Environmental Pollution 21

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Editors

The Plant Family Brassicaceae

Contribution Towards Phytoremediation

Springer

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ENVIRONMENTAL POLLUTION

VOLUME 21

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Foreword

Plant Biology is reaching an extraordinary degree of conceptual complexity, especially since the crucial technological advances during the last two decades, halfway between the twentieth and twenty-first centuries. Integration from the molecular to the whole plant level of the mechanisms governing growth and developmental processes provides now a better and an increasingly diverse vision of the functioning of plants in different habitats and under changing environmental conditions. This is featuring a solid body of scientific knowledge which covers the various levels of plant research: cellular, biochemical, genetic, molecular, physiological, ecological and evolutionary. This plethora of knowledge and methodological consolidation now allows to more precisely understand the physiological and molecular mechanisms, to see the richness of phenotypic responses of plants to the diverse environmental situations, to deepen consistently in the evolutionary lines, to discern the molecular basis of variation for including all within the system's biology, and to consider the diversity and specificities of plant functionalism.

Spectacular progress is being made in the fundamental knowledge of reception of external signals and internal signaling pathways, the vital mechanisms of regulation of homeostasis, the integration of metabolic functions, and the differentiation and development of plants exposed to biotic or abiotic stress factors. As expected, this advanced insight into basic processes governing plants under adverse environmental conditions has substantially improved our expertise in practical applications, not only in the field of agronomy, but also in that of environmental restoration. Phytoremediation as a clean, sustainable technology is becoming an attractive way to recover degraded land. No wonder that research and publications on this topic have increased exponentially during the last years and that we face a rapidly expanding area where basic knowledge is being turned into effective environmental technology.

It is not accidental that this new book entitled '*The Plant Family Brassicaceae: Contribution Towards Phytoremediation*' focuses the issue of phytoremediation on the Brassicaceae. This large botanical family with 360 genus and 3,700 species not only includes important food and industrial plants, but also *Arabidopsis thaliana* the plant model species par excellence. Small genome size, self-compatibility, short life cycle, small biomass, and large production of seeds make this *Arabidopsis thaliana* an ideal research object for studies into functional genomics. Identification of genes involved in metal uptake, transport and compartmentation and knowledge of key enzymes in metabolic pathways able to transform and detoxify organic pollutants provide the fundamental tools for the development of phytoremediation technologies. This extensive basic research in *Arabidopsis thaliana* allowed fast advances in the knowledge of the functioning of *Brassicaceae* species of high interest in phytoremediation processes, such as the close relative *Arabidopsis halleri* and *Thlaspi caerulescens* (*Noccaea caerulescens*), both species with ability to hyperaccumulate cadmium and zinc. Also the nickel hyperaccumulators of the genus *Alyssum* are within the *Brassicaceae*. Furthermore, the non-hyperaccumulating species of the genus *Brassica*, especially *Brassica juncea* and *Brassica napus*, the seed-oil canola which combines both industrial applications and phytoremediation potential, are being explored for phytoremediation technologies. These *Brassica* species have considerably lower shoot metal concentrations than metal hyperaccumulators. This is compensated, however, by the much higher biomass in *Brassica* sp.

Thus, the focus on *Brassicaceae* in this new book is fully awarded by this predominant presence of representatives of this family in current research in phytoremediation. This leads to the question about the characteristics that makes *Brassicaceae* species so outstandingly attractive for recovering contaminated land. To our opinion, two features common to *Brassicaceae* may contribute to this: special pathways of secondary sulphur metabolism for glucosinolate synthesis and, probably in part related to this, the fact that most *Brassicaceae*, are non mycorrhizal. A high tissue level of antioxidants is a further characteristic of importance for performance under stressful conditions.

Brassicaceae have a high sulphur requirement. Sulphur availability in mine spoils uses to range from normal to extremely high and most contaminated soils can fully satisfy the higher sulphur demand of the *Brassicaceae*. Moreover, in *Brassicaceae* the levels of sulphur containing metabolites that are important for basic metal tolerance, such as glutathion and phytochelatins seem to be affected only under extreme sulphur deficiency.

Many *Brassicaceae* species are pioneers on waste areas and seem well-adapted to recently formed, badly structured substrates. No need for mycorrhization under these conditions implies high nutrient efficiency, especially for phosphorous. In *Brassica* crops differences in phosphorus acquisition are closely related to both root architecture traits and internal phosphorus use efficiency. Metal hyperaccumulators like *Thlaspi caerulescens* seem well adapted to the low phosphorus availability of mine waste areas and surplus phosphorus supply may not enhance their biomass production and/or metal extraction potential.

Many species that perform well on mine spoils are mycorrhizal. Considerable amounts of heavy metals can be retained in the cell walls, especially of ecto- and ericoid mycorrhizal fungi, thus limiting metal uptake by the plants. Metallophytes that are non- mycorrhizal, such as most *Brassicaceae* and *Caryophyllaceae* species (e.g. *Silene vulgaris*), have their own mechanisms to exclude metals from either or both roots and shoots. Nonetheless, on polluted soils they tend to take up higher amounts of heavy metals than mycorrhizal plants. Thus tolerance to higher tissue concentrations of metals is a prerequisite for good performance of these species on metal contaminated sites. The evolution of metal hyperaccumulation behavior with an especially high number of representatives in the *Brassicaceae* is a further argument for meriting special attention. These plants do not efficiently exclude metals neither from the roots nor the shoots, but even forage and accumulate metals from soils with low concentrations. Binding of the metals in non-toxic form and efficient compartmentation into cell walls and vacuoles are fundamental mechanisms in the hyperaccumulation life style.

So the great achievement and novelty of this book edited by eminent scientists Naser A. Anjum, Iqbal Ahmad, M. Eduarda Pereira, Armando C. Duarte, Shahid Umar and Nafees A. Khan resides in the focus on phytoremediation referred to the *Brassicaceae* family containing species that are well adapted to harsh conditions on waste land and with efficient metal uptake and tolerance mechanisms. The different chapters written by experienced specialists provide a unique compilation of the dispersed literature on this topic. By sure, the readers of the book will benefit from this joint vision of the actual and the potential contribution of this botanical family to the emerging discipline of phytoremediation.

Therefore, we believe that this book targets a potentially broad spectrum of audience ranging from the researchers in this field of increasing importance to undergraduate and graduate students who want an overview of current knowledge referred to the *Brassicaceae* and its utility in phytoremediation. No less important is this text for all those interested in the agricultural use of the *Brassicaceae* and their contribution to the sustainable production of healthy food.

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Preface

The sustainability of agriculture (the only source of food) and the civilization of mankind rely on two precious natural resources namely land and water. Unfortunately, both land and water have been subjected to maximum exploitation and severely degraded or polluted by kinds of contaminants originating mainly from anthropogenic activities. Majority of contaminants get accumulated in plants and either directly or indirectly, find their way into food web thus cause severe secondary consequences. Above facts together can produce severe deterioration of natural resources, disturbance of ecosystems thus, creating enormous human health and welfare challenges globally. The remediation of varied environmental contaminants using plants (phytoremediation; "phyto" meaning plant, and the Latin suffix "remedium" meaning to clean or restore) have been accepted advantageous over commonly used physical remediation methods in costs, practice and the scale at which the processes operate. This approach is emerging as an innovative tool with greater potential for achieving sustainable development and also to decontaminate air, soils/sediments and water, and for other environmental restoration applications.

The angiosperm family Brassicaceae – commonly termed as the mustard family (or Cruciferae, because of their characteristic flowers consisting of four petals in the form of a Greek cross), is a large dicot plant family comprising nearly 338 genera (assigned to 10–19 tribes) and 3,700 species with worldwide distribution, of major agro-economic and scientific importance. The family Brassicaceae represents hundreds of plant families reported so far for their potential use in the remediation of varied environmental contaminants including toxic metals-metalloids. The most of the members of the Brassicaceae plant family well represent the metal hyperaccumulation among 0.2% of all angiosperms and thus, have key role in phytoremediation technology. Many of the plant species within Brassicaceae family such as *Alyssum, Arabidopsis, Berkheya, Bornmuellera, Cardamine, Cochlearia, Crambe, Peltaria, Pseudosempervivum, Stanleya, Streptanthus,Thlaspi* including oilseed Brassicas grow fast, yield high biomass and are well adapted to a range of environmental conditions. Some species are tolerant to high levels of trace metals, and there is the potential to select superior genotypes for phytoremediation. Of 87 different metals/metalloids-hyperaccumulator plant species in the family Brassicaceae, in particular model metals/metalloids hyperaccumulators *Alyssum* sp., *Thlaspi* sp. and *Arabidopsis* sp. have been studied extensively for their ability to hyperaccumulate varied environmental contaminants including metals and metalloids. In addition, they are well suited to genetic manipulation and in vitro culture techniques and are attractive candidates for the introduction of genes aimed at phytoremediation.

Although, the number of publications focused on plant-based remediation of varied environmental contaminants has been growing exponentially in the last decade but the significant role/contribution of the members of the plant family Brassicaceae for decontaminating varied environmental compartments either remained untouched or little debated.

Written by an international team of authors and as a significant addition to the Springers' Environmental Pollution book series the current volume '*The Plant Family Brassicaceae: Contribution Towards Phytoremediation*' aims mainly to update our understanding on the recent breakthroughs in the area of differential adaptation of the plant species within Brassicaceae family to contaminants through mechanisms involving physiological, biochemical and molecular processes and their cumulative role in contaminants accumulation, tolerance and remediation.

Besides highlighting the taxonomy of the family Brassicaceae, the current status of contaminants (metals/metalloids)-addition to varied environment and its consequences, the information included in this book will bring very stimulating insights into the mechanism involved in the physiological, biochemical and molecular processes responsible for environmental contaminants accumulation, tolerance and remediation in Brassicaceaeans. Additionally, the book also intends to explore the various potential strategies (such as the physiological, biochemical, plant breeding and genetic engineering approaches) for the enhancement of contaminants-tolerance, -accumulation and -remediation potentials of the members of the plant family Brassicaceae. This volume promises to be a useful asset for researchers, students, other academicians and policy makers involved in sustainable remediation of varied environmental compartments.

We are thankful to contributors for their interests, significant contributions and cooperation that eventually made the present volume possible. Thanks are also due to all the well-wishers, teachers, seniors, research students and family. Without their unending support, motivation and encouragement the present grueling task would have never been accomplished.

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Chapter 1
The Plant Family Brassicaceae: An Introduction

Naser A. Anjum, Sarvajeet S. Gill, Iqbal Ahmad, M. Pacheco, Armando C. Duarte, Shahid Umar, Nafees A. Khan, and M. Eduarda Pereira

Abstract This chapter introduces the plant family Brassicaceae (Cruciferae or mustard family) and also summarizes significant roles of some representative plant species from this family for metals and metalloids phytoremediation. Brassicaceae family is one of the largest dicot families of flowering (angiospermic) plant kingdom which comprises 10–19 tribes with a total of 338–360 genera and nearly 3,709 species. The Brassicaceae are easily recognized by having unique

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flowers [with four petals, forming a cross or sometimes reduced or lacking; six stamens, the outer two being shorter than the inner four (however, sometimes only two or four stamens are present) and capsule (having two valves capsule with a septum dividing it into two chambers)]. The plant family Brassicaceae includes several plant species of great scientific, economic and agronomic importance including model species (*Arabidopsis* and *Brassica*), developing model generic systems (Boechera, Brassica, and Cardamine), as well as many widely cultivated species. The well-known model plants from the family Brassicaceae *viz*., Arabidopsis (*Arabidopsis thaliana*) and Brassica species have revolutionized our knowledge in almost every field of modern plant biology. In addition, several representatives of the family Brassicaceae are equally playing significant roles for achieving environmental sustainability.

Keywords Brassicaceae • Biosystematics • *Arabidopsis* spp. • *Brassica* spp. • *Alssum* spp. • *Thlaspi* spp. • Metals • Hyperaccumulation

1.1 Introduction

The Brassicaceae (or Cruciferae/mustard family) is a large angiosperm (flowering plant) dicot family of plant kingdom which belongs to the order Brassicales and has been divided into 10–19 tribes with a total of 338–360 genera and 3,709 species distributed worldwide on all continents except Antarctica (Al-Shehbaz 1973; Appel and Al-Shehbaz 2003; Al-Shehbaz et al. 2006). The **I**nternational **C**ode of **B**otanical **N**omenclature (ICBN) vide Art. 18.5 (Vienna Code) has approved both Cruciferae and Brassicaceae as validly published and thus accepted as names for the family. The largest genera are *Draba* (365 species) followed by *Cardamine* (200 species, but its definition is controversial), *Erysimum* (225 species), *Lepidium* (230 species) and *Alyssum* (195 species). Different taxa of Brassicaceae are distributed differentially in Northern Hemisphere (temperate regions), Southern Hemisphere (such as *Draba, Lepidium and Cardamine*) and also in Southern regions (*e.g*. South African genera: *Heliophila, Silicularia, Brachycarpa, Chamira, Schlechteria*) (Koch and Kiefer 2006). However, the distribution of the Brassicaceae has been shown limited to mountainous and alpine regions in the tropics; where *Arabis alpina* was shown to represent the classical example of a plant with a worldwide northern hemispheric distribution in mountainous, alpine and arctic habitats including East African high mountains in Kenya, Tanzania and Ethiopia (Koch and Kiefer 2006). The plant family Brassicaceae includes several plant species of great scientific (Hall et al. 2002; Koch 2003; Koch and Mummenhoff 2006), economic and agronomic importance including model species (*e.g*., *Arabidopsis* and *Brassica*), developing model generic systems (*e.g*., *Boechera, Brassica, and Cardamine*), as well as many widely cultivated species (*e.g.*, cabbage, broccoli, Brussels sprouts, cauliflower, horseradish, turnip, radish, watercress, etc.) (Bailey et al. 2006). The well-known model plants from the family Brassicaceae *viz.*, Arabidopsis (*Arabidopsis thaliana*) and Brassica species have revolutionized our knowledge in almost every field of modern plant biology. Therefore, Franzke et al. (2011) are of opinion that life without the family Brassicaceae (the mustard family) would be a world without many crop species and the model organisms. Moreover, there are several taxa from the family Brassicaceae which largely stand second to none in terms of their significance in the studies related to trace metal tolerance and hyperaccumulation (*Arabidopsis halleri* and *Noccaea caerulescens*), self-incompatibility and genome evolution (*Arabidopsis lyrata* and *A. suecica*), perennial habit and plant–insect and plant–pathogen interactions (*Arabis alpine*), plant architecture, adaptation along water-usage gradient (*Cardamine hirsute*), self-incompatibility (*Capsella rubella* and *C. grandiflora*), flowering time, floral architecture (*C. bursa–pastoris*), apomixis and plant–insect and plant–pathogen interactions (*Boechera* spp.), mating system changes (*Diplotaxis* spp.), flower and fruit architecture (*Iberis* spp.), seed physiology, fruit structure (*Lepidium* spp.) and/or salt stress (*Eutrema–Thellungiella* ssp.) (see review by Franzke et al. 2011).

Hayek (1911) was the pioneer worker who almost 100 years ago published perhaps the first taxonomic and systematic treatise on the family of the Brassicaceae and thereafter subsequent contributions were made by Schulz (1936) and Janchen (1942) in this regard. Additionally, Vaughan et al. (1976) were among the workers who first attempted to summarize actual knowledge of the family in the book entitled "The Biology and Chemistry of the Cruciferae". This book describes those markers and methods associated with the study of evolution in the Brassicaceae which were the most modern and informative at that particular time. The family Brassicaceae represents a diverse and very interesting group of plants which are well defined by their flower architecture and thus are separated from other families of the Capparales. A number of workers such as Hayek (1911), Schulz (1936), Janchen (1942), Al-Shehbaz (1984) have tried to provide a natural system to divide the family of Brassicaceae into tribes. Based on the studies taking into account a small number of morphological characters such as fruit shape, position of the embryo and cotyledons the Brassicaceae were divided into 10 and 19 tribes by Hayek (1911) and Schulz (1936), respectively; while Janchen (1942) tried to present a comprehensive "natural" system with 15 tribes (Table 1.1). Schranz et al. (2006) summarized recent advances in the understanding of phylogenetics, polyploidization and comparative genomics in the family Brassicaceae in an excellent review which cumulatively may pave the ways for a unified comparative plant genomic framework. Various aspects of biosystematics of the plant family Brassicaceae have either been done or are in progress. The current chapter will update its readers on glimpses of taxonomy of important members of the plant family Brassicaceae and will also explore the significance of important genus for metal hyperaccumulation studies.

Table 1.1 Summary of important features of major tribes within the family Brassicaceae[a,b]

Tribes	Base chromosome[c]	Species[c,d]	Remarks	References
Aethionemeae	7–12	57	Unigeneric Majority of species are endemic to Turkey; Only a few grow as far east as Turkmenistan and west into Spain and Morocco	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Alysseae	8	280+	Includes 15 genera Distributed in Eurasia and N Africa, and only one species (*Alyssum obovatum*) extends its distribution to Canada and Alaska The majority of species in the tribe have stellate trichomes, latiseptate or terete (rarely angustiseptate), mostly few-seeded silicles, often winged seeds, and usually winged, toothed, or appendaged filaments	Koch and Al-Shehbaz (2009); Warwick et al. (2008)
Anchonieae	7	130	Includes 12 genera Distributed primarily in Eurasia and eastern and northern Africa It is distinguished by the presence of multicellular glands on multicellular-multiseriate stalks, 2-lobed stigmas, erect sepals, and often branched trichomes	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Arabideae	8	460+	Includes eight genera It is represented by 119 spp. in North America, 70 in South America, and over 100 in the Himalayas and neighbouring central Asia, but it is absent in Australia and all except NW Africa Primarily have branched trichomes, accumbent cotyledons, latiseptate or terete fruits nonmucilaginous seeds	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Boechereae	7	110+	Includes seven genera Almost exclusively North American, and only *Boechera furcata* grows in the Russian Far East The majority are perennials with well-defined basal rosette	Al-Shehbaz (2005); Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)

Brassiceae	15	230	Includes 46 poorly defined genera Distributed primarily in the Mediterranean region, adjacent SW Asia, and South Africa, and only four species of Cakile are native to North America Includes the most economically important plants in the family Vast majority of species in the Brassiceae have conduplicate cotyledons and/or segmented (heteroarthrocarpic) fruits	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Camelineae	8	240	Recently, reported to be paraphyletic and consists of a heterogeneous assemblage of genera The tribe includes the genera Arabidopsis (10 spp.), Capsella (3 spp.), Catolobus (1 sp.), Camelina (8 spp.), Neslia (2 spp.), Pseudoarabidopsis (1 sp.), and perhaps the Australian-endemic Stenopetalum (10 spp.) The tribe is primarily Eurasian, and only two species of Arabidopsis are native to North America	Bailey et al. (2006); Warwick et al. (2007); Koch et al. (2007); German and Al-Shehbaz (2008)
Cardamineae	8	340	The tribe includes 333 species most of which belong to the genera Cardamine including Dentaria (ca. 200 spp.), Rorippa (86 spp.), and Barbarea (25 spp.) Except for Barbarea, which does not occur in South America, the genera are represented by native species on all other continents. The other genera are Nasturtium (5 spp.; 2 native to Mexico and the United States), and the North American Iodanthus (1 sp.), Leavenworthia (8 spp.), Ornithocarpa (2 spp.), Planodes (1 sp.), and Selenia (5 spp.).	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Chorisporeae	7	47	Includes three genera Is primarily Asian and only four of the 35 species of Parrya are North American Distinguished by the presence of multicellular glands on multicellularmultiseriate stalks, connivent stigmas, and erect sepals, and by the lack of branched trichomes.	Al-Shehbaz and Warwick (2007); Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)

(continued)

Table 1.1 (continued)

Tribes	Base chromosome^{c}	Species^{c,d}	Remarks	References
Cleomaceae	9+ (?)	300	–	Al-Shehbaz et al. (2006)
Cochlearieae	6–7	21	Unigeneric Distributed primarily in Europe, with the ranges of three species extended into northern North America and Asia and one into NW Africa. Exhibit rosulate, undivided basal leaves, white petals, often sessile cauline leaves, terete or angustiseptate silicles, entire stigmas, biseriate seeds, ebracteate racemes, and no trichomes.	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Descurainieae	7	60	Consists of six genera Distributed in three centers: North American (17 spp.), South American (ca. 20 spp.), and Canarian (7 spp.), plus three species in Eurasia. Characterized by the petiolate, 1-3-pinnatisect stem leaves, dendritic or rarely only forked trichomes, incumbent cotyledons, and mostly yellow flowers Descurainia is unique in the Brassicaceae for the presence in some species of unicellular, glandular papillae.	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Euclidieae	7	150	Includes some 25 genera Monophyletic Distributed primarily in Eurasia and northern and eastern Africa. Presence of simple and two to several-rayed (vs. sessile stellate) trichomes	Warwick et al. (2007); Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009); Al-Shehbaz and Warwick (2007)
Eutremeae	7	26	Unigeneric Distributed primarily in Asia, especially the Himalayas and neighbouring central Asia, with two species extending their ranges into North America Members are are glabrous or with simple trichomes and have white flowers, incumbent cotyledons, and often palmately veined basal leaves.	Al-Shehbaz and Warwick (2006), Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)

Halimolobodeae	8	40	Includes five genera A New World tribe Mostly distributed in northern and central Mexico (Bailey et al. 2007), though some genera are disjunctly distributed in northern Argentina, Bolivia, and Peru, the southern United States Members of the Halimolobeae have branched trichomes, white (rarely purplish flowers), seeds mucilaginous when wetted, ebracteate racemes (except two Mancoa), often spreading sepals	Bailey et al. 2002; Fuentes-Soriano 2004; Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Heliophileae	10	82	Unigeneric Are exclusively South African Are easily distinguished by the diplecolobal cotyledons, often appendaged petals and/or staminal filaments, and simple or no trichomes	Mummenhoff et al. (2005), Al-Shehbaz and Mummenhoff (2005); Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009);
Hesperideae	6–10	46	Unigeneric Distributed primarily in the Middle East and Europe, with fewer species in central Asia and NW Africa. Unique in the Brassicaceae for its unicellular glands on uniseriate, few-celled stalks.	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Iberideae	7–11	27	Unigeneric Centered mainly in Europe, with a few species in NW Africa, and SW and central Asia Are glabrous or with simple trichomes and have angustiseptate, two-seeded fruits, zygomorphic flowers, and corymbose infructescences.	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Isatideae	7	90+	Includes two genera Have indehiscent, often pendulous, one- or two-seeded fruits, yellow or rarely white flowers, auriculate stem leaves, and simple or no trichomes.	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)

(continued)

Table 1.1 (continued)

Tribes	Base chromosome[c]	Species[c,d]	Remarks	References
Lepidieae	8	240+	Includes four genera It is represented by native species on all continents except Antarctica Distinguished by the angustiseptate fruits (secondarily inflated in two species formerly assigned to Cardaria), one ovule per locule, often mucilaginous seeds, and simple or no trichomes	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009), Al-Shehbaz et al. (2002)
Noccaeeae	7	85+	Includes three genera Distributed in Europe, Africa, and Southwest Asia, New World and also in the Himalayas	Meyer (2006); Koch and Al-Shehbaz (2004); Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Physarieae	8	150	Includes seven genera Distributed primarily in North America	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Schizopetaleae	14	230	Includes 28 genera Distributed in South and North America	Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Sisymbrieae	7	40	Unigeneric Distributed mainly in North American S. linifolium, the remaining species of the tribe are distributed in Eurasia and Africa Have yellow flowers, pinnately divided basal and lowermost stem leaves, two-lobed stigmas, terete siliques Simple or no trichomes	Warwick et al. (2002, 2005); Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Smelowskieae	6	25	Unigeneric Seven species in North America and 18 in central and eastern Asia. Have branched trichomes, petiolate, pinnatisect cauline leaves, white to purple (rarely cream) flowers, nonmucilaginous seeds, and incumbent cotyledons	Al-Shehbaz and Warwick (2006); Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)
Thlaspideae	7	26+	Includes seven genera Distributed in Europe and Southwest Asia Have striate or coarsely reticulate seeds, undivided cauline leaves, often palmately veined basal leaves, and simple or no trichomes.	(Koch and Mummenhoff 2001; Al-Shehbaz et al. (2006); Koch and Al-Shehbaz (2009)

[a] As proposed by Al-Shehbaz et al. (2006)

[b] Modified after Schranz et al. (2007) and Koch and Al-Shehbaz (2009)

[c] Al-Shehbaz et al. (2006)

[d] Warwick et al. (2008)

1.1.1 General Description of the Brassicaceae

The Brassicaceae consists mostly of herbaceous plants with annual, biennial or perennial lifespans. Although, most of the members of the family exhibit very limited variation in growth forms and are primarily herbaceous with only 5% of the species typically woody (1–9 m tall lianas) such as *Zilla spinosa*, *Ptilotrichum spinosum*, *Dendralyssum* spp., *Cramboxylon* spp., *Dendrosinapis* spp., *Descurainia* spp., *Stanley* spp., *Polypsecadium* spp., *Heliophila scandens*, *Lepidium scandens*, *Cremolobus peruvianus*, *Vella* spp., *Foleyola* spp., *Parolinia* spp., *Farsetia somalensis* (Al-Shehbaz 1984; Franzke et al. 2011). The leaves are alternate (rarely opposite), sometimes organized in basal rosettes; in rare shrubby crucifers of Mediterranean their leaves are mostly in terminal rosettes, and may be coriaceous and evergreen. They are very often pinnately incised and do not have stipules. Moreover, plants from this family are easily recognized also by the pungent taste of its crushed leaves. The presence of thick leaves or leaves with waxes with characteristic trichomes (hairs) (also on cotyledons) are also traits of interest. The structure of the flowers is extremely uniform throughout the family. In fact, the name crucifer comes from the shape of flowers, with four diagonally opposite petals in the form of a cross and/or, because of their characteristic flowers consisting of four petals in the form of a Greek cross this family is also called Cruciferae. As mentioned also above that the floral structure in the family is also highly conserved with four yellow petals arranged in a cross-shape, but exceptions in color (white, pink, purple) and symmetry are known. The other important features of the family may be listed as follows: (a) the number of stamens – usually six (*Exceptions – Lepidium* spp. (2–4), *Megacarpaea polyandra* (8–24)); (b) the type of nectary – varies with species viz., lateral, median, or both, some glucose-, others sucrose dominant (Davis et al. 1998); (c) ovary – superior; (d) inflorescence – ebracteate racemose often apically corymb-like, (e) pollination – entomogamy, nectar is produced at the base of the stamens and stored on the sepals, (f) the type of fruit – highly variable, typically dehiscent bivalvate capsule (silique or silicule; dispersal takes place generally close to the parental plant), can also be indehiscent, becoming lomentaceous or achenelike and only rarely nutlet, samara, schizocarp, or even a drupe; (g) the size of seeds – varies greatly, the smallest in *Mancoa mexicana* (Mexico) and Saharan species of Diplotaxis (at 0.02 and 0.05 mg, respectively) to the largest Megacarpaea gigantea (central Asia) weighing 90 mg; and measuring 1.8 × 1.5 cm (Al-Shehbaz 1984, 1986) and (h) mode of propagation – usually by seeds, only a few crop members of the family have an incredible capacity for vegetative propagation *viz*., *Neobeckia lacustris* (American water cress) and *A. rusticana* (horseradish). Most importantly, many genera of the Brassicaeae have been studied for their chemical constitution, especially for variation in oil content and seed fatty acid and glucosinolates composition. Especially, the glucosinolates (amino acid-derived, secondary metabolites and/or mustard oil glucosides) and hydrolysis products provide the characteristic odors and flavors of crucifers which have immense importance in chemical defence in plants against pathogens, herbivores, and weeds (Angelini et al. 1998; Clauss et al. 2006).

There is a report of more than 96 glucosinolates in the Brassicaceae family, of which majority are unique to certain species and genera (Fahey et al. 2001). Except a few species of the genus *Moricandia with* such as *M. arvensis*, *M. nitens*, *M. sinaica*, *M. spinosa*, and *M. suffruticosa* which are C3–C4 intermediate species (Razmjoo et al. 1996; Apel et al. 1997; Rylott et al. 1998), the majority of the members of Brassicaceae family have typical C3 photosynthesis (Uprety et al. 1995). This is also pertinent to mention here that the major centers of diversity of Brassicaceae family are southwestern and central Asia and the Mediterranean region whereas the arctic, western North America, and the mountains of South America are secondary centers of diversity (Price et al. 1994).

1.1.2 Taxonomy of the Brassicaceae and the Phylogenetic Relationships Within Family

The APG system (Angiosperm Phylogeny Group system) of plant classification has classified the Brassicaceae in to clade – eurosids II, order – Brassicales. The taxonomy of the family Brassicaceae has been plagued by convergent evolution in nearly every morphological feature used to define tribes and genera. Order Brassicales is characterized by the presence of glucosinolates, which contain sulfur. Although, taxonomically the Brassicaceae present a large and rather homogeneous family but the family suffers with problems of classification at suprageneric (where, natural links within the family cause difficulties in grouping of the genera into tribes and subtribes) and generic level (where, taxonomic problems exist in the differentiation of the species within the group of closely related species in large genera) (Anchev and Deneva 1997; Abdel Khalik et al. 2002; Plazibat 2009). Moreover, the taxonomic structure of the whole family is characterized by a large number of monotypic and small genera, mostly with clearly defined taxonomic limits. Controversies also exist in the tribal divisions which range from 3 to 27 tribes (Al-Shehbaz et al. 2006; Bailey et al. 2006; Al-Shehbaz and Warwick 2007; Warwick et al. 2008; Plazibat 2009). As per the opinions of Bailey et al. (2006) and Warwick et al. (2009) classification of Brassicaeae into tribes has long been problematic and not well understood phylogenetically because of the traditionally used few traits (such as orientation of the radicle and cotyledons in the embryo, fruit length to width ratio, fruit compression and dehiscence, number of rows of seeds in each locule, trichome type, and features of the nectarines etc.) to delimit different tribes of the family Brassicaeae.

Hayek (1911), Schulz (1936) and Janchen (1942) were among the pioneer workers who attempted to organize genus of the family Brassicaceae into tribes. Subsequently, based on later research reports by Price et al. (1994), Al-Shehbaz et al. (2006), Warwick et al. (2006) and Warwick and Al-Shehbaz (2006) each tribe has been defined morphologically and cytologically. Altogether, now, based on Internal Transcribed Spacer of nuclear ribosomal DNA (ITS)-based phylogenetic studies and the other recent techniques 33 tribes are recognized in the family on the basis of molecular phylogeny and re-evaluation of morphological features (Al-Shehbaz et al. 2006; Bailey et al. 2006; Beilstein et al. 2006; Al-Shehbaz and Warwick 2007; German and Al-Shehbaz 2008; Warwick et al. 2009). Additionally, molecular phylogenetic explorations at the tribal level was reported by Zunk et al. (1999), Bailey et al. (2002), Warwick and Sauder (2005) and Warwick et al. (2007, 2008); whereas, Koch et al. (2001, 2007), Bailey et al. (2006), Beilstein et al. (2006, 2008), Koch and Mummenhoff (2006), Franzke et al. (2009) explored the molecular phylogeny of Brassicaceae at family level. In this context, readers may consult research paper by Couvreur et al. (2010) and also excellent reviews by Al-Shehbaz et al. (2006), Schranz et al. (2007) and Franzke et al. (2011) for update on genome structure, gene expression and the evolution of specific traits within this important and interesting family. Although, these findings, as well as others on tribes and genera, solved many existing conflicts between molecular and morphological data and established monophyletic taxa that are well-supported molecularly and well-defined morphologically but despite the trend of progress mentioned a large-scale molecular phylogeny in terms of generic sampling and number of genes is still lacking. Therefore, resolving the major phylogenetic relationships within the family has still been problematic (Couvreur et al. 2010; Franzke et al. 2011). The number of monophyletic tribes has now been increased from the original 25 (Al-Shehbaz et al. 2006) to 44 (Al-Shehbaz and Warwick 2007; German and Al-Shehbaz 2008; Warwick et al. 2009, 2010). Based on the above important studies, research reports and/or reviews Table 1.1 summarizes some of these aspects such as the approximate number of genera and species within each tribe, and chief characteristics and distribution of major tribes of the family which have greatly helped researchers on the aspects of the origin, classification, and generic delimitation of the family.

1.2 Brassicaceae, Environmental Contaminants and Metal Hyperaccumulation

Although, immense scientific and technological progress have lead rapid global development in all the major fields but these progress in combination with increase in population, and the inception of Industrial Revolution however, severely contaminated all compartments of biosphere with variety of inorganic and organic contaminants and thus, raised new challenges, in the field of environmental protection and conservation worldwide (Bennett et al. 2003). Various man-made activities have largely accelerated the biogeochemical cycling of potential inorganic and organic pollutants naturally present in the environment; whereas, many xenobiotics have also been discovered, produced and released into the biosphere in recent decades. Moreover, the discovery and utilization of nuclear energy has introduced a large number of radionuclides of potential concern into the environment. Taking together increases in environmental contamination are leading to a progressive deterioration of environmental quality on the globe. It is pertinent to mention here that varying concentrations of hazardous contaminants get accumulated rapidly in the main sink – the soil compared to other environmental compartments (*e.g.*, atmosphere, water). In fact, the soil has been a key component of natural ecosystems because environmental sustainability depends largely on a sustainable soil ecosystem (Adriano et al. 1998). In addition, most of the toxic trace metals and radionuclides cannot be eliminated from the environment by chemical or biological transformation (Cunningham and Ow 1996) but they require a sustainable way of remediation from the sites. Although it may be possible to reduce the toxicity of certain metals by influencing their speciation, they do not degrade and remain persistent in the environment (NRC 1999). In this regard, a number of concerted efforts have been taken into consideration to find effective measures of remediation of varied environmental contaminants to reverse the negative conditions which are severely threatening human and environmental health. Among the major bioremediation techniques in this context, the use of plants (*viz*., phytoremediation) has become an important potential safe, environment friendly and low-cost technology that is currently being investigated for many remediation applications.

Besides benefiting the human society as a major source of edible and industrial oils, vegetable, condiment, and fodder crop species several representatives of the family Brassicaceae are either already known for or have potential to remediate varied environmental contaminants (Milner and Kochian 2008; Cecchi et al. 2010; Krämer 2010). Plants from the family Brassicaceae represent a differential range of tolerance to two important abiotic stresses *viz*. salinity and trace metals. *Cakile* spp. and *Crambe maritima* and *R. raphanistrum* ssp. *maritimus* occurring in coastal strand habitats have been observed to exhibit distinct tolerance to salinity levels (Boyd and Barbour 1986; Megdiche et al. 2007). Moreover, *L. fendleri* and *E. vesicaria* subsp. *Sativa* occur in desert and represent salt tolerant taxa (Ashraf and Noor 1993; Ashraf 1994; Dierig et al. 2004; Warwick 2011); whereas, Inan et al. (2004) reported *Thellungiella salsuginea* (=*T. halophila*) from saline flat habitats. Additionally, a number of plants from the family Brassicaceae can be listed here which have been reported tolerant to varied concentration of different trace metals including Cd, Ni, Pb, Se, Sr, and/or Zn (Boyd et al. 1994; Kruckeberg and Reeves 1995; Boyd and Martens 1998; Palmer et al. 2001; Prasad and Freitas 2003; Ghaderian et al. 2007; Przedpelska and Wierzbicka 2007; Warwick 2011). As reviewed by Prasad and Freitas (2003) and Assunção et al. (2003a, b) to date, 90 species from 11 genera have been reported for their trace metal-tolerance and/or accumulation capacity. Among those important plants, 48 spp. of *Alyssum*, 28 spp. of *Thlaspi*, 4 spp. of *Bornmuellera*, 3 spp. of *Arabidopsis*, 1 each spp. of *Arabis*, *Cardamine*, *Cochlearia*, *Peltaria*, *Pseudosempervivum*, *Stanleya* and *Streptanthus*, and *Brassica juncea* and *T. caerulescens* have been the focus of extensive research and also largely serve as the model plant species for heavy metal tolerance and accumulation studies (Assunção et al. 2003a, b; Belimov et al. 2007).

In relation to metal hyperaccumulation, as stated also above that with 87 species classified as metal hyperaccumulators the family Brassicaceae best represents amongst 34 different metal-hyperaccumulator families (including Asteraceace, Caryophyllaceae, Poaceae, Violaceae and Fabaceae) (see review by Krämer 2010). Of these 87 different metal-hyperaccumulator plant species in the family Brassicaceae, in particular model metal hyperaccumulator plant species *Alyssum*, *Thlaspi* and *Arabidopsis* have been studied extensively for their ability to hyperaccumulate several metals including metals and metalloids. Based on the plethora of recent published reports, the following section discusses chief characteristics of some representative plant species (*Brassica* sp., *Alyssum* sp., *Arabidopsis* sp. and *Thlaspi* sp.) extensively investigated for metal hyperaccumulation.

Regarding the concept of hyperaccumulation, the term first coined by Brooks et al. (1977) (Peer et al. 2003; reviewed in Milner and Kochian 2008) for plants tolerating and accumulating very high concentrations of metals (approx. 100 times that of a nonaccumulator plant species) in their above-ground tissues are called hyperaccumulators (Reeves and Baker 2000; Reeves 2003; Reeves and Adıgüzel 2008; Verbruggen et al. 2009; Krämer 2010). In fact, metal hyperaccumulation occurs in approximately 0.2% of all angiosperms and is particularly well represented in the Brassicaceae. Ni-hyperaccumulation, the majority of the 400 plant hyperaccumulators are Ni accumulators (317 species) (reviewed in Assunção et al. 2003a, b; Reeves and Baker 2000). Over half of all plant Ni hyperaccumulators belongs to the family Brassicaceae the rest are found within 21 other families. Of these some of the most studied include *T. caerulescens*, *Alyssum bertolonii*, *A. lesbiacum* and *T. goesingense* (Küpper et al. 2001). Most of the members of the family Brassicaceae stand second to none in terms of their ability to tolerate very high levels of trace metals in the soil and, more importantly for their metal-hyperaccumulation potential among about known 400 plants from families Asteraceae (including sunflowers and daisies), Caryophyllaceae (includes carnations), Cyperaceae, Cunouniaceae, Fabaceae (including peas and beans), Flacourtiaceae, Lamiaceae, Poaceae (includes grasses), Violaceae (including violets and pansies) and Euphorbiaceae that are able to tolerate high levels of heavy metals in the soil and in their shoots (Prasad and Freitas 2003; Milner and Kochian 2008; Krämer 2010). As mentioned also above that Brassicaceae have the largest number of taxa *viz*. 11 genera and 87 species and different genera of Brassicaceae are known to accumulate toxic trace metals at varied concentrations and are classified as metal hyperaccumulators. Although, the metal-hyperaccumulation ability has been observed in approx. 42 unrelated angiosperm families and was found of polyphyletic origin (Macnair 2003; Krämer 2010). In the context of metal-hyperaccumulation, of the 87 different species in the Brassicaceae, four species in particular, *Alyssym* sp., *Thlaspi caerulescens*, *Thlaspi rotundifolium* and *Arabidopsis halleri* have been studied extensively for their ability to hyperaccumulate several trace metals, including Zn, Cd and Ni. Moreover, the majority of the metal hyperaccumulators from the family Brassicaceae are Ni hyperaccumulators followed by Zn hyperaccumulators while only four species have been described as Cd hyperaccumulators: *T. caerulescens*, *Thlaspi praecox* and *Arabidopsis halleri* (Küpper et al. 2000; Yang et al. 2004; Vogel-Mikuš et al. 2005; Hassan and Aarts 2010). In particular, certain ecotypes of *T. caerulescens* can accumulate as much as 30,000 ppm of Zn and approx. 10,000 ppm Cd in the shoot biomass without any signs of toxicity (typical shoot levels are 100–200 ppm Zn and 0.1–10 ppm Cd). Whereas *Alyssium* spp. has been reported to accumulate Ni in the rage of 1,280–29,400 ppm; *Thlaspi rotundifolium* can accumulate 18,500 ppm (reviewed by Prasad and Freitas 2003; Milner and Kochian 2008). A majority (perhaps 85–90%) of the Ni hyperaccumulators are reported to serpentine-endemic. However, some occur on a variety of substrates with widely differing Ni concentrations, and show a wide range of plant Ni concentrations as a result. Additionally, regarding the hyperaccumulation of Ni, Reeves and Adıgüzel (2008) are of opinion that the term 'hyperaccumulator' should be restricted to plant species showing this behaviour in their natural habitats, *i.e*. this term should not be applied to plants that only accumulate Ni from Ni-rich culture solutions or from soils amended with Ni-rich solids or solutions. Moreover, it was also reported that the family Brassicaceae contains the largest number of Ni-hyperaccumulators in temperate areas of the world (especially Mediterranean Europe and Turkey) while in tropical areas the Euphorbiaceae was found the most important. There are also reports that in Turkey, Ni-hyperaccumulation occurs in the Brassicaceae not only in *Alyssum*, but in species of *Bornmuellera*, *Cochlearia*, *Thlaspi* s.l. (including *Masmenia* F.K.Mey., *Microthlaspi* F.K.Mey., *Noccaea* Moench, and *Thlaspiceras* F.K.Mey.), and *Aethionema* and in *Centaurea* (Asteraceae) (Reeves 1988; Reeves et al. 1983, 2001, 2004; Reeves and Adıgüzel 2004, 2008). Plethora of literature is available regarding the potential exploitation of metal-hyperaccumulation ability including metal homoeostasis and adaptation to extremely hostile environments of variety of plants through molecular explorations (reviewed by Pilson-Smits 2005; Cecchi et al. 2010; Verbruggen et al. 2009; Krämer 2010). It is pertinent to mention here that only a few members of the family Brassicaceae were studied as a model system (such as *Arabidopsis halleri* and Noccaea ('*Thlaspi*') *caerulescens*) at molecular-genetic level and a remarkable proportion (approx. 2%) of hyperaccumulators in the family are still unexloited (Cecchi et al. 2010; Verbruggen et al. 2009; Krämer 2010). Moreover, despite exhaustive molecular research the evolutionary patterns of the metal-hyperaccumulation ability of spp. in the family Brassicaceae are still incompletely known. In this regard, Cecchi et al. (2010), Assunção et al. (2003a, b) and Peer et al. (2003, 2006) argued that the current and future research on metal accumulation must focus on searching new model systems in Brassicaceae consisting of taxa of clear phylogenetic affinity (Assunção et al. 2003a, b; Peer et al. 2003, 2006).

1.2.1 Representative Brassicaceaens with Reported Metal Hyperaccumulation Traits

In addition to representing a number of economically and agronomically important plant species, the family Brassicaceae also encompasses several plant species of great scientific importance including model species significant for contributing to the filed of plant sciences at several arena. Of 87 different metal-hyperaccumulator plant species in the family Brassicaceae, plant species in particular model metal hyperaccumulator plant species *Alyssum*, *Thlaspi* and *Arabidopsis* have been studied extensively for their ability to hyperaccumulate several metals including metals and metalloids. Based on the plethora of recent published reports, the following section discusses chief characteristics of some representative plant species (*Brassica* sp., *Alyssum* sp., *Arabidopsis* sp. and *Thlaspi* sp.) extensively investigated for metal hyperaccumulation.

1.2.1.1 Alyssum

Alyssum is a genus of about 100–170 species of flowering plants in the family Brassicaceae, native to Europe, Asia, and northern Africa, with the highest species diversity in the Mediterranean region. The genus comprises annual and perennial herbaceous plants or (rarely) small shrubs, growing to 10–100 cm tall, with oblong-oval leaves and yellow or white flowers (pink to purple in a few species) (http://en.wikipedia.org/wiki/Alyssum).

The largest genus Alyssum with 172 species classified into six sections, best represents amongst the known plant species with extraordinary Ni-hyperaccumulation potential. All of the Ni-hyperaccumulators belong to section Odontarrhena (C.A. Meyer) Hooker, in which all species are perennials. Hyperaccumulating Alyssum species are distributed mostly on serpentine soils in southern Europe and Asia Minor, extending from Portugal in the west to the western parts of Iran in the east. In fact, in late 1940s, Minguzzi and Vergnano (1948) first observed rather discovered the Ni-concentration of about 10,000 μg g^{-1} shoot dry matter of *Alyssum bertolonii* (Desv). Hyperaccumulators of Ni are the most numerous, accounting for three-quarters of all known hyperaccumulator species (Baker et al. 2000; Reeves and Baker 2000). There are reports of Ni accumulation as high as 3% of shoot dry biomass in more than 50 taxa of the genus Alussum (Brooks et al. 1979; Reeves 1992; Brooks 1998; Reeves and Baker 2000; Reeves et al. 2001; Ingle et al. 2005; Ghaderian et al. 2007).

Both laboratory (herbarium specimens) (Brooks and Radford 1978; Brooks et al. 1979) and field (Reeves et al. 1983; Adıgüzel and Reeves 2002; Reeves and Adıgüzel 2004; Ghaderian et al. 2007) studies have reported varying degree of Ni-accumulation in more than 160 Alyssum species. Ghaderian et al. (2007) analyzed the serpentine soils of two areas (Marivan and Dizaj) in the west/northwest of Iran and also perennial Alyssum plants growing on these soils for Ni and some other metals. Authors observed the highest concentrations of Ni (1,350 μg g^{-1} of soil). Authors noted 3,700 and 8,100 μg g^{-1}, respectively in two Alyssum species, *A. inflatum* and *A. longistylum*, collected from Marivan and Dizaj, respectively. A number species within the genus Alyssum including *A. murale* and *A. corsicum* has now extensively been employed for commercial clean up of soils with varying degree of Ni-contamination (Chaney et al. 1999, 2007). According to recent reports of Li et al. (2003a, b), Broadhurst et al. (2004a, 2009) and Bani et al. (2007) majority of these Ni-hyperaccumulating species are endemic to serpentine (ultramafic-derived) soils throughout Mediterranean Europe. However, these species have also been reported to grow rapidly and prolifically, even in nonnative environments (Li et al. 2003a, b; Broadhurst et al. 2004a, 2009; Bani et al. 2007).

There have been great interests in the understanding of important mechanisms responsible for Ni-hyperaccumulation in different Alyssum species. Regarding localization of Ni in Alyssum species, there are reports of Ni-storage mainly in leaves with significant concentrations in epidermal cell vacuoles, trichome bases, and the lower parts of the trichome pedicle. According to Inamdar and Rao (1983), Oran (1996), Broadhurst et al. (2004b) the upper and lower leaf surfaces of Alyssum sp. are covered with an overlapping network of branching, stalked bifurcate or stellate trichomes. Moreover, trichomes are reported to be anchored in the epidermal layer with a ~20 μm smooth cylindrical pedicle which has a broad fan-shaped basal compartment (Broadhurst et al. 2009). The basal compartment of unicellular trichome and also the epidermal cells adjacent to the trichome basal compartment in *A. murale* have been shown to strongly concentrate Ni (Smart et al. 2007). Histidine has been shown to have a key role as a Ni chelator in both Ni hypertolerance and high root-to-shoot Ni flux in the xylem in *Alyssum lesbiacum* (Ingle et al. 2005). Additionally, Ni hyperaccumulator species of the genus Alyssum have been reported to constitutively harbor high concentrations of the free amino acid histidine, which was shown to act as a ligand for Ni ions in the plants (Krämer et al. 1996; Persans et al. 1999; Kerkeb and Krämer 2003). Moreover, the Ni- and histidine-concentrations in xylem have been reported to exhibit a proportional relationship in Alyssum hyperaccumulators (also addressed as the histidine response), which was interpreted by Kerkeb and Krämer (2003) as indirect evidence suggesting the transportation of Ni-histidine complexes into the xylem. Nedelkoska and Doran (2001) performed studies on transformation of several Alyssum species and reported the Ni-hyperaccumulation and tolerance of hairy root cultures of *A. bertolonii*, *A. tenium* and *A. troodii*. Plants of *A. tenium* regenerated from hairy root cultures were reported more tolerant to Ni and accumulated more Ni than the hairy roots of same species. Vinterhalter et al. (2008) investigated tolerance and nickel hyperaccumulation of *A. murale* under conditions of in vitro culture. Authors obtained hairy root clones by *A. rhizogenes* mediated transformation and checked the plants regenerated from them for tolerance and Ni hyperaccumulation. Authors reported the tolerance and Ni-accumulation up to 24,700 μg g^{-1} dry weight in *A. murale*. In another study, Barzanti et al. (2007) reported the role of an endophytic bacterial flora in *A. bertolonii* for high level of Ni-hyperaccumulation and resistance.

1.2.1.2 Arabidopsis

Arabidopsis (A-ra-bi-dóp-sis, rockcress) is an important genus in the family Brassicaceae. Based on morphological and molecular phylogenies major aspects of Arabidopsis' taxonomical history have been compiled in detail (Al-Shehbaz et al. 1999; Al-Shehbaz and O'Kane 2002) and currently, the genus Arabidopsis has nine species and a further eight subspecies (Koch et al. 2008). Although, Arabidopsis lacks its economic value but because of the small genome size, the ease of genetic approaches to study development, physiology, and gene function coupled with simple transformation methods (rather due to its favorable genetics) *Arabidopsis* (*A. thaliana*) was chosen by the community to be the first plant genome sequenced and for creation of the most advanced set of functional resources. Thale cress, *Arabidopsis thaliana* (L.) Heynh., is a small, 10–40 cm tall, annual herb/weed but one of the best known plants from the mustard family which is being now known as the model organism of flowering plants. The plant is a cosmopolitan generalist whose native range is considered to be Europe and the Middle Asian mountain system. Over 750 natural accessions of *A. thaliana* have been collected from around the world and are available from major seed stock centers. *Arabidopsis thaliana* can be found in a wide range of habitats in the Iberian Peninsula, including agricultural fields, banks and track sides, and openings of deciduous and Mediterranean forests and scrublands. *A. thaliana* exhibits a rapid life cycle (5–7 weeks) and has been reported to flower from April to early June and produce up to 10,000 seeds per plant. The species is self-compatible and self-fertile. The plant has a small genome that has been completely sequenced. Having many available mutants *A. thaliana* can be easily transformed using *Agrobacterium tumefaciens* (http://www.geochembio.com/biology/organisms/arabidopsis/; Al-Shehbaz and O'Kane 2002; Hoffmann 2002; Koornneef et al. 2004).

A. thaliana is currently used in almost every discipline of experimental biology and its completely sequenced genome (The Arabidopsis Genome Initiative 2000) paved the way to a better understanding of every aspect of plant biology (Buell and Last 2010). As a model organism for agricultural biotechnology, Arabidopsis presents the opportunity to provide key insights into the way that gene function can affect commercial crop production. In fact, *Arabidopsis thaliana* was the first plant, and the third multicellular organism after *Caenorhabditis elegans* (The *C. elegans* Sequencing Consortium 1998) and *Drosophila melanogaster* (Adams et al. 2000), to be completely sequenced (The Arabidopsis Genome Initiative 2000; The *C. elegans* Sequencing Consortium 1998; Adams et al. 2000).

Based on molecular analyses, (O'Kane and Al-Shehbaz 1997; Chen et al. 1998; Al-Shehbaz and O'Kane 2002) a number of species (exhibiting the chromosome number $>2n = 10$) have been removed and only nine species have been assigned to genus Arabidopsis where species such as *A. lyrata* and *A. halleri* have been reported to exhibit $2n = 16$. Within this genus, the allotetraploid species *A. suecica* ($2n = 26$) originates from an ancient Moreover, hybridization of *A. thaliana* with *A. arenosa* ($2n = 16$) have yielded the allotetraploid species *A. suecica* ($2n = 26$) (review by Koornneef et al. 2004). *A. halleri*, previously known as *Cardaminopsis halleri* (L.) Hayek, is known to occur on Zn, Cd and Pb contaminated sites and hyperaccumulate Cd and Zn (Brooks 1998; Bert et al. 2000; Küpper et al. 2000). Interestingly, it is closely related to and interfertile with *Arabidopsis lyrata* ssp. petraea (L.) O'Kane and Al Shehbaz that is both non-tolerant and a non-accumulator (Macnair et al. 1999). Identifying the particular gene or genes underlying a specific adaptation has been a major challenge for a wide community of

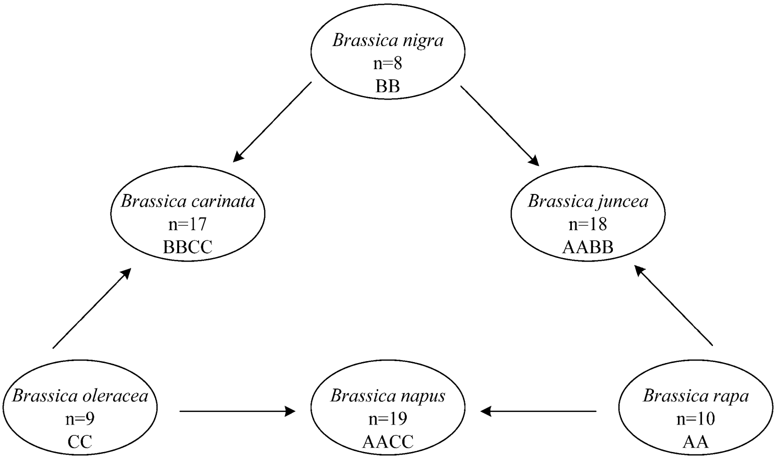

Fig. 1.1 The "Triangle of U" representing the genomic relationships among *Brassica* species (UN 1935)

plant biologists. In this context, with the use of *A. thaliana*, a significant increase in our understanding of the complex response of plants to stress has been achieved (Bressan et al. 2001; Bohnert et al. 2006; Roosens et al. 2008). Considerable variation for adaptive traits have been reported in the accessions of *A. thaliana* so far explored (Alonso-Blanco and Koornneef 2000; Koornneef et al. 2004). The species *Arabidopsis lyrata*, *A. arenosa*, *A. halleri*, and *Thellungiella halophila* were shown to display natural variation for complex traits, namely self-incompatibilty, hybridisation- polyploidization, heavy metal tolerance (Bressan et al. 2001; Clauss and Koch 2006; review by Roosens et al. 2008).

