of species which can grow on these soils (Escarre et al. 2000; Toderich et al. 2002, 2006). It is obvious that conservation and protection of gene pools of such native arid and semiarid plants is basic for understanding the influence of environmental factors on their reproductive systems, as well as enlightening their plasticity and tolerance to the contaminated environments. The current studies on the ion-phytoextraction cellular mechanism, seed reproduction, biochemistry and management of salt tolerance of arid Central Asian plants refers to diverse plant species, which have developed the most unusual strategies for survival and multiplication. The species of genus *Salsola* with extremely variable eco-morphological modes of reproduction and photosynthesis grow well on saline/hypersaline soils (Akhani et al. 2007).

The objective of this chapter is to present the studies on the floral morphology, sexual reproduction system, photosynthesis and biochemistry of desert plants related to their adaptive cellular response strategy to contaminated or salinized arid lands. The sites selected were located inside or close to gas-uranium mining industrial complexes. These are covered by a very poor plant cover. The samples were taken from the sites with an area of 20 x 20 km. These were cut into pieces, dried at 105°C, ashed at 5000°C for 24 h, mixed thoroughly, transferred to plastic containers and measurements taken by using ICP-MS (Perkin Elmer-Sciex, ELAN 6000). The chemical fixation and freeze-drying methods were used for SEM preparations (Bozolla and Russell 1998). These were examined by using JEOL JSM-T330 scanning electron microscope. The preparations are deposited at the Laboratory of Electron Microscopy of A.M. University in Poznan (Poland), as well as at the laboratory of Tree Cell Biology, Kyoto University (Japan). Plant samples (leaves, flowers and fruits) for isotope studies were collected from wild populations in the Kyzylkum Desert. Different floral organs of the species investigated were immersed in 3% glutaraldehyde in sodium cacodylate phosphate buffer (pH 7.2) for 3 h prior to mounting on stubs. The material was then placed in an Edward freeze-dryer for 24 h−55°C. Specimens were coated with carbon. The salt secretions on freeze-dried leaf-like organs (bract/bracteoles and perianth segments) were analyzed by energy disperse X-ray microanalysis (EDX) with a JEOL JSM −T330A SEM. The elemental composition of crystalline deposits associated with salt glands from various ecological types of arid plants was also determined. Anatomical sections of bracts, perianth segments, anthers, embryo and fruits were selective stained with safranin in combination with fast green, haematoxylin or toluidine blue. Samples for anatomical studies of fruit covers were fixed in alcohol: glycerin: water (1:1:1). Sections were stained with methylene blue.

2 Physico-Chemical Characteristics of the Soils

Desert soils of Uzbekistan are of semi-arid and arid origin, characterized by low organic matter (<1.0%), a high level of calcium, often associated with gypsum, and show a low agricultural potential. The soils are composed of particles of varying sizes, generally saline, with unfavourable physico-mechanical properties, poor structural characteristics, and often a high level of compaction. Most of these have evolved from alluvial, colluvial or aeolian loessic deposits with little weathering of the parent rock. Three main groups of soils may be distinguished among all the soil types recorded in Uzbekistan (Gintzburger et al. 2003):

- Sandy aeolian soils (13.3 Mha), sand dunes of the Kyzylkum and some agriculturally important loess deposits (the piedmont of the eastern mountains)
- Grey brown (11 Mha) and sierozem-grey soils (3.8 Mha) of pre-desert and steppe
- Solonchaks and solonets, a zonal soils (1.6 million ha) mostly on depressions, and takyrs (2.8 Mha) with a shallow water table and highly mineralized underground water

The combination of sandy aeolian soils is a common formation occurring in the majority of arid zones in the Kyzylkum desert. The Uzbek desert ecosystem covers the Kyzylkum, the Ustyurt plateau, the Karshi steppe, and the separate sites in the southern part of Uzbekistan and the Fergana valley and is represented mostly by low lying lands with an elevation varying between 100 and 500 m. The soils of Kyzylkym desert and lower streams of Amudarya and Zerafshan River Basins are characterized by low productivity with a predominance of carbonates and gypsum. The humus content is around 0.5% in sandy desert and ranges from 0.7% (grey–brown sites) to 1.2% (virgin and newly irrigated takyrs). Soil type is silty-sandy loam throughout the profile up to the depth of 60 cm. The soil is highly saline in the upper parts as well as lower layers. Their ground water salinity varies between 2000 and 8200 mg L^{-1}, sodium and magnesium are the dominating cations, organic matter ranging from 0.7 to 1.5%, total nitrogen (0.7–5.5 mg kg^{-1}) and phosphorus (10.0–18.26, mg kg^{-1}) contents are low, and available potassium is low or moderate (Shirokova and Morozov 2006).

The soils are generally characterized by low productivity and high salinity (1200–4000 ppm and rarely more than 5800 ppm of soluble salt), with a dominance of carbonates, sulfate, chloride and/or mixed types of salinisation. In the less salinised Central Kyzylkum, the sulfate-potassium-sodium and rarely chloride-potassium-sodium types occur frequently. The uranium production during the last 3 decades has produced devastating effects on the whole Kyzylkum natural environment. There are also surface deposits of approximately 2,424,000 m^3 of sub-economic uranium-bearing material with around 2–5 mg kg^{-1} (0.002 to 0.005%) uranium content. The contaminated material recovered from the surface lies around 3.500.000 m^3 (Solodov 1998). In the southern part of Kyzylkum desert urbanisation, industrialisation, agricultural activities and traffic lead to the pollution of the lands with pesticides, nitrates, organic pollutants and various heavy metals (Goldshtein 1997; Tsukatani and Katayama 2001; Toderich et al. 2001a, b, 2002, 2003, 2005a,

b; Khujanazarov et al. 2007). Thus, the areas suitable for agricultural development in Aral Sea Basin have continuously decreased. The conditions within the core areas of Kyzylkum Desert are getting worse and urgent management practices are needed to protect the biodiversity, resource extraction and communication links.

In the last few years there has been a tendency for fast degradation of floodplain ecosystems of the Amudarya and Zerafshan Rivers delta-marginal territories of Kyzylkum desert. It seems that human induced soil salinisation is the major force for land degradation in the Aral Sea Basin (Kamalov 1995). The productivity of the saline and technogenic contaminated soils; especially in the deltas of the main rivers of Uzbekistan; is rather low and cultivation of most agricultural crops requires high inputs of chemical fertilizers or applying of costly leaching practice. This strategy, however, increases the risk of re-salinization in the root zone of plants and leaching process has to be repeated during every cropping season in order to avoid build-up of high salt concentration in the top soil profile. Therefore appropriate practices for salinity control need to be selected based on the quantification of water and salt movement in the soil, responses and adaptation of crops to water and salinity stress. An efficient system for water use in the irrigation coupled with introduction of modern bio-remediation technologies can help to integrate all interactions and define the best management for crop production under saline environments (Wu et al. 1993; Yensen et al. 2000; Toderich et al. 2006, 2008).

3 Translocation and Cellular Mechanism Involved in the Phytoremediation of Trace Elements

One of the most common feature in the desert/semi-desert plants on contaminated habitats in the area is their lower reproductive capacity. Although these species develop a large amount of flowers, but only a few form viable seeds, the seed germination and seedling survival rate being very low.

Analysis of average values of trace element content in the soils of Central Kyzylkum deserts shows high levels of Hg, Cu, U, As, Zn, Mo, Ni, Sr, Co (Table 12.1) with coefficients of concentration ($K_k = C_f/C_k$) exceeding 1.0. Soils contaminated with As, Zn, Ni, Mn, Cu and Sr are mostly toxic and widely distributed in sandy Kyzylkum Deserts. Nickel is of natural origin and occurs in the form of nickel-cobalt rock type, mainly from Palaeozoic age, and concentrations vary between 60 and 70 ppm. The mobility of As, Cu, Zn (along with other heavy metals and their accumulations) are highly facilitated by chemical properties of soils as well as aridity of the climate of the Kyzylkum deserts.

The soils contaminated with Cd, Cu, Fe, Ni, Mn, Cr, Pb and Zn are colonized by plant and animal species that have developed strategies for avoidance of and/or tolerance to these metals. In the case of plants one possible avoidance strategy is to prevent the uptake of potentially toxic metals. This mechanism is not strongly developed in vascular, arid-inland desert plants, although tolerant species may limit metal uptake to varying degrees. The concentrations of some metals were only unusually high in some of the accumulators.

Table 12.1 Average values (mg kg^{-1}) of trace elements in the aboveground dry matter of field-grown plants of Central Kyzylkum region

Plant species	Fe	Mn	Sr	Pb	Zn	Cu	Mo	Cd	V
Alhagi	315	36.1	170.5	0.0	26.2	8.3	2.7	0.1	0.7
pseudoalhagi	280–350	29–43	85–256	0.0	17–35	7–10	2–3	0–0.1	0.5–0.9
Peganum	865	38.2	234.3	0.3	24.2	7.2	2.5	0.1	2.0
harmala	15–3310	14–86	0–793	0–3.6	14–58	3–13	0–6.4	0–0.2	0–11
Carex physodes	395	22.3	65.7	0.5	17.2	8.9	2.2	0.2	1.8
	250–580	19–26	48–104	0–3.2	11–28	3–21	0–4.6	0.1–0.4	1.4–2.5
Poa bulbosa	280	24.6	53	0.0	18.8	9.1	3.6	0.18	3.1
Carex	685	71.5	57	0.4	16.8	7.6	2.6	0.2	1.5
pahystyllis	500–870	18–25	21–93	0–0.8	16–18	7–9	0–5.2	0.1–0.2	1.2–1.8
Artemisia	932	38.1	123.6	0.4	23.3	11.6	3.1	0.1	2.4
diffusa	117–5020	15–128	13–980	0–7.5	9–72	3–25	0–9.3	0–0.4	0–15
Triticum sp.	2547	62.7	82.3	2.4	25.3	15.3	1.3	0.2	7.1
	440–4650	10–150	27–135	0–7	16–38	6–25	0–3.6	0.1–0.4	1–14
Haloxylon	454	42.0	146.3	0.3	19.2	5.8	2.7	0.1	0.6
aphyllum	100–2600	18–82	53–1041	0–4	11–41	2–13	0–7.1	0–0.2	0–3.4
Salsola	569	41.6	153.6	0.5	20.8	6.7	1.6	0.1	2.0
	160–1880	16–93	21–508	0–7.6	8–50	3–12	0–5.6	0–0.5	0–12
Tamarix	2960	74.0	264	0.0	20.1	6.4	0.9	0.09	8.1
Hispida									
Ferula	755	32.2	147	0.1	21.3	21.4	1.5	0.1	1.8
assa-foetida	510–1100	25–50	116–166	0–0.5	17–33	9–54	1–2	0–0.4	1.1–2.6

Table 12.1 (continued)

	As	Sb	Se	Ni	Co	Cr	Th	Be
Alhagi	0.1	0.0	0.2	2.5	0.3	1.5	0.1	0.0
pseudoalhagi	0–0.2	0.0	0–0.3	2–3	0.3	1–3	0.1	0.0
Peganum	0.1	0.0	0.5	2.0	0.5	3.2	0.3	0.1
harmala	0–0.3	0–0.2	0–0.2	0–6	0.1–2.7	0–11	0–2.5	0–0.2
Carex physodes	0.0	0.1	0.0	1.6	0.2	2.4	0.1	0.0
	0.0	0–0.1	0.0	0.9–2.8	0.2–0.3	0.9–4.7	0–0.2	0–0.1
Poa bulbosa	0.0	0.02	0.0	2.0	0.14	4.4	0.08	0.07
Carex	0.2	0.1	0.5	5.3	0.3	24.6	0.1	0.1
pahystyllis	0–0.4	0–0.1	0.3–0.6	1–9	0.2–0.4	2–47	0.1–0.2	0–0.1
Artemisia	0.2	0.0	0.3	3.3	0.5	3.7	0.3	0.1
diffusa	0–1.9	0–0.5	0–7.6	0.5–12	0.1–1.9	0.5–14	0–1.2	0–0.4
Triticum sp.	0.1	0.2	0.2	5.6	1.1	20.6	0.8	0.1
	0–0.6	0.1–0.5	0–0.5	1–13	0.3–2.2	2–50	0.1–1.5	0–0.3
Haloxylon	0.1	0.0	0.2	3.6	0.2	2.1	0.1	0.0
aphyllum	0–0.4	0–0.2	0–1	0–24	0.1–1.1	0.3–23	0–0.8	0–0.1
Salsola	0.1	0.0	0.9	1.8	0.4	2.0	0.1	0.1
	0–0.5	0–0.2	0–13.6	0–4	0.1–0.8	0.5–5.5	0–0.7	0–0.2
Tamarix	0.0	0.09	0.2	5.7	3.08	8.5	0.87	0.38
Hispida								
Ferula	0.0	0.1	0.0	2.1	0.3	3.2	0.2	0.0
assa-foetida	0.0	0–0.2	0.0	1.2–3.7	0.2–0.4	1.2–3.6	0.2–0.3	0.0

Our studies revealed that very few Kyzylkum desert species have the ability to translocate the metal ions to high concentrations. The values lie between 15–4170 (Fe), 9.0–50.0 (Zn), 0.1–7.6 (Pb), 0.0–3.7 (Ni), 0.1–50.0 (Cr), 0.0–793.0 (Sr) mg kg^{-1}, or in trace levels 0.1–1.9 (As), 0.1–2.7 (Co), 0.1–2.5 (Th), 0.1–0.18 (Cd) mg kg^{-1} (Table 12.1). Analysis of composition of trace elements in the various types of soils of the Central Kyzylkum desert showed high average values of Hg, Cu, U, As, Zn, Mo, Ni, Sr, Co. It was determined that pollution by heavy metals and organic pollutants is concentrated around mining and tailing (waste) deposit zones, and exclusively in the foothill areas of Central Kyzylkum like; Kul'dzhuktau, Auminzatau, Tamdutau, Dzhemtau, Aristantau and Bucantau mountain ranges.

A survey of the Kyzylkum halo-and metallophyte flora has shown that in Uzbekistan mainly species from the following genera are accummulators; *Salsola* (both annual and perennial species), *Haloxylon, Halothamnus (Aellenia), Halostachys, Kalidium, Anabasis, Tamarix, Artemisia, Peganum, Zygophyllum, Aeluropus litoralis, Eremopyrum, Poa, Allysum, Euphorbia, Frankenia,* and *Lycium.* The plant families most strongly represented as accumulators are Euphorbiaceae, Tamaricaceae, Frankeniaceae, Plumbaginaceae, Chenopodiaceae, and Poaceae, while the families Asteraceae, Fabaceae, Cyperaceae and Zygophyllaceae are less represented. Specimens of *Triticum sp*. (Poaceae), growing on the cultivated foothill fields of Central Kyzylkum, show high concentrations of iron (up to 2547 ppm) in the aerial dry matter of the plants. *Artemisia diffusa* (Asteraceae) had remarkably high Zn levels (above 5020 mg kg^{-1}) and it can be classified as a hyperaccumulator. The species *Haloxylon aphyllum*, *Tamarix hispida*, *Artemisia diffusa*, as well as some species of *Salsola* and *Peganum harmala* demonstrate a strong tendency to translocate strontium. However, *Artemisia diffusa* accumulates less than *Tamarix hispida, Carex pahystylis, Triticum sp*. *Salsola spp.*, which show a multi-element accumulation capability with regard to nickel, chromium, strontium and iron. The plant species exhibited differences in the distribution characteristics of ions/metals (Fig. 12.1).

The representatives of genus *Salsola* maintained the ions of Zn in their tissues over a wide range of soil-metal concentrations, indicating hyperaccumulation. Conversely, Zn extraction by *Artemisia diffusa* is relatively high in relation to the comparatively small variation of soil Zn concentrations. The relationship between the concentrations of Co in the plant tissues of *Salsola* species and soil was curvilinear, showing that this taxon is capable of accumulating large concentrations of Co across a wide range of soil concentrations. A few species, among those studied taxons described as ion-accumulators, recorded high survivability and high seed germination rate, but with a low biomass production.

Tamarix hispida as a C_3 salt excluder hyperhalophyte has a remarkably high Fe, Ti, Zn, Cu, Sr and Co levels in the aerial dry matter biomass that it deserves being described as a hyperaccumulator plant. A significant ability for heavy-metal removal has been noted for *Artemisia diffusa*, *A. turanica, Alhagi pseudoalhagi, Alyssum desertorum, Zygophyllum fabago, Suaeda microsperma, S. paradoxa, Frankenia hirsuta, Cressa cretica, Scirpus lacustris, Typha angustifolia, Suaeda spp., Karellinia caspia, Aeluropus litoralis, Dactylis glomerata, Cyperus fusciformis,*

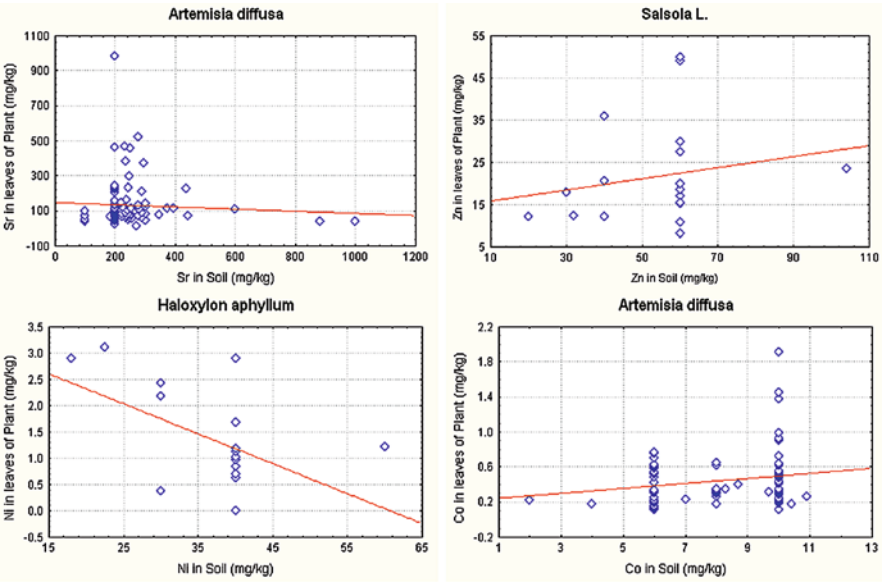

Fig. 12.1 Distribution characteristics of trace elements in various desert taxa

Halopeplis pygmea, Amaranthus retroflexus, Limonium sogdianum, Sonchus maritima Puccinella scleroides, Sorghum bicolor, Peganum harmala, Haloxylon aphyllum, as well as annual and perennial species of the genus Salsola. These pioneer plant species were growing well on mined areas despite unfavourable conditions such as extreme pH, high salinity and phytotoxic levels of several elements.

4 Salt Accumulation, Silicification, and Wax Deposition Associated With Epidermal Structures of Flower

Desert plants successfully growing on metalliferous or salinized soils tend to accumulate the highest ion concentrations in epidermal and subepidermal tissues, including various glandular structures of bracts/bracteoles and perianth segments. Salt glands with varying degree of specialization are actively involved in the elimination of solutes and mineral elements from the surface of the vegetative organs. These are very common in the desert plants of Central Asia. Excretion occurs predominantly on the adaxial surface and is uniformly localized along the lateral walls of the grooves (Figs. 12.2). Salt glands morphology vary in different genera. These can be sunken, semi-sunken or located above the epidermis as in the majority of chenopods and gramineae species. In the latter glandular structures are usually bicellular, comprising a basal and cap cell. Slight variations in morphology of the basal and cap cells of glandular hairs have been observed mostly in the annual and other species such as that of genus *Salsola, Aeluropus litoralis, Tamarix hispida,*

and *Eremopyrum orientale,* which appear to be related to their efficiency of salt secretion (Toderich et al. 2003, 2008).

Our findings showed that epidermal vesicles and papillae in desert species (Figs. 12.2 and 12.3) have a large bladder cell attached to a stalk composed of one or more cells that in turn is attached to an epidermal cell. Comparative study of two annual taxonomically close related *Salsola* species from steppe soils of Europe (Poland) and Kyzylkum metalliferous/salinized sands revealed that salt secretion become prominent and salt glandular structures are formed abundantly only when plants are exposed to high contaminated environments. Under such conditions an evident increase in succulent bracts is a consistency met within Kyzylkum chenopods.

The vesiculate hairs of some annual *Salsola* from Central Asia are considerably involved in the cellular salt secretion. According to Luttge (1971) this might not be taken strictly as a secretor process, because these trichomes are considered

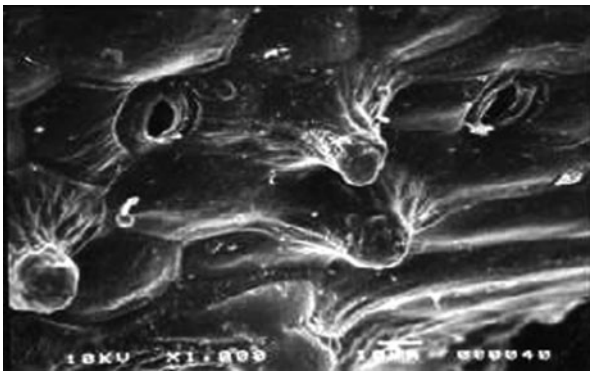

Fig. 12.2 The morphology of Vesicular-and short peltate trichomes on bracts of *Salsola pestifer* (Buchara ecotype)

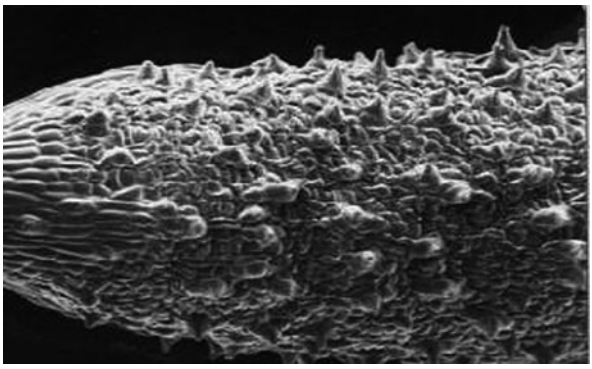

Fig. 12.3 Epidermal surface view of mature bracts of *Salsola pestifer* (Buchara ecotype). Glandular structures have a strong localization, especially on adaxial side, which is mostly exposed to environmental impact. X 750

as salt glands and their function is obviously a specialized mechanism for the removal of salt from the leaves. The emission of salt from these vesiculated hairs is apparently the result of the rupturing and collapse of bladder cells (Gamal 2005; Ottenhof et al. 2007). The presence of papillae on the epidermal cells of *S. praecox*, *S. iberica* and *S. pestifer*, with thick outer walls, cuticle and submerged stomata seemingly protect assimilatory organs against excessive transpiration. C_4- herbaceous annual *Salsola* species differ in the morphology (head shape-mainly clavate or capitate or also in the number of constituent cells composing their stalk) of salt-glands/trichomes and their density on the epidermal surface. Variation in the density of salt glands/trichomes is believed to be mainly due to the effect of stress under desert environmental conditions and even pressure from herbivory (Wahid 2003). These parameters potentially could be used as distinguishing characters between different ecological halophyte groups. For instance the *Climacoptera* complex has unicellular non-glandular trichomes or hairs, smooth or micropapillate (warted surface), whereas the surface of bracts/bracteoles of many dry/sclefiried *Salsola* species have an undulating epidermal surface with numerous salt glandular structures and tall adaxial ridges alternating with deep grooves. On the ridges of annual *Salsola* species we found various papillae and prickle hairs, as well as secreted salts, which appear as crystals. Crystalline deposits were more abundant on the adaxial surface because of higher gland frequency (Fig. 12.4a, b).

It has also been noted that occurrence of calcium oxalate crystals was almost absent in root and stems. An abundance of these crystals was described in the tissue of the seed coats of many xero- and euhalophytes.

Salt glands usually are globose or club-shaped and readily distinguishable from unicellular papillae and sharp-pointed prickles, an ornamented, porous cuticle overlies the epidermis, cuticle is distinctly thicker over the area that adjoins basal and epidermal cells than over the cap or other parts of the epidermis. The cuticle is separated from the outer cap cell wall, resulting in the formation of a salt collecting Chamber (Fig. 12.4b) or cuticular cavity which is similar in the species of *Salsola* (both annual and perennial*), Aeluropus litoralis*, *Eremopyrum orientale*, *Spartina,*

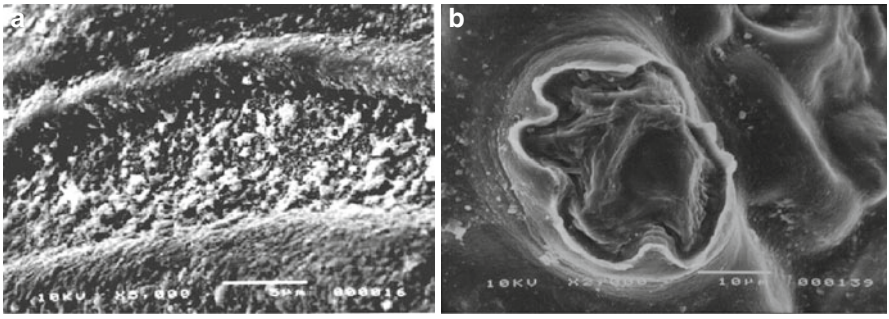

Fig. 12.4 **a** The adaxial surface of bracts of *S. iberica* with ridges and salts which appear as crystals. **b** SEM microgrphs showing the patterns of crystaloid structure in the bract tissues of *S. orientalis*

Cynodon and *Distichlis* (Thomson 1975) and probably represents a temporary collecting compartment where secreted salts accumulate prior to elimination from the leaf. The ions seem to be compartmentalized in small vacuoles and transported to the cuticular cavity, prior to exclusion from the vegetative and reproductive organs either through cuticular pores or by rupture of the cuticle (Yordoan and Kruger 1998; Naidoo and Naidoo 1998; Rozema and Riphagen 2007).

An unusual type of salt glandular structure has been described for *Salsola carinat*a where the terminal cell(s) always ends bluntly. On top of the stalk cell, extremely thin-walled cells form a single originally ornamented ring, while the thick cuticle of the stalk cell remains as a cylindrical scar (Figs. 12.5 and 12.6a, b).

Cross-sections of bracts and bracteoles of many *Salsola* species show that different tissues like swollen epidermal cells (in all species), large–celled hypodermis and water bearing parenchyma carry out water and salt-accumulating functions. Size, shape and/or their density should be recognized by the location and deposits of

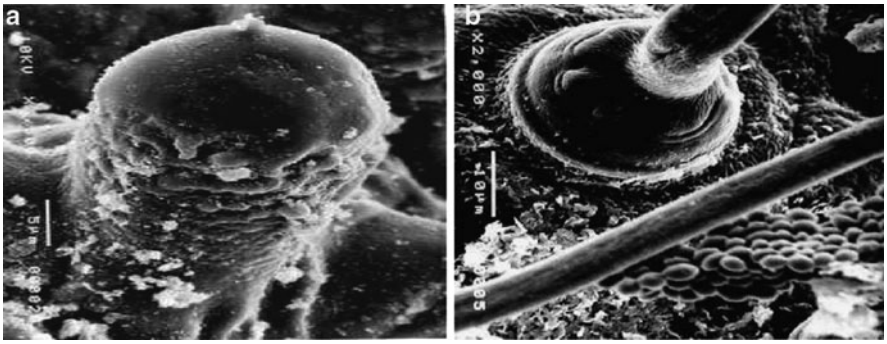

Fig. 12.5 **a** Salt gland of *Salsola paulsenii* comprising flask-shaped basal cell, dome-shaped cap cell and raised cuticular chamber. **b** Micromorphology of glandular hairs of *Salsola sclerantha* and wax-epicuticular inclusions partially surrounding it

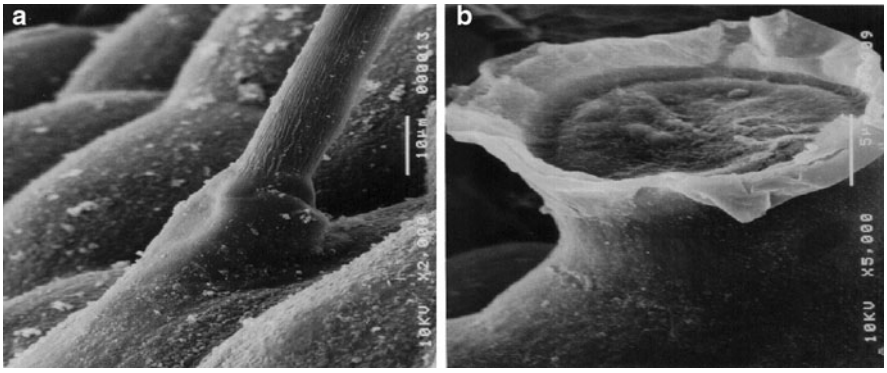

Fig. 12.6 **a** SEM micrograph showing surface features and morphology of non-glandular, unicellular hair of bracts in *Cimacoptera lanata*. **b** Untypical morphology of salt land, occurring on the epidermal bract's surface of *Salsola leptoclada* (Central Kyzylkum ecotype)

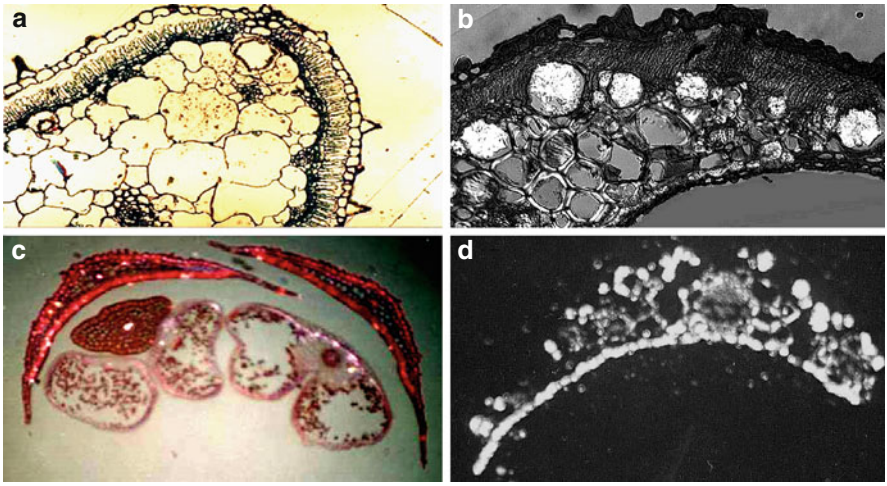

Fig. 12.7 **a** Cross-section of succulent bract of *Salsola praecox*; central part is occupied by 3–4 layers of water–storing parenchyma cells with small salt crystals 10 × 60 (1 μk). **b** Anatomy of bract tissue of *Salsola arbusculiformis*. Different types of crystals in the subepidermal salt-storage cells 10 × 60 (3.0 μk). **c** Cross section of anther in *Salsola arbuscula.* The salt ions location in pollen grains (male gametophyte) is absent 10 × 60 (1 μk). **d** The fluorescent microscopy image of bract of *S. arbusculiformis* with the location of salt/ions in it 10 × 60 (1 μk)

salt/ions in specific (salt- storage) cells. The fluorescent microscopy studies on the displacement of salt ions from the floral organs of some *Salsola* species reveals an abundance of mineral ions in the tissues of sterile organs of flower like sepals and anther connective cells. However ion dislocation has never been observed in male-and female gametophytes or in the embryo tissues (Fig. 12.7a–d).

Occurrence of calcium oxalate crystals in the leaves and seed coats of some plants has been described by Fuller and McClintock (1986). It has been suggested that concentration of oxalate crystals is almost absent in the root and stems. The presence of crystals in the outer covering of seeds may play a role in changing soil pH, thereby providing a more favourable condition for plant survival.

Structurally, SEM studies revealed a high diversity in the micromorphology of epicuticular wax (epicuticular secretion), mostly occurring as specific crystalloids (epicuticular wax crystalloids) on the plant surface of desert plants as proposed by Barthlott et al. (1998). Cuticular wax partially covers the mature prickle-hairs, papillas and long cells of outer epidermis of bracts/bracteoles of some perennial *Salsola* species as is shown at Fig. 12.8a,b.

Their nature and molecular organization of such wax deposits is still unknown for desert plants. The chemical composition of these waxes has been given at length by Barthlott (1994) and Barthlott et al. (1998). However, there are still contradictory opinions concerning waxes deposition. Earlier workers suggest that waxes could be exuded to the outer cuticular surface through pores, while Mahllberg (1991) suggests that there is excretion through lamellate regions onto the cuticle. Glandular trichomes in such case enhance capacity of plant to accumulate large quantities of

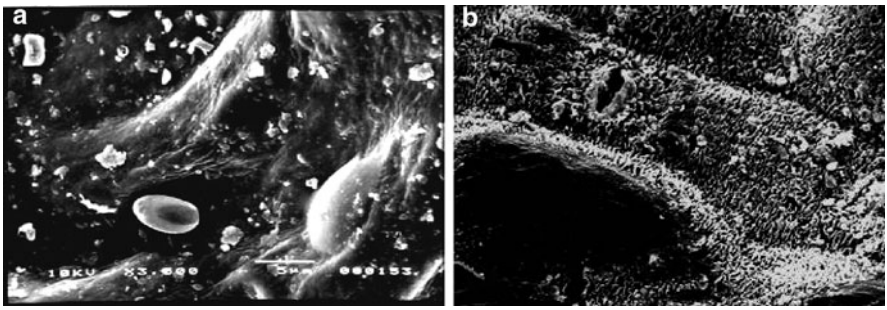

Fig. 12.8 **a** Scanning micrograph of *Salsola orientalis* bract epidermal surface with various salt crystalloids (or epicuticular inclusions) on it. **b** Silicon X-ray distribution image of mature inflorescence bracts in *Eremopyrum orientale (Poaceae)* X 3000

volatile components and transport these to the cuticular surface for vaporization from the gland surface.

A comparative developmental study of floral organs of various chenopods and graminceous species revealed that Si accumulation was greatest on the adaxial trichomes of inflorescence of *Eremopyrum orientale, Bromus tectorum* and *Aeluropus litoralis,* collected from highest contaminated areas of the Bukhara oasis. The localization of small siliceous particles on the inflorescence bracts of *Eremopyrum orientale* is concentrated mostly on the surface of epidermis around stomata. Crystalloid types in *Salsola* taxa are characterized by uniformly distributed small irregular-shaped platelets which occasionally have a parallel orientation around the stomata. In some chenopod species platelets occur in clusters too. A similar silification process associated with trichomes and other epidermal structures of the inflorescence bracts was described for *Phalaris canariensis*. It is said that the silification may be synchronized with the deposition of wall substances, such as lignin, suberin and phenols (Sangster and Wynn Parry 1981). Silicon deposition patterns and localization in bracts has been described for different groups of flowering plants (Sangster et al. 1983; Hodson et al. 1983; Rufus et al. 2007).

Electron microscopic X-ray analysis of secretion products from the salt glands in different representatives of *Salsola* shows a localization of variety of mineral elements and ions. Prismatic crystals secreted by glands primarily contain cations Na, K, Ca, and anions Cl, SO_4, carbonate, although other ions such as Mg, Si, Sr were also detected. These findings require further studies on a wider range of plant materials with respect to structural and genetic variation and their relation to bioremediation of contaminated desert ecosystems.

We can conclude that sandy and saline soils contaminated with Cd, Sr, Cu, Fe, Ni, Mn, Cr, Pb, Zn, and various toxic salts and organic pollutants are colonized by plant species that develop strategies for avoidance and/or tolerance to metals. One possible avoidance strategy is preventing uptake of potentially toxic metals, especially into the reproductive organs like pollen grains and embryo. Although tolerant plants seem to restrict salts and metal uptake to varying degrees this mechanism

still has not been strongly analyzed in arid vascular plants. It was found that salt (minerals and ions) accumulating glands are mostly common in families Poaceae, Tamaricaceae, Chenopodiaceae, and Frankenaciaceae, and occurr only in a few scattered species in the families Plumbaginaceae, Zygophyllaceae, Fabaceae, and Lamiaceae. Many species of these families are known to have glandular structures, but further investigations are needed to determine their secretion products.

5 Diversity in Trichomes, Hairs and Salt Glands (SEM)

Trichomes are highly variable appendages of the epidermis including glandular (or secretory) and nonglandular hairs, scales, papillae etc., varying widely in structure within larger and smaller groups of plants and are sometimes remarkably uniform, and may be used for taxonomic purposes. The glandular forms are structures on the plant leaf/perianth surface, usually in direct contact with surroundings, playing a defensive role against herbivores and pathogens, in the salt secretion, plant pollination and other interactions between plants and environments; due to their morphology and production of different chemical products. Still there is neither a satisfactory nor well-accepted classification of trichomes for higher plants (Behnke 1984). The importance of the micromorphology and distribution of glandular trichomes for the taxonomy of some species and subspecies requires a reconsideration, because morphology and ultrastructure can be used as a valuable marker for the evolutionary level of the taxa. The pronounced variability of glandular structures can also be related to phenotypic responses to salinity or contaminated environments. These have been used in the delimitation of the sub-families of Chenopodiaceae and the categories are fairly homogenous with regard to trichome type. Carolin (1983) has studied the trichome morphology and its classification within Chenopodiaceae and Amaranthaceae. The morphological traits of trichomes and/or hairs provide a key for easier identification and delimitation of the closely related taxa in different flowering plant groups. The herbaceous Central Asian halophytes; well known in the pasture economy of Uzbekistan as “solyanki”; differ from European taxa in the morphology of salt-glands/trichomes (shape of their head, mainly clavate or capitate and its density). An abundant papillae, prickle hairs and salt secretion between ridges on the surfaces of bracts/bracteoles of annual Central Asian *Salsola* species reveals that frequently salt glands are globose or club-shaped and readily distinguishable from unicellular papillae and sharp-pointed prickles. These parameters can be used as discriminating characters between different ecological variants of *Salsola* group. Variation in the indumentum density is believed to be mainly due to the effect of stress under desert environmental factors and/or even herbivory pressure.

An assessment of the validity of trichome characters and their morphological diversity under harsh desert and contaminated environments was evaluated. The main trichome types for the Central Asian species of *Salsola* are schematically shown in Fig. 12.9.

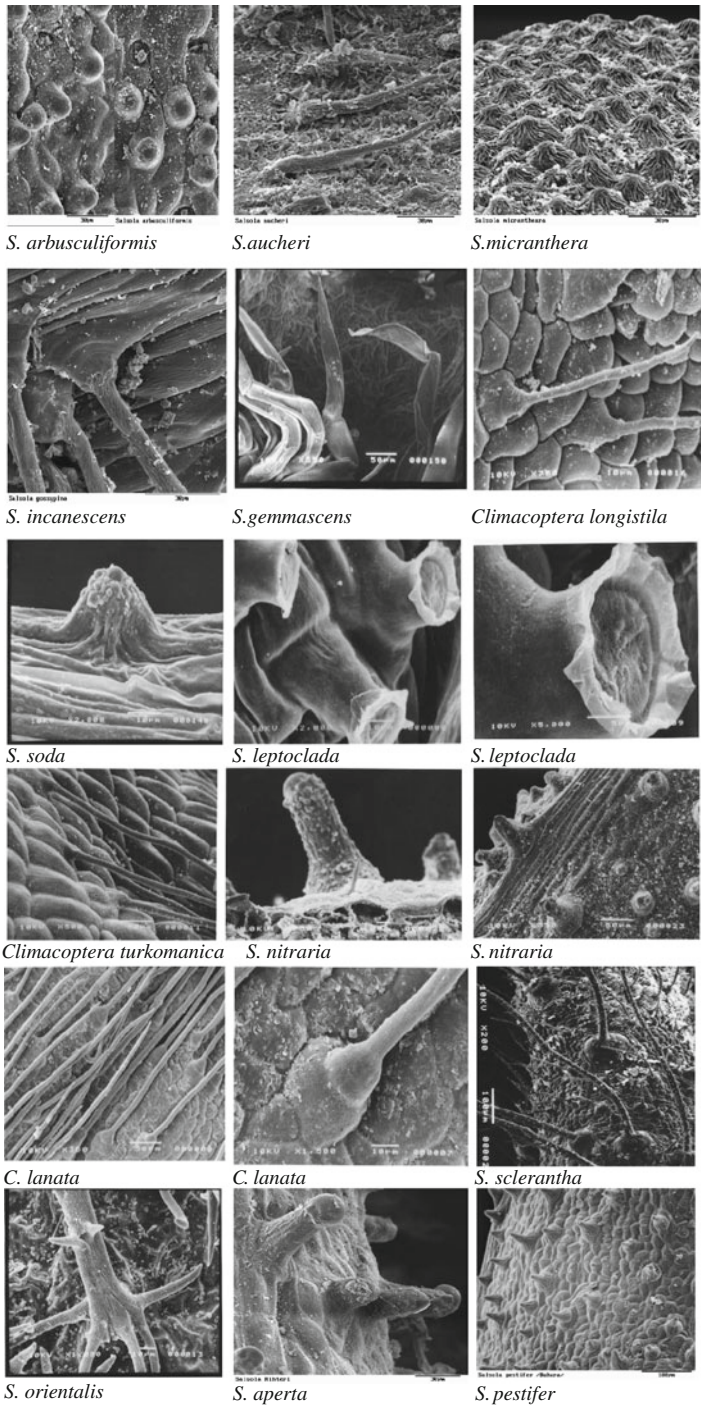

Fig. 12.9 Diversity of trichome morphology (SEM) in some species of genus *Salsola: S. orientalis and S. incanescens* from section *Caroxylon* are clearly separated from all other species of genus *Salsola* by the development of branched and dendric trichomes; malpigian type of hairs are characteristic of *S. gemascens*

The species of Central Asian genus *Salsola* exhibit two unicellular trichome types as described earlier by Carolin (1983) in the families Chenopodiaceae and Amaranthaceae. Using the indumentum characters we found that the *Salsola* species examined by us can be allocated to different sectional groups. A nonparametric analysis of variance of the densities of unicellular/multicellular trichomes on the surfaces of bract/bracteoles, as well as number of cells composing the stalk of multicellular trichomes revealed that the trichome characters studied possess different values for each species and these might be valuable when identification is impossible using macromorphological parameters. Trichomes have highly variable appendages of the epidermis including glandular or secretory and nonglandular hairs, scales, papillae. Trichomes of Central Asian *Salsola* species have been classified by us into a few morphological categories such as; hairs, which maybe unicellular or multicellular; glandular or nonglandular; scales or peltate hairs; water vesicules, which represent enlarged epidermal cells. Glandular hair-a trichome having a unicellular or multicellular head composed of secretor cells, which is usually borne on a stalk of non-glandular cell varying in the degree of differentiation.

For majority of species of *Salsola*-non glandular clothing trichomes, unbranched, uniseriate, multicellular are composed of one or two basal epidermal cells and one or six cells are arranged in one row. Their surface is usually covered by cuticular micropapillae lacking basal part of the trichomes. The glandular structures are usually bicellular, comprising a basal and cap cell, and are referred to as salt glands, trichomes or microhairs.

Based on the analysis given above we propose the following classification of glandular structure for genus *Salsola:*

a. Papillae, the most simple and common type of glandular hairs in the genus *Salsola* scales, huge or sessile glands that can be found in species of section *Salsola* and sect. *Arbuscula* consists of a short stalk of two parallel cells and multicellular glands, often cuticula is removed;
b. Peltate trichomes with one basal cell, one stalk cell, and glandular head; the subcuticular space is remarkably large;
c. Unbranched, short glandular hairs, stalk bi-or multiceseriate, gland spherical, basal biseriate with two very short cells and a few secretory cells;
d. Long capitate trichomes, which have usually one (sometimes two) basal cells; the stalk composed of one to four cells (the upper one is often shorter and marked as neck cell and one cell head; sometimes with small subcuticular space);
e. Simple two-armed, unbranched glandular hairs, stalk cells are usually thin-walled, these types of Glandular hairs could only be found on the bracts/bracteoles and tepals of *S. gemmascens* (sect. *Malpigila*);
f. an unusual type of salt glandular structure was described for *S. carinata*, the terminal cell(s) for many *Salsola* species always end bluntly on top of the stalk cell, extremely thin-walled cells form a single originally ornamented ring, while the thick cuticle of the stalk cell remains as a cylindrical scar.

The unicelled and stiff trichome on multicellular base is one of most frequently found type within genus *Salsola*. This type of uniseriate smooth trichomes are mostly common found in the species of section *Salsola* and *Physurus* and have no noticeable differences in texture between the body and base, which is more or less bulbous. However, it remains to be explained if long (as in the case of species of section *Physurus*) and short (described for *S. paulsenii, S. praecox, S. pestifer, S. iberica*) trichomes represent two different kinds or two different developmental stages of the same trichome. Dense epidermal-cell protrusions or few-celled of well developed smooth trichomes, which were described for some species of sect. *Physurus,* obviously, indicate that these species are tolerant to extreme dry and saline habitats.

Our results showed that Central Asian annual species, especially from sect. *Salsola* subsec. *Kali* can be clearly separated from the annual species of the same section from Europe, not only on the basis of morphology, but also by the density of unicellular trichomes on both bract/bracteoles surfaces. Micropapillate unicellular trichomes are highly specific to *S. paulsenii, S. praecox, S. pestifer, S. iberica.* The closely related annual European species of section *Salsola* subsec. *Kali* in particular *S. ruthenica* and *S. kali* are similar with Asian annual *Salsola* species, except for the density of glandular trichomes on the bract/bracteoles surfaces. *S. ruthenica* and *S. kali* possess smooth bract/bracteoles surface or with a presence of slightly developed papillae-a soft protuberance structures. This probably indicates a co-species relationship between the Asian and European species of genus *Salsola*.

Although an abundant development of various types of trichomes within desert Asian *Salsola* species might be well correlated with the desert ecological factors. Wide morphological variations are exhibited by the species of sect. *Cardiandra* and *Belanthera*, which mostly possess both uni-and multicellular trichome types (bladder cells-structural organization) which are usually globose or club-shaped and readily distinguishable from unicellular papillae and sharp-pointed prickles.The 2-armed or detached smooth trichomes called ‘Malpigilian hairs’ seem highly specific to species of sect. *Malpigila*, while vesicular and various glandular structures are best represented in the species of sections *Cardiandra* and *Belanthera.*

It has been observed that in some cases an accumulation of high concentration in the vacuole of terminal cells of bladder trichomes are released probably by rupture of the cytoplasm and cell-walls (Thomson et al. 1988). In such cases the collapsed cell gives the characteristic mealy appearance of the epidermis in many Chenopodiaceae. Therefore with the help of morphological characters; mainly related to epidermal structures (by SEM analysis); we find that the *Salsola* species complex could in fact be divided into two groups: species with salt-producing trichomes/hairs and salt-accumulating (with specific salt/storage cells) plants. This stresses the fact that different mechanisms and strategies for the sequestration and regulation of the salt ion concentration in the plant tissues are operated in the stem and leaf succulent halophytes and in the recreto-and pseudohalophytes of the Kyzylkum flora. The ability of some desert chenopods to accumulate significant amounts of nitrates and/or oxalates has been reported b y several investigators notable among them being (McWorter et al. 1995; Sandquist and Ehleringer 1997; Judd and Ferguson 1999; Butnik 2001a, b; Wojnicka-Poltorak et al. 2002).

The natural plant-cellular mechanism of salt/metal removal and tolerance presented here shows that more detailed studies are needed for a development and testing of more valid hypothesis regarding the adaptations required for colonization and survival of plants, growing under extremely harsh and simultaneously contaminated desert environments.

It is worth noting here that the multicellular trichomes of vegetative sterile elements of floral bracts, bracteoles and perianth segments of some chenopods and graminaceous plants are related to salt and heavy metal removal. In some cases, it has been observed that a high concentration of various ions accumulates in the vacuole of bladder trichome terminal cells. There are two types of glandular trichomes (salt glands) found by us in *Salsola* species as against the data presented by others related to the absence of salt glands in chenopods (Carolin 1983). The reason may be that they are not strictly homologous, particularly since both occur in annual *Salsola* species. We suggest that the different appearance of terminal cells by these two types is due to differences in function connecting both with the accumulation of various ions and /or secretory processes. A comparative morphological study of closely related annual *Salsola* species from highly contaminated desert soils (Uzbekistan) and unpolluted steppe soils (Europe) shows an increase of succulent bracts/perianth segments consistent with Kyzylkum chenopods, epidermal vesicles were rarely recorded here. The prickles, as single celled hairs with relatively thin cellulose walls and thick cuticles that has been described for some chenopods in some annual chenopods may represent the final stage in the reduction of uniseriate hairs (type 3 and 4) according to the classification presented by Carolin (1983). We are inclined to consider various morphological types of hairs described mostly for *Salsola* species as part of the same transformation series, which probably perform different functions, but little is known about the origin and significance of such transformations, especially when they occur on the same plant.

6 Stomatal Diversity

Stomatal frequency within representatives of genus *Salsola* varies greatly on different parts of the same leaf/or leaf-like organs and on different leaves, bracts/bracteoles of the same plant and is influenced by environmental conditions. In bracts/bracteoles of *Salsola* species stomata occur on both sides or mostly or only on one side, usually lower. Stomata also vary in the level of their position on the epidermis. Some are even with the other epidermal cells; others are raised above or sunken below the surface (as in the case of *S. lanata, S. turkomanica).* The number of stomata per unit area and the positional level of the guard cells with respect to other epidermal cells are so variable that they are of little taxonomic value. The more frequently used taxonomic character is the appearance of the stomata as seen from the surface, especially with reference to the nature and orientation of the neighboring cells). The stomatal counts indicate a great variation in the absolute number per unit area, probably due to differences in variety (species) and ontogenetic stage of leaf-like organs.

A large diversity in the anatomy of assimilatory organs and their photosynthetic pathway has been marked within genus *Salsola*. Two anatomical types, Salsoloid and Sympegmoid occur in the leaves of species of *Salsola* (Toderich et al. 2007; Voznesenskaya et al. 2002; Freitag and Stichler 2002; Akhani et al. 2007). In some species with Salsaloid anatomy NAD-ME C_4 photosynthesis has been reported, whereas others have NADP-ME C_4-subtype (P'yankov et al. 1997, 2001). Plants with Sympegmoid anatomy have C_3-like $^{13}C/^{12}C$ discrimination values (P'yankov et al. 1997, 2000). The variations also occur in structural and biochemical features in cotyledons (P'yankov 1999; Akhani et al. 2007). Two non-Kranz anatomies, Atriplicoid and Salsoloid, are found in *Salsola* cotyledons (Winter 1981; Butnik et al. 1991; P'yankov et al. 2001), such as cotyledons and leaves may or may not contain a hypodermis. The result is a number of unique combinations of structural and biochemical photosynthetic types in leaves and cotyledons in the species of Salsoleae. So, multiple origins of C_4 photosynthesis as described in the families of Poaceae, Cyperaceae, Asteraceae and Zygophyllaceae appear within Chenopodiaceae as well and diversity of photosynthetic types and anatomical structures in the tribe *Salsoleae* suggests a dynamic pattern of photosynthetic evolution within this single tribe.