1.2.1.3 Brassica

The genus Brassica is a monophyletic group within the Brassicaceae which is evolutionarily closely related to model crucifer plant *Arabidopsis thaliana* of the same family Brassicaceae but is reported to have diverged 14.5–20.4 million years ago (Yang et al. 1999; Bowers et al. 2003). An old world genus, Brassica includes about 35 species of mostly annual herbs, with some perennial herbs and small shrubs. Cultivated brassicas are represented by six interrelated species, three of which are diploids [*Brassica rapa* (AA, 2n = 20), *B. nigra* (2n = 16, genome BB), *B. oleracea* (2n = 18, genome CC)] while three are amphidiploid derivatives, derived by hybridization and polyploidization of two of the diploid taxa [*B. juncea* (2n = 36, AABB, brown mustard), *B. carinata* (2n = 34, BBCC), *B. napus* (2n = 38, AACC, oilseed rape)] (Iwabuchi et al. 1991; Lagercrantz and Lydiate 1996; Snowdon et al. 1997). The botanical and genomic relationships between these six species may be represented in the form of a triangle usually known as triangle of U (UN 1935) (Fig. 1.1). *Brassica juncea* is generally thought to have originated in the Middle East, whereas, *B. rapa* and *B. nigra* species overlapped in the wild, but central Asia and China are suggested as the sites of primary origin (Prakash 1980). Hemingway (1976), however, considers that *B. juncea* may also have arisen by independent hybridization at secondary centers in India, China and Caucasus, as *B. nigra* was widely used as the commercial spice from early times.

The oleiferous genus *Brassica*, taxonomically placed within the family Brassicaceae (Cruciferae) is the third most important source of vegetable oil in the world after palm and soybean oil. Several *Brassica* species have been reported to exhibit higher tolerance towards most of the toxic metals/metalloids and majority of *Brassica* species are now known as good accumulators of toxic metals (including Cd, Cu, Ni, Pb, U, Zn) (Kumar et al. 1995) allocating large amounts of majority of these metals into above ground parts; thus stand second to none in terms of their utility in toxic metals-remediation strategies. Moreover, as several species of Brassica are able to produce significant amounts of biomass, (a required trait for phytoremediation) and are adaptable to a range of environmental conditions, there is the potential to develop superior genotypes of *Brassica* sp. for phytoremediation through selection and breeding techniques. Additionally, the adaptability of *Brassica* sp. can also be exploited through *in vitro* culture and transformation techniques to develop plants with range of tolerance capacities to toxic metals/metalloids (Palmer et al. 2001). Among different member of brassicaoilseed species, mustard (*B. juncea* L.), Black mustard (*B. nigra* Koch), Turnip (*B. campestris* L.), rape (*B. napus* L.) and kale (*B. oleracea* L.), *B. juncea* have been shown to exhibit differential accumulation and detoxification attributes which may be employed in the same environment to for affective phytoremediation output (Palmer et al. 2001). Among aforesaid *Brassica* sp., *B. juncea* has been studied most extensively for phytoremediation potential and reported to exhibit superior metal accumulation characteristics (Gleba et al. 1999). Field trials have been conducted for phytoremediation of Cd, Cu, Cr, Ni, Pb, Se, Zn, radionuclides and other metals by using *B. juncea* and *B. oleracea* as well (Ebbs and Kochian 1998; Lasat et al. 1998; Salt et al. 1998). Moreover, several improved *B. juncea* lines have already been developed for Pb accumulation ability by conventional selection methods (Kumar et al. 1995). Substantial removal of Se from contaminated soils has been achieved with *B. juncea* where the element either accumulated in the shoots or was volatilized (de Souza et al. 1999). Brassica species accumulating thallium have been reported (Kemper and Bertram 1991). Under experimental conditions, *B. juncea* has been found to accumulate arsenate mostly in the roots as AsIII triglutathione (Pickering et al. 2000). Readers may consult article by Anjum et al. (2012) to get more information on toxic metals/metalloids accumulation variability in genus Brassica.

1.2.1.4 Thlaspi

According the plant systematics, genus *Thlaspi* L., an important member of family Brassicaceae belongs Kingdom – Plantae, Subkingdom – Tracheobionta (Vascular plants), Superdivision – Spermatophyta (Seed plants), Division – Magnoliophyta (Flowering plants), Class – Magnoliopsida (Dicotyledons), Subclass – Dilleniidae and Order – Capparales. As reviewed by Macnair (2003) within the Thlaspi genus, hyperaccumulation is confined to the Noccaea section where all the members of the section were reported as hyperaccumulators suggesting the occurrence monophyletic hyperaccumulation within this genus. Within the family Brassicaceae the genus Thlaspi has been studied and described mostly in relation to hyperaccumulation of varied metals and metalloids (Peer et al. 2003). In particular, *Thlaspi caerulescens* J. & C. Presl or Alpine Pennycress is the most studied plant species of this genus for Cd-, Zn- and Ni-hyperaccumulation (Assunção et al. 2003a, b; Milner and Kochian 2008). It is important to mention here that Thlaspi caerulescens is a biennial, herbaceous and facultative metallophyte naturally distributed from northern Spain and Italy to the UK, Scandinavia and Poland (Tutin et al. 1993). With some exceptions for incompatibility in inter-population crosses, *T. caerulescens* has been reported to exhibit selfcompatibility (preferentially assured by insects) and free cross-compatibility within populations (Riley 1956). Strong inter-population variations in the out-crossing rate of *T. caerulescens* have been reported extensively (Koch et al. 1998; Dubois et al. 2003; Basic and Besnard 2006). Based on a number of important criteria such as seed morphology (Meyer 1973, 1979) and ribulose-1,5-bisphosphate carboxylase/oxygenase, ITS nuclear ribosomal DNA, and chloroplast DNA restriction-site variation (Mummenhoff and Zunk 1991; Mummenhoff and Koch 1994; Zunk et al. 1996; Mummenhoff et al. 1997), the genus Thlaspi has been divided into several genera/clades (Likar et al. 2010). In the process of the reorganization of this genus, many of the metal hyperaccumulating species (including *T. caerulescens* and *T. goesingense*) have been moved into the Noccaea genus (see Koch and Mummenhoff 2001, for a complete list) and *T. caerulescens* has now been re-named as *Noccaea caerulescens*.

Thlaspi caerulescens has extensively been reported for its extraordinary ability to colonize calamine and serpentine soils containing naturally elevated levels of heavy metals such as Zn, Cd, Ni, and Co it has been the object of interest in the plant biology community for over a century (Assunção et al. 2003a, b; Milner and Kochian 2008). Certain ecotypes of *T. caerulescens* were shown to accumulate Zn and Cd to extremely high levels in the shoot, with Zn reaching levels as high as 30,000 μg g^{-1} (Brown et al. 1995a, b) and shoot Cd concentrations of 10,000 μg g^{-1} (Lombi et al. 2000). By comparison, shoot Zn concentrations in Zn-sufficient nonaccumulator plants are around 100 μg g^{-1}, with 30 μg g^{-1} adequate and 300–500 μg g^{-1} toxic (Mengel and Kirkby 1987); foliar Cd levels above 1–10 μg g^{-1} are usually toxic. Therefore, *T. caerulescens* has been an emerging plant model species to study trace metal hyperaccumulation, homeostasis and tolerance (Deniau et al. 2006; Rigola et al. 2006).

Papoyan et al. (2007) studied the influence of high and low Cd^{2+} and Zn^{2+} status on root and shoot metal accumulation in hydroponically grown *T. caerulescens*. Shoot Cd accumulation was found significantly greater in the high-Zn-grown plants. Using radiotracer (109)Cd root flux experiments authors noticed maintainance of significantly higher root Cd^{2+} influx by high-Zn-grown plants compared to plants grown on 1 microm Zn. Authors concluded that xylem loading may be one of the key sites responsible for the hyperaccumulation of Zn and Cd accumulation in *T. caerulescens* (Piñeros et al. 2007). *T. caerulescens* has been shown to represent a potential source of genes for engineering trace metal phytoremediation in plants. Therefore, molecular genetics of this species has been a subject of intensive research for many years to better understand the mechanism of trace metal hyperaccumulation and tolerance in detail (Assunção et al. 2003b; Cobbett 2003; Deniau et al. 2006). Moreover, the there is a great deal of genetic variation available for metal specificity with regard to accumulation, translocation and tolerance traits between different T. caerulescens accessions (Meerts and van Isacker 1997; Escarré et al. 2000; Lombi et al. 2000; Schat et al. 2000; Deniau et al. 2006). Alonso-Blanco and Koornneef (2000) are of opinion that this intraspecific variation in combinations with its close relation with the genus *Arabidopsis* permits a genetic analysis of these traits in segregating populations generated from intraspecific crosses, including quantitative trait locus (QTL) mapping analysis (Koch et al. 2001; Deniau et al. 2006). Rigola et al. (2006) reported a collection of new resource to unravel the molecular basis of metal homeostasis, tolerance and hyperaccumulation in *T. caerulescens* through analyzing the first expressed sequence tag (EST). In total, authors generated 4289 ESTs originating from Zn-exposed root and shoot tissues, leading to 3709 *T. caerulescens* assembled partial cDNA sequences (unigenes). Moreover, authors opined that a fraction of c. 8% of the *T. caerulescens* unigenes (TcUGs) are *T. caerulescens* specific and have no significant similarity with any known DNA sequence. In addition, they found a relatively large number of *T. caerulescens*-specific transcripts despite the general resemblance of *T. caerulescens* transcriptome with the *A. thaliana* transcriptome. Additionally, *T. caerulescens* also expressed a relatively large number of genes which were expressed at a very low level in *A. thaliana*. Taking together the findings, authors concluded that *T. caerulescens* shares 88% DNA identity in the coding regions with *A. thaliana* (Rigola et al. 2006).

As stated also above that *T. caerulescens* also have ability of Cd-hyperaccumulation (Reeves and Baker 2000). Lombi et al. (2000) reported that *T. caerulescens* may hyperaccumulate Cd (>0.01% Cd in the shoot dry weight) and Ni (>0.1% Ni) as well. However, the accession of *T. caerulescens* namely Ganges (southern France) was shown to exhibit a superior ability to hyperaccumulate Cd with the level of accumulation up to 1% Cd under hydroponic conditions and up to 0.3% Cd under field conditions (Robinson et al. 1998). While, *T. praecox* Wulfen from a trace metal polluted site in Slovenia was reported to hyperaccumulate up to 0.6% Cd in shoots (Vogel-Mikuš et al. 2005), up to 0.07% Cd in flowering and seeding stalks (Pongrac et al. 2007), and up to 0.14% Cd in seeds (Vogel-Mikuš et al. 2007) under field conditions. Tolrà et al. (2006) reported the accumulation of Cd up to 0.3% concentration in shoots of hydroponically grown *T. praecox*; and Vogel-Mikuš et al. (2006) reported up to 0.7% Cd accumulation under greenhouse conditions. However, the capacity of Zn-hyperaccumulation (>1% Zn in the shoot dry weight) in *T. caerulescens* have been found to be a constitutive trait by Escarré et al. (2000) and Assunção et al. (2003a, b). Moreover, metal-hyperaccumulation capacity of Thlaspi spp. has been found differential depending upon the genotype and/or growing conditions. Under field conditions, various accessions of *T. caerulescens* were found to accumulate concentrations of up to 4–5% Zn (Baker et al. 1994; Reeves et al. 2001); whereas, when grown under hydroponics conditions *T. caerulescens* was shown to accumulate up to 2–4% Zn (Brown et al. 1995a, b; Shen et al. 1997). Zn-hyperaccumulation has been reported as a constitutive feature of *T. caerulescens* (Escarré et al. 2000; Reeves et al. 2001), whereas there exists great variation in hyperaccumulation of Ni and Cd (Reeves et al. 2001). Moreover, the accession Ganges within the species, *T. caerulescens* from Southern France was shown to have a superior ability to hyperaccumulate Cd (Robinson et al. 1998; Lombi et al. 2000). There are also reports of high variability in metal accumulation potential in the two populations of *T. praecox* from Slovenia (Vogel-Mikuš et al. 2005). Vogel-Mikuš et al. (2005) reported an accumulation of 1.5% Zn in shoot specimens of *T. praecox* collected from the field while the same group of authors noticed 0.3% Zn under experimental conditions (Vogel-Mikuš et al. 2006). Additionally, in hydroponic experiment using radiolabels ^{109}Cd and ^{65}Zn Xing et al. (2008) showed that the short-term uptake rate of Cd and Zn is higher in *T. caerulescens* (Ganges) than in *T. praecox*, whereas the Cd but not Zn translocation efficiency was higher in *T. praecox*. Exhaustive investigations on metal hyperaccumulation in *T. caerulescens* employing comparative transcriptome, proteome and metabolome analyses, and also for Quantitative Trait Loci (QTL) mapping studies may contribute to the further understanding of hyperaccumulation and tolerance traits also pave the way to the generation of new genotypes with exceptional multi-metals-hyperaccumulation capability.

1.3 Conclusions and Perspectives

The Brassicaceae (or Cruciferae/mustard family) is a large angiosperm (flowering plant) dicot family of plant kingdom which belongs to the order Brassicales and has been divided into 10–19 tribes with a total of 338–360 genera and 3,709 species distributed worldwide on all continents except Antarctica. The major centers of diversity of Brassicaceae family are southwestern and central Asia and the Mediterranean region whereas the arctic, western North America, and the mountains of South America are secondary centers of diversity (Price et al. 1994). The Brassicaceae are easily recognized by having unique flowers [with four petals, forming a cross or sometimes reduced or lacking; six stamens, the outer two being shorter than the inner four (however, sometimes only two or four stamens are present) and capsule (having two valves capsule with a septum dividing it into two chambers)]. Different taxa of Brassicaceae are distributed differentially in Northern Hemisphere (temperate regions), Southern Hemisphere and also in Southern regions. However, the distribution of the Brassicaceae has been shown limited to mountainous and alpine regions in the tropics. The plant family Brassicaceae includes several plant species of great scientific, economic and agronomic importance including model species, as well as many widely cultivated species. The well-known model plants from the family Brassicaceae *viz.*, Arabidopsis (*Arabidopsis thaliana*) and

Brassica species have revolutionized our knowledge in almost every field of modern plant biology. There are several taxa from the family Brassicaceae which largely stand second to none in terms of their significance in the studies related to trace metal tolerance and hyperaccumulation (*Arabidopsis halleri* and *Noccaea caerulescens*), self-incompatibility and genome evolution (*Arabidopsis lyrata* and *A. suecica*), perennial habit and plant–insect and plant–pathogen interactions (*Arabis alpine*), plant architecture, adaptation along water-usage gradient (*Cardamine hirsute*), self-incompatibility (*Capsella rubella* and *C. grandiflora*), flowering time, floral architecture (*C. bursa–pastoris*), apomixis and plant–insect and plant–pathogen interactions (*Boechera* spp.), mating system changes (*Diplotaxis* spp.), flower and fruit architecture (*Iberis* spp.), seed physiology, fruit structure (*Lepidium* spp.) and/or salt stress (*Eutrema–Thellungiella* ssp.) (see review by Franzke et al. 2011).

Hayek (1911) was the pioneer worker who almost 100 years ago published perhaps the first taxonomic and systematic treatise on the family of the Brassicaceae and thereafter subsequent contributions were made by Schulz (1936) and Janchen (1942) in this regard. The family Brassicaceae represents a diverse and very interesting group of plants which are well defined by their flower architecture and thus are separated from other families of the Capparales. A number of workers such as Hayek (1911), Schulz (1936), Janchen (1942), Al-Shehbaz (1984) have tried to provide a natural system to divide the family of Brassicaceae into tribes. The APG system (Angiosperm Phylogeny Group, APG- system) of plant classification has classified the Brassicaceae in to clade – eurosids II, order – Brassicales. The taxonomy of the family Brassicaceae has been plagued by convergent evolution in nearly every morphological feature used to define tribes and genera. Based on Internal Transcribed Spacer of nuclear ribosomal DNA (ITS)-based phylogenetic studies and the other recent techniques 33 tribes are recognized in the family on the basis of molecular phylogeny and re-evaluation of morphological features. It has been argued by recent researchers that resolving the major phylogenetic relationships within the family has still been problematic (Couvreur et al. 2010; Franzke et al. 2011). The number of monophyletic tribes has now been increased from the original 25 (Al-Shehbaz et al. 2006) to 44 (Al-Shehbaz and Warwick 2007; Warwick et al. 2009, 2010).

In addition to representing a number of economically and agronomically important plant species, the family Brassicaceae also encompasses several plant species of great scientific importance including model species significant for contributing to the filed of plant sciences at several arena. In relation to metal hyperaccumulation, as stated also above that with 87 species classified as metal hyperaccumulators the family Brassicaceae best represents amongst 34 different metal-hyperaccumulator families (including Asteraceace, Caryophyllaceae, Poaceae, Violaceae and Fabaceae). Of these 87 different metal-hyperaccumulator plant species in the family Brassicaceae, plant species in particular model metal hyperaccumulator plant species *Alyssum*, *Thlaspi* and *Arabidopsis* have been studied extensively for their ability to hyperaccumulate several metals including metals and metalloids.

Although, there have been major developments in our understanding of phylogenetic relationships within the Brassicaceae, and of genome structure, gene expression and the evolution of specific traits within this family in the past 2 years but a number of issues related to phylogenetic relationships within the Brassicaceae are yet to be resolved. Metal hyperaccumulation is a fascinating phenomenon, which has interested scientists for over a century. Metal hyperaccumulator plants have been shown to constitute an exceptional biological material for understanding mechanisms regulating metal homeostasis and adaptation in plants under extreme metallic environments. Moreover, despite recent advances, the mechanisms underlying molecular and genetic determinants for metal hyperaccumulation trait have not been clearly defined. There is therefore a need to develop a model system for molecular genetic studies of metal hyperaccumulation in plants.

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Chapter 2
Current Status of Toxic Metals Addition to Environment and Its Consequences

Qaisar Mahmood, Audil Rashid, Sheikh S. Ahmad, Muhammad R. Azim, and Muhammad Bilal

Abstract "Heavy metals" are the chemical elements which, in their standard state, have a specific gravity of more than about 5 g cm^{-3} i.e. their densities are five times greater than water. These constitute a very heterogeneous group of elements greatly varied in their chemical properties and biological functions. Heavy metals are kept under environmental pollutant category due to their toxic effects in plants, human and food particularly in areas with high anthropogenic pressure. Heavy metal pollution is one of the most important environmental problems today. Various industries produce and discharge wastes containing different heavy metals into the environment, such as mining and smelting of metalliferous, surface finishing industry, energy and fuel production, fertilizer and pesticide industry and application, metallurgy, iron and steel, electroplating, electrolysis, electro-osmosis, leatherworking, photography, electric appliance manufacturing, metal surface treating, aerospace and atomic energy installation etc. They are widely used in all fields of life i.e. batteries, dyes, alloys, chemical compounds, pharmaceutical and cosmetic products thus suggesting that the risk of pollution is very high. Thus, metal as a kind of resource is becoming shortage and also brings about serious environmental pollution, threatening human health and ecosystem. Three kinds of heavy metals are of concern, including toxic metals (such as Hg, Cr, Pb, Cd, As, etc.), precious metals (such as Pd, Pt, Ag, Au, Ru etc.) and radionuclides

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(such as U, Th, Ra, Am, etc.). The presence of heavy metal in atmosphere, soil and water, even in traces represent a severe risk to all organisms for their long term toxicological effects. Heavy metal bioaccumulation and biomagnifications in the food chain can be extremely dangerous to human health.

Keywords Metal pollution • Arsenic • Cadmium • Lead • Metal toxicity • Environmental consequences

2.1 Metal Pollution – Overview

“Heavy metals” are defined as the chemical elements which, in their standard state, have a specific gravity of more than about 5 g cm^{-3} i.e. their densities are five times greater than water (Holleman and Wiberg 1985). Heavy metals constitute a heterogeneous group of elements greatly varied in their chemical properties and biological functions (Mukesh et al. 2008). They are also called trace element because of their presence in traces (mg kg^{-1}) or in ultra-traces (μg kg^{-1}) quantities in the environmental matrices (Gogoasa et al. 2006).

Heavy metals are considered as environmental pollutants due to their toxic effects in plants, human and food particularly in areas with high anthropogenic pressure (Imran et al. 2008; Mukesh et al. 2008). They are widely used in all fields of life i.e. batteries, dyes, alloys, chemical compounds, pharmaceutical and cosmetic products thus suggesting that the risk of pollution is very high (Reeves and Baker 2000; Alexieva et al. 1981). The presence of heavy metal in atmosphere, soil and water, even in traces represent a severe risk to biotic components of ecosphere for their long term toxicological effects. Heavy metal bioaccumulation and biomagnifications in the food chain can be extremely dangerous to human health (Anastasio et al. 2006; Imran et al. 2008).

2.1.1 Classification of Heavy Metals

The heavy metals can be classified into four major groups based on their requirement and toxicity.

2.1.1.1 Essential Heavy Metals

Major essential heavy metals are also known as micronutrients include Cu, Zn, CO, Cr, Mn and Fe. Small amounts of these heavy elements are common in our environment and diet. These are actually essential for good health, normal growth, disease resistance, strength, vigor production and reproduction. However excessive intake above permissible limits can be toxic and cause damage to the organisms (Blaylock and Huang 2000; Monni et al. 2000; Reeves and Baker 2000; Gogoasa et al. 2006; Aelion et al. 2008).

2.1.1.2 Non-essential Metals

Non-essential metals include metals like Ba, Al, Li and Zr. They are not needed by the living beings for metabolic as well as normal physiology.

2.1.1.3 Less Toxic

The category of less toxic metals includes metals such as Sn and Al which are relatively less toxic than other metals.

2.1.1.4 Highly Toxic

Highly toxic metals are well known as very toxic even at lower concentrations. The examples include Hg and Cd (Mukesh et al. 2008).

2.1.2 Sources of Heavy Metals Pollution

The environmental pollution is a matter of great concern worldwide. The contamination of environment and food chain with heavy metals has become a matter of growing concern in view of its role in human health and nutrition (Sabir et al. 2003; Scheuhammer 1987). Heavy metals are generally introduced to ecosystems via natural processes, such as volcanic activity, erosion, wind-blown dust, rain, snow, and hail and anthropogenic process, such as mining, smelting, industrialization, fuel combustion, increased roadway traffic, and agricultural practices like land application of inorganic fertilizers, bio-solids, , agrochemicals, spread of animal manure and waste dumping (Herawati et al. 2000; Caussy et al. 2003; Licata et al. 2004; Caggiano et al. 2005; Blaylock et al. 2000; Mukesh et al. 2008).

2.1.3 Heavy Metals in Food Chain

Heavy metals enter the human and animals body mainly through inhalation of dust and ingestion of soil and consumption of food plants grown in metal-contaminated soils (Imran et al. 2007). Heavy metals are transferred to animals via direct exposure, polluted water and crops grown on irrigated sewerage water and industrial effluents. Several investigations have studied the transfer of heavy metals from soil to animals either by direct contamination or via the vegetation (Imran et al. 2007). Contaminated animal feed and rearing of livestock in proximity to polluted environment are responsible for heavy metal contamination in milk and meat

Table 2.1 Toxic limits and recommended/safe intake of heavy metals

Heavy metal	Toxic limit	Recommended uptake/safe intake
Arsenic	3 mg/day for 2–3 weeks	15–25 μg/day (adults)
Cadmium	200 μg/kg of fresh weight	15–50 μg/day adults 2–25 μg/day children
Lead	≥500 μg/L (blood)	20–280 μg/day adults, 10–275 μg/day children
Zinc	150 μg/day	15 μg/day

Adapted from Mishra et al. (2008)

(Miranda et al. 2007; Obiri-Danso et al. 2008). Other sources are accidental access to limed field, mineral supplements with high content of trace metal and licking of painted surfaced containing metallic pigments (Mukesh et al. 2008). The excess of heavy metal intake in food may cause severe consequences. The recommended dietary intake of some metals is given in Table 2.1 while Table 2.2 shows typical presentation of the most commonly encountered metals and their treatment.

The presence of heavy metals in excess in the environment can be a serious risk for the stability of ecosystems and living organisms. Some of the heavy metals are cumulative poisons which bio-accumulate and bio-magnify in the bodies of living organisms. They are neither degraded nor metabolized into intermediate compound in the environment. These metals are accumulating in food chain through uptake at primary producer level and then through consumption at consumer level (Mukesh et al. 2008). Thus, the food chain becomes the main gateway for persistent toxic heavy to enter higher organisms. Animals at the top of food chain may generally accumulate a large amount of heavy metals in their tissue, according to the age, size and feeding habits.

2.2 Arsenic

Arsenic has been detected in ground waters from many countries throughout the globe and it has threatened the use of groundwater as major source of drinking water (Bhutta et al. 2002; Harvey et al. 2002; Smedley and Kinniburgh 2002; USEPA 2003; Mroczek 2005; Mohan et al. 2007). Factors such as anthropogenic activities, biological action, and geochemical reactions help to mobilize arsenic into ground waters. Most environmental arsenic problems are the result of mobilization under natural conditions. However, mining activities, combustion of fossil fuels, use of arsenic pesticides, herbicides, and crop desiccants and use of arsenic additives to livestock feed create additional impacts (Mohan et al. 2007). Because of lethality to human health various countries have reduced the maximum contaminant level (MCL) of arsenic from 50 to 10 μg L^{-1} in drinking water (WHO 1993; NHMRC 1996; European Commission Directive 1998). The concentration of arsenic in most rocks ranges from 0.5 to 2.5 mg kg^{-1}, though higher concentrations are found in finer grained argillaceous sediments and phosphorites (Kabata-Pendias and Pendias 2000; Mandal and Suzuki 2002).

Table 2.2 Typical presentation of the most commonly encountered metals and their treatment

Metal	Acute	Chronic	Toxic concentration	Treatment
Arsenic	Nausea, vomiting, “rice-water” diarrhea, encephalopathy, MODS, LoQTS, painful neuropathy	Diabetes, hypopigmentation/hyperkeratosis, cancer: lung, bladder, skin, encephalopathy	24-h urine: $\geq$50 μg/L urine, or 100 μg/g creatinine	BAL (acute, symptomatic) Succimer DMPS (Europe)
Bismuth	Renal failure; acute tubular necrosis	Diffuse myoclonic encephalopathy	No clear reference standard	[a]
Cadmium	Pneumonitis (oxide fumes)	Proteinuria, lung cancer, osteomalacia	Proteinuria and/or $\geq$15 μg/g creatinine	[a]
Chromium	GI hemorrhage, hemolysis, acute renal failure (Cr^{6+} ingestion)	Pulmonary fibrosis, lung cancer (inhalation)	No clear reference standard	NAC (experimental)
Cobalt	Beer drinker’s (dilated) cardiomyopathy	Pneumoconiosis (inhaled); goiter	Normal excretion: 0.1–1.2 μg/L (serum) 0.1–2.2 μg/L (urine)	NAC $CaNa_2$ EDTA
Copper	Blue vomitus, GI irritation/hemorrhage, hemolysis, MODS (ingested); MFF (inhaled)	Vineyard sprayer’s lung (inhaled); Wilson disease (hepatic and basal ganglia degeneration)	Normal excretion: 25 μg/24 h (urine)	BAL D-Penicillamine Succimer
Iron	Vomiting, GI hemorrhage, cardiac depression, metabolic acidosis	Hepatic cirrhosis	Nontoxic: <300 μg/dL Severe: >500 μg/dL	Deferoxamine
Lead	Nausea, vomiting, encephalopathy (headache, seizures, ataxia, obtundation)	Encephalopathy, anemia, abdominal pain, nephropathy, foot-drop/wrist-drop	Pediatric: symptoms or [Pb] $\geq$45 μ/dL (blood); Adult: symptoms or [Pb] $\geq$70 μ/dL	BAL $CaNa_2$ EDTA Succimer
Manganese	MFF (inhaled)	Parkinson-like syndrome, respiratory, neuropsychiatric	No clear reference standard	[a]
Mercury	Elemental (inhaled): fever, vomiting, diarrhea, ALI; inorganic salts (ingestion): caustic gastroenteritis	Nausea, metallic taste, gingivo-stomatitis, tremor, neurasthenia, nephrotic syndrome; hypersensitivity (Pink disease)	Background exposure “normal” limits: 10 μg/L (whole blood); 20 μg/L (24-h urine)	BAL Succimer DMPS (Europe)

(continued)

Table 2.2 (continued)

Metal	Acute	Chronic	Toxic concentration	Treatment
Nickel	Dermatitis; nickel carbonyl: myocarditis, ALI, encephalopathy	Occupational (inhaled): pulmonary fibrosis, reduced sperm count, nasopharyngeal tumors	Excessive exposure: ≥8 μg/L (blood) Severe poisoning: ≥500 μg/L (8-h urine)	[a]
Selenium	Caustic burns, pneumonitis, hypotension	Brittle hair and nails, red skin, paresthesia, hemiplegia	Mild toxicity: [Se] >1 mg/L (serum); serious: >2 mg/L	[a]
Silver	Very high doses: hemorrhage, bone marrow suppression, pulmonary edema, hepatorenal necrosis	Argyria: blue-grey discoloration of skin, nails, mucosae	Asymptomatic workers have mean [Ag] of 11 μg/L (serum) and 2.6 μg/L (spot urine)	Selenium, vitamin E (experimental)
Thallium	Early: vomiting, diarrhea, painful neuropathy, coma, autonomic instability, MODS	Late findings: alopecia, mees lines, residual neurologic symptoms	Toxic: >3 μg/L (blood)	MDAC Prussian blue
Zinc	MFF (oxide fumes); vomiting, diarrhea, abdominal pain (ingestion)	Copper deficiency: anemia, neurologic degeneration, osteoporosis	Normal range: 0.6–1.1 mg/L (plasma) 10–14 mg/L (red cells)	[a]

[a] No accepted chelation regimen; contact a medical toxicologist regarding treatment plan

2.2.1 Occurrence of Arsenic

Arsenic exists in the −3, 0, +3 and +5 oxidation states (Smedley and Kinniburgh 2002). Environmental forms include arsenious acids, arsenic acids, arsenites, arsenates, methylarsenic acid, dimethylarsinic acid, arsine, etc. Arsenic (III) is a hard acid and preferentially complexes with oxides and nitrogen. Conversely, arsenic (V) behaves like a soft acid, forming complexes with sulfides (Bodek et al. 1998). Inorganic forms of arsenic most often exist in water supplies (Bodek et al. 1998). Arsenic is uniquely sensitive to mobilization (pH 6.5–8.5) and under both oxidizing and reducing conditions among heavy metalloids. Two forms are common in natural waters: arsenite (AsO_3^{3-}) and arsenate (AsO_4^{3-}), referred to as arsenic (III) and arsenic (V). Pentavalent (+5) or arsenate species are AsO_4^{3-}, $HAsO_4^{2-}$, H_2AsO^{4-} while trivalent (+3) arsenites include $As(OH)_3$, $As(OH)^{4-}$, AsO_2OH^{2-} and AsO_3^{3-}. Pentavalent species predominate and are stable in oxygen rich aerobic environments. Trivalent arsenites predominate in moderately reducing anaerobic environments such as groundwater (Greenwood and Earnshaw 1984).

2.2.2 Toxicity of Arsenic

Arsenic is a naturally occurring toxic metal and its presence in food could be a potential risk to the health of both humans and animals (Al-Rmalli et al. 2005). Inorganic arsenic occurs naturally in soil, air and water a well as through anthropogenic sources such as mining, agriculture and non-agricultural activities (Smith et al. 2009). Arsenic toxicity is a global health problem affecting millions of people. Arsenic-contaminated groundwater is often used for food and animal consumption, irrigation of soils, which could potentially lead to arsenic entering the human food chain (Al-Rmalli et al. 2005).

Arsenic (As) is commonly associated with sulfides, oxides/hydroxides of aluminum (Al), iron (Fe) and manganese (Mn), other sources are volcanic eruptions, and sea salt sprays (Fitz and Wenzel 2002). Earth crust mainly is made-up of igneous (olivine), metamorphic (granite and limestone) and sedimentary (sandstone) rocks which contain arsenic (As), in sufficient amounts, such as in igneous rocks is in the range of 0.2–10 mg kg^{-1} and in sedimentary rocks up to 0.6 mg kg^{-1} (Zhenli et al. 2005). In soil arsenic (As) is present in the form of oxides, hydroxide, chlorides and sulfides, such as enargite (Cu_3AsA_4), cobaltite (CoAsS), skutterudite ($CoAsS_4$) and it's (As) worldwide found average concentration in different regions is 9.36 mg kg^{-1}, the reason for which is that during Green Revolution, in 1900s, arsenic (As) was being used as major pesticide (Chopra et al. 2007) and after 50 years, was replaced by DDT. Arsenic contamination of soil may result from mining, milling, and smelting of copper, lead, and zinc sulfide ores, coal fly ash, and agricultural use of arsenical pesticides (Adriano 2001; Ng et al. 2003). In the past decade, the global input of arsenic to soils by human activities was estimated in range of 52,000–112,000 ton per year.

Thus, the concentrations of arsenic in soil and environment are both due to natural and anthropogenic activities. Most of the risk from arsenic is associated with the forms of arsenic that are biologically available for absorption, or "bio-available" to humans. A bio-available chemical is the portion of a chemical dose that enters the systemic circulation from an administered dose (Rodriguez et al. 2007). Total As content cannot help, directly in accessing solubility or bioavailability of As, no direct method to quantify bio-available arsenic in soils and assess the risk of incidental injection of arsenic, is available. The only method routinely used to characterize arsenic in soils and other media is by hot acid extraction.

Of 1.4 million worldwide contaminated sites 41% are in the USA and US EPA has recognized that arsenic (As) concentration in Australia was greater than 10,000 mg kg^{-1} (Smith et al. 2009). Arsenic has been found at high levels (10,000–20,000 mg kg^{-1}) in some contaminated areas that results in unacceptable levels of risk to human health from the incidental ingestion of soil (Davis et al. 2001). Arsenic (As) intake with drinking water is a very severe problem in the South East Asia with the Bengal delta being the worst affected area (Sharma 2006). In Warsak Canal, Pakistan, mining has resulted in increased As concentrations in released industrial effluents (Khan et al. 2005). In some areas of Pakistan, a number of shallow aquifers and tube wells are contaminated with arsenic at levels which are above the recommended USEPA arsenic level of 10 ppb (10 μg L^{-1}) (Malik et al. 2009). Large arsenic concentrations such as 0.942, 0.40, 0.38, 0.643 and 0.475 mg L^{-1} were detected in Hattar Industrial Estates, Ghari Rahimabad, Pakha Ghulam, Peshawar Industrial Estate, and Gujranwala Industrial estate, respectively (Rehman et al. 2008) of Pakistan. Chronic exposure to arsenic may result in skin and internal organ cancers, impaired nerve function, kidney and liver damage, or skin lesions. One of the more widespread problems is due to leaching of naturally occurring arsenic into drinking water aquifers (Rathinasabapathi et al. 2006). Thus, ground water contamination with arsenic (As) is most common result of higher concentrations of arsenic (As) in soil. It is estimated that approximately one third of the world's population use groundwater for drinking (UNEP 1999), which ultimately adversely affect human beings such as biggest calamity, was in Bangladesh, where millions of people were dependent on arsenic (As) contaminated drinking water (Chakraborti et al. 2002) and the possible cause of the death of such notables as Napolean and the American president Zachary Taylor (Rathinasabapathi et al. 2006) (Fig. 2.1).

Human exposure to arsenic can take place through ingestion, inhalation or skin adsorption; however, ingestion is the predominant form of arsenic intake. Various effects of As caused by short term and long-term exposure have been described elsewhere (NRC 1999; Abernathy and Morgan 2000; Quamruzzaman et al. 2003; WHO 2004). It was only recently that strong adverse effects on health were found associated with long-term exposure to very low arsenic concentrations. Drinking water is now recognized as the major source of human intake of arsenic in its most toxic (inorganic) forms (UNEP 1999). The presence of arsenic, even at high concentrations, is not accompanied by any change in taste, odors and/or visible appearance of water. The presence of arsenic in drinking water is therefore difficult to detect without complex analytical techniques and hence may present a significant hazard to community health.

Fig. 2.1 An arsenic victim's hand and foot in Bangladesh (http://www.unicef.org/bangladesh/Arsenic.pdf)

Recent studies showed that lung cancer is significantly higher in the areas where arsenic concentration is 1.77 ng m^{-3} or more (Navas-Acien et al. 2005; Yoshikawa et al. 2008). In last decades presences of arsenic and mercury in air/drinking water has become an issue of global concern (WHO 2001; Joo and Cheng 2006). The epidemiological data found that arsenic has a little evidence with cancer risk (Tsuji 2005). Inorganic arsenic has been recognized as a human poison since ancient times, and large oral doses (above 60,000 ppb in water) can result in death. The most characteristic effect of long-term oral exposure to inorganic arsenic is a pattern of skin changes. These include patches of darkened skin and are often associated with changes in the blood vessels of the skin. Skin cancer may also develop. Swallowing arsenic has also been reported to increase the risk of cancer in the liver, bladder, and lungs. The Department of Health and Human Services (DHHS) has determined that inorganic arsenic is known to be a human carcinogen (a chemical that causes cancer) (Eco-USA 1996).

Removal of arsenic from drinking water should be a worldwide priority. Arsenic concentration above acceptable standards for drinking waters has been demonstrated in many countries and thus is a global issue. Arsenic has been reported in groundwater in: Bangladesh, Cambodia, China (including provinces of Taiwan and Inner Mongolia), India, Iran, Japan, Myanmar, Nepal, Pakistan, Thailand, Vietnam, Alaska, Argentina, Chile, Dominica, El Salvador, Honduras, Mexico, Nicaragua, Peru, United States of America, Austria, Croatia, Finland, France, Germany, Greece, Hungary, Italy, Romania, Russia, Serbia, United Kingdom, Ghana, South Africa, Zimbabwe Australia and New Zealand (Borgono and Greiber 1971; Kaneko 1979; Cebrian et al. 1983; Das et al. 1983; Meranger et al. 1984; Chen et al. 1994; Dhar et al. 1997; Burkel and Stoll 1999; Chowdhury et al. 1999; Koch et al. 1999; Karim 2000; Nickson et al. 2000; Chakraborti et al. 2002; Hassan 2005; Hossain 2006; Mondal et al. 2006, 2007). Exposure to high levels of acute arsenic poisoning is relatively less common however; long-term exposure to even low concentrations of arsenic in drinking water also presents a considerable health hazard. Numerous references review the effect of long-term exposure to arsenic on people's health

(NRC 1999; Ahmed 2003). Removal of arsenic from drinking water using low cost, simple and appropriate methods is highly desirable.

Anoliefo et al. (2008) surveyed plant species growing in and around 38 metal welding workshops in Benin City, Nigeria, and provided a list of plants that sprouted and grown in metal polluted soils, which surely were tolerant to metal pollution in soil and can be possible candidates for remediation strategies and management for heavy metals contaminated soils and the frequency of occurrence of any particular plant species was used as an indicator of tolerance to heavy metals. The family Poaceae, was identified in all the sites visited. Cadmium (Cd) and lead (Pb) concentrations occurred in soil samples obtained from the sites. Six of the ten locations (Ekenwan, Plymouth, Siluko, Sapele, Sakponba and Ikpoba Hill Road Quarters) had elevated cadmium concentrations, where the highest concentration of 1.2 mg kg^{-1} was detected at Ikpoba and at Ugbowo lead concentration was highest (53 mg kg^{-1}).

Bhattacharya et al. (2007) found groundwater arsenic contamination (10/50 μg As L^{-1}) in several countries of the world. In Asia, Particularly South Asia, groundwater As toxicity is alarming, as has been found in the Bengal Basin of Bangladesh, West Bengal, India, Terai Belt in Southern Nepal, Pakistan, the Red River Delta, Mekong Basin of Vietnam and Cambodia, thus putting least 100 million people at risk of cancer and other As-related diseases. As contamination could be natural such as due to parent material or anthropogenic which include calcium arsenate, lead arsenate, sodium arsenate in insecticides/pesticides for debarking trees, in cattle and sheep dips to control ticks, fleas, lice, in aquatic weed control and chromated copper arsenate (CCA) and other As-based chemicals in wood preservatives, used in the past. The majority soil and water As pollution occurred in period prior to controls over As emissions. Remediation of As-affected water supplies became important, hence, several technologies such as, coagulation–flocculation, ion exchange and reverse osmosis natural sorbents for As (e.g. laterite, bauxsols, natural red earth or Fe-rich oxisols) are being used worldwide. But we still need to develop these methods further and in a cost-effective way. The concept of phytoremediation of As-contaminated sites was proposed over 20 years ago, has an advantage over conventional remediation methods hence is being adopted as an environmentally friendly and low-cost alternative remediation technique. It is well documented that some tropical and sub-tropical plant species can tolerate and uptake various inorganic and organic forms of As. Reviewing different researches they found that (a) Mesquite (*Prosopis* spp.) treated with As(III) and As(V), accumulated more As concentrations from As(V) were significantly than from As(III) in all portions of the plant and X-ray absorption spectroscopy (XAS) revealed that As(V) was reduced to As(III) inside the mesquite plant which revealed that mesquite could be a good candidate for the uptake of As in contaminated soils in arid regions, and (b) Common marsh fern *Thely palusteris* accumulated As in both roots and fronds and in roots the levels of As were up to 100 times the concentration of treatment solutions of 250 and 500 mg L^{-1} As, but values varied widely and there was no significant difference in As concentrations in fronds between the control (without As) and treatments. However, plants exposed to

500 mg L^{-1} exhibited necrosis in their fronds, suggesting that *T. palusteris* is not a good candidate for phytoremediation of sites with extremely high concentrations of bioavailable As.

Feldmann (2007) elaborated the carcinogenicity of arsenic by telling that exposure to arsenic is correlated with an increased risk to contract skin, bladder and other forms of internal cancer. More than 10 years ago, it has been surfaced that millions of people in Asia have been and are exposed to elevated levels of arsenic in their drinking water and suffer increasingly the detrimental consequences. However, arsenic exposure through food has been neglected in the past decade but not it has been revealed that a major contribution of arsenic exposure can come from rice intake. Thus, in his lecture, through arsenic speciation, using mass spectrometry, coupled with liquid chromatography, he explained arsenic absorption, from the paddy fields into the rice plant and then translocation into the shoots and grains and its accumulation in the different parts of the crop. He also showed arsenic interactions with peptides such as phytochelatins, which was complimentary supported by X-ray absorption spectroscopy (XANES) to identify molecular form of accumulated arsenic in the analysed plant. Fitz and Wenzel (2002) found that there was no evidence that As is essential for plants, but plant growth stimulated when applied at low concentrations. From hydroponic experiments on plant As uptake, they found that the chemical form of supplied As is more important than total As concentrations in solution. In solution culture experiments the phytoavailability of As for two rice cultivars was found as DMAA $<$ As(V) $<$ MMAA $<$ As(III), while for two typical wetland plant species order was DMAA $<$ MMAA ~ As(V) $<$ As(III).and both reports agreed that upon absorption of inorganic species and MMAA were mainly accumulated in the roots. From review they found that phyto-chelatins were involved in As detoxification in *Silene vulgaris* and *Nicotiana tabacum* and most the As in fronds of *P. vittata* and *P. calomelanos* was present as As(III), whereas As(V) dominated in roots. Ghosh and Singh (2005) reviewed the status of phytoremediation technologies with particular emphasis on phytoextraction of soil heavy metal contamination. Unlike organic compounds, metals cannot be degraded, and cleanup usually requires their removal. Most of the conventional remedial technologies are expensive, inhibit the soil fertility; subsequently causes negative impacts on the ecosystem. Phytoremediation proved as a cost effective, environmental friendly, aesthetically pleasing approach and most suited for developing countries. Despite this potential, phytoremediation is yet to become a commercially available technology. They reported the mobility, bioavailability, plant response to presence of soil heavy metals, classified plants according to phytoextraction mechanism and discussed the pathway of metal mobility in soil, water and plant. They gave an insight into the work of authors searched for high biomass producing weeds, discussed various techniques which can enhance phytoextraction and the utilization of by-products and proved phytoextraction more viable for present utilization.

Gonzaga et al. (2006a) examined arsenic uptake by arsenic hyperaccumulator *Pteris vittata* L. and non-arsenic hyperaccumulator *Nephrolepis exaltata* L. through greenhouse experiment and its effect on arsenic chemistry in bulk and rhizosphere soil. The plants were grown for 8 weeks in a rhizopot, with soil containing 105 mg kg^{-1} arsenic. *P. vittata* concentrated 92% of the arsenic in the fronds while N. exaltata 33%. Plant growth has significantly impacted the total arsenic, watersoluble arsenic, as well as arsenic in different fractions in the rhizosphere soil, but not the bulk soil. *P. vittata* had a greater effect on the mobilization of arsenic pools than *N. exaltata*. The soil arsenic was fractionated into five fractions with decreasing availability: non-specifically bound (N), specifically bound (S), amorphous hydrous-oxide bound (A), crystalline hydrous-oxide bound (C), and residual (R). *P. vittata* produced larger plant biomass (7.38 vs. 2.32 mg $plant^{-1}$) and removed more arsenic (2.61 vs. 0.09 mg pot^{-1} arsenic) than *N. exaltata*. Plant growth reduced water-soluble arsenic, and increased soil pH (*P. vittata* only) in the rhizosphere soil. *P. vittata* was more efficient than *N. exaltata* to access arsenic from all fractions (39–64% vs. 5–39% reduction). However, most of the arsenic taken up by both plants was from the A fraction (67–77%) in the rhizosphere soil, the most abundant (61.5%) instead of the most available (N fraction). Arsenic mobilization in the rhizosphere of *P. vittata* followed the order $A >> S = C > N = R$ while in *N. exaltata* it was $A >> S > C > R > N$. Plant growth had no significant effect on the bulk soil pH and DOC but affected these parameters in the rhizosphere. While soil pH was increased by 0.4 units in the rhizosphere of *P. vittata*, no significant change was observed for *N. exaltata*. According to Gonzaga et al. (2006b) Arsenic is a nonessential element for plants, and inorganic arsenic species are generally highly phytotoxic. Its presence at elevated concentrations in soils derives from both anthropogenic and natural inputs. Technologies currently available for the remediation of arsenic contaminated sites are expensive, environmentally disruptive, and potentially hazardous to workers. Phytoextraction, a strategy of phytoremediation, uses plants to clean up contaminated soils and has been successfully applied to arsenic contaminated soils. Under normal conditions, arsenic concentrations in terrestrial plants are usually less than 10 mg kg^{-1}. Several plants contain arsenic in the following order: cabbage (0.020–0.050 mg kg^{-1}) < carrots (0.040–0.080 mg kg^{-1}) < grass (0.020–0.160 mg kg^{-1}) < potatoes (0.020–0.200 mg kg^{-1}) < lettuce (0.020–0.250 mg kg^{-1}) < mosses and lichens (0.26 mg kg^{-1}) < ferns (1.3 mg kg^{-1}). In plants an average toxicity threshold for As is 40 mg kg^{-1}. It is found that in soil chemical behavior of arsenic is almost similar to that of phosphorus and almost so studied plants arsenate arsenate taken up is via the phosphate transport systems. Hence arsenate, As(V) acting as a phosphate analog, can replace phosphate in many biochemical processes. Phosphate replacement, disrupt phosphate metabolism and production of the nucleotide adenosine triphosphate (ATP), main energy source for cells in plants. Whereas, arsenite, As(III) reacts with sulfhydryl groups of enzymes and tissue proteins, resulting inhibition of cellular function and death. They also found that the transfer of arsenic from soil to plant was low for most plant species. The reason for that probably are i) Restricted uptake by plant roots, ii) Limited translocation of arsenic from root to shoot, iii) Arsenic phytotoxicity even at low concentrations in plant tissues, and iv) Low bioavailability of arsenic in soil. Inspite of all, scientists found arsenic hyper-accumulation in ferns, first discovered hyperaccumulating fern was *Pteris vittata* L. followed by *Pityrogramma calomelanos* L. and many others of *Pteris* genus such as *P. cretica* L., *P. longifolia* L., *P. umbrosa* L., *P. argyraea* L., *P. quadriaurita* L., *P. ryiunkensis* L. and, *P. biaurita*. Thus, the plants of *Pteris* genus perhaps have adopted As tolerance and accumulating mechanisms.

Hashmi et al. (2007) collected and analysed data from different departments, field testing and field visits to narrate the hygienic condition of effluent receiving water bodies and found that it is becoming worst, day by day. Water, most valuable resource, is essential to daily life. Growing communities, industries, agriculture, energy production and critical ecosystems depend upon water, if available in adequate quantity and suitable quality. Human activities can negatively impact surface water quality, even when the activity is far removed from the water body. Although fresh stream water, is a valuable resource but the increasing effluent quantity to rivers is alarming. On investigation they found that most of the industries are disposing wastewater without any treatment in natural streams and rivers, few with minor treatment and only very few with proper treatment, and a reasonable amount of water is being used by the agricultural sector in Pakistan. Their research revealed that mixing of effluent in large quantity without any treatment in natural water bodies is a serious threat to the environment and public health in Pakistan. Due to untreated effluent discharge in water bodies' change in temperature between different water layers along depth resulted stratification, decreased simulative capacity and a poor water quality. Thus, water and environmental pollution in the streams can only be controlled by proper wastewater treatment plans, environmental management and powerful environmental legislation.

Krishna and Govil (2008) analysed the top 10 cm of soil around Manali industrial area in Chennai, Southern India, to understand and check status of metal contamination of the area due to industrialization and urbanization. They and assessed geo-accumulation index, enrichment factor (EF), contamination factor and degree of contamination. The area was affected by the industrial activity and saturated by industries like petrochemicals, refineries, and fertilizers generating hazardous wastes. Soil samples were analyzed using Philips MagiX PRO-2440 Wavelength dispersive X-ray fluorescence spectrometry. The data revealed elevated concentrations of Chromium (149.8–418.0 mg kg^{-1}), Copper (22.4–372.0 mg kg^{-1}), Nickel (11.8–78.8 mg kg^{-1}), Zinc (63.5–213.6 mg kg^{-1}) and Molybdenum (2.3–15.3 mg kg^{-1}). The mean concentration of Arsenic was 0.91 mg kg^{-1}. The slight increase in As content in Manali was due to industrial activity. An increase in As content in the environment is caused mainly by the metallurgical industry and coal combustion, hence its content in the areas affected by industrial activity may be elevated. Contamination sites pose significant environmental hazards for terrestrial and aquatic ecosystems. They are important sources of pollution and may result in eco-toxicological effects on terrestrial, groundwater and aquatic ecosystems. In this perspective there is need for a safe dumping of waste disposal in order to minimize environmental pollution.

2.3 Chromium

Chromium is a chemical element (atomic number 24), which belongs to the category of transition metals. This hard, steel-gray colored bright metal is placed in group 6 of the periodic table and is represented by the chemical symbol Cr. It was discovered by Louis Nicholas Vauquelin, in the mineral crocoite, which consists of lead chromate. The name chromium is derived from the Greek word *chroma*, which means color, owing to the different colors produced by its compounds. Chromium is the 21st most abundant element found on the Earth's crust. However, it never occurs as a free metal and is extracted from the mines as chromite ore. Chromium occurs in many oxidative states like +1, +2, +3, +4, +5, +6, out of which +2, +3 and +6 are most common.

Chromium is mainly used in alloys such as stainless steel, in chrome plating and in metal ceramics. Chromium plating was once widely used to give steel a polished silvery mirror coating. Chromium is used in metallurgy to impart corrosion resistance and a shiny finish; as dyes and paints, its salts color glass an emerald green and it is used to produce synthetic rubies; as a catalyst in dyeing and in the tanning of leather; to make molds for the firing of bricks. Chromium (IV) oxide (CrO_2) is used to manufacture magnetic tape.

2.3.1 Chromium Exposure Sources

Much higher exposure to chromium occurs to people working in certain chromium industries (occupational exposure) and to people who smoke cigarettes (www.ideaconnection.com). The two largest sources of chromium emission in the atmosphere are from the chemical manufacturing industry and combustion of natural gas, oil, and coal. Other sources of chromium exposure are as follows: cement-producing plants, since cement contains chromium; the wearing down of asbestos brake linings from automobiles or similar sources of wind-carried asbestos, since asbestos contains chromium; incineration of municipal refuse and sewage sludge; exhaust emission from catalytic converters in automobiles; emissions from air conditioning cooling towers that use chromium compounds as rust inhibitors; wastewaters from electroplating, leather tanning, and textile industries when discharged into lakes and rivers; and solid wastes from the manufacture of chromium compounds, or ashes from municipal incineration, when disposed of improperly in landfill sites. Some consumer products that contain small amounts of chromium are: some inks, paints, and paper; some rubber and composition floor coverings; some leather materials; magnetic tapes; stainless steel and a few other metal alloys; and some toner powders used in copying machines. Occupational sources of chromium exposure mainly occur in industries that produce the following: stainless steel products (from welding); chromates (chemicals made from chromium and used in chemical industries); chrome-plated products; ferrochrome alloys; chrome pigments; and leather (from tanning). Examples of additional occupations that have potential for chromium exposure include: painters; workers involved in the maintenance and servicing of copying machines and in the disposal of some toner powders from copying machines; battery makers; candle makers; dye makers; printers; and rubber makers (www.ideaconnection.com).

2.3.2 Environmental Effects of Chromium

Trivalent and hexavalent Cr are the major stable chemical forms, with Cr(VI) causing the greatest concern because of its carcinogenic properties (Mei et al. 2002). Generally, most Cr(VI) added to soil is promptly reduced to the inert form Cr(III) by several agents among which sulfides, humic compounds and plant and microbial activity. However, Sethunathan et al. (2005) found that soil microorganisms also contribute to the reoxidation of Cr(III) to Cr(VI) and, therefore, both Cr oxidation states should be regarded hazardous for the environment and for humans.

Anthropogenic Cr sources contribute greatly to current Cr pollution, and the global industrial-age cumulative Cr production has been estimated as 105.4 million tonnes, with a significant increase since the 1950s (Han et al. 2004). Though chromium salts are widely used in dyeing, tanning and plating (Dube et al. 2003), Cr pollution has deserved relatively little attention compared to other heavy metals, because this metal is poorly absorbed and translocated by plants, so that both Cr phytotoxicity and accumulation in the food chain rarely occur in field conditions (Barceló et al. 1986; Khan 2001). Nevertheless, laboratory investigations have assessed that both Cr(III) and Cr(VI) are toxic to plant growth and cause disorders in mineral nutrition (Moral et al. 1995; Vajpayee et al. 1999; Dube et al. 2003).

Toxicity of chromium varies with different kinds of chromium and also differs in their effects upon organisms. Chromium enters the air, water and soil in the chromium (III) and chromium (VI) form through natural processes and human activities. The main human activities that increase the concentrations of chromium (III) are steal, leather and textile manufacturing. The main human activities that increase chromium (VI) concentrations are chemical, leather and textile manufacturing, electro painting and other chromium (VI) applications in the industry. These applications will mainly increase concentrations of chromium in water. Through coal combustion chromium will also end up in air and through waste disposal chromium will end up in soils. Most of the chromium in air will eventually settle and end up in waters or soils. Chromium in soils strongly attaches to soil particles and as a result it will not move towards groundwater. In water chromium will absorb on sediment and become immobile. Only a small part of the chromium that ends up in water will eventually dissolve. Chromium (III) is an essential element for organisms that can disrupt the sugar metabolism and cause heart conditions, when the daily dose is too low. Chromium (VI) is mainly toxic to organisms. It can alter genetic materials and cause cancer. Chromium is not known to accumulate in the bodies of fish, but high concentrations of chromium, due to the disposal of metal products in surface waters, can damage the gills of fish that swim near the point of disposal. In animals chromium can cause respiratory problems, a lower ability to fight disease, birth defects, infertility and tumor formation.

2.4 Cadmium

Cadmium (Cd) is a soft, silver-white metal being together with zinc and mercury in group II b in the periodic table. It's melting and boiling point are 320.9 and 765 °C, respectively. Cd is rapidly oxidized into cadmium oxide in the air. Cadmium is a heavy metal that is of great concern in the environment, because of its toxicity to animals and humans. Cadmium is usually rare in natural form and is concentrated in the argillaceous and shale deposits as green rocks (CdS) or otavite ($CdCO_3$) and is naturally associated with Zinc, Lead or Copper in Sulphide form (Cameron 1992). It is a bluish-white soft metal or a grayish powder. At pH 4.5 and 5.5 it is more mobile than zinc but it becomes immobile when pH range is above 7.5. Although it's divalent form is soluble but it is also capable of forming complexes with organics and oxides. Cadmium can be released into the atmosphere naturally through its natural source, volcanoes, thus spreading it over a large area. During the last two decades Cadmium contamination has become a concern because of its wide application in industrial sector mainly in steel plating, pigment stabilization and nickel-cadmium batteries (Fassett 1980). The average content of Cadmium in the soil is lees the 1 ppm whereas the normal range for plants ranges 0.005–0.02 ppm with a toxic level between 5 to 30 ppm. Common sources of Cadmium include alloys, polyvinyl chloride plastic (PVC) manufacture, solders, fungicides, enamels, motor oils, textile manufacturing, electroplating and rubber, sewage sludges and phosphate fertilizers (Bagshaw et al. 1986).

Estimated half-life of Cadmium in soil varies between 15 and 1,100 years with a relative elevated transfer coefficient from soil to plant, which boosts the risk of its increase in the environment and its access into the food chain (Vig et al. 2003). In the United Kingdom a progressive rise in cadmium concentration of soils has been verified over the past 130 years, with the highest increase in the last 20 years (Jensen and Bro-Rasmussen 1992).

2.4.1 Effects of Cadmium on Animals

The quantity of Cd accumulated in organisms varies according to the type of tissue under consideration, as well as diet, age and the conditions of environmental pollution. When dissolved, Cd is absorbed through epithelial membranes, mainly the gills, and conveyed via the blood to the liver, and, to a lesser extent, to other organs (kidney, pancreas, muscles, skin). The magnitude of the environmental toxic accumulation is expressed numerically using the Bioconcentration Factor (BCF), which can be determined under laboratory conditions, and is considered as a quantitative parameter to indicate the potential ecotoxicological risk of such substances (van der Oost et al. 2003).Cadmium (Cd) and Lead (Pb) are heavy metals that can be toxic when introduced into body by ingestion or inhalation in sufficient quantities. They cause various destructive effects (Neathery and Miller 1975). In the liver, Cd induces metallothionein synthesis which is a metal detoxifier or sequester, acting in tissues as a cellular protection against metal toxicity (Linde et al. 1999). It has been proved that one of the effects of heavy metals in aquatic animals is the DNA damage, which can be monitored using a diversity of methods; one of the most important that is used to assess the genotoxic effects of environmental pollutants under field and laboratory conditions is the Micronuclei (MN) Test (Schmid 1975). Except of endocrine and reproductive effects, cadmium exposure has been associated with nephrotoxicity, osteoporosis, neurotoxicity, carcinogenicity, genotoxicity and teratogenicity (EFSA 2009; Kimáková et al. 2006). Selvam and Wong (2008) found that Cd tends to accumulate higher concentrations in the roots than the shoot of *B. napus*. Kachenko and Singh (2006) reported that broad leaf vegetables are hyper accumulators of heavy metals.

2.4.2 Cadmium Pollution and It's Effects on Humans

Lead, cadmium, copper, manganese, etc. were preferred as representative trace metals whose levels in the environment symbolize a trustworthy indicator of environmental pollution. Copper, zinc and manganese are fond to be vital metals since they take an important part in the natural system, while lead and cadmium are non-essential metals as they are noxious yet in traces. The essential metals may be able to have lethal effects when the metal intake is markedly elevated (Krishna et al. 2009).

Cadmium contamination has become a severe environmental as well global issue due to the increasing environmental load from industrial, agricultural, energy and municipal sources. Furthermore, due to neurotoxic, mutagenic and carcinogenic effects, high water solubility and thereby easier entry into human body via food chain render Cd a dangerous environmental pollutant (Sanità and Gabrielli 1999). Cadmium, one of those heavy metals which are characterized to be major pollutants, is also a well-known heavy metal in the environment and it is considered as non-essential element having a long half-life which is exceedingly persistent in the environment (Salt et al. 1995, 1998), through its highly toxic nature and convenient take up by plants (Wu et al. 2003), thus making its entry easy in to the food chain which consequently give rise to serious health problems in both the animals and humans. For that reason, there is a growing awareness into effects of heavy metals taking place on higher plants and their responses to too much metal concentrations as stressors (Grant and Loake 2000).

The mutagenic aptitude of toxic heavy metals cause DNA damage, and probably causes carcinogenic effects in animals and humans (Knasmuller et al. 1998;

Baudouin et al. 2002). Long time contact to high levels of these metals has been associated with the harmful effects on human health and wildlife. Lead poisoning mainly affects the children and cause neurological damage principally to reduced intelligence, loss of short term memory, learning disabilities and coordination troubles. Arsenic contaminations usually cause cardiovascular problems, skin cancer and other dermatological problems, peripheral neuropathy (WHO 1997) and kidney disorders. Cadmium is mostly hoard in the kidneys and is concerned in a variety of kidney diseases (WHO 1997). The prime health perils coupled with mercury are damage to the neurological system, having indications like uncontrollable shaking, muscle wasting, partial blindness, and deformities in children exposed in the womb (WHO 1997).

2.4.3 Effect of Cadmium on Plants

Concentrations of Cd can accumulate in plants that are not toxic to them, yet are toxic to the animals eating the plants. Cadmium toxicity especially affects humans rather than animals, because of their longevity and the accumulation of Cd in their organs by eating Cd-contaminated food (Tudoreanu and Phillips 2004) Anthropogenic pathways by which Cd enters the environment are through industrial waste from processes such as electroplating, manufacturing of plastics, mining, paint pigments, alloy preparation, and batteries that contain cadmium (Adriano 2001; Cordero et al. 2004). Household appliances, automobiles and trucks, agricultural implements, airplane parts, industrial tools, hand tools, and fasteners of all kinds (e.g., nuts, bolts, screws, nails) are commonly Cd coated. Cadmium is also used for luminescent dials, in photography, rubber curing, and as fungicides (Adriano 2001). Tobacco concentrates Cd, leading to human exposure to this carcinogenic metal through smoking (Lugon-Moulin et al. 2004). Heavy metals enter soils through addition of sludge, composts, or fertilizers. Even with the strictest source control, domestic sewage sludge contains heavy metals because they are present in items washed down drains or toilets. For example, Cd is in cigarette butts flushed down toilets. Cadmium is given off from rubber when car tires run over streets, and after a rain, the Cd is washed into sewage systems where it collects in the sludge. Composted sludge can contain high levels of Cd. The composted sludge from Topeka, Kansas, which is applied to crop land, contains 4.2 mg kg^{-1} Cd (Liphadzi and Kirkham 2006). Phosphate fertilizers are contaminated with Cd. Zarcinas et al. (2004) attributed elevated levels of Cd in soil and excessive concentrations of Cd in cocoa (*Theobroma cacao*) in Peninsular Malaysia to input from phosphate fertilizers. People who smoke counterfeit cigarettes, which are packaged in the Far East or the Balkans and made to mimic legitimate brands, are exposed to increased concentrations of Cd. The most likely origin of the excess Cd is from heavy applications of cheap, contaminated phosphate fertilizers. Many researchers demonstrated various plant species capability of heavy metal, cadmium in particular, accumulation and concentration in their roots (Seregin and Ivanov 2001; Prasad

1995; Sanità and Gabrielli 1999). After 6-day-long incubation of young barley plants in the presence of low (30 μ mol L^{-1}), moderate (60 μmol L^{-1}), and high (100 μmol L^{-1}) cadmium doses, metal concentration in the roots was by 25, 35, and 90 times, respectively, higher than in the shoots. This allows referring barley to plants-excluders according to Baker classification (Baker 1981). Root capability of HM retaining in the roots reduces their transport into aboveground organs. We demonstrated that, at lower growth temperature and high metal concentration in medium, the barriers and sites of Cd^{2+} binding could not cope with situation, and cadmium was translocated to the shoots. Cadmium suppressed respiration in the roots stronger than in the leaves. HM are known to affect mitochondrial respiration directly or indirectly. The reductionin the O_2 uptake was shown to be a consequence of direct inactivation of the tricarboxylic acid cycle enzymes (Lösch and Köhl 1999). The cause for this enzyme inactivation is a disturbance in their structure due to the HM interaction with SH groups (Van Assche and Glijsters 1990; Prasad 1995; Sanità and Gabrielli 1999). There is evidence that metal transporters from different families such as ZIP and Nramp are able to transport several divalent cations, including Cd (Korshunova et al. 1999; Pence et al. 2000; Thomine et al. 2000). It has been described that a Ca transport pathway could be involved in Cd uptake (Clemens et al. 1998; Perfus-Barbeoch et al. 2002). Cadmium tolerance in plants is thought to involve internal metal detoxification processes, which may be achieved through both cellular and subcellular compartmentation (Vázquez et al. 1992; Küpper et al. 2000; Ma et al. 2005) and/or complexation with cellular ligands such as phytochelatins, organic acids, cisteine and other low molecular weight thiols (Cobbett and Goldsbrough 2002; Küpper et al. 2000). Although long distance Cd transport also contributes to Cd distribution and accumulation throughout the plant (Petit and van de Geijn 1978; Herren and Feller 1997; Cakmak et al. 2000; Chen et al. 2007), little is known about the chemical form(s) in which this heavy metal is present in xylem and phloem saps. Data available suggest that Cd may be associated in the xylem sap with small molecules such as organic acids (Cataldo et al. 1988; Senden and Wolterbeek 1990). Cadmium treatment led to major effects in shoots and roots of tomato. Plant growth was reduced in both treatments, leaves showed chlorosis symptoms when grown at 10 μM Cd and necrotic spots when grown at 100 μM Cd, and root browning was also observed in both treatments. An increase in the activity of PEPC, involved in anaplerotic fixation of CO_2 into organic acids, was measured in roots of Cd exposed plants. Also, significant increases in the activities of several enzymes from the Krebs cycle were measured in roots of tomato plants grown with Cd. Cadmium excess caused several alterations on photosynthetic rates, photosynthetic pigments and chlorophyll fluorescence as well as in nutrient homeostasis. Seed germination and seedling vigor index of *A. lebbeck* gradually decreased with the increase in concentration of lead and cadmium. Lead and cadmium treatments significantly decreased seed germination as compared to control. Seed germination and seedling growth inhibition by heavy metals has also been reported by many other workers (Morzeck and Funicelli 1982; Al-Helal 1995; Azmat et al. 2005; Shafiq and Iqbal 2005). The decrease in seed germination of *A. lebbeck* can be attributed to the accelerated breakdown of stored food material in seed by the application of lead and cadmium. (Kalimuthu and Siva 1990) found reduction in seed germination in maize treated with 20, 50, 100 and 200 μg/ml lead acetate and mercuric chloride. Excessive amounts of toxic elements usually caused reduction in plant growth (Prodgers and Inskeep 1981). Some elements such as Cu, Co, Fe, Mo, Mn, Ni and Zn are essential mineral nutrients.

2.5 Mercury

Mercury is a chemical element with the symbol Hg and atomic number 80. It is also known as quicksilver or hydrargyrum, from “hydr-” *water* and “argyros” *silver*. Mercury is the only metal that is liquid at standard conditions for temperature and pressure; the only other element that is liquid under these conditions is bromine (Green and Damji 2007). With a freezing point of −38.83°C and boiling point of 356.73°C, mercury has one of the narrowest ranges of its liquid state of any metal. A heavy, silvery d-block metal, mercury is also one of the five metallic chemical elements that are liquid at or near room temperature and pressure (Norrby 1991; Senese 2007), the others being caesium, francium, gallium, and rubidium.

Mercury is used in thermometers, barometers, manometers, sphygmomanometers, float valves, some electrical switches, and other scientific apparatus, though concerns about the element’s toxicity have led to mercury thermometers and sphygmomanometers being largely phased out in clinical environments in favor of alcohol-filled, digital, or thermistor-based instruments. It remains in use in scientific research applications and in amalgam material for dental restoration. It is used in lighting: electricity passed through mercury vapor in a phosphor tube produces short-wave ultraviolet light which then causes the phosphor to fluoresce, making visible light.

Hg in soil is a serious threat for life, evidence of which may be traced in 1950 and 1990s, when in Iraq and Minamata Bay, Japan, 40,000 and 3,000 people were poisoned with methylmercury (CH_3Hg^+) (Eto 2000). Anthropogenic share of Hg to environment is from paper mills, textile, chemical industries and gold mining (Raskin 1996). Rapid industrialization, in Asia, during 1990–1995, resulted in an increase in anthropogenic emissions of Hg from 30% to 56%, almost double, of the total global Hg emissions (Pacyna and Pacyna 2001). In Asia, agricultural fields are adjacent to industrial states and farmers mostly are growing vegetables due to water availability (discharge from industries), greater production and credit incentives.

2.5.1 Effect of Mercury on Animals

Mercury (Hg) is a widespread contaminant of considerable ecological concern due to its toxicity to fish and wildlife (*e.g.,* Scheuhammer and Blancher 1994; Scheuhammer et al. 2007; Wolfe et al. 1998) and its tendency to biomagnify in

Fig. 2.2 The crippled hand of a Minamata disease victim. Tomoko's hand taken by W. Eugene Smith in 1971 (Source: Mercury as a environmental pollutant as seen from the Swedish perspective Copyright belongs to the Aileen Archive)

ecosystems (*e.g.,* Hall et al. 1998). Exposure to certain environmental contaminants, especially neurotoxicants such as Hg, can promote the expression or suppression of behaviors and affect performance (Henry and Atchison 1991), the proficiency with which an organism executes a behavior. The neuron degeneration caused by Hg (Sakamoto et al. 1998) suggests that the metal could potentially disrupt the brain's ability to effectively control motor functions. The resulting behavioral effects may inhibit an organism's ability to capture prey, avoid predators, or successfully compete with others (Little et al. 1990; Walker et al. 2005). Mercury is a potent neurotoxin found in a variety of products. It affects the brain, liver and kidneys and can cause developmental disorders in children. Mercury and its organic compound methyl mercury is recognized as an environmental contaminant and known to be capable of damaging the central nervous system (USEPA 1997; Davidson et al. 2004) (Fig. 2.2).

Minamata disease, sometimes referred to as Chisso-Minamata disease, is a neurological syndrome caused by severe mercury poisoning. Symptoms include ataxia, numbness in the hands and feet, general muscle weakness, narrowing of the field of vision and damage to hearing and speech. In extreme cases, insanity, paralysis, coma and death follow within weeks of the onset of symptoms. A congenital form of the disease can also affect fetuses in the womb (Fig. 2.3).

Minamata disease was first discovered in Minamata city in Kumamoto prefecture, Japan in 1956. It was caused by the release of methylmercury in the industrial wastewater from the Chisso Corporation's chemical factory, which continued from 1932 to 1968. This highly toxic chemical bioaccumulated in shellfish and fish in

Fig. 2.3 Wastewater discharge from the Chisso factory in Minamata (W. E. Smith)

Minamata Bay and the Shiranui Sea, which when eaten by the local populace resulted in mercury poisoning. While cat, dog, pig, and human deaths continued over more than 30 years, the government and company did little to prevent the pollution (http://en.wikipedia.org/wiki/Minamata_disease).

As of March 2001, 2,265 victims had been officially recognized (1,784 of whom had died) and over 10,000 had received financial compensation from Chisso. By 2004, Chisso Corporation had paid $86 million in compensation, and in the same year was ordered to clean up its contamination. On March 29, 2010, a settlement was reached to compensate as-yet uncertified victims.

A second outbreak of Minamata disease occurred in Niigata Prefecture in 1965. Both the original Minamata disease and Niigata Minamata disease are considered two of the Four Big Pollution Diseases of Japan (http://en.wikipedia.org/wiki/Minamata_disease).

2.5.2 *Effect of Mercury on Plants*

Toxic effects of mercury in plants include abscission of older leaves, growth reduction, and decreased vigour inhibition of root and leaf development, decreased chlorophyll content and nitrate reductase activity (Vyas and Puranik 1993). Other adverse effects caused by excessive mercury include membrane structure integrity disruption

(Ma 1998), mineral nutrient uptake reduction (Cho and Park 2000; Patra and Sharma 2000) and photosynthesis and transpiration reduction (Krupa and Baszynski 1995). Higher concentrations ($>$1–2 mg L^{-1}) of mercury decreased the growth of pea (Beaford et al. 1977), tobacco, tomato (Cho and Park 2000) and alfalfa (Zhou et al. 2007). Inhibition of enzymes of different metabolic pathways has also been reported by mercury toxicity (Morch et al. 2002; Shaw and Rout 2002). Considerable amounts of mercury may be added to agricultural land with fertilizers, lime and manures.

2.6 Lead (Pb) Pollution

Lead is a toxic metal that was used for many years in products found in and around our homes. Lead also can be emitted into the air from industrial sources and leaded aviation gasoline, and lead can enter drinking water from plumbing materials. Lead may cause a range of health effects, from behavioral problems and learning disabilities, to seizures and death. Children 6 years old and under are most at risk (http://www.epa.gov/lead).

2.6.1 Sources of Lead in the Environment

In general, the older your home, the more likely it has lead-based paint. Many homes built before 1978 have lead-based paint. The US federal government banned lead-based paint from housing in 1978 (http://www.epa.gov/lead). Some states stopped its use even earlier. Lead can be found in (a) homes in the city, country, or suburbs; (b) apartments, single-family homes, and both private and public housing; and (c) inside and outside of the house.

The soil around a home can pick up lead from exterior paint, or other sources such as past use of leaded gas in cars, and children playing in yards can ingest or inhale lead dust. Household dust can pick up lead from deteriorating lead-based paint or from soil tracked into a home. Pb may also be found in the homes which might have plumbing with lead or lead solder. Food and liquids stored in lead crystal or lead-glazed pottery or porcelain. Food can become contaminated because lead can leach in from these containers. Lead smelters or other industries that release lead into the air.

- Peeling, chipping, chalking, or cracking lead-based paint is a hazard and needs immediate attention.
- Lead-based paint may also be a hazard when found on surfaces that children can chew or that get a lot of wear-and-tear. These areas include:
  - Windows and window sills.
  - Doors and door frames.
  - Stairs, railings, and banisters.
  - Porches and fences.

- Lead dust can form when lead-based paint is dry scraped, dry sanded, or heated. Dust also forms when painted surfaces bump or rub together. Lead chips and dust can get on surfaces and objects that people touch. Settled lead dust can re-enter the air when people vacuum, sweep or walk through it (http://www.epa.gov/lead)

2.6.2 *Effect of Lead on Plants*

Levels of lead in the environment are not stable and vary according to industrial production, urbanization, climate changes and many other factors (Singh et al. 1997). The levels of lead in the environment vary between 4 and 20 mg g^{-1} of dust. Uncontaminated waters contain lead in concentrations ranging from 0.001 to 0.06 mg L^{-1}. In soils, levels of lead reach 5–30 mg $kg^{-1}$of soil. When lead is added into petrol as an additive, the highest lead levels are determined on the surfaces of leaves, from where lead enters the food chain, as well as soil or water. Lead is present in soils as salts in soluble as well as insoluble forms. Lead contamination in the soil is known to inhibit seed germination (Pandey et al. 2007; Sawidis 2008). The inhibition of germination by exogenously supplied Pb^{2+} is a possible effect of interference with some important enzymes involved in the process. Photosynthesis is considered as one of the metabolic processes most sensitive to Pb^{2+} toxicity (Doumett et al. 2008). Closing of the stomata, disruption of the chloroplastic organization, change in the metabolites of photosynthesis and replacement of essential ions like magnesium are the main effects on photosynthesis of lead toxicity (Singh et al. 1997; Malkowski et al. 2002; Stejskal et al. 2007). Plants growing near highways are usually exposed to more Pb than other localities. Sewage sludge containing large quantities of Pb and other metals is regularly discharged on to field and garden soils due to increasing trends in urbanization (Paivoke 2002). Pb-affected soils contain Pb in the range of 400–800 mg Kg^{-1} soil whereas in industrialized areas the level may reach upto 1,000 mg Pb kg^{-1} soil (Angelone and Bini 1992). Half of the Pb-containing particulate matter falls to the ground within 100 ft of roadways and is then washed away and dispersed in the atmosphere and may be carried a considerable distance by air movements before it is eventually deposited. The accumulated Pb on the street and highways is transported to surface streams by rain water and consequently pollutes others surface waterways and soil (Laxen and Harrison 1977).

Mine water also transports a large amount of fine-grained sediments contaminated with Pb (Laxen and Harrison 1977). Pb uptake studies in plants have demonstrated that roots have an ability to take up significant quantities of Pb whilst simultaneously greatly restricting its translocation to above ground parts (Lane and Martin 1977). This notion was overturned by Miller and Koeppe (1971) who demonstrated that *Zea mays* L. plants could translocate and accumulate significant quantities of Pb in the leaves in a concentration dependent manner. The extent to which Pb enters plants via the leaves depends on the ability of leaves to absorb Pb from aerial sources, which in turn depends on the specific leaf morphology. Downy leaves absorb heavy metals from the atmosphere (Godzik 1993). The absorption of Pb (as Pb) in soil follows the Langmuir relation and increases with increasing pH between 3.0 to 8.5 (Lee et al. 1998).

Higher concentrations of Pb cause cell injury and disturb the barrier function of the plasmalemma as well as the selective permeability of the plasmalemma and tonoplast. Seregin and coworkers (2004) demonstrated that a significant amount of Pb was retained at the surface of plasmalemma rather than in the cell walls (Seregin et al. 2004).

2.6.3 *Effects of Lead on Animals and Humans*

The 2009 Berkeley report notes: "Decreased brain function in adults has been associated with blood lead concentrations of 20–50 μg/100 ml." In 2007/2008, official surveillance identified over 3,000 UK workers with blood lead levels at or above 20 μg/100 ml and over 250 of these had lead levels at or above 50 μg/100 ml. In animals exposed to lead in drinking water, lead exposure affects the renin–angiotensin system, inducing sympathetic hyperactivity and increasing sensitivity to stimulation of cardiac and vascular β receptors and dopaminergic receptors (Boscolo and Carmignani 1988; Victery 1988).

There is some evidence in humans that there is an association between low-level lead exposure and blood pressure, but the results are inconsistent. Lead appears to reduce the resistance and increase the mortality of experimental animals (Koller 1985). Exposure to high lead levels can produce renal tubular damage with glycosuria and aminoaciduria (saturnine gout). Some studies have shown a linear correlation between serum creatinine levels and blood lead levels above 40 μg/100 ml while others have shown no effect below 60 μg/100 ml (Gerhardsson et al. 1992; Loghman-Adham 1997). Liver, kidneys and brain have been considered as the target organs for the toxic effects of lead (Sharma and Street 1980). Chronic exposure to this biotoxicant leads to its accumulation in these organs with maximum concentration per gram weight of tissue being recorded in kidneys (Humphreys 1991).

Lead is a poisonous metal that can damage nervous connections (especially in young children) and cause blood and brain disorders. Lead poisoning typically results from ingestion of food or water contaminated with lead; but may also occur after accidental ingestion of contaminated soil, dust, or lead based paint (CABS 2006). Long-term exposure to lead or its salts (especially soluble salts or the strong oxidant PbO_2) can cause nephropathy, and colic-like abdominal pains. The effects of lead are the same whether it enters the body through breathing or swallowing. Lead can affect almost every organ and system in the body. The main target for lead toxicity is the nervous system, both in adults and children. Long-term exposure of adults can result in decreased performance in some tests that measure functions of the nervous system. It may also cause weakness in fingers, wrists, or ankles. Lead exposure also causes small increases in blood pressure, particularly in middle-aged and older people and can cause anemia. Exposure to high lead levels can severely damage the brain and kidneys in adults or children and ultimately cause death. In pregnant women, high levels of exposure to lead may cause miscarriage. Chronic, high-level exposure has shown to reduce fertility in males (Golub 2005). The antidote/treatment for lead poisoning consists of dimercaprol and succimer. Lead is widely used in the production of batteries, metal products (solder and pipes), ammunition and devices to shield X-rays leading to its exposure to the people working in these industries. Use of lead in gasoline, paints and ceramic products, caulking, and pipe solder has been dramatically reduced in recent years because of health concerns. Ingestion of contaminated food and drinking water is the most common source of lead exposure in humans. Exposure can also occur via inadvertent ingestion of contaminated soil/dust or lead-based paint.

The concern about lead's role in cognitive deficits in children has brought about widespread reduction in its use (lead exposure has been linked to learning disabilities) (Hu 1991). Most cases of adult elevated blood lead levels are workplace-related (NIOSH 2007). High blood levels are associated with delayed puberty in girls (Schoeters et al. 2008). Lead has been shown many times to permanently reduce the cognitive capacity of children at extremely low levels of exposure (Needleman et al. 1990).

During the twentieth century, the use of lead in paint pigments was sharply reduced because of the danger of lead poisoning, especially to children (www.derm.qld.gov.au, Lead Paint Information 2007). By the mid-1980s, a significant shift in lead end-use patterns had taken place. Much of this shift was a result of the U.S. lead consumers' compliance with environmental regulations that significantly reduced or eliminated the use of lead in non-battery products, including gasoline, paints, solders, and water systems. Lead use is being further curtailed by the European Union's RoHS directive. Lead may still be found in harmful quantities in stoneware, vinyl (such as that used for tubing and the insulation of electrical cords), and brass manufactured in China. Between 2006 and 2007 many children's toys made in China were recalled, primarily due to lead in paint used to color the product.

Older houses may still contain substantial amounts of lead paint. White lead paint has been withdrawn from sale in industrialized countries, but the yellow lead chromate is still in use; for example, Holland Colours Holcolan Yellow. Old paint should not be stripped by sanding, as this produces inhalable dust.

Lead salts used in pottery glazes have on occasion caused poisoning, when acidic drinks, such as fruit juices, have leached lead ions out of the glaze (CGP 2010). It has been suggested that what was known as "Devon colic" arose from the use of lead-lined presses to extract apple juice in the manufacture of cider. Lead is considered to be particularly harmful for women's ability to reproduce. Lead(II) acetate (also known as *sugar of lead*) was used by the Roman Empire as a sweetener for wine, and some consider this to be the cause of the dementia that affected many of the Roman Emperors (Angier 2007).

Lead as a soil contaminant is a widespread issue, since lead is present in natural deposits and may also enter soil through (leaded) gasoline leaks from underground storage tanks or through a waste stream of lead paint or lead grindings from certain industrial operations.

Lead can also be found listed as a criteria pollutant in the United States Clean Air Act section 108. Lead that is emitted into the atmosphere can be inhaled, or it can be ingested after it settles out of the air. It is rapidly absorbed into the bloodstream and is believed to have adverse effects on the central nervous system, the cardiovascular system, kidneys, and the immune system (Bergeson 2008).

In the human body, lead inhibits porphobilinogen synthase and ferrochelatase, preventing both porphobilinogen formation and the incorporation of iron into protoporphyrin IX, the final step in heme synthesis. This causes ineffective heme synthesis and subsequent microcytic anemia (Cohen et al. 1981). At lower levels, it acts as a calcium analog, interfering with ion channels during nerve conduction. This is one of the mechanisms by which it interferes with cognition. Acute lead poisoning is treated using disodium calcium edetate: the calcium chelate of the disodium salt of ethylene-diamine-tetracetic acid (EDTA). This chelating agent has a greater affinity for lead than for calcium and so the lead chelate is formed by exchange. This is then excreted in the urine leaving behind harmless calcium (Laurence 1966).

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Chapter 3
Cadmium Accumulation by Plants of *Brassicaceae* Family and Its Connection with Their Primary and Secondary Metabolism

Petr Babula, Vojtech Adam, Ladislav Havel, and Rene Kizek

Abstract The mustard family – *Brassicaceae* – is well known as family of plants, metallophytes, which are able to accumulate wide range of heavy metals and metalloids, especially zinc and cadmium, but also nickel, thallium, chromium and selenium. Ecological importance of this process consists partially in plants themselves to survive negative environmental conditions. There are two basic different strategies, how to survive these conditions – accumulation of heavy metals in plants tissues with different intensity in individual cell types, but also organs, which is partially given by chemical composition of cell walls, and ability to synthesize special defensive – detoxification compounds rich on thiol groups – glutathione and phytochelatins, which are able to bind heavy metals and transport them to the "secure" cell compartment – vacuole. The second principle is based on ability to exclude heavy metals. Role of secondary metabolites rich on sulphur in detoxification of heavy metals is still discussed with unclear conclusions. Members of *Brassicaceae* family, especially genera *Thlaspi* and *Brassica*, are well-known hyperaccumulators of heavy metals with possible utilization in phytoremediation technologies. In this review chapter, mechanisms of cadmium uptake and transport and its deposition in various plant cells and tissues are discussed with respect with

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possible utilization in phytoremediation. In addition, role of special sulphur metabolites, which are typical for plants of *Brassicaceae* family – glucosinolates – in detoxification of heavy metals is discussed.

Keywords *Brassicaceae* • Cadmium • Glucosinolates • Metallophytes

Abbreviations

ABC	ATP-binding cassette transporter
APS	Adenosine 5′-phosphosulphate
ATP	Adenosine triphosphate
BSO	Buthionine sulphoximine
EDTA	Ethylenediaminetetraacetic acid
GCS	γ-Glutamyl cystein synthetase
γ-GC	γ-Glutamyl cystein
GSH	Glutathione reduced
GSSG	Glutathione oxidized
MT	Metallothionein
NTA	Nitrilotriacetic acid
OASTL	*O*-acetylserine(thiol)lyase
PC	Phytochelatins
PCS	Phytochelatin synthase
ROS	Reactive oxygen species

3.1 Family *Brassicaceae* – Its Characterization and Specificity

The mustard family – *Brassicaceae* (syn. *Cruciferae*) – is together with next 15 families classified in order *Brassicales*. Other important families are *Capparaceae*, which is sometimes included in *Brassicaceae* as subfamily *Capparoideae*, *Tropaeolaceae* and *Caricaceae*, which is well known for edible fruits – papaya – with interesting pharmacological properties (Breithaupt et al. 2003; Nayak et al. 2007; Okeniyi et al. 2007; Amazu et al. 2009; Abdullah et al. 2011). *Brassicaceae* (mustard family) consists of usually hermaphroditic herbs, sometimes shrubs, with simple lobbed or divided, spiral, exstipulate leaves (Fig. 3.1). Flowers are arranged in inflorescence represented usually by a raceme. Flowers are bisexual, usually actinomorphic, perianth is dichlamydeous, cruciate (cross-shaped). Androecium consists of six stamens (two outer are shorter than four inner), Gynoecium is syncarpous, consisting of two carpels. The fruit is a silique or silicle (Ehrendorfer and Neuffer 2006). Classification of *Brassicaceae* family (338 genera with 3,709 species) is very complicated, still under the discussion, see Table 3.1 (Crespo et al. 2000; Marhold et al. 2004; Johnston et al. 2005; Bremer et al. 2009; Haston et al.

Fig. 3.1 The most important members – hyperaccumulators – of the *Brassicaceae*. (1) *Arabidopsis halleri*, (2) *Arabidopsis thaliana*, (3) *Brassica juncea*, (4) *Thlaspi caerulescens*, (5) *Thlaspi praecox*

Table 3.1 Taxonomic classification of *Brassicaceae*

Phenetic classification[a]	Cladistic classification[b]
Kingdom: *Plantae*	Clade: *angiosperms*
Phyllum: *Magnoliophyta*	Clade: *eudicots*
Class: *Magnoliopsida*	Clade: *core eudicots*
Subclass: *Dilleniidae*	Clade: *rosids*
Superorder: *Capparaneae*	Clade: *malvids*
Order: *Capparales*	Order: *Brassicales*

[a] In accordance with Armen Takhtajan classification system
[b] APGIII classification (Bremer et al. 2009)

2009; Huang et al. 2010; Qiu et al. 2010; Al-Shehbaz et al. 2006; Bailey et al. 2006; Koch and Mummenhoff 2006; German et al. 2009; Endress 2011; Ge et al. 2011).

Family has a worldwide distribution. There are many species of economical value, for example vegetable plants, plants used for obtaining of dyes, oil, etc. There are also many ornamental plants. The best known member of family is *Arabidopsis thaliana*, plant noted as a model for molecular biology. The *Brassicaceae* contains high number of species that are able of hyperaccumulation of heavy metals, especially **nickel** (genera *Thlaspi* and *Alyssum*), **cadmium** and **zinc** (*Thlaspi caerulescens, Thlaspi praecox, Thlaspi goesingense* and *Arabidopsis halleri*). About 25% of all known hyperaccumulators are members of this family (Rascio and Navari-Izzo 2011; Marques et al. 2004; Doran and Nedelkoska 1999).

3.2 Cadmium as an Important Contaminant of Living Environment

Cadmium is soft, bluish-white metal that is chemically similar to zinc and mercury. It occurs as a minor component in zinc ores, no cadmium-containing ores are known. There is only one exception – greenockite (CdS) is closely associated with zinc ore sphalerite (ZnbS) (Criscuolo and Oconnor 1950; Schwartz 2000). Cadmium has many industrial applications. It is used in batteries, in electroplating, as a barrier to control nuclear fission, for manufacturing of pigments, as stabilizers of plastics, etc. Cadmium is in laboratories used for preparation of cadmium-selenide quantum dots, which emit luminescence after excitation (Beri et al. 2011; Fortes et al. 2011; Harris et al. 2011; Kumar and Biradar 2011). Cadmium has no biological role in the living organisms. However, marine diatoms (*Thalassiosira weissflogii*) contain cadmium-dependent enzyme carbonic anhydrase CDCA1, enzyme that catalyses the reversible hydration of carbon dioxide (Cullen et al. 1999; Lane and Morel 2000; Park et al. 2007, 2008). Cadmium performs the same function as zinc in this enzyme. It seems that cadmium is involved in function of this enzyme instead zinc ions, which may be replaced by cadmium ions (Xu et al. 2007; Strmecki et al. 2010). Cadmium is one of the most important contaminants of the environment. The steel industry and waste incineration followed by volcanic action and zinc production are estimated to account the largest emissions of atmospheric cadmium (Bleil and Albers 1964; Boutron et al. 1993; Milacic et al. 1995; Karar et al. 2006; Ostrowska 2008). The main source of soil and water cadmium contamination are especially fertilizers (phosphate fertilizers), but also pesticides, fungicides, and sewage (Mirlean and Roisenberg 2006; Chen et al. 2007; Zarkovic and Blagojevic 2007; Zhao and Masaihiko 2007; Yildiz et al. 2008; Hadlich and Ucha 2010; McGrath and Tunney 2010). Contamination of soil and water by cadmium is connected with its uptake by plants – crops and vegetables – cultivated for human consumption (Jafarnejadi et al. 2011; Khodaverdiloo et al. 2011; Moustakas et al. 2011; Romkens et al. 2011). Smoking represents one of the most important sources of humans' exposure to cadmium (Satarug and Moore 2004; Massadeh et al. 2010; Takeuchi et al. 2010; Lin et al. 2011). Numerous studies are focused on the formation of organic cadmium compounds by organisms, which are more toxic in comparison with inorganic forms, especially dimethyl derivatives (Yannai and Berdicevsky 1995). Toxicity of cadmium is known for many years. Chronic prolonged oral cadmium indigestion is known in Japan as Itai-Itai disease. Human toxic exposure causes damage of kidneys based on kidney tubular damage. Cadmium can also damage bones via direct effect on bone tissue and indirect effect associated with kidney dysfunction (Nishijo et al. 1996; Kasuya 2000; Ogawa et al. 2004; Inaba et al. 2005, 2006; Kobayashi et al. 2009). Just itai-itai disease is connected with damage of bones and is characterized by multiple fractures of the long bones in the skeleton (Wang et al. 1994). Carcinogenicity of cadmium is widely discussed (Barrett 2009; Chen et al. 2009; Strumylaite et al. 2009, 2010; Absalon and Slesak 2010; Gallagher et al. 2010; Julin et al. 2010; Talaat et al. 2010). It has been proposed as a prostate and renal carcinogen, however, available data are still controversial (Kolonel 1976; Il'yasova and Schwartz 2005). Generation of reactive oxygen species, interactions with proteins, and induction of apoptosis by cadmium ions have been described in many *in vitro* studies. Toxicity of cadmium on plants includes inhibition of many enzymes involving in disruption of physiological processes including photosynthesis and growth alterations (Chaffei-Haouari et al. 2009; Hasan et al. 2009; Lopez-Millan et al. 2009; Rodriguez-Serrano et al. 2009; Cherif et al. 2011).

3.3 General Aspects of Cadmium Uptake, Translocation and Accumulation in the *Brassicaceae*

Family *Brassicaceae* contains some members, in which metabolism of heavy metals is intensely investigated. *Arabidopsis thaliana* represents the most important member of family and model plant in molecular biology. Cadmium is rapidly absorbed, translocated and accumulated in the aerial parts of many plants (Fig. 3.2). Tolerance of heavy metals is closely connected to regulation of sulphur uptake. Sulphate transporters are proteins involved in sulphate transport across a membrane. Two classes of sulphate transporters have been established – low -affinity and high-affinity. They differ not only in condition, under they work (sulphur-replete/sulphur/deficient conditions) and in selectivity. Some of them are responsible for uptake of not only sulphate, but also chemically similar, but toxic analogues, such as selenate. High-affinity sulphate transporters are the best known and characterized sulphate transporters. They have been identified especially in model plant *Arabidopsis thaliana* with tissue specificity (Hawkesford 2000, 2003; Buchner et al. 2004; Maruyama-Nakashita et al. 2004; Kumar et al. 2011). Recent works describe newly discovered sulphate transporters in different plants. For example, low-affinity sulphate transporter BnSultr2;2 together with high-affinity sulphate transporter BnSultr1;1 has been identified in *Brassica napus*. Increased tolerance to cadmium ions based on the efficient sulphate uptake and assimilation has been identified (Sun et al. 2007). Some genes involved in cadmium uptake have been identified. BjCdR15 expression was detected mainly in the epidermis and vascular system of cadmium-treated *Brassica juncea* plants (Farinati et al. 2010). Further transport of cadmium (and next metal ions) into aerial parts is significantly affected by: (i) interactions with cell walls, (ii) chelation by thiols, (iii) compartmentation into vacuoles (Nocito: Cadmium retention). Transport of cadmium ions is via cells (across cell membranes) is closely connected with transporters (Fig. 3.2). In addition, a plenty of transporters associated with transport of next heavy metals (zinc, lead, arsenic, etc.) has been described (Song et al. 2010). Cadmium is usually compartmented in vacuoles. However, interaction and deposition in cell walls have been described (Basic et al. 2006). These findings are in agreement with next published data (Ni and Wei 2003). Cadmium accumulators *Arabidopsis halleri, Arabidopsis thaliana, Thlaspi caerulescens*, *Thlaspi praecox* and *Brassica juncea* are intensely studied due to their abilities to accumulate cadmium ions. Individual questions are discussed in independent chapters.

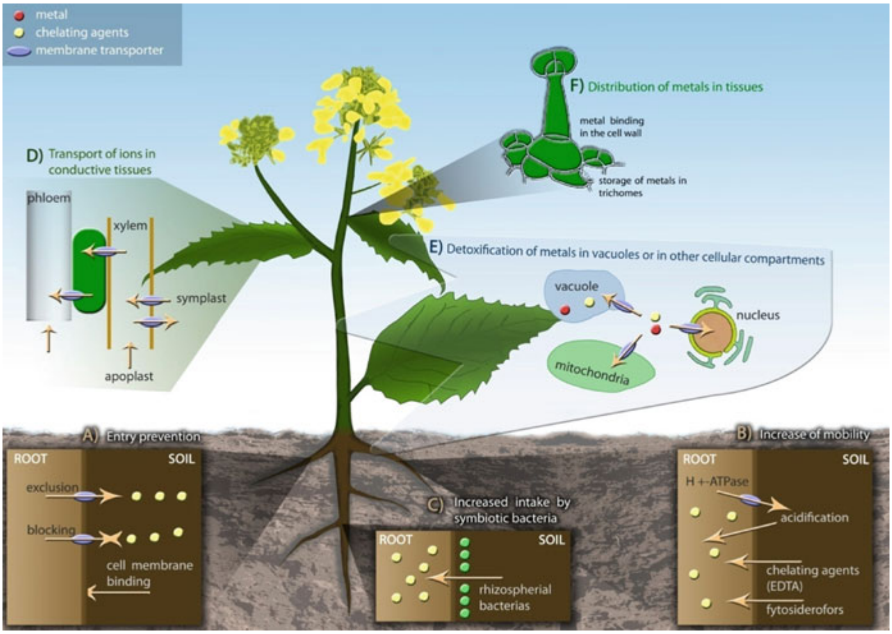

Fig. 3.2 Molecular mechanisms proposed to be involved in transition metal accumulation by plants. (**a**) Metal ions are mobilized by secretion of chelators and by acidification of the rhizosphere. (**b**) Uptake of hydrated metal ions or metal-chelate complexes is mediated by various uptake systems residing in the plasma membrane. Inside the cell, metals are chelated and excess metal is sequestered by transport into the vacuole. (**c**) From the roots, transition metals are transported to the shoot via the xylem. Presumably, the larger portion reaches the xylem via the root symplast. Apoplastic passage might occur at the root tip. Inside the xylem, metals are present as hydrated ions or as metal-chelate complexes. (**d**) After reaching the apoplast of the leaf, metals are differentially captured by different leaf cell types and move cell-to-cell through plasmodesmata. Storage appears to occur preferentially in trichomes. (**e**) Uptake into the leaf cells again is catalysed by various transporters [not depicted in (**f**)]. Intracellular distribution of essential transition metals (= trafficking) is mediated by specific metallochaperones and transporters localized in endomembranes (please note that these processes function in every cell). Abbreviations and symbols: *CW* cell wall, *M* metal, *filled circles* chelators, *filled ovals* transporters, *bean-shaped structures* metallochaperones (Modified according to Clemens et al. 2002)

3.4 Cadmium Uptake, Translocation and Deposition in *Arabidopsis thaliana*

Arabidopsis thaliana tolerates cadmium concentration of 1 µM in substrate (cultivation medium) without visible signs of the toxicity. However, higher concentrations (more than 5 µM) leads to the visible morphological changes including shortening the elongation zone of roots, premature xylogenesis, changes in rhizines formation and their premature elimination, and changes in formation of lateral roots. Changes in root system are closely connected with reduction of aerial parts growth as well as total biomass (Polec-Pawlak et al. 2005; Semane et al. 2007; Isaure et al. 2006; Van Belleghem et al. 2007). However, there are significant differences between individual studied members of the *Brassicaceae* as well as between individual ecotypes. Especially reduction of biomass production under Cd supplementation represents one of the most important restriction of their usage in phytoremediation technologies (Kupper et al. 2000; Bert et al. 2002; Craciun et al. 2006; Zhao et al. 2006).

Cadmium exposure induces expression of about 80–100 genes, which encode especially proteinkinases, transcriptional factors, calmodulin-associated proteins, chaperones, and protective compounds, such as thiols and antioxidant enzymes, such as catalases, ascorbate peroxidases, superoxide dismutases, which are closely connected with cadmium detoxification and elimination of reactive oxygen species (Suzuki et al. 2001; Semane et al. 2007).

Arabidopsis thaliana is cadmium excluder. Roots of experimental plants can contain up to 89% of cadmium in dependence on experimental conditions. Only a minority of cadmium is transported into aerial parts – shoots (Polec-Pawlak et al. 2005; Isaure et al. 2006; Van Belleghem et al. 2007). Similar results have been obtained in related species – *Arabidopsis halleri*. In this species, roots represent the main organs of cadmium accumulation in hyperaccumulating ecotypes (Craciun et al. 2006). This fact is connected with the binding of cadmium ions into cell walls, especially in the lower concentrations (up to 1 μM). The negatively charged carboxyl group of the cell walls provides sites for cation exchange leading to the accumulation of positively charged divalent or polyvalent cations. Experimental conditions play crucial point in these experiments. Hydroponically cultivated *Thlaspi coerulescens* demonstrate the highest cadmium content in roots, however, in soil cultivated plants has only 20% of cadmium in roots, rest of cadmium ions is transported into aerial parts (Perronnet et al. 2003; Roosens et al. 2003). Precipitation of cadmium ions especially with phosphorus under formation of insoluble compounds has been demonstrated. Cadmium ions at concentration about 5 μM are transported into cortex, where form granular deposits in cell walls and intercellular spaces in *Arabidopsis thaliana*. Granular deposits in the cytoplasm are well evident at high concentrations (about 50 μM and more). Deposition of cadmium in cell walls and intercellular spaces is connected with disruption of water transport within roots. Casparian strips present in endodermis contain significantly higher amount of cadmium compared to cell walls of other cells. In addition, granular deposits as a result of cadmium treatment are well evident also in vacuoles. Radial transport of cadmium ions is connected with formation of granular deposits in pericycle and xylem. Higher cadmium content was detected in passage cells, especially in the form of cytoplasmic granular deposits.

In *A. thaliana*, cadmium is transported from roots in the complexes with phytochelatins. On the other hand, transport of cadmium in *A. thaliana* is bidirectional (Gong et al. 2003; Isaure et al. 2006). High content of cadmium in phloem, respectively in cytoplasm of sieve elements and companion cells, is a result of translocation of cadmium from aerial parts – shoots (Van Belleghem et al. 2007). Xylem sap of *A. hallerii* contains cadmium ions in the free form, or in the complexes with citrate (Ueno et al. 2008). In the aerial plant parts, the highest content of cadmium has been found in trichomes in *A. thaliana*. This fact is probably connected with the protection of photosynthetically active tissues – leave mesophyll – against toxic effect of cadmium ions. In comparison with trichomes, epidermis and leaf mesophyll contain undetectable cadmium amount.

Only minimal cadmium levels have been detected in vascular bundles of leaves, especially in vessel elements (Isaure et al. 2006; Van Belleghem et al. 2007). Different situation has been observed in *A. halleri*, whose populations in France may be considered as hyperaccumulators. The highest cadmium concentration was detected in the bases of trichomes (especially in the oxygen compounds as oxides or in the form of citrate) (Kupper et al. 2000). Nevertheless, treatment of experimental plants by high cadmium concentrations leads to its transport into leaf mesophyll vacuoles. Due to fact, that epidermal cells are only weakly vacuolated, they contain only minimal Cd concentrations (Kupper et al. 2000). Different mechanism of cadmium accumulation is presented in *T. coerulescens*, species lacking trichomes, which are usual place of this process. The highest cadmium content was detected in the big epidermal cells (Cosio et al. 2005; Leitenmaier and Kupper 2011) followed by leaf mesophyll (Ma et al. 2005). Thirty-five percent of cadmium ions is bound in cell walls of epidermal/mesophyll cells. The youngest leaves demonstrate the highest accumulation capabilities (Perronnet et al. 2003). Colenchyma cells, which serve as mechanical tissue, contain the highest cadmium content in *T. praecox* leaves (Vogel-Mikus et al. 2008).

Whereas cadmium in roots of experimental *A. thaliana* plants predominates in insoluble form (54% versus 46% of soluble form) due to formation of precipitates with phosphates under formation of $Cd_3(PO_4)_2$ (rhizodermis, apoplast of cortical cells), about 58% of total cadmium in shoots is in water-soluble form (Polec-Pawlak et al. 2005). Phosphate precipitates represent the main Cd form also in *A. halleri*. On the other hand, there are significant differences between individual members of the *Brassicaceae* (Ma et al. 2005). There are also differences between tissues of various onthogenetical ages (Perronnet et al. 2003). However, there is one fundamental question – isn't formation of these precipitates a result of hydroponic cultivation of experimental plants (Zhao et al. 2006)? Granular deposits in symplast are formed by cadmium coordinated to sulphur, which suppose reaction between cadmium ions and sulphur-containing molecules, especially Cd-phytochelatins, Cd-phytochelatins-sulphide and/or Cd-sulphide (Isaure et al. 2006; Van Belleghem et al. 2007). High-molecular ligands (cell walls with carboxyl groups) are responsible for cadmium depositions in shoots (Polec-Pawlak et al. 2005). 75% of total cadmium is in trichomes bound with O and N, probably in pectins and cuticle of cell wall, only 25% of cadmium is bound with sulphur, probably with GSH. This fact corresponds to increased levels of GSH in trichomes (Isaure et al. 2006).