From our phenological observations and experimental results, it seems that structural polymorphism of floral organs and sexual reproduction system in some Asian *Salsola* species are coupled with the diversity of photosynthetic pathways and anatomy of the CO_2 assimilative organs. *S. arbusculiformis* manifests a Sympegmoid leaf and bract anatomy, and non-Kranz bundle sheath cells (Voznesenskaya et al. 2001; Toderich unpublished data). Other species of Section *Coccasalsola* forming a unique "plant functional group" can be united by Salsoloid (with hypodermis both in leaves and reproductive organs) or a "Crownary-centrical Kranz type of photosynthetic cell arrangement (Voznesenskaya and Gamaley 1986). The anatomy of Salsoloid type of Kranz assimilation tissues is always associated with the C_4 syndrome, C_4 like $^{13}C/^{12}C$ carbon discrimination values in leaves, flowers and fruits with a range of 12.0–14.08 (Carolin et al. 1975; Freitag 1997). Such similarity of anatomical and biochemical features is well coordinated with developmental stability of reproductive systems noted by us for *S. arbuscula, S. richteri* and *S. paletzkiana.* However, plants of *S. arbusculiformis* from their natural habitats with Sympegmoid leaf and bract anatomy maintain their $^{13}C/^{12}C$, C_3/C_4 carbon fractionation values in the range from 23.6 to 26.31 throughout their ontogeny, although significant variation was found within plant organs with 2.69% in flowers. This species is also characterized by a set of primitive embryological features such as ana-campylotropous, crassinucellate, bitegmic ovule, autogamy (self pollination /fertilization system), narrow specialization of sexual reproductive system that may be an evidence of lower reproductive plant functional activities leading to the lower level of seed set, seed viability and seed germination. Since C_3 is regarded as the primary type of photosynthesis in relation to C_4, apparently there is a strong connection between structural floral and fruit traits and their physiological and biochemical activity throughout their ontogeny. The anatomy of bracts in different

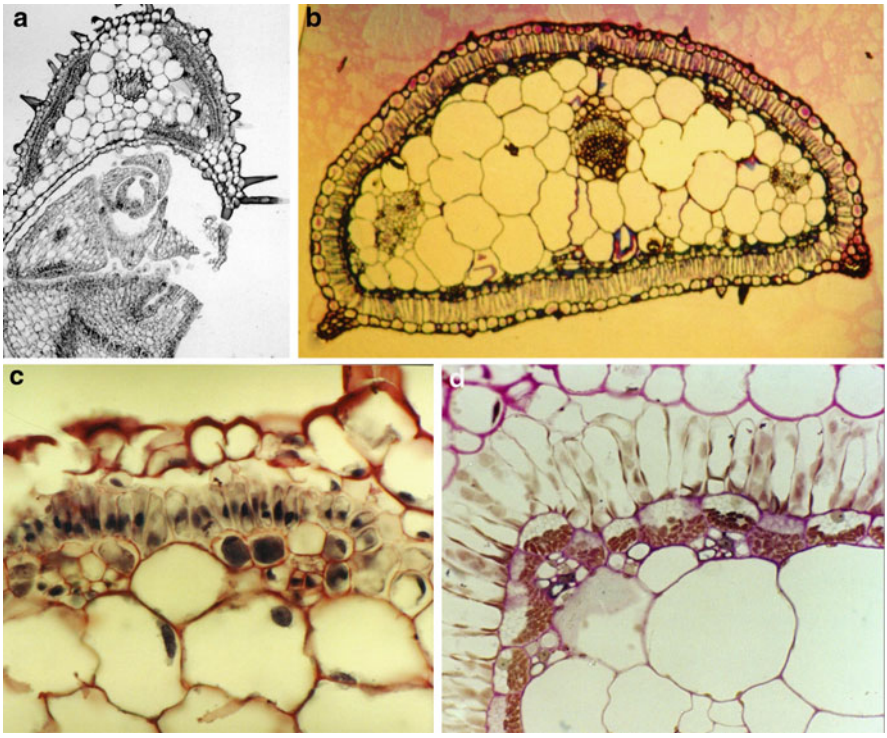

Fig. 12.10 **a** First stage of differentiation of sclerenchyma of *S. praecox*. **b** Anatomy and indefinite Kranz bundle sheath cells of *S. praecos* salsaloid bracts during budding stage. **c** Cross section of *S. praecox* bract during flowering; Kranz bundle sheath cells visible. **d** Cross section of *S. praecox* bract during fruit maturation

Asian species of *Salsola* was studied by us in relation to their photosynthetic activity (Fig. 12.10a–d).

Photosynthetic activity of reproductive organs was insignificant in the budding stage with some increase during flowering process and gradually decreasing during fruit maturation (Figs. 12.10a–d). It was found that *S. pestifer, S. praecox* and *S. paulsenii* are similar in photosynthesis types: C_4-Sals (-H) both in leaves, cotyledons and bracts. Differences were revealed in the anatomy of bracts. All Asian annual *Salsola* species of section *Salsola* subsec. *Kali* have so-called Salsoloid or 'crown centric' Kranz leaf and bract anatomy (Voznesenskaya and Gamaley 1986). The first features of differentiation of chlorenchyma cells in the bracts and bracteoles are marked at the early stage of pollen sac development, reaching a maximum during blooming stage. Cross sections of perianth in the fruits of many annual *Salsola* species during maturity also show an insignificant development of chlorenchyma tissue. Similar situation has been described for the species of section *Belanthera*. In the bracts or fruiting bodies of this type, chlorenchyma is represented by two layers of green cells positioned around the periphery of the organs, the

outer layer composed of palisade mesophyll cells and the inner layer composed of palisade mesophyll cells + inner layer of bundle sheath cells. The main vascular bundle with much thick-walled in the centre, surrounded by the water-storage tissue, and only small peripheral bundles have contact with cholernchyma. In fact all species with Salsaloid Kranz anatomy in photosynthetic organs (irrespective of whether these are leaves, stems, cotyledons or bracts) have C_4 type photosynthesis (Toderich et al. 2007; P'yankov 1999; P'yankov et al. 2000). However, chlorenchyma of *S. ruthenica,* consisting of palisade and Kranz cells, is interrupted by longitudinal colenchymatic ridges.

Diversity in the anatomy of fruits reflects the character of adaptive coevolution of woody *Salsola* taxa and plays a more significant role in the species identification than other elements of floral organs. For instance in *S. richteri* and *S. paletzkiana* the adaptive specialization to the xeric-arid conditions proceeds towards the intensification of sclerification of fruiting perianth and increase in the size and number of cell layers of pericarp and even embryo tissues. The presence of pigments in the fruit covers, singular hydrocytic cells, partial myxospermy and development of membranous layer in the spermoderma intensify the defending function against sun radiation. A fully developed embryo and differentiation of its tissues indicates the complete readiness of embryo of *Salsola* species to the germination. Seed dispersal is manifested by the development of large and wide wings; all elements of fruit cover and embryos of studied species have adaptive value in pigmentation, partial myxospermy, thickenings of external walls, membranous and aleironic layers in the spermoderma, intensification of succulence features as a result of well development of aerial parenchyma, abundance of reserve store nutritional substances, which stimulate the defense mechanism of embryo under extreme desert environments.

The Asian *Salsola* species of section *Arbuscula Coccosalsola* section with both C_3 and C_4 photosynthesis types represent a unique example of the evolutionary convergence of ecological, structural, physiological and biochemical traits. The great range of variation, far more marked in ploidy of genome and fruit structures than in floral and pollen morphology explains the high phenotypic plasticity and good adaptation of *S. richteri* and *S. arbuscula* to various geographical and ecological desert habitats. On the other hand *S. paletzkian*a and *S. arbusculiformis* are characterized by narrow structural specialization of reproductive organs, partly seeds to germinate only on the sandy or stony gypsumferous soils that, perhaps explains the strict local distribution of this species in the Central Asian Flora (Toderich et al. 2008).

An analysis of the carbon isotope ratio ($\delta^{13}C$) of wild Kyzylkum desert species along the salinity gradient revealed significant differences in carbon discrimination between and within C_3 and C_4 species. Within $C_3 \delta^{13}C$ value changes from –30.1‰ (*Zygophyllum fabago, Zygophyllaceae*) to −25.61‰ (*Tamarix hispida,Tamaricaceae*). In general for the C_3 plants investigated by us differences in ^{13}C between different species reached 5.49‰, and within separate species −3.26‰ (*Alhagi pseudalhagi, Fabaceae*). Such changes of carbon discrimination in plants are evidence of change in photosynthetic intensity, as well water use efficiency more than 50%. A 2‰ difference in the discrimination of C_3 species indicates a difference in water-use efficiency of about 30% (Ehleringer and Cooper

1988; Ehleringer et al. 1998). For C_4 species the difference in ^{13}C value was not so significant: from −14.241‰ (*Kochia prostrata, Chenopodiaceae*) to −12.31‰ (*Suaeda arcuata, Chenopodiaceae*).

Stable carbon isotope analysis of different plant communities showed that mean ^{13}C of C_3 species in xerophytes communities was lower, than haloxerophytes and halophytes: −27.39‰, −26.67‰, и −24.79‰ . For C_4 species in the same community follow results were obtained −12,86‰ in haloxerophytes, −12.63‰ xerophytes and −12.16‰ halophytes. It may be due to various salinity levels of soil, because haloxerophyte and halophyte communities occupy soils with moderate and high level of salinity, whereas xerophytic communities grow on non/light saline soils. A negative effect of soil salinity on carbon isotope ratio of desert plants was observed. In general C_3 species are more sensitive to the soil salinity than C_4. Salinity, as stress factor decreases the transpiration and photosynthetic intensity, which leads to a decrease in the rate of biomass accumulation of plants.

7 Conclusion

In conclusion it can be said that desert plants as autotrophic sessile organisms are continuously facing changing and unpredictable environments as well as micro-environmental problems to solve the problems within their organs and cell types which continuously face changing supplies of nutrient ions, sugars, amino acids, gases, light and water. Some major external environmental problems that plants must solve are:

- their biophysical soil and air environments are continuously changing, far beyond normal daily environmental changes;
- their biological environments (microbes, herbivores, and others) change constantly;
- human's particularly move and destroy plants and add both beneficial materials and toxic pollutants to their environments

In the Kyzylkum Desert some plants;characterized as metallohalophytes by us; grow well in either natural or contaminated soils containing salts and metals (Toderich et al. 2004a, b, 2005a, b, 2006). The flora in this desert contains only a restricted number of species capable of removing metal/salts from their habitats. These species can survive and reproduce under these contaminated environments. Some successful species in such habitats produce large quantities of small, easily dispersed seeds, hence facilitating colonization. It is clear from the biochemical and physiological studies that plants have multiple often redundant pathways and mechanisms to accomplish the same function or goal. These genetically built-in mechanisms for redundancy in numerous plant functions act as fail-safe mechanisms. Redundancy apparently gives sessile plants 2 major advantages;

1. their normal developmental ability to form diverse functions in different types of organs, tissues and cells,
2. a very powerful means to adapt the functions of these structures to cope with whatever happens in their biophysical and biological environments (Black 1993; Black et al. 1995).

As external environmental CO_2 levels vary, the internal CO_2 levels in green photosynthetic tissues can be modified to provide this essential nutrient (Toderich et al. 2007).

There are several morphological and anatomical features met within desert plants under natural saline and contaminated environments but most important ones are salt-secretary trichomes and salt glands. These resemble functionally and are associated with the secretion of ions using morphological characters, mainly related to epidermal structures (by SEM analysis). Desert species are developing different mechanisms of adaptation to stress; species with salt-producing trichomes/hairs and salt-accumulating (with specific salt/storage cells) plants. This is an indication that different mechanisms and strategies for the sequestration and regulation of the salt ion concentration in the plant tissues are operated in the stem and leaf succulent halophytes and in the recreto-and pseudohalophytes of the Kyzylkum flora. The existence of great diversity in photosynthetic pathways ofAsiatic *Salsola* species, as well as anatomy and biochermical features in the CO_2 assimilation organs is evidence related to plant growth, survival, and reproduction in such desert plants (Butnik et al. 2001a, b; P'yankov et al. 2001, 2002).

Various morphological types of hairs described mostly for *Salsola* species as part of the same transformation series probably perform different functions. However little is known about the origin and significance of such kind of transformations, especially when they occur on the same plant.

Increasing of sclerification, availability of pigments and tracheids like cells holding moisture, abundance of crystals in the fruit tepals, tissues also promote the protection of embryo from unfavourable conditions (Butnik et al. 2001a, b; Toderich et al. 2008). Some highly adapted metallohalophytes in nature develop a cellular mechanism to partition toxic salts into vacuoles or to exclude salt at the root zone so it does not affect cell metabolism and division, i.e., a high concentration of various ions can accumulate in the vacuoles of bladder-trichome terminal cells which are frequently developed on the adaxial surface of epidermal cells of leaves or bract/bracteoles

The prominent levels of sclerification of perianth segments combined with thickening of pericarp and spermoderma epidermis bearing papillae-shaped protrubarences (*Salsola paulsenii*) are related to the defending of embryo against entrance of toxic elements. Diversities in sexual reproduction mechanisms and CO_2 fixation pathways, for tree-like *Salsola* species, also are important factors regarding reproduction and survival under saline and technogenic contaminated desert environments.

Most essential plant nutrients come from soil-plant interactions via root and microbial contacts; simultaneously essential nutrient uptake must cope with the

presence of any toxicants and non-essential elements in soils. The roles of fungi, bacteria, and other organisms as they interact with plants are crucial. Biological lipid bilayer membranes are essentially impermeable to ions, sugars, and polar molecules; hence channels, pumps, diffusion, solution, and mass flow are used to cross biological membranes. The uptake of mineral ions from soils by plant roots occurs through protein-built channels in a biphasic fashion, first with a strong high affinity active carrier mechanism, followed by a slower diffusion uptake. Such active transport channels and pumps are powered, usually by ATP, and may involve an active co-transport with other ions or an exchange with others ions.

For bioremediation purposes there should be interest in the species which consistently have a metal/salt removal potential. Since several "hyperaccumulators" are characterized by small biomass production, the use of selected metallohalophyte species as phytoremediators capable of accumulating high amounts of toxic ions should be considered. Halophytes and simultaneously metal tolerant arid/semiarid plants may be used for phytoremediation of areas contaminated with toxic salts and heavy metals. However, future work is needed to:

- Select optimal genotypes from Kyzylkum desert flora and to initiate a program of its seed multiplication.
- Determine the mechanisms of their hyperaccumulation and hypertolerance.
- Isolate the genes involved.

It may then be possible to genetically engineer these traits into higher biomass forms and develop more efficient heavy metal phytoextraction processes. Several authors have pointed out that heavy metal hyperaccumulators could prove economically useful as an efficient method for cleaning the soils (Leblane et al. 1999; Escarre et al. 2000; Chaney et al. 2007). Significant progress has been made in recent years in developing native or genetically modified plants for the remediation of contaminated sites (Meagher et al. 2000). The study of chemical compounds (origin, localization etc.) for Asian desert plants are of great interest because they are often specific to a particular plant species or genus and must therefore have been designed to serve a particular protective function. In the case of salt remediation the timing of salt excretion within plant organs is of critical importance, not only for our understanding of the cellular mechanism involved, but also because salt/toxin accumulation could interfere with health problems of other living beings.

The stable recovery of ecosystem functions can be considered best from the viewpoint of development over time. Phytoremediation technology is considered a potentially valuable technique for dealing with heavy metals, which are typically the most difficult pollutants to remove from soils. The use of metallohalophytes from the Central Asian flora to reclaim soils could represent both a practical and economically viable strategy. Even though the scientific technology for molecularly transforming plants is very well established, unfortunately plants that are well adapted to desert environments have not yet been transformed. Plant transformation knowledge needs to be applied immediately to the special needs of desert-adapted plants in Central Asia.

The cultivation of halophytes (C_3 and C_4 plants) can limit long-distance salt spreading and improve the vitality and growth conditions for local species, when cultivated together. Since stress conditions frequently trigger defense mechanisms based on the production of specific biological active metabolites of pharmaceutical or industrial importance, halo-metallophytes of the South part of Aral Sea Basin could constitute a valuable source of cash compounds. These characteristics may offer a new and valuable source of income to local populations.

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Chapter 13
Boron and Plants

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Abstract Boron is found naturally in the earth's crust in the oxidized form as borax and colemanite, particularly in the oceans, sedimentary rocks, coal, shale, and some soils. It is never found in the elemental form in nature possessing a complex chemistry similar to that of silicon, with properties switching between metals and non-metals. Boron has become an important and strategic element in terms of developing technologies. It is released into the environment mainly through the weathering of rocks, volatilization from oceans, geothermal steam, burning of agricultural refuse and fuel wood, power generators (coal/oil combustion), glass industry, household use of boron-containing products (including soaps and detergents), borax mining and processing, leaching from treated wood and paper, chemical plants, and sewage/sludge disposal, but a major proportion originates from the weathering of rocks. Boron is regarded as an essential element for human beings, animals and plants. Boron occurs in soils at concentrations ranging from 10 to 300 mg kg^{-1} depending on the type of soil, amount of organic matter, and amount of rainfall. The treatments lead to significant increases in the productivity of some plants but in certain cases a decrease is seen as the boron level increases with the boron content of irrigation water, in particular on the soils with

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Dedicated to Prof. Dr. Yusuf VARDAR (Ege University) and Prof. Dr. Hubert ZIEGLER (Munich Technical University) on their sad demise in 2009.

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a heavy texture, high $CaCO_3$ and clay content. Lack of boron in plants results in necrosis but excess amounts are said to produce poisonous effects. Turkey produces more than 60% of the world's borax, with important boron reserves located in Susurluk, Bigadic and Sindirgi regions of Balikesir, Kestelek-Bursa, Emet-Kutahya, the largest reserves occur in Kirka-Eskisehir. Therefore, there is a naturally occurring high level of boron in the ground waters in some of these areas due to the excess amounts of boron given out to the environment during washing and purification processes which result in the pollution of cultivated areas. An attempt will be made here to present an overview of the plant diversity on the boron contaminated soils in Turkey, effects of different concentrations of boron on the germination ability of some plants and possible candidates for phytomining of the soils showing boron toxicity symptoms.

Keywords Boron · Toxicity · Phytoremediation · Genotoxicity · *Polygonum*

Contents

1 Introduction

Elemental boron (B) is a member of Group IIIA of the periodic table, along with aluminum, gallium, indium, and thallium, differing distinctly in its chemical properties from aluminum but resembles silicon (Si), arsenic (As), and germanium (Ge) possessing a very complex chemistry (Cotton and Wilkinson 1988; Marschner 1995). Tanaka and Fujiwara (2008) have recorded it as a member of metalloid group of elements belonging to group V, because its characteristics lie between metals and non-metals (Marschner 1995), being a semiconductor rather than a metallic conductor.

It is extensively distributed in low concentrations throughout nature in the form of various inorganic borates constituting about 10 mg kg^{-1} of the Earth's crust, ranging

from 5 mg kg^{-1} in basalts to 100 mg kg^{-1} in shales (Woods 1994), and occurs in soils at concentrations ranging from 10 to 300 mg kg^{-1} (average 30 mg kg^{-1}), depending on the type of soil, amount of organic matter, and rainfall. Economic reserves of borate minerals are rare and are usually found in arid desert regions with a geological history of volcanic and/or hydrothermal activity (Mellor 1980). The majority of the boron occurs in the ocean, at an average concentration of about 4.5 mg L^{-1} (Weast et al. 1985), but is also released from anthropogenic (agricultural, industrial and domestic) sources to a lesser extent (Butterwick et al. 1989). Natural weathering of clay-rich sedimentary rocks, coal and shale on land surfaces accounts for a large proportion of the boron, mobilized into the soils and the aquatic environment, in the form of borates. Boron in soil solution is present as boric acid and easily leached out of the soil due to its high solubility (Shorrocks 1997; Yan et al. 2006). It is adsorbed onto the surfaces of soil particles, with the degree of adsorption depending on the type of soil, pH, salinity, organic matter content, iron and aluminum oxide content, iron-and aluminum-hydroxy content, and clay content (Kekeç 2008; Ayvaz 2002).

The availability of B in soil is limited in many regions in the world with a high rainfall and seasonal water availability. On the contrary, in the arid and semi-arid regions, ground water reaches the topsoil by capillary action and evaporates to leave solutes in soil. In regions with high-boron groundwater, boron concentration in topsoil reaches to a toxic level for plants and reduces crop yields. South Australia, Egypt, Iraq, Jordan, Libya, Morocco, Syria, Turkey, California, and Chile are regions/countries with boron toxicity problems in agricultural lands (Yau et al. 1995).

2 Boron Production and Usage

Borate minerals have been employed in a wide range of uses for many centuries, dating from at least the eighth century when they were used primarily as a flux for assaying and refining gold and silver as well as production of wall plaster and ceramics (Ayvaz 2002; Bayca et al. 2008; Batar et al. 2009). Their valuable properties and relative rarity has stimulated international trade in borates. Marco Polo claimed to have transported Chinese borate minerals from Tibet to Europe and Venice was the center for borate imports (Travis and Cocks 1984). It is wildly used in the industry. A large number of minerals contain boric oxide, but five of them are the most important from a worldwide commercial standpoint. The most widely used commercial productions and materials of boron include borax-pentahydrate, borax, sodium perborates, colemanite, ulexite as well as boric acid. These are produced in a limited number of countries, dominated by the Turkey and United States, which together furnish about 90% of the world's borate supplies (Lyday 1993; Culver et al. 1994). The principal end usage for borate include insulation and textile-grade fiberglass, laundry bleach (sodium perborate), borosilicate glass, fire retardants, chemical fertilizers and herbicides (as a trace element), and enamel coating, frit and ceramic glazes, as well as several other applications (Etiproducts 2005; WHO 1998). Other

minor usage include cosmetics and pharmaceuticals (as a pH buffer), boron neutron capture therapy (for cancer treatment), and pesticides. The cancer treatment application which preferentially accumulates in tumor versus normal tissue, utilizes a boron compound made with ^{10}B isotope, (Barth and Soloway 1994).

3 Boron and Living Beings

The lowest lethal dose for humans exposed to boric acid is reported to lie around 640 mg kg^{-1} body weight by oral exposure, 8600 mg kg^{-1} body weight by dermal exposure, and 29 mg kg^{-1} body weight by intravenous injection (Stokinger 1981). After establishment of essentiality, understanding a role(s) of boron became the major task in boron biology, however, its essentiality in humans has not been established, although its beneficial effect has been reported. Boric acid and borax were widely used in medicine at the beginning of the century for therapeutic purposes, both locally as well as orally. Boric acid was used to treat various diseases, such as epilepsy and infectious diseases. Several case studies reviewed by Kliegel (1980) describe mild to severe responses to boron compounds. Linden et al (1986) have published a retrospective review of 364 cases of boric acid exposure. Vomiting, diarrhea and abdominal pain were the most common symptoms given by the 276 cases exposed.

Boron is also required by animals, including zebrafish, trout (Rowe and Eckhert 1999), and frogs (Fort et al. 1998). Its deprivation causes impaired growth, abnormal bone development, increase in urinary calcium excretion, and change of macromineral status in animals (Devirian and Volpe 2003), also affecting carbohydrate and mineral metabolism, energy consumption, and regulation of the activity of several enzymes; however, the molecular basis of boron function in animals is not well understood (Devirian and Volpe 2003). Excessive boron intake causes acute neurological effects, diarrhea, anorexia, weight loss, and testicular atrophy in mice, rats, and dogs. It also causes decrease in fetal body weight and increase in skeletal malformation and cardiovascular defects in pregnant female animals (Yazbeck et al. 2005; Pawa and Ali 2006). Several investigators have studied the effects of borates on bacteria, protozoa and algae. The effective concentrations for the bacterium *Pseudomonas putida* range widely (Schöberl and Huber 1988; Guhl 1996; Bringmann and Kuhn 1980). Nitrogen-fixing cyanobacteria require boron for proper functioning of the heterocyst cell wall (Bonilla et al. 1990). Mateo et al. (1986) concluded that boron is essential for nitrogen fixation in Anabaena.

4 Boron and Plants

Since the discovery of boron as an essential element for plants, evidence has been accumulating that boron is an essential element not only for vascular plants, but also for diatoms, cyanobacteria, and a number of species of marine algal flagellates (Marschner 1995). Initial phase of the studies was based on the symptoms of

boron deprived plants. It is considered to be involved in the metabolism of nucleic acids, carbohydrates and proteins, indole acetic acid, phenol, cell wall synthesis and structure, membrane integrity and function; however, molecular basis of these roles is mostly unknown (Marschner 1995; Goldbach et al. 2001). It is an essential micronutrient for higher plants, with interspecies differences in the levels required for optimum growth and plays an important role in some plant functions such as metabolic pathways, uptake of Ca^{2+}, sugar translocation, pollen germination, hormone action, root development, flower and fruit formation, normal growth and functioning of the apical meristem, water translocation from roots to the upper portions of the plant body and membrane structure and function (Abdulnour et al. 2000; Liu et al. 2000; Lou et al. 2001). Nobel (1981) studied the effect of several boron compounds on photosynthesis in submerged macrophytes, watermilfoil (*Myriophyllum alterniflorum*), buttercup (*Ranunculus penicillatus*) and waterweed (*Elodea canadensis*).

Early investigation of the effects of boric acid and borax on the field bean (*Vicia faba*) and other plants indicated the role of boron in plant nutrition (Ayvaz 2002). There is an overlap of the beneficial and injurious effects of boron between species; therefore, three broad categories of tolerance (sensitive, semi-tolerant, and tolerant) have been established (Ayvaz 2002). The sensitive species can tolerate 0.5 mg L^{-1} of boron but tolerant species can tolerate up to 4 mg L^{-1} (Batar et al. 2009). Plants in general use less than 5% of boron in the soils (Uygan and Çetin 2004). The tolerant plants endure a wide range of boron concentrations with little effect, and the sensitive plants exhibit a strong reaction to either too much or too little boron. Phytoremediation is the use of plants to make soil contaminants non-toxic and is one form of bioremediation. The term phytoremediation generally refers to phytostabilization and phytoextraction. In phytostabilization, soil amendments and plants are used to alter the chemical and physical state of the heavy metal contaminants in the soil. In phytoextraction, plants are used to remove contaminants from the soil and are then harvested for processing.

Boron is an essential element for higher plants. Many studies have shown that certain boron concentrations are necessary for biochemical, physiological and morphological development of plants. Our studies revealed that boron is an essential requirement for maize. The growth rate of radicule and genomic stability increased at 10 mg L^{-1} boron concentration. Similar findings have been reported by Kocacaliskan and Olcer (2006) and Konuk et al. (2007). Boron toxicity may limit crop productivity in boron rich agricultural soils. In dry seasons/conditions, boron supply to roots is reduced due to reduced mass flow from soil to the root (Shorrocks 1997).

In many countries, the absence of B in the soil causes deficiency problems in plants (Shorrocks 1997). However, in Turkey high levels more commonly end up in the toxicity (Ataslar et al. 1995). According to Ayvaz (2002) and Kekeç (2008) the symptoms of boron deficiency in plants include cessation of root and leaf growth, necrosis of leaf primodia and primary root tips, necrosis of stem and leaf phloem, bark splitting, retardation of enzyme reactions, reduced pollen germination, and even death. Normal growth will usually resume if boron is added to the growth

medium. A boron-deficient nutrient solution also inhibits mitosis in the root tip of the field bean. A 10 mg L^{-1} boron solution produces optimum cell division and elongation of the root tip; however, 50 mg L^{-1} boron causes a reduction in mitosis. The studied on the effects of boron deficiency and toxicity in *Pinus radiata* seedlings grown in water culture have revealed that profound changes occur in cell wall morphology, suggesting that boron is critical to cell wall expansion (Cakmak and Römheld 1997). It has been proposed that this structural, cross-linking function of boron is involved with the pectin fraction, which contains apiose and other hydroxylated fragments amenable to complexation by borate (Loomis and Durst 1992). Hu et al. (1996), studied the fourteen species of crop plants, and it was concluded that high pectin content requires more boron for forming cell walls or that pectin forms a tightly held boron complex that depletes boron availability for other critical functions, thereby increasing the overall demand for boron. Kobayashi et al. (1996) have isolated and characterized a rhamnogalacturonan II/borate complex from enzyme-digested cell wall pectin.

Recently, one of the primary functions of boron in higher plant has been reported at the molecular level. It cross-links pectins in cell walls, and this cross-linking is essential for normal expansion of leaves. Pectins, important components of plant cell wall, are complex polysaccharides, including homogalacturonans and rhamnogalacturonans I and II (RG−I and RG−II). It was demonstrated that the RG−II is cross-linked by a 1:2 borate-diol diester and forms the dimeric RG−II (Kobayashi et al. 1996). O'Neill et al. (2001, 2004) have demonstrated that the cross-link between RG−IIs formed by borate cis-diol ester bonds is essential for normal leaf expansion through analysis of the mur1 mutant in *Arabidopsis thaliana*, which has abnormal sugar composition of RG−II. It is clear that this role of boron in cross-linking of pectin is among the number of roles of boron in plants.

4.1 Boron Tolerance, Deficiency and Toxicity in Plants

Boron is of great importance to plants. However, the amount needed is very little. The amount of boron useful for the growth of plants varies between 0.5 and 2.0 mg L^{-1}. Generally the soils containing less than 0.5 mg L^{-1} of boron are poor in terms of boron and boron deficiency symptoms can be observed in the plants. In the soil where the rate of boron is over 2.0 mg L^{-1} there is boron pollution and consequent decrease in production and defects in the products can be seen (Taiz and Zeiger 1991).

Many studies have shown that certain concentrations of boron are necessary for biochemical, physiological and morphological developments (Hale and Orcutt 1987). There is a very narrow range between boron deficiency and toxicity as more than 5.00 mg L^{-1} available boron can be toxic to many agronomic crops. Lack of boron often limits production of forage legumes (alfalfa, clover, trefoil) and some vegetable crops. The tolerant species are Alfalfa, Beet, Cotton, Grain, sorghum, Oat, Sugar beet and Tomato; moderately tolerant species being Barley, Cabbage, Celery, Corn, Squash, Sweet clover and Turnip, and moderately sensitive species are

Broccoli, Carrot, Cucumber, Pea, Pepper, Potato and Radish. The sensitive species are Avocado, Bean, Grape, Grapefruit, Lemon, Orange and Wheat. The growth of *Vicia faba* grown under a medium without boron supplementation is reduced, but a recovery occurs by supplying boron. It is toxic when present at higher concentrations. Thus, it is essential to maintain concentration of boron in media/soil within an appropriate range for maximum yields. In plant, symptom of boron deficiency occurs mainly in growing or expanding organs in the plant body.

Under boron deficient conditions, leaf expansion and root elongation are inhibited. Apical dominance, flower development, and fruit and seed sets are also inhibited under boron limitation. Thus, boron deficiency causes not only the reduction in crop yield, but also the decrease in the quality. According to Stavrianakou et al. (2006), besides inhibition of growth, boron deficiency causes a notable increase in the relative concentration of 'internal' leaf and root phenolic compounds of *Dittrichia viscosa* (Asteraceae). It does not have any negative effect on parameters related to photosynthesis (such as stomatal density, chlorophyll concentration, photosynthetic capacity and intrinsic photochemical efficiency of PS II). As boron is not efficiently remobilized, i.e., boron tends to stay in organs where it is first distributed, it is important to maintain continuous supply of boric acid for efficient agricultural production (Marschner 1995; Shorrocks 1997; Dell and Huang 1997).

In contrast to the deficiency symptoms, typical boron toxicity symptoms occur in the marginal region of mature leaves, and these portions become chlorotic or necrotic. Boron tends to accumulate in old leaves, especially at the margin of leaves. This is because boron is transported along the transpiration streams and accumulates at the end of transpiration stream. Excess boron also reduces crop yield reduction (Yau et al. 1995). Boron toxicity is an important disorder that can limit plant growth on soils of arid and semi arid environments throughout the world. Soil is generally the primary source of trace elements for plants. However, there are exceptions in which toxic concentrations of trace elements in plants, e.g., B, can be traced directly to water from certain wells, or indirectly to land application of drainage water and soil with high B availability (Kubata 1980). However, the adsorbed and solution phases of B in the soil influence potential B toxicity effects observed in the field (Cartwright et al. 1984; Shani and Hanks 1993); and sometimes lead to decreases in crop yields grown in different regions of the world (Cartwright et al. 1986). There is also a very narrow range between boron deficiency and toxicity as more than 5.00 mg L^{-1} available boron can be toxic to many agronomic crops (Nable et al. 1997). The initial symptom of boron toxicity in plants is chlorosis (yellowing) of the leaf tip, progressing along the leaf margin and into the blade. Necrosis of the chlorotic tissue occurs, followed by leaf abscission. Necrosis of the leaf tissue results in a loss of photosynthetic capacity, which reduces plant productivity (Lovatt and Dugger 1984). Pollen germination and pollen tube growth may also be inhibited (Versar Inc. 1975).

Several investigators have shown a direct relationship between the boron content in leaves (foliar) and the severity of the symptoms of toxicity. Gilliam and Watson (1981) conducted an experiment in which Anderson yews (*Taxus media*) were grown in soil at four boron concentrations (0.5, 5.0, 25.0, or 50 mg kg^{-1}).

Symptoms of toxicity were observed when foliar boron accumulation reached concentrations ranging from 85 to 100 $\mu g\ g^{-1}$ of dry tissue. The observed symptoms included leaf tip yellowing, followed by necrosis and premature defoliation. Suppression of shoot and root growth was observed at 50 mg boron kg^{-1} soil. Shopova et al. (1981) found that concentrations of 16, 24, and 32 mg boron kg^{-1} soil resulted in a decline in plant development, yellowing of leaves, late flowering, reduction of mitotic frequency in root tip cells, and abnormalities during meiosis in the poppy (*Papaver somniferum*). Kluge and Podlesak (1985) found that symptoms due to boron excess begin to develop on the leaves (leaf tip necroses) of pot-grown spring barley (*Hordeum vulgare*) as soon as the boron content of the leaf tissue reaches 60–80 mg kg^{-1} dry weight. Gestring and Soltanpour (1987) grew alfalfa (*Medicago sativa*) in three soil types amended with sodium borate at rates of 0, 10, 20, and 40 mg boron kg^{-1}. Alfalfa yield was significantly reduced by boron application in both the sandy loam and loam soils; however, no yield reduction was observed in the silt loam soil. Soil extractable boron did not adequately assess boron toxicity, whereas plant boron levels were a more reliable index of toxicity. Sage et al. (1989) exposed the rare serpentine plant (*Streptanthus morrisonii*) to boron (0, 20, 60, 240, 650, 1200, or 2400 $\mu mol\ L^{-1}$) via watering. Plants showed mild to moderate toxicity symptoms (older leaves exhibiting chlorosis and necrosis) at boron concentrations of 240 and 650 $\mu mol\ L^{-1}$. Glaubig and Bingham (1985) reported significant linear relationships between both soil and leaf tissue boron concentrations and foliar damage in four tree species endemic to California (digger pine, *Pinus sabiniana*; California laurel, *Umbellularia californica*; madrone, *Arbutus menziesii*; bigleaf maple, *Acer macrophyllum*). Under experimental conditions, Shann and Adriano (1988) demonstrated that chronic foliar aerosol exposures of boron produced phytotoxicity in relation to boron accumulation in the leaves. The authors concluded that the visual damage (leaf tip necrosis) resulting from aerosol exposure was identical to that observed from root boron toxicity for all crops tested. Boron deficiencies in terrestrial plants have been reported in many countries. Boron deficiency is more likely to occur in light-textured, acid soil in humid regions, because of boron's susceptibility to leaching.

In general, there is a small range between deficiency and toxicity. However, considerable variation exists between species in their resistance to boron. Species sensitive to boron are known to include citrus, stone fruits, and nut trees; semi-tolerant species include tubers and cereals; and tolerant species include most vegetables. Toxicity due to excess boron is much less common in the environment than boron deficiency. Amongst a wide variety of plant species, the typical visible symptom of B toxicity is leaf burn-chlorotic and/or necrotic patches, often at the margins and tips of older leaves (Bennett 1993; Bergmann 1992). These symptoms reflect the distribution of B in most species, with B accumulating at the end of the transpiration stream. The chlorotic/necrotic patches have greatly elevated B concentrations compared with the surrounding leaf tissues and some species (e.g., barley) show characteristic patterns for different genotypes. In species in which B is phloem mobile (e.g., *Prunus*, *Malus*, *Pyrus*), in which B accumulates in developing sinks rather than at the end of the transpiration stream, the symptoms of toxicity are

fruit disorders (gummy nuts, internal necrosis), bark necrosis which appears to be due to death of the cambial tissues and stem die back (Brown and Hu 1996).

Although the lack of boron in the soil causes some problems in the plants, excess of boron also causes various physical and biochemical problems. These effects cause defects in the fruits and leaves of the plants (Hartmann 1981). According to researches done on the harmful effects of boron in the sunflower and bean fields the yield of sunflower is high at 0.5 mg L^{-1} (418 kg per 1000 m^2) but the yield decreases as the density increases. The yield decreases down to 306 kg per 1000 m^2 at 16 mg L^{-1}. As for the beans the yield is 180 kg per 1000 m^2 at 0.5 mg L^{-1} but goes down to 73 kg per 1000 m^2 at 16 mg L^{-1} (Şener and Özkara 1989).

Genetic variation in response to high concentrations of boron occurs at both the inter-and intra-specific levels. Boron tolerance of bread wheat (Paull et al. 1992), durum wheat (Jamjod 1996), barley (Jenkin 1993) and field pea (*Pisum sativum*) (Bagheri et al. 1996) is controlled by partially dominant nuclear genes. There have been many investigations on inter-specific variation, with each species or genus represented by a single variety (Maas 1987). All of these have identified a wide range in response to boron, either on the basis of plant growth, or the development of toxicity symptoms, or both. The tolerance to boron toxicity not only operates at the level of whole plants, it also operates at the organ and cellular level (Huang and Graham 1990). In recent studies, it has been reported that high pH can limit boron uptake (Baykut et al. 1987; Hu et al. 1996). The tolerance mechanism appears to be under the control of several major additive genes and specific chromosomal locations have been identified for the genes in some species (Nable and Paull 1991; Nable et al. 1997).

4.2 Boron Uptake By Plants

Boron exists in nature (at neutral pH) primarily as undissociated boric acid-$B(OH)_3$ which is soluble in water and exists a small amount of borate anion, $B(OH)_4^-$ (Bolanos et al. 2004). Plant takes up boron from soil in the form of boric acid (Brown and Shelp 1997). As a result of being a non charged molecule, boric acid is highly permeable to the lipid bilayers and hence, passage is proportionally dependent on the concentration gradient (Brown and Shelp 1997, Tanaka and Fujiwara 2008). In order to reach the aerial parts of the plant, B needs to load xylem and transported towards the upwards proportional with the transpiration rate. Finally, B accumulates into the destination point, mostly tips and margins of the mature leaves (Brown and Shelp 1997). Uptake is reduced when soil pH increases from 4 to 9 and increases by an increase in the light intensity; the rate of boron absorption rapidly increases at temperatures ranging from 10 to 30°C and is sharply reduced above 35°C (Ayvaz 2002).

Membranes are key players during the transport of the elements, solutes and water and possess ion transporters. Common traits of some elements are their low membrane permeability co-efficiencies that make their membrane transport more difficult. But some molecules such as boric acid which are moderately permeable

need a transporter. Recent studies showed that cells do not just need transporters for low permeability coefficient molecule, they also need transporters for solute, uncharged molecules and water even if, these molecules are permeable and require any energy to transport through the membrane (Alberts et al. 2002). Recent studies with artificial membrane and membranes isolated from different species have shown that the membrane permeability coefficient of boric acid is approximately 10^{-7}. According to this data, permeability of boric acid is much higher than tryptophan, glucose and Cl^- but much lower than glucose and urea. However, this value is changeable according to the type of the membrane, like lipid composition, intracellular pH.

4.3 Molecular Basis of Boron Uptake and Transport

Three mechanisms are known for across-membrane transport of boric acid: (1) passive diffusion across lipid bilayer (Dannel et al. 2000; Nuttall 2000; Dordas and Brown 2000; Frommer and von Wirén 2002; Kuchel et al. 2006 and Takano et al. 2002), (2) active transport by BOR transporter (Tanaka and Fujiwara 2008; Takano et al. 2008; Peres et al. 2002; Takano et al. 2002 and Frommer and von Wirén 2002), (3) facilitated transport by nodulin–like intrinsic protein (NIP) channel. All of these are involved in regulation of boron transport in plants.

The theory for boron uptake was that boric acid only entered in root apoplast (extracellular space) by **passive transport**. However, Nuttall (2000), Dordas et al. (2000) and Dordas and Brown (2000) showed that boron absorption can also occur by **facilitated diffusion**, through transmembrane channels- the aquaporins (Chrispeels et al. 1999). It was believed that boric acid does not require assistance of transporter called aquaporins (Benga et al. 1986; Frommer and von Wirén 2002; Kuchel et al. 2006). The findings of Agre and Kozono (2003) concluded that high permeable molecules/solutes (water, urea, glycerol etc.) can pass through the membrane with both passive diffusion and also channel-mediated transport as the membrane includes several transporters to make a rapid flux of molecules/solutes on two sides of the membrane by transporter proteins such as aquaporins (Fig. 13.1). The discovery of BOR1 (Takano et al. 2002), a boron transporter revealed that it is required for xylem loading. Takano et al. (2006) emphasized that the lower permeability of plant membranes imply the need of membrane proteins to satisfy a plant's demand of boron, especially under boron limitation.

Active transport mechanism of boric acid to the xylem and then towards the aerial parts of the plants has been reviewed at length by Tanaka and Fujiwara (2008) and Takano et al. (2008). According to these investigators the xylem loading of boron is achieved by transporter proteins. The boron absorbed by apoplast first needs to enter the cell (symplast) to reach the xylem due to the Casparian band, an apoplast barrier in the endoderm. When these solutes enter the xylem, they return to the apoplast, since vase elements are made of dead cells. The process in which a nutrient leaves symplast and enters the xylem through an ion-efflux channel is called xylem loading (Peres et al. 2002). BOR1, characterized by Takano et al. (2002), is the first protein linked to boron transport in biological systems and is related to boron

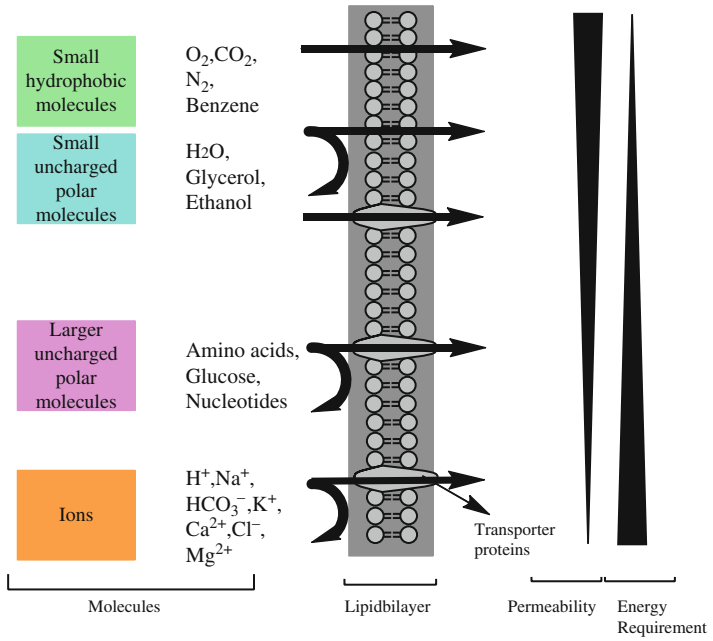


Fig. 13.1 Permeability of biological membranes that allow or prevent the passage of molecules/solutes according to their size, charge, chemical properties, concentration and pressure (Modified from: Alberts et al. 2004)

xylem loading. Among the ten BOR1 hypothetical transmembrane domains, Takano et al. (2002) found a difference of two amino acids in the second transmembrane domain of the putative protein expressed by *Arabidopsis* mutants which requires higher levels of boron. Frommer and von Wirén (2002) suggested that to maintain a boron transport to the xylem, xylem sap requires borate anions. The pH is 5.6 for xylem and 7.5 for cytosole, boric acid inside the cell is converted to borate anion in the cytoplasm because of high cytosolic pH. Therefore boron can easily pass through the membrane as a form of borate anion. Then these borate anions are reconverted in the xylem to boric acid.

Frommer and von Wirén (2002) also proposed three different ways that BOR1 could export borate into the xylem: the first mechanism is diffusion that depends on the concentration gradient for borate (uniport); second is related to borate/chloride exchange coupled to a chloride gradient established by X–QUAC anion channels; and the third one is coupled counter-transport (antiport) of borate with a proton. The proton is exported to the cell wall space by H^+-ATPases inside which generates a negative membrane potential (Frommer and von Wirén 2002).

NIP5;1 is identified as a boric acid channel that resides on the plasma membrane and requires boric acid uptake under boron limitations for normal growth (Takano et al. 2006). Casparian strip has an active role during the boron transport. It blocks the passage of extracellular boric acid from endodermis to the pericyle. Under boron scarcity conditions, NIPs are translated and reside on the plasma membrane of epidermal, cortical and endodermal cells on root and import of boron into the cells

is limited. Boric acid can reach the pericyle and then xylem by means of these importers. The intracellular passage of boric acid between the cells is sustained by plasmodesmata. Hence, boric acid can pass to the Casparian strip and can reach to the destination point-pericyle cells before the xylem loading (Tanaka and Fujiwara 2008). The cellular boric acid needs to efflux from the pericyle cells for xylem loading. According to Tanaka and Fujiwara (2008) BOR1 proteins are expressed somehow, being regulated by posttranscriptional modifications. BOR1 exports the cytosolic boric acid to the pericyclic region under boron limited conditions, but studies have shown that BOR1 proteins are degraded via endocytosis in vacuoles under excess boron supply (30 and 100 μM respectively) (Takano et al. 2005).

4.4 Boron Remobilization

Common idea regarding the boron transport was that it is transported towards the upper parts of the plants as a result of transpiration strength and accumulates on its destination point especially edges of the leaves. Therefore, ideally the older leaves accumulate much more boron than younger. However, studies indicated that for some species, especially significantly sugar alcohol producing species, boron concentration of young leaves is estimated to be higher than older leaves. This stresses that boron can remobilize from the different portions of plants with the help of sugar alcohols especially species that commonly produce significant amount of sugar alcohols (mannitol and sorbitol). Brown et al. (1999) showed that this remobilization is highly related to the sorbitol synthesis. In the case of enhanced production of sorbitol synthase, transport is significantly increased. Tanaka and Fujiwara (2008) have suggested that boron can move along the flow of boron-binding sugar alcohol.

Recent metabolite study for boron toxicity tolerance in plants has shown that glucose level is increased in leaf at high boron exposure levels (1000 μM) compared to low (5 μM) (Roessner et al. 2006). Reid et al (2004) showed in boron intolerant plants, photosynthesis is suppressed by 23% at a high level of boron. Recently Unver et al. (2008) showed a possible role of photosystem II Protein D2 to regulate the boron toxicity in *Gypsophila perfoliata* by comparing the control and high boron exposed (500–1000 μM) leaves. DDRT-PCR results showed that one of the differentially expressed transcript had high level similarity (99% positive score) in the *Triticum aestivum* Photosystem II protein D2. qRT-PCR analysis showed that 500 and 1000 μM boron treated leaf samples showed 10 and 14 fold changes respectively compared to the control groups (30 μM). Thus boron tolerant plants probably tolerate the toxic effects of boron by remobilizing the excess boron between the leaves by forming sugar-boron complexes through phloem. By reverse reaction, deficiency-tolerant plants might tolerate the boron essentiality with the same mechanism and transportation with the same way as of sugar alcohols. However, non-sugar alcohol producing plants can transport boron preferentially to young tissues as observed in *Arabidopsis* (Noguchi et al. 2000), *Brasica napus* (Stangoulis et al. 2001), and *Helianthus annuus* (Matoh and Ochiai 2005) in case of the limited boron exposures (Tanaka and Fujiwara 2008). It is proposed that non-sugar alcohol producing plants have to activate different mechanism to translocate

boron into the young portions of the plants. Boron transporters and channels may be involved in this translocation (Noguchi et al. 2000). Also Tanaka and Fujiwara (2008) hypothesized that plants are capable of sensing boron levels and regulate the transport under limited conditions.

5 Boron Pollution

In recent years, there has been a great increase in the use of boron at the industrial level as well as water desalination processes for healthy irrigation. The mining processes lead to a dramatic increase in the accumulation of boron in agricultural soils (Parks and Edwards 2005). The arid and semiarid regions are potentially having risk with boron toxicity, due to capillary action and evaporation of boron rich ground waters. Under these circumstances boron concentration reaches to a toxic level for plants and reduces crop yields by polluting agriculrural areas (Tanaka and Fujiwara 2008).

Turkey is the important producer of naturally occurring borax fertilizers (Norman 1998). More than 50% of the world boron reserves are found in Turkey (Roskill 1999; Kalafatoglu and Ors 2000). It has become an important and strategic element in terms of developing technologies (Kose et al. 2003; Oren et al. 2006).The proven reserves are 375 million tons, whereas possible reserves are 483 million tons. This is equivalent to the 72.2% of the world reserves (Bayca et al. 2008). These are found in Susurluk, Bigadiç, Sindirgi regions of Balikesir (Fig. 13.2), Kestelek

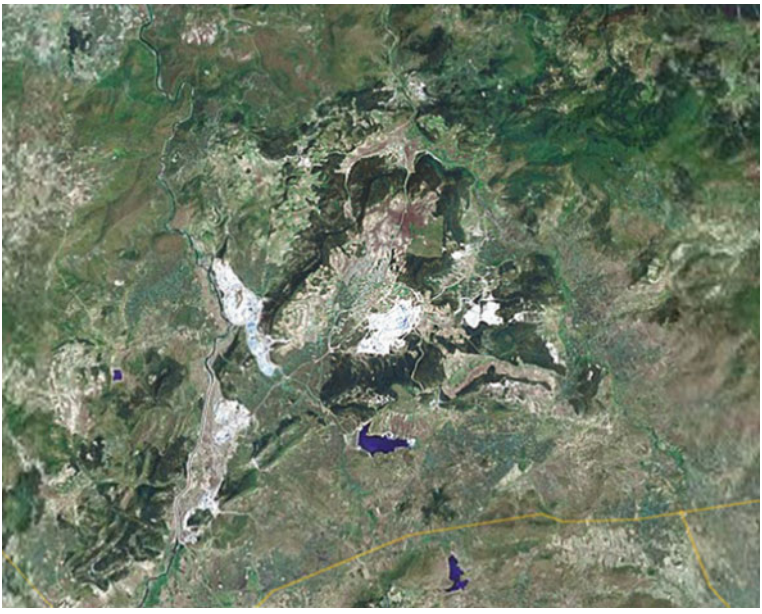


Fig. 13.2 Setallite images of Boron mines in Bigadiç, Balikesir (*White spots* indicate boron mines)

District of Bursa, Emet District of Kütahya and Kirka District of Eskisehir. The largest reserves are found in Emet, Bigadiç, Kirka and Mustafakemalpaşa Districts (72% of the world boron reserves). These are located in an area of 100 × 200 km^2. Mines are situated alongside the drainage areas of Simav and M. Kemalpasa rivers. During the mining processes, boron containing drainage waters, cause pollution of Simav Creek, which is used for the irrigation of nearly 40,000 ha of agricultural area in Balikesir, Kepsut, Susurluk and Karacabey plains (Şener and Özkara 1989; Uygan and Çetin 2004). The boron carried by the Simav Creek is over 2 mg L^{-1} and threatens the fertile agricultural soils (Şener and Özkara 1989). Watery wastes from the mining areas in general contain 14–18% B_2O_3 which flows in to the collection ponds (Kose et al. 2003). A total of 60.000 tons of wastes are produced every year from the boron extraction mining areas (Batar et al. 2009). The boron concentration in the collection ponds is above the limits given by WHO (Oren et al. 2006). Some work has been done to purify these wastewaters (Kalafatoglu et al. 1997). Very few studies have been carried out on the soil-plant interactions in relation to boron in Turkey. Dündar and Çepel (1979) have reported harmful effects of boron on the leaves of some species in the forest vegetation around Emet (Kütahya) Borax Production Plant. Through the wastewaters of the river Simav the boron is spread to a wide area and causes boron pollution in agricultural soils of this area, rendering the soil infertile (Önel 1981).