3.5 Biochemical and Molecular Aspects of Cadmium in *Arabidopsis thaliana*

Exposition of *A. thaliana* to cadmium ions leads to the increased expression of the genes for *O*-acetylserine(thiol)lyase (OASTL), γ-glutamyl cysteine synthetase (GCS) and glutathione synthetase (GS). All these findings support importance of sulphur compounds in cadmium detoxification (Lee et al. 2003). Expressions of GS and GCS are increased in the first step after cadmium exposition. Product of GCS-γ-glutamylcysteine (γ GC) – represents substrate for GSH biosynthesis. These connections have been verified using buthionine sulphoximine (BSO). Application of this inhibitor of GCS biosynthesis leads to the enhancement of sensitivity of *Arabidopsis thaliana* plants to cadmium ions (Wojcik and Tukiendorf 2011). Similar results were obtained using *cad2-1* mutant with altered genes for GCS, which produce only 30–45% of GCS in comparison with wild types (Cobbett et al. 1998). External application of GSH does not lead to the increased tolerance to cadmium, but it is toxic also for plants without Cd exposition. This fact indicates the role of GSH in biosynthesis of phytochelatins. Application of GSH may lead to the disruption of oxidation reduction balance in cells due to reduction of GSSG, which is formed from GSH in cultivation medium (Wojcik and Tukiendorf 2011). Role of phytochelatins (PCs) has been investigated using *Arabidopsis thaliana cad1-3* mutant with mutation in *AtPCS1* gene for phytochelatin synthase. Expression of *TaPCS1* from *Triticum aestivum* in roots of *cad1-3 A. thaliana* mutants led to the reduction of Cd accumulation in roots and enhancement of Cd transport from roots into shoots (Gong et al. 2003). Expression of *AtPCS1* is twofold increased after Cd application, but only in first 5 days (Lee and Korban 2002). Activity of AtPCS1 is after it regulated only on the enzymatic level. Presence of GSH-Cd is limiting factor for PC biosynthesis (Lee and Korban 2002; Lee et al. 2003). However, there are still unanswered questions in the regulation of PCs biosynthesis under Cd exposition (Semane et al. 2007). On the other hand, overexpression of *AtPCS1* does not increase production of PCs and leads to the increased sensitivity of experimental plants to Cd. This fact may be connected with disruption of metals homeostasis, toxicity of PCs excess or depletion of GSH (Lee et al. 2003). Enhanced expression of *AtMT2a* and *AtMT3* for metallothioneins like proteins (MTLs) after Cd exposition is connected with increased tolerance of plants to Cd. Expression of these genes in stomatal cells of *Vicia faba* increased their tolerance to Cd under reduction of ROS formation. MTLs are localised in cytoplasm, they are not translocated into vacuoles after Cd exposition in comparison with PCs. In addition, protective effect of MTLs is based on their antioxidant properties. This fact has been confirmed in experiments with hydrogen peroxide as a generator of ROS (Lee et al. 2004). Transporters of metals of the transport ATPase group – *AtHMA2*, *AtHMA3* and *AtHMA4* plays important role in complexation of heavy metals in cells. Heterological expression of *AtHMA3* in *ycf1* yeast mutants has confirmed role of this transporter in vacuolar sequestration of cadmium ions. Differences in the expression of *AtHMA2*, *AtHMA3* and *AtHMA4* are indicative to their different roles (Gravot et al. 2004). AtHMA2 and AtHMA4 are localised in plasmalemma, whereas AtHMA3 in tonoplast, so, it is well evident that AtHMA3 is involved in sequestration of toxic heavy metal ions into vacuoles. Highest *AtHMA2, AtHMA4* and *AtHMA3* expression was detected in the cells of root stele and in root vascular tissue as well as in stomatal cells and cells of hydatodes. All these cells are in the first contact with heavy metals ions – roots with soil/water and stomata and hydatodes with air polluted by heavy metals.

In addition, these tissues are essential for maintenance of physiological functions, so, detoxification of heavy metals in these tissues is fundamental. AtHMA2 and AtHMA4, which main functions consist in transport of zinc ions to the xylem, have significant affinity to cadmium ions and are able to execute their translocation from roots to shoots. These fact have been confirmed using mutants *hma4* (increased amount of Cd in roots, reduced transport to the shoots), and *hma2 hma4* (Cd accumulation is reduced to only 3% compared to wild type, 2–3-fold higher sensitivity to cadmium ions). HMA2 and HMA4 represent the main mechanism of Cd transport to the xylem. However, in comparison of *hma2 hma4* and *cad1* mutants, sensitivity of *cad1* mutant to cadmium ions is 10–20-fold higher (Wong and Cobbett 2009).

AtNRAMP1, AtNRAMP3 and AtNRAMP4 are the next transporters associated with cadmium transport. *AtNRAMP1*, *AtNRAMP3* and *AtNRAMP4* are expressed in both roots and shoots. Expression of *AtNRAMP3* and *AtNRAMP4* is induced by iron deficiency; it means that their primary function consists in Fe transport. *AtNRAMP3* knock-out does not lead to the reduction of iron absorption (as a result of IRT1 transporter redundancy), but to the increase of tolerance of mutants to cadmium ions. Its overexpression is connected with increased sensitivity of plants to the Cd. In conclusion, AtNRAMP3 is able to transport both Fe and Cd ions, which means that its function consists in iron absorption under presence of Cd, which inhibits other Fe transporters (Thomine et al. 2000). *A. thaliana* genome contains ten *AtPCR* (plant cadmium resistance) genes, which are connected with cadmium resistance. *AtPCR1* and *AtPCR2* expression is induced by Cd exposition. Their expression is not connected with GSH. This fact has been verified by BSO application (Song et al. 2004).

PDR transporters play important role in host-pathogen interactions, enhanced expression of some *PDR* has been recorded under different stress conditions. *AtPDR8* is up-regulated at Cd and Pb exposition. Knockout or silencing of *AtPDR8* increases sensitivity of experimental plants to Cd, on the other hand, its overexpression reduces amount of accumulated Cd in plants and reduce rate of Cd absorption by individual protoplasts. AtPDR8 serves as a pump for transport of Cd ions out of cells (Kim et al. 2007). Mutation of gene *CDR3* – *cdr3-1D* – leads to the enhancement of tolerance of mutants to Cd, Pb, Cu, H_2O_2, and to the reduction of Cd and Pb accumulation. *Cdr3-1D* mutant has increased expression of *AtPDR8*/*AtPDR12*, *AtPCR1* and *GSH1* and increased levels of GSH. *CDR3* is responsible for regulation of above-mentioned genes (Wang et al. 2011). All transporters found in *A. thaliana* are summarized in Table 3.2. In addition, their comparison with next *Brassicaceae* species is presented.

Nitric oxide (NO), important signal molecule, plays crucial role in Cd metabolism in plants. Exposition of plants to cadmium ions leads to the enhancement of NO production in both roots and leaves. On the other hand, supplementation of plants by NO scavengers cPITO and/or L-NAME causes reduction of Cd accumulation in roots under reduction of inhibition of root growth. Nevertheless, cadmium accumulation in aerial parts – shoots – is not altered. All these facts mean that NO is responsible for inhibition of root growth. In addition, NO modulates functions of

Table 3.2 Cadmium transporters connected with its accumulation in the *Brassicaceae*

Transporters	Metals	Plants	Reference(s)
HMA3	Cd, Co, Pb, Zn	*A. thaliana* (AtHMA3) *Thlaspi caerulescens* (TcHMA3)	Gravot et al. (2004), Morel et al. (2009), and Ueno et al. (2011)
HMA2	Cd, Zn	*A. thaliana* (AtHMA2)	Eren and Arguello (2004), Eren et al. (2006, 2007), and Wong and Cobbett (2009)
HMA4	Cd, Cu, Pb, Zn	*A. thaliana* (AtHMA4), *A. halleri* (AhHMA4), *T. caerulescens* (TcHMA4)	Courbot et al. (2007), Hanikenne et al. (2008), and Grispen et al. (2011)
NRAMP1	Cd, Fe, Mn	*A. thaliana*	Curie et al. (2000) and Cailliatte et al. (2010)
NRAMP3	Cd, Fe, Mn	*A. thaliana* (AtNRAMP3) *T. caerulescens* (TcNRAMP3)	Wei et al. (2009) and Oomen et al. (2009)
NRAMP4	Cd, Fe, Mn, Zn	*A. thaliana* (AtNRAMP4) *T. caerulescens* (TcNRAMP4)	Lanquar et al. (2004, 2005, 2010), and Oomen et al. (2009)
NRAMP	Cd, Zn	*B. juncea* (BjNRAMP)	Das et al. (2011)
YSL	Cd, Zn	*B. juncea* (BjYSL)	Das et al. (2011)
PDR8	Cd	*A. thaliana* (AtPDR8)	Kobae et al. (2006) and Kim et al. (2007)
MRP6	Cd	*A. thaliana* (AtMRP6)	Gaillard et al. (2008)
MRP7	Cd	*A. thaliana* (AtMRP7)	Wojas et al. (2009)
IRT1	Cd, Fe	*A. halleri, T. caerulescens*	Zhao et al. (2006), Plaza et al. (2007), and Ueno et al. (2008)
ZNT6	Cd	*T. caerulescens*	Wu et al. (2009)
ZIP9	Cd, Fe, Mn, Zn	*T. caerulescens*	De Mortel et al. (2008)

calcium channels and pumps, because application of NO scavengers does not lead to the reduction of calcium content in roots after cadmium exposition in comparison with NO-scavengers untreated plants (Besson-Bard et al. 2009).

3.6 Connection Between Primary and Secondary Metabolism and Cadmium Hyperaccumulation

Sulphur belongs to the group of the most important essential nutrients in organisms. In plants, sulphur is taken up especially in the form of inorganic sulphate. After reduction, sulphur is incorporated into cysteine in the processes of primary sulphate assimilation. Cysteine serves as a sulphur donor for biosynthesis of both methionine, the major sulphur containing amino acid in plants and precursor of glucosinolates biosynthesis (Fig. 3.3), and glutathione, the most important protective non-protein thiol compound (Mugford et al. 2011). Glucosinolates, mustard oil glycosides, represent one of the bioactive sulphur-containing secondary metabolites in plants, which occur almost only in members of order *Brassicales*

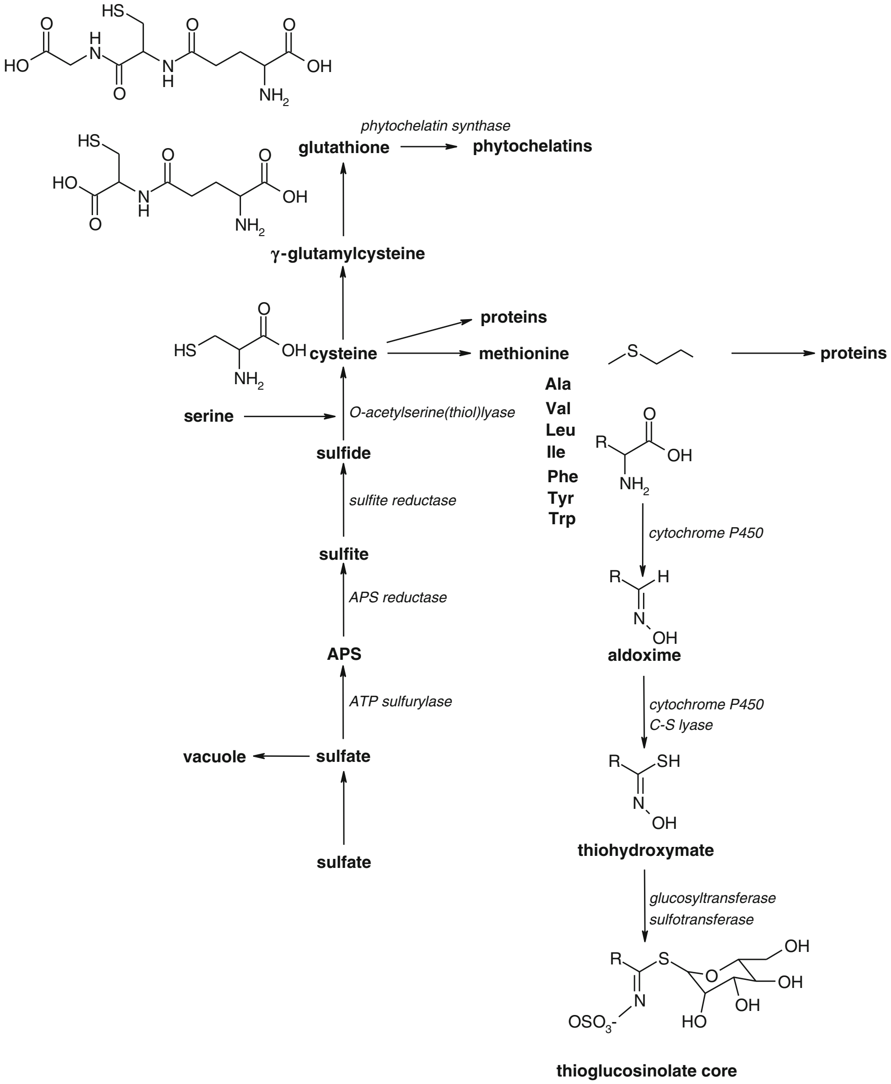

Fig. 3.3 Biosynthesis of thiols and glucosinolates. *APS* adenosine 5′-phosphosulfate, *ATP* adenosine triphosphate

(Fahey et al. 2001; Raybould and Moyes 2001; Redovnikovic et al. 2008; Hopkins et al. 2009; Vig et al. 2009). However, these compounds have been identified in some plants of *Euphorbiaceae* family (Rodman 1991a, b; Rodman et al. 1998; Hu et al. 2010). More than 120 glucosinolated have been identified in the *Brassicaceae*. Some of them and their degradation products, such as sulphoraphane, crambene and indole-3-carbinol, are in the centre of interest due to their chemoprotective effect, especially in connection with malignant diseases (Clarke et al. 2011;

Hamsa et al. 2011; Lubet et al. 2011; Malaguti et al. 2011; Rajendran et al. 2011; Razis et al. 2011; Sharma et al. 2011; Shimamoto et al. 2011; Starrett and Blake 2011; Wiczk and Herman-Antosiewicz 2011). Their structure is based on ά-D-glucopyranose residue, which is linked via a sulphur atom to a (Z)-*N*-hydroximinosulphate ester. Basic structure is modified by variable R group, which is derived from some amino acids. In addition, it seems that sulphur in the glucosinolate structure may be replaced by similar selenium under selenoglucosinolates formation. However, these compounds are still discussed (Bertelsen et al. 1988). Glucosinolates are derived from a limited group of amino acids, including alanine, leucine, methionine, phenylalanine and tryptophan. Their biosynthesis may be divided into three phases: (i) elongation of amino acid chain, in which methylene groups are inserted into the side chain, (ii) conversion of the amino acid moiety to the glucosinolate structure, and (iii) modification of the side chain – oxidation, hydroxylation, or esterification (Du et al. 1997; Ludwig-Muller et al. 1999; de Quiros et al. 2000; Graser et al. 2001; Falk et al. 2004; Field et al. 2004; Hirai et al. 2007; Sawada et al. 2007). However, their biosynthesis is still under the discussion Glucosinolates are stored in the vacuoles. After damage of plant tissue and consecutive exposure to myrosinases, ά-thioglucoside glucosidases, they are hydrolysed to isothiocyanates and other products of toxicological importance, such as nitriles and thiocyanates (Xue et al. 1995; Rask et al. 2000; Andersson et al. 2009). Formation of next product is connected with protein factors called specifier proteins, which are able to promote formation of alternative products on the dependence on the glucosinolate side chain structure (Wittstock and Burow 2007; Burow and Wittstock 2009; Kissen and Bones 2009). Glucosinolates as well as the products of their hydrolysis are connected with the protection against herbivores and pathogens. Glucosinolates as well as product of their hydrolysis induce GST activity in some insect, such as *Myzus persicae* (green peach aphid) feeding *Brassica napus* and next members of the *Brassicaceae* (Vanhaelen et al. 2001; Francis et al. 2005). Defensive role for indole glucosinolates is suggested by the observation that atr1D mutant *A. thaliana* plants, which overproduce indole glucosinolates, are more resistant to *M. persicae*, whereas cyp79B2/cyp79B3 *A. thaliana* double mutants that lack indole glucosinolates, succumb to *M. persicae* more rapidly. Indole glucosinolate breakdown products, including conjugates formed with ascorbate, glutathione and amino acids, are elevated in the honeydew of *M. persicae* feeding from atr1D mutant plants, but are absent when the aphids are feeding on cyp79B2 cyp79B3 double mutants (Hanley and Parsley 1990; Kim et al. 2008).

On the other hand, response of *A. thaliana* to herbivore includes activation of genes involved in oxidative stress, calcium-dependent signalling and pathogenesis-related responses (Moran et al. 2002). Glutathione S-transferases (GST) are mainly cytosolic enzymes that catalyse the conjugation of electrophile molecules with reduced glutathione under formation of less toxic products. Enhanced GST activity was demonstrated in *Episyrphus balteatus* (hoverfly), and *Brevicoryne brassicae* (cabbage aphid). Isothiocyanates as degradation product of glucosinolates were detected in cabbage aphid (Chaplin-Kramer et al. 2011; Khan et al. 2011).

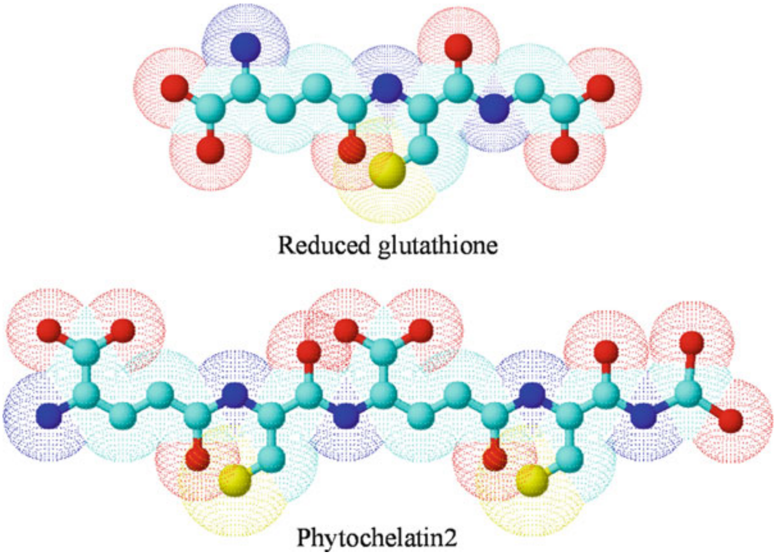

Fig. 3.4 3-D structures of reduced glutathione and phytochelatin2

Nevertheless, this chapter is not focused on biochemistry of glucosinolates and characterization of their breakdown product. For additional information, see published articles (Kim et al. 2008).

In plants, toxic substances become more water soluble (more hydrophilic) and they can subsequently be stored in vacuoles. Compartmentation in vacuoles plays important role in detoxification of many compounds. Tripeptide glutathione as well as phytochelatins as glutathione oligomers play crucial role in detoxification of heavy metal ions and xenobiotics in plants. Cysteine moiety/moieties with sulfhydryl (thiol) groups, which serves as a proton donor, plays essential role in their function.

Glutathione is synthesized from the amino acids L-cysteine, L-glutamic acid and L-glycine (Fig. 3.4). In the first step, γ-glutamylcysteine is synthesized from L-glutamine and L-cysteine via the enzyme γ-glutamylcysteine synthetase (glutamate cysteine ligase, GCL), in the second step, glycine is added to the C-terminal of γ-glutamylcysteine under glutathione formation. This reaction is catalysed by glutathione synthetase (GS). Phytochelatins are synthesized via phytochelatin synthases (PCS) activity (Fig. 3.4). There are many questions in the connection between glutathione and phytochelatins and glucosinolates. However, this relationship can be expected. Treatment of *A. thaliana* by cadmium ions led to the decrease of a content of total glucosinolates, especially indole glucosinolates (De Mortel et al. 2008; Sun et al. 2009). Similar results have been obtained in *Thlaspi praecox* and *T. arvense*. In the case of cadmium-sensitive *T. arvense*, its application led to the shift from alkenyl glucosinolates (mainly sinigrin) to indolyl glucosinolates. Treatment of the second species, *T. praecox*, by cadmium led to the increase of total glucosinolates, especially benzyl glucosinolate sinalbin (Tolra et al. 2006). Connection between glucosinolates biosynthesis, zinc accumulation and organ specificity was demonstrated on *T. coerulescens* in the study of Tolra et al. (2001). Whereas zinc treatment led to the decrease of glucosinolate sinalbin in shoots, its concentrations increased with zinc accumulation. Changes in sinalbin were the most significant, other glucosinolates remained almost unaltered. Only week decrease of indolyl glucosinolates in both shoots and roots and 3-butenylglucosinolate in the roots was observed (Tolra et al. 2001). There are two fundamental questions. Where is a source of sulphur in glucosinolate biosynthesis? Firstly, differences have been observed in the processes of sulphur assimilation. Adenosine 5′-phosphosulphate (APS) kinase limits the availability of sulphur for biosynthesis of sulphur-containing secondary metabolites. Reduction of its activity leads to the reduction of glucosinolates biosynthesis and increase of thiol compounds formation in *A. thaliana*. Overexpression of enzyme APS reductase has no effect on glucosinolates production, but increases production of thiol compounds (Mugford et al. 2011). However, the source of reduced sulphur atom in the core glucosinolate structure remains unknown. Recent evidences show the possible role of glutathione (GSH) in this process. Work of Geu-Flores shows role of GSH as a sulphur donor in glucosinolates biosynthesis. The *A. thaliana* pad2-1 mutant is connected with reduced levels of glucosinolates, respectively camalexin. PAD2 gene encodes a g-glutamylcysteine synthetase that is involved in glutathione biosynthesis, so, pad2-1 mutant contains only 20% of the GSH in comparison with wild plants. Reduced GSH levels correlate with reduction of accumulation of the two major glucosinolates in *A. thaliana* – indolyl-3-methyl-glucosinolate and 4-methylsulfinylbutyl-glucosinolate (Schlaeppi et al. 2008). All these data suggest participation of GSH in glucosinolate biosynthesis and close connection between metabolism of thiol compounds and glucosinolates. The second question consists in involvement of enzymes connected with thiol biosynthesis in the biosynthesis of glucosinolates. Experiments with *A. thaliana* mutants impaired in the production of the γ-glutamyl peptidases GGP1 and GGP3 have demonstrated altered biosynthesis of glucosinolates. GGPs catalyse transfer of γ-glutamyl functional groups of glutathione to acceptors. These peptidases have been found in many plant species and are connected with many physiological processes in plants, including ripening of seeds/fruits and degradation of some biomolecules (Lancaster and Shaw 1994; Shaw et al. 2005; Li et al. 2008; Ohkama-Ohtsu et al. 2008). Function of known cytosolic γ-glutamyl peptidases in the processes of formation of GSH conjugates has been demonstrated in *A. thaliana* plants (Geu-Flores et al. 2011). Role of GG1 was investigated in benzylglucosinolate-producing non-cruciferous plant *Nicotiana benthamiana*. In this study, increased levels of benzylglucosinolate in the presence of GGP1 were accompanied by the high accumulation of the last intermediate desulfobenzylglucosinolate. This fact suggest role of sufotransferase AtSOT16 in the formation of terminal product – benzylglucosinolate (Moldrup et al. 2011). Role of PCS in glucosinolate biosynthesis is widely discussed (Clay et al. 2009). It seems that

PCS serve as a peptidase in indole glucosinolate biosynthesis in *A. thaliana*. Glucosinolate-derived molecules seem to be the most likely candidates as PCS substrates in *A. thaliana* (Clemens and Persoh 2009). All these data suggest role of PCS not only in homeostasis of heavy metals, but also their regulatory role in glucosinolates biosynthesis.

3.7 Impact of Members of *Brassicaceae* Family on Cadmium Elimination from Living Environment – Possibilities of Increasing of Cadmium-Hyperaccumulation by *Brasicaceaeans*

There are different strategies in enhancement of accumulation properties of members of the *Brassicaceae*. The first approach consists in supplementation of soil by additives, compounds that are able to increase uptake of heavy metals including cadmium. There are many compounds, which were tested of possible chelators of heavy metals. However, their usage has many disadvantages including potential toxicity with reduction of biomass production and hazard for the environment due to mobilization of heavy metals. On the other hand, heavy-metals accumulating plants may represent potential risk because of their possible entry the food chain. All these questions must be carefully considered. Chelators in the phytoremediation by the *Brassicaceae* are summarized in Table 3.3.

In the *Brassicaceae*, effect of two different compounds on cadmium uptake – nitrilotriacetate (NTA) and citric acid (CA) – by Indian mustard (*Brassica juncea*)

Table 3.3 Chelators used in members of the *Brassicaceae* in phytoremediation

Chelator	Heavy metal(s)	Plant species	Reference(s)
EDTA, EDDS, histidine	Cu, Pg, Fe, Zn	*Brassica juncea*	Karczewska et al. (2009)
EDTA	Cd, Cr, Ni	*Thlaspi caerulascens*	Munn et al. (2008)
EDTA, DTPA Oxalic acid, citric acid	Cr, Ni	*Brassica juncea*	Hsiao et al. (2007)
EDTA	Cu, Cd, Pb, Zn	*Brassica napus* *Brassica juncea*	Turan and Bringu (2007)
Nitrilotriacetate Citric acid	Cd	*Brassica juncea*	Quartacci et al. (2005)
[S,S]-ethylenediamine disuccinate	Pb	*Thlaspi goesingense*	Finzgar et al. (2005, 2006)
Thiol-rich chelators	As, Hg	*Arabidopsis halleri*	Meagher and Heaton (2005)
EDTA, EDDS, DTPA	Cu	*Brassica rapa* v. *pekinensis*	Kos and Lestan (2004)
NH_4Cl, casein, citric acid	Cd, Cu, Ni, Zn	*Brassica chinensis*	Gramss et al. (2004)
EDTA	Pb	*Brassica juncea*	Blaylock et al. (1997)

were investigated in the study of Quartacci et al. (2005). NTA-treated plants demonstrated twofold increase of cadmium accumulation compared to control plants (Quartacci et al. 2005). EDTA, EDDS and histidine as potential chelators of Cu, Pb, Fe and Zn for *Brassica juncea* uptake were used in experiments of Karczewska et al. (2009). Synthetic aminopolycarboxylic acids ethylenediamine tetracarboxylic acid (EDTA) and diethylenetriamin pentaacetate (DTPA) together with low-molecular organic acids oxalic acid and citric acid were used in experiments with *Brassica juncea* cultivated in serpentine-mine tailings contaminated by Cr and Ni. Authors declare advantages of the use of low-molecular organic acids as chelators due to reduction of environmental risk in phytoremediation (Hsiao et al. 2007). However, further data are still missing.

The second approach is based on construction of transgenic plants. This method is based on an introducing of foreign genes, which are connected with uptake, transport and accumulation of heavy metal/metals. A yeast cadmium factor 1 (YCF1), a member of the ATP-binding cassette (ABC) transporters, is localised at the vacuolar membrane in *Saccharomyces cerevisiae*. YCF1 gene introduced to the transgenic *Brassica juncea* plants showed 1.3- to 1.6-fold tolerance to cadmium ions compared to wild plants (Bhuiyan et al. 2011). Overexpression of BjCdR15 in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* led to the enhancement of cadmium tolerance. *Arabidopsis* tga3-2 mutants demonstrated high cadmium accumulation in roots and inhibition of its transport into aerial parts (Farinati et al. 2010). Possibilities of different approaches including genetic manipulations for improvement of heavy metals tolerance and accumulation are summarized in reviews (Pilon-Smits and Pilon 2002; Singh et al. 2003; Czako et al. 2006; Lynch 2007; Yadav 2010; Anjum et al. 2012).

3.8 Conclusions and Perspectives

An ideal plant for phytoremediation should meet several criteria: (i) to grow rapidly and to produce large quantities of biomass, (ii) to deeply root and to have an easily harvested shoots, and (iii) to accumulate high concentrations of contaminants in the shoots. Presently known hyperaccumulators of metals, in other words plants capable of over-accumulation metals in their tissues, meet the third criterion of an ideal plant for phytoremediation as *Brassicaceae*. In contrast, there other plants as crops meet the first two criteria of an ideal plant for phytoremediation. The combination of these properties by some genetic manipulation could be very promising for the future of remediation of polluted environment by plants.

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Chapter 4
Metal Hyperaccumulation and Tolerance in *Alyssum*, *Arabidopsis* and *Thlaspi*: An Overview

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Abstract Toxic metals (TMs) and metalloids are natural components of environments, but elevated toxic levels and high persistence of TMs and metalloids in major compartments of the biosphere has posed various uncompromising and fatal effects on flora and fauna, and thus, has threatened the stability of the ecosystems as well. In addition, with the rapid increase in anthropological practices, a large number of TMs and metalloids ions are being added to the natural environment disrupting the ecosystem. A plethora of plant species have been identified so far to have potential for the remediation of TMs and metalloids-contaminated sites. Although, a large number of natural metal hyperaccumulator plant species from 34 different plant families including Asteraceace, Brassicaceae, Caryophyllaceae, Poaceae, Violaceae and Fabaceae has evolved the ability to take up, tolerate and accumulate exceptionally high concentrations of metals and metalloids present in the soil (and water) and, more importantly, in their aboveground biomass without visible toxicity symptoms but with 87 species classified as metal hyperaccumulators,

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the family Brassicaceae best represents amongst these metal-hyperaccumulator families. Of these 87 different metal-hyperaccumulator plant species in the family Brassicaceae, plant species in particular model metal hyperaccumutaor plant species *Alyssum*, *Thlaspi* and *Arabidopsis* have been studied extensively for their ability to hyperaccumulate, remove, destroy, degrade, sequester, transform, assimilate, metabolize or detoxify majority of TMs and metalloids in varied environmental compartments. Additionally, significant technological advancements in varied scientific fields have now deciphered important physiological and molecular mechanisms of TMs- and metalloids-remediation processes/intricacies in metal hyper accumulating plant species. Based on the plethora of recent published reports the current chapter critically discusses important strategies adopted by *Alyssum*, *Arabidopsis* and *Thlaspi* for TMs- and metalloids-hyperaccumulation/remediation and tolerance.

Keywords *Alyssum* • *Arabidopsis* • *Thlaspi* • Remediation • Tolerance • Toxic metals • Metalloids

4.1 Introduction

Toxic metals (TMs) and metalloids are important environmental pollutants, and many of them are toxic even at very low concentrations. However, the pollution of environment with toxic trace metals due to humans dates back to Iron age which has been accelerated tremendously as humans increased their numbers, particularly following the industrial revolution (Padmavathiamma and Li 2007; Memon and Schröder 2009; Saier and Trevors 2010). Among the major anthropogenic activities burning of fossil fuels, mining and smelting of metalliferous ores, electroplating, gas exhaust, energy and fuel production, fertilizer and pesticide application, use of sludge or municipal compost, pesticides, fertilizers, and emissions from municipal waste incinerators and car exhausts are adding a great momentum to the soil, water

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and air metal pollution which has become one of the most serious environmental problems today (Alkorta et al. 2004; Wei and Zhou 2008). Due to their immutable nature, metals are a group of pollutants of much concern. Although metals are present naturally in the Earth's crust at various levels and many metals are essential for cells [*e.g.*, copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), zinc (Zn)], all metals are toxic at higher concentrations (Garbisu and Alkorta 2003). Specifically, any metal (or metalloid) species may be considered a "contaminant" if it occurs where it is unwanted, or in a form or concentration that causes a detrimental human or environmental effect (McIntyre 2003; Yang et al. 2005). Arsenic (As), cadmium (Cd), chromium (Cr), Cu, lead (Pb), mercury (Hg), Ni, selenium (Se), silver (Ag), and Zn are among the major metals/metalloids; whereas, other less common metallic species that can be considered contaminants include aluminum (Al), cesium (Cs), cobalt (Co), Mn, molybdenum (Mo), strontium (Sr), and uranium (U) (Long et al. 2002). In context with the metal concentrations in soil, it has been reported that its concentrations in soil typically range from less than one to as high as 100,000 mg kg^{-1}. Over recent decades, the annual global release of heavy metals reached 22,000 t (metric ton) for Cd, 939,000 t for Cu, 783,000 t for Pb and 1,350,000 t for Zn (Singh et al. 2003). Moreover, excessive levels of many metals in soils can result in soil quality degradation, crop yield reduction, and poor quality of agricultural products (Long et al. 2002), pose significant hazards to human, animal, and ecosystem health (Blaylock and Huang 2000).

Majority of TMs can not be degraded into less toxic components but rather only be immobilized in less available forms or physically removed thus, majority of the TMs once released to the environment remain as persistent contaminants in ecosystems (Weis and Weis 2004) and may thus, lead to severe threats to global biotic communities. Because of this grim situation, there is a need to develop strategies to remediate ecosystems that have been injured by metal pollution. A number of concerted technologies have been suggested and/or tried such as soil excavation and either landfilling or soil washing followed by physical or chemical separation of the contaminants to decontaminate metal-polluted soil. But neither of the currently available metal-remediation techniques at hand was found up to the mark in terms of its successfulness, time consumption and cost effectiveness. Therefore, due to the usual enormous costs associated with the removal of metals from soils by means of traditional physicochemical methods explain why most companies tended to ignore the problem. In this regard, the use of natural inherent property of plants and their associated microorganisms to remediate contaminated matrices through the extraction, sequestration, transformation, degradation, stabilization and/or detoxification of organic and inorganic pollutants has received recent attention and is collectively termed as phytoremediation ("phyto" meaning plant, and the Latin suffix "remedium" meaning to clean or restore) (Meagher 2000; Mench et al. 2009). However, the idea that plants can be used for environmental remediation is very old and cannot be traced to any particular source (Padmavathiamma and Li 2007). Phytoremediation takes advantage of the fact that a living plant acts as a solar-driven pump, which can extract and concentrate certain heavy metals from the environment (Raskin et al. 1997). Additionally, this remediation method maintains the biological properties and physical structure of the soil. Phytoremediation has been reported to be environmentally friendly, potentially cheap, visually unobtrusive, and offers the possibility of bio-recovery of the heavy metals, an effective, non-intrusive, inexpensive, aesthetically pleasing, socially accepted technology to remediate polluted soils it is very advantageous over other metal-remediation methods (Pilson-Smits 2005). In addition, phytoremediation strategies can offer suitable approaches for decontaminating polluted soil, water, and air by trace metals as well as organic substances (Yang et al. 2005). Therefore, this unique technology of remediating varied environmental contaminants has now widely been viewed and socially accepted as the ecologically responsible alternative to the environmentally destructive physical remediation methods currently practiced (Meagher 2000).

Phytoremediation encompasses a number of important strategies such as (a) phytoextraction (direct accumulation of contaminants into plant shoots with subsequent removal of the plant shoots), (b) rhizofiltration (phytofiltration) (absorb and adsorb pollutants in plant roots), (c) phytostabilization (root exudates cause metals to precipitate and biomass becomes less bioavailable), (d) phytovolatilization (plants evaporate certain metal ions and volatile organics), (e) phytodegradation (plant-assisted bioremediation) (microbial degradation in the rhizosphere region), (f) phytotransformation (plant uptake of organic contaminants and degradation) and (g) removal of aerial contaminants (uptake of various volatile organics by leaves).

In context with one of the major themes of the current chapter *i.e.*, metal hyperaccumulation, the strategy of ‘phytoextraction’ is being dealt here in detail. ‘Phytoextraction’ (also called phytoaccumulation) refers to the use of plants to remove metals/metalloids from contaminated site by translocating them to their aboveground matter. This method is potentially very efficient. As contaminants (metals/metalloids) are concentrated in above-ground parts, it is easier to handle the total plant dry matter containing the contaminants compared with very hard handling of the tons of soil to be treated by conventional ways of remediation. Moreover, this method is able to maintain the fertility of soils, which otherwise is depleted by removal of top soil through engineering methods (Robinson et al. 2000) and most importantly, valuable metals/metalloids can be recovered from the plant material by incineration, along with the non-conventional advantage of biofuel production (Hassan and Arts 2011). Metal phytoextraction (or metal phytoaccumulation), a sub-class of metal ions resistance and indeed a striking phenomenon exhibited by $<0.2\%$ of angiosperms in fact is, one of the three different strategies developed by different types of plants growing on contaminated or metalliferous soils (Baker and Walker 1990; Baker and Whiting 2002). Metal accumulators are usually referred to as hyperaccumulators that concentrate metals in their aboveground tissues to levels far exceeding those present in the soil or in non-accumulating species growing nearby. It has further been proposed that a plant containing more than 0.1% of Ni, Co, Cu, Cr, and Pb or 1% of Zn on a dry weight basis is called a hyperaccumulator, irrespective of the metal concentration in the soil (Baker and Walker 1990). However, plants can be called hyperaccumulator of specific metals if they exhibit 1,000 μg Ni g^{-1} leaf dry mass, 10,000 μg Zn or Mn g^{-1} shoot dry matter and for Cd 100 μg Cd g^{-1} shoot dry matter, 1,000 μg Co/Cu/Pb or Se g^{-1} shoot dry matter (Brooks et al. 1977; Brooks 1998; Baker et al. 2000). Moreover, according to Chaney et al. (1997), Zhao et al. (2000), McGrath and Zhao (2003), and Alkorta et al. (2004) metal hyperaccumulator plants accumulating metals to concentrations over 100–1,000-folds higher than conventional plants growing on soils with background metal concentrations, and about 10–100-folds higher than most other plants growing on metal-contaminated soils in addition to exhibiting a shoot-to-root metal concentration ratio of >1 can be suitable for phytoremediation purposes.

Although, a large number of natural metal hyperaccumulator plant species from 34 different plant families (including Asteraceace, Brassicaceae, Caryophyllaceae, Poaceae, Violaceae and Fabaceae) has evolved the ability to take up, tolerate and accumulate exceptionally high concentrations of metals and metalloids present in the soil (and water) and, more importantly, in their aboveground biomass without visible toxicity symptoms but the family Brassicaceae best represents amongst these metal-hyperaccumulator families with 87 species classified as metal hyperaccumulators. Of these 87 different metal-hyperaccumulator plant species in the family Brassicaceae, plant species in particular model metal hyperaccumutaor plant species *Alyssum*, *Thlaspi* and *Arabidopsis* have been studied extensively for their ability to hyperaccumulate several metals including metals and metalloids. Based on the plethora of recent published reports the current chapter discusses the basic physiological and molecular mechanisms of metal hyperaccumulation and tolerance.

4.2 Metals/Metalloids Hyperaccumulation

According to Baker (1981), plants colonizing metalliferous soils respond either as accumulators (concentrating metals in plant parts from low or high background levels) or excluders (differential uptake and transport of metals between root and shoot leading to more or less constant low shoot levels over a wide range of external concentration). Moreover, a third type of plants called ‘Indicators’ exhibit proportional relationships between metal levels in the soil, uptake and accumulation in plant parts (Fig. 4.1). The plant kingdom possesses a striking feature of natural hypertolerance towards particular metals/metalloids and, in fewer cases, the property of hyperaccumulation of metals/metalloids in above-ground tissues to sometimes extraordinary quantities (Baker and Brooks 1989; Chaney et al. 1997; Reeves and Baker 2000; Clemens 2006; Krämer 2010). In addition, the metal hyperaccumulation trait of plants has recently gained considerable interest because of its potential use also in phytomining (Li et al. 2003) and food crop biofortification (Broadley et al. 2007; Palmgren et al. 2008). Therefore, studying heavy metal hyperaccumulation is becoming more and more interesting for ecological, evolutionary, nutritional, and environmental reasons. Brooks et al. (1977) first coined the term hyperaccumulation for plants that are endemic to metalliferous soils and are able to tolerate and accumulate metals in their above-ground tissues to very

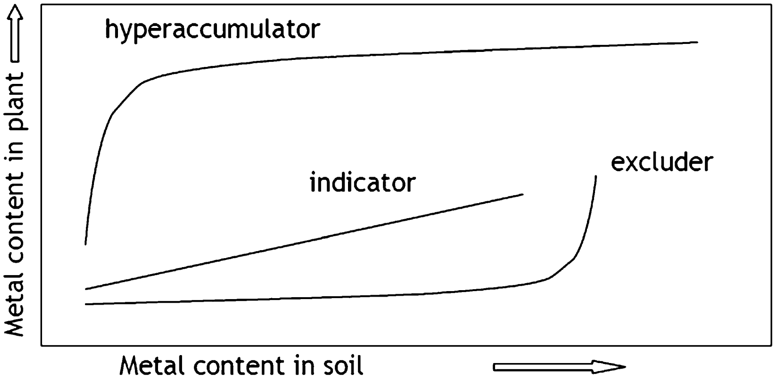
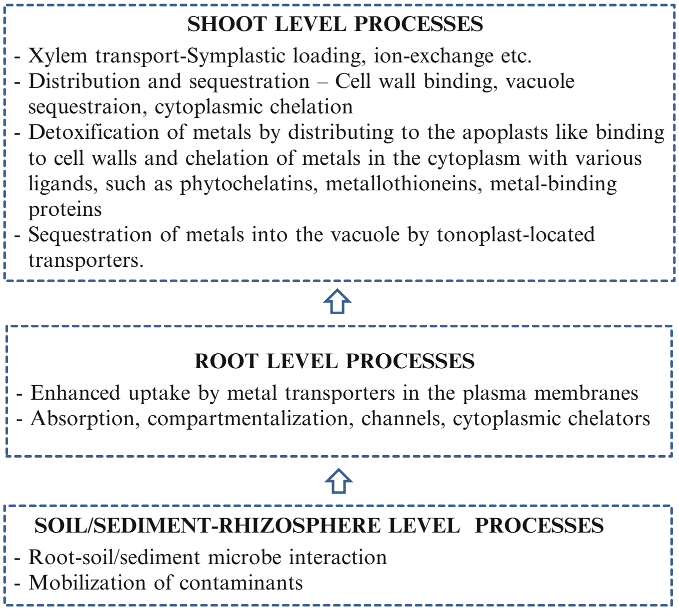


Fig. 4.1 Responses of plants to increased soil metal concentrations (Baker 1981)

high concentrations (≈100 times that of a non-accumulator plant species). It is important to mention here that there exists a poor understanding of the 'why and how' questions for plants' striking property of metals/metalloids hyperaccumulation. In this context, Hanson et al. (2003) reported that metals/metalloids hyperaccumulation may be related to plants strategy to deter herbivores by making leaves unpalatable or toxic, or by reducing pathogenic infection; whereas, Huitson and Macnair (2003) found no effects such as Zn in deterring insect herbivores. Although, hyperaccumulation and tolerance are clearly correlated, the causal connection between the two is obscure. It has been argued that hyperaccumulation may directly confer tolerance or, conversely, that tolerance permits hyperaccumulation to evolve. Nevertheless, plant metal hyperaccumulation has been shown to be associated with a strongly enhanced ability of plants to detoxify the metal accumulated in their aboveground tissues, and thus with metal hypertolerance (review by Krämer 2010). The accumulation of metals to a high level (usually called hyperaccumulation), in fact, is a sub-class of metals/metalloids resistance and/or the protection/defensive strategy of plants against these metals, thus to avoid these metals/metalloids-accrued different secondary consequences inside (Baker and Whiting 2002). However, the accumulation and/or hyperaccumulation trait(s) of plants largely benefit us by remediating the TMs and metalloids-contaminated sites. Although, a large number of plant species from 34 different plant families including Asteraceace, Brassicaceae, Caryophyllaceae, Poaceae, Violaceae and Fabaceae possess the ability to tolerate very high levels of TMs and metalloids present in the soil (and water) and, more importantly, in the plant shoot but the family Brassicaceae best represents amongst these metal-hyperaccumulator families with 87 species classified as metal hyperaccumulators. The majority of these hyperaccumulator plant species (approximately 320 species) are Ni hyperaccumulators (Baker et al. 2000). It has been reported that these species can accumulate concentrations of Ni in excess of 2% on a dry matter basis in their foliage and have been shown to occur on serpentine soils, derived from ultramafic rocks, typically containing 0.1–1% (w/w) Ni. Additionally, Zn hyperaccumulators represent the



SHOOT LEVEL PROCESSES

- Xylem transport-Symplastic loading, ion-exchange etc.
- Distribution and sequestration – Cell wall binding, vacuole sequestraion, cytoplasmic chelation
- Detoxification of metals by distributing to the apoplasts like binding to cell walls and chelation of metals in the cytoplasm with various ligands, such as phytochelatins, metallothioneins, metal-binding proteins
- Sequestration of metals into the vacuole by tonoplast-located transporters.

ROOT LEVEL PROCESSES

- Enhanced uptake by metal transporters in the plasma membranes
- Absorption, compartmentalization, channels, cytoplasmic chelators

SOIL/SEDIMENT-RHIZOSPHERE LEVEL PROCESSES

- Root-soil/sediment microbe interaction
- Mobilization of contaminants

Fig. 4.2 Summary of major processes (at soil/sediment-rhizosphere-, root- and shoot-levels) involved in metals/metalloids-hyperaccumulation in plants, *See text for details*

second largest group of hyperaccumulators where 15 Zn-hyperaccumulator plant species have been identified yet growing mainly on calamine soils enriched in Zn, Pb and Cd, either naturally, or due to human activities such as mining and metal smelting. Among the Zn-hyperaccumulators, only four species have been described as Cd hyperaccumulators namely *Thlaspi caerulescens*, *T. praecox*, *Arabidopsis halleri* and *Sedum alfredii* (Küpper et al. 2000; Lombi et al. 2000; review by Maestri et al. 2010). As also mentioned above that the most hyperaccumulators grow on soils enriched in heavy metals but some species including *T. caerulescens* and *A. halleri* have been classified as facultative metallophytes, with both metallicolous and non-metallicolous populations as well. Moreover, of these 87 different metal-hyperaccumulator plant species in the family Brassicaceae, plant species in particular, *Alyssum*, *Thlaspi* and *Arabidopsis*, have been studied extensively for their ability to hyperaccumulate several metals including TMs and metalloids. Figure 4.1 summarizes major processes (at soil/sediment-rhizosphere-, root- and shoot-levels) involved in metals/metalloids hyperaccumulation in plants (Fig. 4.2).

4.2.1 Rhizosphere Processes

Metals hyperaccumulators make use of conventional rhizosphere mechanisms to improve their trace element accumulation (*i.e.*, the same mechanisms as other plants) but also may have novel ways in which they manipulate their rhizospheres (Alford et al. 2010). Moreover, specific rhizosphere conditions potentially modify the bioavailability of varied metals/metalloids to hyperaccumulator plants when growing in different contaminated media. In fact, root proliferation and effective root uptake mechanisms are among the key processes in the rhizosphere that distinguish metal hyperaccumulators from normal plants. However, considerably less information is available on the processes in the rhizosphere of hyperaccumulators. Moreover, the role of rhizosphere processes in the phytoremediation of inorganic pollutants, in particular metals/metalloids, is much less investigated and only a few specific reviews are available on this topic (McGrath et al. 2001; Fitz and Wenzel 2002; Wenzel et al. 2004; Wenzel 2009). Most importantly, establishment of vital plants with sufficient shoot and root biomass growth, active root proliferation and/or root activities have been shown to support the growth of microbial consortium which in turn significantly affect the efficiency of phytoremediation in the rhizosphere. Therefore, rhizosphere interactions are of much importance for the establishment of functioning plant–microbial phytoremediation consortia in adverse, toxic environments (Wenzel 2009). In addition, plants can control metal/metalloid bioavailability in their rhizosphere via uptake mechanisms, properties of their root system, and root activities. Metal hyperaccumulator (*T. caerulescens*) and non-hyperaccumulator (*T. arvense*) have been reported to differently sense and actively forage the metal-rich patches in soil and accordingly to restrict and/or promote the root growth (Schwartz et al. 1999; Whiting et al. 2000). With numerous fine roots and dense root hairs, *T. caerulescens* has been shown to positively respond Zn in soil and allocation of about 70% of their total root biomass and root length, and about 70% of the current assimilate (^{14}C) into the metal-enriched soil. In contrast, *T. arvense* was reported to restrict its root growth in the metal enriched soil (Whiting et al. 2000). Root growth and proliferation in polluted soils has also been shown to increase in the presence of ACC (1-aminocyclopropane-1-carboxylate) deaminase-producing bacteria (Arshad et al. 2007). In Ni hyperaccumulator *T. goesingense*, indigenous ACC deaminase producing bacteria were found in the rhizosphere (Idris et al. 2004), indicating their potential role in metal resistance of this hyperaccumulator species. *T. caerulescens* roots have been reported to actively fetch metal/metalloids polluted soil patches (Schwartz et al. 2003). The pH of the rhizospheric zone has been shown to largely modulate the metal uptake in hyperaccumulator plants (Wieshammer et al. 2007). However, typically small changes in rhizosphere pH could not be related to hyperaccumulation (McGrath et al. 2001). Earlier, McGrath et al. (1997) reported that rhizosphere acidification may not be the responsible factor for efficient mobilization of Zn and Cd from soil in *Thlaspi caerulescens*, a Zn/Cd hyperaccumulator. A slight increase in rhizosphere pH of *T. caerulescens* was reported by Luo et al. (2000). The initial soil pH was shown considerably affecting metal uptake in *T. caerulescens* (Wieshammer et al. 2007); whereas, decreasing pH was argued as an effective strategy to enhance phytoextraction (Wang et al. 2006). Bernal and McGrath (1994) and Brown et al. (1994) reported that both *A. murale* and *T. caerulescens* can potentially thrive under neutral to slightly alkaline conditions where, decreasing pH from neutral to the acidic range was shown to depress the growth of the two species thus, indicating the rhizosphere acidification-independent hyperaccumulation of both Ni and Zn in these two species. Additionally, these authors argued that rhizosphere pH changes can be related to the balance of cation and anion uptake, but not to the specific metal being hyperaccumulated. Moreover, the changes in rhizosphere pH did not significantly differ between hyperaccumulators and non-hyperaccumulator species (McGrath et al. 1997). Hyperaccumulator species have been reported to release root exudates containing chelators with the potential to enhance heavy metal uptake, translocation and resistance. Although, root exudation has been reported to affect trace element solubility but information is meager on the involvement of root exudates in metal hyperaccumulation. Root exudates (and/or microbial activity) were proposed to be an integral part of Ni accumulation in *T. goesingense* where organic acids may participate in dissolution of Ni-bearing mineral surfaces (Puschenreiter et al. 2005). There are reports of production of comparatively less reductant (to increase metal solubility) by the Ni-hyperaccumulator Alyssum murale than a nonhyperaccumulator (Bernal et al. 1994). Most surprisingly, Salt and Kramer (2000) did not find any high affinity Ni-chelating compounds (citrate and histidine) in the root exudates of the Ni hyperaccumulator *T. goesingense*. In addition, root exudates of the Cd/Zn hyperaccumulator *T. caerulescens* (Zhao et al. 2001) also could not explain the excessive metal uptake and/or mobilization compared to a closely related non-accumulator. In this context, although some progress has been made towards an understanding of the rhizosphere processes associated with metal hyperaccumulation, still there is a considerable debate and uncertainty about the mechanisms by which hyperaccumulators activate and take up metal from the rhizosphere (Wenzel et al. 2003; Gonzaga et al. 2009). The association of increased levels of dissolved organic carbon with enhanced Ni concentrations in soil solutions from depleted rhizospheres of *Thlaspi goesingense* has been reported by Wenzel et al. (2003). Based of field data authors concluded that exudation of organic ligands may contribute to enhanced solubility and replenishment of metals in the rhizosphere of hyperaccumulating species. Salt and Kramer (2000) have shown in a hydroponics experiment that the release of citrate and histidine did not appear to be involved in Ni-hyperaccumulation in *T. goesingense*. Similarly Zhao et al. (2001) found root exudates of the hyperaccumulator *T. caerulescens* do not enhance metal mobilization.

Rhizosphere-microorganisms interactions have been shown to affect and control metals/metalloids bioavailability in plants (review by Wenzel 2009). In fact, microorganisms are ubiquitous in soils with high concentrations of metals to which hyperaccumulators are native (Ghaderian et al. 2000). A number of root parameters, such as root morphology and growth has been shown to be influenced by a variety of microbes. An increase in root exudation of organic solutes could affect the rate of phytosiderophore release. In turn, rhizosphere microoganisms may interact symbiotically with roots to enhance the potential for metal uptake (Burd et al. 2000). In this context in *Alyssum murale*, rhizobacteria were shown to play an important role in increasing the availability of Ni in soil, thus enhancing

Ni hyperaccumulation by the plant (Abou-Shanab et al. 2003). Authors studied the effect of three bacteria namely *Sphingomonas macrogoltabidus*, *Microbacterium liquefaciens*, and *M. arabinogalactanolyticum* isolated from the rhizosphere of *A. murale* on their ability to affect Ni solubilization, as measured by extraction, in soil and to affect Ni uptake into *A. murale*. It was revealed that *Sphingomonas macrogoltabidus* significantly reduced Ni extraction by 10 mMSr$(NO_3)_2$ from soil, *M. arabinogalactanolyticum* significantly increased Ni extraction, whereas *M. liquefaciens* exhibited no effect. Extractability of few other metals was observed affected by inoculation. When authors added these bacteria to surface-sterilized seeds of *A. murale* grown in non-sterile soil, Ni uptake into the shoot increased by 17% (*S. macrogoltabidus*), 24% (*M. liquefaciens*), and 32.4% (*M. arabinogalactanolyticum*) were noted when compared with uninoculated controls. In another Ni hyperaccumulator *Thlaspi goesingense*, Idris et al. (2004) characterized indigenous bacterial communities. Using cultivation and cultivation-independent techniques authors reported the production of siderospores by majority of bacterial strains. Ma et al. (2009) examined rhizosphere isolates from the Ni hyperaccumulators *Alyssum serpyllifolium*. Moreover, endophytic bacteria of *A. bertolonii* and *Thlaspi goesingense* were also reported to produce siderophores (Idris et al. 2004; Barzanti et al. 2007). The potential of microbial inoculation in the rhizosphere for the enhancement Zn hyperaccumulation was reported in *T. caerulescens* by Lodewyckx et al. (2002). Rhizosphere bacteria increased concentrations of Zn in the hyperaccumulator species *Thlaspi caerulescens* (Whiting et al. 2001). Similarly, Abou-Shanab et al. (2006) reported the benefits of the inoculation of several rhizobacteria strains obtained from the rhizosphere of *A. murale* grown on a serpentine site in the enhancement of Ni extractability from soil and increase (40%) in Ni uptake in *A. murale* (compared to non-inoculated controls). It is worthy also to mention here that the tolerance/resistance of bacteria in the hyperaccumulator rhizospheres largely differ from bacteria in the bulk soil (Aboudrar et al. 2007; Becerra-Castro et al. 2009). Delorme et al. (2001) reported higher Cd- and Zn-resistance in rhizosphere bacteria and fungi isolated from *T. caerulescens* than from a non-hyperaccumulator. Therefore, it would be interesting to investigate the mechanisms behind the exhibition of increased trace element resistance in rhizosphere bacteria.

4.2.2 Uptake and Transport of Metals-Metalloids

Despite the toxic nature of majority of trace metals and metalloids, a large number of these metal ions enter plant cells via specific transporters and uptake systems for essential cations. When within the plant (in excess of some times even in fewer concentrations) these metals inhibit vital enzymes activities, growth, development, metabolism and mineral nutrition. In context with hyperaccumulation of toxic trace metal ions by plants it is thought to be dependent on three physiological mechanisms namely (a) high rates of uptake from the soil, (b) efficient translocation

Table 4.1 Summary of tissue/cellular level metals/metalloids-distribution in *Alyssum*, *Arabidopsis* and *Thlaspi* sp.

Plant species	Metals	Tissue/organ	References
Alyssum lesbiacum	Ni	Trichome, epiermis	Kramer et al. (1997)
Arabidopsis halleri	Zn, Cd	Trichome, mesophyll	Küpper et al. (1999)
Thlaspi caerulescens	Zn	Epidermis, vacuole	Vazquez et al. (1994)
T. goesingense	Ni	Cell wall	Krämer et al. (2000)

from the roots to the shoots, and (c) safe deposition of heavy metals in appropriate compartments of the shoot (Lasat et al. 2000). Thus, as a whole, hyperaccumulation is necessarily coupled to mechanisms of tolerance. Therefore, Zhao et al. (2002) and Pollard et al. (2002) are of the opinion that hyperaccumulating plant species exhibit striking feature of rendering excessive foliar metal burdens harmless in addition to enhanced rates of root metal uptake and root to shoot metal translocation. Considerable progress has been made in understanding the mechanisms of metal hyperaccumulation at the physiological and molecular levels, although the full picture is far from complete. Hyperaccumulators are also tolerant to metals, but hyperaccumulation and tolerance are genetically independent traits (Macnair et al. 1999; McGrath and Zhao 2003). Metal hyperaccumulators are characterized by an enhanced translocation of the metal from the root to the leaves resulting in high metal concentrations in the xylem sap and shoot to root metal concentration ratios higher than unity (Lasat et al. 1996, 1998; Krämer et al. 1996; Shen et al. 1997; Schat et al. 2000). Although, the underlying mechanisms and the transporters involved are incompletely known but both apoplastic and symplastic pathways have been proposed for the efficient transport of inorganic ions in plants (White et al. 2002) (Table 4.1). Exhaustive physiological studies related to metals-hyperaccumulation in plants have recognized three main steps in the process involving symplastic transport namely (i) metal active transport across plasma membranes in roots, (ii) metal entry in the symplast during translocation from root to shoot, and (iii) metal chelation and sequestration in specific cell compartments within leaves (mainly relevant for metal detoxification and tolerance) (review by Maestri et al. 2010). Furthermore, it is also pertinent to mention here that metal hyperaccumulators ensure enhanced metal accumulation in their aerial parts and maintain metal homeostasis employing special mechanisms where the enhanced metal influx into the root cells is the first step in the metal accumulation process. According to recent review by Hassan and Aarts (2011) As metals cross the plasma membrane of the root endodermal cells to enter the plants through the symplast and/or by crossing the root apoplast through the intercellular spaces (review by Hassan and Aarts 2011). Moreover, the role of a number of factors such as the chemical environment in the plant rhizospheric zone (Zhao et al. 2001; Puschenreiter et al. 2005; Xie et al. 2009) and the presence of specific members of metal transporters (review by Maestri et al. 2010) has been recognized having prime importance in impacting to a great extent the metal bioavailability, mobility, uptake and its translocation in hyperaccumulator plant species. In *T. caerulescens*, the chemical form of N in the soil has been shown to affect plant uptake of Cd and Zn (Xie et al. 2009). The availability of metals in the rhizosphere of hyperaccumulators has usually been reported to be higher than in non-hyperaccumulating plants (Puschenreiter et al. 2005). In addition, the rhizosphere of hyperaccumulators has been shown to be very rich in metal-resistant bacteria (Whiting et al. 2001; Abou-Shanab et al. 2003) which significantly increases the uptake of metals and also enhance root elongation (Xiong et al. 2008). The proliferation of root has been observed enhanced in *T. caerulescens* grown in metal-enriched soil patches (Whiting et al. 2000), but at the same time not all populations were shown to behave in this way (Dechamps et al. 2008). Zhao et al. (2001) observed that root exudates from *T. caerulescens* however did not increase the mobility of Cd and Zn in the rhizosphere in a significant way as compared to non-accumulating plants. However, it is opined that there should be more exhaustive studies on the comparison of the root environment for hyperaccumulator and non-accumulator plant species to ascertain the different effects of these factors on metal bioavailability and root uptake.

4.2.3 Metal Transporters Genes – Role for Hyperaccumulation and Tolerance

Entry of both essential and non-essential metal ions in plant cells have been shown to be mediated by a number of metal transporters such as ZIP1-4, ZNT1, IRT1, COPT1, AtVramp1/3/4 and LCT1 (a nonspecific transporter for Ca^{2+}, Cd^{2+}, N^{a+} and K^{+}) on the plasma membrane-cytosol interface; ZAT, ABC type, AtMRP, HMT1, CAX2 seen in vacuoles; RAN1 seen in Golgi bodies; CPx-ATPases, CDF (cation diffusion facilitator); Nramps (natural resistance-associated macrophage proteins), ZIP (ZRT/IRT like protein) and the P_{1B}-type subfamily of P-type ATPases (HMA, heavy metal ATPase) were first identified in *A. thaliana* ; MATE (Multidrug And Toxin Efflux) family of small organic molecule transporters; the *Thlaspi caerulescens* heavy metal ATPase, TcHMA4, a novel family of cysteine (Cys) rich membrane proteins that mediate Cd resistance in *A. thaliana* and AtMRP3, an ABC transporter (Guerinot 2000; Hirschi et al. 2000; Williams et al. 2000; Clemens 2001; Hall 2002; Papoyan and Kochain 2004; Tong et al. 2004; Rascio and Navari-Izzo 2011) (Table 4.2). A number of above mentioned metals transporters has been shown to be involved in metal uptake, homeostasis and tolerance in plants. Manipulation of these transporters to achieve removal of metal ions from the cell holds great potential (Tong et al. 2004). The following sections deal mainly the significant role of above mentioned different transporters for major process of metals/metalloids hyperaccumulation in plants viz., metal uptake and root sequestration, root-to-shoot transport and metal storage in detail.

Regarding Zn-hyperaccumulation in *T. caerulescens*, it was revealed through molecular studies that this species exhibits about 4.5-fold higher maximum initial velocity of Zn influx in roots cells compared to the related non-hyperaccumulator

Table 4.2 Genes and ligands-mediated metals/metalloids-transport and detoxification in *Thlaspi caerulescens* (Modified after Clemens 2001; Shah and Nongkynrih 2007; Mench et al. 2009)

Gene and ligands	Mechanisms	References
ZIPs, IRTs	Zn, Cd, Mn uptake	Hassinen et al. (2007)
HMA4	Cd, Zn xylem loading	Bernard et al. (2004) and Courbot et al. (2007)
AtMRP4, AtMRP7, ATH12	Cd root-to-shoot	Plaza et al. (2007)
TcZNT1	Zn root-to-shoot	Kamińska et al. (2008)
NAS, TcNAS4	Ni, Zn chelation	Kamińska et al. (2008)
Metallothioneins	Cu, metal chelation	Hassinen et al. (2007)

Thlaspi arvense thus suggesting the importance of the increased root absorption of Zn as a major factor in the mechanism of Zn hyperaccumulation (Lasat et al. 1996; Lasat and Kochian 2000). Although, through Zn kinetic studies, the presence of similar Km values in roots of *T. caerulescens* and *T. arvense* (Lasat and Kochian 2000) was revealed but compared to *T. arvense* a much higher Vmax was observed in *T. caerulescens* thus suggesting the occurrence of the fact that transporters with very similar function in the roots of both species, while a higher expression of these transporters play role in driving Zn in larger amounts into the hyperaccumulator *T. caerulescens* compared to *T. arvense*. In this context, van de Mortel et al. (2006) reported the role of Zn transporters from the ZIP family. Members of the ZRT-IRT-like Protein (ZIP) family were the first metal transporters to be identified in plants (Eide et al. 1996). Fifteen ZIP genes have been identified in *A. thaliana* so far, based on whole genome sequencing and in general, the ZIP transporter proteins have the capacity to transport a variety of divalent cations including Zn^{2+}, Fe^{2+}, Mn^{2+} and Cd^{2+} (Guerinot 2000) and the expression of ZIP genes is regulated by plant metal status due to environmental metal levels (Grotz et al. 1998). There are reports of Zn deficiency-induced strong induction of these ZIP transporters in *A. thaliana*. Although, similar induction has been reported in *T. caerulescens* but overall expression levels are much higher than in *A. thaliana* thus reflecting the constitutive nature of Zn uptake in this hyperaccumulator. ZIP1, ZIP2 and ZIP5 are among the other ZIP genes that are higher expressed in *T. caerulescens* roots than in *A. thaliana* which are induced by Zn deficiency in *A. thaliana* (van de Mortel et al. 2006). According to recent review by Verbruggen et al. (2009) striking convergence can be observed between the genes identified in *T. caerulescens* and *A. thaliana* (Table 4.3). Recently, Wu et al. (2009) reported the cloning of two new ZIP genes from *T. caerulescens* accession La Calamine (LC), which were named TcZNT5-LC and TcZNT6-LC based on their high similarity to the *A. thaliana* genes AtZIP5 and AtZIP6 respectively. Authors compared the transcription levels of the two *T. caerulescens* genes with their presumed *A. thaliana* orthologues and by comparing the *T. caerulescens* accessions LC and GA differing in Cd hyperaccumulation potential. Authors reported that expression of TcZNT5-LC in *A. thaliana* did not modify Cd or Zn tolerance, but mildly affect the root and shoot Zn concentrations towards a hyperaccumulator shoot to root concentration ratio. Further, *A. thaliana* zip5 knock-out mutants were found exhibiting increased tolerance to Cd and

Table 4.3 Genes more expressed in *Arabidopsis halleri* (vs. non-accumulator relative *A. thaliana*) or *Thlaspi caerulescens* (vs. non-accumulator relative *T. arvense*) (Modified after Roosens et al. 2008 and Verbruggen et al. 2009)

	A. halleri root or shoot	References	*T. arvense* root or shoot	References
Genes from ZIP family of metal transporters; functionally related to metal uptake into cells				
ZIP4	Root and shoot	Becher et al. (2004) and Weber et al. (2004)	Root and shoot	Hammond et al. (2006) and van de Mortel et al. (2006)
ZIP6	Root and shoot	Becher et al. (2004) and Filatov et al. (2006)	Root and shoot	Hammond et al. (2006) and Talke et al. (2006)
ZIP7	–	–	Shoot	Hammond et al. (2006)
ZIP10	Root; lower in shoot	Talke et al. (2006)	Root; lower in shoot	Hammond et al. (2006) and van de Mortel et al. (2006)
IRT1	Root	Becher et al. (2004)	Root	van de Mortel et al. (2006)
IRT3	Root and shoot	Becher et al. (2004) and Talke et al. (2006)	Root and shoot	van de Mortel et al. (2006)
Genes from cation diffusion facilitator family; functionally related to metal vacuolar sequestration				
MTP1	Root and shoot	Becher et al. (2004) and Weber et al. (2004) and Talke et al. (2006)	Root and shoot	van de Mortel et al. (2006)
MTP8	Root and shoot	Talke et al. (2006)	Root and shoot	Hammond et al. (2006) and van de Mortel et al. (2006)
MPT11	Shoot	Talke et al. (2006)	–	–
Genes from NRMP, natural resistance associated macrophage; functionally related to metal remobilization from the vacuole				
NRAMP1	–	–	Shoot	Hammond et al. (2006) and van de Mortel et al. (2006)
NRAMP3	Root and shoot (expressed only in *A. haleri*)	Weber et al. (2004) and Filatov et al. (2006)	–	–
NRAMP5	–	–	Shoot	Hammond et al. (2006) and van de Mortel et al. (2006)

Gene from P-type metal ATPase; functionally related to xylem loading/unloading of metal/ligands/metal–ligand complexes				
HMA4	Shoot	Becher et al. (2004), Weber et al. (2004), Filatov et al. (2006), and Talke et al. (2006)	Shoot	Hammond et al. (2006) and van de Mortel et al. (2006)
Gene from multidrug and toxin efflux family transporter; functionally related to xylem loading/unloading of metal/ligands/metal–ligand complexes				
FRD3	Root and shoot (expressed only in *A. haleri*)	Talke et al. (2006)	Root and shoot	Hammond et al. (2006) and van de Mortel et al. (2006, 2008)
Genes from yellow-stripe-like transporter; functionally related to xylem loading/unloading of metal/ligands/metal–ligand complexes				
YSL3	Root and shoot	Gendre et al. (2007)	Root and shoot	Gendre et al. (2007)
YSL6	Shoot	Talke et al. (2006)	–	–
YSL7	Shoot	Gendre et al. (2007)	Shoot	Gendre et al. (2007)
Genes from nicotinamine synthase; functionally related to synthesis of metal ligands				
NAS2	Root	Weber et al. (2004) and Talke et al. (2006)	Root	van de Mortel et al. (2006)
NAS3	Shoot (expressed only in *A. haleri*)	Becher et al. (2004) and Talke et al. (2006)	Root (expressed only in *T. caerulescens*)	Hammond et al. (2006) and van de Mortel et al. (2006)
NAS4	Root and shoot	Weber et al. (2004)	Root and shoot (expressed only in *T. caerulescens*)	Hammond et al. (2006) and van de Mortel et al. (2006, 2008)
Genes from S-adenosyl-methionine synthetase; functionally related to synthesis of metal ligands				
SAMS1	Shoot	Talke et al. (2006)	–	–
SAMS2	Shoot	Talke et al. (2006)	–	–
SAMS3	Root and shoot	Talke et al. (2006)	–	–

decreased seed mineral concentrations. Although, enhanced the Cd sensitivity of *A. thaliana* was observed expressing TcZNT6-LC, but no phenotype was observed for the zip6 mutant. Authors concluded that the changes in expression of TcZNT5-LC and TcZNT6-LC upon changes in Zn or Cd exposure indicate both genes act in metal homeostasis, but their CaMV 35Smediated expression in *A. thaliana* does not create *T. caerulescens* hyperaccumulator phenotypes. There are reports of TcZNT5 clones (ZIP5-orthologue) from *T. caerulescens* (Plaza et al. 2007; Küpper and Kochian 2009; Wu et al. 2009). Even at different Zn exposures, significantly higher expression of ZIP4, ZIP10 and especially IRT3 were observed in *T. caerulescens* roots than in *A. thaliana* roots. Moreover, Assunção et al. (2001) cloned the *T. caerulescens* orthologues of the AtZIP4 and AtIRT3 genes (namely TcZNT1 and TcZNT2). However, no expression of AtZIP4 was observed in the root epidermis, but in the endodermis and pericycle (Milner and Kochian 2008). In a recent study, Shanmugam et al. (2011) evaluated the role of differential expression and regulation of Fe-regulated metal transporters for Zn tolerance in *A. halleri* and *A. thaliana*. Authors uncovered the difference of a cross-homeostasis system between Fe and Zn in dealing with Zn excess in the Zn hyperaccumulator *A. halleri* ssp. gemmifera and non-hyperaccumulator *A. thaliana*. *Arabidopsis halleri* showed low expression of the Fe acquisition and deficiency response-related genes IRT1 and IRT2 compared with *A. thaliana*. In *A. thaliana*, lowering the expression of IRT1 and IRT2 through the addition of excess Fe to the medium increased Zn tolerance. It was revealed through this study that excess Zn induces significant Fe deficiency in *A. thaliana* and reduces Fe accumulation in shoots. By contrast, the accumulation of Fe in shoots of *A. halleri* remains stable under various Zn treatments. Additionally, root ferric chelate reductase activity and expression of FIT were found low in *A. halleri* compared with *A. thaliana*. Overexpression of a ZIP family member IRT3 in irt1-1 was revealed to rescue the Fe-deficient phenotype. Authors concluded that a fine-tuned Fe homeostasis mechanism in *A. halleri* maintains optimum Fe level by Zn-regulated ZIP transporters and prevents high Zn uptake through Fe-regulated metal transporters, and in part be responsible for Zn tolerance.

TcZNT1, TcZNT2, TcZNT5, IRT1 (At4g19690), AtNramp4 (At5g67330), AtNramp5 (At4g18790) and AtNramp2 (At1g47240) are among the most important and studied metal transporters in hyperaccumulator plants. TcZNT1, a member of the ZIP family of transport proteins, has been identified and extensively studied in *T. caerulescens* (Pence et al. 2000) has been shown to exhibit homology to ZRT1 (yeast high-affinity Zn uptake transporter) and IRT1/ZIP4 (*A. thaliana* Fe transporter, At1g10970). According to Pence et al. (2000) TcZNT1 is a putative plasma membrane localized transporter which has been observed to act both as a high- and as a low-affinity Zn transporter in complementation assays using a yeast zrt1 zrt2 mutant (zhy3). The level of Zn in the growing medium has been shown to modulate the expression of TcZNT1 in Zn-hyperaccumulator plants including *T. caerulescens*, *T. arvense* and *A. thaliana*. Although, the gene has been shown to express at very low levels in roots and shoots grown on sufficient and high Zn both in *T. arvense* and *A. thaliana* as well but Grotz et al. (1998) has earlier shown the increased expression under condition of Zn deficiency presumably thus to facilitate Zn uptake through the synthesis of more transporters. Moreover, the expression of TcZNT1 in *T. caerulescens* has been shown to be very high in both Zn-deficient and Zn-sufficient plants, while at very high Zn levels there was evidence of a down regulation of its expression (Pence et al. 2000; Assunção et al. 2001). Additionally, authors reported higher TcZNT1 expression than that of its homologues in non-accumulator plants even at very high Zn levels. There are recent reports of expression of TcZNT1/AtZIP4- homologues in roots of *A. halleri* but not in *A. thaliana* where also authors noticed a decreased level of expression under excess Zn (Chiang et al. 2006; Talke et al. 2006; Weber et al. 2006). As noted also above the plant-Zn-status-induced modulation of the expression of TcZNT1 in Zn-hyperaccumulator plants ZNT1/ZIP4 has also been observed to follow the same trend in hyperaccumulator plants but in different ways as it happens in non-accumulator plants. ZIP4, the closest homologue to TcZNT1, shares 90% DNA sequence identity and 81% homology at the amino acid level in *A. thaliana* and has been shown be involved in the transport of different metals (Wintz et al. 2003). There are evidences that clearly suggest the involvement of *A. thaliana* ZIP4 (AtZIP4) in Cu uptake rather than in Zn/Cd uptake in addition to the cell specific expression of these transporters in different plant species. The deficiency of both Cu and Zn has been shown to induce the expression of AtZIP4 in *A. thaliana* (Wintz et al. 2003). According to Pence et al. (2000) AtZIP4 does not complement with the zrt1 zrt2 Zn uptake deficient yeast mutant as does TcZNT1. Instead, Wintz et al. (2003) reported that AtZIP4 complements the ctr1 yeast mutant (defective in Cu uptake) thus restoring the growth of this yeast mutant under limited Cu conditions. Moreover, there are reports of the localized expression of AtZIP4 (in the stele of *A. thaliana*) (Milner and Kochian 2008) and TcZNT1 (in leaf mesophyll, bundle sheath and guard cells of *T. caerulescens*) (Küpper et al. 2007) thus strongly proving the existence of the differences in behaviour basically due to a different function rather than to a different regulation. However, microarray analyses have highlighted the overexpression of more ZIP members in *A. halleri* and *T. caerulescens* (the homologs of AtZIP3, AtZIP6, AtZIP9, AtZIP10 and AtIRT3), but their roles in plants and in Zn hyperaccumulation remain to be established (Becher et al. 2004; Weber et al. 2004, 2006; Filatov et al. 2006; Hammond et al. 2006; van de Mortel et al. 2006, 2008; Talke et al. 2006; reviewed in Krämer et al. 2007; review by Verbruggen et al. 2009).

Regarding the P_{1B}-type subfamily of P-type ATPases (also known as HMA, heavy metal ATPase)-metal transporters it has been reported that HMAs translocate metal cations out of the cytoplasm across membranes using energy from the hydrolysis of ATP. Transport proteins of the P-type ATPase superfamily are characterised by the formation of a phosphorylated intermediate during the transport reaction cycle. The members of this family have 8–12 transmembrane domains and a number of conserved sequence motifs, including sites for ATP binding, phosphorylation and dephosphorylation (review by Cobbett et al. 2003).

HMA4 was the first gene encoding a plant P1B-type ATPase of the divalent transport group to be cloned and characterized in *A. thaliana* (Mills et al. 2003). Localized at the plasma membrane HMA4 plays significant roles in metal hyperaccumulation in general and Zn homeostasis, Cd detoxification, and in the translocation of these metals from the root to the shoot in particular has been demonstrated in *A. thaliana*, where its expression seems to be confined to the stele (Mills et al. 2003, 2005; Hussain et al. 2004; Verret et al. 2004, 2005). Moreover, reports of Bernard et al. (2004), Papoyan and Kochain (2004), Hammond et al. (2006), van de Mortel et al. (2006), Talke et al. (2006), and Courbot et al. (2007) on the expression of HMA4 in roots and shoots of both *A. halleri* and *T. caerulescens* compared with Cd/ Zn-sensitive close relatives strengthen the idea of the role of this gene in tolerance and/or accumulation of both metals. HMA4, encoding a P1B-type ATPase, has been shown to be essential for root-to-shoot transport of Zn and Cd in general (Papoyan and Kochain (2004); Verret et al. 2004; Wong and Cobbett 2009) and plays a crucial role in metal hyperaccumulation of *A. halleri* (Hannikenne et al. 2008).

The strong expression of the genes encoding HMA3 and HMA4 have been reported in *A. halleri* and *T. caerulescens*, as compared to *A. thaliana* (Becher et al. 2004; Talke et al. 2006; van de Mortel et al. 2006; Hammond et al. 2006). In addition, Hannikenne et al. (2008) reported the requirement of a high level of expression of HMA4 for the high rate of Zn translocation in *A. halleri* when compared to *A. thaliana*; however, it is doubtful, whether enhanced expression of HMA4 alone is sufficient to produce the full hyperaccumulator translocation phenotype. Moreover, using radiotracer efflux analysis, Xing et al. (2008) reported no correlation of the variation in Zn and Cd translocation among *T. caerulescens* accessions with HMA4 expression but instead, at least for Cd, correlated with vacuolar retention in root cells. HMA4 in *A. thaliana* (AtHMA4) and *A. halleri* (AhHMA4) have been reported to be involved in Zn xylem loading and localized to the plasmamembrane by several workers including Hussain et al. (2004), Verret et al. (2004), and Hannikenne et al. (2008). Although, Gravot et al. (2004) have reported AtHMA3 as the major transporter of Cd and Pb rather than Zn but Becher et al. (2004) speculated the possibility of AhHMA3 involvement in vacuolar sequestration of Zn. Moreover, both in *A. halleri* and *T. caerulescens* HMA4 expression has been reported 2–3 times higher in roots than in shoots. Whereas the expression levels of HMA4 in *A. halleri* was noticed relatively constant over Although, different external Zn concentrations have been shown to modulate the expression levels of HMA4 in plants but its expression levels was noticed relatively constant in *A. halleri* whereas, an increased level of expression was observed in *T. caerulescens* in response to high Zn and Cd and also under Zn deficiency (Bernard et al. 2004; Papoyan and Kochain 2004; Talke et al. 2006). There are also reports of the HMA2 and HMA4 expression in the xylem parenchyma of *A. thaliana* where they play a role in root to shoot translocation of Zn probably through mediating xylem loading (Mills et al. 2003; Hussain et al. 2004).

ATP-binding cassette (ABC) family transporters represent one of the largest protein families in living organisms ranging from bacteria to humans (Hall and Williams 2003). In plants, ABC transporters are the best-characterized system to transfer toxic organics out of root cells and into vacuoles after conjugation by GSTs (Meagher 2000; Dixon et al. 2002). Approximately a number of 130 ABC transporters with precise functions have been reported in *A. thaliana* and *O. sativa* as well (Sanchez-Fernandez et al. 2001; Rea 2007). Recently, some Arabidopsis ABC transporters were found to participate in detoxification processes as well as in plant growth and development (Campbell et al. 2003; Geisler et al. 2005). Kim et al. (2006) reported mitochondrial origin of Arabidopsis AtATM3 ABC transporter which is largely involved in the biogenesis of iron–sulfur clusters and iron homeostasis in plants and has also been to be upregulated in roots of plants when exposed with Cd (II) or Pb (II). Additionally AtATM3 overexpressed in Arabidopsis plants has been shown to enhance Cd and Pb resistance compared to wild type. Moreover, authors observed Cd-sensitive phenotypes in AtATM3 knockout plants which were found to resist Cd and Pb after wild-type AtATM3 overexpression.

Transporters of the cation diffusion facilitator (CDF) family have been named Metal Tolerance Proteins (MTPs) and also have been shown to mediate the cytoplasmic efflux of transition metal cations, such as Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+} or Mn^{2+}. High expression of MTPs such as MTP1, MTP8 and MTP11 have been reported in *A. halleri* and *T. caerulescens*, compared to non-hyperaccumulator species. Through transcriptomic approaches a number of workers have reported MTP1 as a candidate tolerance gene in shoots and roots of *A. halleri* and in roots of *T. caerulescens* (Becher et al. 2004; van de Mortel et al. 2006; Talke et al. 2006). The expression of MTP1 has been reported to be differentially modulated by external Zn concentrations where this gene helps plants in Zn-accumulation and tolerance as well to varied extent. In *A. thaliana*, ectopic over-expression has been reported to enhance Zn tolerance and increased root-Zn accumulation (Van der Zaal et al. 1999). In *A. halleri*, expression of AhMTP1 was reported to be constitutively high over a range of external Zn treatments, especially in leaves. Whereas, in *A. thaliana*, Dräger et al. (2004) and Krämer (2005) reported the contribution of a much lower expression of AtMTP1 to basic levels of Zn tolerance and Zn accumulation in leaf tissue. The (over-)expression of the other MTPs genes such as MTP8 and MTP11 in yeast or *A. thaliana* has been speculated to contribute to the tolerance and homeostasis of metals other than Zn, such as Fe, Cu and Mn (Delhaize et al. 2003). Additionally, there are reports on the potential roles of both MTP8 and MTP11 in the adjustment of the homeostasis of other metals in Zn hyperaccumulators (Becher et al. 2004; Talke et al. 2006; van de Mortel et al. 2006).

MATE (Multidrug And Toxic compound Extrusion Transporters or Multidrug And Toxin Efflux) is another important family of small organic molecule transporters seems to be another kind of transport proteins that are active in heavy metal translocation in hyperaccumulator plants. MATE transporter was first reported from bacteria (Morita et al. 1998) and its homologs were later reported from plants and animals. MATE transporters are involved in the transport of plant secondary metabolites. They consist of 400–700 amino acids with 9–12 transmembrane domains (Yazaki et al. 2008). Fifty six MATE transporter genes have been reported *A. thaliana* plant genome (Li et al. 2002; Yazaki et al. 2008). In *Arabidopsis thaliana* plants, MATE Transporter TT12 acts as a vacuolar flavonoid/H+-Antiporter (Marinova et al. 2007). AtDTX1 (*Arabidopss thaliana* detoxification 1) is a member of the MATE family and serves as a carrier for a range of toxic compounds and is also capable of the detoxification of Cd^{2+} (Li et al. 2002). Whereas, FRD3 (ferric reductase defective 3), a gene encoding a member of MATE family, have been reported to be localized at root pericycle plasma membranes. FRD3 is thought to have an important role in Fe homeostasis in Arabidopsis where it usually operates in the xylem influx of citrate, which is required as a ligand for Fe homeostasis and transport (Rogers and Guerinot 2002; Durrett et al. 2007), but Krämer et al. (2007) reported its potential play a role in translocation of other metals, such as Zn. in addition, Magalhaes et al. (2007) reported a gene of the MATE family in sorghum that confers Al tolerance. Yellow Stripe-like (YSL) proteins are thought to play a role in long-distance transport of metals that are complexed with plant-derived phytosiderophores (PS) or nicotianamine (NA) (Didonato et al. 2004). According to Colangelo and Guerinot (2006) mediate the loading into and unloading out of xylem of nicotinamine–metal chelates. Eight predicted YSL proteins were reported in Arabidopsis and were thought to transport metal-NA complexes (Curie et al. 2001). Gendre et al. (2007) reported the constitutive overexpression of three genes namely TcYSL3, TcYSL5 and YSL7 in roots and shoots of *T. caerulescens* where the YLS proteins were confirmed to participate in vascular loading and translocation of NA–metal (especially NA–Ni) complexes.

4.3 Metals-Metalloids Tolerance

In general, some level of metal tolerance is exhibited by almost all plants where plants may exhibit 'basic metal tolerance' or 'metal hypertolerance'. According to the classification of Ernst et al. (2008), plants with 'basic metal tolerance' are those living on non-metal enriched soils (also called 'non-metallicolous' by Meerts and Van Isacker 1997), whereas metal hypertolerant (or merely called 'tolerant') plants can survive and reproduce on highly metal-enriched soils. Literatures are available on the involvement of many cellular mechanisms in both the basic and hyper metal tolerance in plants (Hall 2002; Clemens 2006; Krämer 2010). Metals/metalloids tolerance in plants in general, has been reported to be largely based on the principle of storage of metals/metalloids (once translocated to shoot cells) in cellular locations hence to avoid metals/metalloids-accrued damage to the vital cellular processes. In this perspective, a series of important mechanisms such as uptake, translocation, chelation/complexation (through synthesis of metallothioneins and phytochelatins), metal efflux and/or cellular/vacuolar compartmentalization works to achieve the ultimate aim of metals/metalloids tolerance. The basic mechanisms of metal/metalloids uptake and translocation have been already critically reviewed above. A review of the significance of metallothioneins (MTs) and phytochelatins (PCs) for metal/metalloids tolerance in *Alyssum*, *Arabidopsis* and *Thlaspi* sp. will be covered in the section detoxification and sequestration hereunder.

4.3.1 Detoxification and Sequestration

Despite the huge concentrations of damaging metals/metalloids in plant organs hyperaccumulators have unique features of detoxification, sequestration and/or binding metals/metalloids to molecules or structures to limit their otherwise negative effects. Intracellularly, metals can be complexed with ligands involving chelation by peptide ligands such as metallothioneins (MTs) and phytochelatins (PCs). However, in addition to MTs and PCs, a number of amino acids and organic acids are among the other chelators/high affinity ligands playing important roles for buffering metals/metalloids in cytosol (Rauser 1999; Clemens 2001; Hall 2002). Ni-hyperaccunulating plants has been shown to detoxify metals/metalloids using the low-Mr chelators His (Krämer et al. 1996) and citrate (Lee et al. 1978). Moreover, exposure of the hyperaccumulator *Alyssum lesbiacum* to nickel (Ni) is known to result in a dose-dependent increase in xylem sap concentrations of Ni and the chelator free histidine (His). In this context, Kerkeb and Krämer (2003) studied the role of free histidine in xylem loading of Ni in *Alyssum lesbiacum* and *Brassica juncea*. Authors concluded that enhanced release of Ni into the xylem was associated with concurrent release of His from an increased root free His pool in both *A. lesbiacum* and *B. juncea* (Kerkeb and Krämer 2003). Another Ni-hyperaccumulator *Thlaspi goesingense* exhibited increased root His concentrations when compared with *T. arvense*. However, none of the three genes of histidine biosynthesis studied was regulated by Ni (Persans et al. 1999). Besides Ni, Salt et al. (1999) identified Zn–His complexes in the roots of Zn-hyperaccumulator *T. caerulescens*. The beneficial role of high histidine levels has been shown in transgenic *A. thaliana* expressing a *Salmonella typhimurium* ATP phosphoribosyl transferase enzyme (StHisG) (Wycisk et al. 2004). Recently, Richau et al. (2009) compared the distribution of Ni over root segments and tissues in the hyperaccumulator Thlaspi caerulescens and the nonhyperaccumulator *Thlaspi arvense*, and investigated the role of free histidine in Ni xylem loading and Ni transport across the tonoplast. Authors observed Ni accumulation in mature cortical root cells in *T. arvense* and in a high-Ni-accumulating *T. caerulescens* accession, but not in a low-accumulating *T. caerulescens* accession. In addition, compared to *T. arvense*, the concentration of free histidine in *T. caerulescens* was noticed tenfold enhanced in roots regardless of Ni exposure. In MgATP-energized root- and shoot-derived tonoplast vesicles of *T. caerulescens*, Ni uptake was almost completely blocked when Ni was supplied as a 1: 1 Ni-histidine complex, but surprisingly it was observed uninhibited in *T. arvense*. The exogenous application of histidine lead to enhancement in Ni xylem loading in *T. caerulescens* but not in *T. arvense*. Authors concluded that the high rate of root to shoot translocation of Ni in *T. caerulescens* compared with *T. arvense* seemed to depend on the combination of two distinct characters, that is, a greatly enhanced root histidine concentration and a strongly decreased ability to accumulate histidine-bound Ni in root cell vacuoles. To sum up, the process of high affinity ligands-mediated chelation and subsequent cellular/vacuolar compartmentalization have been shown to be involved in metal detoxification and sequestration in hyperaccumulator plants

(Clemens et al. 2002; Hall 2002; Schat et al. 2002; Assunção et al. 2003). Epidermis (Ma et al. 2005; Asemaneh et al. 2006), trichomes (Küpper et al. 2000) have been reported to be as preferential principal organs for metals/metalloids detoxification and/or sequestration (review by Rascio and Navari-Izzo 2011). MTs (first identified in mammalian tissues as Cd-binding peptides) (Kawashima et al. 1992; Cobbett 2000) and PCs (first identified in yeast, *Schizosaccharomyces pombe*, and termed cadystins) (Kondo et al. 1984) are respectively gene-encoded and enzymatically synthesized. Later, a number of MTs and PCs genes and proteins have been identified in different plants species (Gekeler et al. 1989; Kawashima et al. 1992; Cobbett 2000) suggesting that the PCs/MTs-producing pathways evolved very early in order to maintain the endurance of vascular plants growing under potentially hostile and toxic environments (Kawashima et al. 1992; Cobbett 2000; Kondo et al. 1984; Gekeler et al. 1989). This section will briefly discuss the role of PCs and MTs in the tolerance to, detoxification, sequestration and/or cellular/vacuolar compartmentalization of major metals/metalloids.

4.3.1.1 Phytochelatins

Phytochelatins (γ-glutmylcysteinyl-isopeptides; PCs) are small metal binding peptides first isolated from cell suspension cultures of a higher plant after exposure to Cd (Grill et al. 1985). PCs are synthesized in vivo within minutes of exposure to metals or metalloids from glutathione (GSH) by a γ-Glu-Cys dipeptididyl transpeptidase (EC 2.3.2.15), commonly referred to as phytochelatin synthase (PC synthase) (Grill et al. 1989). Although, PCs are synthesized in response to exposure to various physiological and non-physiological metal ions but Cd^{2+} ions are the most potent activators of PCs-production. Among the other important metal ions Pb^{2+}, Zn^{2+}, Cu^{2+}, $Sb^{3+,}$ Ag^{+}, Hg^{2+}, AsO_4^{3-} and a few others have also been show to activate PCs synthesis in plants where PCs thus, formed in the presence of excess metals create metals-PCs complexes and provide tolerance to these metals (Clemens 2006; Tennstedt et al. 2009). Moreover, PC-mediated detoxification is unique to plants and a few other PC-producing organisms but different from that of budding yeast or humans, in which PCs or PC synthase (PCS) do not exist. However, there is a great deal of literature that whether PCs actually provide metals/metalloids tolerance in plants or is the secondary consequence of metals/ metalloids stress. In this context, the metals/metalloids-mediated modulation of GSH, the precursor of PCs has been extensively reported in plants where at one hand the maintenance of high levels of PCs or its precursor, GSH have been shown to provide metals/metalloids tolerance. There are reports that the deficiency of PCs leads to diminished Cd accumulation in plants (Howden and Cobbett 1992; Larsson et al. 2002). Higher levels of GSH may contribute to metal detoxification either by direct binding or by synthesis of PCs. Thus, Canovas et al. (2004) are of the opinion that Cd-treated plants can withstand Cd toxicity by increasing the GSH pool (the precursor of PCs) to maintain the high levels of PCs. But this is not the case always, because *Arabidopsis* mutants or transgenic plants with elevated GSH content or PC synthesis did not show enhanced Cd-resistance in comparison with wild type plants

(Xiang et al. 2001). There are instances to conclude the fact that different populations rely on different strategies in Cd detoxification using PCs. For example, Ebbs et al. (2002) found that PCs may not contribute to the hyperaccumulation phenotype and do not appear to be involved in metal tolerance in the hyperaccumulator Gange ecotype. While leaf and root PC levels for metal tolerance in *T. caerulescens* and the related Prayon ecotype showed a similar positive correlation with tissue Cd, the total PC levels in the Gange ecotype were generally lower, despite correspondingly higher metal concentrations. Moreover, when PC accumulation in *T. caerulescens* was blocked by buthionine sulfoximine (BSO) treatment, Cd sensitivity was affected neither in the accession 'Plombières' (Wójcik et al. 2005a, Wójcik 2005b), nor in 'Ganges'. However, Cd sensitivity was increased in accession 'Prayon' (Hernandez-Allica et al. 2006), and, more strongly so, in 'Monte Prinzera', originating from serpentine soil (Schat et al. 2002). In general, there seems to be an inverse correlation between the PC concentrations, or plants-internal PC-thiol to Cd molar ratios, and the Cd tolerance level among the accessions, which is also found among accessions of nonhyperaccumulator metallophytes. As mentioned also above that phytochelatin synthase (PC synthase) catalyzes the synthesis of PCs in vivo within minutes of exposure to metals or metalloids from glutathione (GSH). However, In Arabidopsis, PC synthase expression has been shown independent of trace metal exposure (Howden et al. 1995). There are conflicting reports regarding the induction of transcript levels in response to trace metal exposure. PC biosynthesis probably varies between different plant species, being regulated at transcriptional or post-transcriptional levels, or both. This suggests that PC synthase activity is regulated differently in different plant species. Howden et al. (1995) reported that Arabidopsis mutants lacking PC synthase were reported unable to synthesize PCs and thus were hypersensitive to Cd and Hg. Moreover, authors predicted cad 1 as the structural gene for PC synthase (Howden et al. 1995). The Arabidopsis cad 1 gene (referred to as AtPCS1) (Howden et al. 1995; Ha et al. 1999; Vatamaniuk et al. 1999) has been shown to confer resistance to Cd when expressed in the yeast *S. cerevisiae* (Clemens et al. 1999; Cobbett and Meagher 2002). In addition, Gasic and Korban (2007) reported exhibition of increased tolerance to and accumulation of Cd and As by transgenic *Brassica juncea* plants expressing Arabidopsis PC synthase. Although, these studies suggest the fact that GSH and PC concentrations can be manipulated to enhance trace metals accumulation potential of plants but reports are available where overexpression of PC synthase in plants was observed paradoxically leading to hypersensitivity to metals/metalloids. In this perspective, a critical level of AtPCS1 cDNA has been shown important in heavy metal detoxification (Lee et al. 2003a) in a study, using few transgenic Arabidopsis lines generated following transformation with a construct containing AtPCS1 cDNA under the control of cauliflower mosaic virus (CeMV) 35 S promoter (35::AtPCS1). Among the transgenic Arabidopsis lines studied 14-fold increase in AtPCS1 transcript and 30% increase in PC level were observed in two transgenic lines where these were found more Cd-sensitive than wild type. While twofold Cd-tolerance and slight increase in AtPCS1 expression and 15% increase in PC content were noted in remaining transgenic lines. Although, a critical level of

AtPCS1 were shown important in heavy metal detoxification but the question of AtPCS1 protein's sole responsibility for Cd sensitivity was clarified by another study of the authors (Lee et al. 2003b). Accumulation of 12–25-fold higher AtPCS1 mRNA and 1.3–2.1-fold increased PC concentration was reported in Arabidopsis plants overexpressing Arabidopsis PC synthase (AtPCS1), designated PCs lines when compared to wild type. Furthermore, they were more sensitive to Cd stress than a PC-deficient Arabidopsis mutant, cad1-3 overexpressing AtPCS1 to similar levels as those of PCs lines, supplemented with low GSH levels. In addition, increased GSH levels in the medium was observed nullifying Cd hypersensitivity of PCs lines. It was therefore argued that Cd hypersensitivity in PCs lines appeared not due to toxicity of AtPCS1 protein but due to toxicity of PCs as they have high Cys content and existed at supraoptimal levels when compared with GSH levels. There have been contradictory results on the effect of PC synthase overexpression in plants, ranging from an increased Cd tolerance to no effect on Cd tolerance, or, paradoxically, even Cd hypersensitivity. Expression of PCS gene TcPCS1 isolated from *T. caerulescens* in wild-type *Nicotiana tabaccum* has been shown to increased PC production, Cd accumulation and enhanced metal tolerance (Liu et al. 2011). Whatever the case may be, but the occurrence of PC synthase in different higher plants has been confirmed (Clemens et al. 1999; Cobbett 2000) and gel filtration analysis revealed Cd-PC interactions as low molecular weight (LMW) and high-molecular-weight (HMW) complexes (Howden et al. 1995; Rauser 2000). The LMW Cd-binding complex has been reported to contain shorter PCs occurring in cytosol; whereas, HMW complex was reported by Ortiz et al.(1995) and Rauser (2000) to occur in vacuol containing longer PCs, acid-labile sulfide, and usually dominating over that of the LMW complex when plants were exposed to Cd for longer time. Furthermore, Howden et al. (1995) and Ha et al. (1999) confirmed the essentiality of HMW Cd–PC complex-accumulation for Cd detoxification in *A. thaliana*. Most importantly, aside from detoxification, PC plays a role in homeostasis of metals/metalloids in plants thus regulating the metal ions availability in plant cells (Guo et al. 2008). Long-distance transport (either from shoot to root or vice-versa) of PCs has been reported in recent studies (review by Pal and Rai 2010). Gong et al. (2003) reported increased increased Cd^{2+} transport in stems and rosette leaves of transgenic Arabidopsis expressing *Triticum aestivum* TaPCS1 cDNA. Using a shoot-specific, light-induced regulatory cassette Li et al. (2006) showed the long-distance movement of thiol-peptides from shoots down to roots by expressing a bacterial γ-glutamylcysteine synthetase (ECS) in the shoots of an Arabidopsis ECS-deficient mutant. Additionally, expression of TaPCS1 gene under the control of a shoot-specific promoter (CAB2) in an Arabidopsis PC-deficient mutant cad1-3 (CAB2::TaPCS1/cad1-3). CAB2:: TaPCS1/cad1-3 plants was shown to exhibit higher Cd accumulation in roots and lower in shoot compared to wild type.

4.3.1.2 Metallothioneins

Metallothioneins (MTs) are gene-encoded low molecular weight cystein-rich polypeptides, first identified in mammalian tissues as Cd-binding peptides

(Salt et al. 1995). However, MTs are found throughout the animal and plant kingdoms. Although, MTs have been shown to play a role in the detoxification of heavy metals in animals and fungi but their exact function is not completely understood (Robinson et al. 1993; Hall 2002; Memon and Schröder 2009). Correlation between MT RNA levels and naturally observed differences in the tolerance to heavy metals in plants such as Arabidopsis ecotypes has been reported, suggesting a role in metal homeostasis (Murphy and Taiz 1995; Murphy et al. 1997; Guo et al. 2008). Moreover, MTs have been indicated to play roles for metal detoxification (Roosens et al. 2005; Domenech et al. 2006), development (Ledger and Gardner 1994), senescence (Coupe et al. 1995; Hsieh et al. 1995), and in protection against abiotic stress (Zhou et al. 2005). Several MT genes, and also proteins have been identified in plants where MTs can form mercaptide bonds with various metals and thus act as metal protective proteins. Based on different expression patterns in plant tissues during development fulfilling different important functions Cobbett and Goldsbrough (2002) grouped plant-MTs into four subfamilies namely MT1, MT2, MT3, MT4.

Thus far, no correlations between the levels of MT proteins and the metal concentrations in the plants have been established. However, some differences, at least in the expression levels, between hyperaccumulators and non-accumulators have been found where overexpression of MTs-genes involved in the synthesis of metal chelators have been reported to lead both enhanced/reduced metal uptake, enhanced metal translocation and/or sequestration (Cherian and Oliveira 2005). Arabidopsis has been reported to harbor four MT types with seven members expressed, namely MT1a, MT1c, MT2a, MT2b, MT3, MT4a and MT4b (Zhou and Goldsbrough 1994; Cobbett and Goldsborough 2002; Guo et al. 2003). Depending upon the type/forms and/or concentrations of metals/metalloids and also on plants parts both hyperaccumulators and non-accumulators may exhibit differential patterns of MTs-genes. For example, Garcìa-Hernàndez et al. (1988), Zhou and Goldsbrough (1994), and Kohler et al. (2004) have reported high expression levels of MT1a and MT1b in non-accumulator *A. thaliana* roots exposed to Cd, Cu and Zn. Although, using RT-PCR and in situ hybridization Garcia-Hernandez et al. (1998) detected both mRNA species in root maturation zones and leaf trichomes, the expression of only MT1a was revealed in the vascular tissue and the mesophyll cells. Additionally, Murphy and Taiz (1995) correlated the Cu-induced induction of MT2a gene with Cu tolerance of the Arabidopsis ecotypes. In contrast to the mentioned reports, the levels of MT1mRNA were found in leaves constitutively higher than in roots in *T. caerulescens* where the levels were also found increased with exposure to Cu (Roosens et al. 2005). In this context, constitutive high expression of MT2 has been shown in *A. halleri* and *T. caerulescens* by Roosens et al. (2005), Chiang et al. (2006), and van de Mortel et al. (2006). In addition, there is report of dual role of MT1 for conferring both tolerance to Cd and Zn homeostasis in Arabidopsis (Zimeri et al. 2005). The MT1a loss-offunction plants were reported to exhibit reduced Cu concentrations in the roots, thus implying that MT1a is important in the Cu-accumulation and homeostasis in roots as well (Guo et al. 2008). In the study, even the mt1a mt2b double mutant exhibited normal

Cd and Cu tolerance (Guo et al. 2008). The MT2 protein has been reported to be localized in the epidermis and root hairs of both *T. caerulescens* and *A. thaliana*. Moreover, MT2 and MT3 were reported to contribute to the metal-adapted phenotype but were not directly linked to Zn accumulation in *T. caerulescens* (Hassinen et al. 2009).

Regarding metals/metalloids-mediated modulation of MT3 expression in hyperaccumulator and non-hyperaccumulator plants there exist contradictory reports on contribution of MT3 expression toward tolerance to and/or homeostasis of varied metals/metalloids. In *T. caerulescens*, Roosens et al. (2004) reported the Cd-induced induction of MT3 but authors reported much greater levels of tolerance of yeast to Cu than to Cd overexpressing TcMT3. Latter, studies by the authors (Roosens et al. 2005) leading to the discovery of a smaller cavity adapted for Cd chelation in the MT3 protein of *A. thaliana* as compared with the analogous protein from *T. caerulescens* confirmed the role of TcMT3 for the maintenance of a normal Cu homeostasis under conditions of high Cd and Zn in the cytoplasm. The role of high expression of MT4 gene in metal homeostasis has been reported in *A. thaliana* during seed development and seed germination (Guo et al. 2003).

4.4 Summary, Conclusions and Perspectives

Elevated toxic levels and high persistence of TMs and metalloids in major compartments of the biosphere has posed various uncompromising and fatal effects on flora and fauna, and thus, has threatened the stability of the ecosystems as well. Unlike organic contaminants, TMs and metalloids are not biologically degradable, and hence, remain persistent in environmental bodies for a long time. In this perspectives, an innovative, economic, environmental-friendly process named phytoremediation utilizes the natural property of plants for taking up/removing, destroying, degrading, sequestering, transforming, assimilating, metabolizing, or detoxifying hazardous chemical pollutants including TMs and metalloids from soil, aquatic and atmospheric environments. Phytoremediation encompasses number of important strategies such as (a) phytoextraction (direct accumulation of contaminants into plant shoots with subsequent removal of the plant shoots), (b) rhizofiltration (phytofiltration) (absorb and adsorb pollutants in plant roots), (c) phytostabilization (root exudates cause metals to precipitate and biomass becomes less bioavailable), (d) phytovolatilization (plants evaporate certain metal ions and volatile organics), (e) phytodegradation (plant-assisted bioremediation) (microbial degradation in the rhizosphere region), (f) phytotransformation (plant uptake of organic contaminants and degradation) and (g) removal of aerial contaminants (uptake of various volatile organics by leaves). Metal hyperaccumulation, the strategy of ‘phytoextraction’ is being dealt here in detail. ‘Phytoextraction’ (also called phytoaccumulation) refers to the use of plants to remove metals/metalloids from contaminated site by translocating them to their aboveground matter. This method is potentially very efficient. As contaminants (metals/metalloids) are concentrated in above-ground parts, it is easier to handle the total plant dry matter containing the contaminants compared with very hard handling of the tons of soil to be treated by conventional ways of remediation. Metal phytoextraction (or metal phytoaccumulation), a sub-class of metal ions resistance and indeed a striking phenomenon exhibited by <0.2% of angiosperms in fact is, one of the three different strategies developed by different types of plants growing on contaminated or metalliferous soils. As stated also above that metal accumulators are usually referred to as hyperaccumulators that concentrate metals in their above-ground tissues to levels far exceeding those present in the soil or in nonaccumulating species growing nearby. Metal hyperaccumulator plants accumulating metals to concentrations over 100–1,000-fold higher than conventional plants growing on soils with background metal concentrations, and about 10–100-fold higher than most other plants growing on metal-comtaminated soils in addition to exhibiting a shoot-to-root metal concentration ratio of >1 can be suitable for phytoremediation purposes.