Especially in the areas around the boron reserves in Turkey industrialization and urbanization have developed dramatically and this pollution can be seen intensively. The wastewater with a high boron content flowing into the rivers like Simav adversely affects the agricultural areas in the region (Şener and Özkara 1989). The washing waters, rich in boron which are released from boron mines are collected in the Çamköy Dam (Fig. 13.3). However, other waters rich in boron from inactive

Fig. 13.3 The wastewater from the Boron mines flown into the Çamköy Collector Dam

Fig. 13.4 Boron mines which are not used but cause environmental pollution through rain and underground waters

and closed boron mines are flown into the river Simav which reach the agricultural areas through rain as well as underground waters (Fig. 13.4).

According to Uslu and Türkmen (1987) boron levels recommended for permanent usage should be up to 0.75 mg L^{-1}, and 2 mg L^{-1} for short term usage. The samples taken from Simav Creek and its environs in Bigadiçshowed boron levels as 22.56 (open mine surface water); 22.85 (Çamköy Dam water); 23.07 (water taken after ore washing); 23.07 (water from collected pools); 11.35 (water from Simav River); 1.64 (water from the Simav River-500 m away from the mine); and 16.89 mg L^{-1} (open mine surface water). Soils associated with these reserves are high in boron and host a plant diversity with tolerance to high levels of boron.

The natural plant cover of the boron mining areas around Kirka–Eskişehir is represented by the taxa like (Türe and Bell 2004); *Gypsophila perfoliata* L. var. *Perfoliata. Catapodium rigidum* (L.) C.E. Hubbard ex Dony subsp. *rigidum* var. *rigidum; Juniperus oxycedrus* L. subsp. *oxycedrus; Adonis flammea* Jacq.; *Glaucium leiocarpum* Boiss.; *Papaver rhoeas* L.; *Hypecoum imberbe* Sibth. & Sm.; *Alyssum pateri* Nyâr. subsp. *pateri*; *Reseda lutea* L. var. *lutea*; *Chenopodium album* L. subsp. *album* var. *album*; *Melilotus officinalis* (L.) Desr.; *Medicago sativa* L. subsp. *sativa*; *Potentilla recta* L.; *Carduus nutans* L. subsp. nutans; *Centaurea solstitialis* L. subsp. *solstitialis*; *Centaurea depressa* Bieb.; *Centaurea virgata* Lam.; *Tragopogon latifolius* Boiss. var. *angustifolius* Boiss.; *Convolvulus lineatus* L.; *Quercus trojana* P. B. Webb. T; *Galium verum* L. subsp. *verum*; *Allium atroviolaceum* Boiss.; *Aegilops cylindrica* Host.; *Aegilops triuncialis* L. subsp. *triuncialis*; *Hordeum distichon* L.; *Hordeum murinum* L. subsp. *leporinum* (Link) Arc. var. *leporinum*: *Chrysopogon gryllus* (L.) Trin; *Stipa lessingiana* Trin. & Rupr.; *Pinus nigra* Arn. subsp. *pallasiana* (Lamb.) Holmboe; *Neslia apiculata* Fisch.; *Matthiola longipetala* (Vent.) DC. subsp. *longipetala*; *Helianthemum canum* (L.)

Baumg.; *Polygala pruinosa* Boiss. subsp. *pruinosa*; *Dianthus crinitus* Sm. var. *crinitus*; *Paronychia carica* Chaudhri; *Hypericum avicularifolium* Jaib. & Spach. subsp. *depilatum*; *Linum hirsutum* L. subsp. *anatolicum* (Boiss.) Hayek var. *anatolicum*; *Haplophyllum thesioides* (Fisch. ex DC.) G. Don; *Genista aucheri* Boiss.; *Astragalus vulneraria* DC.; *Coronilla varia* L. subsp. *varia*; *Onobrychis gracilis* Besser; *Sanguisorba minor* Scop. subsp. *muricata* (Spach.) Briq.; *Sedum sartorianum* Boiss. subsp. *sartorianum*; *Eryngium campestre* L. var. *virens* Link.; *Morina persica* L.; *Scabiosa argentea* L.; *Anthemis tinctoria* L. var. *pallida* DC.; *Achillea wilhelmsii* C. Koch.; *Onopordum tauricum* Willd.; *Jurinea consanguinea* DC.; *Centaurea urvillei* DC. subsp. *stepposa* Wagenitz; *Leontodon asperrimus* (Willd.) J. Ball.; *Asyneuma limonifolium* (L.) Janchen subsp. *limonifolium*; *Asyneuma virgatum* (Labill.) Bornm. subsp. *virgatum*; *Onosma bracteosum* Hausskn. & Bornm.; *Anchusa officinalis* L.; *Anchusa stylosa* Bieb.; *Convolvulus compactus* Boiss.; *Convolvulus holosericeus* Bieb. subsp. *holosericeus*; *Lappula barbata* (Bieb.) Gürke; *Linaria corifolia* Desf.; *Orobanche alba* Stephan; *Acanthus hirsutus* Boiss.; *Globularia orientalis* L.; *Teucrium chamaedrys* L. subsp. *chamaedrys*; *Teucrium polium* L.; *Scutellaria orientalis* L. subsp. *pinnatifida* Edmonson; *Phlomis armeniaca* Willd.; *Marrubium parviflorum* Fisch. & Mey. subsp. *parviflorum*; *Sideritis montana* L. subsp. *montana*; *Stachys byzantina* C: Koch; *Thymus leucostomus* Hausskn. & Velen var. *argillaceus* Jalas; *Salvia sclarea* L.; *Salvia cryptantha* Montbret & Aucher ex Bentham; *Acantholimon acerosum* (Willd.) Boiss. var. *acerosum*; *Plantago lanceolata* L.; *Euphorbia macroclada* Boiss.; *Quercus pubescens* Willd.; *Cruciata taurica* (Pallas ex Willd.) Ehrend.; *Asphodelina damascena* (Boiss.) Baker subsp. *damascena*; *Muscari neglectum* Guss.; *Koeleria cristata* (L.) Pers. and *Puccinella convoluta* (Homem.) P. Fourr.

The plant taxa recorded from Bigadiç, Balikesir are (present study);

Pinus nigra Arn.; *Juniperus oxycedrus* L. ssp. *oxycedrus; Delfinum peregynium; Amaranthus retroflexus* L.; *Chenopodium album* L. ssp. *album* var. *album; Polygonum lapathifolium L.; Polygonum aviculare L.; Polygonum equisetiforme* Sibth. & Sm; *Rumex Pulcher* L.; *Quercus ilex* L.; *Quercus pubescens* Willd.; *Silene otites; Lavatera punctata; Tamarix sp.; Sinapis arvensis L.; Neslia Apiculata* Fisch.; *Reseda lutea* L.*; Anagallis aquatica; Rosa canina* L.; *Malus sylvestris* miller ssp. *orientalis (*A. Uglitzkich*) Browicz* var. *orientalis; Crateagus monogyna* Jacq. *ssp. monogyna; Spartium junceum* L.; *Trifolium angustifolium* L. var. *angustifolium; Trifolium hybridum* L. var. *hybridum; Ononis spinosa; Lythrum salicoria* L.; *Pistacia terebinthus* L. ssp. *terebinthus; Pistacia* vera; *Ruta montana* (L.) L.; *Tribulus terrestris* L.; *Linum bienne* Miller; *Eryngium campestre L. var. visens; Eryngium creticum; Bupleurum odontites; Ammi visagna; Bupleurum tenuissimum; Papaver rhoeas* L.; *Olea Europea* L. var. *europea; Phillyrea latifolia* L.; *Solanum nigrum.* L. ssp. *nigrum; Convolvulus arvensis* L.; *Ballota nigra* ssp. *anatolica; Mentha spicata* ssp. *spicata; Stachys byzantina; Teucrium polii; Thymbra spicata; Plantago major* L.; *Plantago lanceolata* L.; *Rubia tinctorum* L.; *Paliurus spina–christi; Viscum album; Osyris alba; Scabiosa columbaria* L. ssp *columbaria* var. *Columbaria; Dipsacus laciniata; Xanthium spinosum* L.; *Pallenis spinosa* (L.) Cass.; *Picnomon acarna* (L.) Cass.; *Carduus nutans* L.; *Centaurea solstitialis* L. ssp. *solstitialis; Centaurea ibericaTrev.* ex Sprengel; *Centaurea virgata; Cardopatium*

corymbosum (L.) Pers.; *Echinops ritro* L.; *Scolymus hispanicus* L.; *Cichorium intybus* L.; *Picris altissima* Delile; *Helminthotheca echinoides* (L.) Holub; *Carthamus Lanatus; Xeranthemum annuum; Hordeum murium* L.; *Hordeum bulbosum* L.; *Lolium perenne* L.; *Dactylis glomerata* L.; *Cynosurus echinatus* L.; *Phragmites australis* (Cav.) Trin. ex Steudel; *Cynodon dactylon* (L.) Pers.; *Elymus elongatus* ssp. *eloggatus; Juncus conglomeratus; Cyperus longus* L.; *Draculus vulgaris; Ruscus aculeatus* L. var. *angustifolius* Boiss.; *Asparagus acutifolius* L.; *Asphodelus aestivus* Brot.; *Allium neapolitanum* Cyr. and *Tamus communis* L. ssp. *communis.*

The plant diversity of the areas shows variation depending upon the boron content of the soils. The soils with lower boron concentrations (0.1–2 mg kg^{-1}) show a rich species diversity (84 species), whereas those with higher levels (10 mg kg^{-1}) are poor in the plant cover (28 species). According to Babaoglu et al. (2004) only five species *Catapodium rigidum* ssp. *rigidum* var. *rigidum* and *Gypsophila perfoliata* var. *perfoliata* show resistance to boron levels in excess of the accepted toxic levels (35 mg kg^{-1}); these species are reported to flourish in the zone with highest boron concentration. Our investigations revealed that in Bigadiç, Balikesir boron mining area *Polygonum equisetiforme* was tolerating high levels of boron.

6 Phytoremediation

Plants which uptake high levels of an element from the soil are called hyperaccumulators; these are now being closely investigated, both by molecular techniques and by soil/plant analyses, at the sites where they occur (Karenlampi et al. 2000). The term hyperaccumulator was first used in relation to plants containing more than 1000 $\mu g\ g^{-1}$ (0.1%) Ni in dry tissue (Jaffre et al. 1976; Brooks et al. 1977). A later publication (Baker and Brooks 1989) extended the use of the term to include plants containing more than 1% Zn or Mn, or more than 0.1% Cu, Co, Cr and Pb. The ability of *Thlaspi caerulescens* to accumulate Zn to more than 10,000 $\mu g\ g^{-1}$ (1%) in dry tissue has been known since the 1860s, but it has become apparent from more recent work that several species of this genus can also hyperaccumulate (Reeves and Brooks 1983; Reeves 1988) from metal-rich soils and can hyperaccumulate a wider variety of metals (including Cd, Mn and Co) from amended nutrient solutions (Baker et al. 1994). There has also been recent interest in high-Cd populations of *T. caerulescens* from mine soils (Robinson et al. 1998; Reeves et al. 2001). A recent study of hyperaccumulators for some metals (Zn, Cd, Pb, Ni, Cu, Se and Mn) has been published (Reeves and Baker 2000). This list did not include several other elements, such as B, As and Al. As accumulation by ferns has been studied by Ma et al. (2001), and also Kochian et al. (2002) reported a plant which accumulates 3000 mg kg^{-1} Al, nevertheless there is not much information about boron accumulation in plants.

Recently, Gezgin et al. (2002) surveyed the boron content of 898 soil samples from 7 States in Turkey. These States include 3.5 million ha of cultivated land in Central Southern Anatolia. However, nearly 50% of soils in these areas contained low levels of available boron which can be corrected by external boron applications

in the form of borax or boric acid. However, another 18% of soils contain boron at more than the critical upper level for available soil born, which is considered to be 3 mg kg^{-1} (Keren and Bingham 1985) for most crops. These areas can be released from this abiotic stress by phytoremediation using boron accumulating species. Soil amendments by conventional techniques such as leaching or increasing pH by liming (Nable et al. 1997) for increased boron adsorption on soil seem not to suit Central Anatolian conditions due to its low annual rainfall and water shortages, and the high lime content of the soils. For this reason, boron accumulating species appear as a solution to this problem.

First hyperaccumulation studies of boron in Turkey were undertaken by Babaoglu et al. (2004) on different taxa of *Gypsophila sp*. commonly growing on the boron rich areas around Kirka, Eskisehir–Turkiye. *Gypsophila sphaerocephala* var. *sphaerocephala*, *G. perfoliata*, *Puccinellia* ssp. *distans* and *Elymus elongatus* ssp. *turcicus* species were found in the highest boron containing sections of the mine. Out of these species, *G. sphaerocephala* was able to accumulate extraordinarily high concentrations of boron (Babaoglu et al. 2004). The species were found growing successfully under high total (8900 mg kg^{-1}) and available (277 mg kg^{-1}) soil boron concentrations. *G. sphaerocephala* contained considerably higher boron concentrations in its above-ground parts (2093 $\pm$ 199 SD mg kg^{-1}, seeds; 3345 $\pm$ 341 SD mg kg^{-1}, leaves), compared to the roots (51 $\pm$ 11 SD mg kg^{-1}) and organs of the other species.

We also determined a boron tolerant species during our studies undertaken during 2000–2003 namely; *Polygonum equisetiforme*, which showed luxuriant growth over boron mining areas in the Balikesir region. It appears to us as one of the candidates as for phytoremediation of boron contaminated soils. It is a perennial deciduous taxon, with procumbent to erect stems, up to 100 cm tall, and few flowering shoots bearing pink or white flowers and distributed in Canakkale, Istanbul, Izmir, Antalya, İçel and Gaziantep. Water samples were taken from waste water of the collecting dam as well as Simav creek near the mining area.

The samples were collected around the Etibor mining area of Bigadic, Balikesir, one of the richest boron mines in the world. Plant samples along with their representative soils (0–50 cm deep) were collected from the area. Samples of surface soils were collected from pits measuring 20 $\times$ 20 $\times$ 20 cm.

All samples were put into plastic bags and directly brought to the laboratory for analyses. The plant samples were carefully washed with water to remove any traces of soil, then oven-dried at 70°C for 48 h before measuring dry weights. Samples (0.5 g) of finely ground plant material were digested with concentrated HNO_3 in a microwave system (CEM). Boron in the extracts was analyzed by ICP–AES (Varian-Vista model) (Nyomora et al. 1997) in at least 4 plant samples with 3 replicates. The boron standard used was from Merck, Germany. The extractable boron concentrations in soil were determined according to the method of Cartwright et al. (1984) by extraction with 0.01 M mannitol plus 0.01 M $CaCl_2$ using a soil solution ratio of 1:5 and a shaking time of 16 h. Boron extracted was determined by ICP–AES (Bingham 1982). The results of boron content of the soils and plants from the sampling sites is presented in Table 13.1.

Table 13.1 Boron content of the soils and plants from the sampling sites

Sampling Sites	Boron content (ppm)			
No	Soil B	SD	Plant B	SD
1	6.84	0.56	150.22	2.52
2	6.80	0.38	112.26	1.81
3	6.91	1.05	156.44	3.14
4	6.96	0.95	155.29	2.52
5	6.91	0.35	144.54	1.96
6	6.95	0.46	150.36	4.13
7	6.87	0.39	146.89	2.69
8	6.78	0.45	147.99	3.41
9	6.84	0.78	151.53	2.48
10	6.79	0.95	160.15	1.82
11	6.85	0.16	154.64	2.74
12	6.81	1.05	156.02	2.61
13	1.39	0.12	146.36	1.94
14	6.81	0.35	146.24	1.30
15	6.81	0.42	153.14	2.28
16	1.48	0.08	145.35	1.28

7 Boron and Seed Germination

The studies undertaken by us on the germination behavior of bean, chickpea, maize, wheat, barley and tomato revealed that there is a significant difference ($p < 0.001$) between control and 1000 mg L^{-1} boron exposure of seeds. The growth rates and measurements of radicle and plumule lengths were calculated for all crop seedlings in response to different boron concentrations (control, 10, 50, 100, 250, 500, 750, 1000 mg L^{-1}) and hormones (10 mg L^{-1} GA_3, IAA, ABA, KIN). After seven days of germination, bean root length was 8.6 cm in control. It decreased to 7.1 cm at 10 mg L^{-1}, and increased to 9.2 cm at 50 mg L^{-1} boron. However, the length of radicle decreased gradually to 1.05 cm at the concentrations above 50 mg L^{-1}. The length of plumule was 11.6 cm in control, but decreased gradually to 2.4 cm for increasing boron concentrations (Fig. 13.5).

The chickpea radicle length was 3.5 cm in control and decreased to 1.8 cm at 10 mg L^{-1} boron, but increased to 8.8 cm at 50, 100 mg L^{-1}. For other concentrations, the radicle length decreased gradually to 1.61 cm. The plumule length was 1.8 cm in control but increased to 3.4 cm at 10, 50, 100 mg L^{-1} boron and decreased gradually to 0.5 cm for other concentrations (Fig. 13.6).

The maize radicle length was 20 cm in control. It decreased to 7.5 cm at 10 mg L^{-1} boron, and increased to 13.7 cm at 50, 100 mg L^{-1}. For other concentrations, the radicle length decreased gradually to 2.1 cm. The plumule length was 7.5 cm in control and decreased gradually to 2.1 cm as the boron concentrations increased (Fig. 13.7).

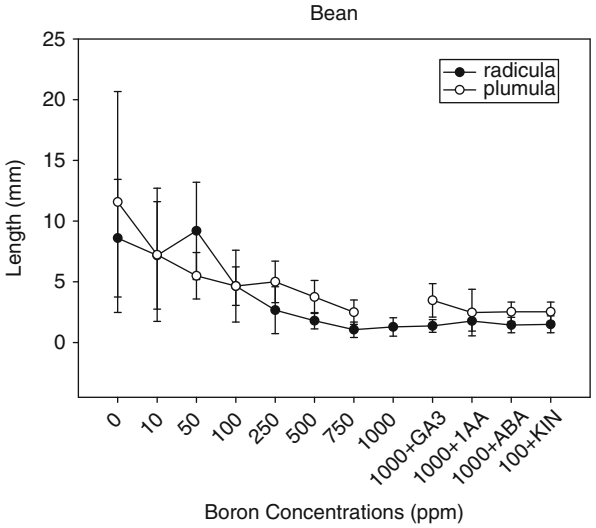


Fig. 13.5 Radicle and plumule length of bean seedlings under different boron concentrations and plant hormones

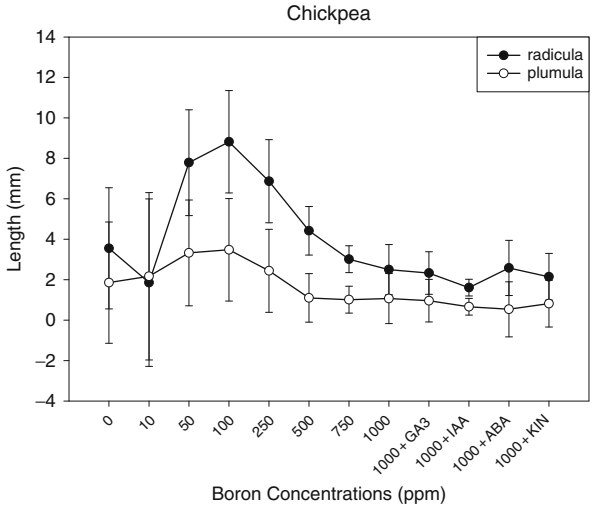


Fig. 13.6 Radicle and plumule length of chickpea seedlings under different boron concentrations and plant hormones

The wheat radicle length was 11 cm in control, increased to 13.2 cm at 10 mg L^{-1} boron and decreased gradually to 1.4 cm at 50, 100 250, 500, 750, 1000 mg L^{-1} and GA_3, but increased to 6.7 cm under IAA, ABA and KIN exposures. The plumule length was 11.3 cm in control. It decreased gradually to 5.6 cm for different boron concentrations (Fig. 13.8).

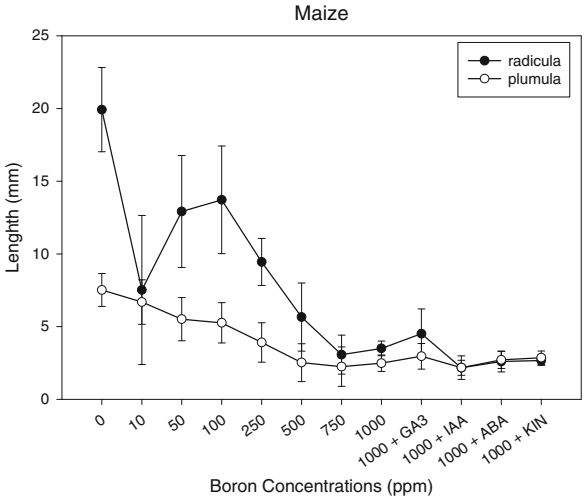


Fig. 13.7 Radicle and plumule length of maize seedlings under different boron concentrations and plant hormones

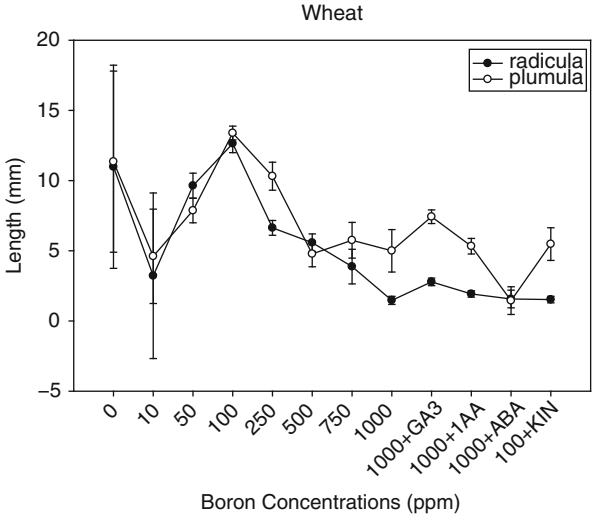


Fig. 13.8 Radicle and plumule length of wheat seedlings under different boron concentrations and plant hormones

The barley radicle length was 14.7 cm in control, increased to 15.4 cm at 10 mg L^{-1} boron, but decreased gradually to 0.9 cm at other concentrations. The plumule length was 8.2 cm in control. It increased to 8.6 cm at 10 mg L^{-1}, but decreased gradually to 3.5 cm at 50, 100, 250, 500, 750 mg L^{-1} of boron. It abruptly increased to 7.1 cm at GA_3, it decreased gradually to 1.3 cm under IAA, ABA exposures and abruptly increased to 3.72 cm with KIN (Fig. 13.9).

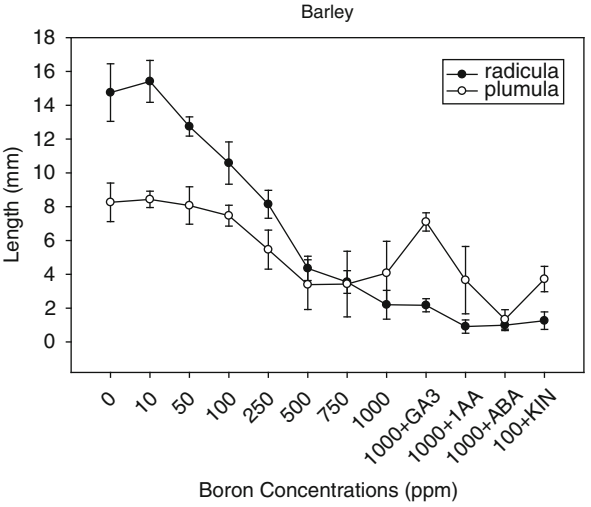


Fig. 13.9 Radicle and plumule length of barley seedlings under different boron concentrations and plant hormones

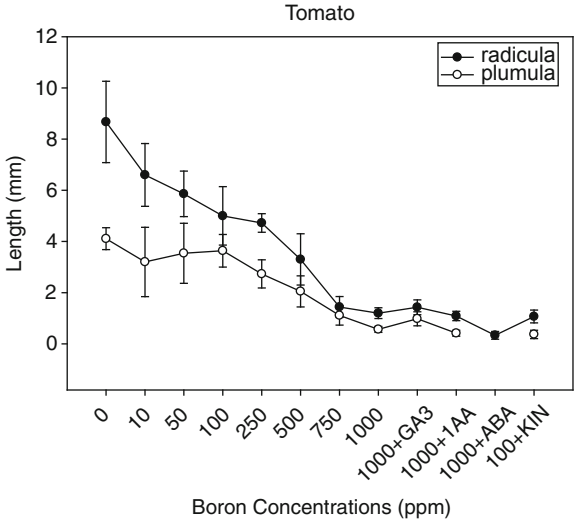


Fig. 13.10 Radicle and plumule length of tomato seedlings under different boron concentrations and plant hormones

The tomato radicle length was 8.7 cm in control. For the following concentrations it decreased gradually to 0.33 cm. The plumule length was 4.1 cm in control and decreased gradually to 0.3 cm under all concentrations (Fig. 13.10).

After seven days of varying amounts of boron and hormone applications, at 50 mg L^{-1} germination inhibitory rate in beans was calculated as 7%, at other concentrations it decreased gradually from (−) 19 to (–) 86 ($p < 0.001$). A highly significant correlation was observed between boron concentrations and inhibitory rates. At 10 mg L^{-1} GA_3, IAA, ABA and KIN applications the inhibitory

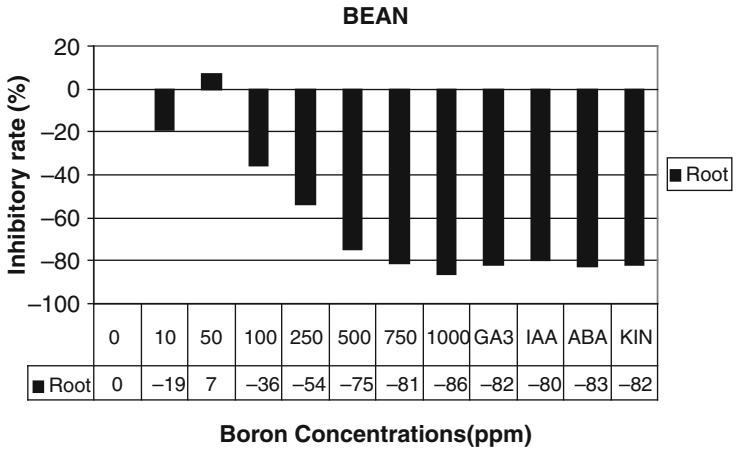


Fig. 13.11 Comparison of radicle growth inhibition in bean seedlings under different boron concentrations

rate was calculated as (–) 86, (–) 82, (–) 80, (–) 83, (–) 82% respectively (Fig. 13.11).

The germination inhibitory rate of chickpea was calculated as 54, 60, 49, 20% at 50, 100, 250, 500 mg L^{-1} boron exposures respectively. It decreased gradually from (−) 23% to (−) 91% at other concentrations ($p < 0.05$) (Fig. 13.12). The germination inhibitory rate of maize was calculated as 10% at 50 mg L^{-1} boron but decreased gradually from (−) 9 to (−) 90 ($p < 0001$) (Fig. 13.13). The germination inhibitory rate of wheat was calculated as 13% at 10 mg L^{-1} boron and other concentrations (50, 100, 250, 500, 750, 1000 mg L^{-1} boron and GA_3) but decreased gradually from (−) 13% to (−) 87% ($p < 0.001$). With IAA, ABA and KIN inhibitory rate of germination in wheat was calculated as 17.5, 14.2, 13.8% respectively ($p < 0.05$) (Fig. 13.14).

The germination inhibitory rate of barley was calculated as 4.4% at 10 mg L^{-1} boron and the other concentrations decreased gradually from 14 to (–) 94% ($p < 0.001$) (Fig. 13.15). The germination inhibitory rate of tomato decreased gradually at all concentrations from (−) 31 to (−) 100% ($p < 0.001$) (Fig. 13.16).

The results confirmed that boron is indeed an essential micronutrient element (at 10 and 50 mg L^{-1} concentrations) but when it is in excess it is toxic for plants as (Kocacaliskan and Olcer 2006; Konuk et al. 2007). GA_3, IAA, ABA and KIN did not alleviate the boron induced growth inhibition effect significantly.

8 Boron and Genotoxicity in Plants

Plant mutagenicity bioassays have been in existence for many years. The plant bioassays are now well-established systems, used for screening and monitoring of

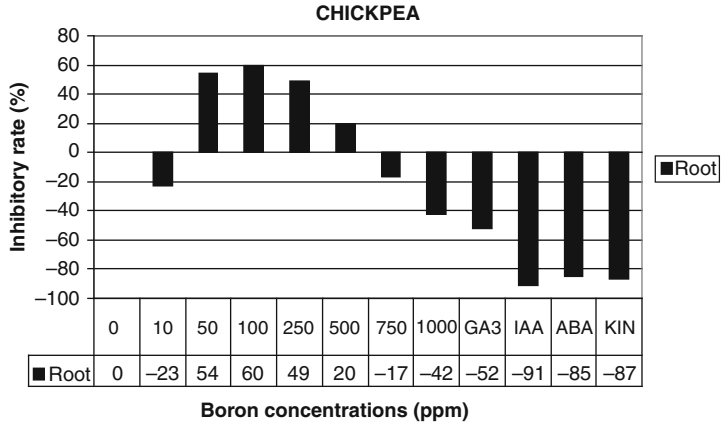


Fig. 13.12 Comparison of radicle growth inhibition in chickpea seedlings under different boron concentrations

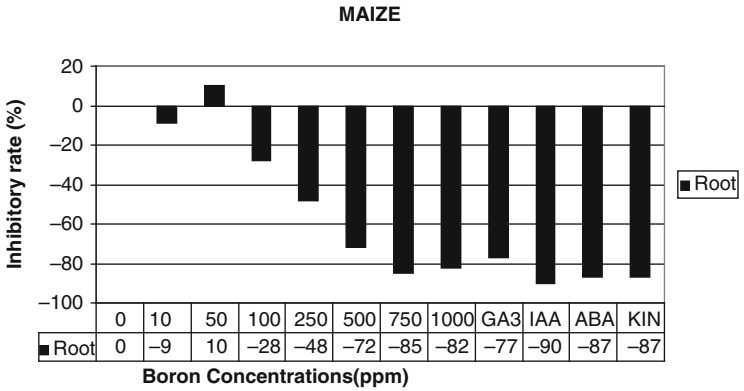


Fig. 13.13 Comparison of radicle growth inhibition in maize seedlings under different boron concentrations

environmental chemicals with mutagenic and carcinogenic potential (Knasmuller et al. 1998; Ma 1999). Genotoxicity of environmental exposures is hard to elucidate by one-way approaches, but requires multi-step methods, both deductive and inductive, at the same environmental design. Most higher plant bioassays are based on the detection of chromosomal aberrations, sister chromatid exchanges, and recently, on the analysis of DNA strand breaks. The cytogenetic tests analyze the frequency and type of chromosome aberrations in mitotic cells and the frequency of micronuclei in interphase cells (Uhl et al. 2003). Several studies have used the comet assay, micronucleus assay or chromosome aberration assay to measure the genotoxic effect of metals on plants (Steinkellner et al. 1999; Angelis et al. 2000). The advantages of measuring effects of genotoxic chemicals directly on DNA are mainly related to the sensitivity and short response time. The advances in molecular biology have led to

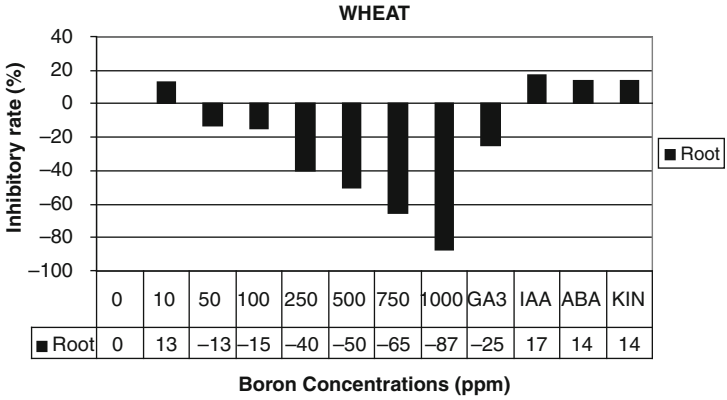


Fig. 13.14 Comparison of radicle growth inhibition in wheat seedlings under different boron concentrations

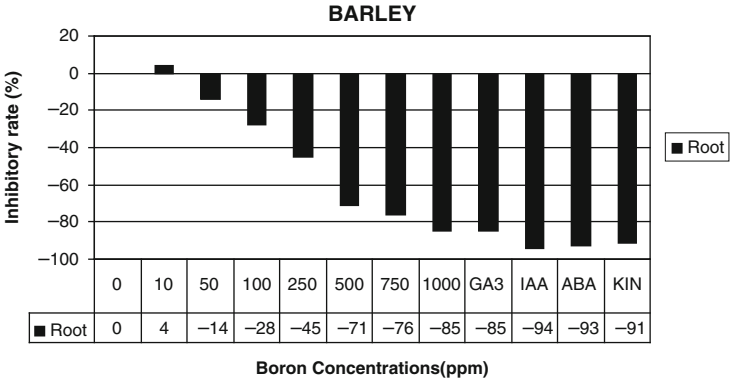


Fig. 13.15 Comparison of radicle growth inhibition in barley seedlings under different boron concentrations

the development of a number of selective and sensitive assays for DNA analysis in the field of genotoxicology. RAPD, developed by Williams et al. (1990) and Welsh and McClelland (1990), is a PCR-based technique that amplifies DNA fragments of genomic DNA with single short primers of arbitrary nucleotide sequence under low annealing conditions. This technique is used extensively for species classification, genetic mapping and phylogeny etc. In addition, their use in surveying genomic DNA for evidence of various types of DNA damage and mutation shows that RAPD may potentially form the basis of novel biomarker assays for the detection of DNA damage and mutational events in cells of bacteria, plants, invertebrate and vertebrate animals (Savva 1996; Savva 1998; Atienzar et al. 2000). RAPD assay has proved useful to detect genomic instability manifested such as point mutations, genetic and chromosomal rearrangements, deletion and insertions (Liu et al. 2005, 2007). Mutations can only be responsible for the appearance of new bands if they occur

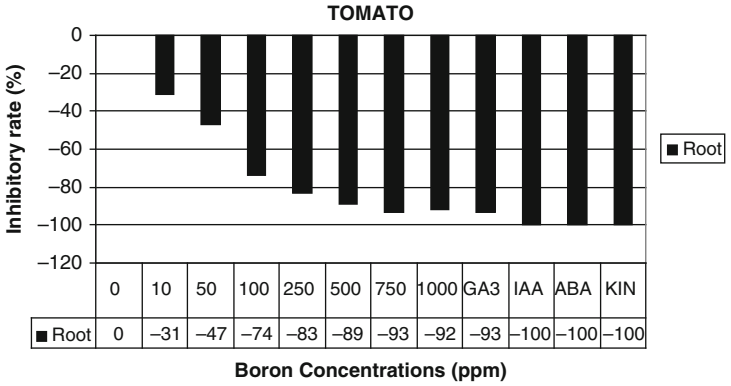


Fig. 13.16 Comparison of radicle growth inhibition in tomato seedlings under different boron concentrations

at the same locus in a sufficient number of cells (a minimum of 2% of mutations may be required to get a new PCR product visible on agarose gel) to be amplified by PCR. RAPD is likely to detect genomic instability as the newly growing and developing cells will produce a clone of dividing daughter cells. Thus the proportion of cells presenting the same genomic instability is high and easy to detect. In the field of genetic toxicology most RAPD studies describe changes such as differences in band intensity as well as a gain/loss of RAPD bands, defined as diagnostic RAPD.

Boron can result in the physiological and metabolic problems related to genotoxicity thus limiting crop productivity. In some recent studies the genetic and epigenetic aspects of boron toxicity have been evaluated together with a reference to the mitotic index in some plant species where mitotic abnormalities have been recorded (Papadakis et al. 2004; Konuk et al. 2007). Konuk et al. (2007) has reported that boron inhibits mitosis in *Allium cepa* at doses of 100 mg L^{-1} and above. However, according to Karabal et al. (2003) and Cervilla et al. (2007) although boron causes oxidative damage, but its genotoxic effect is still unclear. In some recent studies, leaf cupping, a specific visible symptom of boron toxicity in some species, has been suggested to result from inhibition of cell wall expansion, through disturbance of cell wall cross-links (Loomis and Durst 1992). The nutritional importance and toxic effects of boron on plant growth have been investigated at length in different maize cultivars (Goldberg et al. 2003). These studies revealed that in general boron tolerance of cultivars varied from high to low and boron concentrations of low tolerant cultivars were higher than those of high boron tolerant cultivars. A considerable genotypic variation in susceptibility to boron toxicity has been identified for agronomic species like wheat and barley (Nable and Paull 1991; Paull et al. 1992). Donghua et al. (2000) investigated the effects of boron ions on root growth and cell division of broadbean. The results indicated that boric acid has a stimulatory effect on root growth at concentrations of 10^{-6} and 10^{-3} M, and

an inhibitory effect at higher concentrations. Boric acid has toxic effects on the root tip cells during mitosis, forming chromosome bridges, chromosome fragments, chromosome stickiness, and micronuclei. Ayvaz (2002) investigated the genotoxic effects of 500, 750 and 1000 mg L^{-1} boron concentrations on barley. He recorded the germination percentage, root length, mitotic index and mitotic abnormalities. These findings point out that a decrease in the mitotic index level is due to mitodepressive effect which leads to an inhibition of cell access to mitosis, stressing the fact that boron disrupts the normal cell cycle process by preventing biosynthesis of DNA and microtubule formation.

During oxidative stress, the excess production of reactive oxygen species (ROS) causes membrane damage that eventually leads to cell death. As in most ionic stresses, toxic levels of boron cause the formation of ROS. Karabal et al. (2003) observed in barley cultivars that its toxicity induced oxidative and membrane damage in leaves. Recently it has been reported in apple and grapevine that boron toxicity induces oxidative damage by lipid peroxidation and hydrogen peroxide accumulation (Molassiotis et al. 2006; Gunes et al. 2006). Cervilla et al. (2007) too found that high boron concentration in the culture medium provokes oxidative damage in tomato leaves and induces a general increase in antioxidant enzyme activity, in particular increasing ascorbate pool size. It also increases the activity of L-galactose dehydrogenase, an enzyme involved in ascorbate biosynthesis, and the activity of enzymes of the Halliwell-Asada cycle. This work therefore provides a starting point towards a better understanding of the role of ascorbate in the plant response against boron stress.

Takano et al. (2005) demonstrated that boron regulated endocytosis and degradation of BOR1, a plasma membrane transporter for boron in plant. They monitored BOR1 activity and protein accumulations in response to various boron doses. They found that the posttranscriptional regulation was a major regulatory mechanism in this connection. Their findings proved that endocytosis and degradation of BOR1 are regulated by B availability in order to avoid accumulation of toxic levels of boron in shoots under high-boron supply, while protecting the shoot from boron deficiency under limited boron supply.

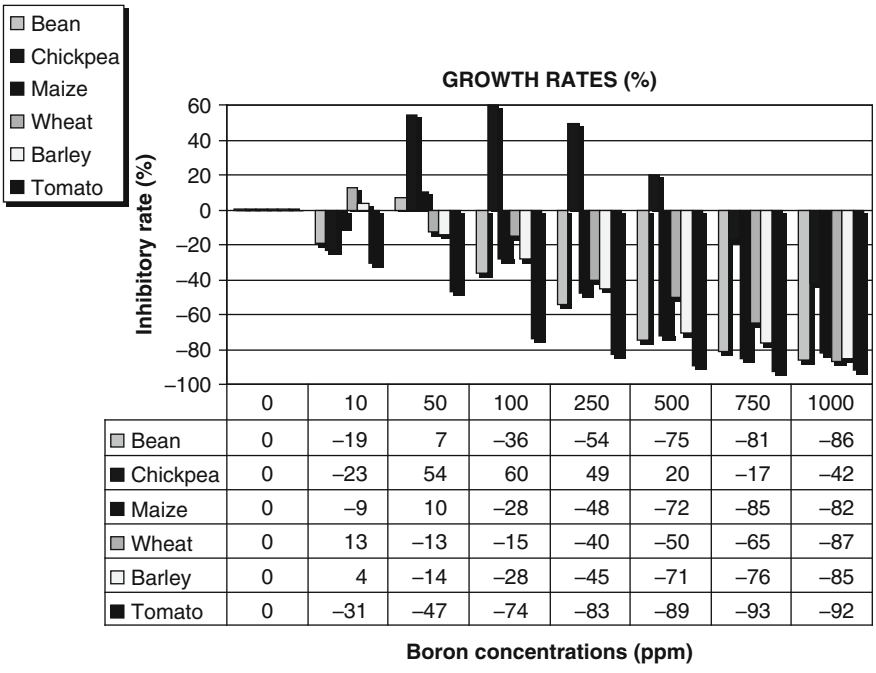
9 Conclusion

In conclusion this overview on the interrelations of plants and boron stresses the following points; using plants for phytoremediation should possess (a) targeted metal(s) accumulating capability, preferably in aerial parts; (b) tolerance to the accumulated metal concentrations; (c) fast growth of the metal accumulating biomass; and (d) ease of cultivation and harvesting (Baker and Brooks 1989).

This study has also revealed that the boron concentrations in plants are 20 times more than in the soils around Bigadiç-Balikesir. *Polygonum equisetiforme* appears as a hyperaccumulator of boron. Its wide distribution in the region implies that it can be used for restoration of desertified agricultural lands. Biochemical and molecular studies on this plant will enlighten the mechanisms of growth of hyper-boron

accumulating species on boron rich soils. These findings can be used in the molecular and genetic studies in agricultural plants. This study stresses the fact that this plant can be used to evaluate the boron polluted agricultural soils irrigated by Simav stream which contains high boron levels. In this way more than 3 million ha of boron polluted soils can be again used for agricultural productivity. At the same time it can be used as a fertilizer in the boron poor soils.

Germination results indicate that some of the plants show sensitivity and some are tolerant. For example; in bean the inhibitory rate is (−) 19% at 10 mg L^{-1} boron whereas it is (−) 86% at 1000 mg L^{-1}, indicating its sensitivity. In chickpea the inhibitory rate was (−) 23% at 10 mg L^{-1} boron and (−) 42% at 1000 mg L^{-1}, depicting a high tolerance. Our data confirms the fact that maize is a semi−tolerant species. The inhibitory rate of maize is (−) 9% at 10 mg L^{-1} boron but (−) 82% at 1000 mg L^{-1}. Barley has been reported as a semi tolerant species (Maas 1987) but in our studies it appears percent at 1000 mg L^{-1}. Wheat also has been recorded as a sensitive species but it was reasonably tolerant and growth rate was 13% at 10 mg L^{-1} boron and (−) 87% at 1000 mg L^{-1}. Finally tomato was highly sensitive, the inhibitory rate was (−) 31% at 10 mg L^{-1} boron and (−) 92% at 1000 mg L^{-1} (Fig. 13.17). Bean and tomato are sensitive, maize is semi tolerant, chickpea, wheat and barley are tolerant species on the basis of germination results.



	0	10	50	100	250	500	750	1000
Bean	0	–19	7	–36	–54	–75	–81	–86
Chickpea	0	–23	54	60	49	20	–17	–42
Maize	0	–9	10	–28	–48	–72	–85	–82
Wheat	0	13	–13	–15	–40	–50	–65	–87
Barley	0	4	–14	–28	–45	–71	–76	–85
Tomato	0	–31	–47	–74	–83	–89	–93	–92

Fig. 13.17 Comparison of radicle growth inhibition in crop seedlings under different boron concentrations

Boron induced polymorphism is higher than many chemicals like mercury, chromium and zinc (Cenkci 2009). The RAPD-PCR method can be used as an investigational tool for boron induced genomic alterations. RAPD-PCR fingerprinting in conjugation with physiological parameters can be a powerful strategy for assessing boron exposure. OPA−08 primer is informative and may have great potential for detecting boron-induced specific genomic alterations, but the nature and amount of DNA impact in RAPD band can only be obtained by sequencing or probing (Atienzar and Jha 2006). Genomic targets of boron exposure should further be assessed with systematic sequencing to make RAPD-PCR assay a quantification method rather than a qualification method.

Changes in the boron-exposed maize genome observed in the present study is mainly variations in RAPD band intensity in the profiles. Short-term treatment with boron did not seem to induce many permanent genomic mutations or changes in oligonucleotide priming sites that would mainly produce new or result in lost RAPD bands. In this study the appearance of new PCR products was detected at 25 mg L^{-1} and at 50 mg L^{-1} respectively (Tables 13.2 and 13.3). Appearance of bands may be a result of the genomic instability related to DNA damage. These damages may be induced directly as seen in aflatoxins or indirectly as seen in oxidative stress (Risom et al. 2005). Many studies show that toxic levels of boron influence the excessive production of ROS in different plants (Cervilla et al. 2007; Ardic et al. 2009). Oxidative stress induces ROS production and may cause chromosomal aberrations and DNA damages (Martindale and Holbrook 2002; Risom et al. 2005). The potential for genotoxicity of boron comes either through the production of ROS via oxidative stress or toxicity determination parameters (Beddowes et al. 2003). The RAPD technique is promising for the detection of boron-induced DNA effects but requires further experimentation and validation. The first thing to evaluate should be the innate genetic variation of the organism and then the acquired and additional genotoxic factors.

Table 13.2 Permeability coefficient of boric acid on artificial and natural membranes, isolated from different species

Permeability coefficient of Boric acid	Organism	Reference
8×10^{-6} cm s^{-1}	Theoretical	Raven (1980)
4.9×10^{-6} cm s^{-1}	Artificial liposome consisting of phosphatidylcoline	Dordas and Brown (2000)
3.9×10^{-7} cm s^{-1}	Membranes isolated from Squash roots (*Cucurbita pepo*) − plasma membrane	Dordas et al. (2000)
2.4×10^{-8} cm s^{-1}	Membranes isolated from Squash roots (*Cucurbita pepo*) – plasma membrane deplated vesicles	Dordas et al. (2000)
4.4×10^{-7} cm s^{-1}	Plasma membrane of the giant internodal cells of charophyte alga *Chara coralline*	Stangoulis et al. (2001)

Table 13.3 Boron transporter–like protein encoding genes identified in different species

Organism	Genes	Locus identifier	Reference
Rice (Oryza sativa)	OsBor1	Os12g37840	
	OsBor2	Os01g08040	
	OsBor3	Os01g08020	
	OsBor4	Os05g08430	Takano et al. (2005)
Arabidopsis thaliana	AtBOR1	At2g47160	
	AtBOR2	At3g62270	
	AtBOR3	At3g06450	
	AtBOR4	At1g15460	
	AtBOR5	At1g74810	
	AtBOR6	At5g25430	
	AtBOR7	At4g32510	
	AtNIP6;1	At1g80760	Tanaka and Fujiwara (2008)
	AtNIP5;1	At4g10380	Takano et al. (2006)
Hordeum vulgare	HvBOR2-BOT1	LOC100127239	Reid et al. (2004); Sutton et al. (2007)
Triticum aestivum	TaBOR2	ABX26206	Zhao and Reithmeier (2001)
Physcomitrella patens	PpBOR1	EDQ69077	Shelp et al. (1998)
	PpBOR2	EDQ75588	Stangoulis et al. (2001)
Chlamydomonas reinhardtii	BOR1	EDP05760	Matoh and Ochiai (2005)
	Atr1	YML116W	Kaya et al. (2009)
Saccharomyces cerevisiae	BOR1	EDN62551	Takano et al. (2007)
Citrus macrophylla	Bor1	EF581174	Canon et al. (unpublished)
Homo sapiens	NaBC1	SLC4A11	Frommer and von Wiren (2002)

These results may suggest that short-term (1 week) boron treatment induces mainly DNA damage, which causes the specific RAPD band intensity to either increase or decrease. Although our results strongly suggest that boron-induced genomic DNA instability is reflected by the RAPD-PCR method, it is important to note the change of RAPD band patterns do not show a dose-dependent tendency to boron exposure. This might be explained with the short exposure time which may not be enough for the toxic effects to develop. The target tissue for the ultimate genotoxic effects of boron might not be the root tissue, that needs further work to clarify the target tissue of boron. Its concentrations in agricultural soils hardly exceed 1000 mg L^{-1}, however, the accumulation of boron in various plant species can even be above 2000 mg L^{-1} e.g., *Gypsophila sphaerocephala* (Babaoglu et al. 2004) accumulating in leaves. Further studies should focus on the correlation

between the accumulation of boron in indicator species and the target tissues of boron in comparison to genomic instability.