Although, a large number of natural metal hyperaccumulator plant species from 34 different plant families has evolved the ability to take up, tolerate and accumulate exceptionally high concentrations of metals and metalloids present in the soil (and water) and, more importantly, in their aboveground biomass without visible toxicity symptoms but the family Brassicaceae best represents amongst these metal-hyperaccumulator families with 87 species classified as metal hyperaccumulators. Of these 87 different metal-hyperaccumulator plant species in the family Brassicaceae, plant species in particular model metal hyperaccumutaor plant species *Alyssum*, *Thlaspi* and *Arabidopsis* have been studied extensively for their ability to hyperaccumulate several metals including metals and metalloids. Hyperaccumulation of heavy metals by higher plants is a complex phenomenon. It involves several steps such as (a) transport of metals across the plasma membrane of root cells (b) xylem loading and translocation, and (c) detoxification and sequestration of metals at the whole plant and cellular levels. Metals hyperaccumulators make use of conventional rhizosphere mechanisms to improve their trace element accumulation. Moreover, specific rhizosphere conditions potentially modify the bioavailability of varied metals/metalloids to hyperaccumulator plants when growing in different contaminated media. Microorganisms are ubiquitous in soils with high concentrations of metals to which hyperaccumulators are native where rhizophere-microorganisms interactions have been shown to affect and control metal/metalloid bioavailability in the plant. The tolerance/resistance of bacteria in the hyperaccumulator and non-hyperaccumulator rhizospheres largely differs from bacteria in the bulk soil. Therefore, it would be interesting to investigate the mechanisms behind the exhibition of increased trace element resistance in rhizosphere bacteria.

Despite the toxic nature of majority of trace metals and metalloids, a large number of these metal ions enter plant cells via specific transporters and uptake systems for essential cations. Entry of both essential and non-essential metal ions in plant cells have been shown to be mediated by a number of metal transporters. Majority of these metals transporters has been shown to be involved in metal uptake, homeostasis and tolerance in plants. Manipulation of these transporters to achieve removal of metal ions from the cell holds great potential. Metals/metalloids tolerance in plants in general, is largely based on the principle of storage of metals/metalloids (once translocated to shoot cells) in cellular locations hence to avoid metals/metalloids-accrued damage to the vital cellular processes. In this perspective, a series of important mechanisms such as uptake, translocation, chelation/complexation (through synthesis of metallothioneins and phytochelatins), metal efflux and/or cellular/vacuolar compartmentalization works to achieve the ultimate aim of metals/metalloids tolerance. Intracellularly, metals can be complexed with ligands involving chelation by peptide ligands such as metallothioneins (MTs) and phytochelatins (PCs). However, in addition to MTs and PCs, a number of amino acids and organic acids are among the other chelators/high affinity ligands playing important roles for buffering metals/metalloids in cytosol. These processes and subsequent cellular/vacuolar compartmentalization have been shown to be involved in metal detoxification and sequestration in hyperaccumulator plants.

The use of naturally occurring metal hyperaccumulator plant species as a phytotechnology for metals/metalloids-contaminated-site-cleanup has been reported to be strongly limited due to due to slow metal extraction coupled with low biomass production and metal recovery process. In this context, concerted efforts should be made to manipulate the microorganism-rhizosphere relationships to enhance the metals/metalloids-availability, solubility, uptake, sequestration, detoxification and subsequent compartmentalization in known and/or potential metal hyperaccumulator plant species. Moreover, since, it is an established fact that PCs and MTs are involved in metals/metalloids detoxification and/or accumulation in plants, PCs-MTs-biosynthetic genes may be engineered in common plants capable of growing fast and producing large biomass. Additionally, as the major targets of phytoremediation technology are larger metal-polluted natural-systems having multimetal conditions; therefore, to achieve its successful application at commercial scale, it is imperative to know the exact nature of PCs produced and their interaction with metals independently and in combinations. As mentioned above that major investigations have focused on uptake into root cells, apoplastic distribution, symplastic element pool remobilization, xylem loading, xylem unloading/cellular uptake in shoots, and vacuolar sequestration in shoot, storage, and ligands. But little is known about the regulation of metals/metalloids tolerance mechanisms (sensing, signal transduction, etc.) while tolerance and accumulation are often linked. Moreover, in this context, information has been gained mainly for a limited number of trace metals such Cd, Zn, Ni, and As, but less is known about other elements. Therefore, potential “Omics” technologies in phytotechnologies must be exploited to investigate the metals/metalloids contaminants-uptake, transfer, and distribution under realistic environmental conditions. Integrated studies on the role of transporters, ligands, and storage of parent compounds and metabolites may yield some promising results in this context. As the mechanisms for metals/metalloids-solubility, availability, uptake, detoxification, sequestration and/or compartmentalization greatly depend on plant species and ecosystems several models other that known metals/metalloids hyperaccumulation plants are needed. In this perspective, knowledge gained with Arabidopsis, Thlaspi and/or Alyssum sp. must be extended to other species including macrophytes that display traits absent in these model species. Stacking of modified genes capable of increasing toxic metals/metalloids tolerance and accumulation in transgenic lines represents a highly promising new tool for use in phytoremediation especially in nature where often multimetal conditions prevail. Biochemical, molecular, and physiological analysis of interesting and important traits for improving phytoremediation such as enhanced growth rate and biomass production, enhanced root depth, stimulation of rhizosphere microflora, biodegradative enzymes, changes in metals/metalloids redox state, and enhanced metals/metalloids transporters and chelators in subcellular compartments should be thoroughly investigated. Further identification of genes able to promote such traits, including the regulatory steps in terms of upregulation and downregulation of responsive genes, the signal transduction pathway, and responsive transcription factors, will be very useful for bioremediation studies and help to develop suitable biological materials. The biochemical and molecular-genetic characterization of various components of PCs-MTs biosynthetic pathways in tissues of metal-hyperaccumulator plant species may provide additional insights for designing and/or improving phytoremediation technology for its success enabling wider application.

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Chapter 5
Brassica Rhizosphere-Microbe Interactions and Their Role in Phytoremediation

Mushtaq Ahmed, V.K. Singh, and R.S. Upadhyay

Abstract Numerous toxic metals such as cadmium, selenium, lead, zinc etc. released from industrial production, mining, smelting and traffic contaminate the agricultural soils. This has raised concerns not only for crop quality but also for human health. Engineering and/or microbial based technologies are used to remove the toxic metals from contaminated soils. But these approaches are costly and less efficient in comparison to phytoremediation technique which has emerged as more efficient and cost effective method for decontamination of the toxic metal affected sites. There are highly specialized plants that have the ability to accumulate and tolerate high concentrations of toxic metals from soils and may provide the basis for remediation of heavy metal contaminated sites. Members of the family Brassicaceae have a key role in phytoremediation technology. Metal uptake, sensitivity and sequestration have been extensively investigated in *Arabidopsis thaliana*. There are a number of *Brassica* and related crop species that have been reported as the potential candidates for phytoremediation. *Brassica* spp. display a great diversity of morphological form and many are of economic value as oilseeds, vegetables and forages. They are well suited to genetic manipulation and *in vitro* culture techniques and are attractive candidates for the introduction of genes aimed at phytoremediation. *Brassica* oilseeds are adaptable to a range of environmental conditions. Moreover, rhizosphere of *Brassica* spp. is colonized by several beneficial microbes that can be isolated, mass cultured and utilized to maximize the biomass of this crop plant for use in phytoremediation technology. These microbial inoculants are cheaper as compared to the chemical fertilizers, and are not hazardous to the environment.

Keywords *Brassica* spp. • Rhizosphere • Plant growth promoting soil microbes • Phytoremediation

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5.1 Introduction

Toxic metals such as cadmium, selenium, lead, zinc etc. released from industrial production, mining, smelting and traffic contaminate agricultural soils and threaten crop production by their toxicity to plants (Wang et al. 2009). Heavy metals have been found to accumulate in human body through ecological food chain and eventually pose serious health hazards (Sanita-di Toppi and Gabbrielli 1999). Phytoremediation *i.e.* the use of plants to remove toxic metals from contaminated sites is generally viewed as more efficient and cost effective technique compared to other methods of decontamination such as engineering and/or microbial approaches (Vickers and Lemaux 1998). With an extensive root system for absorption and transport, large amounts of heavy metals can be directed to the shoots and removed by biomass harvesting (Palmer et al. 2001). The metal-enriched plant material can then be removed from the site, the contaminants concentrated, disposed or if possible, the metal element recovered and valuable metal recycled (Palmer et al. 2001). Plants therefore, represent natural environmentally safe way to remediate contaminated sites (Palmer et al. 2001). There are highly specialized plants that have the ability to accumulate and tolerate high concentrations of toxic metals from soils and may provide the basis for remediation of heavy metal contaminated sites (Anderson et al. 1998). Members of the family Brassicaceae have a key role in phytoremediation technology (Palmer et al. 2001). There are a number of *Brassica* and related crop species that have been reported as the potential candidates for phytoremediation (Palmer et al. 2001). The oleiferous genus *Brassica,* a member of the family Brassicaceae includes over 150 species of annual, biennial or rarely perennial herbs, mostly native to the north temperate parts of the Old World, especially the Mediterranean region. It is the third most important source of vegetable oil in the World after palm and soybean oil and has a key role in phytoremediation technology. *Brassica* spp. that are adaptable to a range of environmental conditions, are well suited to genetic manipulation and *in vitro* culture techniques and are attractive candidates for the introduction of genes aimed at phytoremediation (Palmer et al. 2001). Moreover, rhizosphere of *Brassica* spp. is colonized by several beneficial microbes such as *Trichoderma atroviride, Trichoderma harzianum*, *Bacillus megaterium*, etc. that can be isolated, mass cultured and utilized to maximize the biomass of this crop plant for use in phytoremediation technology (Brown 1975; Adams et al. 2007).

5.2 Role of *Brassica* Species in Phytoremediation

Numerous species of *Brassica* and related cruciferous crops have been recognized as potentially useful candidates for phytoremediation (Palmer et al. 2001). Several species of *Brassica* are able to produce significant amounts of biomass, which is of definite advantage in phytoremediation (Palmer et al. 2001). With rapidly growing

Brassica spp., multiple cropping can be achieved during the growing season and since flowering and seed production is not essential, contaminant recycling by leaf fall can be avoided (Palmer et al. 2001). *Brassica* spp. are adaptable to a range of environmental conditions and therefore, there is the potential to develop superior genotypes for phytoremediation via through selection and breeding techniques (Palmer et al. 2001). The adaptability of *Brassica* species can also be exploited through *in vitro* culture and transformation techniques to develop plants with range of tolerance capacities to toxic metals (Palmer et al. 2001). Different *Brassica* species with different accumulation and detoxification attributes may be employed in the same environment to for affective phytoremediation output (Palmer et al. 2001). Among several *Brassica* species, *Brassica juncea* has been studied most extensively for phytoremediation potential (Banuelos et al. 1992, 1996, 1997a, b, 1998). It exhibits superior heavy metal accumulation characteristics (Blaylock et al. 1997; Ebbs et al. 1997; Gleba et al. 1999) and improved lines have already been developed for Pb accumulation ability by conventional selection methods (Nanda Kumar et al. 1995). Field trials have been conducted for phytoremediation of lead (Pb), selenium (Se), cadmium (Cd), zinc (Zn), radionuclides and other metals by using *Brassica juncea* and *Brassica oleracea* (Salt et al. 1998). A number of *Brassica* species have been studied for their ability to absorb and transport lead to the shoot system (Nanda Kumar et al. 1994). It has been found that Pb accumulation in these plants varies from 1,416 to 18,812 μg^{-1} dry weights (Nanda Kumar et al. 1994). *Brassica juncea* was found to be superior to the other species in the transport of absorbed lead to shoots, while *B. nigra* showed the least efficiency (Nanda Kumar et al. 1994). Selected genotypes of *B. juncea* have been reported to accumulate Pb in the roots 563-fold over the concentration present in nutrient solution (Dushenkov et al. 1995). Dushenkov et al. (1995) studied the potential of *B. juncea* for removal of cadmium (Cd) from contaminated sites and found that the roots of this plant were able to concentrate Cd 134-fold in the shoot tissues under hydroponic conditions. Efficient Cd removal required intact roots as derooted plants did not accumulate the metal (Raskin et al. 1997). Gleba et al. (1999) reported the shoot accumulation of Cd in B. *juncea.* This plant has also been demonstrated to show substantial tolerance to zinc and copper (Ebbs and Kochain 1997) and significant amounts of Zn have been found to accumulate in shoots of the plant grown in contaminated sites (Ebbs and Kochain 1998). *Brassica* species are known as selenium hyperaccumulators and most extensive studies on phytoremediation using these plants have been conducted with this element (Rosenfield and Beath 1994). Analysis of Se content in a number of plant species such as *Astragalus, Atriplex* and *Brassica* showed the highest level in *Brassica*, with *B. oleracea* having 1,200 $\mu g\ g^{-1}$ in the florets (Banuelos and Schrale 1989). Most of the Se content was found to accumulate in the foliage indicating efficient transport from the roots (Banuelos and Schrale 1989). Substantial removal of Se from contaminated soils has been achieved with *B. juncea* where the element either accumulated in the shoots or was volatilized (de Souza et al. 1999). This species coupled with high tissue accumulation has commercial potential for phytoremediation (Brooks 1998). In addition to Pb, Cd, Zn and Se, the species of

Brassica have been found to remove Cu, Cr, Ni and the radionuclides such as ^{137}Cs and ^{90}Sr from the contaminated soils (Salt et al. 1995b; Lasat et al. 1998). *Brassica* species accumulating thallium have been reported (Kemper and Bertram 1991). Under experimental conditions, *B. juncea* has been found to accumulate arsenate mostly in the roots as As^{III} triglutathione (Pickering et al. 2000). Arsenate chelators have been reported to enhance arsenate transport to the shoots (Pickering et al. 2000). To be affective in phytoremediation and removal of specific elements, *Brassica* species must exhibit significant tolerance to a number of heavy metals or xenobiotics and have the ability to grow under varied growth conditions (Palmer et al. 2001). While many species of plants show sensitivity to increased levels of toxic metals (Pahlsson 1989), *B. juncea* appears to be very tolerant under experimental conditions, even though biomass and root growth were reduced (Ebbs and Kochain 1997). This plant has also been found to be tolerant to selenium under saline conditions (Banuelos et al. 1996). Such tolerance potential of this important plant may be related to enhanced levels of detoxifying enzymes in response to toxic metal ions (Prasad et al. 1999), and the production of metal complexing peptides (Speiser et al. 1992). Such peptides have been demonstrated to be sulphur rich and there is evidence that indicates the induction of sulphate-metabolizing enzymes in response to toxic metals (Lee and Leustek 1999).

5.3 Plant Growth Promoting Soil Microbes and Phytoremediation Potential of *Brassica* Species

Numerous microorganisms indigenous in the rhizosphere of Brassica species such as *Phyllobacterium brassicacearum, Serratia plymuthica, Comamonas terrigena, Stenotrophomonas maltophilia, Agromyces cerinus, Acinetobacter rhizosphaerae, Microbacterium oxydans, Paenibacillus lautus, Arthrobacter globiformis, Pseudomonas fluorescens, Variovorax paradoxus Trichoderma* spp. etc. have been reported to influence the growth and phytoremediation potential of this crop plant (Larcher et al. 2008; Adams et al. 2007). Many studies have demonstrated that application of beneficial microorganisms colonizing the rhizosphere of plants considerably reduce heavy metal toxicity to plants and enhance the accumulation of heavy metals in the plants (Wang et al. 2009). Sheng and Xia (2006) reported that inoculation of cadmium resistant plant growth promoting bacteria enhance dry weight and Cd uptake of oilseed rape in Cd contaminated soils. Growth conditions, root surface area and ion exchange capacity of the roots have been advanced as the factors affecting uptake and removal of toxic metals by the plants (Salt et al. 1997). For instance, etiolated *B. juncea* has been found to accumulate Pb 1,250-fold from solution compared with lower values for green seedlings (Raskin et al. 1997). This was related to the larger root surface area and greater cell wall ion exchange capacity of etiolated seedlings (Salt et al. 1995a). Therefore, the process of phytoremediation depends on the extent of root development, biomass production and adaptability for growth in a particular soil environment (Salt et al. 1999). For enhancing the root development, biomass production and consequently phytoremediation potential of *Brassica* species, the modern agriculture requires the use of chemical fertilizers. But, these chemical fertilizers are expensive, hazardous to the environment and also are available in limited supply. Therefore, to explore the possibility of supplementing the inorganic chemical fertilizers with organic ones such as the biofertilizers of microbial origin is the need of the day. A reliable approach is the sustainable agricultural system which maintains and improves human health, benefits producers and consumers both economically and spiritually, protects the environment, and produces enough food for an increasing world population (Higa 1991). Sustainable agriculture that integrates environmental health, economic profitability and social or/and economic equity, is based on substantial use of beneficial soil microorganisms that hold tremendous potential for use to enhance plant growth and yield at a low cost (Higa 1991). Microbial inoculants are cheaper as compared to the chemical fertilizers, and are not hazardous to the environment. There are many species of beneficial soil microbes such as *Trichoderma* spp., *Bacillus megaterium*, *Verrucomicrobium spinosum, Phyllobacterium brassicacearum, Variovorax paradoxus* etc. flourishing in the rhizosphere of *Brassica* plants that, have immense potential to enhance plant growth by a plethora of mechanisms including phytohormone production (Mehnaz et al. 2001), complex substrate degradation and/or siderophore production (Nautiyal et al. 2000; Masalha et al. 2000) etc. and are therefore, of agricultural importance. There are techniques for the production of inoculum of the desired microbial strain and its application in soil to cause positive effect on plant growth and yield (Malik et al. 2005; Flores-Vargas and O’Hara 2006). The most reliable approach is to select microorganisms that are physiologically and ecologically compatible with each other, introduce them into the soil as part of a mixed culture at a sufficiently high inoculum density to have desirable positive effect on plant growth and yield (Higa and Wididana 1991; Parr et al. 1994).

Plant growth promoting activity of beneficial soil microbes has been attributed by several workers to their ability to enhance the availability of nutrients in the rhizosphere by mineralizing complex substrates and/or producing siderophores which facilitate the transport of certain metal ions, notably Fe^{3+} ions (Wang et al. 1993; Glick 1995; Kim et al. 1998; Rodriguez and Fraga 1999; Hyakumachi 2000). Some rhizospheric bacteria have been reported to produce siderophores which bind with ferric (Fe^{3+}) ions to form Fe^{3+}-siderophore complexes that can be easily absorbed by the root system of a number of plant species (Bar-Ness et al. 1991). Masalha et al. (2000) suggested that uptake of ferric ions as bacterial Fe^{3+}-siderophore complex by the plant roots plays a vital role in the overall iron requirement of the plants especially, in calcareous soils. Phosphate-solubilizing bacteria which occur commonly in the rhizosphere of many crop plants (Vazquez et al. 2000b; Nautiyal et al. 2000), have been reported to secrete organic acids and phosphatases that aid in the conversion of insoluble forms of phosphorous to plant-available forms (Kim et al. 1998) and enhance nutrient availability to host plants resulting in their better growth as well as yield (Richardson 2001). *Bacillus megaterium, Azotobacter chroococcum, Enterobacter agglomerans, Pseudomonas chlororaphis* and *Pseudomonas putida, Rhizobium* sp., *Bradyrhizobium japonicum* etc., residing respectively, in the rhizospheres of *Brassica* (Brown 1975), wheat (Kumar and Narula 1999), tomato (Kim et al. 1998), soybean (Cattelan et al. 1999) and radish (Antoun et al. 1998) have been reported to solubilize the phosphates and promote the growth of these crop plants. Altmore et al. (1999) investigated the capability of *Trichoderma harzianum* Rifai 1295-22 (T-22) to solubilize some insoluble or sparingly soluble minerals *in vitro* and reported that T-22 was able to solubilize MnO_2, metallic zinc and rock phosphate (mostly calcium phosphate) in a liquid sucrose-yeast extract medium. This phosphate solubilising activity of *T. harzianum* might be responsible for its plant growth promoting ability. Kang et al. (2002) reported the ability of *Fomitopsis* to solubilize tri-calcium phosphate. Richa et al. (2007) tested the efficacy of *Aspergillus tubingensis* and *A. niger* to solubilize rock phosphate and found that both these fungi had the ability to solubilize rock phosphate and also enhanced the growth and yield of maize in rock phosphate amended soil. El-Azouni (2008) tested the efficacy of *Aspergillus niger* and *Penicillium italicum* to solubilize tri-calcium phosphate (TCP) *in vitro* as well as their effect on the growth of soybean (*Glycine max*) *in vivo* and reported that both these fungi showed high TCP solubilizing ability on agar plates and their dual inoculation in pot experiments significantly increased the yield and dry matter of soybean plants.

Plant growth promoting activity of microorganisms residing in the rhizosphere of crop plants may be related to their ability to produce phytohormones including auxins, gibberellins, cytokinins and ethylene (Barazani and Friedman 1999; Unyayar et al. 2000) which in most cases are believed to change assimilate partitioning patterns in plants, affect growth patterns in roots resulting in bigger roots system with greater surface area enabling the plant to access more nutrients from the soil and consequently enhance their ability to accumulate heavy metals from the contaminated sites (Salisbury 1994). Barazani and Friedman (1999) observed that Indole-3-acetic acid (IAA) which induces root initiation, cell division and cell enlargement in plants, is very commonly produced by Plant Growth Promoting Rhizobacteria (PGPR) such as *Agrobacterium* sp., *Alcaligenes piechaudii*, *Comamonas acidovorans* associated with the rhizosphere of lettuce. Mehnaz et al. (2001) reported IAA production by the PGPR *Aeromonas veronii* and *Enterobacter cloacae* which are associated with the root system of *Oryza sativa*. The other plant growth promoting rhizobacteria which have been reported to produce IAA include *Azospirillum brasilense, Enterobacter* sp. residing respectively in the rhizosphere of wheat (Kaushik et al. 2000) and sugarcane (Mirza et al. 2001). *Rhizobium leguminosarum* and *Pseudomonas fluorescens* which stimulate the growth respectively, of rape or lettuce (Noel et al. 1996) and soybean (de Salammone et al. 2001) have been found to produce cytokinins which are known to induce cell division and cell enlargement (Salisbury 1994). *Bacillus* sp. that stimulate the growth of alder, have been reported to produce gibberellins (Gutierrez-Manero et al. 2001) which induce modification of plant morphology by the extension of plant tissue (Salisbury 1994). *Aspergillus niger, Aspergillus flavus, Penicillium corylophilum, Penicillium cyclopium*, *Penicillium funiculosum* and *Rhizopus stolonifer* have been reported to produce gibberellin (Hasan 2002). Plant growth stimulating effect of some rhizobacteria such as *Alcaligenes* sp., *Bacillus pumilus, Enterobacter cloacae, Pseudomonas* sp., *Variovorax paradoxus* has been attributed to their ability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that breaks down ACC, the immediate precursor for ethylene in the biosynthetic pathway in plants thereby decreasing ethylene production in the roots of host plants resulting in root lengthening, better pant growth and increased phytoremediation potential of the plants (Glick et al. 1998; Belimov et al. 2001; Saleh and Glick 2001). Elad (2000) studied the biological control of foliar pathogens of cucumber by means of *T. harzianum* and found that four foliar pathogens namely *Botrytis cinerea, Pseuperonospora cubensis, Sclerotinia sclerotiorum* and *Sphaerotheca fusca* causing grey mould, downy mildew, white mould and powdery mildew diseases of cucumber, respectively, were suppressed by *T. harzianum* under greenhouse conditions. Narisawa et al. (2002) reported that *Verticillium dahliae* causing wilt disease of eggplant was suppressed by *Heterconium chaetospira, Phialocephala fortinii, Penicillium* sp. and *Trichoderma* sp. *T. harzianum, T. viride* and *T. virens* have been found to suppress the mycelial growth of *Fusarium oxysporum* f. sp. *ciceris* and enhance the growth and yield of this crop plant (Dubey et al. 2007).

Plant-microbe symbiosis plays an important role in Phytoremediation (Wang et al. 2009). Plant rhizosphere microbe symbiotic association has been reported to be often more efficient in remediation of heavy metal polluted soil than single treatment (Labean et al. 2008; Wang et al. 2009). The microorganisms residing in the rhizosphere of plants have been reported to enhance phytoextraction of target heavy metals by promoting the growth of host plants or/and increasing toxic metal uptake in plant tissues (Wang et al. 2009). The rhizosphere microorganisms appear to be important in the removal of selenium because the treatments that reduce fungal or bacterial populations reduce plant uptake and accumulation of Se (de Souza et al. 1999). Successful manipulation of the rhizospheric environment to suppress pathogenic soil microbes, to optimize mineral uptake or to improve soil-plant water relationships can be achieved through a better understanding of the ecological complexity of the root environment (Macrae et al. 2000) The addition of soil amendments has been shown to facilitate the removal of this element from contaminated soils (Banuelos and Meek 1990; Banuelos et al. 1990). *Trichoderma harzianum* strain T22 has been reported to significantly promote the growth of willows that consequently resulted in increased phytoextraction of heavy metals by the tree (Adams et al. 2007). *Trichoderma atroviride* has been reported to affectively stimulate the growth of *Brassica juncea* in contaminated soils and also significantly increase the accumulation of cadmium and nickel in plant tissue (Cao et al. 2008). *Phyllobacterium brassicacearum* that resides in the rhizosphere of *Brassica* spp. has been reported to stimulate root morphogenesis in *Brassica napus* (Larcher et al. 2008).

5.4 Prospects and Challenges

Plants offer a potentially more cost-effective means of remediating contaminated sites (Palmer et al. 2001) as compared to engineering or other methods such as microbial remediation and chemical treatments (Palmer et al. 2001). Current research has emphasized the desirability of plants as candidates for the removal of both organic and inorganic pollutants (Cunningham et al. 1996; Salt et al. 1998). For plant species to be effective in phytoremediation, they must have a high rate of growth and biomass accumulation and be able to remove large amounts of contaminants and thus affect phytoremediation within a reasonable period of time (Palmer et al. 2001). They must be tolerant to high levels of such contaminants and be well adapted to polluted environments (Palmer et al. 2001). For phytoremediation of heavy metals, metal tolerance and hyperaccumulation may be more important than high biomass (Chaney et al. 1997). Genetic manipulation is likely to be of increased value in the production of plants with phytoremediation attributes and such species should be amenable to biotechnological techniques (Raskin 1996). In an evaluation of the potential of genetic engineering in plant improvement for phytoremediation, some workers have indicated the need to modify both metal tolerance and accumulation characteristics of the plant (Karenlampi et al. 2000). It has been suggested that the lack of well-characterised metal-resistance systems in plants was a limitation to genetic engineering aimed at phytoremediation (Karenlampi et al. 2000). A limitation to the use of resistance genes from microbial systems is the complexity and multigene nature of such resistances (Palmer et al. 2001). Even with such limitations genetic manipulation is still a very useful approach to understanding the basis of metal tolerance and accumulation (Palmer et al. 2001). In this review impact of plant growth promoting soil microorganisms on growth and phytoremediation potential of *Brassica* spp. was emphasized. A number of these plants are hyperaccumulators of heavy metals, and while they may not be suitable for direct use because of low biomass and slow rate of growth, they are a valuable source of genes for transfer to more desirable species (Palmer et al. 2001). Climate change at global level has led to drastic reduction in agricultural soil fertility. Because of this, modern agricultural practices require the use of chemical fertilizers that are of particular concern because of their high cost, development of resistance in the pathogens and most importantly the chemicals pose environmental hazards of various kinds. Twenty-first century agriculture needs to be more productive, environmentally benign, robust in the face of climate change and socially beneficial. A reliable approach is the sustainable agricultural system which maintains and improves human health, benefits producers and consumers both economically and spiritually, protects the environment, and produces enough food for an increasing world population (Higa 1991). Therefore, to explore the possibility of supplementing the inorganic chemical fertilizers with organic ones such as the biofertilizers of microbial origin, is the need of the day. There are many species of bacteria, actinomycetes, blue green algae (BGA), fungi including VAM (vesicular arbuscular mycorrhizae) and viruses that flourish in diverse habitats, have immense potential to enhance plant growth by a plethora of mechanisms including biological nitrogen fixation (Hashem 2001), phytohormone production (Mehnaz et al. 2001), complex substrate degradation and/or siderophore production (Nautiyal et al. 2000; Masalha et al. 2000) etc. and are therefore, of agricultural importance. There are techniques for the production of inoculum of the desired microbial strain and its application in soil to cause positive effect on plant growth and yield (Malik et al. 2005; Flores-Vargas and O'Hara 2006). The most reliable approach is to select microorganisms that are physiologically and ecologically compatible with each other, introduce them into the soil as part of a mixed culture at a sufficiently high inoculum density to have desirable positive effect on plant growth and yield (Higa and Wididana 1991; Parr et al. 1994). Microbial inoculants are cheaper as compared to the chemical fertilizers, and are not hazardous to the environment. Numerous microorganisms colonizing the rhizosphere of *Brassica* species such as *Phyllobacterium brassicacearum, Serratia plymuthica, Comamonas terrigena, Stenotrophomonas maltophilia, Pseudomonas fluorescens, Variovorax paradoxus Trichoderma* spp. etc. have been reported to enhance the growth, health, biomass and phytoremediation potential of this crop plant (Adams et al. 2007; Larcher et al. 2008). Among crucifer crop species, *B. juncea* exhibits superior heavy metal accumulation characteristics under experimental conditions of heavy metal stress and such features can be improved through conventional or molecular plant breeding techniques (Palmer et al. 2001). There is also the potential to exploit the adaptability of crucifer crop species to in vitro cell culture and transformation techniques to develop plants with a range of tolerance to heavy metals and organic pollutants (Palmer et al. 2001).

5.5 Conclusions

Toxic metals are known to contaminate agricultural soils and threaten crop production by their toxicity to plants (Wang et al. 2009). Heavy metals accumulate in human body through ecological food chain and eventually pose serious health hazards (Sanita-di Toppi and Gabbrielli 1999). Members of the family Brassicaceae have a key role in phytoremediation technology (Palmer et al. 2001). There are a number of *Brassica* and related crop species that have been reported as the potential candidates for phytoremediation (Palmer et al. 2001). The ability of *Brassca* spp. to complex heavy metals and detoxifies organics is related to specific sulpher-rich peptides or small proteins (Palmer et al. 2001). Although the role of metal complexing peptides such as phytochelatins in heavy metal tolerance has been questioned, there is enough positive evidence of their participation in resistance to metal toxicity to warrant manipulation of these components in *Brassica* species as part of a phytoremediation strategy (Palmer et al. 2001). Metal binding peptides and proteins have been well studied in microorganisms (Mejare and Bülow 2001). For effective phytoremediation a combination of plant species and microorganisms may be desirable, as all the requirements for remediation may not be found in a single organism (Palmer et al. 2001). Plant growth promoting soil microbes have been reported to play an important role in phytoremediation (Wang et al. 2009). The microorganisms residing in the rhizosphere of plants have been reported to enhance phytoextraction of target heavy metals by promoting the growth of host plants or/and increasing toxic metal uptake in plant tissues (Wang et al. 2009). *Trichoderma atroviride* has been reported to affectively stimulate the growth of *Brassica juncea* in contaminated soils and also significantly increase the accumulation of cadmium and nickel in plant tissue (Cao et al. 2008). *Brassica* spp. is colonized by several beneficial microbes such as *Trichoderma harzianum*, *Bacillus megaterium* etc. that can be isolated, mass cultured and utilized to maximize the biomass of this crop plant for use in phytoremediation technology (Brown 1975; Adams et al. 2007).

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Chapter 6
Phytoremediation of Heavy Metals by *Brassica juncea* in Aquatic and Terrestrial Environment

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Abstract Plant-based environmental remediation has been widely pursued by academic and industrial scientists as a favorable low-cost clean-up technology. Phytoremediation is being developed as an alternative technology for removing or, more accurately, reducing the concentration of toxic pollutants to clean up the environment. In the present research, potential of green plants have been screened for phytoremediation of heavy metals both from aquatic and terrestrial environment. Indian mustard *(Brassica juncea)* has been found as a potential candidate for phytoremediation of heavy metals. *B. juncea* has been used for remediation of Cd, Pb and Zn at varying concentrations, *viz*., 0, 5, 10, 20 and 50 ppm. The depletion of heavy metals was observed at the intervals of 0, 1, 3, 7, 14 and 21 days and metal uptake was studied in the roots/shoots of the plants. The percentage removal of Cd, Pb and Zn was found 88.9%, 80% and 89.8%, respectively at the higher exposure concentration (50 ppm). Similarly *B. juncea* has also been used for phytoremediation of heavy metals (Cd, Pb and Zn) at varying concentrations, *viz*., 0, 5, 10, 20 and 50 mg/kg from mycorrhizal soil in pot culture technique and uptake was studied in the roots/shoots; after harvesting the plants. The uptake of metals in roots was found 25,000 μg g^{-1} – Cd, 32,750 μg g^{-1} -Pb and 30,550 μg g^{-1} –Zn; whereas uptake in shoots was found 4,596 μg g^{-1} Cd, 3,469 μg g^{-1} Pb and 15,878 μg g^{-1} Zn at higher exposure concentration (50 ppm). The research study has proved effective remediation of heavy metals (Cd, Pb and Zn) by *B. juncea* in water-soil environment.

Keywords *Brassica juncea* • Heavy metals • Metal uptake • Phytoremediation

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Abbreviations

Cd	Cadmium
cm	Centimeter
kg	Kilogram
μg	Micro gram
μl	Micro liter
mg	Milli gram
ml	Milli liter
MS	Mycorrhizal soil
Pb	Lead
ppm	Parts per million
VAM	Vesicular arbuscular mycorrhiza
Zn	Zinc

6.1 Introduction

The industrialization and urbanization of the modern world has led to the proliferation of many different metals and compounds in our environment. Metals such as zinc, copper, nickel, manganese, magnesium, iron etc. are essential in very low concentrations for the survival of all forms of life, but are toxic in excess. Metal concentrations have a range of 1–100 mg kg^{-1} soil. If the concentration of a particular metal crosses the normal threshold, then it becomes potentially toxic and may lead to lethal changes. They build up in biological systems through food chain and become a significant health hazard. The most common heavy metal contaminants are: cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), nickel (Ni) and zinc (Zn) (USEPA 1997; Lasat 2002).

Natural contamination originates from either excessive weathering of mineral and metal ions from rocks or from displacement of certain contaminants from the groundwater or subsurface layers of the soil. The most common anthropogenic sources are human activities such as mining, smelting, electroplating, energy and fuel production, power transmission, sludge dumping, and melting operations, disposal of industrial effluents, deposition of air-borne industrial wastes, military operations, land-fill operations, industrial solid-waste disposal and the growing use of agricultural chemicals such as pesticides, herbicides and fertilizers (Okoronkwo et al. 2005; Jing et al. 2007; Igwe and Abia 2006; Lone et al. 2008). The threat of heavy metals to human and animal health is aggravated by their long-term persistence in the environment (Gisbert et al. 2003). There is an urgent need to develop multifaceted approaches for cleaning environment, which consume lesser resources and would be eco-friendly (Fulekar 2005a). Currently, conventional remediation methods of heavy metal contaminated soils include electrokinetical treatment, chemical oxidation or reduction, leaching, solidification, vitrification, excavation, and off-site treatment. These clean up processes of heavy metal pollution are expensive and environmentally destructive (Bio-Wise 2000; Aboulroos et al. 2006). In order to overcome some shortcomings of conventional methods, phytoremediation is introduced into remediation field (Meagher 2000; Wei et al. 2008).

Phytoremediation is defined as the use of plants to remove pollutants from the contaminated environment (Cunningham et al. 1995; Flathman and Lanza 1998; Salt et al. 1998; Weber et al. 2001). Actually, with some added advantages such as environmental beautification, easy acceptance by the public and potential application to a relatively large pollution area, phytoremediation of heavy metal contaminated soils is widely considered a promising remediation approach in the future (Chaney et al. 1997; Lewandowski et al. 2006; McGrath et al. 2006; Lai and Chen 2009). It utilizes the remarkable ability of the plants to concentrate or remove heavy metals from the environment. Plants are unique organisms equipped with remarkable metabolic and adsorption capabilities, as well as transport systems that can take up nutrients or contaminants selectively from the soil and water. Phytoremediation methods offer significant potential for certain application and permit a much larger site to be restored would generally be possible using more traditional remediation technologies (Fulekar 2005b). There are over 400 different plant species considered suitable to be used as phytoremediator. Indian mustard has been identified as a higher biomass producing plant with the capacity to accumulate zinc and cadmium at higher concentrations in plant cells (Kumar et al. 1995; Salt et al. 1995). After the completion of phytoremediation, the plants used for the study, can be harvested, incinerated and followed by recycling of the metals or as disposal in a landfill (Bennett et al. 2003; Angel and Linacre 2005). It results in reduction of metal contamination from the polluted site.

In the present study, Indian mustard plant has been screened and selected for the phytoremediation of heavy metals such as cadmium, lead and zinc in aquatic and terrestrial environment. Studies are conducted to determine the potential and uptake capacity of cadmium, lead and zinc by *Brassica juncea* from aquatic and terrestrial environment under controlled environmental conditions.

6.2 Screening of Plants

The success of phytoremediation depends on screening and selection of an ideal plant. A plant suitable for phytoremediation possesses the characteristics: ability to tolerate/accumulate metals, fast growth, effective accumulating capacity, high biomass and easily harvestable. Researchers have reported that plant species that are long-term competitors and survivors under adverse conditions normally have an advantage for phytoremediation. They adapt self operative defensive mechanism to survive in adverse environmental conditions. A screening for phytotoxicity and effectiveness of plant's cultivars/varieties is required on a site-specific basis as an initial step in plant selection (Pivetz 2001; Schnoor 2002). The plants which have potential to survive and grow in the contaminated environment are screened and reported by the Scientists for phytoremediation of heavy metals and radionuclides. The green plants which survive in such adverse conditions, but not exploited for phytoremediation of heavy metals have been studied and screened for the present research work. After studying the toxicity of heavy metals at varying concentrations, *Brassica juncea* has been screened as suitable plant for phytoremediation of heavy metals. Indian mustard (*Brassica juncea*), also known as mustard greens, and leaf mustard, is a species of mustard plants and belongs to the family Brassicaceae. The plant was selected for the phytoremediation study based on their fast growth rate/ high biomass along with the tolerance potential to thrive in heavy metals contaminated environment. This plant is used to remove heavy metals from the soil in the hazardous waste sites because it has a higher tolerance towards most of the toxic metals and very high capability of storing them in its parts. *Brassica juncea* is a good candidate for efficient phytoextraction of heavy metals such as Cd from polluted soils (Schneider et al. 1999). The plant is then harvested and disposed off properly. This method is easier and less expensive than traditional methods for the removal of heavy metals. It also prevents erosion of soil from these sites preventing further contamination.

6.3 Methodology Adapted

6.3.1 Plant Materials

Healthy seeds of Indian mustard (*Brassica juncea* L. Czern and Coss) var. TM4 (Trombay Mustard 4) were selected for the research studies. Mustard seeds were obtained from gamma field, Bhabha Atomic Research Centre, Mumbai. Seeds were pre-soaked in soap and dettol solution for 15 min and thoroughly washed in running tap water until the soap solution was completely removed. Then the seeds were sterilized with 70% ethanol for 30 s followed by sterilization with 0.1% mercuric chloride for 3–5 min. The seeds were thoroughly rinsed 5 times with sterile distilled water. These sterilized seeds were inoculated in test tubes containing MS (Murashige and Skoog 1962) basal medium supplemented with 3% sucrose. Seedlings were allowed to grow for 1 month under *in vitro* condition.

6.3.2 Hydroponic Experimental Setup

Uniform plants were selected for the uptake study. MS medium was drained off and replaced with hydroponics media i.e. Hoagland solution (Hoagland and Arnon 1938) containing nutrient solution for the acclimatization for 1 week prior to the experiment. They are then transferred to another Hoagland solution which contained each of the following heavy metal in a separate set up: Cadmium supplied as Cd $(NO_3)_2$. $4H_2O$; lead supplied as Pb $(NO_3)_2$; and zinc supplied as $ZnSO_4$. The different concentrations of metals used in these studies were 5, 10, 20, and 50 μg ml^{-1} with control. Each experiment was carried out in triplicates. Plants grown in nutrient solution without metals served as control. The sampling from aqueous solution containing metals has been carried at an interval of 0, 1st, 3rd 7th, 14th and 21st days *in vitro* condition to ensure that uptake of each metal is being taking place (Anamika et al. 2009). 500 μl of aliquots were withdrawn from each concentration with increasing period. These samples were used for the analysis of cadmium, lead and zinc content. The reduction in concentration of these metals in the medium was attributed to their uptake by the plants. At the end of the experiment the plant samples were collected and washed with de-ionized water twice and rinsed with distilled water. Each sample was divided into root and shoot and oven-dried at 60°C until completely dry. Dry weights of roots and shoots were determined and noted.

6.3.3 Soil Sampling and Characterization

The alluvial soil used in the pot experiment, was collected from a depth of about 0–15 cm along the banks of Surya River, Palghar (located 100 km away from Mumbai). The soil was air-dried and then passed through 2 mm sieve, and large stones and plant root debris were removed. This prepared soil was stored in a plastic bag at room temperature (27–30°C) until the use. The physico-chemical characteristics of the soil were measured by standard methods (Table 6.1). The content of heavy metals (Cd, Pb and Zn) in soil was estimated by atomic absorption spectrophotometer (APHA 1998).

6.3.4 Mycorrhizal Inoculum

Soil based mycorrhizal inoculum was developed by pot-culture technique at laboratory scale with the help of starter inoculums and using sorghum as a host plant. A starter culture of mycorrhizal fungi (VAM) was procured from Division of Microbiology, IARI, New Delhi. The physico-chemical parameters (Table 6.1) and microbial characterization (Table 6.2) of soil were done after the development of mycorrhizal soil. The developed mycorrhizosphere provide a direct link between soil and roots, and are renowned for their ability to increase plant mineral nutrients, notably P and enhance phytoremediation (Leyval et al. 1997; Gaur and Adholeya 2004; Bush 2008; Anamika and Fulekar 2010).

Table 6.1 Physico-chemical characterization of Alluvial soil and mycorrhizal soil[a]

Parameters	Methods used	Alluvial soil	Developed mycorrhizal soil
pH	APHA (1998)	7.2	7.3
Electrical conductivity (mMohs)	APHA (1998)	0.2	0.34
Moisture (%)	APHA (1998)	35	42.2
Water holding capacity (%)	APHA (1998)	65	67
Organic carbon (g/kg)	Walkley-Black method (Jackson 1973)	72	259
Nitrogen (g/kg)	APHA (1998)	5.8	8.4
Phosphorus (g/kg)	APHA (1998)	0.72	0.81
Sodium (mg/kg)	APHA (1998)	23	32
Potassium (mg/kg)	APHA (1998)	21	22
Heavy metal (ppm)	APHA (1998)		NA
Zn	APHA (1998)	10.5	
Cd	APHA (1998)	BDL	
Pb	APHA (1998)	BDL	

APHA American Public Health Association, *BDL* Below Detection Limit

[a] All the values are mean of three replicates

Table 6.2 Microbial status of developed mycorrhizal soil

Microorganisms	Species
Bacteria	*Alcaligens* spp. *Bacillus* spp. *Pseudomonas* spp. *Sarcina* spp. *Serratia* spp. *Streptococcus* spp.
Fungi	*Asergillus flavus* *A. fumigatus* *A. niger* *Penicillium* spp. *Rhizopus* spp. *Mucor* spp.
Actinomycetes	*Micromonospora* spp. *Nocardia* spp.

6.3.5 Pot Culture Experiment

Pot culture experiments were conducted in the green house. The growth medium in the pots consisted of alluvial soil and mycorrhizal inoculum (5:1) and treated as Mycorrhizal soil (MS). The alluvial soil without the mycorrhizal inoculum was treated as control. Mycorrhizal soil filled in each 1 kg capacity of pot (having perforated base for proper aeration and drain connecting system) and amended with heavy metals: Cd as $Cd(NO_3)_2 \cdot 4H_2O$; Pb as $Pb(NO_3)_2$ and Zn as $ZnSO_4$, separately. The various concentrations applied for each heavy metal was; 0, 5, 10, 20, 50 mg kg^{-1}.

The healthy seeds of Indian mustard (*Brassica juncea* L. Czern and Coss) var. TM4 (Trombay Mustard 4) were surface-sterilized with 0.1% mercuric chloride for 5 min and subsequently washed several times with distilled water to avoid fungal contamination. Six seeds were sown into each pot and the pots were randomly placed in a green house at an average diurnal temperature of 25–27°C, and a relative humidity 40–60%. Plants were watered to maintain soil moisture at 60–70% of water holding capacity by adding water during the experiment. One hundred milli liter of Hoagland solution (Hoagland and Arnon 1938) was given to growing plants once in a week. The drainage collected at the bottom of the pot was also added in the pots to avoid the loss of metals through leachate. The plants were grown for a period of two and half months.

6.3.6 Analytical Methods

Each sample (dried root and shoots) was digested with 10 ml of perchloric acid: nitric acid ($HClO_4$: HNO_3- 1:5 v/v) mixture separately. Acid digestion was carried out on hot plate at 70–100°C until yellow fumes of HNO_3 and white fumes $HClO_4$ were evolved. The digestion process was continued until a clear solution remains after volatilization of acids. The digestion was stopped when the residue in the flask was clear and white. The digested sample was dissolved in distilled water, filtered for the removal of impurities (APHA 1998) and made up to the desired volume. The samples were analyzed by GBC 932 B+ Atomic Absorption Spectrophotometer (Australia) using air-acetylene flame to estimate cadmium, lead and zinc contents in the plant samples.

6.3.7 Data Analysis

The heavy metals are taken up by the plants through their roots from the solution. Experimental data were analyzed for uptake of cadmium, lead and zinc by Indian mustard plants. The experiment was carried out in triplicates and average values are reported. Data were analyzed for mean and standard deviation (X ± S.D.) using standard statistical methods (Mahajan 1997).

6.4 Results

6.4.1 Phytoremediation of Heavy Metals in Aquatic Environment

The remediation of heavy metals (Cd, Pb and Zn) were carried out using *Brassica juncea* (Indian Mustard) from aquatic environment at the concentrations ranging from 0, 5, 10, 20 and 50 ppm for a period of 21 days. The healthy plants of *B. juncea* were grown in Hoagland solution spiked with various concentrations of Cd, Pb and Zn, separately. After the growth, the plants were harvested and analyzed for biomass and metal uptake of heavy metals in the roots/ shoots.

6.4.1.1 Depletion of Metals from the Solution

Metal depletion was studied to understand the potential of plant- *B. juncea* at various exposure levels. Figure 6.1a–c demonstrates the depletion of Cd, Pb and Zn from aquatic environment after 21 days of exposure period. *B. juncea* has remediated 35.2–88.9% Cd, 26–80.1% Pb and 30–89.8% Zn from lower to higher concentration of heavy metals. Pb uptake was found lower than Cd and Zn by *B. juncea*.

6.4.2 Dry Biomass Analysis

Brassica juncea was harvested after 21 days of phytoremediation of heavy metals from aquatic environment. Biomass of plant was found to be 0.009 g in roots and 0.063 g in shoots for Cd, 0.028 g in roots and 0.107 g in shoots for Pb and 0.015 g in roots and 0.061 g in shoots for Zn at 50 ppm exposure; after 21 days period. The significant difference in biomass was found for each metal exposure with increasing period as demonstrated in Table 6.3. The statistical analysis has been given for each heavy metal (Cd, Pb and Zn) for minimum 0–50 ppm concentrations. Result shows that *B. juncea* has tolerance potential to grow in contaminated environment and effectively phytoremediated metals.

6.4.3 Bioaccumulation of Metals in the Roots and Shoots of Plants

Bioaccumulation of metals has been studied in root/shoots of plant- *B. juncea*. Bioaccumulation of Pb, Cd and Zn in roots was found to be 12,264, 18,419 and 26,517 μg g^{-1}, respectively at higher exposure concentration (50 ppm), after 21 days exposure period. Whereas bioaccumulation of Pb, Cd and Zn in shoots of *B. juncea* was found 2,477, 3,349 and 2,585 μg g^{-1}, respectively at 50 ppm after phytoremediation of heavy metals in aquatic environment. Result shows that all the metals studied have bioaccumulated more in roots as compared to shoots (Fig. 6.2a–c) of *B. juncea*.

Phytoremediation studies show that Pb- 1,258 μg g^{-1}, Cd- 2,230 μg g^{-1} and Zn- 2,250 μg g^{-1} in roots of the plants at lower exposure level (5 ppm); whereas bioaccumulation of metals in shoots was recorded 254 μg g^{-1} Pb, 344 μg g^{-1}

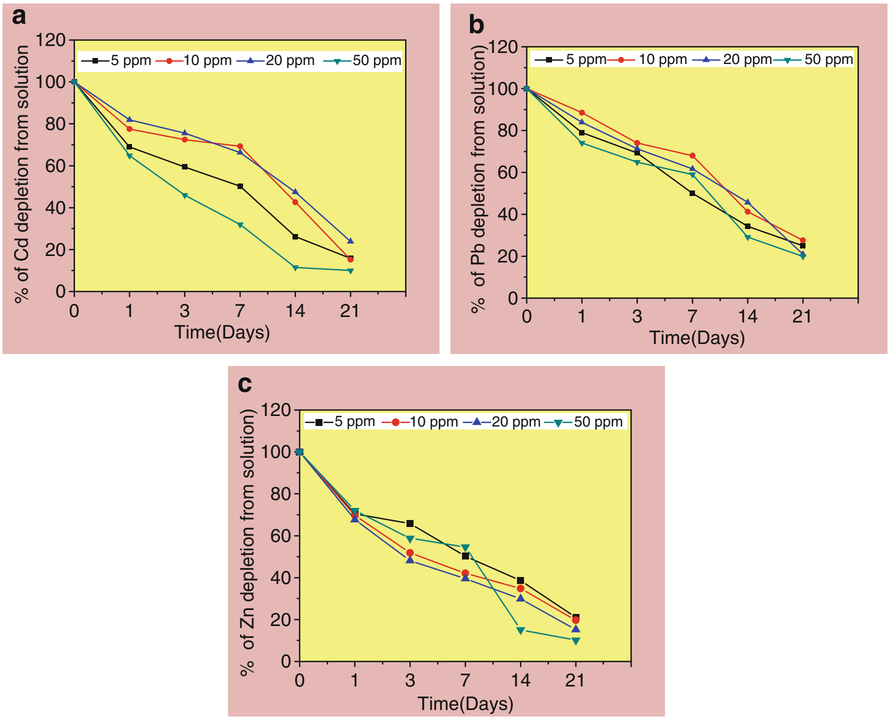

Fig. 6.1 Depletion of (**a**) Cd, (**b**) Pb and (**c**) Zn from the solution during phytoremediation experiments. The results show the depletion at various concentrations of Cd, Pb and Zn. All the values are mean of three replicates

Table 6.3 Dry biomass of *B. juncea* after 21 days of exposure to the heavy metals contaminated Hoagland solution[a]

Metal	Concentration (ppm)	Dry weight (g)	
		Roots	Shoots
Zn	Control	0.020 ± 0.008	0.063 ± 0.012
	5	0.016 ± 0.003	0.057 ± 0.007
	10	0.014 ± 0.002	0.056 ± 0.003
	20	0.013 ± 0.004	0.057 ± 0.008
	50	0.015 ± 0.004	0.061 ± 0.009
Cd	Control	0.011 ± 0.002	0.080 ± 0.017
	5	0.008 ± 0.004	0.076 ± 0.019
	10	0.005 ± 0.002	0.055 ± 0.006
	20	0.007 ± 0.003	0.063 ± 0.014
	50	0.009 ± 0.004	0.079 ± 0.009
Pb	Control	0.037 ± 0.007	0.124 ± 0.007
	5	0.022 ± 0.003	0.064 ± 0.009
	10	0.014 ± 0.004	0.072 ± 0.013
	20	0.017 ± 0.002	0.095 ± 0.017
	50	0.028 ± 0.005	0.107 ± 0.014

[a] Values are averages of three replicates ± S.D.

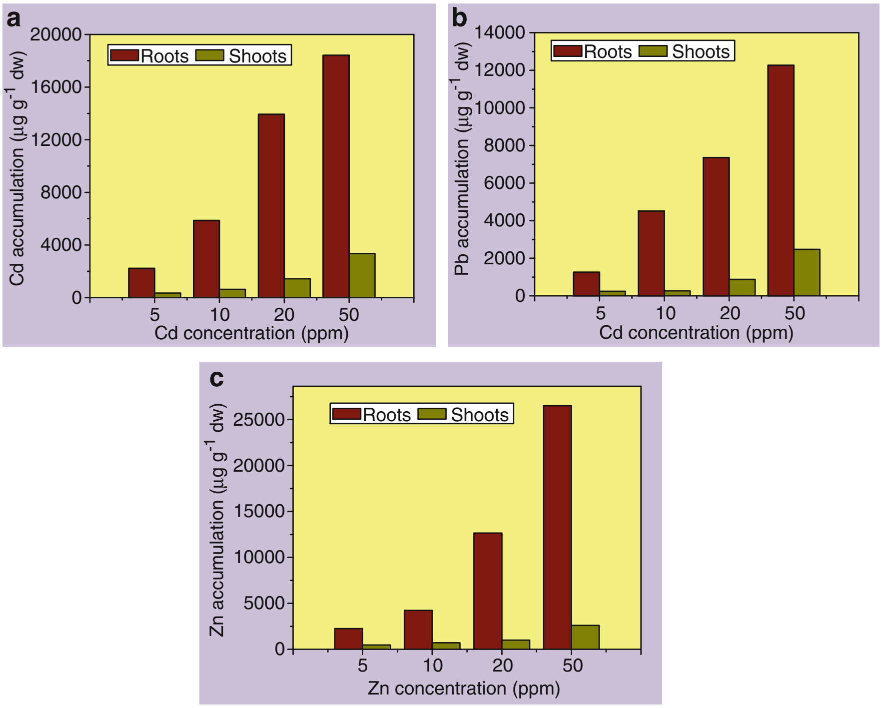

Fig. 6.2 Accumulation of (**a**) Cd, (**b**) Pb and (**c**) Zn in the roots and shoots of *B. juncea*. All the values are mean of three replicates

Cd and 471 µg g^{-1} Zn. The bioaccumulation of metals increased with increasing concentrations and found 12,517 µg g^{-1} Pb, 18, 419 µg g^{-1} Cd and 26,517 µg g^{-1} Zn in the roots of *B. juncea;* whereas 2,477 µg g^{-1} Pb, 3,349 µg g^{-1} Cd and 2,585 µg g^{-1} Zn in shoots at higher exposure level (50 ppm). The comparison of data analysis revealed that Cd was more accumulated in shoots than roots as compared to Zn and Pb. Metal accumulation in *B. juncea* was found to be 5.4, 4.9 and 5.96 times higher in roots as compared to the shoots in case of cadmium, lead and zinc respectively. The data showed that the heavy metals accumulation by *B. juncea* were found in order of Zn $>$ Cd $>$ Pb. The comparison has been made to demonstrate concentration of Pb, Cd and Zn in roots and shoots of *B. juncea* at varying concentrations. The results of the present study have shown that *B. juncea* has the potential for the uptake and accumulation of cadmium and lead. This plant may be of practical use for the decontamination of polluted water and soil containing these metals.

Brassica juncea – an identified potential green plant has been used for phytoremediation of heavy metals from aquatic environment. The depletion of heavy metals in aquatic solution and uptake by plants with increasing exposure period have been observed. After the proper growth, the plant biomass, uptake of each selected heavy metals in roots/shoots of the plants including their translocation factor to assess bioaccumulation potential of plants have been studied. The biomass of *B. juncea* was found higher upto the concentration of 10 ppm in case of Cd and Pb, and thereafter decreased as the exposure levels of Cd and Pb increased i.e. 20 and 50 ppm. Zn has shown positive effect on plant biomass. Heavy metals were found efficiently taken up mainly by the roots of *B. juncea* plants at all the evaluated concentrations. Similar findings were reported by Jadia and Fulekar (2008) for uptake of heavy metals (Cd, Cu, Ni, Pb and Zn) by fibrous root grass. Once metal ions are absorbed, they can be accumulated in the roots or be exported to the shoots via the transpiration stream (Ximenez-Embun et al. 2001).

Research has shown that metal concentration in plant tissues is a function of the heavy metals content in the growing environment (Cui et al. 2004), and that the uptake and accumulation of different metals may vary from plant to plant species. Kim et al. (2003) suggested that such discrepancies arise due to variation in type of heavy metals, its concentration, form of metal present and plant species. Different metals are differently mobile within a plant; cadmium and zinc are more mobile than lead and copper (Greger 2004). Cd is one the most toxic heavy metals due to its high mobility and the small concentration at which its effects on the plants begin to show (Vázquez et al. 1992).

Our results further showed that Pb is accumulated more in roots as compared to the other two metals (Zn and Cd). Pb uptake studies in plants have demonstrated that roots have an ability to take up significant quantities of lead whilst simultaneously greatly restricting its translocation to above ground parts (Lane and Martin 1977). Liu et al. (2000) have reported that *B. juncea* has considerable ability to remove lead from solutions and accumulate it in roots. Kumar et al. (1995) have also reported the higher accumulation of lead in roots of sorghum species, with indications that lead can be found on the outer surface of plant roots, as crystalline or amorphous deposits, and could be deposited in the cell walls or in vesicles.

The Cd and Zn uptake were found to be higher in shoot as compared to Pb. Zn and Cd have many physical and chemical similarities as they both belong to Group II of the periodic table. They are usually found together in the ores and compete with each other for various ligands. Thus the interaction between Zn and Cd in the biological system is likely to be similar. The fact that cadmium is a toxic metal and Zn is an essential element makes this association interesting as it raises the possibility that the toxic effects of cadmium may be preventable or treatable by Zn (Chowdhury and Chandra 1987).

6.4.4 Phytoremediation of Heavy Metals in Mycorrhizosphere

The research work carried out for phytoremediation of heavy metals by alfalfa in mycorrhizal soil as discussed above has been followed for *Brassica juncea*. Phytoremediation of cadmium, lead and zinc at varying concentrations viz. 0, 5, 10, 20 and 50 ppm using *B. juncea* in the mycorrhizospheric soil for a period of two and half months has been studied.

Table 6.4 Dry biomass of *B. juncea* after two and half months exposure to the mycorrhizal soil enriched with heavy metals[a]

Metal	Concentration (mg/kg)	Dry weight (g)	
		Roots	Shoots
Zn	Control	0.031 ± 0.003	0.472 ± 0.030
	5	0.050 ± 0.005	0.506 ± 0.043
	10	0.059 ± 0.004	0.622 ± 0.047
	20	0.045 ± 0.003	0.533 ± 0.024
	50	0.037 ± 0.004	0.486 ± 0.030
Cd	Control	0.031 ± 0.003	0.472 ± 0.030
	5	0.049 ± 0.004	0.522 ± 0.029
	10	0.045 ± 0.008	0.472 ± 0.041
	20	0.034 ± 0.005	0.326 ± 0.019
	50	0.021 ± 0.004	0.297 ± 0.046
Pb	Control	0.031 ± 0.003	0.472 ± 0.030
	5	0.045 ± 0.004	0.500 ± 0.018
	10	0.049 ± 0.002	0.466 ± 0.035
	20	0.036 ± 0.002	0.396 ± 0.013
	50	0.027 ± 0.005	0.288 ± 0.038

[a] Values are averages of three replicates ± S.D.

6.4.4.1 Dry Biomass Analysis

The dry biomass of *B. juncea* was recorded and presented in Table 6.4 for Cd, Pb and Zn metals at varying concentrations ranging from 5 to 50 ppm. Biomass of *B. juncea* was found to be 0.021 g in roots and 0.297 g in shoots for Cd, 0.027 g in roots and 0.288 g in shoots for Pb and 0.037 g in roots and 0.486 g in shoots for Zn at 50 ppm of exposure level over a period of two and half months in mycorrhizosphere pot culture experiment. *B. juncea* has produced high biomass in case of Zn as compared to Cd and Pb. Biomass decreased gradually as the concentration of Cd and Pb increased (i.e. 50 ppm) in mycorrhizal soil. The plant's biomass yield affected at the higher ppm levels exposure of Cd. Lead showed low effect on plant biomass. The positive effect has been seen in Zn concentrations exposure from lower to higher, resulting into high biomass yield.

6.4.4.2 Bioaccumulation of Metals in the Roots and Shoots of Plants

The study shows that heavy metals were efficiently taken at all concentrations using high biomass producing plant *B. juncea* grown in mycorrhizal soil. The mean uptake of metals Cd, Pb and Zn by *B. juncea* increased as the concentrations of these metals in mycorrhizal soil increased. In plant, shoots and roots were observed to have a characteristic uptake capacity for different metals. The research findings demonstrated that uptake of Cd from mycorrhizosphere by *B. juncea* was 2,783 μg g^{-1} at 5 ppm and 25,000 μg g^{-1} at 50 ppm in roots and 838 and 4,596 μg g^{-1} at minimum to maximum concentrations (0–50 ppm) in shoot, respectively. The uptake of Pb in roots was recorded 2,938 and 32,750 μg g^{-1} at

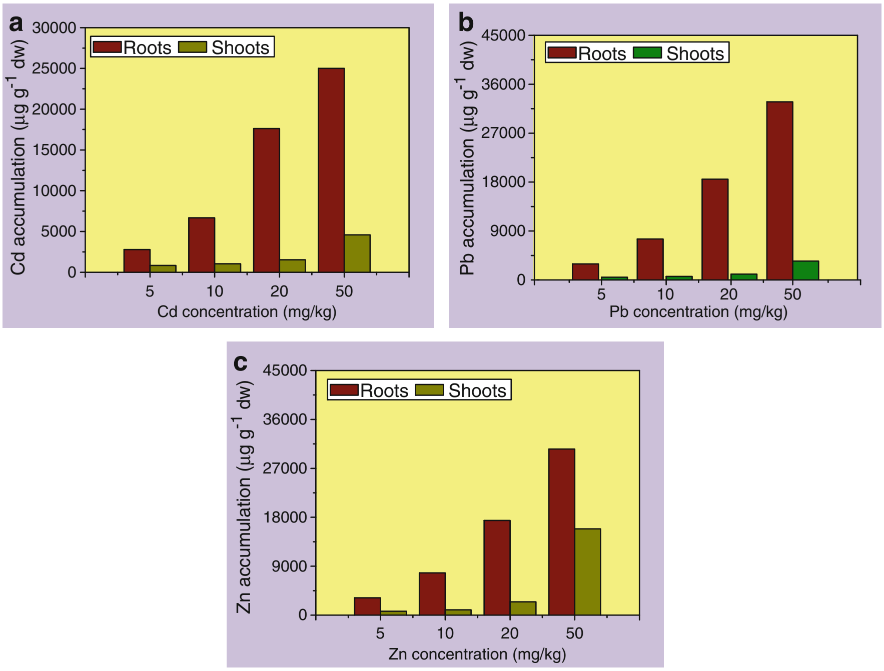

Fig. 6.3 Accumulation of (**a**) Cd, (**b**) Pb and (**c**) Zn in the roots and shoots of *B. juncea* during metal remediation from mycorrhizosphere soil. All the values are mean of three replicates

5 and 50 ppm, while in shoots it was found 486 µg g^{-1} at 5 ppm and 3,469 µg g^{-1} at 50 ppm. However, the uptake of Zn was found more as compared to Cd and Pb, reached highest accumulation, i.e. 30,550 µg g^{-1} in roots and 15,878 µg g^{-1} in shoots at highest concentration (50 ppm). The heavy metals were taken by the *B. juncea* in the following order: Zn > Cd > Pb. These results could be seen in Fig. 6.3a–c.

The accumulation of Cd, Pb and Zn was compared in roots and shoots of *B. juncea*. The metal uptake in roots was found 2,783 µg g^{-1} (Cd), 2,938 µg g^{-1} (Pb) and 3,175 µg g^{-1} (Zn) at lower doses (5 ppm). The uptake of metals in roots was increased upto 25,000 µg g^{-1} – Cd, 32,750 µg g^{-1} -Pb and 30,550 µg g^{-1} -Zn at higher concentration- 50 ppm. Similarly, the uptake of metal in shoots was found to be 838 µg g^{-1} Cd, 486 µg g^{-1} Pb and 705 µg g^{-1} Zn at lower doses (5 ppm); whereas uptake increased to 4,596 µg g^{-1} Cd, 3,469 µg g^{-1} Pb and 15,878 µg g^{-1} Zn at higher doses (50 ppm). The comparison of data analysis shows that *B. juncea* was found to accumulate 5.4 times more Cd, 9.4 times more Pb and 1.9 times more Zn in roots as compared to the shoots. The research findings show that Pb was bioaccumulated more in roots than shoots of plants as compared to Cd and Zn.

Phytoremediation of heavy metals using *B. juncea* has been studied in mycorrhizal soil using pot culture technique. The results showed an increasing trend for biomass production (Cd and Pb) as the concentrations increased from 5 to 10 mg/kg.

The positive effects have been seen in case of Zn concentrations. However, the biomass yield was found only affected at the higher concentrations, i.e. 20 and 50 mg/kg of Cd and Pb that shows inhibitory effect on plant growth. Higher doses of heavy metal can affect physiology, reduced plant growth and dry biomass. It is reported that Mycorrhizal fungi helps/ protects the plants against metal toxicity, however restricts the translocation of metals from root to shoot. Therefore, in the present research there were no toxicity symptoms like chlorosis, retarded growth etc. observed in case of *B. juncea* plants. In the present research, the significant effect on plant growth in mycorrhizosphere was observed. *M. sativa* plants have produced better biomass which results in higher uptake of heavy metals in mycorrhizosphere. The results showed the enhanced nutrients availability as well microbial communities in mycorrhizal soil, which favour the uptake of heavy metals by green plants. Higher levels of organic matter and nutrients content in the mycorrhizosphere had beneficial influence on soil chemical and biochemical properties and plant growth, thus increasing biomass yields. Various researchers have reported the similar findings where the mycorrhizosphere was shown to change soil structure by stabilizing aggregates (Miller and Jastrow 1990; Bearden and Petersen 2000; Augé et al. 2001), thereby enhancing soil-HM retention capacity in the plants.

The results showed that *B. juncea* efficiently bioaccumulated heavy metals at the varying concentrations range of 5, 10, 20 and 50 mg/kg. The accumulation of Cd, Pb and Zn was found increased with increasing concentrations of metals in the mycorrhizosphere. Root of the plants is the primary source of metals accumulation followed by shoot. The root of plants is being in the direct contact with mycorrhizal soil able to take up the heavy metals, along with their nutrients uptake ability. Once the metal is taken by the root of plants, it translocated to shoot via vascular tissues. In the soil-mycorrhizosphere, the mycorrhizal hyphae also help to take up the heavy metals from contaminated environment.

6.5 Conclusions

The green plant *Brassica juncea* has been identified as potential candidate for remediation of heavy metals – Cd, Pb and Zn from aquatic/ terrestrial environment. The research study shows that the plant has grown in contaminated environment and produced significant biomass (roots/shoots) which has found directly proportionate to uptake of heavy metals. The bioaccumulation of heavy metals was found higher in roots than the shoots of *B. juncea* for each of the heavy metal studied. The present study has proved the effective remediation of heavy metals by the green plant which could be buried or disposed off at safe environment. The higher concentrations of heavy metals bioaccumulated in plants can also be recovered and reused. Therefore, phytoremediation study will be beneficial for decontamination of heavy metals contaminated environment and/or extraction of metals for beneficial purpose.

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Chapter 7
Toxic Metals Accumulation, Tolerance and Homeostasis in Brassicaoilseed Species: Overview of Physiological, Biochemical and Molecular Mechanisms

Vinay Kumar, Monika Mahajan, and Sudesh K. Yadav

Abstract Brassicaoilseed and related cruciferous crop species of economic importance are identified as metal hyper accumulator with high biomass. These species possess genetically inherited traits of metal hyper accumulation and tolerance. They have been reported to store metal in their upper ground part with the character of metal tolerance. These species has been adapted with various mechanisms to counter metal toxicity. This adaption attracted everyone to understand range of mechanism in these plants with relation to accumulation of metal ion and tolerance to nullify metal ion mediated toxicity. Toxic metal influenced the various physiological processes such as growth, photosynthesis, ion and water uptake and nitrate assimilation in plant. At the cellular level, they have been reported to cause damage including blocking functional groups of enzymes, denaturing or inactivating enzymes, disturbance in the function of polynucleotide, transport mechanism for nutrient ions and disrupting cell and organelle membrane integrity. These symptoms might occur due to interaction of biomolecules with excessive amount of toxic metals. In addition, toxic metal excess stimulates the formation of free radical and reactive oxygen species. *Brassica* species and related cruciferous crop evolved to survive and thrive in metal toxicity and adapt a range of mechanisms that may be involved in the detoxification and tolerance. Plant antioxidant system scavenged free radicals ion induced by toxic metal exposure. Such tolerance has also been related to increased level of antioxidant molecules and detoxifying enzymes in response to toxic metal ions. Plants are synthesizing a variety of metal chelating legends including phytochelatins, metallothioneins and organic acids. These legends ensure metal detoxification by complexation and vacuolar sequestration. A whole range of metal transporter families have been identified in plant that

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could play a key role in tolerance and metal homeostasis. These plants possess genes for resistance to toxic effects of a wide range of metals. Apart from tolerance to metal toxicity, these plants also have fine balance of metals that are regulated either by preventing or reducing the entry into the cell or through efflux mechanisms. So, *Brassica* utilized the mechanism of accumulation, translocation and uptake of toxic metal more efficiently for tolerance. *Brassica* has been proposed as a natural environmentally safe option to clean contaminated sites. These species are well adapted to a range of environmental conditions and suitable for phytoremediation due to adequate accumulation with highly regulated translocation and uptake of toxic metal. These species are likely source of genes for phytoremediation. These plants will be playing a key role in phytoremediation technology and can be used for remediation of polluted areas. The adaptation ability of *Brassica* to toxic metals can be utilized to understand the mechanism of tolerance to toxic metals and development of toxic metal restricted plants in metalliferous soil.

Keywords Brassicaoilseed • Compartmentation • Homeostasis • Metal-toxicity • Phytoremediation • Tolerance

7.1 Introduction

Some heavy metals such as Fe, Co, Cu, Mo, Ni and Zn are essential for the growth and development of organism upto a limit (Ferreira 2011). While Pb, Cd and As are not required for living organism’s growth and development but these are used widely. Plants have highly developed physiological and molecular mechanisms to make them tolerate against these toxic metals. Plant absorbs, accumulates and detoxifies them from their sources (soil, water and air) through physical, chemical and biological processes. The ability of plant to absorb toxic metal contaminants from polluted soil has been used for remediation technique. Among all remediation techniques, attention has been diverted toward plant-mediated bioremediation (phytoremediation) due to cost effectiveness, non-intrusive and eco friendly nature. Efficiency of phytoremediation depends upon several soil and plant factors including nature of contaminant, chemical and physical properties of soil and plants ability to nullify metal toxicity. The plant ability accounts the uptake, accumulation, translocation, sequestering and detoxification for remediate metal mediated damage.

Phytoremediation involves mainly phytoextraction, rhizofiltration, phytostablization, phytovolatization, and rhizodegradation for remediation purpose from polluted soil (Mukhopadhyay and Maiti 2010). Phytoextraction involves harvesting and removal of aerial part of plant that accumulates metal ions. This practice helps the permanent removal of metals from soil. Plant can grow only on soil, which have low to moderate level of metals. But plant cannot sustain and perform their task of accumulation of metals in their aerial tissue in highly polluted environment. Plants that are used for phytoextraction have been reported to possess high biomass, fast growth rate and a well-developed root system. They have well-established network of biochemical and molecular mechanism for high metal accumulation and tolerance. The soil contaminated by Pb, Cd, Ni, Cu, Cr and V is detoxified using this process. The remediation from radionucleotides and uranium has been done with the help of *Helianthus annus* and *Brassica. juncea* by using this technique (Tome et al. 2009). Rhizofiltration utilizes the ability of plant root to uptake metal from aqueous medium. Plants that have significant amounts of root biomass, root surface area and ability to tolerate high metal concentration are suitable for this task. Rhizofiltration can be conducted *ex-situ* and *in-situ*. Indian mustard (*B. juncea*) and sunflower (*H. annuus*) are good examples of plants used for this purpose. They absorb Cd, Cr, Cu, Ni, Zn and Pb, U elements from hydroponics and soils medium, respectively (Tarun and Esringu 2007; Huhle et al. 2008). Phytostablization technique exploits plant potential to transform toxic metal in soil to less toxic forms. This technique stabilizes wastes and prevents their exposure. Further they alter physical, biological and chemical conditions of polluted soil to convert toxic form of metals in less or non-toxic forms. *Agrostis tenius* and *Festuca rubra* are used for stabilization of Pb, Zn and Cu polluted soils (Prasad and Freitas 2003). Phytovolatization uses the ability of plant to absorb metal contaminants from transpiration and convert them into less toxic volatile forms. This technique is suitable for metals like As, Hg and Se, which may exits as gaseous species in the environment. A number of plants such as *Chara canescens*, *B. juncea*, *Arabidopsis thaliana*, *Nicotiana tobaccum* and *Astragulus bisculatas* are used for absorbing metal vapors, from leaf inlets and help in reducing their toxicity (Cherian and Oliveira 2005). All those plants, that release enzymes in the rhizosphere to degrade organic pollutants are also, consider under phytodegradation. The released plant enzymes such as dehalogenase, nitroreductase, peroxidase, laccase and nitrilase in plant sediments and soils transform toxic metal ions into less or non-toxic ones.

Plants are divided into three types on the basis of their ability to accumulate toxic metal as accumulator, indicator and excluder plants. Plants, involved in different modes of phytoremediation, are generally termed as hyperaccumulator. Hyper accumulators are evaluated on the basis of their ability to absorb elements from contaminated soil, termed as biological absorption coefficient (BAC). The BAC is the ratio of element concentration present in plants to concentration in soil. Higher value of BAC for a single or group of elements indicates the plant as good hyper accumulator for that single or group of elements. More than 500 plant species comprising of 101 families have been identified as hyperaccumulators, including members of Asteraceae, Brassicaceae, Caryophyllaceae, Cyperaceae, Cunouniaceae, Fabaceae, Flacourtiaceae, Lamiaceae, Poaceae and Euphobiaceae families (Sharma 2011). Brassicaceae is known to posses highest number of taxa *i.e.* 11 genera and 87 species that are identified as hyperaccumulators of metals. Different member of Brassicaceae are promising candidates for phytoextraction of metals from metalliferous soils. At present Brassicaoilseed species are most viable candidates for the phytoextraction of a number of heavy metals such as Cd, Cu, Ni, Pb, U, Zn (Kumar et al. 1995). Among different member of brassicaoilseed species, mustard (*B. juncea* L.), Black mustard (*B. nigra* Koch), Turnip

(*B. campestris* L.), rape (*B. napus* L.) and kale (*B. oleracea* L.), *B. juncea* have great phytoextraction potential than others.

This article provides the understanding of different mechanisms of toxic metal accumulation; tolerance and homeostasis in plant and implication of various strategies in brassicaoilseed species to enhance their phytoextraction potential with latest updates. We also highlighted the use of chemical and biological approaches for optimization the toxic metal remediation by brassicaoilseed species.

7.2 Metals Bioavailability in Soil

Plants assimilate toxic metals through foliar or root absorption processes from natural and pollute environment. Plants efficiency to absorb toxic metals depends upon bioavailability of metals (Jing et al. 2007). The rate and extent of bioavailability of toxic metals in soil depend upon various parameters such as physiological properties of the deposited material, soil processes, soil properties, various plant factors, physical parameter of ion replenishment, kinetic parameters, regulating metal absorption by plant and metabolic behavior of metals (Cataldo and Wilddung 1978; Chamon et al. 2005; Lehto et al. 2006).

For successful uptake of metal from soil, a soluble species make close proximity with root membrane for some defined period. The interaction of metal ion with root membrane influences longevity and mobility of complex in soil (Wenzel 2009). This interaction also controls the rate and extent of uptake. The absorption of different toxic metal from environment and their mobility also depends upon the nature of elements. Only a small fraction of endogenous elements are soluble and available for absorption. So, bioavailability of metals in soil is generally indicated by the concentration ratio (CR values). The CR values are noted as total endogenous soil concentration of each element and on total metal amended (Cataldo and Wilddung 1978). The amended metals are also soluble in soil for availability. The absorption of metals from both pools is defined by their CR values. Only that pool provides the availability of element for plant absorption that has high CR values corresponding to that element. The metal after absorption is accumulated primarily in rhizodermis, cortex as well as in root hair. The uptake mechanism of metal ions is multiphase and metabolically regulated in plants. The absorption of metal ion by higher plant represents a series of distinct isotherms and each isotherm has different kinetic characteristics. The kinetics constants, K_m and V_{max} also govern the efficiency of transport mechanism (Lasat 2000). Each metal ion represents distinct kinetic phase based upon their corresponding K_m values. Concentration of metal ions also directly influences the uptake of ions. The absorption of metal ions are much more efficient in lower concentration than higher concentration of metal ions in soil. However the uptake mechanism for an individual metal ion may show competition with closely related anions and cations.

7.3 Metals-Mediated Toxicity

Elevated concentration of both essential and non-essential metals in soil mediates toxicity and inhibits plant growth. All metals are toxic at source level of solubility. But only a few metals in soil have been observed to cause toxicity in plants. Zn, Cu and Ni toxicities have been occurred frequently. Presently, research related to Zn, Cu, Ni, Cd, Pb and Hg mediated toxicity of more concern.

Excess of metal ions induce the generation of free radicals and cause oxidative burst. The oxidative stress includes a series of biochemical and physiological alteration in plants. There are three different mechanisms of metal mediated toxicity that can be distinguished at the cellular level for biochemical alteration:

1. Generation of free radicals and reactive oxygen species
2. Blocking of essential functional groups in biomolecules
3. Displacement of essential metal ions from biomolecules

The first mechanism to mediate metal toxicity involves free radicals and reactive oxygen species. The chemical properties of various metals determined the toxicity to living organisms. Metals of biological significance are divided into redox active and inactive metals. Only those metals participate in biological redox reaction that has redox potential higher then biological molecules such as Fe^{2+} and Cu^{2+}. These redox active metal ions undergo biological redox reactions and generate reactive oxygen species (Shah et al. 2001; Sharma and Dietz 2006). Reactive oxygen species comprises $^{\cdot}O_2^{-}$, H_2O_2 and $^{\cdot}OH$, having strong oxidizing activities. These ROS are intermediates generated during the successive reduction of O_2 to H_2O during metal toxicity. Metal ions shift the balance of free radicals towards an accumulation of H_2O_2. Redox active transition metals such as Cu^{+} and Fe^{+2} also activated the conversion of H_2O_2 into $^{\cdot}OH$ via Fenton reaction. Other metal such as Cr, V and Co also undergoes redox-cycling reaction (Fenton reaction and Haber Weiss reaction) and generate free radicals.

$$Fe^{+3}/Cu^{2+} + O_2^{-} \leftrightarrow Fe^{2+}/Cu^{+} + O_2 \tag{7.1}$$

$$H_2O_2 + Fe^{2+}/Cu^{+} \rightarrow \cdot OH + OH^{-} + Fe^{+3}/Cu^{2+}(\text{Fenton reaction}) \tag{7.2}$$

The overall reaction of combined steps is called Haber Weiss reaction

$$O_2^{-} + H_2O_2 \leftrightarrow O_2 + \cdot OH + OH^{-}\ (\text{Haber Weiss reaction}) \tag{7.3}$$

$$O_2 + Fe^{3+}/Cu^{2+} \rightarrow Fe^{2+}/Cu^{+} + O_2 \tag{7.4}$$

The oxidized metal ions further undergo re-reduction with superoxide radicals. These ROS caused irresponsible chemical modifications of various cellular components. As a result of oxidative stress, increased concentration of carbonylated proteins, malondialdehyde and increased production of ethylene has been reported in cell.

Second mechanism of metal mediated toxicity involves formation of strong bond with essential metal ions such as oxygen, nitrogen and sulphur of biomolecules (Schutzendubel and Polle 2002). These interaction blocks the essential group in biomolecules to perform normal function (Mishra and Dubey 2006). This binding strength is related to free enthalpy of the formation of the product of metal and ligands. Toxic metal ions arranged with increasing affinity for sulphides as Ni>PB>Mn>Zn>Cd>Co>Cu>HG>Ag (Schutzendubel et al. 2001). Due to this interaction, toxic metal block enzymes by binding to cysteine residues. Thus, interaction of metal with native protein resulted into denatured protein and do not function properly.

The position of essential metal ion in various enzymes and others are very important due to their functions. Toxic metal displaces essential metal ion and leads to inhibition or loss of enzyme activities (John et al. 2009). Toxic metals on the basis of their ability to seek out particular essential metal ions from biomolecules are classified into class A, class B and boardline. The class A (Al *etc.*) involves oxygen seeking metal ions while class B (Hg, Pb (IV) and Cu (I) *etc.*) comprises nitrogen and sulphur seeking metal ion. The boardline class consists intermediate toxic metal ions. Class B elements as Co, Ni, and Zn seek out the Mg^{2+} in RUBISCO and resulted into its loss of activity. The Cd displaces Ca^{2+} from calmodulin lead to inhibition of calmodulin dependent phosphodiestrase activity in radish (Garnier et al. 2006). The toxic metal ions also caused the metal catalyzed oxidation of proteins due to oxidation of amino acids such as Arg, Lys, Pro and His (Kasprzak 2002). The metal catalyzed oxidation has resulted in irreversible formation of reactive carbonyls on amino acid side chains.

As above discussed three mechanisms collectively caused biochemical alterations as lipid peroxidation, inhibition of enzymes, DNA damage, disturbance of the ionic homeostasis and increased ATP content. Metal mediated toxicity has also influenced various physiological processes such as growth, photosynthesis, ion and water uptake and nitrate assimilation in plant (Lagriffoul et al. 1998; Gajewaska et al. 2009).

Toxic metals act on membrane biogenesis and integrity, considered as primary sites of metal injury. This occurred due to altered lipid metabolism that inhibited the activities of enzymes such as fatty acids synthase and oleoyl-ACP desaturase (Chaffai et al. 2007). Toxic metal ions leaked out K^+ from the plasmalemma and decreased K content in root cells. The toxic metal ions not only influence K^+ and H^+ extrusion, functions of membrane carriers and ion channels but also alter the permeability of membranes for water. Toxic metal ions such as Cd^{2+} reduces ATPase activity of plasma membrane, changes the lipid composition of membranes and increases the contents of palmitic, linoleic and linolenic acids. Cu^{2+} toxicity leads the efflux of K^+ from root by changing cell permeability, damage cell membrane by oxidation of proteins, inhibits key membrane proteins, change the composition and fluidity of membrane lipids. The non-transition metal ions such as Cd^{2+}, Ni^{2+} and Hg^+ generated ROS by inhibiting the activities of antioxidative enzymes glutathione reductase and decreased the concentration of GSH. Arsenic (As) directly binds to thiols. Among nucleic acids, RNA is more severity affected due to its occurrence in protoplasm as compared to the DNA in nucleus. Toxic metals such as Cr^{2+} and Ni^{2+} have been reported to concentrate in ribo-nucleo-protein fractions. A similar report has been documented the influence of toxic metal on the structure of deoxyribonucleic acid (Kasprzak 2002). Reduction of DNA and RNA has been reported in germinating rice seedling (Gautum et al. 2011). Cd^{2+} has been reported to influence the level of nucleic acid synthesizing enzymes and nucleases, indirectly imparting negative influence on cellular metabolism. Zn^{2+} and Al^{3+} ions have been studied with their effect on DNA replication by influencing the DNA polymerase activity and increasing the rigidity of the DNA double helix, respectively (Meriga et al. 2010).

The high concentration of toxic metals has also been reported to ionic imbalance, loss of turgor and breakdown of cellular metabolism (Bertrand and Poirier 2005). Toxic metals affect the uptake of cations (K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Fe^{3+}) and anions (No_3^-) via diverse mechanism. Competition of Cu^{2+}, Zn^{2+}, Cr^{2+} and Ni^{2+} with Fe^{3+} for uptake from contaminated soil caused iron deficiency in plants. This type of competition also occurred for all other mineral nutrients because all of them are chemically similar (size of metal ion radii) and have similar uptake mechanisms. The toxic metal induced alterations in nature of membrane, and shifts the ionic homeostasis in cell (Hall 2002). Similarly, Mg^{2+} ion competes with K^+ for uptake and caused the K^+ deficiency. The acidic nature of soil caused by toxic metals is also responsible for nutrient deficiency. Cd^{2+} inhibits the transport of electrons and protons in the mitochondria, disorganizes the electron transport chain and decreases ATP formation.

Toxic metals decrease the photosynthetic rate of treated plants by influencing all the component of photosynthetic apparatus**.** The toxic metal ions in excess interfere with chloroplast replication and cell division. Toxic metals reduce the number of chloroplasts per cell and causes chlorosis due to low chloroplast density. This occurs either due to iron deficiency or influence caused by other metal ions. Toxic metal ions have been reported to disturb the chloroplast ultrastructure like composition of thylakoid membranes and electron transport (Bertrand and Poirier 2005). It has been reported that toxic metals affect both light and dark reactions of photosynthesis and declines the photosynthetic rate (Bertrand and Poirier 2005). The substitution of Mg^{2+} by Hg^+, Cu^{2+}, Cd^{2+}, Ni^{2+}, Zn^{2+} and Pb^{2+} in chlorophyll leads to the breakdown of photosynthesis as explained by Solymosi et al. (2004). This leads to the inactivation of photosystem II donor side (oxygen evolving centers), disturbs electron transport and reduces the demand of ATP and NADPH in Calvin cycle. The phosphorylation rate in presence of toxic metals is also decreased. In toxic metal polluted plant the CO_2 uptake by the stomata is rapidly decreased due to less number of stomata or closed stomata. Toxic metal ions also negatively influence the RUBISCO and PEPcase activity (Chaffai et al. 2007). The various enzymes of chlorophyll biosynthesis such as S-amino-levulinic acid dehydratase and NADPH: Pchlide oxidoreductase is also influenced by toxic metal ions due to blocking of sulfhydryl group (Wang et al. 2009). Toxic metal ions such as Cr (IV) also oxidize NADPH and influence the chlorophyll biosynthesis. The photosynthetic apparatus is more sensitive to the toxicity of Cr^{2+} toxic metal. Cu^{2+} has also been found to reduce the amount of chlorophyll-a and total chlorophyll in lichens and hamper photosynthesis. Ni^{+} is known to suppress photosynthesis electron flow and to impair photosynthesis activity by substituting Mg^{2+} in chlorophyll molecules.

Prolonged exposure of toxic metal ions resulted retardation in growth of treated plant. This occurred due to metabolic disorder and direct effect on growth. The toxic metals interact with cell wall polysaccharides and decrease cell wall plasticity. The root growth is much sensitive to toxic metal toxicity than shoot growth of plants (Kopittke et al. 2007). The impact of metal toxicity in plants is described by the root morphology due to decline in the rates of cell division and elongation in the root. The elongation in roots is inhibited by toxic metal mediated enhanced rigidity of root cell wall. Binding of toxic metal ions with HGA (Homogalacturonan) domains cause the rigid nature of cell wall. The retardation of root growth in presence of toxic metals occurred due to direct binding to DNA, metal induced aberrations, expansion of the mitotic cycle, cease microtubule development and reduction in glutathione pool. The growth of main root, the number and growth of lateral roots, leaf area, dry mass and plant height are some physiological indicators showing the influence of metal ion toxicity.

Excess toxic metals have been reported to affect the plant-water relations via modification of cell wall properties and growth retardation. Consequently, decreasing cell wall elasticity and reducing number and diameter of woody vessels. Further, they decreased stem hydraulic conductivity (Mckenna et al. 2010). The transpiration rate and water content in treated plant have been reported to decrease. This process occurred due to reduction in leaves area due to lesser growth, smaller guard cells, alteration in metabolites and ions responsible for maintaining turgor pressure. Toxic metals increase the ABA (Abscisic acid) content that is responsible for stomata closure, disordered respiration and oxidative phosphorylation and disturbs plant-water relations. Cu^{2+} has been reported to altered long-distance transport of water in plants (Pandey and Sharma 2002). Zn^{2+} has been reported to bind with water channel proteins of plasma membrane leading to reduce water uptake.

Toxic metals caused the considerable change in nitrogen metabolism, specifically the amino acid biosynthesis (Sutter et al. 2002). The first step of nitrogen assimilation is uptake of nitrate from soil by plasma membrane of root cells. Nitrate is then converted to ammonia via nitrite by the action of enzyme nitrate reductase and nitrite reductase subsequently. Ammonia is later assimilated into organic forms as glutamate and glutamine with the help of glutamine synthase and glutamate 2-oxoglutarate aminotransferase. The toxic metals influence all the levels of nitrogen assimilation. Initially, they restricted the uptake of nitrate ions from the soils. Cd^{2+} is also reported to reduce NR activity whereas insignificant change has observed in GS activity in pea plant (Kevresan et al. 2001) Toxic metals decrease the uptake of nitrate and disturbs the nitrogen metabolism. Al^{3+} affect the nitrate uptake is concentration dependent manner.

At low concentration of Al^{3+}, nitrate uptake is reported to increase but at high concentration nitrate uptake is restricted. Al^{3+} also acts directly on nitrate assimilation by inhibiting NR activity.

7.4 Plants Tolerance to Metal Toxicity

Plants tolerance to toxic metals is defined the ability to survive in toxic soil in which other plant species cannot survive. Plants tolerance includes all sensitive changes that occur due to metal mediated toxicity and further helps to provide the viability to plant upto an extent. At cellular level, plants have wide range of mechanisms that are involved in the detoxification, provide tolerance against toxic metal stresses such as sequestering of toxic metals in plant cell wall, formation of inactive metal ions form by chelating them using secreted organic compounds in soil, complexation of toxic metal ions in plant tissue and these transport to specialized cells or cellular compartments such as vacuole. Plants mechanism against toxic metal also includes chelating cysteine rich peptides and small proteins such as phytochelatins and metallothionenins. The phytochelatins show dual function of long distance translocation of toxic metal ions and protection of plant against metal mediated toxicity (Cobbett 2002; Chen et al. 2006). So, plant tolerances to toxic metals depend upon an interrelated network of physiological, biochemical and molecular mechanisms at cellular level (Table 7.1). Some well-studied mechanisms are listed as-

1. Extracellular exudates assisted uptake, accumulation of metal and cell wall constituents
2. Activation or modification of plant metabolism to provide tolerance and rapid repair of cell structure
3. Chelation of toxic metal ions with various chelating legends inside the cell
4. Compartmentation of toxic metal from cytoplasm to extra nuclear compartments such as vacuoles or trichomes

These mechanisms collectively maintain internal concentration of essential metals between limits of deficiency and toxicity, and have non-essential metals below their toxicity thresholds.

Metal tolerant plants have two basic strategies- metal exclusion and metal accumulation. Exclusion strategy includes the mechanism of extracellular exudates assisted uptake, accumulation of metal ions and cell wall constituents. This strategy of plant depends upon their ability to prevent the entry of metals inside the cell and excluded from cells to outside. While all other mechanisms are considered under accumulation strategy of plants. Plants to mitigate the influence of entered metal ions in cell adopt accumulation strategy. This includes different mechanisms as modulation at metabolism level by alteration of gene expression, rapid repair damage proteins, compartmentation of metals in specific compartments of cell etc.

Table 7.1 Summary of potential components of tolerance mechanism against toxic metals in plant

Mechanism	Significant feature/phenomenon suitable for remediation	Component/phenomenon to provide tolerance	Tolerance against toxic metal	References
Physiological	Longevity	Short life span	All major toxic metals	Kumar et al. (1995a)
	Phenotypic traits	Extensively network of root system		Kumar et al. (1995a)
		High biomass		Nehnevajova et al. (2007)
Biochemical	Extracellular uptake of metal	Root exudates	Ni and Al	Salt et al. (2000)
	Cell wall constituents	Polysaccharides	Various metal ions	Krzeslowska (2011)
	Plasma membrane	Reduced uptake by metal ion carriers or channels	As and Ni	Jabeen et al. (2009)
		Active efflux by efflux transport system and activation of signal cascade pathway	Various metal ions	Migeon et al. (2010)
	Altered metabolism	Activation of antioxidant system, glyoxalase pathway and synthesis of osmolytes	Various metal ions	Chao and Kao (2010) and Yadav et al. (2005a)
		Up regulation of Pentose phosphate pathway and down regulation of starch mobilization pathway	Various metal ions	Herbette et al. (2006)
	Repair mechanisms	Repair DNA damage	Various metal ions	Hamid et al. (2010)
	Complexation of metal ions	Phytochelatins	Cd, Al and Cu	Cobbett and Goldbrough (2002)
		Metallothioneins	Cu	Karenlampi et al. (2000) and Upadhyaya et al. (2010)

		Organic acids and amino acid	Various metal ions	Qiu et al. (2009) and Kerkeb and Kramer (2003)
		Metal binding proteins/Heat shock protein	Cd, Hg, Fe and Cu	Suzuki et al. (2001) and Lewis et al. (2001)
		Phenolic compounds	Ni and Al	Michalak (2006) and Winkel-Shirley (2002)
	Compartmentation of metals ions	Vacuole and trichome	Zn, Cd, Ni and Fe	Tennstedt et al. (2009), Carrier et al. (2003), and Choi et al. (2001)
Molecular	Modification at transcriptional level	Modulation of transcription factor interaction at upstream or downstream of gene	Al	Yamaji et al. (2009)
		Activation or inhibition of gene’s transcription by promoter modification such as DNA methylation	Cd and Pb	Aina et al. (2004) and Kovalchuk et al. (2005)
	Modification at posttranscriptional level	Silencing of specific gene by miRNA regulatory pathway	Cd and Cu	Huang et al. (2010) and Ding and Zhu (2009)
	Modification at translational level	Inactivation or activation of specific protein activity	Cd and Cu	Vassilev et al. (2003)
	Modification at posttranslational level	Inactivation or activation of specific protein activity	Cu, Cd and Ni	Janika-Russak et al. (2008)

7.4.1 Extracellular Exudates Assisted Uptake and Cell Wall Constituents

The entry of toxic metal in cell is prevented by two different strategies. First strategy involves the release of chemical by root to prevent the entry of metal ion. Second strategy involves the modification of cell wall constituents to make them less permeable for in migration of toxic metals inside the cell.

7.4.1.1 Root Exudates Assisted Uptake

Root exudates have a variety of roles, Extracellular exudates that are secreted either actively or passively by root of some plant species to absorb toxic metals of the soil and prevent them to enter inside the plant cells (Bertrand and Poirier 2005). These root exudates consist of various types of organic molecules such as reducing sugars, amino acids, phenolics and organic acids; such molecules cause the detoxification of metal ions externally and internally. At the root surface, mucilage uronic acid binds with toxic metals. The ability of uronic acid to bind with metals depends upon the type of toxic metal. These compounds decrease the pH of the soil, act as chelating molecules for toxic metals and reduce them (Weis and Weis 2004). As a result, these compounds influence the availability of micronutrients. In Al-tolerant plant species, plants release phosphate, organic acids and mucilage in with response to high concentration of Al^{3+} and prevent their uptake. They are also involved in Ni-detoxification strategy (Salt et al. 2000). Oxalic acid is secreted by the root of buckwheat in response to Al and has been reported to accumulate non-toxic Al-oxalate in the leaves (Hall 2002).

The bioavailability and absorption of toxic metals by the root are also influence by various other factors such as pH, redox potential, soil type, cation exchange capacity, metal concentration, and competing ions. Bioavailability of toxic metals in soil regulates the metal uptake by the plants (Lasat 2000). Most of the toxic metals enter a cell as cationic elements (Zn^{2+} etc.) while some enters as anionic group (As^{2+} etc.) and organic compounds (CH_3Hg). The uptakes of toxic metals by plants depend upon the root interception of metal ions, entry of metal ions into the roots and their translocation to the shoot by mass flow and diffusion. Root exudates, specifically metal reductase and proton extrusion from roots assisted the uptake of metal ion from soil into plant. The bioavailable metal ion enters inside the plant either through intercellular (symplast) and extra-cellular (apoplast) transport. The root epidermis and root cortex are part of extracellular transport. This transport system is unregulated and limited by high cation exchange capacity of cell wall (Jabeen et al. 2009). In extracellular transport, membrane provides strong electrochemical gradient for the inflow of metal ions (Ghosh and Singh 2005).

7.4.1.2 Cell Wall Constituents – Barrier of Metal Uptake

Toxic metal ions interact with cell wall during water solution uptake from the soil and are also excluded by specific transporter proteins or by exocytosis mechanism from the protoplast (Krzeslowska 2011). Cell wall behaves as defensive during toxic metal ion stress. It acts as sink and actively gets modified during metal ion stress. The modified cell wall has been reported to accumulate more toxic metal ions and decrease permeability against migration of metal ions in cytosol. Polysaccharides, constituent of cell wall binds significantly with divalent and trivalent metal cations due to presence of –COOH, –OH and –SH functional groups (Krzeslowska 2011). So, the nature of polysaccharides in cell wall determines the capacity of cell wall to bind with metallic cations. Polysaccharide pectins are major constituent of cell wall of higher plants. Pectins consist of four major domains, but HGA domain is well studied with regard to its binding ability with toxic metal cations due to its presence of free carboxyl group (Krzeslowska 2011). The interaction between the carboxyl groups of HGA domain with toxic metal cations depends upon the charge density of pectins and ionic strength of the toxic metal cations. The Cu^{2+} and Pb^{2+} cations bound strongly to pectins whereas Zn^{2+}, Ni^{+} and Ca^{2+} have been reported to bind less strongly with pectins (Krzeslowska 2011). Further the calcium ion is replaced by other strong cations such as Al^{3+}, Pb^{2+}, Cu^{2+} Cd^{2+} and Zn^{2+}. By this mode of action, cell wall participates in the formation of inactive form of cations, accumulation of toxic metal cations to high concentration and reduces the uptake of active metal ions inside the cell upto an extent. Hyperaccumulator plants adopt this strategy to grow in toxic metal enriched soils. Cell wall undergoes modification in response to the influence of heavy metal ions. This modification includes increased polysaccharide especially pectin constituents of cell wall and induction of callos synthesis. An increase in pectin level under toxic metal stress has been reported in the roots of variety of plant such as *Cucurbita maxima*, *Triticum aestivum*, *Z. mays* and *Solanum tuberosum* (Le Van et al. 1994; Hossain et al. 2006; Schmohl and Horst 2000; Schmohl et al. 2000). The elevated polysaccharide as pectin during toxic metal stress is used to increase the accumulation capacity of cell wall. Callos (ß-1, 3-glucan) acts as physical barrier against the penetration of metal ions and others inside protoplast. Callos synthesis under heavy metals has been observed under a wide range of plant species (Krzeslowska et al. 2009). Callose is accumulated in the cell wall around plasmodeta in response to heavy metal treatment in roots. Callose caused the blockage of cell-to-cell transport by blocking plasmodesmata resulted into prevention of a wide incursion of excess or toxic metal ions. However, in *Lemna minor*, callos did not provide evidence of protection against the entry of metal ions to cell protoplast (Krzeslowska 2011).

The anatomy of root is prevents the entry of metal ions through cell wall. The multilayer cortex appears to nullify the metal toxicity on other tissues by binding most of these in the cell wall and acts as barrier for inside movement of metal ions.

7.4.1.3 Modulation of Plasma Membrane Composition

Plasma membrane is the prime target of toxic metal attack. Toxic metal ions caused ions leakage from cells and rapidly influence the function of plasma membrane. They cause the oxidation and cross linking of protein thiols, inhibition of key membrane protein and change the composition and fluidity of membrane (Hall 2002). These entire disturbances collectively affect the permeability of the ion uptake. Maintenance of plasma membrane integrity is one of the specific features of toxic metal tolerant Brassicaoilseed species. Toxic metal ions cause alterations in the composition of the plasma membrane and change the membrane permeability (Hall 2002). The degree of peroxidation further alters many metabolic processes. Among Brassicaoilseed species, *B. juncea* posse more stable cellular membrane to cadmium stress as compared to *B. napus* (Nauairi et al. 2006). Besides having more resistant plasma membrane or improved repair mechanism, tolerant species has been reported to maintain the ionic homeostasis by efflux mechanism, preventing and reducing entry of metal ions into the cell. The complete exclusion of metal ions is not possible because some are essential ions. The efflux of toxic metal ions is energy dependent process in bacterial system. Efflux transport systems are also reported in animal cells. A number of transporters are identified to participate in the efflux of toxic metal ion outside in plant. Tolerant species also prevent the entry of toxic metal ions by releasing some chemical in soil that make these metal ions them unavailable for uptake and reduce their concentration inside the cells by using transporter proteins.

7.4.1.4 Metal Ion Carrier Proteins of Plasma Membrane

Once metal ions are absorbed, they are partitioned between the root system and the shoots. The ability root to retain toxic metals and than pass it to the shoots also depend upon various factors such as plant species, nature of metal, and the concentration of metal ions. Brassicaoilseed species have the ability to transfer ions to shoot with higher extent and shows one of the best features for phytoremediation.

Root to shoot movement of ions depends upon the transporters for absorption, sequestration and storage of essential and toxic metals in plant. The movement of metal ions in plant cell is assisted by a number of metal ion carriers or channels (Jabeen et al. 2009). A number of metal transporters have been identified to play a crucial role in tolerance during heavy metal stress. The ZIP1-4, ZNT1, IRT1, 2, COPT1-5, AtNramp1/3/4, LCT1 and CNGC metal transporters are present on the plasma membrane-cytosol interface (Hall and Williams 2003). However, transporter proteins present across the membrane of plastid, vacuole, golgi and ER have been studied with details. The ZIP4 and PAA1 transporter proteins assisted the accumulation of Zn^{2+} and Cu^{+} from cytosol to plastid, respectively (Hall and Williams 2003). Vacuolar transportation of toxic metals is done with the help of

AtNramp3, ZAT, ABC-type and CAX2 proteins (Hall and Williams 2003). RAN1 and ECA1 transporter family have been reported to assist accumulation of toxic metals in Golgi and ER (Hall and Williams 2003). These transport systems are involved in acquisition, distribution and homeostasis of toxic metals in plants. The gene families corresponding to these transporters are quite large and this diversity helps the plant to cope with various type of stress, for movement of ions at both cellular and sub cellular levels. These gene families also provide low and high affinity transport systems for metal ions in plants.

ATP binding cassette (ABC) superfamily is involved in a wide range of transport functions (Martinoia et al. 2002). They are powered by ATP hydrolysis and transport varieties of substrate including ions, sugars, lipids, peptides, xenobiotics, antibiotics and toxic metals. The *Arabidopsis* genome encodes about 130 ABC proteins however most of these proteins are not fully understood.

Natural resistance associated macrophage proteins (Nramps) is a family of integral membrane proteins. It is widely distributed in living organisms and is involved in transport of broad range of divalent metal cations (Forbes and Gros 2001). Nramps genes are distributed among all plant families and show as high number of homologues per species. In plants, these appear as two distinct subfamilies independent from clusters formed by animals, bacterial and yeast sequence (Migeon et al. 2010). The Group I includes AtNramps 1 and 6 (*Arabidopsis*), LeNramp1 (Tomato) and OsNramps (Rice). A GROUP II includes AtNramps 2–5 (*Arabidopsis*) and OsNramps (Thomine et al. 2000; Mäser et al. 2001). Both group I and group II are required for proper homeostasis of essential metal ions and transport of toxic metal ions in all plant organs such as Fe^{3+}, Mn^{+} and Cd^{2+} (Curie et al. 2000; Thomine et al. 2000). They also perform different physiological functions. In *Arabidopsi*s, Nramps family members such as AtNramps, AtNramp3 and AtNramp4 are up regulated in root system of iron deficient plants. The AtNramps 3 and AtNramp4 have also been reported in the shoots and have indicated their involvement in the translocation of metals between different organs.

Cation diffusion facilitators (CDF) family members are present in all type of organisms such as bacteria, yeast, animals and plants (Migeon et al. 2010). These are involved in Co^{2+}, Cd^{2+} and Zn^{2+} toxic metals transport. CDF proteins are involved in various physiological processes such as toxic metals uptake and catalyse efflux presence in both plasma membrane and in intracellular membranes. These have been well characterized as ZnT zinc efflux transporters of human and rodents. Four genes of ZnT transporter, ZnT 1-4 have been identified in mammals. In *Arabidopsis*, ZAT (zinc transporter as ZAT1) has been reported. ZAT1 and ZAT2 showed homology with ZnT-2 and ZnT4, respectively. In plant, transporters of this family have a role in zinc sequestration. Overexpressing *ZAT1* in plants, increased zinc content of the root under high zinc exposure (Van der Zaal et al. 1999). This is expressed in all part of plants and has not been reported with increasing zinc concentration. H^{+} coupled cation antiporter (Cation/H^{+} antiporter) have been identified at the vacuolar (tonoplast) membrane, and driven by proton electrochemical gradient. They play various roles in cell such as controlling cytosolic levels of Ca^{2+} post signaling, vacuolar sequestration of toxic metal ion and vacuolar storage of essential metal ions. In *Arabidopsis*, two subfamilies of cations exchanger genes have been identified as CAX1 and CAX2. These have high and low efficiency for Ca^{2+}/H^{+} exchanger, respectively (Hirschi 2001; Gaxiola et al. 2002). CAX1 have properties similar to native plant vacuolar H^{+}/Ca^{2+} exchanger. Two domains in CAX1 have been identified to modulate their activity as Ca^{2+} domain and autoinhibitory domain. The Cd^{2+}/H^{+} antiporter might be encoded by CAX2 and involved in accumulation of Cd^{2+} into the vacuole. *CAX2* overexpressing tobacco has been reported to accumulate more Ca^{2+}, Cd^{2+} and Mn^{2+} ions. These transporters are also involved in transport of metal ions in organelles such as chloroplast and mitochondria. The Cu^{2+} influx transporter (COPT1) has been reported in plants, which are believed to be homologous in function with the CTR1 copper transporter of yeast (Pulg and Thiele 2002). COPT1 has been reported to be present in flowers, stems and leaves but not in roots. It may be possible that another homologous of COP1 is present in roots. LCT1 cloned from wheat root is non-selective transmembrane transporter involved in uptake of Na^{+}, Cl^{+} and Cd^{2+} in cell (Clemens et al. 1998; Amtmann et al. 2001). Normally, LCT1 has been reported to mediate transport of calcium transport but also transports toxic metals.