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Chapter 14
Potential for the Use of Rhizobacteria in the Sustainable Management of Contaminated Soils

Vincenza Andreoni and Patrizia Zaccheo

Abstract The removal of contaminants from the environments has become a crucial problem that requires a variety of approaches to reach suitable solutions. This review will focus on the use of rhizobacteria for restoration of sites co-contaminated with organic pollutants and heavy metals. While the first contaminants can be biodegraded to innocuous end products, metals are not biodegradable and must either be removed or stabilized within the site. Plant growth promoting rhizobacteria (PGPRs) represent a wide variety of soil bacteria which, when grown in association with a host plant result in stimulation of growth of their host also in a stressed environment. Plants, especially dicotyledons that are treated with ACC deaminase-containing PGPRs are more resistant to the deleterious effects of ethylene synthesized as a consequence of stressful conditions. In this review the use of PGPRs to assist plants in remediation processes is examined by discussing recent advances in bioaugmentation efforts. The effectiveness of the external manipulation of rhizosoil to overcome physical and chemical constraints to root establishment and to enhance pollutant removal is also examined. Finally, it is provided a summary of the recent advances in the potential for the use of transgenic plants and/or microorganisms to remediate environmental contaminants. The complexity and diversity of plant/soil/microorganism systems require an integrated approach involving basic and applied researches in order to establish phytoremediation as a viable and attractive technology for efficient restoration of co-contaminated soils

Keywords Rhizoremediation · Plant tolerance · Plant growth promoting rhizobacteria · Detoxification genes · ACC deaminase activity

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Contents

1 Introduction

Rapid industrialization coupled with increased urbanization and changing agricultural practices have resulted in the non-judicious production and use of chemical compounds. Consequently, the environment has become heavily contaminated with pollutants that are toxic to both the environment and human health. Many sites are currently co-contaminated with organic pollutants and heavy metals. Therefore, the removal of contaminants has become a crucial problem that requires a variety of approaches to reach suitable solutions.

Phytoremediation, which is the use of plants to remove pollutants or to render them harmless through physical, chemical and biological processes (Cunningham and Ow 1996; Pilon-Smits 2005), is a low-cost and ecologically accepted technology for *in situ* decontamination of soil and water. During phytoremediation the soil biological properties and physical structure are maintained and soil fertility and biodiversity can be improved. Moreover, well-planted phytoremediation site prevent landscape destruction while garnering strong public support due to the aestetic appearance of the plants. As shown in Fig. 14.1, phytoremediation includes different processes, among which rhizoremediation and phytoextraction represent more challenging techniques for remediating soil that has been contaminated with organic and inorganic pollutants. Additionally, microbe-assisted phytoremediation has recently been employed by exploiting the symbiotic plant-microbe relationship in a rhizosphere (Chaudhry et al. 2005; Gerhardt et al. 2006). Plant roots provide a large surface area for a large population of bacteria and transport the colonizing bacteria to a depth of 10–15 m in the soil. During rhizoremediation, the root system distributes microorganisms through the soil and penetrates otherwise-impermeable soil layers while drawing soluble forms of the pollutants in the soil water phase towards the plant and the microorganisms. Moreover, the plant roots help increase the availability of the pollutant by breaking apart and aerating soil particles as well as by pumping water to the root-colonizing bacteria which helps improve their survival.

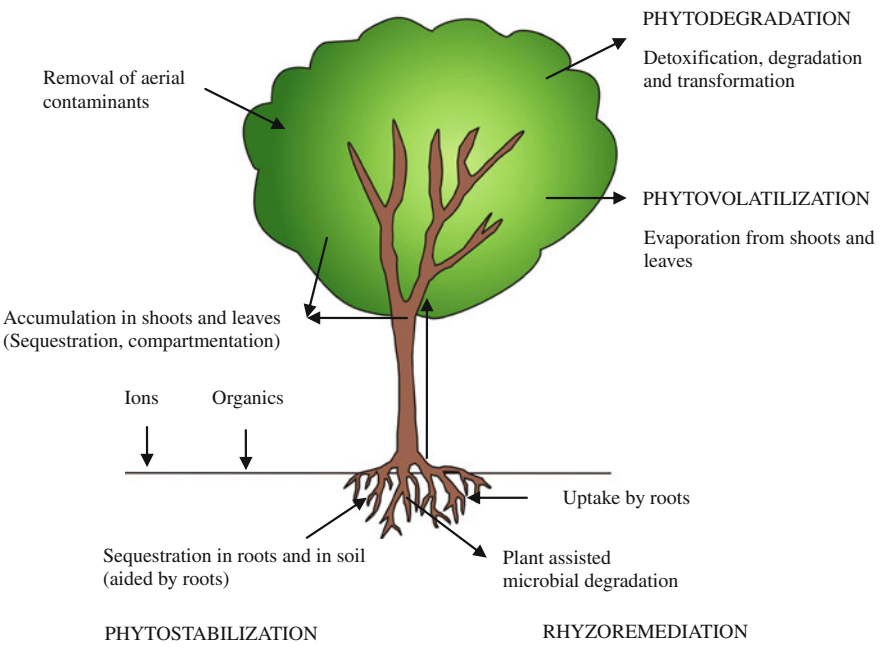

Fig. 14.1 Mechanisms involved in inorganic and organic pollutant decontamination / degradation in phytoremediation processes

Xenobiotic pollutants that can be remediated/metabolised include thrichloroethylene (TCE), polychlorinated biphenyls (PCBs), pesticides, explosives, trinitrotoluene (TNT), petroleum hydrocarbons (PHC), polycyclic aromatic hydrocarbons (PAHs) and detergents (Macek et al. 2000; Newman and Reynolds 2004). Soils that have been contaminated by weathered hydrocarbons and heavy metals (Palmroth et al. 2006) have been effectively treated with rhizoremediation. Processes involved in the phytoremediation of xenobiotic pollutants are microbial transformation and/or mineralization and plant uptake, translocation, transformation and compartmentalization of the contaminants. Rhizosphere factors play an important role in phytoremediation efficiency during successful rhizoremediation projects. Indeed, N- and P-fertilizers, root exudation and chelating agents can enhance plant uptake and accumulation of contaminants by improving the availability of the pollutants to the plants.

Some naturally occurring plants, known as hyperaccumulators have the potential to bioconcentrate metals to 10–500 times higher than non-accumulator species do. Despite this capacity, most hyperaccumulator plants are not suitable for field phytoremediation due to their small biomass production (Shen and Liu 1998).

2 Fate of Contaminants in the Rhizosphere

Once introduced into soil, organic and inorganic contaminants interact with the soil solid phase through many chemical, physical and biological processes (sorption/desorption, precipitation/dissolution, microbial immobilization/ mineralization). As shown in Table 14.1, pH, redox and dissolved organic matter play a fundamental role in controlling the fate and bioavailability of inorganic pollutants (Kabata-Pendias 2004).

PAHs tend to be strongly adsorbed to soil colloids, particularly organic matter, and the hydrophobicity of PAHs result in their having a high persistence in soil. Additionally, xenobiotics can undergo to an ageing process or be sequestered with time in microsites, which result in their becoming more tightly sorbed and less bioavailable (Ruggiero et al. 2002). In the rhizosphere, PAHs are strongly adsorbed to the roots, and this effect is more pronounced with increasing plant age (Schwab et al. 1998).

Table 14.1 Bioavailability of inorganic pollutants under different soil conditions

Condition		**redox**		**pH**	
		low reducing	*high* oxidizing	*low* acid	*medium-high* neutral-alkaline
Increasing metal mobility ↑	*high*	As	Zn	Zn, Cu, Co, Ni, Hg	
	medium		Cu, Co, Cd, Ni	Cd	Cd
	low		Pb	Pb	Pb
	very low	Cu,Co,Ni, Zn, Hg, Cd, Pb	Fe, Mn, Al, Sn, Cr		Cu, Co, Ni, Zn, Hg

Rhizospheric soil has chemical, physical and biological properties that are quite different from bulk soil due to the root activity and the presence of free enzymes and rhizobacteria (Hinsinger et al. 2003). In the rhizosphere, the mobility of heavy metals and redox sensitive elements such as arsenic (As), copper (Cu) and mercury (Hg) may increase greatly, leading to the contamination of crop plants. For example, a sixfold increase in bioavailable Cu in the rhizosphere of maize grown in a fungicide polluted soil was reported by Cattani et al. (2006). However, little Cu uptake by maize occurred, presumably due to the sequestration of Cu by dissolved organic carbon (DOC), which was present in the rhizosphere in levels three-fold greater than that of bulk soil. Enhancement of soluble Ni driven by the formation of Ni-organic

complexes and the dissolution of Ni-bearing minerals through ligands was observed in the rhizosphere of Ni hyperaccumulator plants (Krämer et al. 1996; Wenzel et al. 2003). The ability of *Pteris vittata* L. to hyperaccumulate arsenic is related to a fern-mediated increase in rhizosferic soil pH of 0.4 units and a DOC concentration of 33–40% (Silva-Gonzaga et al. 2006). However, *Thlaspi caerulescens* L., which is a well known Zn hyperaccumulator plant, does not mobilize Zn through soil acidification and root exudation (Luo et al. 2000; Zhao et al. 2001; Whiting et al. 2001).

Also soil microorganisms can modify chemical properties of the rhizospheric soil, thus affecting inorganic contaminant bioavailability. While bacteria may enhance the ion bioavailability by exuding a variety of organic compounds or stimulating the release of exudates by the plants (Salt et al. 1995), mycorrhizae may reduce metal phytoavailability by sequestering these compounds in the hyphae (Lasat 2002).

3 The Interactions Among Bacteria and Organic and Inorganic Pollutants

Organic-degrading microorganisms and a number of metal-resistant microorganisms that are known to detoxify metals/metalloids have been isolated from impacted soils and characterized (Daane et al. 2001; Singer et al. 2004; Cavalca et al. 2004; Dell'Amico et al. 2008). Bacteria degrade xenobiotics through a variety of enzymes including peroxidases, monooxygenases and dioxygenases, laccases, phosphatases, dehalogenases, nitrilases, and nitroreductases (Siciliano et al. 2001; Gibson and Parales 2000; Gianfreda and Rao 2004; Andreoni and Gianfreda 2009).

Although some microorganisms can completely degrade a specific xenobiotic, individual species generally do not contain entire degradation pathways. Rather, microbial consortia in the rhizosphere work synergistically to effectively degrade the pollutants (Chaudhry et al. 2005; Yateem et al. 2007). For example, the synergistic degradation of naphthalene by two *Pseudomonas fluorescens* strains in the rhizosphere of a grass was reported by Bloemberg et al. (2000). Moreover, by labelling the strains with different autofluorescent protein markers, the authors observed the frequency of the appearance and distribution of pure and mixed microcolonies along the root and found that mixed colonies only occurred in the presence of naphthalene, presumably because one strain secreted naphthalene intermediates that were used by the other strain when they were close to each other on the root.

It is also becoming clear that the horizontal transfer of genes plays a large role in the spread of functional abilities within communities and in enabling the adaptation of organisms to changing niches by allowing the acquisition of new metabolic potential for degradation of recently introduced xenobiotics (Janssen et al. 2005; Phale et al. 2007) or for detoxification of inorganic pollutants.

Genes located on chromosomes, plasmids or transposons encode specific resistance to a variety of inorganic elements. The most frequent mechanism of arsenic,

cadmium and mercury resistance is the energy dependent pumping out of these compounds, via membrane efflux pumps. Prominent examples include inducible plasmid-encoded resistance for Cd by the *cad* operon in *S. aureus* and *Bacillus sp.* or by the *czc* operon found in *Alcaligenes eutrophus* (Nies 2003), as well as resistance for Hg encoded by the *mer* operon found in Gram-negative and Gram-positive bacteria (Barkay et al. 2003) and resistance for As, and antimonite (Sb) mediated by the *ars* operon in *E. coli* (Rosen 2002) and *S. aureus* (Messens et al. 1999). Each *ars* operon has two essential components: the arsenate reductase (arsC, ACR2) and an arsenite-specific efflux pump (ArsB, ACR3) (Silver and Phung 2005). Although arsenic resistance is not directly involved in arsenate respiration and arsenite oxidation, *ars* operons have been found in arsenate-respiring bacteria (Saltikov and Newman 2003) as well as and in many arsenite-oxidizing bacteria, providing the latter the ability to both oxidize and reduce arsenic (Macur et al. 2004). The *Mer* operon generally contains a mercuric reductase (merA), but in some organisms the operon also contain an organonomercurial lyase (merB) that cleaves certain organomercuric compounds (Barkay et al. 2003). An overview of membrane associated uptake, efflux, reduction and oxidation of the cited ions is reported in Fig. 14.2.

While organic contaminants can be biodegraded to innocuous end products (CO_2, cell mass, water), metals are not biodegradable and must either be removed or stabilized within the site.

Co-contaminated soils, which are widespread throughout the world, are still considered difficult to remediate due to the mixed nature of the contaminants (Sandrin and Maier 2003). The presence of metals can impact both the physiology and ecology of organic degrading microorganisms. Metals may inhibit pollutant degradation through interaction with enzymes involved in biodegradation (e.g., pollutant-specific oxygenases) or with enzymes involved in general metabolism (Angle and Chaney 1989).

Metal toxicity is related to the concentration of bioavailable ionic species rather than the total metal concentration. Usually, inhibition of biodegradation increases progressively as the concentration of bioavailable metal in a co-contaminated environment increases. When considering the impact of metals on organic biodegradation, the effects of metals on populations other than degraders of the parent compound must be also considered. Reduced microbial activity may also originate from changes in the microbial community structure after long-term exposure to heavy metals. Doelman et al. (1994) observed that metal-contaminated soil contained more metal-resistant microorganisms, but with a restricted ability to degrade organic pollutants. The presence of multiple contaminants may present extreme challenges to the maintenance of a phylogenetically and functionally diverse microbial community. In soils contaminated with both heavy metals and hydrocarbons, only those that tolerate both contaminants may survive. Shi et al. (2002) when examined microbial community composition and activity after long-term exposure to Pb, Cr, and hydrocarbons, found that the soil microbial community was not affected by metals but predominantly by hydrocarbons.

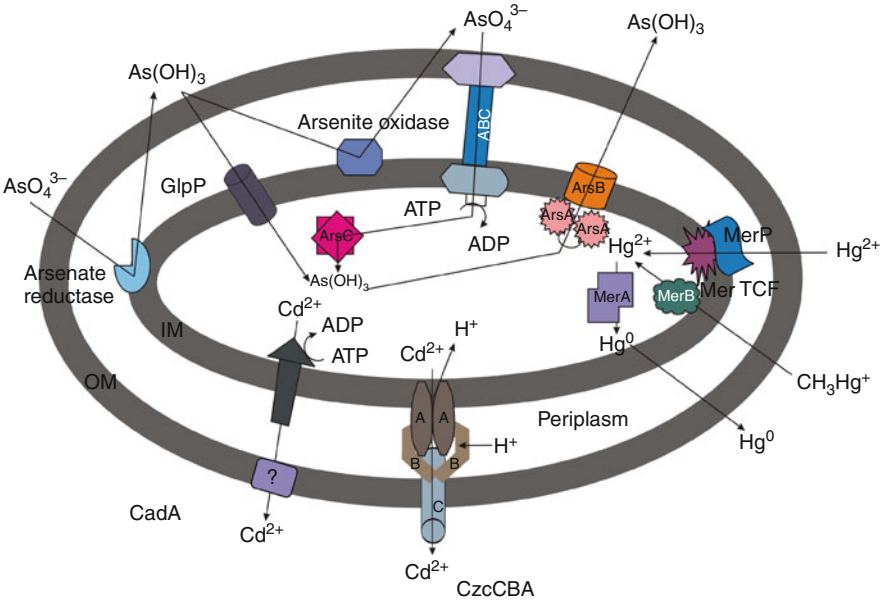

Fig. 14.2 Overview of membrane associated uptake, efflux, reduction and oxidation of arsenic, mercury and cadmium. For cadmium, a schematic presentation of the efflux systems is given due to the complexity of CadA P-type ATPases and chemiosmotic CzcCBA systems. Arsenic and mercury are given more emphasis, as peryplasmic and cytoplasmic enzymes are included as well as the class of transporters. GlpP, aquaglycerolporine; ABC, multicomponent Pst-like ATPase uptake system; ArsA/B, two component ATPase efflux pump; ArsC, small intracellular arsenate reductase; MerP, peryplasmic protein that binds Hg^{2+}; MerT/C/F, alternative membrane uptake proteins; MerB, organomercurial lyase; MerA, mercuric reductase; CzcCBA, three polypeptide chemiosmotic complex that function as an ion/proton exchanger to efflux Cd^{2+}; CadA, P-type membrane efflux ATPase for Cd^{2+} (large single polypeptide) (Adapted from Silver Phung 2005; Barkay et al. 2003; Nies 2003)

The influence of heavy metals on PAH degradation in polluted soils has recently been emphasized, and the effect of various metals on the degradation of phenanthrene has been thoroughly investigated. The degradation of phenanthrene was found to be retarded by the presence of Cu, and high levels of the metal caused incomplete mineralization and accumulation of phenanthrene metabolites (Sokhn et al. 2001). A marginal stimulation of the phenanthrene biodegradation rate in soil occurred when 140 mg kg^{-1} phenanthrene was in the presence of 40 mg kg^{-1} Zn. However, phenanthrene degradation was inhibited at Zn concentrations at or above the "action" values (i.e., the level of a contaminant at which soil quality is deemed to impair the soil functional properties) (Wong et al. 2005).

Stimulated biodegradation at low metal concentrations and inhibition at high metal concentrations has also been observed. The addition of hexavalent chromium (0.01 ppm total chromium) was found to increase the biodegradation rate of phenol by 177% and that of benzoate of 169% over controls without metals (Kuo and

Genthner 1996). Similar results were obtained by Hughes and Poole (1989). These responses suggested that the stimulatory effect could be due to metals competition for reducing equivalents or nutrients between metal-resistant degrading bacteria and non degrading bacteria that are metal-sensitive. However, Roane and Pepper (1997) found that a population of 2,4-D degrading bacteria in a Cd contaminated soil showed higher resistance at 40 mg L^{-1} than at 20 mg/L and that the higher Cd concentration inhibited less the biodegradation. This response can be explainable by microbial community dynamics wherein high metal concentrations create selective pressure for metal-resistant degraders. Specifically, a reduction in the competition of metal-sensitive non degrading microorganisms may have led to increased biodegradation at higher metal concentrations.

Dual bioaugmentation appears to be a viable approach in the remediation of co-contaminated soils. A dual bioaugmentation that employed metal-detoxifying and organic-degrading bacteria to remove 2,4-D from co-contaminated soils in the laboratory and a pilot field experiment was found to be effective (Roane et al. 2001). The success of the bioremediation strategy, which required a 48-hour time interval between inoculation with a cadmium-detoxifying population of bacteria (*Pseudomonas* spp. H1) and inoculation with a cadmium sensitive 2,4-degrader (*Ralstonia eutropha* JMP 134), was attributed to metal detoxification as the primary mode of bacterial action, which resulted in organic degradation no longer being inhibited. Indeed, some microbial mechanisms of resistance to metal, such as metal sequestration and precipitation, can reduce the toxicity toward organic degrading microorganisms.

Aerobic degradation of TCE can occur through many different oxygenases, including toluene *ortho*-monooxygenase (TOM) (Mars et al. 1996). The stable integration of the *TOM* gene of *Burkholderia cepacia* G4 into naturally occurring rhizobacteria that had colonized the roots of a poplar tree such as *Pseudomonas* Pb2-1 and *Rhizobium* strain 1032D was found to enable the establishment of a bacterium-plant-soil microcosm in which 63% of the TCE was degraded in 4 days (Shim et al. 2000). The subsequent introduction of a gene coding for the metal-binding peptide EC20 in the Pb2-1 and 10320D strains gave rise to strains with both metal accumulation (extracellularly) and TCE degradation capabilities (Lee et al. 2006). Thus, the bioaugmentation of the rhizosphere with a microorganism that is capable of both organic degradation and metal resistance may represent another means of bioremediation.

4 Rhizospheric Microbial Populations

The rhizosphere is an area encircling the plant root system that is characterized by enhanced microbial biomass productivity. Rhizobacteria obtain nutrients excreted from roots, such as organic acids, amino acids, enzymes and complex carbohydrates. The enhanced growth of microorganisms also depends on microenvironmental conditions (chemical factors, pH, O_2 content and redox potential).

In return, rhizobacteria that promote plant growth (PGPR) convert nutrients into available minerals for the plants, synthesize compounds that protect the plants against stress hormone levels and plant pathogens, and degrade and/or immobilize contaminants before they can negatively impact the plants (Hontzeas et al. 2004; Chaudhry et al. 2005; Liu et al. 2007). PGPR are fast-growing bacteria that include numerous genera such as *Bacillus*, *Pseudomonas*, *Erwinia*, *Flavobacterium, Caulobacter, Serratia, Arthrobacter, Comamonas, Alcaligenes, Agrobacterium* and free-living nitrogen fixing bacteria (Gray and Smith 2005). Among these bacteria, *Pseudomonas* spp. predominate rhizosperic soil and discontinuously colonize root surfaces, resulting in random distribution on roots. For example, *P. putida* are species that respond rapidly to the presence of root exudates in soil, converging through chemotaxis and motility mechanisms at root colonization sites, where they establish stable biofilms (Broek and Venderleyden 1995; Espinosa-Urgel et al. 2002). Numerous bacterial traits, such as production of thiamine and biotin, synthesis of the O-antigen of lipopolysaccharide and cellulose, production of amino acids and the presence of an efflux pump induced by isoflavonoids are required for effective root colonization. Flavonoids and coumarins are an important group of plant compounds that are structurally similar to many xenobitics such as PCBs, PAHs, and PHC, thereby stimulating the growth and activity of PHC, PAH and PCB degrading bacteria (Chaudhry et al. 2005; Leigh et al. 2006).

The successful application of rhizoremediation is largely dependent on the capacity of degrading bacteria or PGPR to efficiently colonize growing roots. Moreover, many PGPR play an important role in metal solubilisation, which is a prerequisite for rhizoremediation and/or phytoremediation, by producing indoleacetic acid or metal-chelating compounds such as siderophores that release metal cations from soil particles (Khan 2005) and thus favour metal uptake.

5 Methods for Assessing and Monitoring Rhizospheric Bacteria

It is essential to thoroughly understand the role that bacteria play in phytoremediation to maximize the sustained bioremediation that occurs under natural conditions and to monitor the presence, survival and activity of degrading or detoxifing micro-organisms. Until recently, studies of *in situ* bioremediation were primarily based on cultivation techniques. However, pure culture isolation, biochemical testing using methods such as BIOLOG and counting methods (plate counts or most probable number, MPN) are not well suited for the estimation of total microbial biomass or the assessment of community composition within environmental samples. Accordingly, culture-independent methods, that rely on the isolation of signature biomarkers, such as DNA, RNA and phospholipid fatty acid (PLFA) have been used to provide a quantitative measure of the rhizosphere microbial biomass, community composition, nutritional status, relative frequency of specific functional genes and, in some cases, the community metabolic activity. PLFA provides a broad-scale diversity index that can be used to evaluate the number of bacterial families

present in the samples. Additionally, combinations of BIOLOG and PLFA have been used to demonstrate differences in the microbial composition of bulk and rhizospheric soil (Soderberg et al. 2004). However, these methods are inadequate to describe the abundance and diversity of microbial communities in the environment or to relate a microbial species to the ecosystem function, but these limitations can be overcome by using a number of culture-independent approaches.

Polymerase chain reaction (PCR) to amplify selected fragments of DNA isolated from soil microorganisms or environmental DNA samples, combined with fingerprinting techniques, such as ribosomal intergenic spacer analysis (RISA), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment analysis (T-RFLP), amplified rDNA restriction analysis (ARDRA), cloning and sequencing can provide detailed information about the species composition of communities (Spiegelman et al. 2005). As a result, detection of specific nucleic acid sequences and nucleic acid hybridization, using specific probes for a functional gene involved in a degradation pathway (i.e., *nah* and *nod* gene sequences encoding for naphthalene dioxygenase and *phen* gene sequences for phenanthrene dioxygenase) or for metal resistance genes, or gene messages, are indispensable for the identification of microorganisms in environmental samples for the evaluation of their bioremediation potential. For example, PAH-degrading bacteria have been detected and characterized in salt marsh rhizospheres using a variety of phenotypic and molecular properties (Daane et al. 2001). In a total of five different plant samples, the primary bacterial groups were Gram-negative pseudomonads, Gram-positive (predominantly nocardioform), and the Gram-positive, spore forming group, *Paenibacillus*. Furthermore, 75% of the pseudomonad isolates hybridized to the classical *nah* gene from *P. putida* NCIB9816-4, while approximately the same number hybridized to the *nag* genes cloned from *C. testosterone* GZ42, whereas the *Paenibacillus* isolates were not found to be homologous with any of the tested gene probes (Daane et al. 2001). Siciliano et al. (2001) observed that naphthalene dioxygenase (*ndoB)* catabolic genotypes were enriched in the rhizosphere of *Scirpus pungens* in response to pollution in a contaminant-dependent manner.

DGGE has been used to demonstrate that different plants supported different bacterial, archeabacterial and fungal communities (Griffiths et al. 2003; Nicol et al. 2003; Gomes 2003). Furthermore, the addition of Hg^{2+} to a silt loam was found to cause an increase in the abundance of two RISA bands that were subsequently identified as a *Clostridium*-like organism and a *Ralstonia*-like organism (Ranjard et al. 2000). However, it is important to note that these techniques result in destruction of the samples.

To study the pattern of microbial plant-root colonization, microscopy, microscopy combined with the use of marked strains, or strains equipped with reporter genes can be used. Reporter technology has been used to assess several functions in the rhizospheric soil including gene expression even at the single cell level. The increasing knowledge of the promoter and regulator genes along with the refinement of reporter gene insertion techniques will allow to use this technique for monitoring induction, expression and regulation of virtually any

gene in the rhizosphere (Jansson 2003). The *Gfp* and *lux* genes are examples of common reporter genes that encode green fluorescent protein (GFP) or bioluminescence, respectively, and can be used to tag environmental bacteria with degrading or detoxifying capabilities. The visual reports of *gfp* and *lux* can be assayed non-destructively, without supplying external cofactors or substrates to cells. For example, *Comamonas* sp. strain CNB-1 isolated from an activated sludge and capable of degrading 4-chloronitrobenzoate (4-CNB) was applied for the rhizoremediation of 4-CNB-polluted soil through association with the alfalfa plant (Liu et al. 2007). The inoculation of CNB-1 in the rhizosphere was evaluated by constructing a GFP-expressing strain CNB-1: *gfp2* and then monitoring the colonization of alfalfa roots by CNB-1: *gfp2* and the formation biofilms on the surface and within roots by confocal laser scanning microscopy. Additionally, a *Pseudomonas fluorescens* F113rifpcb bioreporter, utilizing a chlorobenzoate-responsive promoter was used to monitor the cell-activity in alfalfa rhizospheric soil contaminated by PCBs. In particular, the fluorescence-emitting cells of the modified bacterium F113rifpcb were found to be located in microcolonies, occurring all along the root (Boldt et al. 2004). Finally, Tom-Peterson et al. (2001) determined the amount of Cu bioavailable in a soil amended with complex organic material using a specific Cu reporter construct harboured by an indigenous soil bacterium, *P. fluorescens* DF57.

One drawback of techniques based on probes is that investigations are limited to the identification of known groups and may fail to capture the presence of truly novel organisms. Fluorescent *in situ* hybridization (FISH) allows the phylogenetic identification of uncultured bacteria in natural environments using fluorescent group specific phylogenetic probes targeting rRNA and fluorescence microscopy. Combining FISH with microautoradiography or with immunodetection of bromodioxyuridine allows the detection and quantification of the active population utilizing a specific substrate (Cottrell and Kirchman 2000; Pernthaler and Amann 2004).

The extraction and characterization of mRNA from soil can provide data on activity of certain genotypes in polluted soils. Naphthalene degradation for example has been monitored by quantification of mRNA transcripts of naphthalene dioxygenase gene (*nahAC)* (Sanseverino et al. 1993–1994). Microarrays are increasingly being used to analyse microbial communities (phyogenetic oligonucleotide array), to characterize microorganisms in environmental samples and to monitor gene expression under different growth conditions (functional genes and expression arrays) (Zhou 2003). For example, Mark et al. (2005) used DNA microarrays to identify unique *P. aeruginosa* genes expressed during growth in artificial medium containing sugarbeet exudates from two beet cultivars.

Stable isotope probing (SIP), which involves tracking of a stable isotope atom from a substrate into components of microbial cells, provides phylogenetic and functional information, such as lipid content and DNA and RNA sequences. Butler et al. (2003) reported the use of PLFA-SIP to reveal spatial and temporal differences in microorganisms utilizing root exudates in the rhizosphere of ryegrass. More recently, Rangel-Castro et al. (2005) applied RNA-SIP to a $^{13}CO_2$-pulsed labelled grassland microbial community to determine the effect of liming on the structure of the rhizosphere microbial community metabolizing root exudates.

6 PGPR with ACC Deaminase Activity

PGPR are free-living saprophytic bacteria that inhabit the plant rhizosphere and colonize the root system. PGPR have long been used as plant growth promoters to increase agricultural production and as biocontrol agents against plant diseases (Zehnder et al. 2001). Recently, the application of PGPR has been extended to the remediation of contaminated soils in association with plants due to their catabolic versatility, excellent root colonizing ability and the capacity to produce a wide range of enzymes and metabolites that favour the plants under varied stress conditions (Ramamoorthy et al. 2001; Mayak et al. 2004).

For many plants, a burst of ethylene is required to break seed dormancy; however, following germination, a sustained high level of ethylene would inhibit root elongation. In addition, ethylene is synthesized in plant tissues from the precursor 1-aminocyclopropane-1-carboxylic acid (ACC) during biotic and abiotic stress conditions, which can depress growth and causes senescence in crop plants (Ma et al. 2003). Many PGPR strains and some fungi possess the enzyme ACC deaminase (Shah et al. 1998; Glick et al. 2007) which can cleave the plant ethylene precursor ACC, thereby lowering the level of ethylene in a developing seedling or stressed plant. The gene encoding ACC deaminase has been found in a variety of soil bacteria (Glick 2003; Madhaiyan et al. 2007; Dell'Amico et al. 2008; Saravanakumar and Samyappan 2007) and more than one type of ACC deaminase gene may exist (Shah et al. 1998; Babalola et al. 2003; Blaha et al. 2006). Plants, especially dicotyledons that are treated with ACC deaminase-containing PGPR are dramatically more resistant to the deleterious effects of stress ethylene synthesized as a consequence of stressful conditions. The formation of longer roots through the action of ACC deaminase may facilitate the survival of plant seedlings under various stress conditions, such as flooding (Grichko and Glick 2001), phytopathogens (Wang et al. 2000), drought and high salt concentration (Mayak et al. 2004), and heavy metals (Grichko et al. 2000). For example, ACC deaminase rhizobacteria have the potential to protect canola and tomato seeds from Ni toxicity (Burd et al. 1998) and Indian mustard, rape and canola from Cd toxicity (Belimov et al. 2005; Dell'Amico et al. 2008).

Prolific root growth may also enhance the rates of rhizoremediation. For example, a multi-component phytoremediation system of soil that combined land farming, bio-augmentation with PAH-degrading bacteria and the growth of plants (*Festuca arundinacea*) with PGPR containing ACC deaminase activity under laboratory conditions led to improved effective removal of 16 persistent and soil-bound PAHs, when compared to the results of treatment with any of these methods alone (Huang et al. 2004). Phytoremediation was successful because the plant species were able to grow in the presence of high levels of contaminants and the strains of PGPR increased plant tolerance to PAHs and accelerated plant growth in heavily contaminated soils.

Liu et al. (2007) demonstrated that the inoculation of alfalfa with *Comamonas* sp. CBN-1 eliminated the phytotoxicity of 4-CNB by completely removing it from soil within 1 or 2 days. However, the presence of ACC deaminase activity in this

bacterium was not investigated. Besides the role that ACC deaminase activity plays in alleviating ethylene-mediated stresses, the addition of other traits, the ecology of the bacterium and the physiology of the plant may also have interacted with the plant system to increase resistance to stress.

To date, very little work has been conducted to evaluate the use of ACC deaminase containing bacteria in rhizoremediation of organic-contaminated soil. Wu et al. (2006) also found that the inoculation of sunflower roots with the engineered rhizobacterium, *P. putida* 06909, caused a marked decrease in Cd phytotoxicity and a 40% increase in Cd accumulation in the roots. However, they did not investigate the ACC deaminase activity of the bacterium. A comparison of the efficiency of transgenic bacteria that carry ACC deaminase and control bacteria at promoting seed germination and root elongation in soils contaminated by copper and PAHs revealed that both native and transformed *Pseudomonas asplenii* AC equally promoted seed germination and root elongation under stress conditions (Reed and Glick 2005). Moreover, the efficiency of transgenic inoculated strains was found to be affected by soil pH, temperature, moisture content and competition with native microflora and microfauna.

Additionally, according to Burd et al. (1998) and Belimov et al. (2005), PGPRs containing ACC deaminase have great potential for use in the development of bacterial inocula for improvement of plant growth under unfavourable environmental conditions, particularly for hyperaccumulator plants. Furthermore, the plant growth promotion observed in response to inoculation with ACC deaminase-containing bacteria has been found to stimulate the development of transgenic plants that express ACC deaminase genes, thus protecting them from some of the deleterious effects of metals.

7 Plant Tolerance to Toxic Compounds and Transgenic Plants with Detoxification Genes

The potential for the use of transgenic plants and/ or microorganisms to remediate environmental contaminants has been extensively explored in the laboratory. Strategies feasible for the transformation and engineering of microorganisms or plants include the introduction of genes encoding functions to enhance resistance to contaminants or to environmental stressors, to overexpress enzymes involved in degradation pathways, to release specific exudates that can act as inducers for microbial degradation, and to increase the plant capacity for the uptake, transport and sequestration of contaminants. For example, for metal phytoremediation purposes transgenic plants may be manufactured to synthesize a product that alters metal tolerance or uptake that decreases ethylene synthesis to reduce the deleterious plant response to metal stress.

Transgenic tomato plants and transgenic canola plants expressing bacterial ACC deaminase were found to grow in soil in the presence of cadmium, copper, cobalt, nickel, lead or zinc, to accumulate high amounts of metals and to proliferate in the

presence of high levels of arsenate (Grichko et al. 2000; Nie et al. 2002). In the presence of arsenate, transgenic canola plants grew to a significantly greater extent than non-transformed canola plants, regardless of whether plant growth-promoting bacteria were present. Additionally, plants accumulated similar amounts of arsenate whether or not they were treated with *E. cloacae* CAL2. Moreover, transgenic canola shoots contained less arsenate than non-transformed canola shoots, suggesting that a limited translocation of arsenate from roots to shoots occurred, which may have lowered arsenate toxicity, even if the reason for this decreased translocation in transgenic plants was unknown. When biomass was considered in calculating the arsenate accumulation, transgenic canola plants accumulated approximately four times as much arsenate as non-transformed canola. The higher rate of germination of transgenic canola also contributed to the total amount of arsenate accumulation. The use of transgenic canola in conjunction with plant growth-promoting bacteria made phytoremediation much more efficient (Nie et al. 2002). Similar results were reported by Stearns et al. (2005) and Farwell et al. (2006) in the phytoremediation of nickel contaminated soils.

Meagher and Heaton (2005) evaluated the capability of *Arabidopsis* transgenic plants expressing bacterial metal resistance genes (*merA, merB and arsC*) to take up and transform levels of mercury and arsenic several times higher than the lethal level for most plant species. *Mer* plants, which are modified plants expressing the bacterial *merB* gene encoding an organomercury lyase, were found to grow on

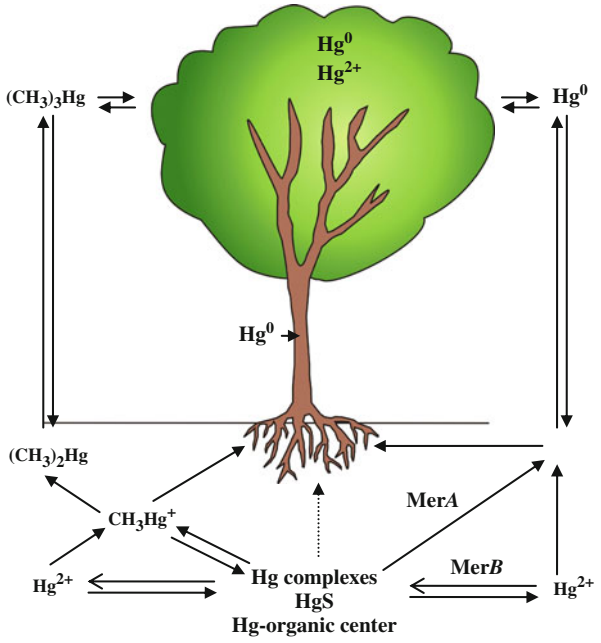

Fig. 14.3 Role of microorganisms and plants in the biogeochemical cycle of Hg

0.1–1 μM of methylmercury or phenylmercuryacetate in agar medium, which are levels high enough to kill native plants. Plants expressing the *merA* gene which encodes mercuric reductase, detoxify Hg^{2+} by reducing it to Hg^0, allowing the plants to grow in soil containing concentrations of Hg^{2+} of 100 ppm or higher (Rugh et al. 1996). Finally, combining the transgenic expression of *merA* and *merB*, enabled plants to detoxify organic mercury more efficiently and to be resistant to 2–10 μM of phenylmercuryacetate (Bizily et al. 1999; Bizily et al. 2003).

In theory, plants engineered with both genes should extract organomercurials from soils and transpire Hg^0 into the atmosphere using the same mechanisms as bacteria (Fig. 14.3). However, from a regulatory perspective, the release of Hg into the atmosphere is not acceptable, therefore, the use of plants genetically transformed with *merA* and *merB* gene is not permitted.

8 Strategies for Enhancing Phytoremediation

Studies of *in situ* application of rhizoremediation have provided contradictory results because several biotic and abiotic factors may severely limit the establishment of vegetation, microbial growth and contaminant mobility. The ability of plants to enhance rhizospheric activity and to extract contaminants from the soils can be drastically reduced by contaminant phytotoxicity or by unsuitable soil physical and chemical properties such as acidity, compaction, and anoxic conditions. The consequent reductions in root development represent severe constraints in phytoremediation because contaminants are often heterogeneously located in soil and a limited root system cannot gain access to niches with a high degree of pollution. These constraints can be partially overcome by selecting tolerant plants, and/or by applying agronomic techniques to amend soil properties and modify contaminant bioavailability. For example, nitrogen and phosphorus fertilization increased the rhizobacteria-assisted phytoextraction of As (Jankong et al. 2007), as well as pyrene rhizodegradation (Thompson et al. 2008). Additionally, soil amendments with humified organic matter enhanced the biodegradation of PCBs (Smith et al. 2007) and of aged hydrocarbons and heavy metals in co-contaminated soils (Palmroth et al. 2006)

There is evidence that the external manipulation of bulk and/or rhizospheric soil pH can improve the phytoremediation of metal polluted soils in cases of low metal concentration in soil solution due to strong binding to the solid phases. Conversely, it is still a matter of debate if plants can transfer high amounts of metals from soil into the shoots by adopting rhizosphere strategies such as acidification and exudation. A decrease in bulk soil pH can be achieved through application of mineral acids, organic acids and acid-producing fertilizers (Cui et al. 2004; Kayser et al. 2001). Acidification of the rhizosphere may be obtained by modulating the nitrogen nutrition, and supplying $N\text{-}NH_4$ to plants has been found to induce rhizosphere acidification, thereby enhancing Cd and Zn uptake by tobacco and

sunflowers (Loosemore et al. 2004; Zaccheo et al. 2006). Conversely, a N-NO_3 supply promoted growth and phytoextraction of Cd and Zn by *Thlaspi caerulescens* (Xie et al. 2009).

There is evidence that organic acids released from the roots of some plants can provide the impetus for movement of PAHs from bulk soil to the rhizosphere and accelerate PAH mobilization (Liste and Alexander 2000). Root exudation of chelators may be mimicked by the addition of natural and synthetic compounds (i.e., citric acid, NTA) to enhance heavy metal solubility and phytoextraction efficiency of several plant species like willow, Indian mustard, corn and sunflower (Schmidt 2003). It is, however, important to minimize the ecological hazards connected with chelate-assisted phytoextraction, as phytotoxicity or metal leaching. The amendment of soil with some organic compounds was found to be effective at enhancing phytoremediation and biodegradation of co-contaminated soils in pot experiments in which *Alyssum lesbiacum* was grown in nickel and PAH spiked-soil (Singer et al. 2007). In that study, treatment with a combination of a surfactant (sorbitan trioleate), a PAH biodegradation inducer (salicylic acid) and a Ni-chelator (histidine) induced high biomass production by *Alyssum lesbiacum*.

Finally, rhizobacteria can be stimulated by the addition of agrowaste residues (Azcon et al. 2009) or chelates to ameliorate plant growth and metal phytoextraction. For example, Chen et al. (2006) found that microbial communities of *Elsholtzia splendens* and *Trifolium repens* grown on Cu contaminated soil amended with glucose and citric acid facilitated Cu solubilisation without inhibiting the microbial community.

9 Conclusions

Rhizoremediation and phytoextraction might be effective approaches to the remediation of soils contaminated by metals and organics. The exploitation of symbiotic relationships between plants and rhizobacteria should lead to better clean-up of polluted soils. However, the complexity and heterogeneity of co-contaminated soils require integrated approaches of the rhizosphere management. In particular, concerted efforts should be focused on the development of suitable environmental and agricultural engineering techniques that will have a major impact on the efficiency of plant cultivation. The selection of more efficient plant varieties and soil amendments and the optimization of agronomic practices should provide improved phytoremediation. The combined use of phytoextraction and rhizodegradation crops, the inoculation of roots or seeds of hyperaccumulator plants, the genetic manipulation of hyperaccumulators expressing ACC deaminase and other specific organic-degradative genes may be a breakthrough in the enhanced removal of heavy metals and organics from the soil environment.

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Chapter 15
Phytoremediation of Saline Soils for Sustainable Agricultural Productivity

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Abstract Salinization of soils is one of the major factors which severely affect the agricultural productivity worldwide. Due to salinity, more than half a billion hectares of land are not being properly used for crop production. Thus, there is a need to search means to improve saline soils so that such soils could support highly productive and meaningful land-use systems to meet the current challenges of global food security. Although permanent solution of soil salinity problem necessitates a sound drainage system to manage the rising water table, this option, being energy- and cost-intensive cannot be employed on a large scale on vast areas. Phytoremediation or biological approach, i.e., plant-based strategies for improvement of deteriorated soils is an appropriate option. Phytoremediation of saline soils can be done by cultivating suitable plant species as well as by Exploiting the ability of plant roots to improve the dissolution and enhance levels of Ca in soil

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solution to efficiently remove Na from the soil cation exchange complex and leach it from the root zone. During the amelioration process, soil-aggregates stability, root proliferation, soil hydraulic properties and availability of nutrients to plants are also improved. Such improvement in soil properties facilitates cultivation of less tolerant plants, improves the environment in general, and the climatic conditions by enhancing carbon sequestration.

Keywords Salt removal · Salt tolerance · Plant productivity · Soil properties · Halophytes · Carbon sequestration

Contents

1 Introduction

Salinization is one of the most intriguing and fundamental problems for agriculture particularly in the semi-arid and arid regions of the world (ICARDA 2002). It prevails in more than half of the irrigated areas (Cheraghi 2004) and is a major constraint for the agricultural productivity in Pakistan, where more than 6.3 million ha (Mha) of land is salt-affected (Khan et al. 1998). The contamination of soils due to salinization hampers the balance between the functions (goods and services) supplied by the natural resources (land and water) and the demands of societies which ultimately affects the livelihoods of the population of that area (Abdel-Dayem 2005). Salt-contaminated soils are increasing due to intensive cultivation with high input demanding crops (Akhter et al. 2003), lack of drainage system in the farmers fields in irrigated areas, as well as discharge of soap, leather and oil industries in irrigated water (Pitman and Läuchli 2002). It has also been observed that excess of salts reduce the permeability of soils (Ashraf 2007). Salt-affected soils usually contain a variety of inorganic salts with cations like Na^+, Ca^{2+}, Mg^{2+}, and K^+, and anions like Cl^-, $SO_4{}^{2-}$, $HCO_3{}^-$, $CO_3{}^{2-}$, and $NO_3{}^-$ (Tanji 2002) which adversely affect plant growth and productivity due to causing ion toxicity or osmotic effect on plants (Parida and Das 2005; Läuchli and Grattan 2007).

With the increase in world population, food, feed and industrial material resources are shrinking day by day. This urges the utilization of salinized wastelands for plant production. Different approaches for remediation of these lands are being used for the last few decades which include construction of drainage system,

chemical amendments, tillage operations, crop-assisted interventions etc. (Oster et al. 1999). So, identification of remediation techniques for salt-contaminated soils which are environment friendly is necessary. Phytoremediation i.e., utilization of plants to remediate contaminated soils, is one of these techniques which is environmental friendly. The cultivation of salt tolerant plants having ability to absorb excessive salts from root zone and accumulate them in plant body is an effective low cost option. These plants not only remediate the salt-contaminated soils but also provide food, fodder, fuel wood and industrial raw material and increase the income of the farmers owning salt-affected lands.

Plants having ability to remove salts from contaminated soils have been identified by many workers (Ashraf et al. 2005a). Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan is playing a key role in disseminating these plants all over the world especially in under-developed and developing countries through different national and international projects. Most of the selected plants are introduced on salt-affected soils for cultivation. The field experiments related to this topic have been conducted at two Biosaline Research Stations (BSRS) of NIAB, one located at Rakh Dera Chal near Lahore (BSRS−I) at longitude 74° 7′ E and latitude 31° 6′ N and the other at Pakka Anna near Faisalabad (BSRS−II) at 73° 05′ E longitude and 31° 24′ N latitude where average annual rainfall is 500 mm. *Suaeda fruticosa*, *Atriplex lentiformis* and *Kochia indica* (Chenopodiaceae), and Kallar grass (*Leptochloa fusca*) and *Sporobolus arabicus* (Poaceae) were grown on both the stations using brackish groundwater for irrigation (Table 15.1). Soil samples (0–20, 40–60 and 80–100 cm depths) were collected before and after the cultivation of above mentioned plant species and analyzed for various physico–chemical properties (Table 15.2) using mostly the methods described by the US Salinity Laboratory Staff (1954).

Table 15.1 Characteristics of tube–well water at BSRS–I and II

Characteristics	Values	
	BSRS–I	BSRS–II
EC (dS m^{-1})	0.14	4.97
pH	7.6	8.2
TSS (mg L^{-1})	89.6	3878
SAR	7.8	40.5
SAR adj	19.8	101.25
RSC	9.7	21.60
Soluble ions (me L^{-1})		
Na^+	10.4	51.2
K^+	0.2	0.4
Ca^{2+}, Mg^{2+}	3.6	3.21
Cl^-	0.7	13.75
CO_3^{2-}	–	1.5
HCO_3^-	12.8	21.75
SO_4^{2-}	0.4	17.35

Table 15.2 Characteristics of soil of BSRS–I (Lahore), and II (Faisalabad)

Soil characteristics	Range	
	BSRS–I	BSRS–II
Soil texture	Sandy clay loam	Sandy loam
Clay (%)	18–23	12.5–15.5
Silt (%)	52–57	16.5–19.5
Sand (%)	23–25	65–71
EC (dS m^{-1})	1.25–2.22	12–27.24
pH	10.4–10.5	7.82–8.92
Bulk density (g cm^{-3})	–	1.38–1.58
$CaCO_3$ (%)	–	12–23
$CaSO_4.2H_2O$ (%)	0.065–0.189	2.56–4.15

2 Changes in Soil Physical Characteristics

Plants generally influence the physical properties of the soils like soil porosity, soil hydraulic permeability (Kfs), bulk density, soil water retention and soil structural stability (Marschner 1995). These properties can be improved by the cultivation of salt tolerant plants through their different activities.

Roots of plants are necessary to maintain the soil structure and cultivation of plants having lower depths are responsible in developing macropores in the soil profile, due to which soil porosity improves (Czarnes et al. 2000; Yunusa and Newton 2003). Roots are also responsible in removing the entrapped air from the soil pores (Tisdall 1991). They also facilitate the Na leaching and replace it with other cations from the deeper layers of the soil which is triggered by deep-rooted vegetation that can withstand different levels of salinity during phytoremediation. Akhter et al. (2004) has reported 15% increase in soil porosity by the cultivation of Kaller grass for five years on salt-affected soils. Similarly, Yunusa and Newton (2003) have reported that cultivation of salt tolerant plants improves the physico-chemical properties of soil and help remove subsoil salt contamination. Soil porosity is significantly enhanced by the rooting system of these plants. It has been proved that deep tillage is beneficial in ameliorating subsoils having low porosity, but these benefits are not permanent without vegetation cover (Cresswell and Kirkegaard 1995). Roots of some plant species have potential to act as tillage tools which is called biological drilling. It is proving as a promising alternative to deep tillage necessary to ameliorate the dense subsoils. Biological drilling has two stages: (a) creation of macropores in the subsoil by the penetration of roots in the compact soil layers followed by their decay resulting in an improvement of gaseous diffusion and water movement (b) benefits for the subsequent crop(s) after improvements in subsoil macroporosity (Cresswell and Kirkegaard 1995). Some plants like *Atriplex*, *Suaeda fruiticosa*, *Paspalum notatum* and *Festuca arundinacea* have ability to grow on compact soil layers due to their deep and strong root systems as a result of which soil porosity is improved. Field experimentation with *Atriplex* and *Suaeda* showed that their cultivation on salt-affected soils is beneficial because of their strong rooting system

which penetrates into soils with low-permeability (Ashraf 2007). A proper rooting system in guar changes the physical properties of salt-affected soils as reported by Ashraf et al. (2005b). Studies on guar indicated that rotation of plants with high root volume, dry weight and high tap root diameter and length are tolerant to salinity; they have certainly high ability for deep tillage (Ashraf et al. 2006b). Rotation of salt tolerant plants like *Sesbania* with crops like wheat is also beneficial for phytoremediation. A field study conducted at BSRS–II, (NIAB, Faisalabad, Pakistan), indicated that rotation of the deep rooted plants species such as *Brassica* with Kaller grass improves porosity of the saline soil (Ashraf et al. 1999, 2006b). So the plants with active and strong rooting system can be perfectly used for the remediation of salt-affected soils.

Deep-rooted perennial grasses (such as *Cenchrus* and *Pennisetum* spp.) and legumes (*Acacia*) can improve the hydraulic properties of saline soils (Akhter et al. 2004). Observations from the field studies have revealed beneficial effects of root growth in saline soils during phytoremediation. Ashraf et al. (2006a) found that deep-rooted *Acacia* species ameliorate a low-permeability hard saline sodic soil which results in an increase in saturated hydraulic conductivity. *Acacia* roots penetrated as deep as 2 m as compared to 1.1 m in *Atriplex*. They proposed inclusion of deep-rooted crops such as *Acacia*, *Atriplex*, *Suaeda fruticosa* in mixed cropping systems as a potential biological drilling strategy to improve subsoil permeability (Table 15.3).

Studies conducted by Akhter et al. (2004) to examine cultivation of Kallar grass for different periods (from 1 to 5 years) on the soils with different characteristics has enlightened the fact that hydraulic permeability of the soil enhances in the upper depth (0–20 cm). The maximum value for hydraulic permeability is 55.6 mm d^{-1} after five years of cultivation of Kallar grass while it is minimum (0.35 mm d^{-1}) in uncropped plots. The increase is 159 fold after 5 years followed by 101.6, 43.8, 25.1, and 6.1 after 4, 3, 2 and 1 year of cropping, respectively. The increase in soil hydraulic permeability (K_{fs}) is due to the improvement in soil structural stability and porosity along with reduction of sodium adsorption ratio (SAR). After 5 years the K_{fs} rate of soil was the maximum with structural stability index value of 96%, porosity 42% and SAR value of 29. The main reason for these changes is due to the extensive root system Kallar grass possesses, which has the capacity to penetrate into the soil up to 1 m deep. In calcareous sodic soil, hydraulic permeability is maintained only during cropping and it decreases in non-cropped soil. A significant increase in hydraulic conductivity was noted by Gupta et al. (1989) who planted rice in highly alkaline soil. Meek et al. (1990) recorded higher infiltration rates with alfalfa as compared to cotton. To improve the physical properties of highly saline sodic soils, planting Kallar grass and other such salt tolerant plant species is recommended by many workers (Akhter et al. 1988; Gupta et al. 1989; Meek et al. 1990). Ilyas et al. (1995), however, reported that irrigation with poor quality caused adverse effects on hydraulic permeability of good soil. All these findings stress the fact that plantation with salt tolerant plant species on salt-affected soils is beneficial for improving hydraulic permeability of the soil due to which interactive processes like soil porosity, structural stability, organic matter and leaching of salts to lower surface of the soil increases.

Table 15.3 Influence of growing Kallar grass for different time periods on physical properties of a saline sodic soil

Growth year	Soil depth (0–20 cm)	(40–60 cm)	(80–100 cm)	Mean (years)
Available water				
0	0.155	0.151	0.153	0.153
1	0.175	0.173	0.170	0.173
2	0.184	0.183	0.183	0.183
3	0.195	0.191	0.199	0.195
4	0.216	0.199	0.211	0.212
5	0.214	0.203	0.212	0.210
Mean (depths)	0.19	0.185	0.188	
Stability index				
0	31.9	18.6	32.6	27.7
1	57.6	36.0	34.1	42.6
2	66.8	64.7	71.2	67.6
3	68.4	50.5	55.4	58.1
4	119.4	66.8	76.5	87.6
5	150.6	47.3	90.8	96.2
Mean (depths)	82.5	47.3	60.1	
Bulk density (Mg m^{-3})				
0	1.62	1.73	1.68	1.68
1	1.61	1.72	1.60	1.64
2	1.58	1.65	1.59	1.61
3	1.55	1.59	1.56	1.56
4	1.54	1.53	1.55	1.54
5	1.53	1.53	1.54	1.53
Mean (depths)	1.57	1.62	1.59	
Porosity (%)				
0	28.9	34.6	36.5	36.7
1	29.1	35.3	39.7	38.0
2	40.4	37.7	40.0	39.4
3	41.5	40.1	41.3	41.0
4	42.3	41.5	41.9	41.9
5	42.8	42.2	42.4	42.2
Mean (depths)	40.8	40.8	40.3	

Values for different depths are means of three determinations

Due to root proliferation, soil bulk density (BD) reduced in all soils studied by us. However, Miyazaki (1996) has reported that soil BD is also changed by natural processes such as shrinkage with drying, consolidation with drainage and swelling with infiltration. He has pointed out that greater the BD of a soil (or alternately less the soil porosity) smaller is the saturated hydraulic permeability. According to Meek et al. (1992) an increase in BD from 1.7 to 1.89 Mg m^{-3} decreases the infiltration rate by four times and increases resistance to penetration by three times under cropping. A linear relationship has been found between log(K_{fs}) and total porosity

of the soil. The effectiveness of the biological model for improving soil physical properties such as soil bulk density is well documented (Toy and Shay 1987; Glauser et al. 1988; Costa et al. 1991). The cropping practices with Kaller grass for 5 years reduces the BD of soil by 8.9% compared to the uncropped plot. The BD reduction percentages are 2.3, 4.2, 7.1, 8.3 and 8.9% after 1 to 5 years respectively, compared to the uncropped plots (Table 15.3). Generally, growing of Kallar grass for 5 years has more pronounced effect on soil BD than other treatments. The values for soil BD decrease gradually from 1.67 to 1.52 Mg m^{-3} (Akhter et al. 2004). Our results indicated that change in soil BD differed with depth. The highest reduction of 3.1% was recorded for soil layer at 80–100 cm. The activity of rooting system is largely dominant in the upper depths (0–20 cm) after 1 year and roots are well distributed through the deepest soil depth (80–100 cm) during subsequent growth periods. Reduction in soil BD is a beneficial character for remediation of salt-affected soil as a result of which soil becomes suitable for conventional food crops.

Akhter et al. (2004) found that cultivation of Kallar grass enhances the water retention by soil at various tensions. More prominent effect is observed in the upper soil layers as compared to the deeper layers (Table 15.3). This is due to larger root activity in top layer of soil which leads towards the improvement in soil porosity, organic matter and other soil characteristics at a faster rate in the upper than the deeper layers of soil. Increase in soil water retention enhances availability of water for plants during cropping. For plants, water is available between field capacity and permanent wilting point (Cassel and Nielsen 1986), which can be estimated by measuring relative differences within and among soils. Availability of water (AW) significantly increases with cultivation of Kallar grass compared to uncultivated control (Akhter et al. 2004). The AW has a positive relationship ($r = 0.922^{**}$) with soil organic matter content. Water retention of 2 mm sieved soil samples increases with increasing organic carbon content at suctions from 10 to 1500 KPa. With an increase in organic matter (OM) soil water holding capacity increases, consequently AW increases (Bauer and Black 1992; Darwish et al. 1995). Results of Akhter et al. (2004) show strong correlations between AW and soil porosity, structural stability and hydraulic permeability which may affect the soil AW indirectly. Querejeta et al. (2000) has reported that addition of organic matter and mechanical terracing with sub-soiling increased the water storage of the soil profile which is due to improvement in soil structure and permeability.

There are many reports (Haynes and Francis 1993; Chenu et al. 2000) which indicate increases in aggregate stability by growing different crops in different types of soils. A positive relationship between soil carbon and increase in stable aggregates under cropping has been reported by Bruce et al. (1992). Considerable improvement has been recorded in soil structure by growing forages (Perfect et al. 1990; Haynes and Francis 1993). The reason for this is high root biomass, root length and dense rooting system. Akhter et al. (2004) studied effect of Kallar grass cultivation on structural stability of salt-affected soils which was measured as stability index in Kallar grass plots and noted that it was 54% of uncropped control plots after one year and increased up to 247% after 5 years (Table 15.3). Structural stability index increase rate was 13.4 per year after growing Kallar grass. However,

soil depth significantly affected the soil structural stability index which was 82.5, 47.3 and 60.1 for the soil depths i.e., 0–20 cm, 40–60 cm and 80–100 cm, respectively (Table 15.3). Caron et al. (1992) also noted large increase in soil aggregate stability by growing bromegrass for 3 years. Different studies indicate that plantation of salt tolerant plants significantly influences the structural stability of the soils, particularly the water stable aggregates (Tisdal and Oades 1980; Ried and Goss 1981).

3 Changes in Soil Chemical Characteristics

Plants influence the chemical characteristics of the soils like soil pH, electrical conductivity (EC), sodium adsorption ratio (SAR) and soil organic matter. Cultivation of salt tolerant plants improves all these characteristics. For example, Kallar grass grown on salt-affected soil up to 5 years significantly reduced the soil salinity up to 71% compared with control (Table 15.4). The highest reduction is 87% after 5 years of growth followed by 80, 84, 65 and 42% after 4, 3, 2 and 1 year, respectively, as compared to uncropped plots. Soil salinity markedly reduces from 16.2 to 2.1 dS m^{-1} (Akhter et al. 2003). The reduction in EC occurs chiefly due to the leaching of salts to deeper layers of the soil (Bhatti and Wieneke 1984). In contrast to above findings, field studies of 3 years with *Acacia* species and *Atriplex lentiformis* indicate that EC of the soil gradually decreases within 2 months after cultivation of *Acacia nilotica* and *Atriplex lentiformis*, while it increases in the case of *Acacia ampliceps* (Ashraf et al. 2006b). After 20 months of growth period, the highest EC values have been recorded for *Acacia ampliceps* followed by *Atriplex lentiformis* and *Acacia nilotica*. After 2 years of planting, a significant decrease in soil EC has been recorded which is maximum under *Acacia nilotica* plantation. Salinity of the soil fluctuated up to 36 months of planting but was lowest under *Acacia nilotica* (Ashraf et al. 2006a). Shekhawat et al. (2006) reported that the cultivation of *Haloxylon recurvum* reduced soil EC by 56 to 85% which varied with the depth of soil. Maximum decrease in soil EC took place in the upper layer (10–20 cm depth) and minimum at 40–50 cm soil depth. They reported that by cultivating *Suaeda nudiflora*, 60 to 85% change in soil EC took place. These changes were again higher in 10–20 cm soil layer and lower in 40–50 cm soil layer. Ashraf (2007) has also reported similar observations for *Suaeda fruiticosa* and *Atriplex*. Another study conducted by Yensen and Biel (2006) on the soil remediation through salt-conduction plants indicates that cultivation of *Distichlis*, *Spartina*, *Aeluropus* is beneficial to reduce the soil EC. Due to root activities, improvement in soil permeability has been recorded in soil under cultivation of all the plants mentioned above due to decreased EC in the upper soil layers. Therefore in order to reduce the soil salinity, cultivation of *Leptochloa fusca*, *Haloxylon recurvum*, *Suaeda nudiflora*, *Distichlis*, *Spartina* and *Aeluropus* can be recommended.

Results of the experiments on Kallar grass showed that soil pH decreases due to Kallar grass plantation (Table 15.4). The maximum decrease of 14.4% in pH was observed after 5 years with an average decrease rate of 0.229 units per year in case of

Table 15.4 Changes in chemical properties of a saline sodic soil by growing Kallar grass for different time periods

Growth year (T)	Soil depth (0–20 cm)	(40–60 cm)	(80–100 cm)	Mean (years)
Electrical conductivity (dS m^{-1})				
0	22.0	22.2	12.5	18.9
1	12.6	14.0	6.3	11.0
2	7.4	9.7	3.1	6.7
3	3.2	3.8	2.4	3.1
4	2.8	3.8	4.8	3.8
5	2.0	2.1	3.2	2.4
Mean (depths)	8.3	9.3	5.4	
Soil pH				
0	10.4	10.5	10.4	10.4
1	9.3	9.2	9.5	9.5
2	9.1	9.4	9.3	9.3
3	9.2	9.5	9.4	9.4
4	9.1	9.6	9.7	9.3
5	8.9	8.9	9.0	8.9
Mean (depths)	9.3	9.5	9.6	
Sodium adsorption ratio				
0	185.5	187.2	114.7	162.5
1	70.6	97.6	78.7	82.3
2	65.9	91.5	74.1	77.2
3	32.5	53.0	35.8	40.4
4	25.8	47.5	25.0	32.8
5	20.7	41.2	25.4	29.1
Mean (depths)	66.9	86.4	59.0	
Organic matter (g kg^{-1})				
0	3.3	1.9	1.8	2.3
1	3.2	8.9	2.8	4.9
2	5.5	11.7	3.4	6.8
3	7.3	10.7	2.6	6.9
4	6.3	11.9	2.9	7.0
5	7.4	13.3	3.8	8.2
Mean (depths)	5.6	9.6	2.9	

Values for each depth are means of three replicates

growing Kallar grass. Usually, soil pH is different at different depths of soil profile which generally increases with increase in soil depth. Another study conducted with different species of *Acacia* and *Atriplex* indicated that soil pH did not change with the passage of time, which was alkaline at the outset of the trial, and was similar after three years of continuous cultivation (Ashraf et al. 2006a). However, reduction in soil pH was noted by Helalia et al. (1992) due to plantation with *Echinochloa stagninum* on saline soil. The reduction in soil pH may be directly related to root H^+, OH^-, HCO_3^- and organic anions which react with soil exchangeable ions or

complexes, consequently disturbing cations and anions equilibria in the soil (Helyar and Poster 1989). It has also been observed that microbial activity increases by root respiration and by root exudates, as a result of which organic matter is added by vegetation which is responsible for change in soil solution quality thus changes in soil pH occur (Dormaar 1988). Uptake of NH_4^+ by the plants could also decrease soil pH considerably (Gorham et al. 1985). Efflux of H^+ from roots is commonly observed in roots of the plants growing under saline conditions which results in the reduction of soil pH. This reduction facilitates uptake of macro- and micro-nutrients due to which increase in growth and yield of the crop is expected when grown on these soils after phytoremediation.

Studies with Kallar grass indicated that SAR of soil decreases with the growth, however, reduction is more prominent in the upper layers of soil. Use of saltish water particularly in saline sodic soils raised the soil SAR at lower depths (80−100 cm) due to leaching of Na from upper layers and its ensuing accumulation in the middle soil depths (Table 15.4). Reduction in SAR in upper (0–15 cm) soil layers as compared with lower soil layers by cropping system has been found by many workers (Hussain et al. 1994; Chang and Leghari 1995). Kallar grass plantation up to 3 years significantly decreased the soil SAR, therefore, its cultivation was continued up to 5 years as a result of which a further reduction in SAR value of highly saline sodic soils was recorded. So, cultivation of Kallar grass on salt-affected soils is beneficial in removing and leaching of Na^+ from soil solution and exchange complex (Akhter et al. 2003). In contrast to these findings, Shekhawat et al. (2006) did not find any appreciable change in SAR with the cultivation of *Salsola baryosma*, *Haloxylon recurvum* and *Suaeda nudiflora*. However, studies with different species of *Acacia* and *Atriplex* also indicated that soil SAR decreased with cultivation of these species and effect of *Acacia nilotica* was more pronounced than others (Ashraf et al. 2006a). Similarly, results of the experiments conducted with *Sporobolus arabicus*, *Leptochloa fusca*, *Suaeda fruticosa*, *Atriplex lentiformis* and *Kochia indica* also confirmed that cultivation of salt tolerant plants is effective in reducing the soil SAR (Ashraf 2007).

It is a well established fact that vegetation cover on any type of soil increases organic matter (OM) content of the soil. So any type of vegetation on salt-affected soils is effective in enhancing its OM content. Akhter et al. (2004) reported that cropping with Kallar grass increased the OM of salt-affected soil significantly. Nelson et al. (1996; 1997) found that retention of OM would be improved if added after the reduction in soil SAR or ESP. Barzegar et al. (1997) observed improvement in soil aggregates stability due to the addition of plant residues which increased the OM content and reduced the soil sodicity. According to Akhter et al. (2004) addition of OM (8.2 g kg^{-1} of soil) after the cultivation of Kallar grass for 5 years, it reduced with the soil depth. Another study with *Acacia* species indicated that their cultivation on salt-affected soils increased the soil OM (Ashraf et al. 2006a). Similarly, Aganga et al. (2003) found an increase in soil OM with cultivation of *Atriplex* on salt-affected soils. *Suaeda salsa* plantation has been found effective in enhancing the OM content of saline soils (Zhao 1991). So, the cultivation of salt tolerant plants is beneficial in improving the OM of salt-affected soils.

4 Removal of Salts from Soil

Phytoremediation means the introduction of salt removing plant species on salt-affected soils to reduce salt content and to improve sustainability of salt affected soils. Salt-affected soils contain excessive Na^+ which is toxic to plants. A single plant of *Suaeda fruticosa* can remove 100 g of salt mainly by accumulating high amount of salts in its aerial parts. Shekhawat et al. (2006) conducted experiments with salt tolerant plants viz. *Salsola baryosma*, *Haloxylon recurvum* and *Suaeda nudiflora* and reported that after 3 months of growth period *Haloxylon recurvum* removed the highest Na^+ (17 g plant^{-1}) and maintained the highest biomass followed by *Suaeda nudiflora* (15.6 g plant^{-1}) and *Salsola baryosma* (9.6 g plant^{-1}). Zhao (1991) has reported that reduction in Na^+ was higher in the upper soil layer (20–30 cm) by *Salsola salsa*.

The root activity of halophytes in saline soils may affect the mobilization of native lime in the soil. Robbins (1986) reported that CO_2 produced due to the root respiration may be a one of the primary factors contributing to remediation of salt-affected lands, because in the presence of H_2CO_3, solubility of $CaCO_3$ increases. The released Ca^{2+} thus replaces the Na^+ from the soil exchange complex. Later along with other salts present in excessive amounts in the soil may be carried away from the root zone through excessive supply of good quality water. Shekhawat et al. (2006) have reported that cultivation of *Suaeda nudiflora* is effective in increasing the exchangeable Ca^{2+} in the soil, but most effective plant is *Haloxylon recurvum* followed by *Suaeda nudiflora* and *Salsola baryosma*. Plantation of these halophytes is effective in changing the EC, pH, exchangeable Na^+ and Ca^{2+} and exchangeable sodium percentage of the soils.

Qadir and Oster (2004) conducted 14 experiments to compare the remediation of salt-affected soils by chemicals and through vegetation and reported that soil amendment with gypsum reduced 62% of sodicity levels while it was 52% by phytoremediation. The reduction in sodicity due to phytoremediation of salt-affected soils may be less due to off season cultivation of salt tolerant plants. The change in results may be due to availability of limited irrigation during growth period, which is necessary for the downward movement of Na^+ otherwise phytoremediation is more effective than chemical amendments in reducing the soil salinity or sodicity.

Akhter et al. (2003) reported that cultivation of Kallar grass on salt-affected soils significantly reduces the soil Na^+ content (Table 15.5). The reduction in soil Na^+ is 70.5% after 5 years cultivation with Kallar grass when compared with uncultivated control plots. They reported that soil Na^+ content significantly decreases by 38.0, 62.0, 81.3, 86.6 and 84.5% as compared with control after 1, 2, 3, 4 and 5 years, respectively. The cation (Ca^{2+}, Mg^{2+} and K^+) content of soil also significantly reduces after 5-year growth of Kallar grass (Table 15.5). Before the cultivation of Kallar grass soil Ca^{2+}, Mg^{2+} and K^+ are 56, 16.8 and 28.5 mg kg^{-1} which reduces to 20.0, 3.6 and 11.7 mg kg^{-1} after 5 years of cropping with Kallar grass which are earlier 64.3, 78.6 and 80% respectively. However, reductions in these ions vary with soil depths which were 35.3, 40.0 and 45.5% Ca^{2+}, Mg^{2+} and K^+, respectively at 80–100 cm of soil depth and 11.8, 20.0 and 36.4% at 40–60 cm of soil depth higher

Table 15.5 Concentration of soluble cations in saturation extract of soil at different depths as a function of growing Kallar grass for different time periods

	Soil depth			
Growth year	(0–20 cm)	(40–60 cm)	(80–100 cm)	Mean (years)
Na^+ (me L^{-1})				
0	207	226	128	187
1	116	136	96	116
2	73	101	40	71
3	26	38	40	35
4	18	38	16	25
5	23	46	18	29
Mean (depths)	77	98	56	
Ca^{2+} (me L^{-1})				
0	3.7	2.6	2.0	2.8
1	2.0	2.0	1.9	2.0
2	1.3	1.4	0.4	1.0
3	0.9	0.7	1.2	0.9
4	0.6	1.0	0.5	0.7
5	1.4	1.0	0.6	1.0
Mean (depths)	1.7	1.5	1.1	
Mg^{2+} (me L^{-1})				
0	1.8	1.3	1.0	1.4
1	0.5	1.0	0.6	0.7
2	1.2	1.1	0.2	0.8
3	0.4	0.3	1.3	0.7
4	1.1	1.5	0.4	1.0
5	0.4	0.3	0.3	0.3
Mean (depths)	1.0	0.8	0.6	
K^+ (me L^{-1})				
0	1.3	1.8	1.3	1.5
1	3.0	0.5	0.5	1.3
2	0.7	0.7	0.7	0.7
3	0.7	0.7	0.7	0.7
4	0.3	0.5	0.3	0.4
5	0.4	0.2	0.2	0.3
Mean (depths)	1.1	0.7	0.6	

Values are means of three replicates

in the levels of Ca^{2+}, Mg^{2+} and K^+, respectively, in comparison with surface layer (0–20 cm).

In another study conducted by Ashraf et al. (2006a) with five *Acacia* species and *Atriplex* as check indicated that Na^+ decreased with the cultivation of different species which increased with increase in time and was maximum after 3 years while increase in Ca^{2+} and K^+ was observed in soil due to the cultivation of different *Acacia* species which was the highest after 3 years of their cultivation. Krishnapillai and Ranjan (2005) found reduction in soil Na^+ contents due to the cultivation of *Atriplex* in salt-affected soils. Some reports (Hussain et al. 1994; Chang and Leghari

1995) show that cultivation of salt tolerant plants helps restore soil structure and permeability. They do this through deep penetration of their roots and solubilization of soil $CaCO_3$ thereby leading to enhanced salt leaching and reduced salinity and alkalinity of saline or sodic soil. These reports also indicate that many economic crops and native halophytic plant species resulted in high removal of soil Na^+, effective in mobilization of naturally occurring insoluble $CaCO_3$, reduces the soil pH by increasing CO_2 solublization and its release due to the activities of plant roots. So, cultivation of halophytes and grasses are effective in reducing the soil salinity and alkalinity through different mechanisms.

Salt-affected soils in addition to cations also contain excessive anions like Cl^-, SO_4^{2-} and HCO_3^- which are toxic to plants and reduce growth and plant productivity. Cropping with halophyte grasses and other plants is helpful in removing these anions from soil profile (Crescimanno et al. 1995). It was found that cultivation of Kallar grass is effective in reducing the anions significantly (Akhter et al. 2003). The reduction in Cl^-, SO_4^{2-} and HCO_3^- was 88.4, 88.6 and 90.9% respectively after 5 years of Kallar grass cultivation. Levels of Cl^-, SO_4^{2-} and HCO_3^- in soil solution

Table 15.6 Concentration of soluble anions in saturation extracts of soil at different depths as a function of growing Kallar grass for different time periods

	Soil Depth			
Growth year	(0–20 cm)	(40–60 cm)	(80–100 cm)	Mean (year)
Cl^- (me L^{-1})				
0	62.1	72.5	40.7	58.4
1	32.7	44.7	33.6	37.0
2	20.3	29.3	16.6	22.1
3	9.7	12.6	9.0	10.4
4	8.2	11.6	7.5	9.1
5	6.0	8.0	6.0	6.8
Mean (depth)	23.2	29.8	18.8	
SO_4^{2-} (me L^{-1})				
0	46.7	76.0	28.8	50.8
1	55.2	71.3	24.2	50.2
2	22.2	39.4	13.5	25.0
3	12.5	16.2	9.4	12.7
4	10.2	13.8	10.0	11.3
5	5.6	10.1	4.7	6.8
Mean (depth)	25.4	37.8	15.1	
HCO_3^-(me L^{-1})				
0	103.4	101.4	68.3	91.0
1	36.1	50.5	15.4	34.0
2	23.1	37.8	11.0	24.0
3	12.3	15.8	14.8	14.3
4	6.8	8.0	13.4	9.4
5	6.2	3.6	15.0	8.3
Mean (depth)	31.3	36.2	22.9	

Values are means of three replicates

reduced at rates of 0.449, 0.435 and 0.467 me L^{-1} $year^{-1}$. Reductions in concentration of these anions varied with the soil depth and the highest reduction 36.9, 60.1 and 36.7% (Cl^-, SO_4^{2-} and HCO_3^-, respectively) was noted at 80–100 cm soil depth followed by 22.1, 32.8 and 13.5% at soil depth of 40–60 cm when compared with the soil depth of 0–20 cm (Table 15.6). According to Qadir and Oster (2004) soil chemical properties have significant correlations with the removal of salts by the planting of salt tolerant plants, because the changes in both soil EC and pH depend on the concentrations of Na^+, Ca^{2+}, Mg^{2+}, K^+ and Cl^-, and HCO_3^- present in the salt-affected soils. Similarly a high correlation of SAR exists with most of the soil chemical properties indicated above. However, Akhter et al. (2003) have reported a negative correlation between all chemical properties and soil organic matter content mentioned above.

5 Improvement in Soil Fertility

There are many reports (Ashraf et al. 2006a, b; Qadir et al. 2006; Shekhawat et al. 2006) which indicate that phytoremediation of salt-affected soils improves their fertility. After phytoremediation, availability of nutrients to the subsequent crop should increase. Qadir et al. (2006) compared the effect of phyto- and chemical remediation of salt-affected soils for nutrient availability to the subsequent crops and reported that cropping with Kallar grass, *Sesbania* and sordan grass cultivation for 15 months significantly increased P, Zn and Cu availability while addition of gypsum in the non-cropped soil reduced the availability of these inorganic elements. Nitrogen (N) contents of the soil increased where *Sesbania* was cultivated which was due to nitrogen fixing ability of the crop. According to their findings, the amount of N increased from 0.49 to 0.53 g kg^{-1} in 15 months, however, they did not note any appreciable change in soil K^+ contents. Cultivation of *Sesbania* for 45 days and then its use as green manuring, enriched the salt-affected soils by adding up to 122 kg N ha^{-1} which was available for the next crop. Evidence of N conservation has also been provided by other phytoremediation-oriented crops like Kallar grass (Malik et al. 1986). However, N losses via NO_3 leaching were recorded during the remediation of saline sodic soils with chemical amendment like gypsum (Qadir et al. 1997). Singh and Gill (1990) conducted experiments with tree species viz. *Prosopis juliflora*, *Acacia nilotica*, *Eucalyptus tereticornis*, *Albizia lebbeck*, and *Terminalia arjuna* and reported that a considerable reduction in pH and increase in organic matter (organic C) content, and available P and K content in 0–15 cm soil depth occur due to the cultivation of these species.

Appreciable changes in soil microbial biomass were recorded by the plantation of halophytes especially leguminous plant species like *Acacia* and *Sesbania* (Batra et al. 1997; Ashraf et al. 2006a). Addition of microbial biomass in soils is beneficial to increase the soil organic matter and nutrients. The microbial activity in the salt-affected soils is often very low due to the absence of vegetation cover. It is measured as dehydrogenase activity (DHA) showing how much microbial population is present. As a result of CO_2 generation through respiration and decomposition of organic matter, overall microbial activity can also be estimated

in the soils under cultivation (Włodarczyk et al. 2002). Batra et al. (1997) studied chemical and phytoremediation effects on DHA of the saline soil. They used gypsum (14 Mg ha^{-1}), Karnal grass, sorghum + gypsum, rice + Sesbania + gypsum for sodic soil, and found that DHA was greater in those soils where Karnal grass was cultivated. Earlier, Rao and Ghai (1985) also reported significant increase in DHA of sodic soil by the permanent cultivation of grasses. Rao and Pathak (1996) reported that green manuring with *Sesbania* increased DHA and urease activity of salt-affected soils. Garg (1998) studied changes in sodic soil with the cultivation of four tree species, i.e., *Acacia nilotica*, *Delbergia sissoo*, *Prosopis juliflora* and *Terminalia arjuna* and reported that *Delbergia sissoo* and *Prosopis juliflora* were more effective in terms of producing high biomass production and reducing soil sodium contents. Higher microbial activity in the upper 0–60 cm soil depth was recorded due to cultivation of these species because of humus accumulation by the decay and decomposition of plant litter and root decay that led to increase in soil organic carbon. The rate of soil carbon increase was low for the first 2–4 years, thereafter it was exponential from 4–6 years and a plateau during the period from 6 to 8 years. Bhojvaid and Timmer (1998) also found that cultivation with *Prosopis juliflora* on sodic soil increased organic carbon of the upper 1.2 m soil layer from 11.8 to 13.3 Mg C ha^{-1} after 5 years, 34.2 Mg C ha^{-1} after 7 years and 54.3 Mg C ha^{-1} after 30 years. Average annual increase rate in soil organic carbon was 1.4 Mg ha^{-1} in 30 years. Plants used for phytoremediation of salt-affected soils showed wide range in their decomposition and turnover rates as a result of C stored in the soil (Torn et al. 1997; Kiem and Koegel-Knabner 2003; Sahrawat 2004; Sariyildiz and Anderson 2003; Six et al. 2002). Sahrawat (2003) and Sahrawat et al. (2005) reported that green manuring in salt-affected soils also increases the soil organic matter through microbial activity which affects the C sequestration via soil inorganic C.

6 Selection of Plants for Phytoremediation

Plants having capability to remove the maximum quantity of salts by producing higher biomass with some economic importance are mainly selected for phytoremediation (Qadir and Oster 2002). This selection is by and large based on their ability to resist to the high levels of soil salinity. Several plant species including, grasses, shrubs and trees are being used for phytoremediation of salinised soils. The plants identified at NIAB, Faisalabad, Pakistan are summarized in Tables 15.7 and 15.8. Kallar grass has been widely recommended by many workers (Kumar and Abrol 1984; Malik et al. 1986; Akhter et al. 2003), however, different workers recommend different plants keeping in view the soil texture, and physico-chemical properties of salt laden soils. Oster et al. (1999) and Robbins (1986) worked on grasses and recommended Bermuda and Sordan grasses respectively, similarly *Sesbania* and alfalfa have been recommended by Ahmad et al. (1990) and many others. These produce high biomass and have high salt tolerance and are recommended for the remediation of salt-affected soils. Other species are shrubs like *Kochia scoparia* (Garduno 1993), *Atriplex* and *Maireana* (Barrett-Lennard 2002), *E. crusgalli* (Aslam et al.

Table 15.7 Salt tolerant grasses and shrubs identified for phytoremediation

Plant species	Root zone salinity causing 50% yield reduction	
	EC (dS m^{-1})	% salt
Grasses		
Leptochloa fusca	22.0–14.6	1.41–0.93
Sporobolus arabicus	21.7	1.39
Cynodon dactylon	21.0–13.2	1.34–0.84
Hordeum vulgare	19.5–10.0	1.25–0.64
Sorghum. vulgare	16.7–15.0	1.07–0.96
Panicum antidotale	16	1.02
Echinochloa crusgalli	15.9	1.02
Polypogon monspeliensis	13.7	0.88
Avena sativa	11.8–9.1	0.76–0.58
Lolium multiflorum	11.2	0.72
Echinochloa colona	11.2	0.72
Desmostachya bipinnata	9	0.64
Panicum maximum	9.0–8.5	0.58–0.54
Sorghum halepense	7	0.45
Shrubs		
Suaeda fruticosa	48	3.07
Kochia indica	38	2.43
Atriplex nummularia	38	2.43
Atriplex amnicola	33	2.11
Atriplex lentiformis	23	1.47
Atriplex undulate	22.5	1.44
Atriplex crassifolia	22.5	1.44
Sesbania Formosa	21.4	1.37
Beta vulgaris	19	1.22
Lotus carniculatus	16.7	1.07
Trifolium alexandrinum	15.8	1.01
Sesbania aculeate	13	0.83
Hasawi rushad	12.5	0.8
Medicago sativa	13.2–12.2	0.84–0.78
Sesbania rostrata	12	0.77
Macroptilium atropurpureum	12	0.77
Trifolium resupinatum	11.6	0.77

1987), *Portulaca oleracea* (Grieve and Suarez 1997), *Salicornia bigelovii* (Glenn et al. 1996), and *Glycyrrhiza glabra* (Kushiev et al. 2005). Many trees have also been recommended. Phytoremediation with trees and grasses is beneficial because these can be utilized as fodder, timber, fuel (Chaudhry and Abaidullah 1988; Sandhu and Qureshi 1986). Qureshi et al. (1993) suggested agroforestry systems consisting of mainly tree species and cultivation of salt tolerant crop varieties, as the most economically viable approach for phytoremediation because production of fuel-wood, and timber is a demand of local market and cultivation of grasses can fulfill fodder shortage and fetch reasonable prices in local markets.

Table 15.8 Salt tolerant vegetables and trees identified for phytoremediation

	Root zone salinity causing 50% yield reduction	
Plant species	EC (dS m^{-1})	% salt
Vegetables		
Aster tripolium	31.7	2.03
Brasssica napus	19.5	1.25
Trigonella foenum–graceum	19.2	1.23
Spinacea oleracea	14.8	0.94
Medicago falcata	13.4	0.86
Brassica carinata	12.5	0.8
Brassica juncea	12.4−8.44	0.79−0.54
Lactuca sativa	9.9	0.63
Brassica campestris	9.8	0.63
Eruca sativa	9.4	0.6
Coriandrum sativum	5.7	0.37
Trees		
Acacia sclerosperma	38.7	2.48
Acacia ampliceps	35.7	2.28
Prosopis juliflora	35.3	2.26
Prosopis chilensis	29.4	1.88
Casuarina obesa	29.2	1.86
Acacia victoriae	28.3	1.81
Eucalyptus microtheca	27.9	1.78
Acacia nilotica	27.9	1.78
Acacia acuminate	27.7	1.77
Acacia cambagei	27.7	1.77
Eucalyptus striaticalyx	26.2	1.68
Acacia salicina	24.5	1.57
Prosopis cineraria	24.4	1.56
Casuarina glauca	24.4	1.56
Prosopis tamarugo	22.7	1.45
Acacia calcicola	19.9	1.27
Acacia coriacea	18.2	1.16
Cassia nemophila	16.8	1.07
Cassia sturtii	15.8	1.01
Acacia saligna	15.7	1
Acacia bivenosa	13.7	0.88
Acacia subtessarogna	13.7	0.88
Leucaena leucocephala	12.4	0.79
Acacia kempeana	11	0.7
Acacia aneura	9.5	0.61
Acacia cunnighamii	9.4	0.6
Acacia holosericea	9	0.78
Acacia adsurgens	4.3	0.27
Acacia validinervia	1.7	0.11

7 Conclusion

Most of the relevant literature and experiments conducted by different scientists have shown that phytoremediation is the most economical approach through which salt-affected wasteland can be successfully utilized for plant production. Toxic ions like Na^+ and Cl^- are removed by the salt tolerant plants used for phytoremediation, and addition of Ca^{2+}, K^+, P and N in the salt-affected soils occurs thereby improvement in the soil physico-chemical properties takes place and soils become fertile for subsequent crops.

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Chapter 16
Salts as Potential Environmental Pollutants, Their Types, Effects on Plants and Approaches for Their Phytoremediation

Murat Dikilitas and Sema Karakas

Abstract Demand for food dramatically increases as the world gets populated, and this problem is of central attention all over the world. Under these circumstances, the balance between food production and consumption depends on the agricultural productivity. However, an increase in the world population and decrease in the agricultural areas due to many reasons such as industrializations, global warming, use of marginal water etc. have been forcing us to use arable lands efficiently as well as saline-prone areas. Low fertile agricultural areas or non-agricultural areas have to be included into agricultural areas if the food production is to be increased. For this reason, many breeding and amelioration strategies have been evaluated so far, however, a few of them have been found successfully in achieving the goals. Physiological, genetical and biochemical mechanisms in plants are quite complex, therefore, it is very difficult to breed a resistant or tolerant plant against stress. To date, breeding or amelioration strategies have followed one direction, either chemical or biological, they then concentrated on either soil or plant itself, have been tested on a few plants in a few local research stations, e.g., use of mycorrhiza. An amelioration strategy both on soil and plant, which could possibly increase the crop production in saline or polluted areas, enable us to improve soil conditions for a long period of time with little effort and expenses. Salt concentration in the soil could be reduced via drainage as well as using high quality water. On the other hand, economically important crop plants have been bred for their resistance to disease and non-pathogenic stress agents such as drought and salinity and some of them have been made commercially available. However, this has not solved the problem globally, especially for the many crop plants which have to be grown in moderate saline conditions, therefore, an effective alternative approach must be found. In recent years, a new method called "bioreclamation" or "phytoremediation" has been introduced in many scientific works

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and reports. It is one of the efficient methods to improve crop production and quality in saline areas aiming to grow halophytes as companion plants with the crop plants. In this chapter, the effect of salt on plants and plant metabolisms and their phytoremediation strategies have been evaluated so that halophytes could possibly be used as companion plants with crop plants without retarding their growth in saline areas.

Keywords Salinity · Phytoremediation · Companion Plants · Halophytes · Salsola

Contents

1 Introduction

Many civilizations are dependent on crops such as rice, wheat, barley or corn for their survival. People not only rely on plants for their own food but they also use them for animal feed. So, everything we do is affected either directly or indirectly by plants. However, today, *ca*. 7% of the world's cultivated land and nearly half of all irrigated lands are affected by salinity (Dajic 2006). This is a substantial portion of the world's land. Additionally, secondary salinization also occupied 20% of the world's land. On the other hand, semi-arid regions in Asia, such as those irrigated by the Indus in Pakistan, the Tigris and Euphrates flowing through Syria and Iraq, and the Ganges system in the North-West of India have the worst salinity problems (McWilliam 1986; Dajic 2006). These figures might increase in the future and indicate the magnitude of the problem that must be tackled if future global food needs are to be met.

2 Soil Salinity

A soil is considered to be a three dimensional piece of landscape having shape (form), area, and depth (Soil Survey 1951). The concept of a soil as a profile

having depth but necessarily shape or area is also a common use of the term. Scofield (1942) and Campbell and Richards (1950) considered a soil to be saline if the electrical conductivity of a solution, extracted from a saturated soil paste, has a value of 4 mmhos cm^{-1} ($\cong$ 2.56 g L^{-1} dissolved salt, Maas and Hoffman 1977; Abrol et al. 1988), or more at 25°C, and the exchangeable-sodium-percentage is less than 15. Generally, the pH would be less than 8.5.

Saline soils are recognizable by the presence of white crusts of salts on their surface. The kinds and amount of salts present mainly determine the chemical characteristics of saline soils. The soluble salt consists of various proportions of the cations; Na^+, Ca^{2+}, and Mg^{2+} and the anions; Cl^-, and $SO_4{}^{2-}$. The cation K^+, and the anions $HCO_3{}^-$ and $CO_3{}^{2-}$ and $NO_3{}^-$ occur in minor amounts. Despite the essentiality of Cl^- as a micronutrient for all higher plants and Na^+ as a mineral nutrient for many halophytes, an increase in their concentration will result in toxicity to non-salt tolerant plants. So, Na^+, especially as NaCl, is the most significant of the salts causing salt stress in plants (Levitt 1972; Dikilitas 2003; Zapata et al. 2008). Other cations such as Ca^{2+} and Mg^{2+} are usually present in sufficient quantities to meet the nutritional needs of crops; they sometimes contribute to the salinity especially at the later stages of soil development (Flowers and Yeo 1986; Taiz and Zeiger 1991; Yilmaz and Kina 2008).

3 Salt Types and Reasons

Salts are a common and necessary component of soil, and many salts (nitrates and potassium) are essential plant nutrients. The salts that contribute to the problem of soil salinity are derived from various sources. Firstly, water that evaporates from the sea includes salt, which then falls as rain over inland areas and may deposit these 'cyclic salts' (Teakle 1937) in coastal regions. This source is considered to be the major cause of salt accumulation in the soil and groundwater of inland areas. Secondly, soils derived from inland seas that retreated about ten million years ago naturally contain large quantities of salts. Thirdly, the continued weathering of rocks, which involves hydrolysis, hydration, solution, oxidation, carbonation and other processes, release salts that become soluble (Abrol et al. 1988). These salts move from the more-humid- to the less-humid- and relatively arid areas, by means of ground-and stream water. In arid areas, over millions of years, they gradually concentrate due to lack of leaching and so produce salt affected areas. This may result in a salt desert. However, under humid conditions these soluble salts are transported to the oceans (Abrol et al. 1988). Tidal inundation of seawater also causes salinity in the low-lying areas of the world (Rowell 1994). Soil salinity in some areas results from the restricted drainage caused by the construction of roads and rail lines, or other developmental activities. Such activities may cause a high-ground water table or low permeability of soil (Abrol et al. 1988; Zhang et al. 2009). In addition to that, important source of salts may come from ice-melters used on roads and sidewalks. Marine salts may also be brought by an underground infiltration of sea-water (infiltrating salts) (Waisel 1972).

Accumulation of excess salts in the root zone causes partial or complete loss of soil productivity and this is the oldest and most serious environmental problem (McWilliam 1986; Zhu 2001). For example, the collapse of the Babylonian Empire is considered to be partly the result of failure of irrigated crops resulting from accumulation of salts (Hillel 1992). Although irrigation practices have increased agricultural productivity, it is now widely recognised that it has also contributed to the increasing salinization of agricultural lands (Sinha and Singh 1976; Boyer 1982; Shannon 1997; Zapata et al. 2008). For example, irrigation of crops with water of marginal quality due to competition between agriculture and demand by cities and industries for high quality of water also caused soil salinity (Wainwright 1984). The presence of even small concentration of salts in good quality irrigation water leads to salt accumulation in soils unless leached away by rain or irrigation water. On the other hand, intensive irrigation without adequate drainage results in a rise in the ground water level and capillary action draws up salts through the soil profile (Bridges 1997). It has been reported that there is more land going out of irrigation world-wide because of salinity then there is new land coming into irrigation (Vose 1983).

Salinity may also occur in soils or compost in glasshouses in the form of potassium, nitrate and chloride, resulting from the application of water that contains fertilizers, or from the accumulation of residues of fertilizers and liquid feeds in excess of crop needs (Epstein et al. 1980; Flowers 2004).

Salinity, whether natural or induced by man, is a widespread environmental stress that can limit growth and development of salt-sensitive plants. As salinity levels increase, plants extract water less easily from soil, thus aggravating water stress conditions and resulting in accumulation of elements that are toxic to plants. An increase in salinity causes nutrient imbalances and reduction in water infiltration.

The salinity problem is primarily associated with the arid and semi-arid regions of the world, where there is insufficient rain to leach away soluble salts (Fisher and Turner 1978). Most of the salts are left behind after the extraction of water by the root, which leads to an increase in concentrations of salts that contribute to salinity in the soil. In addition to that, evaporation from the soil surface will remove water and leaves the salt behind in the soil, which eventually reaches toxic levels in the root zone.

In humid areas, the soil solution is concentrated very little; consequently root zone salinity in humid regions is rarely a problem (Abrol et al. 1988; Dajic 2006).

4 Effects of Soil Salinity on Plant Growth

There are many symptoms caused by salinity, some of these symptoms include; increased succulence of leaves or stems, leaf chlorosis and necrosis, leaf drop, root death, nutrient deficiency symptoms, and wilting (Johnson 2000; Dikilitas 2003). Most of these symptoms may be mixed with the symptoms caused by the microorganisms. Salinity limits both plant growth and yield to different extents, depending on the plant species involved, salinity levels and the ionic composition of the salts.

Plants exposed to saline environments are subjected to several adverse conditions, which can be categorized as shown in Table 16.1 (FAO 1988).

Table 16.1 General guidelines for plant response to salinity (Adopted from FAO 1988)

Soil salinity class	Conductivity of the saturation extract (EC, dS m^{-1})	Effect on crops
Non saline	0–2	Salinity effects negligible
Slightly saline	2–4	Growth of sensitive plants may be restricted
Moderately saline	4–8	Growth of many plants are restricted
Strongly saline	8–16	Only tolerant plants grow satisfactorily
Very strongly saline	>16	Only a few very tolerant plants grow satisfactorily

There is no critical point of salinity where plants fail to grow. As the salinity increases growth decreases until plants become chlorotic and die. Plants differ widely in their ability to tolerate salts in the soil. Salt tolerance ratings of plants are based on yield reduction on salt-affected soils when compared with yields on similar non-saline soils.

Salinity has three common effects on plant growth as described below (Levitt 1980; Fitter and Hay 1987; Romero and Maranon 1994; Romero et al. 1994).

1. Direct toxicities of ions (excessive ion accumulation) e.g., Na^+ and, Cl^-, Boron.
2. Ion-specific effects (ion imbalance in the plant).
3. Osmotic effects (a reduction in the availability of water resulting from salt).

An increase in the external salinity decreases water flow into the plant and limits water uptake to cells. It also causes a reduction in turgor potential and reduces cell volume (Tal 1984). This has been termed physiological drought, because plants are affected by a lack of water even though the water content of the soil is apparently adequate for crop needs (Greenway and Munns 1980). There is a close correlation between salt concentration and growth. For optimal growth, plants must receive all the required elements, in a form that is easily available and must absorb them in the right proportions. When the concentration of the salt in the surrounding medium is increased, water absorption is reduced, and as a consequence, growth tends to diminish. Consequently, plants have to acclimatize to the lowering of water potential in order to survive in a saline environment. For example, *Avicennia germinans*, a maritime halophyte, grows in a soil where the salinity can vary from less than half the concentration of sea water, during the rainy season, to more than double that in the dry season (Smith et al. 1989).

In the past, there has been considerable argument as to whether the primary injury caused by salt stress was mediated through ion toxicity or osmotic effects. While Bernstein and Hayward (1958) emphasized osmotic stress as the primary cause of growth reduction, later workers considered toxicity of Na^+ and Cl^- ions to

be more important (Al-Rawahy 2000, Reezi et al. 2009). Santa-Cruz et al. (1997) compared the effect of salinity and non-ionic osmotic stress induced by mannitol on the growth of several tomato species. They concluded that the primary stress induced by salinity was osmotic stress; hence both stresses had similar effects in the short-term. Continual exposure to high salt concentrations in the root zone has been shown to cause a build-up of potentially toxic ions within the plant cells, and to disrupt the uptake of other essential micronutrients, so limiting plant growth and in severe cases resulting in necrosis (Passioura 1986). However, in many herbaceous crop species growth inhibition and injury occurs even at low levels of NaCl salinization (Maas 1993; Flowers 2004). Under this condition, water deficit is not a constraint (Greenway and Munns 1980). Certainly, there is good evidence for ion toxicity having a major effect on plant growth in some species. In a number of species, such as avocado (Downton 1978), growth is reduced by concentrations of NaCl (20 mmol L^{-1}) that are so low to cause osmotic stress. In these species at least ion toxicity must be a major stress. For example, Strogonov (1964) found that NaCl depressed the germination of lucerne (*M. sativa*) much more than iso-osmotic solution of mannitol. The growth of beans, maize and barley was much better in polyethylene glycol (PEG) solutions than in iso-osmotic salt solutions (Greenway and Munns 1980; Dikilitas 2003). According to Levitt (1980), different salts supplied at iso-osmotic concentrations often inhibit growth of plants at different threshold osmotic concentrations. This again indicates that ion toxicity plays a part in overall stress. Especially, high concentrations of Na^+ and Cl^- may cause disruption in membrane function, protein synthesis, enzyme activity, and assimilation and photosynthesis (Flowers et al. 1977; Patrick and Biber 2006).

One of the negative effects of salt stress, which might be responsible for the reduction in growth, is induction of deficiencies in other essential nutrients, or imbalances in ionic content. For example, high external sodium reduces the activity of Ca^{2+} ions in the root medium and so decreases the quantity of Ca^{2+}, which is available for uptake by the plant (Cramer et al. 1986; Flowers 2004). As a result, root growth and function may be inhibited and the translocation of Ca^{2+} from root to shoot may be impaired (Grieve and Maas 1988; Dajic 2006). In addition to that ionic imbalance, particularly Na^+:Ca^{2+} and Na^+:K^+ ratios may affect cell metabolism and function and impairs the membrane integrity causing cell death (Cuartero et al. 1992; Perez-Alfocea et al. 1996; Rodriguez-Rosales et al. 1999; El-Iklil et al. 2002). It has often been observed that salt stress causes a decline in the potassium concentrations of various plants (e.g., *Agrostis stolonifera*, tomato, cucumber (Ahmad et al. 1981; Del Amor et al. 2001; Alpaslan and Gunes 2001). It is possible that tissue potassium concentration declines to the extent that potassium deficiency causes growth reduction in some cases. It has also been reported that salinity increased the Cl^- content of the leaves (Inal et al. 1997; Del Amor et al. 2001; Essa 2001; Inal 2002). Thus, it causes a reduction in uptake of $NO^-{}_3$by replacing it.

Salinity can cause changes in photosynthetic pigment composition. High concentrations of NaCl were responsible for the inhibition of photochemical reactions of isolated chloroplast (Reddy et al. 1992; Patrick and Biber 2006). In halophytes and

salt tolerant species, the chlorophyll content increased (Reddy et al. 1992) while in salt sensitive species it decreased (Salma et al. 1994; El-Iklil et al. 2002). The reduction in chlorophyll in salt sensitive species was correlated with Cl^- accumulation (Velagaleti et al. 1990). It has been reported that in salt sensitive cultivars of *M. sativa* at 170 m*M* NaCl treatment, photosynthesis was reduced by the accumulation of Cl^- in the chloroplast (Seemann and Chritchley 1985) and as a result of that productivity and quality of the crops decreased (Satti and Yahyai 1995; Stoop et al. 1996; Jumberi et al. 2002; Patrick and Biber 2006).

Salinity occurring during the day or in the spring or summer cultivation causes higher reductions in yield than if it occurs during the night or in autumn cultivation (Van Ieperen 1996). This results, because the higher temperatures and illumination and lower relative humidity in summer time lower water potential in the plant by inducing faster transpiration. As well as high transpiration affecting water potential, high salinity also lowers it, which will reduce the water flow into the fruit and therefore, the rate of fruit expansion (Johnson 2000; Johnson et al. 1992; Del Amor et al. 2001).

Nitrogen uptake by tomato plants is not affected at relatively low salt concentrations (70 m*M* NaCl) but at 140 and 200 m*M* NaCl, nitrogen uptake drops to a one third of that observed in non-saline conditions (Pessarakli and Tucker 1988). It has also been reported that uptake of NO_3^- from the root solution is strongly inhibited by salinization; consequently NO_3^- concentration in leaf and stems as well as nitrate reductase activity within the leaves are lower in salinized than in control plants (Cramer et al. 1995; Flores et al. 2002).

Salinization has been observed to alter the hormone balance in plants. An increase in salinity caused a decreased transport of kinetin from roots to leaves, and an increase in leaf content of abscisic (ABA) acid. Both changes decrease stomatal aperture (Aspinall 1980; Vaidyanathan et al. 1999). ABA appears to modulate the response of plants to a variety of stresses (Zeevaart 1988; Parida and Das 2005). Drought, NaCl, and 'cold tolerance' induce a two to four fold increase in the ABA content of tomato leaves (Plant et al. 1991; Yurekli et al. 2001; Parida and Das 2005). This similarity in the response suggests that ABA may be a common signal for mediating the response to all three environmental stresses in tomato. The increase in ABA can be due either to higher ABA production in the roots or by a decrease in ABA metabolism in leaves (Jackson 1997).

The (IAA) content either decreases or remains unchanged under saline conditions (Wang et al. 2001). The hormone causes reduction of the movement of water in the roots and therefore, it may play a role in protecting tomato plants from water deficit and decreasing plant turgor (Tal and Amber 1971; Vaidyanathan et al. 1999). Plants might respond to salinity-mediated water stress by reducing water losses through ABA-regulated stomatal closure while IAA may perform independently (Dunlap and Binzel 1996; Wang et al. 2001; Parida and Das 2005). Besides stomatal closure, the increased ABA concentration in leaves causes a reduction in leaf expansion while lower root IAA content promotes root growth. These two effects would partially explain the increased root/shoot ratio in the plants grown in saline conditions. On the other hand, ethylene was also detected in tomato

plants that were exposed to salinity (Jones and El-Beltagy 1989; Parida and Das 2005).

Salinity also causes blossom end rot (BER) in plants such as tomato, which makes fruits unacceptable for both the fresh market and the processing industry. BER symptoms begin with slight browning at the distal placental tissue, which progressively invades the pericarp; the fruit stops growing and starts ripening too early. The main cause of symptoms is a local Ca^{2+} deficiency as a result of excessive salinity in the irrigation water or growing media. This is made worse in high temperatures because, under saline conditions, the increased transpiration causes more Ca^{2+} to move the leaves and less to the fruit (Adams and Ho 1993; Gao et al. 1998).

Salinity also has a detrimental effect on germination. It may affect germination in two ways; by creating a low osmotic potential which reduces or prevents water uptake; or by providing conditions for the entry of ions which may be toxic to the embryo or developing seedlings (Bewley and Black 1982; Bliss et al. 1986a; Sosa et al. 2005). In many studies, it has been reported that a low osmotic potential or the toxicity of the ions involved had a detrimental effect on the germination of seeds (Emmerich and Hardgree 1990; Johnson 2000; Essa 2001; Esechie et al. 2002, Sosa et al. 2005). Bliss et al. (1986b) showed that inhibitory effect of NaCl and betaine (a non-toxic solute) were similar before germination began, but they were different subsequently. They proposed that the difference between isotonic betaine and NaCl might be the toxic effect of NaCl, which is obvious after the hydration threshold had been surpassed. It has also been reported that salinity not only causes a reduction in germination but also delays the germination (Kent and Lauchli 1985; Sosa et al. 2005). It appears, then, that all three main components (osmotic effects, ion toxic effects and nutritional effects) are responsible for reduction of growth of plants in saline conditions.

The effects of salinity are not always negative; salt treatment has also been shown to improve tomato fruit quality (Mirzahi et al. 1988; Del Amor et al. 2001). The improvement of quality through irrigation with saline waters has also been reported in grape vine (Watzman 1999) and celery (Pardossi et al. 1999). The application of brackish water (2 dS m^{-1}) to vines was reported to result in an increase in wine quality whilst maintaining the crop yield (Watzman 1999). It was also reported that the application of moderate salinity during the development of fruit, such as melon and tomato, caused an increase in soluble solids. Shannon and Grieve (1999) concluded that a small decrease in crop yield resulting from salinity might be partially offset by the increased marketable quality of the fruit.

4.1 Effects of Salt Stress on Cell Membranes

It has been reported that many adverse effects of salinity are related to the structural and functional integrity of membranes (Laszlo et al. 1980; Balsamo and Thomson 1995; Rodriguez-Rosales et al. 1999, Parida and Das 2005). For example, Na^+ increased the permeability of cell membrane and caused K^+ leakage from barley, bean roots (Nassery 1975; 1979) and even from rose (Reezi et al. 2009). Leopold and

Willing (1984) reported that the leaked organic solutes from salt-stressed soybean leaves increased with the increase of NaCl concentration, while almost no leakage was observed resulting from osmotic effects caused by sorbitol. On the other hand, Reezi et al. (2009) demonstrated that the increased membrane permeability in *Rosa xhybrida* plants due to salt effect was recovered with the application of various concentrations of silicon.

4.2 The Role of Proline Accumulation Under Salt Stress

Proline accumulation has occupied a special position in plant physiological research, particularly in response to different stresses. Its accumulation at whole plant level under salt stress in halophytes has been reported by many workers such as Smirnoff and Stewart, 1985; Aghaleh et al. 2009) in coastal plants; Stewart and Lee (1974) in *Triglochin maritima* and *Armeria maritima*. Proline accumulation has been reported under salt stress in glycophytes such as *Hordeum vulgare* (Buhl and Stewart 1983), wheat (Arfan 2009), *Medicago media* (Chaudhary 1996), *Agrostis stolonifera* (Ahmad et al. 1982) and tomato (El-Iklil et al. 2002; Claussen 2005).

Several hypotheses have been put forth to explain the role of proline accumulation in stress metabolism. Proline acts as a compatible solute regulating and reducing water loss from the cell during episodes of water deficit. Proline may have also a role as a sink for the nitrogen from nitrogenous compounds derived from the net loss of protein, and lastly it may represent merely a manifestation of the damaging effects of stress (Aspinall and Paleg 1981; Arfan 2009) and may act as a substrate for respiration that might provide energy needed for recovery from stress (Hare and Cress 1997). Proline accumulation may be a general response to stress, especially under salinity, water or temperature stress (Stewart 1981; Heuer 1994; Aziz et al. 1999; Claussen 2005). For example, salinized tomato plants are able to produce osmotically active organic substances that help alleviate the salinity-mediated osmotic stress. Proline accumulation in salt-stressed plants could be due to the low activity of the oxidizing enzymes (Sudhakar et al. 1993) and its accumulation in leaves and particularly in roots is considered as a salt sensitive trait in tomato that may be used to select plants with different degrees of tolerance (Bolarin et al. 1995).

Some workers suggested that proline accumulation is neither a sensitive indicator of salinity nor of protective value, but merely a symptom of injury (Hadson and Hitz 1982). However, most investigations have indicated a positive correlation between proline accumulation and adaptation to salt or drought stress (Rhodes et al. 1986; Aghaleh et al. 2009). Under salt stress conditions, a salt marsh ecotype of *A. stolonifera* accumulated more proline in roots and shoots than an inland ecotype (Ahmad 1978). In the apices of maize seedlings growing at −1.6 MPa, proline accumulation reached 120 mmolal, accounting for almost 50% of the total osmotic adjustment (Voetberg and Sharp 1991). Such observations clearly suggest that in some plants, proline accumulation may play a direct, adaptive role in countering the effects of osmotic stress.

Addition of proline to salt supplemented medium has also been shown to enhance the growth and survival of unselected cells in a number of species (Pandey and Ganapathy 1985; Handa et al. 1986; Van Swaaij et al. 1986, Al-Rawahy 2000). For example, exogenous proline showed beneficial effects during recovery of barley plants from water stress (Itai and Paleg 1982), and in cultured tomato cells during water stress (Handa et al. 1986). Proline application also increased the production of superoxide dismutase and peroxidase enzymes in stressed plants such as *Glycine max* (Hua and Guo 2002). In another study, exogenous application of proline resulted in mitigating the deleterious effect of NaCl on cell membrane (Mansour 1998). Similarly, proline (10 m*M*) in the external medium of NaCl-selected and unselected cell lines of *Cicer arietinum* under 100 m*M* NaCl stress, increased fresh and dry weights (Pandey and Ganapathy 1985). A similar treatment increased the growth of salt-unadapted callus of rice (Kishor 1988).

Synthesis and accumulation of proline also occur in cell suspension cultures of both glycophytes and halophytes. For example, increased proline accumulation in response to NaCl stress was found in suspension cultures of *Mesembryanthemum crystallinum* (Thomas et al. 1992), while a positive correlation was found between proline accumulation and the capacity of cell cultures from chili pepper (a mesophyte) and creosote bush (a xerophyte) to grow under water stress (Santos-Diaz and Ochoa-Alejo 1994).

5 Mechanism of Salt Tolerance

Although plant responses to salinity are one of the most widely researched subjects in plant physiology, the mechanisms that impart salt tolerance are still unresolved (Cheeseman 1988; Munns 1993; Ashraf and Foolad 2007).

Plants which were able to obtain more water than others from a soil under low water potential would grow better in saline conditions (Cruz and Cuartero 1990). So, plants have developed various mechanisms for survival under high salinity stress. Some tolerate high concentrations of toxic ions present in their root environment by exclusion or compartmentation of ions into the vacuole, and the production of high concentrations of organic solutes in the cytoplasm that lower the osmotic potential (Greenway and Munns 1980, Parida and Das 2005). These organic solutes such as proline (Perez-Alfocea et al. 1993; Ashraf and Foolad 2007) and *myo*-inositol (Sacher and Staples 1985) are generally non-toxic to enzymes.

It has been reported that Na^+ and Cl^- ions were accumulated in the vacuolar sap of halophytes (Austin 1989; Aghaleh et al. 2009). As a result of this, plants become succulent. Succulence is usually defined as the thickening of the leaves of the plants exposed to salinity, although this condition is also applicable to the stem and the root. It is expressed as an increase of water content per unit dry weight, fresh weight or water content per unit area (Jennings 1976). It has been proposed that increases in succulence in response to salinity could be a characteristic indicative of an increased degree of salt tolerance (Tal and Shannon 1983; Dikilitas 2003).

An increase in salt uptake generally depends on transpiration loss, because the water loss will increase the flux of saline water into the root system. Consequently, most plants, especially halophytes, show morphological features that prevent water loss, such as increased succulence, a thick cuticle on leaves, a reduced number of stomata, or sunken stomata, altered stomatal distribution and rolled leaves (Begg 1980; Flowers et al. 1986; Cruz and Cuartero 1990; Ashraf and Foolad 2007), which would thereby reduce the uptake of ions and would improve salinity tolerance. Preventing water loss, by this way, might also reduce the toxic effect of excessive ion concentration (Flowers et al. 1991).

Climate and irrigation also influence salinity tolerance. As the soil dries, salts become concentrated in the soil solution, increasing salt stress. Therefore, salt problems are more severe under hot and dry conditions than under cool and humid conditions. Detailed description of adaptation to salinity is given in Fig. 16.1 following Waisel (1991).

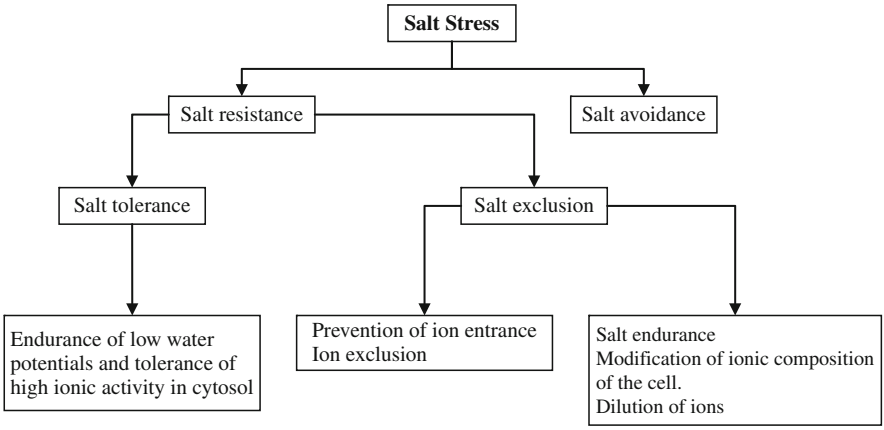

Fig. 16.1 Modes of plant adaptation to salinity (Waisel 1991)

5.1 In Vitro Selection for Salt Tolerance

The generation of salt tolerant plants has potential application to semi-arid and arid soils. Plant tissue cultures techniques have been used successfully to develop variant lines from somatic cell cultures (Ben-Hayyim and Kochba 1983; Ben-Hayyim et al. 1985; Rumbaugh and Pendery 1990; Al-Rawahy 2000). Many salt tolerant somatic cell lines have been isolated in a number of plant species, including *Nicotiana sylvestris* and *Capsicum annum* (Dix and Street 1975), Citrus (Ben-Hayyim and Kochba 1983), *Cicer arietinum* (Pandey and Ganapathy 1984), *Lycopersicon peruvianum* (Hassan and Wilkins 1988). It is generally accepted that a mechanism regulating Na^+/K^+ selectivity exists in plant cells, which show salt tolerance (Chaudhary 1996; Sosa et al. 2005).

Many countries depend heavily on irrigation for food production; however, much of the food productivity is affected by soil salinity (Brown 1981). If the problem of soil salinity decreases food production, whilst the population growth increases, then the rate of food production cannot keep the pace with the growth of the population for the world as a whole. Therefore, improvement of salt tolerance in crop plants is an important challenge to biotechnology.

Salt tolerant cell lines of lucerne have been selected in several laboratories (Shah et al. 1990; Al-Rawahy 2000; Shah et al. 2002; Dikilitas 2003). Studies with the first salt-tolerant cell line of lucerne showed a halophytic type of salt tolerance which was selected in the cell line that required salt for optimal growth (Croughan et al. 1978). In some cases, the selected lucerne cell lines were maintained "in vitro" for several years and the plants were finally regenerated; the somaclones were so stunted that whole-plant tolerance was not determined (Stavarek and Rains 1984). Similarly, one disappointing example has been with *Pennisetum purpureum* Schum, where plants regenerated from NaCl tolerant callus were even more NaCl sensitive than plants regenerated from unselected callus (Chandler and Vasil 1984). Smith and McComb (1981) screened four lucerne cultivars at the whole-plant and cellular level. One cultivar W75RS (Regen S), which showed "in vitro" tolerance also had a higher level of whole-plant tolerance. However, following selection of a NaCl cell line capable of plant regeneration, it was found that the regenerated plants were as salt sensitive as the initial plants (Smith and McComb 1983). This may have resulted from loss or interchange of chromosomal segments during the cellular selection process, a process that was observed "in vitro" (McCoy et al. 1982). However, in one study, the salt-tolerant lucerne plants that were regenerated from salt-adapted cell lines apparently showed dominant salt tolerance and it was transmissible through seed (Winicov 1991).

As it was seen from the previous works, biochemical or genetical approaches have not always brought the success for the crop plants exposed to saline conditions. Those plants either lost their tolerance to salt after some time following their generation or they did not show high tolerance to salt as desired. Therefore, a new approach or a new alternative method should be introduced to the agricultural sciences for the crop plants exposed to salinity. One of the methods for the crop plants under saline conditions is to grow them with halophytes, thus allowing crop plants to use more energy to elaborate substances for the fruit or crop development, instead of building up mechanisms of tolerance (Graifenberg et al. 2003).

5.2 Mechanism of Salt Tolerance in Glycophytes and Halophytes

On the basis of their tolerance or sensitivity, plants are commonly distinguished as halophytes or glycophytes. Glycophytes ("sweet" plants) tolerate only low concentrations of salt, while halophytes (halas = salt, salt plants) tolerate relatively high concentrations of salt (Flowers and Yeo 1986; Flowers and Yeo 1988). It was estimated by Flowers et al. (1986) that there were at least 800 species of halophytic angiosperms in more than 250 genera. This illustrates the point that there are many

species of plants that possess the necessary features to enable them to grow and survive in a saline environment (Austin 1989).

Some halophytes possess glands and bladders, which actively excrete excess salts. Examples of these are *Spartina, Armeria, Limonium* and *Glaux* and *Mesembryanthemum* (Long and Mason 1983; Agarie et al. 2007). Each gland may excrete up to 0.5 μl of salt solution in an hour. Obligate halophytes, for example *Halogeton glomeratus*, only grow in saline soil, and *Salicornia europaea* grows well in the presence of NaCl (Wainwright 1984; Aghaleh et al. 2009). For example, a salt bush (*Atriplex halimus*) indigenous to Australia, has developed a mechanism to control the $Na^+$and Cl^- ion concentration of its tissues. The epidermal bladders on the surface of the aerial parts of the plant are specialized cells that accumulate salt. As the leaf ages the salt concentration in the cell increases and eventually the cell bursts or falls off the leaf, releasing the salt outside the leaf (Troughton and Donaldson 1972).

In non-halophytes, resistance to salinity is commonly correlated with the ability to restricted entry of ions into the shoot. Their growth will be retarded when the salt content of the soil exceeds a rather low value. Glycophytes lack specialized anatomical features as well as tolerance to ions accumulated in the tissues. Typical of glycophytic dicotyledons is the uptake of ions from the external medium but the upward movement of these ions through the shoots is restricted by mechanisms of varying effectiveness (Greenway and Munns 1980; Dajic 2006).

In most halophytes osmotic adjustment results from the increase in concentrations of Na^+ and Cl^- in the tissue. In glycophytes, tolerance to salinity is related to the exclusion of these ions from tissues. This became clearer by comparing ionic concentrations in the tissues of salt-tolerant and non-salt tolerant cultivars of the same species. Many salt tolerant non-halophytes tend to restrict Na^+ uptake and take up more K^+ than do the less tolerant ones (Greenway and Munns 1980). For example, salt tolerant clones of *Agrostis stolonifera* contained lower Na^+ in the shoots than a salt-sensitive inland clone (Ahmad et al. 1981). This showed that restricted Na^+ uptake and maintenance of high Na/K ratios were features of salt tolerance in *A. stolonifera*, a result later confirmed by Hodson et al. (1981).

However, Na^+ "exclusion and accumulation" have often been implicated, as mechanisms of salt-tolerance in non-halophytes, but this conclusion cannot be generalized. The wild maritime tomato species *Lycopersicon chesmanii* was a salt accumulator but the commercial species *L. esculentum* exhibited salt exclusion (Rush and Epstein 1976; Santa-Cruz et al. 1999: Rajasekaran et al. 2000).

The high concentrations of the ions in the tissues of halophytes suggest that their metabolic process may be tolerant to salt stress when compared to glycophytic metabolism. However, comparison shows the enzymes of halophytes and glycophytes have a similar degree of sensitivity to salt (Gibson et al. 1984). The sensitivity of enzymes from halophytes to salt, despite the presence of high ionic concentrations, suggests that plant cells have the capability to compartmentalize the toxic ions away from sensitive metabolic sites (Flowers et al. 1977). Most importantly, halophytes have developed 'controls' in Na^+ influx strategy in roots to lower Na^+ accumulation compared to glycophytes (Wang et al. 2006). Halophytes also

have a capacity for osmotic adjustment in that these plants accumulate osmolytes such as glycine betaine and proline that maintain the osmotic balance disrupted by the presence of ions in the vacuole (Wang et al. 2004). Halophytes can maintain high metabolic activity even at inhibitory concentrations of intracellular Na^+ and possess enhanced antioxidant mechanism (Fang et al. 2005). On the other hand, Jithesh et al. (2006) concluded that the antioxidant enzymes protected halophytes from deleterious ROS production during salt stress. It is clear that salinity induces oxidative stress in plants. Therefore, increases in malondialdelyde and lipid peroxidation are generally used as indicators for ROS production during or after salt stress conditions. Works with halophytes suggested that maintenance of malondialdehyde levels after salt stress and the induction of antioxidant enzymes confirmed the role of antioxidants in salt tolerance trait in halophytes (Parida et al. 2004; Fang et al. 2005). In these circumtances, induction of antioxidant enzymes was shown to protect halophytes against ROS, thus preventing lipid peroxidation during salt stress. This suggests that the antioxidant enzymes are essential components of an adaptive defense mechanism against salt stress in halophytes (Jithesh et al. 2006).

6 Phytoremediation Strategies for Overcoming Salinity Problems and Use of Halophytes as Companion Plants

In recent years, salinity has become the most important issue in fields, gardens and greenhouses as well. This, of course, has forced us to control saline areas, and therefore, many control mechanisms that have been put forward. Many of them (genetics, biochemical and physical) have not brought the desired success. Since salt is due to irrigation and natural causes, so, alternative control mechanisms should be provided. Recent advancement in this area is to obtain quick results from saline-affected areas without damaging the environment, and add these areas into the arable lands. One of those amelioration procedures is phytoremediation, which is an environmental-friendly green technology that is cost-effective and energetically inexpensive (Shah and Nongkynrih 2007). This procedure is generally performed by using halophytes which are known for their ability to adapt to salinity by altering their energy metabolism (Winicov and Bastola 1997). Adaptation of halophytes to salinity is generally associated with osmotic adjustment that leads to the accumulation of several organic solutes, such as free proline and sugars (Bohnert et al. 1995). Halophyte species (*Atriplex* spp., *Suaeda* spp., *Salsola* spp., *Chenopodium* spp., *Portulaca* spp.) could uptake the salt ions through their roots and metabolize or store in leaves (McKell 1994; Grieve and Suarez 1997). Therefore, they have potential to desalinize the salt-affected areas. Due to their biology and physiology, they could possibly be used as companion plants with crop plants. According to Qadir et al. (2002) phytoremediation has two main advantages for the farmers: Firstly, no fnancial outlay to purchase chemical amendments, and secondly, financial or other benefits from the crops grown during the amelioration process.

The salt uptake and accumulation performed by the halophytes can reduce the severity of the stress at a rhizospheric level, providing better conditions for the

growth of the agricultural species and, in conclusion, better yields (Zuccarini 2008). He also concluded that consociation with *Portulaca oleracea* gave the best results in terms of increase of tomato growth and yields. Similar results were also obtained from the work of Zhao (1991) who worked on *Medicago sativa* and *Suaeda salsa*. He concluded that *S. salsa* accumulated Na^+ during a 120-day growing period and caused a net reduction in the Na^+ content of the soil. However, the Na^+content was decreased by only 1% with *M. sativa*. In another study, *S. salsa* did not prevent suppression of growth of tomato plants by NaCl either. In fact, it reduced blossom end rot of tomato fruit but did not significantly affect fruit weight, number or yield (Albaho and Green 2000). A greenhouse experiment also confirmed the positive effect of *S. soda* used as a desalinating companion plant on growth, yield, mineral composition, and fruit quality of pepper grown under moderate (EC = 4.0 dS m^{-1}) and high salt concentration (EC = 7.8 dS m^{-1}). The presence of *S. soda* decreased the EC of the medium by 45% and increased the total yield, marketable yield, and total biomass of pepper by 26%, 32%, and 22%, respectively, in comparison with those grown without *S. soda* (Colla et al. 2006). They demonstrated that using *S. soda* as a companion plant under moderate saline concentrations would be an attractive strategy in limiting yield reduction. Graifenberg et al. (2003) stated that the companion plants such as *S. soda* and *P. oleracea* did not only reduce the Na^+ or Cl^- content of the soil, but they also reduced the Na^+ concentration in tomato leaves by 39.6% and 35.6%, respectively. On the other hand, *P. oleracea* showed less reduction in saline condition when tomato was grown with both halophytes. A higher Ca content was also observed in tomato leaves in the presence of companion plants under saline stress. Graifenberg et al. (2003) concluded that the higher yield obtained in tomato growing with companion plants under salt-stress might be due to a reduction in Na^+ absorption and an increase in P and Ca uptake. Companion plants could also be used to desalinize the saline soils under non-leaching conditions. For example, in a study carried out by Rabhi et al. (2008), *Arthrocnemum indicum*, *Suaeda fruticosa* and *Sesuvium portulacastrum* species significantly decreased the soil electrical conductivity by absorbing soluble salts, mainly sodium ions. Similar findings were also made by Hamidov et al. (2007) who stated that when the water table remained at a depth of about 1.1 m, the capillary rise from the groundwater played a significant role in meeting the demand of plants for water to remove the soil salts and obtain the biomass production of *Portulaca oleracea*. The highest salt accumulation was 497 kg ha^{-1}, which eventually, removed about 16.8% of the total soil salts, at a depth of 10 cm. Similarly, Akil (2008) stated that *Atriplex canescens* and *Festuca arundinacea* were found successful to lower the EC and ESP of the soil.

As it was seen from the recent works, companion halophytes would be promising in reducing the salinity levels in dryland areas as well as in greenhouses (personal communication with Dr. Manzoor Qadir, ICARDA-Syria, 2009, Dr. Paolo Zuccarini, Pisa Univeristy-Italy, 2009 and Dr. G Colla, Universita della Tuscia-Italy, 2009. Biochemical, biological and genetical traits of these plants should be extensively evaluated and their ion absorbant capacity should be increased through biotechnological works. The determination of genetical and biochemical traits of these plants in every aspect would enable us to know how the ions are stored or

metabolized in the cell and how these traits would be improved through biochemical or genetical ways. It is also important to determine the level of salt tolerance and antioxidant capacity of these plants, By this way, suitable companion plants would be selected to improve the saline areas. For example, Dikilitas et al. (2007) reported the possible use of *Peganum harmala* as companion plants by determining the antioxidant capacity and ion absorption rate at various NaCl concentrations. They concluded that the halophyte *P. harmala* was more tolerant to salt than that of the glycophytes.

Seeds of halophytes have the unique property of surviving at extremely high salinity during the storage in the seed bank (Khan and Ungar 1997) and they germinate readily when soil salinity is reduced.

The strategy of the remediation of saline soils with the use of halophytes is quite new, it could be used with success especially where genetical and biochemical approaches are expensive. However, one should note that the ability to accumulate toxic ions varies significantly between species and between cultivars within a species. With the use of these plants, saline and polluted areas would be ameliorated and with the help of other amelioration techniques the amelioration process would be fast, reliable and sustainable. Local authorities, private companies and other bodies involved in the remediation of contaminated land should be encouraged to use phytoremediation, especially if budgets are limited and the alternative is that no treatment is carried out.

Phytoremediation has also limitations. The plants that mediate the clean-up have to be where the pollutant is and have to be able to act on it. Therefore, the soil properties, toxicity level, and climate should allow their growth. Phytoremediation is also limited by root depth because the plants have to be able to reach the pollutant (Pilon-Smits 2005). Apart from these issues, soil texture, pH, salinity, concentrations of other pollutants and the presence of other toxins must be within the limits of plant tolerance. Phytoremediation is also frequently slower than physicochemical processes, and may need to be considered as a long-term remediation process.

However, phytoremediation process can be improved by identifying candidate proteins and transporter genes for transfer and/or over-expression of a particular gene in halophytes (Fulekar et al. 2009). One of the promising improvement methods is through recombinant DNA technology. Fulekar et al. (2009) described the steps in detail. According to these procedures, the technology involves the introduction of DNA encoding enzymes or other proteins from other living organisms, or even completely synthetic genes designed to encode enhanced enzymes. DNA or gene of interest is spliced into a small, circular carrier DNA molecule known as a vector. The vector is introduced into plant cells either by physical means or biological means. Upon entry into the cell and integration into the plant chromosome, the desired gene is “expressed” in a subset of the cells, these cells are selected in tissue culture and used to regenerate whole plants for subsequent breeding.

7 Conclusion

Phytoremediation has many advantageous; its major advantages are the low cost and environment-friendly sites. Because of these features, this new technology needs to be promoted and expanded in developing countries. On the other hand, there is a significant demand for applied and fundamental research since we do not know the limiting factors in increasing uptake, translocation and tolerance of soil contaminants by plants. The biochemical and molecular mechanisms of companion plants should be thoroughly understood that their sequestration or translocation rates of toxic substances should then be increased. Therefore, a multidisciplinary approach is required such as plant biology, agronomy, agricultural engineering, biochemistry, molecular biology, soil science, microbiology and genetic engineering to improve our understandings.

Acknowledgments We dedicate this chapter to the memory of our deceased son Fecri Sami Dikilitas.

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Chapter 17
Phytoremediation of Toxic Explosives

Nand Lal and Neerja Srivastava

Abstract Widespread contamination of the environment by explosives resulting from the manufacture, disposal and testing of munitions is becoming a matter of increasing concern. Most explosives are considered to be a major hazard to biological systems due to their toxic and mutagenic effects. Interest on the bioremediation of lands contaminated with explosives has recently been focused on phytoremediation. Unfortunately, whilst plants have many advantages for the remediation of contaminated land and water, they lack the catabolic versatility which enables microorganisms to mineralize such a wide diversity of xenobiotic compounds. This raised the interesting question as to whether the impressive biodegradative capabilities of soil bacteria could be combined with the high biomass and stability of plants to yield an optimal system for in situ bioremediation of explosive residues in soil. During the last few years, plants have been genetically modified to overcome the inherent limitation of plant detoxification capabilities, following a strategy similar to the development of transgenic crops. Bacterial genes encoding enzymes involved in the breakdown of explosives have been introduced in higher plants, resulting in significant enhancement of plant tolerance, uptake and detoxification performances. Transgenic plants exhibiting biodegradation capabilities of microorganisms bring the promise of an efficient and environmental-friendly technology for cleaning up polluted soils.

Keywords Explosives · Phytoremediation · Detoxification · RDX · TNT · PETN · Transgenic plants

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1 Introduction

Industrial and military activities have led to widespread contamination of the environments, including thousands of sites termed as Superfund sites that are severely polluted. The concentrations of the contaminants can vary from highly toxic concentrations from an accidental spill to barely detectable concentrations that, after long term exposure can be detrimental to human health (Doty 2008).

The cost of cleaning up contaminated sites is extremely high. The global cost of cleaning of these sites annually is in the range of $25–50 billions (Doty 2008). Engineering methods for the remediation of contaminated sites include excavation, transport, soil washing, and extraction, pumping and treating of contaminated water, addition of reactants such as hydrogen peroxide or potassium permanganate, and incineration. A serious consequence of the high cost of remediation technologies is that polluted sites are often abandoned rather than clean up.

Another popular clean-up method involves augmented bioremediation with the addition of specific microbial strains known to degrade the pollutants. Bacteria and fungi collectively can utilize a vast range of organic molecules. But for bioremediation using microbes at a particular site to be successful, many conditions must be met. These include the ability of the microbes with the desired metabolic activity to survive in that environment, the accessibility or bioavailability of the chemicals, and the presence of inducers to activate expression of the necessary enzymes. Many organic pollutants are recalcitrant to degradation and cannot be used as sole carbon sources. The pollutants are sometimes metabolized by enzymes with other natural substrates, therefore, these substrates sometimes need to be present in order for the genes to be expressed. This requirement is problematic if the inducing chemical is itself a harmful pollutant. Bioremediation also depends on the presence of sufficient carbon and energy sources. Often, thousands of gallons of a food source such as molasses must be pumped down into the site to allow bacterial growth. The use of microorganisms in engineered bioremediation systems has a mixed response.

Phytoremediation is the use of plants to treat/clean contaminated sites. This technology has been extensively reviewed by several scientists (Schnoor et al. 1995; Salt et al. 1998; Meagher 2000; Dietz and Schnoor 2001; McCutcheon and Schnoor 2003; Newman and Reynolds 2004; Suresh and Ravishankar 2004; Pilon-Smits and

Freeman 2006). Phytoremediation takes advantage of the natural ability of plants to extract chemicals from water, soil and air using energy from sunlight. Its some of the advantages are that it is less expensive, is passive and solar driven, has high public acceptance, retains topsoil, and has less secondary waste generation.

Phytoremediation has been used to treat a variety of pollutants including metals, petroleum, solvents, explosives, polycyclic aromatic hydrocarbons and other organic contaminants. Phytoremediation involves different processes depending on the type of pollutant. Phytoextraction refers to the method of removal of contaminants from the soil and translocation to the foliage. It is an effective means of remediating a site because it reduces the overall mass to be treated from tons of widespread contaminated soil to plant tissues that can be dried to small volume. Plants that are especially good at concentrating the pollutants are termed hyperaccumulators. Phytodegradation involves the metabolic degradation of organic pollutants. In this process, plants break down the pollutants through either internal or secreted enzymes. Phytodegradation of chlorinated hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), and explosives has been studied most exclusively.

A very important class of environmental pollutants for which plants can be used for remediation includes explosives including trinitrotoluene (TNT) and Royal Demolition Explosives (RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine). TNT is toxic to humans, causing aplastic anemia and hepatitis (Rosenblatt 1980). More than 100 military bases and explosive-manufacturing facilities in the U.S.A. are contaminated with these chemicals. The groundwater at these sites is contaminated, increasing hazard that the health risk will spread beyond the military bases. Research with aquatic plants demonstrated that TNT can be metabolized in the absence of microorganisms (Hughes et al. 1997). Both poplar and willow have been used in munitions remediation research. Hybrid poplar (*Populus deltoids* x *P. nigra*) was able to take up TNT from hydroponic solution, but the trees only translocated about 10% of it to the foliage (Thompson et al. 1998). In a study comparing phytoremediation of TNT by hybrid willow (*Salix* clone EW-20) and Norway Spruce (*Picea abies*), it was shown that both tree species readily metabolized TNT (Schoenmuth and Pestemer 2004). A serious problem with phytoremediation of TNT and RDX is that the contaminated soil and water at military firing ranges can contain concentrations of these chemicals that are phytotoxic. Obviously, only healthy and actively growing plants would be effective in taking up pollutant and metabolizing it fully.

Although much research has been done to demonstrate the success of phytoremediation, resulting in its use on many contaminated sites, the method still lacks wide application. Its primary disadvantage when compared with engineering methods is that it is often considered too slow or only seasonally effective. Regulatory agencies often require significant progress in remediation to be made in only a few years, making most phytoremediation applications unsuitable. Plant species with the ability to treat a particular pollutant are often either unable to grow under the environmental conditions of the contaminated site or are too small to be useful, such as many of the hyperaccumulators. In some contaminated sites, the pollutants can be at phytotoxic concentrations, as in the case of TNT at military firing ranges, or recalcitrant to degradation by plants, as in the case of PAHs. For these reasons, attention

has recently focused on ways to enhance the phytoremediation capacity of plants using either transgenic methods or endophytes.

A direct method for enhancing the effectiveness of phytoremediation is to overexpress in transgenic plants the genes involved in metabolism, uptake, or transport of specific pollutants (Stomp et al. 1994; Rugh 2004; Cherian and Oliveira 2005). The introduction of these genes can be readily achieved for many plant species using *Agrobacterium tumefaciens*-mediated plant transformation. Phytoremediation is generally more effective with use of large size, high biomass and fast growing plants. In this category, willow tree seems to best fit but its transformation protocols have not yet been published, therefore the focus has been on poplar. Depending on the hybrid and particular clone, reasonable transformation frequencies can be achieved in poplar trees (Han et al. 2000).

2 Explosives as Pollutants

The term *explosive* refers to prepared chemicals subject to a rapid chemical reaction that produce or cause explosions. The three main classes of explosives are nitroaromatics, nitramines and nitrate esters. Nitroaromatics are characterized by an aromatic ring and nitro groups. The electronegativity of the nitro groups prevents explosives from readily falling under electrophilic attack. For this reason they are generally non-hygroscopic, insoluble in water and do not readily react with metals. Common uses of explosives include military weapons and pyrotechnic shows. Table 17.1 lists common explosives and some of their properties whereas their structures are illustrated in Fig. 17.1.

Table 17.1 Some common explosives and their properties

Compound Name	Chemical formula	MW (g mol^{-1})	Density (g mL^{-1}-20ºC)	Solubility (g 100 mL^{-1}-20ºC)
2,4-Dinitrotoluene (2,4-DNT)	$C_7H_6N_2O_4$	182.1354	1.521	0.027
2,6-Dinitrotoluene (2,6-DNT)	$C7H_6N_2O_4$	182.1354	1.2833	0.0182
2-nitrotoluene	$C_7H_7NO_2$	137.1378	1.163	0.06
4-nitrotoluene	$C_7H_7NO_2$	137.1378	1.392	<0.1
Hexahydro-1,3, 5-trinitro-1,3,5-triazine (RDX)	$C_3H_6N_6O_6$	222.117	1.82	Insoluble
Octahydro-1,3,5, 7-tetranitro-1,3,5, 7-tetrazocine (HMX)	$C_4H_8N_8O_8$	296.156	1.90	Insoluble
Tetryl	$C_7H_5N_5O_8$	287.1452	0.02	
2,4,6-trinitrotoluene (TNT)	$C_7H_5N_3O_6$	227.133	1.64	0.01

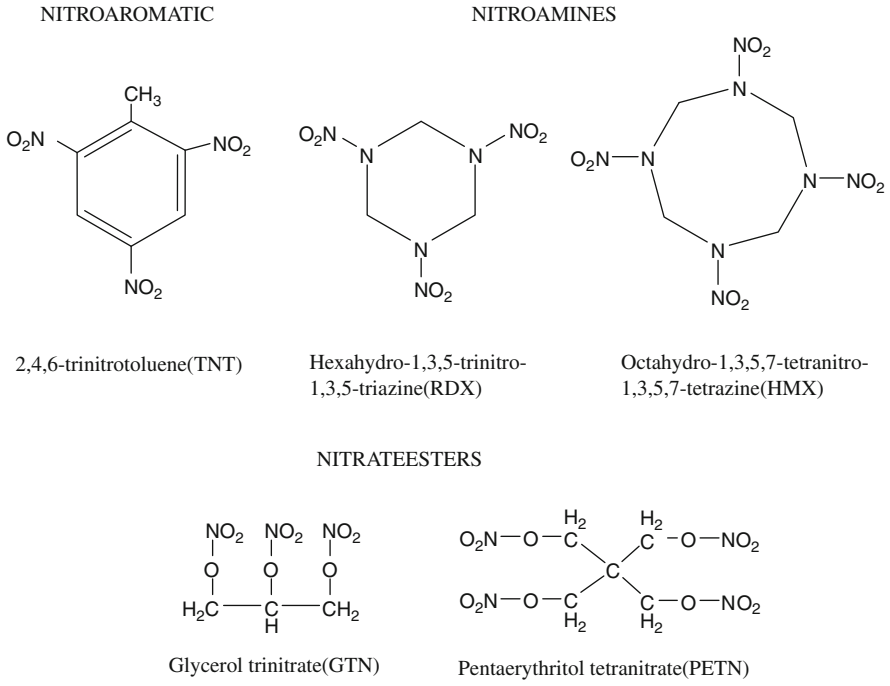

Fig. 17.1 Some common explosives and their properties

Contamination of soil with explosives is largely due to manufacturing, storage, testing and inappropriate waste disposal of explosive chemicals. The primary explosives at hazardous waste sites are 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (Royal Demolition explosive-RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazine (High Melting explosive-HMX). TNT is a nitroaromatic constituent of many explosives. In a refined form, TNT is stable and can be stored over long periods of time. It is relatively insensitive to blows or friction. It is readily acted upon by alkalis to form unstable compounds that are very sensitive to heat and impact. Health effects due to exposure to TNT include anemia, abnormal liver function, skin irritation, and cataracts (ASTDR 2004). RDX is a nitramine widely used as an explosive and as a constituent in plastic explosives. RDX can cause seizures when large amounts are inhaled or eaten. Long-term health effects on the nervous system due to low-level exposure to RDX are not known. HMX is a nitramine that explodes violently at high temperatures. It is used in nuclear devices, plastic explosives and rocket fuels. Insufficient studies on the effects of HMX to the health of humans and animals have been performed.

Incineration, landfilling, and pump and treat systems are traditional methods applied to remove explosive contamination from soil and groundwater. These approaches are expensive and can cause air pollution with generation of ash. Phytoremediation mechanisms that have been successful in containing and/or

remediating explosive contamination include phytoextraction, phytodegradation, and phytostabilization using tobacco, periwinkle, and parrot feather plants in constructed wetlands (Bhadra et al. 1999b; Wayment et al. 1999; Hughes et al. 1997).

To address this issue, Travis et al. (2007) further investigated whether plants could be genetically engineered to yield an optimal system for in situ bioremediation of toxic explosive residues in soil. A significant progress has been made towards this goal by successfully combining the biodegradative capabilities of soil bacteria with the high biomass, stability, and sequestration properties inherent in plants.

3 Phytoremediation: Detoxification of Explosives by Plants

High explosives such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and 2,4,6-trinitrotoluene (TNT) are important contaminants in the environment and phytoremediation has been viewed as a cost-effective abatement. There remains, however, an insufficient knowledge-base about how plants respond to explosives, especially in the steady state (Rao et al. 2009).

The two greatest advantages of phytoremediation compared with traditional abatement methods are: (1) cost effectiveness, and (2) soils remain in place thereby causing less ecosystem disruption. Cropping systems with costs ranging between US $200 and US $10,000 ha^{-1} would correspond to a remediation cost of US $0.02–1.00 m^{-3} of soil per year, which is a saving of many orders of magnitude when compared to costs associated with physicochemical remediation technologies (Cunningham et al. 1995). There are several studies which show that plants, in general, readily take up RDX and TNT. For example, recently Vila and others reported that agronomic plants (maize, soybean, wheat, and rice) could grow on soils containing RDX and TNT and were able to uptake these compounds (Vila et al. 2007). In another recent study, it was reported that maize (*Zea mays* L.) and broad beans (*Vicia faba* L.) were able to remove TNT from soils (Van Dillewijn et al. 2007). Also, *Catharanthus roseus* (*Vinca*) hairy root cultures, *Myriophyllum aquaticum* (parrot feather) plants, and hybrid poplars have been reported to take up RDX (Bhadra et al. 2001; Thompson et al. 1999). Harvey and others have reported bioaccumulation of RDX in bush bean plants grown in hydroponic cultures (Harvey et al. 1991). However, unmodified plants are typically not very efficient in their accumulation and degradation of explosives. Therefore, genetic engineering might help increase phytoremediation capacity and certainly would be required for phytosensing, i.e., using plants to report the presence of contaminants. In this regard, plants have been genetically engineered to phytoremediate explosives (French et al. 1999; Hannink et al. 2001, 2007; Rylott et al. 2006; Van Dillewijn et al. 2008; Rylott and Bruce 2009; Van 2009; Eapen et al. 2007), but there is no published report on phytosensors for explosives. Understanding plant transcriptional responses to explosives is thus necessary and useful for developing phytosensors or phytoremediators.

Based on studies published to date, a working hypothesis for how plants deal with organic chemical contaminants such as RDX and TNT is based on three phases (Harvey et al. 1990; Sandermann 1992; Coleman et al. 1997; Best et al. 1999, 2005, 2006; Bhadra et al. 1999a, b, 2001; Larson et al. 1999; Hannink et al. 2002; Ekman et al. 2003, 2005; Just and Schnoor 2004; Van et al. 2004; Van Dillewijn et al. 2008; Rylott and Bruce 2009): phase I (transformation or activation) – a transformation phase of metabolism of the chemical, phase II (conjugation) – conjugation of the chemical contaminant to endogenous hydrophilic molecules to facilitate compartmentalization of the contaminant, and phase III (compartmentation) – movement of the contaminants and breakdown products into vacuoles to reduce their toxicity.

Activation or transformation generally involves oxidation or hydrolysis or reduction type of reactions, where functional groups such as hydroxyl (−OH) and carboxyl (−COOH) are added to the contaminant with enzymatic involvement of cytochrome P_{450} monooxygenases, esterases, reductases, dehalogenases, and dehydrogenases. The products of phase I (activation) are more hydrophilic and sometimes more toxic than the parent compound. In the phase II (conjugation), the activated contaminant undergoes deactivation by the formation of covalent linkages with endogenous hydrophilic molecules such as glucose, malonate, glutathione (GSH), or carboxylic acids using glucosyltransferase-, glutathione-S-transferase-, and acyltransferase-mediated reactions that result in water soluble conjugates that are less toxic compared to the parent compound. Phase III (compartmentation) involves exporting conjugates to either the vacuole or apoplast using ABC transporters or multidrug and toxic compound extrusion (MATE) transporters (Sandermann 1992; Ishikawa 1992; Ishikawa et al. 1997; Rea et al. 1998; Coleman et al. 1997; Schaffner et al. 2002). Several genes induced by RDX treatment in this study suggest RDX detoxification via the three phases. Functional categorization by loci of the genes upregulated in this study revealed that several genes had transferase activity and transporter activity, further supporting the notion of potential RDX detoxification in *Arabidopsis*. Also, there were nine expressed genes with unknown function from two-color experiment (greater than 1.5 fold upregulation) and 20 from the Affymetrix experiment (greater than 2.0 fold upregulation) identified, some of which might be involved in RDX metabolism. There is no earlier report on whole genome expression studies in response to RDX except a serial analysis of gene expression (SAGE) study (Ekman et al., 2005), where gene expression in *Arabidopsis* roots was characterized. These authors reported three cytochrome P_{450}s (At1g16400, At3g20940, At4g13310), induced greater than fivefold in their study, to be possibly involved in phase I transformation of RDX in *Arabidopsis*. They also speculated about a putative peroxidase (At1g49570) and α-hydroxynitrile lyase-like protein (At5g10300) to be involved in RDX metabolism. Incongruence of lists of differentially upregulated genes of Rao et al. (2009) and Ekman et al. (2005) can possibly be attributed to organs used in the respective studies: roots (Ekman) vs. whole plants (Rao et al. 2009). As also suggested by Ekman et al. (2005), since RDX is readily translocated and accumulated in leaf tissues (Best et al. 1999; Harvey et al. 1991; Thompson et al. 1999), gene expression in shoots is highly relevant. With

respect to TNT, plants readily take up and accumulate TNT in roots (Burken et al. 2000; Harvey et al. 1990; Hughes et al. 1997; Larson et al. 1999). Several studies in plants have been reported supporting the three phase detoxification of TNT in plants (Rylott and Bruce 2009).

Arabidopsis had apparent differences in transcriptional regulation from RDX and TNT treatments. Few significant genes were commonly up- or down regulated among RDX and TNT-treated plants suggesting that plants cope with these compounds differently. This lack of overlap was also observed by Ekman et al. (2005) who studied transcriptional responses to RDX in *Arabidopsis* roots and compared it to transcriptional responses to TNT in *Arabidopsis* roots studied earlier by Ekman et al. (2003). One possible explanation could be that these two compounds differ chemically and in their metabolic pathways (Hawari et al. 2000; Hannink et al. 2002; Rylott and Bruce 2009). TNT belongs to the nitroaromatics group and consists of an aromatic ring with three nitro-groups. TNT in plants is probably detoxified using phase I reductive transformation to 2- and 4-hydroxydinitrotoluene isomers by means of nitrosodinitrotoluene, followed by phase II conjugation with endogenous plant compounds such as sugars or glutathione, and finally phase III sequestration into the apoplast or vacuole (Hannink et al. 2002; Rylott and Bruce 2009). RDX is classified as a cyclic nitramine explosive and consists of N-nitro groups (Hannink et al. 2002; Hawari et al. 2000). The RDX detoxification mechanism, as proposed in poplar, involves reduction of RDX to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) followed by light-mediated cleavage of heterocyclic ring of RDX, MNX, and DNX generating formaldehyde and methanol and a final light-independent plant cell mediated mineralization to carbon dioxide (Van et al. 2004). Therefore, common phytoremediation or phytosensing strategies between these two explosives are likely not feasible. While TNT and RDX are often used together in landmines, phytoremediation would require consideration of both compounds, but phytosensing for landmine detection might be accomplished by detection of either TNT or RDX.

4 Bacterial Genes Involved in Phytoremediation of Explosives

TNT is one of the most toxic explosives known to man, affecting plants, animals and most microorganisms. *Enterobacter cloacae* PB2, a Gram-negative bacterium, is able to utilize TNT as a sole source of nitrogen (Binks et al. 1996). The *nsfI* gene, isolated from *E. cloacae*, encodes the enzyme nitroreductase (NR), which is responsible for the reduction of the nitro groups of TNT (Fig. 17.2), producing hydroxylamino- and amino-dinitrotoluenes (French et al. 1998). Hannink et al. (2001) transferred the bacterial *nsfI* gene into tobacco via *Agrobacterium*-mediated gene transformation. Transgenic tobacco plants expressing the bacterial NR enzyme tolerated TNT concentrations up to 0.5 m*M*, which is the solubility limit of TNT in aqueous solution. In a different study, tobacco plants were transformed with the *E. cloacae onr* gene, which encodes the enzyme pentaerythritol tetranitrate (PETN)

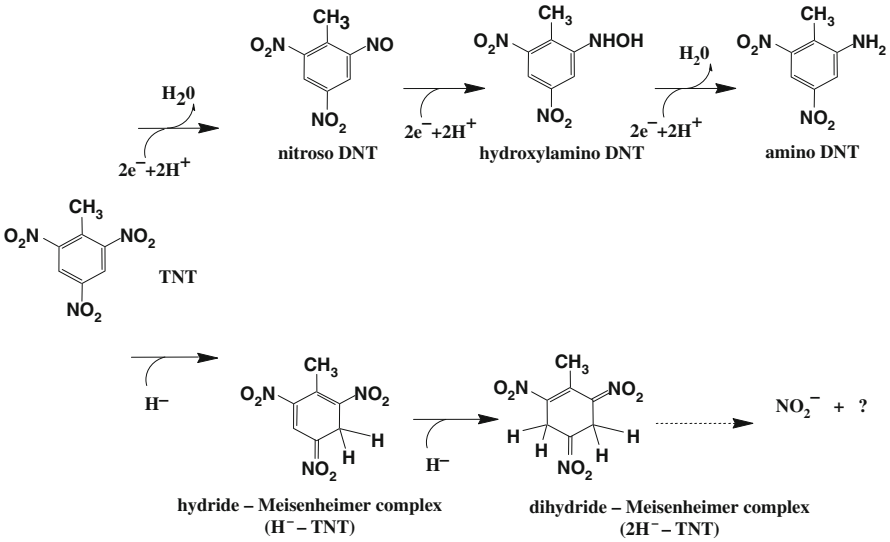

Fig. 17.2 Metabolism of TNT by *Enterobacter cloacae*

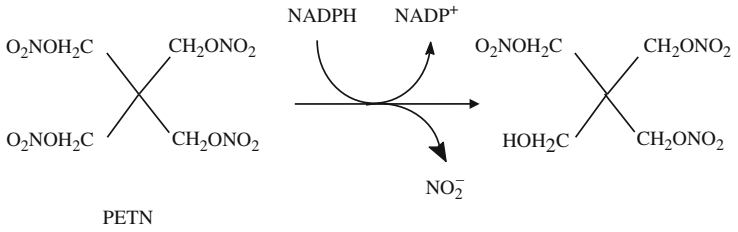

Fig. 17.3 Metabolism of PETN by *Enterobacter cloacae*

reductase (French et al. 1999). PETN reductase reduces PETN and glycerol trinitrate (GTN) to nitrite (Fig. 17.3). Seeds from transgenic tobacco plants carrying the *onr* gene germinated and grew in media containing 1 m*M* GTN, which was toxic to untransformed seeds. The researchers showed that transgenic tobacco plants expressing microbial NR and PETN reductase could not only tolerate high amounts of TNT, GTN, and PETN but also uptake and degrade them, making phytodetoxification a possibility in the cleanup of fields contaminated with nitroaromatic and nitrate ester explosives (Jube and Borthakur 2007).

Schnoor et al. (2006) investigated several genes encoding for enzymes known to be involved in the detoxification of xenobiotic pollutants, such as glutathione *S*-transferases (GSTs), cytochrome P_{450}s (CYPs), NADPH-dependent reductases, and peroxidases. Starting from *A. thaliana* TNT-inducible genes, corresponding *Populus* sequences were retrieved from the JGI Poplar Genome Project database and they were used to design gene-specific primers. The 18S ribosomal DNA

(rDNA) was used as an internal standard and recorded gene expression levels were normalized by reference to non-exposed plants. In three separate experiments, 5 genes were found to be significantly amplified in leaf tissues by exposure to RDX, including GST (9.7 fold), CYP (1.6 fold), reductases (1.6 to 1.7 fold), and peroxidase (1.7 fold). In root tissues, only a single GST gene was found to be significantly amplified by exposure to RDX (2.0 fold). These results show for the first time that exposure of poplar plants to RDX results in the induction of several genes potentially involved in explosive detoxification.

5 Transgenic Plants for Phytoremediation of Explosive Compounds

Although plants are capable of reducing the concentrations of some organic environmental pollutants, the activity is often too slow to be of practical value. Because phytoremediation proceeds primarily only during the growing season, substantial remediation must be achieved during a limited time period. The effectiveness of phytoremediation can be greatly enhanced by introducing genes known to be involved in metabolism of pollutants in other organisms (Table 17.2). For example, the nitroaromatic explosives TNT and RDX are phytotoxic and cannot be effectively treated by using conventional phytoremediation. By introducing bacterial genes involved in the metabolism of TNT and RDX, the tolerance and uptake of these pollutants by transgenic plants were considerably improved (Doty et al. 2007).

Phytoremediation of nitroaromatics was significantly improved with transgenic plants (Rosser et al. 2001; Hannink et al. 2002). As nitroaromatic explosives are phytotoxic, phytoremediation of these pollutants using nontransgenic plants is severely hindered. However, when bacterial genes involved in degradation of the nitroaromatics were expressed in plants, the plants became more tolerant of the pollutant and could more readily remove it. In the first paper on this strategy, French and colleagues introduced pentaerythritol tetranitrate (PETN) reductase into transgenic tobacco (*Nicotiana tabacum*), resulting in increased tolerance to trinitroglycerin and TNT (French et al. 1999). This paper in 1999 was the first published case of plants being genetically modified to actually detoxify a xenobiotic pollutant (Hooker and

Table 17.2 Microbial genes capable of utilizing explosives and their transgenics

Gene	Source organism	Gene product capable of utilizing	Transgenic produced in
RDX gene cluster	Rhodococcus rhodochrous 11Y	RDX	*Arabidopsis*
nfsI	Enterobacter cloacae PB2	TNT	*Nicotiana*
xplA/xplB	*Rhodococcus rhodochrous*	TNT, RDX	*Arabidopsis, Populus*
onr	Enterobacter cloacae PB2	PETN, GTN	*Nicotiana, Populus*

Skeen 1999). The PETN reductase is the only enzyme known to remove nitrate from TNT, degrading it to nontoxic compounds. The gene was isolated from the soil bacterium *Enterobacter cloacae* PB2, which can utilize the explosives as a sole nitrogen source (Binks et al. 1996). Transgenic tobacco seedlings containing the PETN reductase gene germinated on medium containing 1 m*M* glycerol trinitrate while the nontransgenic seedlings failed to germinate. In a later work, a bacterial nitroreductase (NR) was overexpressed in tobacco plants. These transgenic plants were more tolerant to higher concentrations of TNT and metabolized it at far greater rates than the control plants (Hannink et al. 2001). Wild-type plants exposed to 0.25 m*M* TNT became chlorotic and lost mass, while the NR transgenic plants continued to grow. When 20-d-old seedlings were exposed to 0.1 m*M* TNT, wild-type seedlings failed to grow at all, whereas the NR transgenic plants still looked healthy. At that concentration, wild-type plants had a root tolerance index of 3%, and transgenics had an index of 68%. For phytoremediation of explosives to be successful, the plants must be healthy and have effective root systems. By expressing bacterial genes for the degradation of TNT, the transgenic plants overcame some of the phytotoxic effects and removed TNT more rapidly than the wild-type plants. In addition, the transgenic plants benefited the soil microbial community (Travis et al. 2007). NR transgenic tobacco had increased tolerance to soil contaminated with TNT even to the limits of its solubility (130 mg L^{-1}). The transgenic plants decreased the TNT concentration surrounding the roots, allowing the microbial community to survive, unlike the wild-type plants which had a dramatic reduction in colony-forming units and in microbial diversity at the higher TNT concentrations. In military training ranges and production facilities for explosives, the areas are contaminated not only with TNT but also with other explosives such as RDX. Using a similar approach as that used for TNT, genes were isolated from an RDX-utilizing bacterium and overexpressed in transgenic plants. The required genes consisted of an unusual microbial P_{450} system with two components: a flavodoxin reductase (*xplB*) and a fused flavodoxin cytochrome P_{450} (*xplA*). Transgenic plants expressing *xplA* showed enhanced removal of RDX (Rylott et al. 2006). When transgenic *Arabidopsis* seedlings were exposed to RDX at 40 mg L^{-1}, a concentration three times as high as those found in waste at manufacturing plants, the best-performing line removed all the RDX within 5 days. By contrast, the wild-type plants did not reduce the concentration at all. The transgenic plants did not exhibit any of the signs of RDX toxicity present in the wild-type plants. These studies demonstrate the potential for enhancing phytoremediation of explosives using genetic engineering. Similar studies with poplar and range grasses are in progress (Doty 2008).

6 Conclusions

A rapidly expanding literature documents phytoremediation to be an effective method in treating hazardous sites. Yet the method is not used as widely as it could be to restore thousands of contaminated areas. Over the past several years, a significant progress has been made to increase the effectiveness and efficiency of

phytoremediation. The use of genetic engineering has especially helped to step up removal rates of hazardous pollutants. However, it may be the judicious combination of engineering methods and enhanced phytoremediation that will provide the ultimate solution to cleaning up heavily contaminated sites. Genetic engineering of plants for enhanced phytoremediation has obvious environmental benefits, yet some would see therein potential risks. This is especially true when using genetically altered trees. Their long life cycle makes risk assessment more challenging and thus more specific research is needed. In a commentary on this topic, Nicholas Linacre and colleagues describe a risk assessment scenario for enhanced metal remediation. They state that the risk of contamination of food with an engineered metal hyperaccumulator, for example, is low because plants used for phytoextraction would be in isolated, industrial-type areas, not in agricultural areas. Furthermore, crops used for phytoextraction would be harvested before seed set, thus reducing the threat of crossing with other crops intended for food, or entering the food supply. Plants engineered to hyperaccumulate toxic metals in foliage could be harmful to wildlife; however, studies have demonstrated that such foliage is not appealing in taste and is avoided. The best way to determine the ecological impact of transgenic plants for phytoremediation is by conducting field trials designed to assess risks. Opposition to using transgenics, even in field trials, based on the fear of unknown risks may well interfere with the potential removal of the known risks of having carcinogens and other harmful pollutants in our environment.

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Chapter 18
Phytoremediation of Cyanide

Avinash C. Srivastava and Rajasekhara Reddy Duvvuru Muni

Abstract Free cyanide and complex cyanide, including HCN and CN^- is the most reactive and toxic substance of all industrial and anthropogenic pollutants. Many studies till date have proved that cyanide can be efficiently removed by plants. From the economic point of view, phytoremediation could be an attractive and useful technology in dealing with this dangerous pollutant. Phytoremediation of complex and free cyanide include removal of cyanide by terrestrial and aquatic plants. Experiments using free and complex cyanide have shown that many terrestrial and aquatic plants including willow, sorghum, cassava and water hyacinth can remove free cyanide from the hydroponic media. Cyanide uptake in plants can be associated with very complex physiological mechanisms which include transport and assimilation of cyanide within the plants for catering plant's nitrogen needs. Transport and metabolism of different chemical species of cyanide differ in various plant species including trees, grasses and aquatic plants. Again uptake of cyanide by roots is depending on its form and condition. A detailed insight of uptake, transport and assimilation of cyanide compounds in plants is discussed here. In this chapter, chemical nature of cyanide, possible industrial pollutant sources, various phytoremediation approaches, mechanism of cyanide assimilation in plants, and genomics of cyanide remedy are evaluated.

Keywords Cyanide · Genetic engineering · Metabolism · Phytoremediation · Uptake

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Contents

1 Cyanide as a Pollutant

Cyanide is a nitrile, an organic compound that contains a triple-bonded carbon nitrogen functional group. Most such compounds are highly toxic, carcinogenic, and mutagenic (Banerjee et al. 2002). Common symptoms of cyanide poisoning include gastric problems, vomiting, respiratory distress, convulsions, and coma (Banerjee et al. 2002). The toxicity of cyanide is quite high due to its ability to poison the respiratory system by inhibiting the final transport of electrons from cytochrome C oxidase to oxygen, preventing production of ATP.

Cyanide can be found naturally in soils or can result from contamination from industrial processes such as gas plant sites, salt storage facilities, electroplating facilities, and gold mining operations (Kjeldsen 1998). Hydrogen cyanide (HCN), cyanide anion (CN^-), inorganic salts (e.g. NaCN), ferrocyanide ($Fe(II)(CN)_3{}^6-$), ferricyanide ($Fe(III)(CN)_4{}^6-$), thiocyanates ($-SCN$), and nitriles (organic materials with CN group) are typical cyanide-bearing environmental contaminants (Ebbs et al. 2008). At contaminated manufactured gas plant sites, iron cyanide, primarily Prussian blue, is the predominant form of cyanide contamination. Hydrogen cyanide is formed during the gasification of the coal, and the toxicity of the gas required its removal prior to natural gas distribution (Riesenfeld and Kohl 1974).

1.1 Physical and Chemical Forms of Cyanide

The specific form of cyanide determines the environmental fate and transport of cyanide, as well as its toxicity. Cyanide occurs as various physical metal-cyanide complexes and metal-cyanide solids in water and soil. Figure 18.1 has described distribution of various forms of cyanide in aqueous, solid and gaseous phases.

Chemically, cyanide can be classified into inorganic and organic forms, as indicated in Fig. 18.1. Inorganic forms, which occur in all three physical states, include free cyanide, weak metal-cyanide complexes, strong metal-cyanide complexes, thiocyanate and metal-thiocyanate complexes, cyanate and metal-cyanate complexes and cyanogen halides. The cyanide anion, CN^-, and HCN, are very volatile under environmental conditions, and occur as both aqueous and gaseous species (Dzombak et al. 2006). The cyanide anion is a versatile ligand that reacts with metal cations to form metal-cyanide complexes. Dissociation of these complexes can yield free cyanide. These are again subdivided into weak and strong metal cyanide complexes. Free cyanide can also be oxidized to form cyanate, CNO^-, which is less toxic than free cyanide. Free cyanide can react with sulphur to form thiocyanate, SCN^-, which is almost nontoxic. Metal-thiocyanate and organocyanate are other aqueous forms of cyanide present in the atmosphere.

There are three major solid forms of cyanide that exist in the nature. These are metal::cyanide, metal::metal-cyanide and alkali earth metal::metal solids.

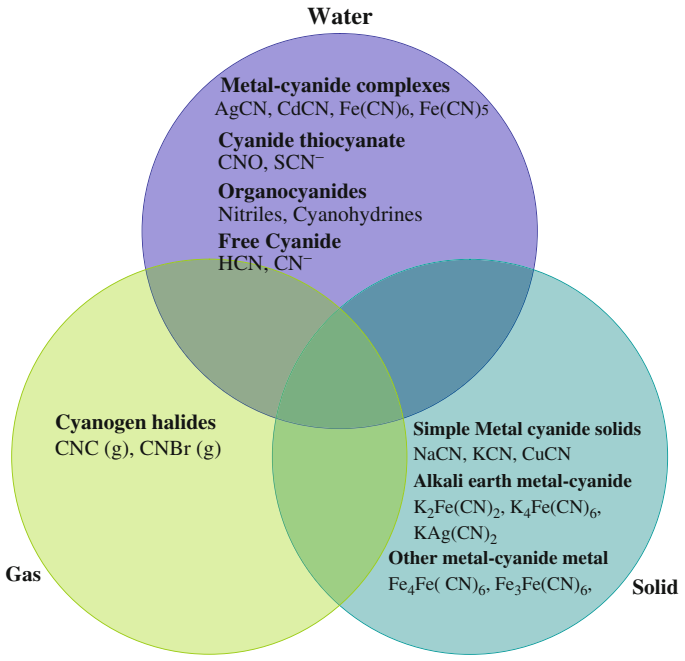

Fig. 18.1 Various forms of cyanide distributed in solid, liquid and gaseous phases

Metal::cyanides are simple structures consisting of metal and cyanide (NaCN and KCN are common examples). Bonding of another metal with the previous form of complex gives birth to metal::metal-cyanide complexes. In the third category, one or more alkali earth metal cations are ionicaly bonded to an anionic metal-cyanide metal complexes. A common example is potassium ferrocyanide ($K_4Fe(CN)_6(s)$). This form can readily dissociate into aqueous solution, releasing alkali metal cyanide and anion metal complexes (Ghosh et al. 2006).

1.2 Industrial and Natural Sources of Cyanide

Cyanide can be released by various industrial and natural sources. For example, thiocyanate, SCN^-, is present in a variety of industrial wastewater discharge. The cyanogen halides, CNCl and CNBr, form upon chlorination or bromination of water containing free cyanide. These compounds are volatile under environmental conditions and thus occur as aqueous and gaseous phases. Cyanide is also used as a raw material during the production of chemicals (nylon and plastic), pesticides, rodenticides, gold, wine, anticaking agent, fire retardents, pharmaceuticals, painting inks, and other materials. Cyanide can also be used directly in a variety of processes, including electroplating and hydrometallurgical gold extraction. Current industries that produce cyanide as a by-product include chemical manufacturing, iron and steel making, petroleum refining, and aluminum smelting (Wong-Chong et al. 2006).

As far natural resources of cyanide are concerned, cyanide has been shown to form nutrient "microcycle" in the environment (Wong-Chong et al. 2006). These micro-cycles involve both cyanide producing (cyanogenic) and cyanide assimilating organisms. Various natural and anthropogenic activities discharge a wide range of cyanide to the environment. Over 3000 species of plants (130 families) produce cyanogenic glycosides as part of natural defense mechanism (Table 18.1). Upon stress or injury, cyanogenic glycosides are hydroloyzed by a coexisting plant enzyme and release HCN. In addition, almost all fruit bearing plants release HCN during ethylene synthesis which aid in the fruit ripening. Table 18.1 shows a partial list of cyanogenic plants. Critical analysis of the table reveals that plants of different families contain different quantities of cyanogenic material and plants within the same family or within the same species, contain different quantities of cyanogenic material. Different tissues can contain different concentrations of cyanogenic compounds. These variations in cyanogenic material could be due to different soil conditions, cultivation conditions, life stage of the plant, physiological status of the plant, and some other factors (Dzombak et al. 2006). Plants containing cyanide occur mostly in the rose family and in particular, trees and shrubs from the genus *Prunus* including choke cherry, pin cherry, domestic cherry, apricot, peach, apple, and to a lesser degree elderberry (*Sambucus*), flax (*Linum*), Sudan grass, Johnson grass (*Sorghum*) and Serviceberry (*Alelanchier*). Elderberry plants are known to contain the purgative alkaloid sambucine as well as hydrocyanic acid in the seeds, stems, roots, and unripe fruit, but significant ingestion of this compound is needed to cause problems. Most plants in the *Prunus* genus have edible fruit, but the other

Table 18.1 Cyanide content in various tissues of cyanogenic and non-cyanogenic plants

Plant species	Tissue analyzed	Cyanide concentration (mg HCN kg fw^{-1})
Cassava (Bitter)	Whole root	530
Cassava (Bitter)	Root pulp	310
Cassava (Bitter)	Root peel	650
Cassava (Bitter)	Root cortex	2450
Cassava (Bitter)	Whole root	395
Cassava (Bitter)	Leaves	310
Cassava	Fresh leaves	80–4000
Cassava (sweet)	Root pulp	38
Cassava (sweet)	Root peel	200
Cassava (sweet)	Whole root	462
Cassava (sweet)	Leaves	468
Bamboo	Immature shoot tip	8000
Sorghum	Whole immature plant	2500
Sorghum	Dried pulp	249
Sorghum	forage	100–800
Lima beans		100–3200
Almonds (bitter)	Leaves	1059–1807
Almonds (bitter)	Unripe pod	882
Acacia erioloba	Leaves	1059–1807
	Unripe pod	882
Acacia glaucescens	Leaves	2513
Eucalyptus polyanthemos	Leaves	0–181
Eucalyptus yarraensis	Leaves	39–113

Sources: Data from Dzombak et al. (2006)

parts of the plant including the leaves, bark, wood, and seeds contain hydrocyanic acid (Poulton 1990). The cyanide component is readily absorbed through the rumen where it binds quickly to the ferric ion in cytochrome oxidase, which prevents the release of oxygen from the hemoglobin in the blood thus resulting in the suffocation of the ruminants. For their defense, ruminants contain an antitoxic enzyme which prevents poisoning from hydrocyanic acid until a toxic threshold is reached, upon which poisoning occurs rapidly. Cyanide sugar is always present and usually the ruminants can detoxify small amounts of the toxin. Although, when the plant is exposed to frost, drought, and intense summer storms, concentration of cyanide sugar can be very high resulting in poisoning. Therefore, cyanogenic plants are also known as goat killers (Poulton 1990).

1.3 Cyanide in Water and Soil

Cyanide is an industrial byproduct or residual as solid phase iron-cyanide compounds. Leaching of soluble metal-cyanide complexes from these materials can

eventually result in cyanide accumulation in groundwater. At some sites, such as electroplating and ore heap leaching sites, other metal-cyanide complexes can be formed due to the presence of other metals like Cu and Ni. The specific distribution and specification of cyanide at an industrial site is a function of the characteristics of the existing production processes, as well as it determines past and present environmental conditions at the site (Ghosh et al. 2006).

Cyanide impacts in soil have been observed in many industrial sites. At many sites, the predominant forms of cyanide compounds are iron-cyanide solids (Prussian blue or ferric-ferrocyanide). On the contrary, at ore heap leaching and electroplating spill sites, the cyanide in soil is usually dominated by a mixture of iron-cyanide and other metal-cyanide compounds (Ghosh et al. 2006). The sources of cyanide production at coke production site is usually oxide-box residuals that were managed onsite as fill (Ghosh et al. 2006) which comes from "spent oxide". The spent oxide material contained double-iron-cyanide compounds, like Prussian blue, which formed over time as the free cyanide reacted with the iron. The presence of spent oxide in soil is readily apparent from the intense blue color of the Prussian blue. The double-iron-cyanide salts, like Prussian blue and turnbull's blue have very low solubility under acidic to neutral in nature (Ghosh et al. 2006). As a result, these compounds dominate cyanide-impacted soils that are acidic to neutral in nature. In addition to the cyanide source materials, dissolved metal-cyanide complexes can also absorb various natural soil adsorbents (Ghosh et al. 2006). Natural organic matter can also act as an important adsorbent for both ferro- and ferricyanide complexes over a range of pH conditions (Ghosh et al. 2006). This information suggests that in addition to dissociation of source materials such as spent oxide, desorption of iron-cyanide complexes should be considered for certain soil types.

2 Cyanide Detoxification

2.1 Mechanical Processing of Cyanide Waste

The majority of processes used for remediation convert cyanide into one or more less toxic compounds through an oxidation reaction (Akcil 2003). Sulfur dioxide/air is a common process developed by the International Nickel Company (INCO) more than two decades ago. This process uses SO_2 or a derivative along with air in the presence of a soluble copper catalyst. This causes oxidation of cyanide to the less toxic cyanate (Akcil 2003).

In the second process, hydrogen peroxide is used in place of SO_2 and air. The hydrogen peroxide process is primarily used for solutions, whereas the SO_2/air process can be used in both the treatment of slurry and solutions (Akcil 2003). The SO_2/air and the hydrogen peroxide processes, both catalyzed by copper, are the most successful of the non-biological processes (Akcil and Mudder 2003).

A third and the important process in the destruction of cyanide waste is alkaline and breakpoint chlorination. The first step of this two-step process is the

conversion of cyanide to cyanogenic chloride. This compound is then hydrolyzed into cyanate (Akcil 2003). Although chemical and physical treatments provide more rapid detoxification and are less susceptible to environmental upsets (Akcil and Mudder 2003), biological alternatives are more economical and good for ecological balance.

2.2 Phytoremediation

Phytoremediation is the use of vascular plants, algae, or fungi to metabolize, sequester, or to induce contaminants breakdown in soil or other plant growing medium (McCutcheon and Schnoor 2003). Plant sequestration of contaminants is important as an alternative to physically based treatment approaches. The use of plants for remediation seems less expensive, but it depends on certain factors. Phytoremediation has gained importance in the last one decade and approximately US $6–8 billion per year have been spent for environmental cleanup in the United States, and $25–50 billion per year worldwide through phytoremediation (Glass 1999; Tsao 2003).

Phytoremediation effectively removes cyanide pollutants, but in many cases the underlying biological mechanisms remain largely unknown. To increase the efficiency of phytoremediation technologies, it is important that we learn more about the biological processes involved. These include plant-microbe interactions, rhizospheric processes, plant uptake, plant chelators, translocation mechanisms, tolerance mechanisms (compartmentation, degradation), and assimilation processes related to cyanide detoxification. It is also important to understand fundamental knowledge of the physicochemical mechanisms that influence cyanide fate processes in the environment. In this chapter, we have tried to focus on basic and advanced physiological and molecular processes involved in cyanide metabolism based on the results available from laboratory and field studies.

To elucidate further about phytoremediation, it is very important to understand the basic background of natural production of cyanide and its relation with plants. Cyanogenic plant species have the capability to convert certain amino acids to cyanogenic glycosides, a simple sugar bonded to a cyanide molecule. More than 500 genera and 100 families of plant species are cyanogenic (Seigler 1998), and cyanide is released from the cyanogenic glycosides (a process called cyanogenesis) in response to tissue disruption (Selmar et al. 1990).When tissue injury occurs, cyanoglycosides are hydrolyzed to a sugar, HCN, and a keto or aldehyde compound (Kobayashi and Shimizu 2000). Although there are many organisms and plants that produce cyanogenic compounds, these quantities are very less compared to cyanide produced by industrial processes. Natural development of detoxification system of cyanide in plants is very obvious if plants are cyanogenic. Cyanogenic glycosides may be utilized as a nitrogen source within the plant and as a precursor for amino acid and protein synthesis during seedling development (Niedzwidez-Siegien, 1998). This same mechanism can be used to detoxify CN within cyanogenic species. Not only this but excised leaves of maize and 28 Chinese vegetations

also degrade CN (Yu et al. 2004; Yu and Gu 2007) which proves that plants can digest cyanide even if they are not cyanogenic. Decrease of cyanide compounds in sorghum over time has also been reported and correlated with plant maturity providing evidence of the potential for contaminant removal. In addition, willows and other species have also shown transport and metabolism of free cyanide and iron cyanide complexes (Ebbs et al. 2003; Yu et al. 2005a, b, 2006; Larsen and Trapp 2006). All these examples very clearly indicate that cyanide can be very effectively removed from the pollution zone by plants if we can develop through understanding of cyanide detoxification processes in plants.

2.3 Phytoremediation – Case Studies

A large number of studies have proved that free cyanide can be rapidly biodegraded by micro-organisms (Knowles and Bunch 1986; Kunz et al. 1994; Fernandez and Kunz 2005), however, many cyanide complexes including iron cyanide complexes tend to be resistant to microbial degradation (Aronstein et al. 1994). There are only few reports of microbial (Cherryholmes et al. 1985; Dursun et al. 1999) and fungal (Barclay et al. 1998) biodegradation of metal cyanide complexes, but this has only been observed during *in vitro* studies, and most often with strains isolated from contaminated sites. Plants as discussed above can deal with various forms of cyanide including cyanide complexes and free cyanide.

Several experiments, mostly hydroponic experiments have been conducted for phytoremediation of cyanide. Investigation on the potential of Chinese vegetation to degrade cyanide revealed that detached leaves (1.5 g fresh weight) from 28 species of Chinese vegetation plants in aqueous solution spiked with potassium cyanide can remove cyanide from the solution in variable amounts (Table 18.2) (Yu et al. 2004). Cyanide concentrations ranged from 0.83 to 1.0 CN mg L^{-1}. The fastest cyanide removal reported is for Chinese elder, *Sambucus chinensis*, with a removal capacity of 8.8 mg CN kg^{-1} h^{-1}, followed by upright hedge-parsley (*Torilis japonica*) with a value of 7.5 mg CN kg^{-1} h^{-1}. The lowest removal capacity of cyanide has been noted for snow-pine tree (*Credrus deodara* (Roxb.) Loud). Results from various studies have indicated that a wide range of plant species can efficiently metabolize cyanide. It is therefore, cyanide elimination with plants is a feasible option for cleaning soils and water contaminated by cyanide from gold and silver mines or from other sources (Yu et al. 2004, 2005a, b).

Metabolic responses of hydroponically grown weeping willow (*Salix babylonica* L.) to cyanide were not only positive but it also showed growth-promoting effects on plants. For example, plants grown under cyanide had higher transpiration rates, chlorophyll contents and soluble protein contents compared with the non-treated control plants. Superoxide dismutase (SOD), catalase (CAT) and peroxidase activities in leaves also changed due to cyanide application. These changes due to cyanide application in plants indicate that willow and similar plants can detoxify cyanide contaminations from any solutions and this cyanide can be further metabolized. Although experimental evidence also suggests that small amounts

Table 18.2 Calculated cyanide removal capacity per plant mass (mg CN kg^{-1} h^{-1})

Species	CN removal capacity (mg CN kg^{-1} h^{-1})	Plant family
Sambucus chinensis Lindl.	8.77	Caprifoliaceae
*Torilis japonica (*Houtt.) DC	7.52	Umbelliferae
Prunus persica Sleb.	2.83	Rosaceae
Prunus pseudocerasus Lindl.	6.28	Rosaceae
Salix babylonica L.	6.08	Salicaceae
Glycine max (L.) Merr.	3.45	Leguminosae
Chimonanthus praecox (L.) Link	5.97	Calycanthaceae
Liquidambar formosana Hance	5.22	Hamamelidaceae
Metasequoia glyptostroboides Hu & Cheng	5.32	Taxodiaceae
Capsicum frutescens L.cv. 'Hexiniujiaojiao'	4.93	Solanaceae
Euonymus alatus (Thunb.) Sieb.	4.27	Celastraceae
Zea mays L.	4.75	Poaceae
Cudrania tricuspidata (Carr.) Bur.	4.33	Moraceae
Sorghum vulgare Pers.	3.98	Poaceae
Calendula officinalis L.	4.02	Compositeae
Nymphea teragona	3.6	Nymphaceae
Salix matssudana alba	3.15	Salicaceae
Alternanthera philoeroides Griseb	3.97	Amaranthaceae
Populus deltoides Marsh.	3.43	Salicaceae
Iris tectorum Maxim	3.05	Iridaceae
Prunus persica (L.) Batsch	2.83	Rosaceae
Buxus sinica (Rehd. & Wils.) M. Cheng	2.72	Buxaceae
Trachycarpus fortunei (Hook.f.) H. Wendl	1.63	Areacaceae
Viburnum odoratissimum Ker-Gawl	2.28	Caprifoliaceae
Gossypium hirsutum L.	1.52	Malvaceae
Pterocarya stenoptera C.DC.	1.75	Juglandaceae
Podocarpus macrophyllus (Thunb.)	0.6	Podocarpaceae
Credrus deodara (Roxb.) Loud	0.23	Pinaceae

Data from Yu et al. (2004)

of cyanide can be detected in the plant tissues after cyanide consumption at contaminated sites, recovery of cyanide in different compartments of plants varies significantly and root is the dominant sink for cyanide accumulation (Yu and Gu 2009). It has been observed that >97% of the applied cyanide can be metabolized during transport through weeping willow and the metabolic rates of cyanide by plants is linearly increased with increase in cyanide applied in the growth medium. These findings strongly indicate that phytoremediation is a desirable solution of treating environmental sites contaminated with cyanide (Yu and Gu, 2009).

Aquatic plants can also play an important role in cyanide detoxification. Cyanide in the effluents from the gold mines can be removed by water hyacinth (*Eichhornia crassipes*). Sodium cyanide phytotoxicity and removal capacity of cyanide by *E. crassipes* has been found to be 10 mg L^{-1} and cyanide can be completely eliminated within 23–32 h by using *E. crassipes.* After feeding $K^{14}CN$, it was observed that about 40% of the radioactivity from solution was converted into $^{14}CO_2$ within 28 h (Ebel et al. 2007). In response to cyanide application, *E. crassipes* can also maintain its high biomass production. Due to wide distribution and also tolerance to toxic metals other than cyanide (CN), *E. crassipes* can be considered as a very important aquatic plant in cyanide detoxification. These results indicate that *E. crassipes* could be very useful in treating cyanide effluents from small-scale gold mines (Ebel et al. 2007).

In addition to the plant's ability to detoxify cyanide, this whole process is also greatly influenced by soil microorganisms and their interactions with plant roots. Symbiotic fungi are an important component of soil microbes. In nature majority of plants live in symbiotic association with different types of mycorrhizal fungi. During successful symbiosis, mycorrhizae provide selective advantage to the plant not only by enhanced supply of water and nutrients with increased root surface area but also support the plants by detoxifying certain harmful chemicals and compounds. From several studies, it is now clear that mycorrhizal fungi can contribute to the heavy metal detoxifiction directly by phytostabilisation, phytoextraction or by phytodegradation, and indirectly by increasing plant ability to withstand phytotoxicity. In this regard, the systems that incorporate microbes can form robust and stable associations with plant roots and is another useful tool of phytoremediation. In this regard, example of *Trichoderma harzianum* strain T22 is very important which, in synergy with plants can hyper-accumulate heavy metals and arsenic, and can remove various toxicants from soils or water. These microbes can also produce enzymes that degrade cyanide when associated with plant roots (Ebbs 2004). *Trichoderma* has been used in agriculture, and it has been shown to be a plant symbiotic, safe and nontoxic. In another case, *Gloeocercospora sorghi,* the cause of zonate leaf spot of sorghum, is adaptively tolerant of HCN (Fry and Evans 1977; Fry and Munch 1975). The effectiveness of the bioremediation techniques depends on the appropriate selection of both the plant and the fungal partners. Many plants conventionally introduced in polluted places disappear relatively soon, while those appearing during natural succession are better adapted to harsh conditions. Symbiotic partners selected on the basis of such research are often the best choice for future phytoremediation technologies.

3 Uptake and Transport of Cyanide By Plants

Uptake of pollutants by plant roots is different for organics and inorganics. Organic pollutants are usually manmade, and xenobiotic to the plant. As a consequence, there are no transporters available for these compounds in plant membranes. Organic

pollutants therefore tend to move into and within plant tissues driven by simple diffusion, depending on their chemical properties. An important property of the organic pollutant for plant uptake is its hydrophobicity (Briggs et al. 1982; Trapp and McFarlane 1995). Depending on the phytoremediation strategy, cyanide uptake into the plant may be desirable (e.g., for phytoextraction) or not (e.g., for phytostabilization).

Phytoremediation, may provide opportunity to remediate cyanide and iron cyanide contamination, provided that these compounds can be transported and assimilated by plants after passive or active? uptake. Translocation from root to shoot requires a membrane transport step from root symplast into xylem apoplast. The impermeable suberin layer in the cell wall of the root endodermis (Casparian strip) prevents toxic substances from flowing straight from the soil solution or root apoplast into the root xylem (Taiz and Zeiger 2002). Organic pollutants pass the membrane between root symplast and xylem apoplast via simple diffusion. When pollutants are sequestered in tissues, they are often bound by chelators or form conjugates. Chelators that are involved in metal sequestration include the tripeptide GSH (γ - glu-cys-gly) and its oligomers, the phytochelatins (PCs) (Pickering et al. 2000). After chelation, an ABC-type transporter can actively transport the metal-chelate complex to the vacuole, where it is further complexed by sulfide (Cobbett and Goldsbrough 2000). For example, ferritin is an iron chelator in chloroplasts (Theil 1987). Several studies have demonstrated transport of organic contaminants and metal-chelate complexes by plants (Burken and Schnoor 1997; Thompson et al. 1998; Vassil et al. 1998; Epstein et al. 1999; Thompson et al. 1999) via ATP binding cassette (ABC) transporters (Mäser et al. 2001). A mitochondrial inner membrane anion channel has also been shown to transport ferrocyanide as well as a variety of other anions (Beavis and Vercesi 1992). There is still much to be discovered about the roles of different chelators in transport and detoxification of various pollutants including cyanide.

Possibility of cyanide transporting mechanism in willow (*Salix eriocephala* L. var. Michaux) was suspected as these trees can grow very well in close proximity to iron cyanide contamination site (Reeves and Baker 2000). Various studies indicate that willow plant is capable of phytoremediation of iron cyanide complexes by cyanide assimilation and not by cyanide accumulation. This is mainly evidenced by KCN consumption in plants. It has been observed that by providing KCN at 2 mg L^{-1}, no change in transpiration, water content, or plant biomass was noticed with time and across treatments (Trapp et al. 2003). Further, only negligible amount of cyanide content was detected in any plant tissue despite having significant enrichment in KCN application (Trapp et al. 2003). Evidence that cyanide is actually assimilated in plants comes from detection of various complex forms of cyanide in different tissues despite applying free cyanide to the roots. It is important that free cyanide can be detected only in roots, but majority of the leaf cyanide was in complex form. Additional efforts to fully elucidate the potential pathways of cyanide metabolism have proved importance of cyanide assimilation in plant nitrogen metabolism, and the efficacy of cyanide phytoremediation. Greenhouse experiments using stable isotope labeled (^{15}N) free cyanide

(as KCN) and ferrocyanide were conducted to examine the transport and biological fate of these compounds in willow, two species of *Eucalypts*, and several grass species. The results to date suggest that these species are capable of transporting both chemical forms of cyanide. Both chemical species are also apparently metabolized by plants, although the dissociation of the ferrocyanide complex appeared to be a rate limiting step for the metabolism of this compound. Thus far, neither cyanide accumulation in above-ground tissues nor cyanide volatilization have been observed, suggesting that cyanide phytoremediation would not pose an ecological risk. The results from these physiological studies have been combined with those from studies of the geochemical studies of cyanide in soil-water systems to develop an integrated computer model which quantifies partitioning of free cyanide and ferrocyanide in a soil-water-plant system. This robust model has been extended to a constructed wetland scenario to evaluate the engineering feasibility of cyanide phytoremediation. Transport of cyanide and ferrocyanide by plants, coupled with the potential metabolism of these compounds suggests the possible application of phytoremediation to these contaminants (Trapp et al. 2001). Whether the pathway of metabolism in the KCN- and ferrocyanide-treated plants is the same, it is yet to be determined and more experimental evidence is needed.

3.1 Factors Affecting Uptake and Transport

There are several factors which could affect cyanide uptake:

1. For phytoextraction and phytostabilization, selection of plant species with the desired properties is useful. Screening studies under uniform conditions will be a supportive strategy to compare cyanide uptake characteristics of different species.
2. Agronomic practices may also be employed to maximize cyanide uptake. Plant species may be selected for suitable rooting depth and root morphology (Negri et al. 2003) and plant roots can be guided to grow into the polluted zone via deep planting in a casing, forcing the roots to grow downward into the polluted soil and to tap into cyanide polluted water rather than rainwater (Negri et al. 2003; Elizabeth 2005).
3. Phytosiderophores are chelators that facilitate uptake of various metals in grasses. They are biosynthesized from nicotianamine that chelate metals and may facilitate their transport (Taiz and Zeiger 2002). Chelation in roots can affect phytoremediation efficiency as it may facilitate root sequestration, translocation, and/or tolerance. Root sequestration may be desirable for phytostabilization (less exposure to wildlife), whereas export to xylem is desirable for phytoextraction. Bioavailability of various toxic substances including cyanide can be enhanced by using chelators that are released by plants and bacteria. Chelators such as siderophores, organic acids, and phenolics can release various metal cations from soil particles. This usually increases availability of the toxic substances for

plant uptake (Taiz and Zeiger 2002) although in some cases it can also prevent uptake. Furthermore, plants extrude H^+ via ATPases, which replace cations at soil CEC sites, making metal cations more bioavailable (Taiz and Zeiger 2002). This strategy can be very useful in detoxification of cyanide-metal complex.

4. Cyanide bioavailability could also be affected by various plant and/or microbial activities. Some bacteria are known to release biosurfactants (e.g., rhamnolipids) that make hydrophobic compounds more water soluble (Volkering et al. 1998). Plant exudates or lysates may also contain lipophilic compounds that can increase solubility or promote biosurfactant-producing microbial populations in cyanide pollutant water (Siciliano and Germida 1998). Furthermore, plant and microbe derived enzymes can affect the solubility and thus the bioavailability of cyanide via modification of side groups (Siciliano and Germida 1998) is possible.
5. In a nutshell, rhizosphere processes that favor phytoremedation can be optimized by the choice of plant species, e.g., plants with large and dense root systems for phytostimulation, or aquatic plants for metal precipitation. Secondly, if a certain exudate compound is identified to enhance phytoremediation (e.g., a chelator or a secondary metabolite that stimulates microbial degradation) plants can be selected or genetically engineered to produce large amounts of this compound. In one such study, overexpression of citrate synthase in plants conferred enhanced aluminum tolerance, probably via enhanced citrate release into the rhizosphere, which prevented Al uptake due to complexation (Elizabeth 2005). If the microbial consortia responsible for the remediation process are known, it may be possible to increase the abundance of these species by the choice of vegetation. An alternative approach is to grow these microbial isolates in large amounts and add them to the soil, a process called bioaugmentation (Elizabeth 2005).

4 Cyanide Assimilation and Metabolism

In phytoremediation, cyanide uptake is not always associated with cyanide assimilation but sometimes after cyanide uptake, this toxic substance can be accumulated in certain plant species. In all cases where potentially cyanide pollutants are accumulated in plant tissues, phytoremediation in the field should include a risk assessment study because the plant material may pose a threat to wildlife. The degree of toxicity depends on leaf concentration and also on the form of the cyanide pollutant that is accumulated. During accumulation, the toxicity of the cyanide pollutant may change. To test the potential toxicity of the plant material, a laboratory digestibility test using model organisms would be helpful.

Cyanide assimilation is a complex metabolic process, where phyto-degradative plant enzymes act on cyanide pollutants and catabolize them, or degrade them partially to a stable intermediate that is stored in the plant (McCutcheon and Schnoor 2003). This enzymatic degradation can happen in both root and shoot tissues. Degradation within plant tissues is generally attributed to the plant, but in some

cases involves endophytic microorganisms (Barac et al. 2004). Phytodegradation involves some of the same classes of enzymes responsible for accumulation in tissues. The modifying enzymes that create side groups on organics increase solubility and enable conjugation playing a role in the initial steps of phytodegradation.

More than 1000 plant species have been demonstrated as cyanogenic and they also possess detoxifying enzyme systems (Seigler 1998; Raquel et al. 2008). The mechanism for the production of HCN, in most species, is the degradation of cyanogenic glycosides (Conn 1980). Several studies have investigated the occurrence and distribution of cyanide metabolizing enzymes in a variety of higher plants, including both cyanogenic and noncyanogenic species.

The HCN potential is a reflection of the concentration of cyanogenic glycosides in the plant which, upon degradation, leads to the release of HCN. Four major enzymes for cyanide degradation and cyanide metabolism are known in plants and microbes-

1. β-cyanoalanine synthase (EC 4.4.1.9)
2. Rhodanese (EC 2.8.1.1)
3. Formamide hydrolyase (FHL3- EC 4.2.1.66)
4. Cyanide dihydratase

The reactions they catalyze are shown in Eqs. (18.1)–(18.4), respectively:

$$HSCH_2CHNH_2CO_2H + HCN \rightarrow NCCH_2CHNH_2CO_2H + H_2S \tag{18.1}$$

$$S_2O_{32^-} + CN \rightarrow SO_{32^-} + SCN^- \tag{18.2}$$

$$HCN + H_2O \rightarrow HCONH_2 \tag{18.3}$$

$$HCN + H_2O \rightarrow HCOOH + NH_2 \tag{18.4}$$

The enzyme β-cyanoalanine synthase has been shown to be present in several plant species (Blumenthal et al. 1968; Floss et al. 1965; Miller and Conn 1980), insects (Ogunlabi and Agboola 2007), and in some bacteria (Dunhill and Fowden 1965; Castric and Strobel 1969; Castric 1975). The utilization of cyanoalanine synthase by plants for the metabolism of cyanide could also be advantageous since many plants (Castric et al. 1972) can further metabolize the product, cyanoalanine to asparagine which can then be incorporated into the general metabolism of the plant. The ubiquity of this enzyme suggests that plant species should also be capable of metabolizing cyanides. In all vascular plants investigated so far, β-cyanoalanine synthase (CAS) catalyze cyanide and cysteine to β-cyanoalanine and sulfide (Miller and Conn 1980; Maruyama et al. 2001). In the following metabolic step, the enzyme β-cyanoalanine hydrolase produces asparagine, an amino acid important for nitrogen storage (Castric et al. 1972). Because of its exclusive localization in mitochondria, the main physiological role of CAS has been considered

to be the detoxification of toxic cyanide (Hendrickson and Conn 1969; Manning 1988).

Rhodanese, however, has been extensively investigated from animal sources (Jones 1998), and some bacteria (Westley 1973), but only little studied in higher plants (Chew 1973; Tomati et al, 1972). Various studies also indicated that rhodanese occurs far less commonly in plants (Miller and Conn 1980). Rhodanese is also reported in mature leaves of *Brassica. oleracea* var. capitata (Tomati et al. 1972), in *Sorghum* sp. (Myers and Fry 1978) and cassava (Emmanuel and Emmanuel 1981).

Formamide hydrolyase (FHL) has been found in several fungal species (Fry and Evans 1977; Fry and Millar 1972) and reported from only two plant sources, Japanese apricot (*Prunus mume*, Sieb. et Zucc.) and loquat (*Eryobotrya japonica*, Lindl) (Shirai 1978; Miller and Conn 1980). Shirai (1978) proposed that HCN is first converted to formamide by FHL and that the latter is then converted to formic acid and NH_3, possibly via formaldoxime as an intermediate. Another possibility is the direct hydrolysis of formamide to formate and NH_3 by the enzyme formamide amidohydrolase. The proposed theory of formamide to formate and NH_3 was later not confirmed by the enzymatic conversion of HCN to formamide or NH_3 as reported by Shirai (1978). Since the enzymatic conversion of HCN to either formamide or NH_3 was not observed in any other plant species, significance of FHL in the metabolism of HCN by higher plants is questionable.

Cyanide hydratases (CHT) and cyanide dihydratases exist in numerous plant pathogenic fungi such as *Fusarium solani* (Barclay et al. 1998), *Gloeocercospora sorghi* (Wang et al. 1992), *Fusarium lateritium* (Cluness et al. 1993; Nolan et al. 2003), and *Leptosphaeria maculans* (Sexton and Howlett 2000). CHT converts cyanide into formamide (Cluness et al. 1993). The related cyanide dihydratases (CynD) convert cyanide to formate and ammonia (Watanabe et al. 1998a) and are found in *Alcaligenes xylosoxidans* subsp. *dentrificans* (Ingvorsen et al. 1991.), *Bacillus pumilus* (Meyers et al. 1993), *Pseudomonas stutzeri* AK61 (Watanabe et al. 1998b) and in *Pseudomonas fluorescens* NCIMB 11764 (Fernandez and Kunz 2005).

Every plant investigated so far is capable of metabolizing HCN by one or more pathways and the pathway common to all plants tested was that involving β-cyanoalanine synthase. A general trend has been noticed between cyanide metabolizing activity and HCN potential in higher plants; the higher the HCN potential, in general, the higher the cyanide metabolizing activity (Miller and Conn 1980). Since the degradation of cyanogenic glycosides leads to the release of HCN, it may therefore be advantageous for plants which contain cyanogenic glycosides to be capable of metabolizing cyanide, and for those plants which contain high levels of cyanogenic glycosides to have high levels of cyanide metabolizing activity. In general, free cyanide (CN^-, HCN) in plants is rapidly removed by the cyanoalanine and the sulfur transferase pathway (Maruyama et al. 1997).

The extensive metabolism of HCN by higher plants indicates importance of cyanide assimilation process. There is evidence that cyanide produced on the breakdown of cyanogenic glucosides in *Lotus* seedlings and shoots of *Nandina domestica*

(Blumenthal et al. 1968) is readily metabolized and converted into asparagine (Peiser et al. 1984). In the cyanophoric plant, the conversion of HCN to asparagine could be a detoxification mechanism. Larsen et al. (2004) also found that in European woody plants cyanide can be rapidly metabolized. Studies on cyanide assimilation in fungi and other microorganisms (Dunhill and Fowden 1965) also suggest that detoxification is a possible role for this process in higher plants. This may be a metabolic activity acquired early in evolution and retained by species that no longer have any need for such a process (Dunhill and Fowden 1965).

A different role for cyanide assimilation has been proposed for plants which contain y-glutamyl-/3-cyanoalanine or other lathyritic compounds. Cyanoalanine, a cyanogenic glycoside would constitute an alternate source of HCN and perhaps the only source of carbon for the nitrile group of 3-cyanoalanine. Phenylalanine-2-^{14}C fed to *Vicia angustifolia* seedlings formed *p*-cyanoalanine labeled predominantly in the nitrile carbon. This vetch species contains the cyanogen vicianin which is derived from phenylalanine, and Tschiersch (1964) postulated that the glycoside is an intermediate in the formation of 3-cyanoalanine from phenylalanine but not all plants known to contain lathyritic agents. While the cyanogenic glycosides could constitute prime candidates for this role, the distribution of these compounds in plants is not known to be ubiquitous (Blumenthal et al. 1968).

Another theory highlighting the significance of cyanide assimilation suggested the importance of CAS in cyanide metabolism during active ethylene biosynthetic conditions such as in fruit ripening, organ senescence, auxin-induction, and in various stress conditions (Yip and Yang 1988). The increase in ethylene production that occurs during the senescence of certain flowers and the ripening of climacteric fruit is accompanied by cyanide production which is detoxified by β-cyanoalanine synthase and this process also produces β-cyanoalanine, a compound that is widely spread in higher plants and neurotoxic to many animals. β-cyanoalanine serves as a plant defense molecule against predators in many plants (Piotrowski et al. 2001). A semi-quantitative relationship between the activity of β-cyanoalanine synthase and ACC (1-aminocyclopropane-1-carboxylic acid) oxidase, the last enzyme in the ethylene pathway in stigmas and styles of *Petunia* flowers has been found. ACC is oxidized by ACC oxidase to form one molecule of ethylene and one molecule of cyanoformic acid, and the latter is decomposed to CO_2 and HCN (Peiser et al. 1984; Pirrung 1985). This cyanide is subsequently detoxified by CAS, which uses HCN and L-cysteine to produce 3-cyanoalanine (Blumenthal et al. 1968; Hendrickson and Conn 1969).

Plants are also inherently more resistant to low concentrations of free cyanide, due to the presence of the alternative oxidase in the mitochondrial electron transport chain along with endogenous plant cyanide-detoxifying enzymes (Hatzfeld and Saito 2000; Aichi et al. 1998; Hasegawa et al. 1995, 1994). Cell respiration study in *Neurospora* proved that respiration in wild type proceeds via a cytochrome chain that is similar to that of higher organisms and sensitive to antimycin A or cyanide. On the other hand, its mutant (Poky) respires by means of two alternative oxidase systems. One of these is analogous to the wild-type cytochrome chain. The second oxidase system is unaffected by antimycin A or cyanide at concentrations which

inhibit the cytochrome chain maximally. It can, however, be specifically inhibited by salicyl hydroxamic acid. The cyanide-resistant oxidase is not exclusive to the mutant but it is also present in small quantities in wild type grown under ordinary circumstances. These quantities may be greatly increased (as much as 20-fold) by growing wild type in the presence of antimycin A, cyanide, or chloramphenicol (Alan and Slayman 1971). These results again strongly indicate the existence of cyanide assimilation mechanism in plants for valuable reasons.

5 Genetic Diversity for Cyanide Assimilation in Plants

Capacity of each plant is different for cyanide assimilation (Table 18.1). For example, external cyanide concentration of 1 μM as free cyanides is nontoxic for *Arabidopsis thaliana* (McMahon and Arteca 2000). However, bush bean can sustain 25 mg NaCN (DW) for 3-days and only then it shows wilting. A complete loss of whole plant turgor caused bush bean to collapse 1 day after the addition of 100 mg NaCN, with 100% mortality after 9 days (Wallace et al. 1977). Willow (*Salix* spp.) is a promising plant for cyanide phytoremediation and is more resilient (Trapp et al. 2001). Diamond willow (*Salix eriocephala* var. Michaux) can grow normally after exposing plants to 2 mg L^{-1} free cyanide for 20 days (Ebbs et al. 2003). Wheat (*Triticum aestivum* L.) treated with ammonium thiocyanate at a rate of 900 kg ha^{-1} showed no adverse effects and expressed a stimulation of growth after 69 days. Plants also use thiocyanate as a central component of the glucosinolate metabolism in cabbage, broccoli, turnip, and Indian mustard (Knowles and Bunch 1986). Sodium cyanide phytotoxicity of cyanide by *E. crassipes* has been found to be 10 mg L^{-1} (Ebel et al. 2007).

6 Cyanide Phytoremediation Technologies

Phytoremediation is a family of emerging biotechnologies that utilize plants for the remediation of environmental contamination. Bushey et al. (2006) described five important steps for phytoremediation:

1. Phytoextraction
2. Rhizofiltration
3. Phytostablization
4. Phytovolatilization
5. Phytodegradation

Phytoextraction is the use of plants to remove metals, or other contaminants from soil and concentrate those contaminants in above-ground plant tissues and finally these contaminants are removed by harvesting the aerial tissues.

Rhizofiltration is the use of plant roots to remove contaminants from polluted waters that can be achieved on small bodies of water. Sunflower and Indian mustard are most often used for this purpose. Since plant cell walls are negatively charged and have large sorption capacity, rhizofiltration can be highly effective in the removal of cationic contaminants. Phytostabilization is the use of plants to reduce the solubility of contaminants in soils, primarily through modification of the physicochemical condition in the rhizosphere thereby reducing contaminant solubility, modify, or toxicity (Lytle et al. 1998). Another aspect of phytostabilization involves the use of metal-tolerant grasses that preferentially sequester metals in their roots (Salt et al. 1995).

Phytovolatilization uses plant that removes contaminants from terrestrial or aqueous systems and facilitates their conversion to volatile forms for release to the atmosphere (Meagher et al. 2000). Phytodegradation involves the use of plants to metabolize mutable contaminants. The extent of degradation varies by contaminant, with some studies showing incomplete degradation of the contaminant (Burken and Schnoor 1997).

7 Genomics and Proteomics of Cyanide Assimilation in Plants

More than 1000 plant species have been demonstrated to have cyanide detoxifying enzyme systems (Seigler 1998; Raquel et al. 2008) and many bacteria and fungi also showed cyanide degrading activity (Westley 1973; Fry and Evans 1977; Fry and Millar 1972; Barclay et al. 1998; Wang et al. 1992; Nolan et al. 2003; Sexton and Howlett 2000; Fernandez and Kunz 2005). Here we have discussed enzyme kinetics and recent progress of certain important enzymes and genes involved in cyanide detoxification.

7.1 β-cyanoalanine Synthase (EC 4.4.1.9)

β-cyanoalanine synthase (CAS; EC 4.4.1.9) catalyzes the conversion of cyanide and cysteine to β-cyanoalanine. CAS has been characterized, purified and cloned from several plants and it has been found that it is a homolog of mitochondrial cysteine synthase (Blumenthal et al. 1968; Hendrickson and Conn 1969; Maruyama et al. 1998; 2000, 2001; Hatzfeld et al. 2000; Warrilow and Hawkesford 2000). Purification and kinetic studies on CAS from plants has been carried out by Hendrickson and Conn (1969). They demonstrated that CAS is a pyridoxal-5#-phosphate (PLP)- dependent enzyme which also possesses cysteine synthase activity but favors CAS activity. By contrast, a closely related group of PLP-dependent O-acetylserine sulphydrylases (OASS: EC 4.2.99.8) are homologous with CAS (Hatzfeld et al. 2000; Maruyama et al. 2001), but favours cysteine synthase activity (equivalent to OASS activity) and also CAS activity (Saito et al. 1993).

Subcellular localization of various OASS has been found in organelles including cytosol, chloroplast, and mitochondrion in plants (Takahashi and Saito 1996; Hesse et al. 2004; Kuske et al. 1996). CAS has been determined in mitochondria exclusively by mitochondrial fractionation in barley leaves (Wurtele et al. 1985) and blue lupin seedlings (Akopyan et al. 1975). Due to the main difference in sub-cellular localizations among these two proteins, it is believed that CAS is localized in mitochondria for effective removal of cyanide and to protect the oxidative phosphorylation process. The optimal pH for CAS activity is also around 8.5 which is also the pH in the matrix of mitochondria. Earlier it was hypothesized that CAS is OASS-like protein located in mitochondria, but in *Arabidopsis* two different genes coding for CAS and OASS co-exist in the mitochondria (Jost et al. 2000). CAS removes cyanide by combining it with cysteine to form β-cyanoalanine, and the displaced sulphide being recycled back to cysteine by the action of OASS. It is widely accepted that cysteine synthesis is a highly regulated process that is catalysed by a cysteine synthase complex comprising of serine acetyltransferase (SAT) and OASS in bacteria and plants (Droux et al. 1998; Liszewska et al. 2005). Based on the OASS/CAS mutant study in *Arabidopsis*, K Saito's group speculated that some OASS proteins in cytoplasm can function in cyanide detoxification (Saito et al. 1993). Importance of CAS in cyanide detoxification during ethylene biosynthesis has been postulated (Yip and Yang 1988), and keeping that in mind, a tryptic sequence of the partially purified CAS preparation from rice seedlings having high ethylene biosynthetic rate have been identified. By visualizing the recombinant OsCAS protein expressed in *Arabidopsis*, the authenticity of CAS has been proved by observing a high CAS to OASS activity ratio with m*M* to sub-m*M* range Km for cyanide (Hatzfeld et al. 2000; Maruyama et al. 2001; Han et al. 2007), a severe inhibition on CAS activity at <10 m*M* HCN (Jost et al. 2000; Warrilow and Hawkesford 2000) and localization of recombinant CAS in mitochondria.

CAS and two kinds of cysteine synthases (CS-1 and CS-2) have also been purified from potato tubers. Cysteine synthase (CS; EC 4.2.99.8) catalyzes the formation of cysteine from O-acetyl-L-serine and H_2S. The molecular masses of CAS, CS-1 and CS-2 have been estimated to be 37, 39 and 34 kDa, respectively. The purified CAS had CS activity, and both CS-1 and CS-2 also showed CAS activity. However, kinetic characteristics of CAS and both CS are significantly different. The molecular mass and the partial amino acid sequence of CS-2 are similar to those of the cytosolic CS, whereas the molecular mass of CS-1 is similar to that of the plastidic CS. The partial amino acid sequence of CAS is similar to those of CS isozymes, especially the mitochondrial CS isolated from spinach (Maruyama et al. 2000, 2001).

7.2 Rhodanese (EC 2.8.1.1)

An alternative pathway of cyanide detoxification could be carried out by rhodanese (EC 2.8.1.1) (thiosulfate:cyanide sulfurtransferase) and the phylogenetically related mercaptopyruvate sulfurtransferase (mercaptopyruvate:cyanide sulfurtransferase).

Both catalyze the formation of thiocyanate from cyanide and a sulfur donor (thiosulfate and mercaptopyruvate, respectively). Rhodanese is a mitochondrial thiosulphate sulphurtransferase involved in the formation of iron-sulphur complexes. This enzyme is a single polypeptide of 293 residues and 33 kDa, composed of two globular domains of the same size separated by a connecting loop. A conserved cysteine is involved in the binding of sulfane moiety of thiosulfate at the active site (Ploegman et al. 1978). Rhodanese regulates the respiration rate, through the control of the status of the iron–sulfur centers of enzymes of the respiratory chain (Ogata and Volini 1990). Two isoforms of rhodanese AtRDH1 and AtRDH2 have also been found in *A. thaliana*, where AtRDH1 is mitochondrial, while AtRDH2 is cytosolic. *AtRDH1* and *AtRDH2* genes originated from the duplication of a large genomic region in chromosome 1 which took place before the appearance of genus *Arabidopsis* (Hatzfeld and Saito 2000). In animals, cytosolic mercaptopyruvate:cyanide sulfurtransferase (EC 2.8.1.2; MST), catalyzes cyanide by using mercaptopyruvate as a substare and produces pyruvate and thiocyanate. MST is also closely related to mitochondrial rhodanese (Nagahara et al. 1995; Scott and Wright 1980). The sequence of the active site of plant rhodanese is closer to that of animal MST (Hatzfeld and Saito 2000) and two mercaptopyruvate sulfurtransferases have also been cloned from *A. thaliana* (Hatzfeld and Saito 2000; Nakamura et al. 2000; Papenbrock and Schmidt 2000), however, their role in cyanide detoxification, remains to be clarified (Meyers et al. 2003).

7.3 Formamide Hydrolyase (FHL3- EC 4.2.1.66)

Formamide hydrolyase is a constitutive or inducible protein, which is induced by HCN. Mostly it is found in fungal pathogens of cyanogenic plants and also in non-pathogenic fungus (Fry and Evans 1977). Maximum FHL activity can be observed by the addition of 1–5 m*M* HCN and within 12–18 h after addition of HCN. Pathogens of cyanogenic plants produced moderate to high amounts of FHL after induction by HCN. The specific activities of FHL3 range between 4 and 66 μmoles min^{-1} mg^{-1} protein. The range of FHL-specific activities induced in pathogens of cyanogenic plants and in pathogens of non-cyanogenic plants is very wide (Fry and Evans 1977).

7.4 Cyanide Dihydratase (CynD)

Cyanide-degrading enzyme has been isoalted from *Bacillus pumilus* C1. The enzyme consisted of three polypeptides of 45.6, 44.6, and 41.2 kDa and the molecular mass is 417 kDa. CynD is a multimeric, rod-shaped protein approximately 9 by 50 nm. Cyanide can be rapidly degrades into formate and ammonia by this enzyme. Enzyme activity is optimal at 37°C and pH 7.8 to 8.0. Enzyme activity can be enhanced by Sc^{3+}, Cr^{3+}, Fe^{3+}, and Tb^{3+} and enhancement is independent of metal

ion concentration at concentrations above 5 μM. Kinetic studies have indicated a Km of ~2.56 m*M* for cyanide. The Km increased approximately two fold in the presence of 10 m*M* Cr^{3+} to 5.28 m*M* for cyanide (Meyers et al. 1993). CynD from the fungus *Gloeocercospora sorghi*, and from the bacterium *Pseudomonas stutzeri*, and *Bacillus pumilus* strain C-1 have been cloned (Jandhyala et al. 2003; Watanabe et al. 1998b Wang et al. 1992).

8 Transgenics for Cyanide Remedy

Phytodegradation of cyanide can be optimized by selecting or engineering plant species with higher activities of the enzymes thought to be involved and rate-limiting in cyanide detoxification. There are some examples of promising transgenic approaches which have been used in other cases. For example, the expression of bacterial enzymes in plants involved in reductive transformation of TNT (tetranitrate reductase or nitroreductase) resulted in enhanced plant tolerance and degradation of TNT (Hannink et al. 2001; French et al. 1999). Also, the constitutive expression of a mammalian cytochrome P_{450} in tobacco resulted in an up to 640-fold higher ability to metabolize TCE (Doty et al. 2000). Similar approach can be applied for cyanide detoxification.

After decades of accumulating evidence for the existence and importance of various enzyme complexes involved in cyanide detoxification, the ability to model these systems in three dimensions will be an approaching reality. As described in previous sections that a significant progress have been made for the various enzymatic pathways involved in cyanide detoxification and this information can be very useful in making genetically engineered plants for cyanide remediation. For example, cyanoalanine hydratase (E.C. 4.2.1.65) is involved in the cyanide detoxification pathway of higher plants and catalyzes the hydrolysis of β-cyano-L-alanine to asparagine. The isolated cyanoalanine hydratase has already been sequenced and it was shown to be a homolog of *A. thaliana* and *Nicotiana tabacum* NIT4. Full-length cDNA sequences for two NIT4 homologs from blue lupine have also been obtained. The recombinant LaNIT4 enzymes, like *Arabidopsis* NIT4, hydrolyze cyanoalanine to asparagine and aspartic acid, but show a much higher cyanoalanine-hydratase activity. Data also indicated that the cyanoalanine hydratase of plants is not a bacterial type nitrile hydratase but a nitrilase enzyme which can have a remarkably high nitrile-hydratase activity (Piotrowski and Volmer 2006). Recently, a putative plant-induced nitrilase gene (*pinA*) in *Pseudomonas fluorescens* SBW25 was expressed in the rhizosphere of sugar beet plants. *P. fluorescens* SBW25 is a plant growth-promoting bacterium that efficiently colonises the leaf surfaces and rhizosphere of a range of plants. *pinA* is also a NIT4-type nitrilase that catalyses the hydrolysis of β-cyanoalanine, which is a common nitrile produced during cyanide detoxification in plants. In *P. fluorescens* SBW25, *pinA* can be induced by β-cyanoalanine, and the β-cyanoalanine precursors cyanide and cysteine. *pinA* also allows *P. fluorescens* SBW25 to use β-cyanoalanine as a nitrogen source and to tolerate toxic

concentrations of this nitrile. In addition, *pinA* also complements a *NIT4* mutation in *Arabidopsis thaliana*, enabling plants to grow in concentrations of β-cyano-l-alanine that would otherwise prove lethal (Howden et al. 2009). This potentially proves that transgenic approach dealing with cyanide detoxification is a practical approach and it can be a very useful technique in future for dealing with cyanide phytoremediation.

9 Conclusion

Plants and their associated microbes can remediate cyanide via cyanide uptake, transport, degradation and assimilation in plants. Experiments using free cyanide have shown that many terrestrial and aquatic plants including willow, sorghum, cassava and water hyacinth can remove cyanide from the growing medium. Cyanide uptake in plants can be associated with a very complex physiological mechanism which includes transport and assimilation of cyanide within the plants for catering plant's nitrogen needs. Phytoremediation offers a cost-effective and environment-friendly alternative or complementary technology for conventional remediation methods. Although phytoremediation can work effectively, the underlying biological processes are still largely unknown in many cases. Some important processes that require further study are plant-microbe interactions, detailed cyanide transport, chelation and degradation mechanisms in plants. Collection of this information would be useful in developing cyanide detoxification efficiency and for developing transgenic plants that can thrive well in cyanide pollution zone.

Phytoremediation has advantages but also limitations. The plants that mediate the cleanup have to be in pollution zone and it should act on cyanide. Therefore, the soil properties, toxicity level, and climate should allow plant growth. Phytoremediation may also be slower than the more established remediation methods like excavation, incineration, or pump-and-treat systems. Flowthrough phytoremediation systems and plant degradation of pollutants work fairly fast (days or months), but soil cleanup via plant accumulation often takes years. Phytoremediation may also be limited by the bioavailability of the cyanide pollutants. Non-biological remediation technologies and bio/phytoremediation are not mutually exclusive. Cyanide distribution and concentration are also heterogeneous for many sites. In future, mining of the genomic sequences from *A. thaliana,* rice, sorghum and willow and availability of new genomic technologies will lead us to identify novel genes important in cyanide remediation, including regulatory networks (e.g., transcription factors) and tissue-specific transporters. The expression of these genes may then be manipulated in high biomass species for use in phytoremediation.

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Chapter 19
Herbicides and Pesticides as Potential Pollutants: A Global Problem

Bushra Rashid, Tayyab Husnain, and Sheikh Riazuddin

Abstract Herbicides and pesticides have been used to control, eliminate or destroy pests in order to protect human being's food. This technology could be economical and effective if the selection of herbicides and pesticides is based on its mode of action, chemical nature, method and time of application and nature of crop. They have been extensively studied for their toxic potential to biological systems. Herbicides and pesticides are gradually more water soluble, polar and heat stable, therefore it is difficult to reduce their lethalness and to fade away them from the atmosphere. They are highly selective, and found to be toxic to a number of people in industry, agriculture and public health work places. They have harmful effects directly or indirectly on soil, environment, surface and ground water natural flora and fauna, aquatic life which will ultimately adversely influence the human beings and livestock. So, likely impact of herbicides and pesticides on atmosphere and community health is of great significance regardless of their noticeable benefits. It is likely to reduce the selection of pest resistance by preventing the contact between pesticide which act in a particular way and the pest population and to subsequently apply pesticides from diverse classes of compounds having dissimilar modes of accomplishment. Integrated Pest Management (IPM) is intended to protect the maximum likely risks to agriculture as well as environment by using cost-effective measures and pest management will prolong for improvements with the advent of new and improved technologies.

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Contents

1 Introduction

Herbicides and pesticides have been used to control, eliminate or destroy pests in order to protect human being's food. The term pesticide in a broader way includes insecticides, herbicides, fungicides, rodenticides and algicides. They include compounds labeled as (i) insecticides such as organophosphates, organochlorines and carbamates, (ii) rodenticides such as anticoagulants, (iii) herbicides such as paraquat, diquat and 2,4–dichlorophenoxyacetic acid (Ellenhorn et al. 1997). These may include the substances or chemicals applied to crops in different forms during cultivation, even after harvest to protect them from decline while storage and transportation. The selection of herbicides and pesticides depends on the mode of action, its chemical basis, method and time of application and nature of crop, so this technology is found to be economical and effective.

It is evident that herbicides and pesticides are used for a variety of benefits to human beings. They are the chemicals that are intentionally released into the environment during agricultural activities. Because of their recognized potential to

adversely affect biological systems, they have been extensively studied for their toxic potential (Abdollahi et al. 2004). There are a number of undesirable and unwanted effects of their usage such as severe water and environmental pollution and hazards to health. Comprehensive documentation for the implications of pesticide residues for human health is required to be done. This article mainly aims at highlighting the chronic and acute effects that arise from the use of herbicides and pesticides.

2 The Active Ingredients of Herbicides and Pesticides

The modern era of pest control by chemicals began by the development of synthetic organic chemical industry. The original and pure form of a pesticide is formulated to technical grade materials that can be used directly. They are amenable to storage, handling and application, and can be used in an effectively and safely manner. They are supplied in many forms like liquid sprays, powders and dusts, oil solutions and aerosols etc. There are several classes of herbicides and pesticides but only few examples have been outlined here.

Organochlorine compounds, such as dichlorodiphenyltrichloroethane (DDT) were the first synthetic organic pesticides. By that time it was considered as a wonderful invention because it was very toxic to insect pests and less toxic to mammals. Therefore, this has been used for many years as a broad-spectrum insecticide. Benzene hexochloride, an insecticide and chlorine containing benzene having several isomers, named after Greek letters, alpha, beta, gamma, delta and epsilon but only the gamma isomer has insecticidal properties and the remaining serve as inactive filler ingredients. Lindane is the product containing 99% gamma isomer of benzene hexochloride and is most active against several insects. Biodegradation process of lindane is mediated by the activity of *Clostridium* and *Escherichia*. The dehalogenation process converts lindane to 2, 3, 4, pentachloro-l cyclohexane. Another group of organochlorine insecticides comprises Aldrin, Dieldrin, Heptachlor, Endosulfan, Chlordane, and is generally known as dieneorganochlorine insecticides or cyclodienes–cyclo.

Malathion is an example of organophosphates derived from phosphoric acid in combination with alcohol esters. These esters of phosphorus have varying combinations of oxygen, carbon, sulphur and nitrogen attached to phosphorus. They are biodegraded by *Torulopsis, Chlorella, Pseudomonas, Thiobadllus* and *Trichoderma*. Parathion is also an example of organophosphates belonging to phenyl derivatives. Another synthetic pyrethroid pesticide was derived from naturally occurring pesticide pyrethrin.

Herbicides are classified as selective when they kill weeds without causing harm to the main crop and non-selective when used to diminish all vegetation. When herbicides are in direct contact, they will kill parts of the plant to which the chemical is applied, whereas translocated herbicides are absorbed by roots or above-ground parts of plants and then transported to different tissues. Application of herbicides may be done at different plantation stages by banding, broadcasting, spot treatments or direct spraying.

The two major classes of herbicides are inorganic and organic. Most extensively investigated and well known is 2,4-dichlorophenoxy also known as phenoxyalkanoic acids or phenoxy herbicides They are degraded by microorganisms of the genera *Pseudomonas, Achromobacter, Flavobacterium, Cornebacterium, Arthrobacter and Sporocytophaga.* It is a potential weed killer and is active against broad leaved weeds. Dicamba is another example of aliphatic acids or carbon chain acids, whose action may be similar to 2,4-D. Simazine and Atrazine are examples of widely used 5-triazine herbicides. Linuron belongs to phenylureas that have the hydrogen atoms replaced by various carbon chain and ring structures to form compounds that are primarily used as selective pre-emergence herbicides. These are applied to soil primarily for their post-emergence action through inhibition of photosynthesis. Biodegrading process occurs by the genera *Aspergillus, Rhizopus, Fusarium, Penicillium and Trichoderma*. Trichloroacetic acid (TCA) and Dalapon are two widely used herbicides, known as chlorinated aliphatic acids to remove grasses. Chloropham are esters of carbamic acid known as carbamates or carbanilates used as an inhibitor of sprouts in potatoes.

Persistence of organophosphorus insecticides as well as other banned organochlorine insecticides was detected in the aquatic environment. These pesticide/herbicide classes have been extensively used for the production of corn, cotton and rice. Most frequently detected compounds from herbicides were atrazine, simazine, alachlor, metolachlor and trifluralin, from insecticides were diazinon, parathion methyl and from pesticides were lindane, endosulfan and aldrin. Pesticidal residues were detected more in rivers as compared to the pollution in lakes. The detected residues of most pesticides followed seasonal variation, as maximum values were detected during the late spring and summer durations followed by decrease in winter. However, increased level of concentrations was observed in areas of maximum pesticide use and heavy agricultural practices (Konstantinou et al. 2006). Several compounds are non-toxic but may be converted to toxic products by the process called ‘activation’ mediated by microbial activities. This leads to the formation of carcinogens, teratogens, neurotoxins, phytotoxins, and insecticidal or fungicidal chemicals.

3 Adverse Effects of Herbicides and Pesticides on Ecosystem

3.1 Effects on Structure and Functions of Ecosystem

Application of herbicides and pesticides is meant to reduce the effect of pests to below economically acceptable threshold, estimated on the basis of the amount of damage that can be tolerated to crops. However, sometimes their application can adversely affect the invertebrate species especially within arthropoda (Schluz 2004). The structure as well as the function of microbes may be imbalanced by toxicity of herbicides and pesticides. Studies show that spray drift and surface water runoff cause heavy losses (Wauchope 1978; Van der Werf 1996; Shreiver and Liess 2007).

During application pesticides also enter the atmosphere by evaporation and spray droplets that remain in the air. After application they may be volatilized from crop or soil and ultimately wind erosion can affect soil and dust particles with pesticides to enter the atmosphere (Van Dijk and Guicherit 1999). Generally, insecticides are more toxic to the environment, followed by fungicides and herbicides. But there are certain herbicides that may be exceptionally highly toxic and much more hazardous than the insecticides. They can be identified on the basis of solubility. If the compounds are water soluble they can easily be moved out of the target area into ground water, lakes, rivers or streams. If the compounds are fat soluble then they will be absorbed into the bodies of insects, fish, and other animals, which ultimately persist in food chains.

The most hazardous pesticides to ecosystem are: insecticides (DDT, dieldrin, diazinon, parathion, and aldicarb), herbicides (2,4-D, atrazine, paraquat, and glyphosate) and fungicides (benomyl, captan, mercury, copper, and pentachlorophenol). They may pollute the environment, which in turn causes decline in the natural flora and fauna. Sometimes, it may result to the contamination of agricultural products which leads to a decrease in biological diversity. If the biological diversity level gets upset then ecological imbalance may occur. This ultimately may lead to other problems like weed and pest infestation. Farming applications may affect biological diversity, e.g., removing natural flora and fauna which may result in weed invasion, diminishing of naturally occuring predators from an ecosystem which may lead to an outbreak of pests and weed species. Species and habitat diversity is needed to be maintained for conservation of biodiversity. Therefore good farm management applications are necessarily important for maintaining species diversification.

Important issues must be considered when methods are designed to assess the ecological effects of pesticides. Many species and processes interact so assessment of toxic effects of pesticides on ecosystems is difficult. Significant changes in important ecological parameters are undetectable in short term experiments. They may become apparent only after a long time period. Sensitive and careful measurements are required to observe naturally occurring variations in ecological parameters. Sampling from different places and at high frequency is also required because sampling parameters observed at one place or location may be different or may not be applicable to other locations. Good structured ecosystems may be quite susceptible to a pesticide effect than a naturally occurring ecosystem in the same location.

3.2 Risks for the Species

Herbicides and pesticides have been used for many centuries to reduce pests in order to protect mankind, livestock and plants. A few of these chemicals are selective for special purposes, while others demolish the rest of population in an area to clear ground for further life survival. Unfortunately, these herbicides and pesticides leave residual effects on plants, environment, ground water they protect, which ultimately can cause dangerous harm in humans, who work with them or consume them. Generally, herbicides are found to be more toxic to phototrophic

microorganisms as being toxic by interrupting their photosynthesis system. Atrazine is an example of widely used and extensively studied herbicide which causes losses to the microorganisms (Delorenzo et al. 2001).

Microorganisms play a significant role to primary production in the ecosystems; so, injurious effects of pesticides on microbes may eventually affect on secodary living beings. Pesticides may have hazardous effects on microorganisms in different ways. Mechanism of toxicity depends on the nature of chemical and the type of microbial species. Normally, conventional methods have been used to study the effects of herbicides on soil microbial species. These methods illustrate the fostering of the microorganisms and estimation of their metabolic nature (Fantroussi et al. 1999). Effects of pesticide mixtures and their interactions with different nutrients are required to be considered to describe their toxicity level in the ecosystems (DeLorenzo et al. 2001). Chemical and biological methods have been developed for the assessment of water quality to maintain the aquatic life. Environmental pollution has been observed when residual contamination damages the single organisms or at population level and or biological community (Sbrilli et al. 2005). Therefore, bioassays have been developed for evaluation of the damages of contaminants on an organism (Moriarity 1983). Algae are known as primary producers in freshwater systems, therefore, they have frequently been studied to envisage the environmental impact of herbicides (Peterson et al. 1994; Carrasco and Sabater 1997; Ma et al. 2001).

3.3 Pollution Levels in Plants and Animals

Basically, when you use something with nature, either with animals, insects, or humans; nature gets used to what you're using and that means that it won't be as effective. Herbicides and pesticides are present in our food supply (plants, animals, fish and grains), although some of these are directly carcinogenic. Organic farms free of herbicides and pesticides naturally promote a healthy environment as it encourages wildlife. Indeed, the potential health effects are of great concern for long-term exposure of herbicides and pesticides to human beings, animals/livestock and crops (Igbedioh 1991). Plants are the major ultimate recipients of herbicides and pesticides, either from direct application, soil uptake, or atmospheric drift. These may reside on the surface of plants or by their lipophilicity they may penetrate the cuticle of leaves, fruits, stems, roots, or seeds (Finlayson and MacCarthy 1965). Animals, in part, due to an efficient circulatory and excretory system tend to eliminate biotransformation products primarily in urine and feces. Furthermore, contact with a pesticide is usually of short duration or of a transitory nature. Plants usually are in contact with the pesticide for longer periods of time, especially if they grow in a treated soil. Furthermore, due to a less efficient circulatory system and limited excretion, the pesticide may reside for a longer time in a plant (Menn 1978).

Both of the plant metabolism and nutritional patterns have been affected by pesticides and herbicides. Due to these changes ecology may have further deteriorating effects like stable mutagenic and toxic metabolites are formed. Residues in plants

can be accumulated that could be hazardous to humans and animals if exposed through the food web. Bound residues are formed in plants and integrated into lignin, hemicellulose and carbohydrate components of the cell wall that are generally less harmful to the ecosystem. Plants and other organisms get some essential elements from the atmosphere for throughout use in an ecosystem. Other supplements may directly be obtained from the environment, and are recycled through the biosphere. If the pesticides reduce the accessibility of one or more organisms participating in the recycling process in an ecosystem to a large extent, it may function at such a condensed rate as to make threat to the entire web chain.

Cumulative multifactorial hazardous impacts (10–100 folds) resulted from coca production on amphibian populations by the applications of herbicides directly or indirectly. Insecticides are also of great concern, as they selectively target the primary food source of amphibians, which may indirectly affect cultivation of crops (Brain and Solomon 2009). Defining and applying the principles of pest toxicology are critical to food production and human health. Current insecticides act primarily on four nerve targets, i.e., acetylcholinesterase, the voltage-gated chloride channel, the acetylcholine receptor, and the gamma-aminobutyric acid receptor, systems which are present in animals but not in plants. Herbicides act mostly on plant specific pathways by blocking photosynthesis, carotenoid synthesis, or aromatic and branched chain amino acid synthesis essential in plants but not in mammals (Casida 2009).

Glutamate and ammonia form glutamine with associated hydrolysis of ATP by ligation of catalyzing glutamine synthetase. The binding site of amino acid substrate is highly conserved in bacterial and eukaryotic GSs as compared to the nucleotide binding site which varies and thus offers target for specific drug design. Designing of herbicides targeting glutamine synthetase are of great concern as mammalian and plant enzymes are much more restrained (Krajewski et al. 2008). Bioavailable and non-available fragments of bound pesticidal residues could be distinguished in the soil (Khan 1982). Fraction of a compound, which, the plant or animals residing in soil could take up from the soil are the bioavailable bound residues but the non-available fraction cannot be taken up by the soil borne animals/ plants. Increase or decrease in pH also plays an important role for the pesticide solubility. If the pH of rhizosphere is increased, the pesticide solubility will also be increased and eventually pesticide absorption by plants increases. Similarly decline in pH may cause dissolution of the pesticide accumulation at the soil or roots boundary and subsequently discharge the toxins.

4 Effects of Herbicides and Pesticides on Soil and Microbes

4.1 Structure of Soil

Soils are very diverse in composition and nature. They consist of mineral particles and organic matter. Plant roots and microbial population complete the system. The fate of pollutants is affected by all the components of soil. Soil solid phase starts

degradation of organic pollutants in the soil by affecting its water/air ratio in the system and ultimately biological activity of the soil is affected. Therefore, soil constituents characterized by surface area should be considered while discussing soil pollution (Yaron et al. 1996). Presently, a number of herbicides and pesticides are in use to protect the crops and livestock. These are classified on the basis of physical and chemical properties. These properties are specific for each of the pesticides and control its bioactivity and behaviour into the soil. Among these properties, solubility, size, polarization and volatility are the main and are influential but some of the properties show major and more dominating effect. There are types/classes of pesticides which interact with the soil and form biologically/environmentally bound pesticide residues in soils which may not be recovered from soil even by extensive extraction (Gevao et al. 2000). Bioavailability of a bound residue has environmental significance (Khan 1982; Calderbank 1989). Biological degradation of microbes is mostly available in the upper layer of the soil surface, whereas the organic matter is the source of nutrient supplements (Navarro et al. 2004).

The fate of herbicides and pesticides is mainly dependant on the soil type, climate of a particular location and the farm practices used for a specific crop in an area. These pesticides may be destroyed after a short period of a few days by soil microbes or they may be restored for many years (Rosales-Conrado et al. 2002; Perrin-Ganier et al. 2001). Concerns have been growing increasingly about the possibilities for the release of residues of bound pesticides from the soil. The importance of the release depends upon the nature of released residues i.e. whether they are significant for toxicological or of ecological importance. Different soil processes, aquatic living beings, crop plants and soil microbes are the components which are directly affected by soil bound residues because they are closely related to each other through the food web. Soil bound residues can naturally be released by physical, biochemical and chemical processes. Although, It has been documented that the activities of soil microorganism are primarily responsible for the release of bound residues from the soil but the other factors like agronomic applications and application of some of the chemicals that are involved to change the chemical nature of soil may lead to the release of soil bound residues. This may cause the recycling of the compounds into the soil solution which may ultimately be taken up by the plants. These well established, improved, conventional and classical methods resulted in high sample throughput and still describe that there is a further need to improve the investigation of pesticide effects in soil (Andreu and Pico 2004).

4.2 Herbicides and Pesticide Pollution on Microbial Activities

Unfortunately, modern agriculture is increasingly dependant on extensive use of pesticides. Soil organisms are an integral part of the soil and promote an interaction among all soil populations (bacteria, fungi, algae and fauna). Pesticides are organic chemicals, which vary greatly in chemical structure and are highly toxic to biota. Soil surfaces treated with pesticide sprays are affected by ultraviolet photons with an outcome of breakdown of the molecule. Toxic effects of the degradation

products to the soil microorganisms have been investigated and found that the pesticides degraded by ultraviolet radiations may cause considerable changes in soil microsphere and eventually form biological injurious residues (Burrows et al. 2002, Bonnemoy et al. 2004; Bartos et al. 2005; Virag et al. 2007). Therefore, there is a need to study the impact of pesticides on microbial activities measuring the susceptible soils in lieu of nastiest circumstances (Greaves 1982; Lynch 1995). Structure of soil bacterial population may distinctly be changed if the pesticide is not affected by nitrogen and carbon metabolism. Some of the microbes may be concealed while others may be propagated in the available ecological sites (Johnsen et al. 2001). Cyanobacteria are important species of microbes which live in both aquatic and soil environments. They help plants to alter atmospheric nitrogen into nitrate compounds, thus the plants can use these compounds and play an important role in nitrogen fixation. Application of trifluralin renders the development of commonly useful cyanobacteria (Kobbia et al. 1991).

Expected effect of a pesticide on soil organisms is of enormous importance and depends on its availability. A number of crop and soil related components such as how much crop area is covered by soil, its sorption, leaching and biodegradation of the residual compound are involved to determine the availability of a pesticide to soil microbes. Among these components, soil area covered by a crop is of maximum importance for the calculation of actual dosage reaching to the soil microbial community. The application of insecticides is usually done on intense and standing crops at different intervals, therefore direct contact of insecticide to the soil is not too much. Therefore, it has been concluded that higher the concentrations of compounds, more will be their toxic effect on the degradation of soil microorganisms (Gan et al. 1995; Gevao et al. 2000).

4.3 Persistence in Soil

Herbicides and pesticides are usually classified on the basis of their persistence in the soil/environment. They are applied mostly in the field on crops to control pests and during storage in the homelands. Therefore, they have been studied more in soil as compared to the other contaminants in the environment. Their transformation products in the soil are expected to be prevalent at higher levels rather than the original pesticide and normally they are not as much toxic as their parent compounds (Nawab et al. 2003). The prevalence of pesticides in the environment and soil is restricted by physical, chemical and biological status of the atmosphere. Increase in pesticide concentration may increase its persistence in soils. From the agricultural point of view, accumulation of residues in soil may lead to increased absorption of toxic chemicals by plants to a level at which the consumption of plant products may prove deleterious to livestock and human beings.

It is a difficult task to develop methods which determine the pesticide itself in the soil and its metabolites, therefore general characteristics of these samples should be taken into account. If the concentration of concerned analytes is extremely low then analytical methods should be developed and provide sensitivities and precision for

the quantification and detection of analytes and their metabolites in the soil. Strong binding of analytes to soil requires special extraction techniques for analysis of these compounds (Lerch et al. 2003). Soil organic matter content is also responsible for retention of pesticides and their metabolites into the soils. This depends upon the interactions between soil and pesticide properties as organic matter contents and adsorption of pesticides are directly proportional.

5 Effects of Herbicides and Pesticides on Environment

5.1 Environmental Fate of Herbicides and Pesticides

When the contamination crosses considerable/measurable threshold, it damages biological communities at single organism or population level, then environmental pollution may occur (Moriarity 1983). It is a human action capable to make modifications to the properties of environment or availability and quality of resources over a given space and timeframe and is called as environmental contamination (Bacci 1994). Unfortunately, the extensive reliance on herbicides and pesticides in recent agricultural system is increasing. Since, they are widely used for pest control in corps/livestock, so their behavior in the environment is vitally important. The parameters like type/nature of chemical and soil, climate, number of pesticide application (single or multiple) are responsible for shelf life of a pesticide. Environmentally suitable chemical pest control adoption strategies need knowledge of the fate and behavior of pesticides.

Human beings are exposed to pesticides through environment or through their occupation/workplace. Environmental exposure is expected to be very high and may be the outcome of contamination through food, air or drinking water. Occupational contact to agricultural workers is mainly related to handling of pesticides such as mixing and filling of chemicals to the equipment, and their application to the target area. Cleaning of equipment and disposing off the empty packing also cause exposure to the workers which may affect the injurious hazards. Generally, exposures while mixing and loading of chemicals are considered to be more severe as compared to the application, because of the use of undiluted and strong pesticides. An occupational exposure like skin contact is much higher than inhalation. Another key cause of exposure is the entrance to the treated field after application because residues are still persistent in the field.

When a parent pesticide itself and its transformation products are compared, it has been observed that differences in the environmental performance of many transformation products of the pesticides raised their mobility in soil. The transformation products have the potential to turn out an adverse effect on the environment even if it is found to be less toxic than its parent pesticide (Sinclair and Boxal 2003; Papadakis and Papadopoulou-Mourkidou 2002). Therefore, transformation products are required to be considered while taking the environmental risk estimation. In the European law it has been documented that, before introduction of a new

pesticide in the market, environmental data related to all amounts of metabolites, degradation and possible reaction products must be provided (Barcelo and Hennion 1997). Herbicide compounds are known to target the photosynthesis pathway and energy transporting enzymes. Algae are of key importance as primary producers in freshwater food chain systems. They are likely to be more susceptible to herbicides than other aquatic living beings (Galassi et al. 1993). Therefore algae have been commonly studied for bioassays applied to forecast the herbicides environmental impact on wastes and receiving waters (Peterson et al. 1994; Carrasco and Sabater 1997; Ma et al. 2001). They are well responsive to stimulation and inhibition as the level of concentration changes. Therefore they are valuable indicators for detection of probable pollution at a specific place.

5.2 Effects of Herbicides and Pesticides on Aquatic Life

Pollution to aquatic life is mostly land based and caused by agricultural overspill and waste materials carried by wind. The surface flow can contaminate water sources as 1–6% of the applied pesticides may be lost to the aquatic environment by runoff and drainage depending on the slope of the field, agronomic practices, presence or absence of subsurface drains, and the quantity and timing of rainfall after applications. Sometimes these deposited chemicals react in such a way that they may cause scarcity of oxygen which may lead to the aquatic environment hazardous for the living beings there. Moreover, they may be the base for mutations, or harmful diseases for mankind and even for the whole food web. So it is now well understood that marine food web is contaminated by the accumulation of harmful pesticide residues which may have been entered and released into the marine surroundings. Introduction of toxic metals is another cause of marine food web contamination. These metal toxins in aquatic life may be the source of changes to biochemical metabolism, reproductive system, tissue structure and restrained growth. Some of the marine life products are used to prepare land animal/bird feeds. In this way, these toxins from marine life are shifted to land animals/birds, whose meat and dairy products are eventually be taken up by man.

Chlorpyrifos, diazinon, trifluralin, oryzalin, ronstar and roundup are herbicidal and pesticidal contaminants and generally found highly toxic to fish and kill them in water-channels passing through treated farms or buildings (Cooperative Extension Service Pesticide Information Project 1993; EPA 2000; Cox 2000; Extoxnet 1996; Extoxnet 1996). Other marine or freshwater animals (newts, frogs crabs, shellfish) are endangered contamination of 2,4-D or its products (Zaffaroni 1986; Suwalsky 1999; Caldwell 1979). Trifluralin and diuron have been found toxic to shrimps, mussels and aquatic invertebrates.

Production of agricultural lands is affected very adversely if the resources are not managed properly. Moreover reduction in biodiversity of natural flora and fauna, production capabilities of polluted waterways and aquatic ecosystems are the problems created by poor resource management. In modern science there are tremendous developments in the concern to study the effective use of different categories of

farms and availability of improved farm practices for a successful future. Future research needs much attention to study the toxic effects of pesticides on marine microbes, specifically bacteria and protozoa.

5.3 *Effects on Surface and Ground Water Quality in Agricultural Areas*

As the toxic effects of herbicides and pesticides have been detected, there is an alarming situation regarding contaminated water resources. Pesticides can enter and contaminate water resources frequently by escapes, erosion, run-off, drift, and rarely, unintentional or intentional discharge. Contamination of ground and surface waters is a major concern because these are used as drinking water (Karcher and El Rassi 1999). If the half life of a pesticide is long, aqueous solubility is high and sorption rate is low, they can contaminate groundwater gradually more (Barcelo and Hennion 1997). In agricultural areas, most herbicides do not leave the field, either leaches to the subsurface or becomes surface flow in soluble forms or as insoluble forms bound to soil particles. Persistence, hydrophobic nature, and bio-accumulative characteristics of a pesticide make it capable to strongly bind to soil. Most of the pesticides such as organochlorine, DDT, endosulfan, endrin, heptachlor, and lindane are currently forbidden for agricultural use. Their strong soil bound residues and transformed products are still detected nearby. Polar pesticides generally represent the herbicides but they also include carbamates, fungicides and some organophosphorus insecticide transformed products. They are removed from the agricultural lands by overspill and forceful discharge, but in this manner trouble for the drinking water would be expected. Pesticide polluted irrigation water can contaminate the agricultural farms where they have not been applied. Quality of ground or drinking water underneath those agricultural areas may be affected by the irrigation of pesticide polluted water.

It is evident that lager amounts of pesticides can be moved quickly away from the plants' root region in the soil (Kladivko et al. 1991; Johnson et al. 1994; Brown et al. 1995; Kolpin et al. 1998). As the EU directive set the maximum concentration of a particular pesticide in drinking water comparatively lower i.e. 0.1 mg L^{-1}, this may make threats to the quality of drinking groundwater sources for leaching of soil bound pesticide residues from agricultural lands (Papadopoulou-Mourkidou et al. 2004). Major parameters related to pesticides leaching are: soil properties, physicochemical characteristics of pesticides, environmental climate and farm management (Nicholls 1988; Van der Werf 1996; Carter 2000, Van der Linden et al. 2009). However, limited data is available on studies of impact of variable farm practices on probable leaching of pesticides.

Agricultural practices have the positive impact to increase the probability for contamination of ground water sources through pesticides. Some of the pesticides such as prometryne, alachlor, atrazine and carbofuran are intended for their leaching prospective. Pesticides, revealed underneath agricultural farms are in concurrence with their agricultural exploitation, but this proportion is limited to only a few of

them. Most of the pesticide residues are evenly distributed to the surface water utilized for irrigation of agricultural areas. Sometimes, the value of groundwater may have adverse impact due to very frequently used farm management applications. However, an extra comprehensive investigation is required to understand the complex mechanism, action and effects of pesticides in farming areas.

6 Pest Resistance to Herbicides and Pesticides

Pesticides are used to control pests which may cause economic losses to agricultural products and livestock. The widespread use of herbicides and pesticides may cause weeds and insects to eventually develop resistance to particular chemicals which ultimately compel growers to apply yet multiple or more dose. It is evidently expected that if impacts of herbicides and pesticides are neglected, it will commonly lead to an extensive and long-term effects on mankind and other living beings. Some of the constituents in some specific pesticides are noticeably poisonous which are supposed to be immobile. Occasionally, prior to pesticide application, there is a natural resistance in a small number of individual's pest population against some specific pesticides. After the treatment of pesticide, a number of vulnerable pests have been expectedly executed while natively resistant may perhaps stay alive, reproduce and may multiply their population. Therefore, continued application of similar group of a chemical compound on a specific place may ultimately develop considerable resistant/challenging pest populations.

With the advancement in performance of pesticides in environmental and awareness for their toxicity, improvements have been made to pest management technologies. There is need for continuous development of better and safer technologies for pest management rather than the application of wide spread pesticides. Thus, integrated pest management (IPM) is the approach which may come together with different methods putting emphasis on prevention. This may be the most successful approach for pest control. Selectivity of pesticides to beneficial arthropods is a key for the implementation of IPM program. Fungicides and herbicides are compatible with IPM programs. For foliar insecticides, some treatments are required to be used carefully according to the selectivity, but for soil insecticide treatments, their toxicity raise the question regarding their residues to the soil and ground water, so it is important to use them with proper management in IPM programs in vegetables and there is need of new compounds or development of alternative pest control programs (Hautier et al. 2007). So far, an IPM is intended to protect the maximum likely risks to agriculture as well as environment by using cost-effective measures.

7 Effects on Human Health

Most of the pesticides are not highly selective, and found to be toxic to other non-target species, including human being. Hundreds of these pesticides have been produced all over the world and continuously been applied by a number of people

in industry, agriculture and public health work places. Therefore, a large number of people may have been in contact with such toxic compounds on a broad-spectrum (Aprea et al. 2002). Poisoning with pesticides is a global health problem and accounts for deaths worldwide. Exposure to organophosphate compounds inhibits acetylcholinesterase resulting in acute toxicity. Intermediate syndrome can be developed and may lead to respiratory paralysis and death. Immunotoxic and genotoxic responses have been observed in animals and humans after exposure to organophosphates which could lead to the development of cancer (Galloway and Handy 2003). Glyphosate is a broad-spectrum non-selective herbicide and levels of cytotoxicity and genotoxicity of glyphosate occurring in mammalian cells suggests that its mechanism of action is not limited only to plant cells (Monroy et al. 2005). Pesticides can also cause neurotoxic effects as those insecticides, which kill insects by damaging their nervous system. They can also have neurotoxic effect on mammals (Soderlund and Bloomquist 1989). Parkinson's disease, a neurodegenerative disorder, is caused by some herbicides and fungicides (Costa et al. 2008). One of the agricultural health study reported that wives and the applicator farmers of fungicides observed the risks of their retinal deterioration (Kamel et al. 2000; Kirrane et al. 2005; Hines et al. 2008).

Organochlorine group of pesticides are found toxic to the central nervous system and sensitize the myocardium to catecholamines. Ingestion of paraquat can be harmful in a number of ways: (i) severe inflammation of the throat, (ii) corrosive injury to the gastrointestinal tract, (iii) renal tubular necrosis, (iv) hepatic necrosis, and (v) pulmonary fibrosis. Barium carbonate ingestion can cause severe hypokalaemia and respiratory muscle paralysis (Goel and Aggarwal 2007). Aluminium phosphides are effective for protection of grains in stores and during transportation against insects and rodents. These compounds can cause acute poisoning by direct intake or otherwise indirectly due to unintended breathing of fumes produced throughout permitted exploitation. These poisoning is mediated by phosphine which inhibits cytochrome C oxidase and breakdown of cellular respiration. Phosphine is responsible to disturb the morphology of mitochondria very quickly and turn out into a rigorous fall in mitochondrial membrane prospective in the nematodes. Phosphine and hydrogen peroxide react to create hydroxyl radical which is exceedingly reactive. While this reaction, the enzymes (catalase and peroxidase) are inhibited that may cause the lipid peroxidation. Intake of phosphides and the emergence of toxicity take place in a very short time period. The most important toxic effects due to phosphide intake are blood circulation failure, damage to heart muscles, body fluid losses, and adrenal gland damage due to its corrosive actions. Most of the patients suffered by toxins from phosphine or metal phosphide pass away even with exhaustive treatment (Proudfoot 2009).

Although the number of pesticides detected in environment varies, the concentration was generally very low to which individuals, agricultural lands and further ecosystems are in contact in an ambient environment. The occurrence and concentrations usually can be correlated with local use, and high levels of the pesticides in the atmosphere can occur (Majewski et al. 1998). According to global estimates, an array of drawbacks is expected to provide incorrect approximation caused by

application of herbicides and pesticides. Therefore it is urgently needed to collect accurate data on severe toxicity of pesticides and to be in command of this; such data should be looked upon as the preliminary point for such program (Jeyaratnam 1990).

8 Integrated Weed Management System

Defining most commonly, weeds are the plants growing where they are not wanted. According to weed experts, weeds are the plants which flourish and sustain their larger quantities even under circumstances of frequent troublesome. Therefore, defining weeds more precisely, they are the plants supposed to have accustomed properties that let them to occupy, continue to exist and replicate agricultural farming. There are improvements in the profitable, ecological and health troubles associated with conservative agricultural systems (Liebman and Davis 2000). Continuous implementation/addition of input to crop cultivation is a part of these improvements. Herbicides have been considered economically important, but they may perhaps interrupt the adjacent surroundings. Although effective, but they are expensive and their use on crops causes environmental concerns more importantly. Herbicide residues are frequently found in rivers, streams and lakes and can build up resistance in weeds (Chikowo et al. 2009). Thus, to avoid maximum reliance lying on herbicides, growers and researchers are in search of other approaches to control weeds.

Agricultural systems largely managed with scientific and technological methods are mostly been depending on heavy applications of chemicals in the form of a variety of herbicides and pesticides. There are possible health risks directly or indirectly to mankind. Ecosystem can also be adversely endangered through habitat and non-target organisms. Therefore, it is urgently required to introduce directly pest-specific alternatives to solve these problems of continuous dependence on herbicides and pesticides in agriculture.

Integrated Weed Management technology could be adopted to extend agricultural productivity rather to depend much more on herbicides and tilling practices. This comprises the collective application of cultural, biochemical, chemical and mechanical procedures which eventually decrease weeds' occurrence, persistence and their adverse impacts on crops. The main goals and ultimate impacts of an IWM program are: avoidance for sustainability of weed which may not be deleterious for the crop, development of system which may be helpful to increase crop production, reduction of those weeds which affect crops very adversely. All these practices ultimately will have positive impact as they may generate improved agricultural revenues by increasing crop production and making improvements to the environment and ecosystem. Therefore, a complete knowledge of biochemical and environmental issues regarding different dynamics of weeds in a cropping system should be accomplished for successful implementation of Integrated Weed Management systems (Smith and Menalled 2006). This would involve durable developmental plan,

awareness regarding weed metabolisms and the suitable weed removal technologies. Different farming practices can contribute to weed suppression such as crop rotation, cover crops and intercrops, making improvements to soil with previous crop remains, and addition and improvements to soil organic matter. Seed selection and seed mass may also represent as one of the key sources for suppression of weeds to protect crops from expected dangers. There is need to make improvements for the implementation of this technology efficiently which is not easy, but it is critical for development of sustainable farming systems.

9 Benefits vs Risks to Use Herbicides and Pesticides

There has been an increasing reliance on herbicides and pesticides leading to minimize the need for traditional farming system. Ultimately, cropping patterns have been adapted, driven to further increase crop output, to rely more on these products, which in return are rewarding economically to farmers. Pesticide and herbicide use is not only limited to the agricultural community, there are a number of lawn, garden and home care chemicals that help to get rid of unwanted plants and animals. Likely impact of herbicides and pesticides on atmosphere and community health is of great significance regardless of their noticeable benefits. Concerns related to community strength settlement such as increased crop production to supply safe and enough food and considerable reduction for the incidence of vector borne disease is being recognized by applying pesticides (Laws and Hayes 1991). Likewise, approximately three–fourth of the pesticides has been applied for crop production in the USA and the left over quantity was utilized to apply in housing (Lang 1993). According to Environmental Protection Agency (EPA) nearly 85% of the residents in USA store up things and making safe with pesticides application. As the pesticides are now used extensively, so it has been impractical to circumvent their contact at some specific intensity (Morgan 1992). Pesticides have made a vital contribution to the quality and quantity of food and overall to health both in developed and, most significantly, developing countries, so that their sudden withdrawal would present far more serious health problems than do their potential long-term toxic effects (Blain 1990). It has been well known that pesticides are gradually more water soluble, polar and heat stable, therefore, it is difficult to reduce their lethalness and to vanish off them from the atmosphere, but instantly for adequate pest management, they are required to endure for extended periods of time.

Some negative consequences due to pesticides and herbicides have emerged which is now needed to be addressed in the interest of long-term sustainability. Existence of toxic pesticides all over the atmosphere possibly will put in danger other organisms as well as humans, because they are not exclusively precise only for the targeted species. Someone should be aware of the dosage penetrating into the body. This awareness about the dose introducing into the body is the initial point

for the possible risk assessment (Barr and Needham 2002). Increase in agricultural production and profitability has been obtained with broad-spectrum application of pesticides.

10 Phytoremediation of Herbicides and Pesticides

Overall, herbicides and pesticides have harmful effects on soil and pesticide-soil interactions need future research. Herbicide and pesticide contamination is usually too expensive to clean up with current mentioned technologies. Studies have shown that certain tolerant plants and microbes can be used in biological remediation which can be cost-effective and simpler, due to the in-situ advantage, pollution can be exacerbated by the pesticides/herbicides involved, but these, too can be remediated through biological remediation (Perkovich et al. 1996; Kruger et al. 1997; Anderson et al. 1995). Most of the naturally occurring microorganisms are selected on conventional culture media which has many drawbacks for the selection. To prevail over these drawbacks, molecular biological technology has been introduced.

Herbicides and pesticides have been considered for phytoremediation by means of conservative plants. Now it is possible to isolate genes concerned for metabolic pathways from different species such as bacteria, fungi, plants, and even from animals. These genes are then cloned into a suitable vector and then possibly by introducing into the plants applied with any of the suitable methods. Presently, there are different methods available for transformation. Herbicide and persistent toxin resistant transformed plants have to be capable of phytoremediation of toxins present in polluted soil and water. Resistance and phytoremediation action for intended herbicides have been observed by plants transformed for expression of mammalian P_{450}s gene. Pollutants or toxins have been detoxified or absorbed and eventually contaminated atmosphere is phytoremediated (Kawahigashi 2009; Magaña-Gómez and de la Barca 2009). It is concluded that natural agricultural cropping pattern proposes the practical application of economical and sustainable food provision system which is exclusive of the employment of risky pesticides. Now, we have to choose to confront the advances of biotechnology and agricultural production for understanding of this imagination.

11 Conclusion

Herbicides and pesticides are used to increase the agricultural products and it is based on the effective use of technology and inputs. Their direct or indirect toxic potential to biological systems has been proven extensively. This technology could be economical and effective if a number of factors are considered before selection

and application of herbicides and pesticides. Further improvement is required to progress for the appliance of this technology to analyze the herbicides and pesticides in existent situation.

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