Magnesium transporters are also involved in metal transport. AtMHX transporter from Arabidopsis functions as a H^{+} exchanger with Mg^{+} and Zn^{+} and localized in the vacuolar membrane (Hall and Williams 2003). Another family of Mg transport (AtMGT) gene has been identified in *Arabidopsis* (Li et al. 2001). The AtMGT1 protein from AtMGT family has been reported from plasma membrane and showed high affinity with Mg^{2+} ion. AtMGT1 also helped in the transport of Ni^{+}, Ca^{2+}, Fe^{3+}, Mn^{+} and Cu^{2+} when they are present in high concentration. The YSP1 and FRD3 transporter from maize and *Arabidopsis* has been reported to involve in iron homeostasis (Curie et al. 2001; Rogers and Guerinot 2002). A putative cation transporter family homologous to the animal cycle nucleotide-gated channels has been reported in *Arabidopsis* (Mäser et al. 2001). This protein contains six transmembrane domains and a calmodulin-binding site. These proteins are plasma membrane located and permeable for both monovalent and divalent cations (White et al. 2002).

7.4.2 Metabolism Alterations to Provide Tolerance and Rapid Repair of Cellular Structure

The cell wall acts as primary toxic metal sensing site, where a variety of signaling molecules are generated in response to extracellular stimuli. After sensing the toxic metal, plant cell activates specific genes to counteract the stress stimuli. Plants make sure tolerance against toxic metal by different activities such as biochemical and physiological reactions, and by activation of repair mechanism.

7.4.2.1 Metabolic Alterations to Provide Tolerance Against Metal Toxicity

Toxic metal transmitted the information within individual cells and throughout the plant by activating signal transduction pathways. The signals mediated by toxic metal initiate a variety of pathways that involve receptor-coupled phosphorylation, phospho-inositol-coulped Ca^{2+} changes, mitogen-activated protein kinase cascades and transcription activation of stress-responsive genes (Xiong and Zhu 2001). The signal generated by these pathways lead to expression of a range of genes and further leads to modification of various metabolic activites. Toxic metals also induce the signal by calcium and calmodulin as secondary messenger, reducing glutathione-oxidized ratio, generating by ROS and regulating the hormonal synthesis. The protein posttranslational modification and protein-protein stress interactions are also involved in signaling during toxic metal stress.

Receptor coupled phosphorylation receptor like kinases are also involved in the perception and transduction of extracellular signals during toxic metal stress. Receptor-like protein kinase HvLysMR1 of barley has been reported to be induced during toxic metal stress (Quelhadj et al. 2007). Intracellular parts of receptor-like kinases undergo auto phosphorylation of the intracellular part that is required for interaction with downstream regulatory factors in the connected signaling pathway. Phospho-inositol derived second messenger play an important role during the transmission of signal within the cells. Binding of toxic metals to the receptor sites on the membrane releases inositol phosphate generated from the hydrolysis of membrane bound phospho-inositol bisphosphate. One of the inositol phosphates, inositol 1, 4, 5-trisphosphates is mediator of Ca^{2+} release from intracellular stores that helps in the signaling cascades. Mitogen-activated protein kinase cascade is involved in the intracellular signal transduction process during biotic and abiotic stresses. The sequential phosphorylation- activation of different components transmits and amplifies the signal from the MAPKKK to the targets. Toxic metals have been reported to activate MAPKs in several plant systems such as rice and alfalfa (Liu et al. 2010). MAP kinase activities has been reported higher in Cd^{2+}-tolerant than Cd^{2+}-sensitive cultivars of rice (Yeh et al. 2007). Arsenic has been reported to activate MAP kinase in rice roots and leaves (Rao et al. 2011). Cyclic nucleotide gated channels (CNGCs), permeable to monovalent and divalent cations, contains a cytoplasm-localized cyclic nucleotide binding domain and an overlapping calmodulin binding site at the C-terminus of the polypeptide. The over expression of *NtCBP4* in tobacco confirms tolerance to toxic metals (Sunkar et al. 2000).

The toxic metals disturb the glutathione-oxidized ratio. This disturbed ratio activates other defense mechanism due to their involvement in redox signaling (Meyer 2008). During this signaling pathway, glutathione interacts with ROS, redox molecules and plant hormone. Decreased reduced glutathione-oxidized ratio by toxic metals directly influenced the regulation of growth, development, cell cycle, gene expression and protein activity due to their effect on the redox state of the cells (Ogawa 2005; Yadav 2010; Anjum et al. 2010, 2011, 2012). The disturbance in this ratio also influences the transfer and storage of sulfur (Szalai et al. 2009). ROS are common byproducts of aerobic metabolism. In photosynthetic tissues, chloroplast and in non-photosynthetic tissue mitochondria and peroxisomes are prime source of ROS generation. The toxic metal stress increased the rate of ROS production. A number of enzymes are assigned to maintain the ROS within a limit to prevent the cell damage. Overproduced ROS triggered signal transduction mechanisms by antioxidant activity and metabolic adjustment to maintain ROS within a limit. ROS act as signals of oxidative stress in toxic metal stress response (Jaspers and Kangasjarvi 2010). NADPH oxidases (Rbohs for respiratory burst oxidase homologs) are identified as important ROS generating system in plant. Role of Rbohs has been reported to generate ROS during toxic metal stress (Pourrut et al. 2008). The central stress signaling molecule in plant, Ca^{2+} is also associated with ROS.

The glyoxalase system consists of two enzymes, glyoxalase I and glyoxalase II to maintain the level of cytotoxic methylglyoxal in plants. The up regulation of glyoxalase I activity has been reported in response to toxic metal stress treatment in *brassica* (Veena et al. 1999; Yadav et al. 2005). The elevated levels of glyoxalase I activity is required for the removal of methylglyoxal. The up regulation of mitochondrial glyoxalase II has been also reported in response to Zn treatment from *B. juncea* (Saxena et al. 2005). The overexpression of glyoxalase pathway genes in transgenic plant, maintain the MG levels under stress conditions, regulate glutathione homeostasis and help the plants to tolerate metalliferous soils (Singla-Pareek et al. 2006; Yadav et al. 2007).

Toxic metals have been reported to modulate the hormonal balance inside the cell. The ethylene synthesis has been induced by toxic metal treatment. The activated ethylene level causes the induction of senescence process and rigidity of cell wall. The Cd^{2+}, Cu^{2+}, Fe^{3+}, Zn^{2+} toxic metals have been reported to induce ethylene production. The Cu^{2+} has been reported to stimulate ethylene production via increase of ACC synthase activity and its gene expression. Toxic metals induced jasmonate pathway and with increased ethylene level also have induced lipooxygenase activity (Maksymiec 2007). The jasmonate concentration has been reported to accumulate in plant treated with Cd^{2+} or Cu^{2+}. Jasmonate acts as stress molecule to adversely affect apparatus and growth while accelerate the senescence process. The ability of toxic metals to induce signaling pathway can be utilized to activate plant resistance pathway against other stress. The mild dose of Cd^{2+} has been reported to be increase plant resistance against viral and fungus infections (Maksymiec 2007). This occurred due to induction of defense pathways against pathogens by toxic metals. It helps to understand the common role of defense enzyme in both toxic metal and pathogen stress. Toxic metals also induce the NO signaling inside the cell. NO is a widespread intracellular messenger with regulating various physiological processes. The NO might act as antioxidant by scavenging the ROS inside the cell; the depletion of NO by cadmium stress is correlated with antioxidant role of NO (Rodriguez-Serrano et al. 2006). The NO act as antioxidant agent and protect from oxidative stress in Cd^{2+} treated sunflower leaves and Ni^{+} treated *B. napus*. (Laspina et al. 2005; Kazemi et al. 2010). Cd^{2+} and Cu^{2+} induced the accumulation of NO in *B. juncea* and *Pisum sativum* (Bartha et al. 2005). Oxylipins as jasmonic acid and its immediate precursor

12-oxo-phytodienoic acid (OPDA) are accumulated with response to toxic metal stress. In addition, a number of oxylipins are also induced non-enzymatically during oxidative burst by ROS by toxic metal treatments. Oxylipins act as signaling molecules and induce the expression of genes. Brassinosteroids act as phyto-harmone and play ameliorative role as plant to cope with biotic and abiotic stresses (Hayat et al. 2006). 28-homobrassinolide has been reported to enhance the antioxidants level under cadmium stress in *B. juncea* (Hayat et al. 2006). 28-homobrassinolide at 10^{-9} M concentration lowered Zn uptake and bioconcentraction factor (Sharma et al. 2007). The pre-sowing treatments of 28-homobrassinolide decreased the uptake of metals and enhanced the activities of antioxidant enzymes in *B. juncea* L. seedlings. The 24-epibrassinolide has been reported to regulate the diminution of Cr metal toxicity in *B. juncea* L. plants (Arora et al. 2010). Brassinosteroids bind with membrane proteins and help in detoxification of toxic metal stresses (Arora et al. 2010).

The toxic metal stress induced signals are transmitted by number of pathways that overlap and cross talk. Activation of phosphorylation cascades, Ca-calmodulin system, ROS signaling and stress-related hormones eventually regulate the transcription factor. These transcription factors further activate a number of gene sets to make response against toxic metal stress. The toxic metal altered plant metabolism either by modulation of transcription factors or transcription activation of stress responsive genes. For varieties of metal and plant species, it has been reported that tolerance is controlled small number of major genes with additional modifiers (Bert et al. 2003). Now with the help of cDNA microarray and gene chips, it is possible to investigate the response of several genes at transcriptional level against toxic metal stress. They are involved in different cellular response and metabolic processes including transcriptional regulation, stress response, nutrient and ion transport, cell metabolism, hormone homeostasis, and detoxification and programmed cell death. Expressions of these genes form a complex regulatory network to adapt plants against metal toxicity. The gene expression analysis of *Arabidopsis* plant with Cd^{2+} and Pb^{2+} exposure has been carried out (Kovalchuk et al. 2005). This analysis revealed much more information about the up regulation and down regulation of genes. In root, genes related to sulfur assimilation-reduction and glutathione metabolism has been reported to induce on Cd^{2+} exposure. In leaves, Cd ions exposure induced several genes encoding enzymes involved in biosynthesis of phenylpropanoids metabolite. A number of transcription factors including *bZIP*, *WRKY*, *MYB*, *ADR6*, and *NAc* have been reported to up regulate in Al^{3+} stressed plant. Gene expression of many transporters in plant is regulated at the transcription level by extracellular metal concentration via transcription factor proteins. Toxic metals also induce specific DNA demethylation in roots of *Trifloium repens* L., and *Cannabis sativa* L. (Aina et al. 2004). This phenomenon might permit plants to survive during metal stressed condition. The protein-protein interaction during toxic metal exposure also plays important role in signaling cascade.

The identification of miRNAs in response to toxic metals in plants; act as integral component of regulatory mechanisms of plant tolerance to toxic metals.

MicroRNAs are non-coding small RNAs (22 nucleotides), complementary to their target genes to halt their translation or induce their degradation. They plays crucial role in the regulation of plant responses to toxic metal stress. A number of conserved and non-conserved miRNAs from a range of plants as rice, *Medicago truncatula*, *B. napus* and *Arabidopsis* has been reported in response to toxic metal (Khraiwesh et al. 2012). The list of miRNAs has been updated recently in *B. napus* with response to sulphate deficiency and cadmium stress (Huang et al. 2010). MicroRNAs as miR398 has been reported to regulate the copper homeostasis by negatively regulating the expression of CU, Zn-superoxide dismutase. MicroRNAs such as miR393 and miR171 has also been reported to play an important role during cadmium stress (Ding and Zhu 2009). Seven miRNA families belonging to different cellular responses and metabolic process in rice have been reported to show differential express ions during oxidative stress. This indicates that diverse miRNAs from complex regulatory networks coordinate plants responses of plant against toxic metal stress.

Toxic metals directly or indirectly influence the expression of gene related to nutrient and ion transport to provide tolerance to plants. The Cd/Zn treatment in plants also causes significant changes in the uptake of other mineral nutrients (Kupper and Kochian 2009). With increased concentration of Zn, Mn concentration is decreased. With addition of Cd, the uptake of Mg, Ca, and S has enhanced in shoot in treated *Thlaspi caerulescens* (Kupper and Kochian 2009). The iron concentration is decreased under Cd toxicity in *T. caerulescens*. Toxic metals also influence expression of various genes related to normal cellular activities.

A number of metabolic changes have been observed in plants with response to toxic metal stress. The alteration in the activity of acid phosphatase has been reported in response to by a number of metal ions. Metal ions act as activator or inhibitor for acid phosphatase activity. The phosphate analogues metal ions such as arsenate, molybdate and vanadate act as potent competitive inhibitor of plant acid phosphatase. Toxic metals such as Cd, Cu and Ni have been studied to alter the H^+-ATPase activity at post-translational level in *Cucumis sativus* roots (Janicka-Russak et al. 2008). Cd has been reported to down regulate the genes encoding enzymes of chlorophyll synthesis pathway and protein PS1 and PS11 in *Arabidopsis*'s leaves. Some enzymes involved in calvin cycle as ribose-5-phosphate isomerase and fructose-bisphosphate aldolase has been reported to downregulated in response to Cd stress in *Arabidopsis* leaves (Herbette et al. 2006). Genes encoding enzymes of pentose-phosphate pathway such as glucose-6-phosphate-1-dehydrogenase, gluconolactonase, phosphogluconate dehydrogenase, 3-hydroxy-iso butyrate dehydrogenase, transketolase also has been reported to decrease in Cd stress in *Arabidopsis* (Herbette et al. 2006). The genes encoding the α- and β-amylases have been also observed to up regulate during Cd stress. The down regulation of pentose phosphate pathway and up regulation of starch mobilization pathway might be providing the compensation for decrease in carbohydrate synthesis (Herbette et al. 2006). Sulphur transport system is also induced due to increase in sulphur containing phytochelatins in toxic metal treated plants.

Toxic metals also cause change in hormonal level of plants. An increase in ethylene production has been reported in Cu treated barley plants (Vassilev et al. 2003). Cu and Cd induced the expression of ACC synthase gene, increased activity of these protein and increased ethylene production. The ethylene enhances inhibitory effect of toxic metals especially Cu on different part of dicotyledons plants (Maksymiec and Krupa 2006). Toxic metal stress is involved in the accumulation of jasmonic acid while ABA and cytokine content has been altered in plants treated with toxic metal (Prasad 1995). The altered phytoharmonal levels during toxic metal stress further induce changes in gene expression. A number of genes related to antioxidant defense mechanisms and detoxification has been unregulated with respect to toxic metal stress. The activity of various proteins involved in defense and detoxification mechanism has been reported during toxic metal stress. Toxic metals result into oxidative stress and subsequently ROS- dependent programmed cell death. Cadmium has been reported to cause oxidative burst and eventual cell death in tobacco (Garnier et al. 2006). Toxic metal caused the release of calcium from internal pool and further accumulation of NADPH oxidase-dependant hydrogen peroxide leading to cell death. The membrane peroxidation and superoxide radical generated by toxic metals also contributed to cell death. Cd, Al, Cu and Mercury have been reported to cause the ROS-dependent cell death (Garnier et al. 2006; Ortega-Villasante et al. 2007).

Modulation of expression of various genes resulted into alteration of various enzymes involved in biosynthesis of primary and secondary metabolites in plant cells. Plants treated with toxic metal showed differential responses in synthesis and accumulation of various biomolecules. The deficiency of œ-tocopherol causes the accumulation of ascorbate and glutathione in *vte1* mutants of *Arabidopsis* (Kanwischer et al. 2005). On the contrary, Asc and glutathione levels have been observed to decline in response to accumulation of œ-tocopherol in *vte1* overexpressing plants (Kanwischer et al. 2005). In *B. juncea*, œ-tocopherol plays an important role in alleviation of metal toxicity. Osmolytes such as proline and glycinebetaine have been identified to play role during oxidative detoxification in plant (Kumar and Yadav 2009). These osmolytes such as proline and betaine also acts as protein stabilizers, metal chelators, inhibitors of lipid peroxidation and free radical scavengers. Glycinebetaine acts as osmoregulator and accumulate naturally in plants. Glycinebetaine also stabilizes photosynthetic reactions and cell membranes. Proline has been reported to accumulate under metal toxicity in many plant species viz. *Oryza sativa*, *Triticum aestivum*, *Cajanus cajan*, *Vigna mungo*, *Vigna radiata*, *B. juncea* and *H. annus* belonging to distinct families. Proline is accumulated in plant because of toxic metal stress but as a result of the water stress induced by these metals. These osmolytes also alleviates the damaging effects of oxidative stress in Plants. Secondary metabolites biosynthesis has been reported to induce in many plant species with toxic metal treatment. The plant yield has been reported to enhance with toxic metal treatment in some plant species. Co and Ni at low doses have been reported to increase uptake of both macro and micronutrients. Thus, they affect plant height, number of branches, and shoot biomass, size and number of leaves per plant. The elevated levels of glutathione and phytochelatins during metal toxicity enhanced the net photosynthesis rate and stimulated nitrogen metabolism in plants.

7.4.2.2 Activation of Repair Mechanism

Toxic metals induced altered growth patterns and metabolic profile of plants by making direct interaction with nucleic acids (Hamid et al. 2010). ROS is the primary cause of single-strand breaks in the DNA either directly or by covalent modification of bases. Repair of these mutagenic modified bases is carried out by excision repair pathway. So, repair of DNA damage is very crucial for maintenance of genomic integrity and for providing tolerance against toxic metal exposure As a result of ROS action on DNA during toxic metal exposure, the cell cycle is halted to same time for DNA repair and induction of genes required for repair and protection of cellular components from metal toxicity.

The first group includes proteins that likely function by protecting cells from dehydration, such as the enzymes required for the biosynthesis of various osmoprotectants, late-embryogenesis abundant proteins, antifreeze proteins, chaperones, and detoxification enzymes. The second group of gene products includes transcription factors, protein kinases, and enzymes involved in phosphoinositide metabolism.

7.4.3 Conjugation of Toxic Metal Ion Inside the Cell

Detoxification via conjugation is one of the known defense mechanism of higher plants. The toxicity of conjugates compared to patterns compounds is significantly decreased because of binding with non-toxic cellular compounds (Yadav 2010). Conjugates are kept in cell for a certain periods of time without causing visible pathological deviation from cell homeostasis. Enzymes such as oxidases, reductase, esterase and transferase are involved in conjugation of toxic metal ions to cell endogens compounds by covalent bond formation.

After metal ion uptake by roots is either stored in roots or translocated to shoot via xylem. The pace of metal ions translocation determined by the concentration of accumulated metal ions in roots (Jabeen et al. 2009). After ions entered in the cell, chelators and chaperones are associated with metal ion mobility. Chelators help in metal detoxification by maintaining metal ions in cytosol. The immobilized metals with chelator in form of metal chelators complexes are very stable and less toxic than free metal ion. Chaperones transport metal ion to organelles and metal requiring proteins (Clemens et al. 2001). Chelators include amino acids (free histidine acids), organic acids (malate, oxalate and citrate) and peptides (phytochelatins and metallothioneins). Phytochelatins and metallothioneins are enzymatically synthesized and genetically encoded peptides. Cells avoid the cytotoxic effects of toxic metal ions by sequestering the ions in stable and intracellular macromolecular complexes with the help of chelators. Among toxic metal binding chelators, phytochelatins and metallothioneins are characterized in plants.

7.4.3.1 Amino Acids and Organic Acids

Plants synthesize a set of diverse metabolites on exposure to toxic metals such as amino acids proline and histidine, peptides such as glutathione and phytochelatins, and amines such as spermine, spermidine, putrescine and nicotianamine.

7.4.3.2 Peptides

Metal binding proteins and peptides increase the ability of metal tolerance/accumulation in plants. These metal binding peptides or proteins are preferentially metal specific. Plants produce variety of ligands for Cd, Cu, Ni, and Zn. The phytochelatins and metallothiones are very important part of metal detoxifying mechanisms in higher plants.

Toxic metal ions form complexes with cysteine rich small peptides called phytochelatins that has been reported in a wide variety of plant species and organisms. Phytochelatins are considered an important part of detoxifying mechanism in the chelation of heavy metals by a family of peptides ligands. They are made up of only three amino acid residues- Gly, Cys and Glu. The structure of the phytochelatins (Y-Glu-Cys) $_n$-Gly in which n is normally 2–5 and reported as high as 11. The Glu and Cys residues are linked through a Y-carboxyamide bond. The C-terminal amino acid may be glycine, alanine, serine or glutamate (Oven et al. 2002). Glutathione, homo glutathione, hydroxymethyl glutathione or y-glutamylcysteine acts as substrate for the synthesis of phytochelatins and this reaction is catalyzed by phytochelatin synthase (PCS) (Blum et al. 2007). This enzyme is activated at post translation level by toxic metal ions both in vivo and in vitro. This gene has been successfully characterized in *Arabidopsis* and yeast. Phytochelatins are structurally related to their precursor glutathione (GSH; Y-Glu-Cys-Gly). However, structurally variant phytochelatins such as (Y-Glu-Cys)$_n$-B Ala, (Y-Glu-Cys)$_n$-Ser and (Y-Glu-Cys)$_n$-Gly have also been reported in some plants. The cysteine residues and high percentage of glutamic acid residues ensure metal binding (Bertrand and Guary 2002). Metals such as Cd, Hg, Ag, Cu, Ni, Au, Pb and Zn act as inducer for the biosynthesis of phytochelatins but Cd has been reported to act as strong inducer of phytochelatins. Thus, plants can withstand with Cd toxicity by maintaining high levels of phytochelatins or its precursor glutathione. Like Cd, phytochelatins are also involved in response against Cu toxicity. Like Cd, Cu is also very strong activator of PC biosynthesis both in vivo and vitro and can form stable complexes with phytochelatins (Cobbett and Goldsbrough 2002). Toxic metal exposure, decrease glutathione concentration as a result phytochelatins biosynthesis is started. The expression of genes involved in

GSH synthesis (gsh1 and gsh2) and phytochelatins synthase has been reported to induce Cd treated leaves of *Arabidopsis* (Semane et al. 2007). The nature of toxic metals to form complex also depends upon the age of tissue (Kupper et al. 2004). The Pc-metal complexes also undergo long distance transport from roots to shoots. A gene PaPCS1 has been identified to be involved in long distance root to shoot Cd^{2+} transport in *Arabidopsis* (Gong et al. 2003). The phytochelatins also help in sulphur metabolism and antioxidant defense of the plant.

Metallothioneins (MTs) is defined as a group of low molecular weight cysteine-rich, metal binding proteins varying from range 5–20 kDa. In their reduced state, MT provides thiols for metal chelation. They are broadly distributed in prokaryotes, eukaryotic microorganisms, animals and plants. They can be divided into two different classes on the basis of their cysteine content and structure (Mejare and Bulow 2001). In plants, MT genes encode peptide that contains 60–80 amino acids, rich in cysteine residues. The metal ions are coordinated with cysteine residues of MTs protein through mercaptide bonds. On the basis of location of Cys residues, the MT-like proteins are divided into two types, Type I and II (Vasak 2005). Type I contained Cys-x-Cys motifs while type II contained Cys-Cys and a Cys-x-x-Cys pairs in the N-terminal domain. The type I gene have been reported, out of which three are identified to plant of Brassicaceae family. They have seven amino acid links in-between one and two domains and seventh residues is predicted in domain 2. Type II class contains 27 genes in which domain one and two contains eight and six cysteine residues, respectively. The introduction of mammalian MT gene confirms Cd tolerance in *B. comestris*, *B. napus* and *N. tabacum* by increasing chelation of metal with MTs (Karenlampi et al. 2000). MTs forms strong complex with Cu that are induce due to Cu exposure (Upadhyaya et al. 2010). The MT2 mRNA level has been reported to strongly induce by Cu treatment. In *P. sativum*'s, *PsMTa* gene have been identified to regulate the synthesis of MT-like proteins (Karenlampi et al. 2000). The MTs also act as antioxidant and decrease level of MT has been reported to increase the level of free transition metal ions (Wong et al. 2004; Briat 2002). In addition, MTs also show NO- scavenging capacity and are involved in plasma membrane repair.

7.4.3.3 Glutathione

Glutathione is non-protein sulphur containing tripeptides. It is related to sequestration of toxic metals, xenobiotics and is actively involved in cellular antioxidant defense system (Yadav 2010; Anjum et al. 2010, 2011, 2012). Plants detoxify many organic contaminants by conjugating them or their metabolites to GSH for storage or for further metabolism. This conjugation is catalyzed by the glutathione-S-transferase (GSTs). Glutathione is also the precursor of toxic metal chelating compounds such as phytochelatins and glutathione oligomers. Glutathione exits in reduced and oxidized states. In a healthy cells and tissue, more than 90% total glutathione pool is in reduced form. Reduced form of glutathione reacts with unstable molecules such as reactive oxygen species and converts them into reduced form and gets oxidized it (Tausz et al. 2004). The conjugation of glutathione is very effective mechanism for the cellular detoxification during toxic metal stress (Nagalakshmi and Prasad 2001). The conjugation of glutathione can occur spontaneously but increase many folds by the activity of glutathione-S-transferase enzyme.

7.4.3.4 Citrate, Malate, Histidine and Others

The carboxylic acids and amino acids such as citric, malic and histidine are also involved during tolerance and detoxification of toxic metals. Organic or amino acids have been studied to involved in the chelation of metal ions within plant cells and in xylem sap (Gasic and Korban 2006). The exogenous application of citric acid and malic acid reduced Ni uptake by the roots of leaf mustard (Qiu et al. 2009). The supplement of citric, oxalic and malic acid during Cd treatments enhanced the accumulation of Cd but decreased the toxicity symptoms (Aravind and Prasad 2005). Cd increased lipid peroxidation that is also effectively controlled by organic acid supplements in Cd-10 μm treated *Ceratophyllum demersum* (Aravind and Prasad 2005). The role of organic acid as detoxification agent has been studied in *Triticum aestivum* (Nigam et al. 2000). These studied revealed that organic acids are integral part of detoxification mechanism during toxic metal stress. Histidine has been reported to be increased 36-folds of the xylem sap on exposure of Ni in the Ni-hyperaccumulators plant *Alyssum lesbiacum* (Hall 2002). The exogenous application of histidine to the non-accumulator plants increased Ni tolerance and enhanced capacity of Ni transport to the shoots. Ni-hyperaccumulators depend upon the histidine-dependent root to shoot translocation of Ni. With exogenous application of histidine has been reported to provide Ni tolerance in *A. montanum* and *B. juncea* (Kerkeb and Kramer 2003).

Nicotianamine is well studied in plants in context to Fe nutrition. It is synthesized from three molecules of methionine by nicotianamine synthase (NAS). The three carboxylic groups in each molecule bind with Fe and other transition metals. The nicotianamine are involved in the delivery of metals in plant at different stages especially at the reproductive stage (Takahashi et al. 2003). Nicotianamine is also involved in toxic metal detoxification. Nicotianamine also form complex with toxic metals but not like phytochelatins and toxic metal. Nitrogen donor centre and carboxyl groups of nicotianamine form complex with the toxic metal. These complexes help to maintain in metal homeostasis within the cell and different tissue of hyperaccumulator plants. The nicotianamine is also involved in transportation of excess metal ions from root to shoots (Sharma and Dietz 2006).

Polyamines are distributed in all organisms. Polyamines such as putrescine, spermidine and spermine and their biosynthetic enzymes have been reported to during different environmental stresses in plant. The polyamine levels are disturbed during exposure to toxic metals. The role of polyamine in plants during toxic metal is not yet revealed. The polyamine might be involved in protection of membrance against toxic metal toxicity acts as inhibitor of lipid peroxidation (Sharma and Dietz 2006).

The polyamine also involved to decrease ion conductance at the vacuolar membrane for facilitate compartmentation of metal ion (Sharma and Dietz 2006).

7.4.3.5 Heat Shock and Metal Binding Proteins

Heat shock proteins are expressed in organisms at temperatures above then the optimum growth temperatures. These are present in all type of organisms and classified according to their molecular weight. An increased expression of these proteins has been observed in response to toxic metal stresses. During toxic metal stress, they are involved in protection and repair of the cellular proteins. HSP-17 has been reported in the roots of *Armeria maritime* plant in response to Cu (Hall 2002). HSP-70 has been observed in seaweed *Entormporpha intestinalis* on Cu exposure (Lewis et al. 2001). HSPs is also playing role to protect membrane against damage.

Toxic metal triggers the synthesis of certain set of novel proteins to detoxify the metal ion toxicity and provide tolerance against toxic metal stress. AtPcrs, a Cys-rich membrane protein has been identified to play an important role in Cd resistance in *Arabidopsis*. Cdl19, another protein has been identified to play important role during Cd, Hg, Fe and Cu stress in *Arabidopsis* (Suzuki et al. 2001). The amount of apoplastic polypeptides has been observed to be increase in response to heavy metal stress (Zhang et al. 2009). The Pathogen related protein (PR proteins) and germin-like proteins (GLPs) have been reported during metal toxicity (Edreva 2005; Alvarez et al. 2009).

7.4.3.6 Phenolic Compounds

The intermediate of lignin biosynthesis, soluble phenolics was induced in response to toxic metals (Michalak 2006). Phenolic compounds such as flavonoids, phenylpropanoids and phenolic acids have been reported to induce in response to toxic metals (Michalak 2006; Keilig and Ludwig-Muller 2009). The biosynthesis of phenolic compounds was reported to induce in response to Ni and Al metal ion toxicity in wheat and maize respectively (Winkel-Shieley 2002; Michalak 2006). The accumulation of phenolics has been also reported in *Phaseolus vulgaris* and *Phyllantus tenellus* in response to Cd and Cu treatments, respectively (Winkel-Shieley 2002). Under toxic metal stress, accumulation of phenolics occurred either by de novo biosynthesis or by conjugate hydrolysis (Michalak 2006). Exogenous application of phenolics such as flavonoids has been observed to restore the partially growth of metal treated *Arabidopsis* (Keilig and Ludwig-Muller 2009). The phenolic compounds have multiple functions under toxic metal stress in plants. These are used in creation of physical barriers, in conjugation and sequestration process for detoxification, and act as scavenger of free radicals in antioxidant system. The phenolics such as flavonoids act as chelating agent for metal ions. Anthocyanins are used to form conjugates with metal ions and help in sequestering them into vacuole. Flavonoids also act as scavenger of free radicals induced by metal ion toxicity. The antioxidant potential of flavonoids are due to their ability to donate electrons or hydrogen atoms. Their mode of action is similar to ascorbate and glutathione. Anthocyanins are also involved in photo-protection of photosynthetic pigments during toxic metal stress.

7.4.4 Sequestration of Toxic Metal from Cytoplasm to Extra Nuclear Compartments

Sequestration or compartmentation is the final step of conjugation involving storage of conjugates during metal toxicity. Vacuole is considered as the main storage site for metals in plant cells (Tennstedt et al. 2009). Phytochelatin-metal complexes are pumped into vacuole of plant cells. Sequestration of toxic metal ions to vacuole reduces the level of toxic metal ions in cytosol and acts as a potential mechanism of tolerance. Soluble conjugates toxic compounds are sequestered and accumulated in vacuoles while insoluble conjugates are moved out of cell via exocytosis and stored in the apoplast. The vacuolar compartmentalization mechanism directed metals to their final location as vacuoles within a cell. This has been carried out by transporter regulatory mechanism present in the internal membranes of organelles. Zn and Cd had been reported to accumulate in vacuoles (Tennstedt et al. 2009). Transport of Cd occurred by Cd^{2+}/H^{+} antiport mechanism across the tonoplast (Carrier et al. 2003). A vacuolar metal transport proteins termed as metal-tolerance proteins (TgMTPs) has been identified to involve in compartmentalization of Ni in vacuoles in shoots of Ni-hyperaccumulator plant *Thalspi goesingense* (Persans et al. 2001). The vacuolar transporter YCF1 is well known for their role in metal tolerance in yeast. It is a MgATP transporter responsible for vacuolar sequestration of GSH-organic complexes and GSH-metal complexes. This protein catalyzed the transport of GSH-metal complexes in Cd, As and Hg toxicity (Tamas et al. 2006). The *YCF1* overexpression in *Arabidopsis* provides tolerance against high concentration of Pb and Cd (Song et al. 2003). The long-term tolerance of *B. napus* against Cd has been reported due to efficient distribution and microlocalization of Cd ion in the plant soils (Carrier et al. 2003). The more progress has been achieved on the role of vacuole in transport, homeostasis and detoxification of micronutrient Zn and iron. Plant maintains a limited concentration of them in cytosol and these metals are released in a very controlled way to play enzymatic functions. Vacuole acts as internal iron reservoir. Iron and nicotianamine complex has been found in vacuole during iron overload or while accumulating iron present in excess (Pich et al. 2001). In yeast, iron is transported by carrier named CCC1p and stored as a ferric hydroxide in the lumen (Martinoia et al. 2007). A gene has been reported to highly homologous to CCC1p and encodes the vacuolar Fe^{2+} transporter. The ABC-type transporter has been involved in transport of iron-nicotianamine and phytochelatins-Cd complex inside the vacuole. Metallochaperones, a family of soluble metal receptor proteins has been identified for intercellular metal trafficking. These proteins are involved in guiding and protecting the metal during its delivery to appropriate receptors.

The glandular trichomes and epidermal structures are also involved in chelation, sequestration and detoxification of the metals. The number of trichomes has been increase in Cd-exposed tobacco seedlings (Choi et al. 2001). A significant amount of toxic metal as Ni ad Zn has been reported in trichomes of *Alyssum lesbiacum* and *Arabidopsis halleri* (Sarret et al. 2002; Mishra and Dubey 2010). The head cells of tobacco trichomes actively exclude toxic Cd by forming and excreting Cd/Ca crystals containing (Choi et al. 2001). All these studies suggested the role of trichomes for accumulation as well as detoxification of toxic metal ions. A number of studies also revealed the role of hydropotes in detoxification of metals in semi aquatic and aquatic plants.

7.5 Different Approaches Implicated in Brassicaoilseed Species for Remediation of Metalliferous Soils

Hyperaccumulators are generally identified as slow growing and low biomass plants and not relevant to be used in remediation systems (Brown et al. 1995). Therefore, the crops with the ability to significantly uptake higher concentrations of metals and producing high biomass as well are required for metal extraction from soils within a reasonable time frame like *H. annuus* L., *N. tabacum*, *B. juncea* or *Z. mays* L. (Nehnevajova et al. 2007). The search of plants for phytoremediation is centered on Brassicaceae family (Crucifer family) because many hyperaccumulators belong to this family (Palmer et al. 2001). The members of *Brassica* are identified as potent accumulation of toxic metals in shoots *Brassica*, *Thlaspi* and other genus of Brassicaceae family have been the model plant of phytoremediation. Plants from *Thlaspi* genus are identified with high foliar metal concentrations but their annual biomass production is not too high. On other side, Brassica genus plants have lower metal concentration but a large biomass production (ten times higher than all other hyperaccumulators). They also show fast growth rate. So, *Brassica* genus plants such as *B. juncea* removing capability of high amounts of metal as compared to other plants (Palmer et al. 2001). *B. juncea* accumulates Cd approximately 400 $\mu g g^{-1}$ d. wt in their aerial parts (Hall 2002). This species also show high biomass production and fast growth rate. The lower and intermediate level of metal ions in contaminated soils improves the growth of *B. juncea* (Singh and Tewari 2003). Six particular agricultural importance species such as *B. carinata*, *B. juncea*, *B. oleracea*, *B. napus*, *B. nigra* and *B. rapa* are derived from three earlier species by the combination of chromosomes, as described by the Triangle of U theory. All these species have very close relationship and identified as hyperaccumulators. Kumar et al. (1995) tested these species of *Brassica* for their ability of metal tolerate and accumulation. Among these, *B. juncea* showed

Table 7.2 List of toxic metal contents in Brassicaoilseed species and their threshold level in plant

Metals	Metal accumulation in Brassicaoilseed species (μg g^{-1}d. wt)	Thresholds level of metals in plant (μg g^{-1} d. wt)	References
Cd	400	100	Hall (2002)
Cr	135	100	Shahandeh and Hossner (2000)
Co	238	100	Weerakoon and Somaratne (2009)
Pb	1,471	100	Blaylock et al. (1997)
Ni	1,100	1,000	Shevyakova et al. (2011)
As	2,100	1,000	Reisinger et al. (2008)
Se	1,565	1,000	Euliss and Carmichael (2004)
Cu	3,600	1,000	Ariyakanon and Winaipanich (2006)
Zn	9,089	10,000	Ghnaya et al. (2011)

strong potential to accumulate and translocate Cu, Cr (IV), Cd, Ni, Pb, and Zn in shoots. *B. rapa, B. napus* and *B. juncea* are most common source of oil worldwide and are collectively called as Brassicaoilseed species. But *B. juncea* and *B. napus* Brassicaoilseed species are well known for phytoremediation research and showed strong ability to accumulate in root and translocate Cu, Cr (IV), Cd, Ni, Pb, Zn and Se to their stem (Table 7.2). This ability has cleared their use for phytoextraction at large scale. These species are thus likely, a source of genes for phytoremediation. They are perennial fast growing plant with a well-developed root system and luxuriant biomass above ground. These can grow in both warm and cold conditions. This adaption has attracted everyone to understand wide range of mechanisms going on in these plants in relation to the accumulation of metal ions and tolerance to nullify metal ions mediated toxicity.

All these characteristics of Brassicaoilseed species especially *B. juncea* have proved their ability as good source for phytoremediation. At presently, *B. juncea* has been given considerable attention by researcher, geneticists and plant breeders because of their potential in phytoextraction of toxic metals. In the near future, these plants will be playing a key role in phytoremediation technology and remediation of polluted areas. There are different approaches for utilization of brassicaoilseed species ability to remove metal from contaminated soils such as non-genetics and genetics. The non-genetic approaches involve physical, chemical and physiochemical technologies. These include lowering pH, increasing electrode potential, use of chelating agents and use of microorganism and plant growth promoting rhizobacteria. The genetics methods involve plant breeding and biotechnological approaches.

Increasing the bioavailability of toxic metals in soil is vital for the success of phytoremediation. Lowering pH by using either sulphuric acid, organic fertilizers or chelating agents increases the bioavailability of toxic metals in soils to plants that causes effective accumulation of metal ions in plant tissue. The accumulation of toxic metals in the shoots of *B. juncea* can be enhanced through using above said discussed techniques. The addition of lime and organic amendment has achieved higher plant biomass production. Elevated levels of Zn, Cu and Pb have been reported in leaves of *B. Juncea* in low pH soils (Clemente et al. 2005). The contaminants such as Pb have limited bioavailability in soils. The high accumulation of Pb has been reported in the shoots of *B. juncea* from soils containing 600 mg of Pb/kg amended with EDTA (Blaylock et al. 1997). The bioavailability of lead has been increased for effective accumulation in *B. juncea* by applying EDTA and an electric field simultaneously in contaminated soil (Lim et al. 2004). The classical fertilizers and organic acids also used for effective accumulation of metal ions in plant tissue either by increased bioavailability of toxic metal in contaminated soils or enhanced biomass of plants. Addition of fertilizers in combination of low N and high P produced a high yield in *B. juncea*, resulting into highest Cu removal from copper-polluted soil, (Wu et al. 2004). An organic acid such as citric acid has been reported most effective in enhancing Uranium accumulation in *B. juncea* and *B. chenensis* for phytoextraction purposes (Huang et al. 1998). Plant growth promoting rhizobacterias (PGPRs) are also used to increase the plant yield and provide tolerance against heavy metal contaminated soils (Ali and ZulkifliHj 2010). But the applications of PGPRs depend upon their ability to tolerate the concentration of toxic metals. The levels of PGPRs population depend upon the amount, type of heavy metals, nature of organic acids secreted by the plant roots. A number of PGPRs have been reported that favors increased biomass production and metal accumulation of brassicaoilseed species in metelliferous soil.

Classical genetics has also been used to generate metal ions tolerant brassicaoilseed species. Plant breeding, in which different mutants or variants of a given plant species are mated and desired traits are selected, is well used for improving phytoremediation. Several accessions of *B. juncea* have been identified as accumulators of toxic metal. The genetic integrity of these accessions is maintained by using appropriate breeding techniques. The toxic metal-contaminated soil is also remediated with a combination of chemical treatments and plants. Brassicaoilseed species are also accompanied by other enhancement methods to increase the amount available of toxic metals. The biotechnological approaches such as tissue culture, somaclonal variations, in vitro selection and generation of transgenics to understand and enhance the potential of brassicaoilseed species against toxic metals. Somaclonal variations and in vitro selection technology are also used for development of new plant variants with enhanced metal accumulation and extraction properties. Plant species within the *Brassicaceae* family has been reported to grow and manipulate in vitro. Callus cultures have been used for selection and biochemical characterization of zinc and manganese adapted lines in *Brassica* species (Rout et al. 1999). Nehnevajova et al. (2007) has achieved the regeneration of shoots of *B. juncea* variant from Cd-tolerant selected cell lines. Several attempts have been made to alter expression levels of enzymes involved in the detoxification and tolerance mechanism in hyperaccumulator and enhance their growth in metalliferous soil. A number of transgenics have been generated. Brassicaoilseed species genetic resources have also been used for this purpose. Selection of brassicaoilseed species to generate transgenic plants is preferred due to following characteristics:

1. Ability to grow faster under environments that require remediation.
2. Ability to take and concentrate the toxic metals into their harvesting biomass
3. Avoid growth of uncontrolled weeds via vegetative means or pollen
4. Do not create the problem for animals and humans

There are several strategies for generation of transgenics of *B. juncea* to improve the capacity of phytoremediation. One approach is to increase the ability to accumulate metals ions by introducing genes encoding transporter molecules. Second strategy is to increase proteins, peptides or other molecules within *Brassica* plant cells that have high affinity for entered metal ions. Third strategy is to change the oxidation state of toxic metals by introducing genes encoding enzymes of changing the redox state of metal ions. Fourth strategy is to enhance the antioxidant capacity of *Brassica* plants by introducing the genes of antioxidant or detoxification system. These strategies for enhancing phytoremediation capacity of brassicaoilseed species are straightforward. The source of transgene for this purpose can be of any origin- bacterial, plant or even animal. Some examples of for metabolic engineering for toxic metal stress tolerance in Brassicaoilseed species have been summarized (Table 7.3). These examples demonstrate that potential of brassicaoilseed to detoxify metal ions and provide tolerance to plant against metal ions mediated toxicity.

7.6 Conclusions and Perspectives

The elevated concentrations of toxic metals in environmental causes negative effects on living organisms. The ability of some plant species to accumulate toxic metals without any adverse effect on them upto a limit provide a direction for remediation of polluted environments. To survive in contaminated soils, plants possess a range of interlinked mechanisms to provide tolerance and homeostasis of toxic metals inside the plant cells. *Brassica* has been proposed as a natural, environmentally safe option to clean contaminated sites. Brassicaoilseed species utilize the mechanism of accumulation, translocation and uptake of toxic metals more efficiently for tolerance. The adaptation abilities of brassicaoilseed species to tolerate metal mediated toxicity have been enhanced by number of chemical and biochemical approaches. Brassicaoilseed species are also identified as source of genes used for generating more phytoremediation sources for other plants. Further enhancing the tolerance and accumulation potential of Brassicaoilseed species by several physiological, biochemical and molecular approaches can effectively emerge out as a solution for phytoremediation.

Table 7.3 List of genes and corresponding transgenics involved in different mechanism of phytoremediation providing tolerance against toxic metals

Gene	Product	Transgenic plant	Source	Tolerance against	References
BjCdR15	bZIP transcription factor	Tobacco	*B. juncea*	Cd	Farinati et al. (2010)
CUP1	MT	*B. oleracea*	*Sacchromyces cerevisiae*	Cd	Hasegawa et al. (1997)
AtPCS1	Phytochelatins synthase	*B. juncea*	*Arabidopsis*	As and Cd	Gasic and Korban (2006)
APS1	ATP sulfurylase	*B. juncea*	*Arabidopsis*	Sn	Pilon-Smits et al. (1999)
CSase	GSH synthase	*B. juncea*	*E. coli*	Cd	Zhu et al. (1999a, b)
y- ECS and *GS*	Y-Glu-Cys synthase	*B. juncea*	Bacteria	As, Cd, Zn and Pb	Reisinger et al. (2008)
CGS	Cystathionine-gamma synthase	*B. juncea*	Bacteria	Sn	Van Huysen et al. (2003)
BjCET2	Cation- efflux transporter	*B. juncea*	*B. juncea*	Cd/Zn	Xu et al. (2009)
Gly I	Glyoxalase I	Tobacco	*B. juncea*	Zn	Singla-Pareek et al. (2006)
Gly II	Glyoxalase II	*B. juncea*	–	Zn	Saxena et al. (2005)
BiMnSOD	MnSOD	*B. napus*	Wheat	Al	Basu et al. (2001)
GR	Glutathione reductase	*B. juncea*	Bacteria	Cd	Pilon-Smits et al. (2000)
BjCAT3	Catalase	Tobacco	*B. juncea*	Cd/ Zn	Guan et al. (2009)
BjDHN2/ *BjDHN*3	Dehydrin gene	*Tobacco*	*B. juncea*	Cd and Zn	Xu et al. (2008)
AnnBj1	Annexin protein	Tobacco	*B. juncea*	Cd	Jami et al. (2008)
ACC deaminase gene	ACC deaminase	*B. napus*	*Pseudomonas putid*	Ni	Stearns et al. (2005)
BjPIP1	Aquaporin	Tobacco	*B. juncea*	Cd	Zhang et al. (2008)
γ-TMT	Gamm-tocopherol methyl transferase	*B. juncea*	*Arabidopsis*	Cd	Yusuf et al. (2010)
BnHO	Heme oxygenase	*B. napus*	*B. napus*	Hg	Shen et al. (2011)

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Chapter 8
The Role of Plant Growth-Promoting Rhizosphere Bacteria in Toxic Metal Extraction by *Brassica* spp.

Oguz C. Turgay and Serdar Bilen

Abstract *Brassicaceae* are scattered all over the world, where they exclusively grow on serpentine rocks in Western Australia, New Zealand, South Africa, Japan, Philippines, Brazil, Portugal, Italy, Turkey, Cuba, eastern Canada, and western north America. Although serpentine rocks cover only less than 1% of the earth's surface their worldwide distribution has recently attracted many researchers in exploring their distinctive potential for phytoremediation plant communities, mainly members of *Brassicaceae* plant family inhabiting on serpentine rocks of these countries. On the other hand, the majority of *Brassicaceae* plant family are slow-growing plants producing little biomass and their use for phytoextraction purposes may not be practical, especially when bioavailable metal concentration is high in the contaminated conditions. Therefore, recently emerging practices in the field of phytoremediation have pointed out various focuses such as the utility of high-biomass crops such as maize, peas, oats and Indian mustard and associated soil practices including application of synthetic chelators such as ethylenediaminetetraacetic acid and nitrilotriacetate and elemental sulphur to enhance metal uptake by these plants. These approaches may meet the conditions required for the phytoremediation. However, one of the most critical components of phytoextraction process is the bioavailability of heavy metals meaning the portion of the metals that is available for absorption into living organisms such as plants.

It has been known that various plant growth-promoting rhizobacteria (PGPR) associated with plant roots may provide some beneficial effects on plant growth and nutrition through a series of well known mechanisms, namely, nitrogen fixation, production of phytohormones and siderophores, and transformation of nutrients

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once they are either applied to seeds or incorporated into the soil. Similarly, heavy metal mobility and availability can substantially be driven by PGPR populations through their release of chelating agents, acidification, and phosphate solubilization in rhizosphere. Miscellaneous PGPR were also shown to tolerate heavy metals in different ways including the mechanisms of exclusion, active removal, biosorption, precipitation, and extra- or intracellular bioaccumulation. Since these processes may affect the solubility and the bioavailability of heavy metals to the plant and hence modifying their toxic effects, interactions between hyperaccumulator plants such as *Brassicaceae* spp., and metal tolerant or resistant PGPR are considered to have an increasing biotechnological potential in the remediation of anthropogenically polluted soils. Present chapter/review considers the role of PGPR on soil-heavy metal-plant interactions and more specifically bioaccumulation of toxic metals by *Brassicaceae* plant family.

Keywords *Brassicaceae* • Metal uptake • Plant growth promoting bacteria • Phytorextraction

Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
IAA	Indole-3-acetic acid
PGPR	Plant growth promoting rhizobacteria

8.1 Introduction

The heavy metal uptake by plants is an expanding research interest because the soil contamination with metals has became a major environmental issue and the strategy of phytoremediation, which is based on the ability of plants (i) to absorb metals from soil, (ii) transfer them to their aboveground components and (iii) hyperaccumulate to higher levels, gained an increasing popularity due to its lower cost and environment friendly nature, during the last few decades (Kumar et al. 1995).

On the other hand, the majority of hyperaccumulators such as *Brassicaceae* spp. are slow-growing plants producing little biomass (Khan et al. 2000; Puschenreiter et al. 2001) and their use for phytoextraction purposes may not be practical, especially when bioavailable metal concentration is high in soil (Jing et al. 2007; Turgay et al. 2011). Therefore, recently emerging practices in the field of phytoremediation have pointed out various focuses such as the utility of high-biomass crops such as maize, peas, oats and Indian mustard (Ebbs and Kochian 1998; Bryson and Barker 2007; Brunet et al. 2008; Li et al. 2009) and associated soil practices including application of synthetic chelators such as ethylenediaminetetraacetic acid and nitrilotriacetate and elemental sulphur (Puschenreiter et al. 2001; Chen et al. 2004) to enhance metal uptake by these plants.

These approaches may meet the conditions required for the phytoremediation. However, one of the most critical components of phytoextraction process is the bioavailability of heavy metals (Kayser et al. 2000) meaning the portion of the metals that is available for absorption into living organisms such as plants. It has been known that various plant growth-promoting rhizobacteria (PGPR) associated with plant roots may provide some beneficial effects on plant growth and nutrition through a series of well known mechanisms, namely, nitrogen fixation, production of phytohormones and siderophores, and transformation of nutrients once they are either applied to seeds or incorporated into the soil (Kloepper et al. 1989; Glick 1995; Glick et al. 1999). Similarly, heavy metal mobility and availability can substantially be driven by PGPR populations through their release of chelating agents, acidification, phosphate solubilization, and redox changes in rhizosphere (Smith and Read 1997; Abou-Shanab et al. 2003a). Many microorganisms were shown to tolerate heavy metals in different ways including the mechanisms of exclusion, active removal, biosorption, precipitation, and extra or intracellular bioaccumulation (Silver 1996; Whiting et al. 2001; Lasat 2002; Gadd 2004) Since these processes may affect the solubility and the bioavailability of heavy metals to the plant and hence modifying their toxic effects, interactions between hyperaccumulator plants, metals and PGPR have gained increasing attention for the last two decades. Today metal tolerant or resistant bacteria are considered to have an increasing biotechnological potential in the remediation of anthropogenically polluted soils (Rajkumar et al. 2009). Plants inoculated with metal-resistant rhizosphere bacteria have reported to show higher metal bioaccumulation (Zaidi et al. 2006; Jiang et al. 2008) or reduced metal toxicity (Madhaiyan et al. 2007).

Soil bacteria isolated from rhizosphere of *Brassicaceae* spp. inhabiting various countries such as India, Portugal, America, Italy, Austria have been studied by many authors and well summarized by Rajkumar et al. (2009). Despite these valuable efforts, soil microbial diversity of serpentine soils is still a black box due to occurrence of high genetic diversity and heterogeneity of the bacterial communities present in different conditions (Mengoni et al. 2004). This should not be surprising because weathering of serpentine rocks depends on the degree of physical breakdown which allows larger surface areas for chemical weathering, liberating soil elements to different extents and hence having marked influence on organisms (Proctor and Woodell 1975). Therefore, serpentine soils reserve not only metal hyperaccumulating plants species i.e. *Brassicaceae* spp. but also a great potential for the exploration of metal-resistant/tolerant rhizosphere microorganisms. In the present chapter, we discussed the role of rhizosphere bacteria on toxic metal movement from soil to plant with a particular emphasis on those inhabiting rhizosphere of *Brassicaceae* spp.

8.2 Rhizosphere as a Microhabitate for Plant Growth Promoting Rhizobacteria (PGPR)

In 1904, the German Scientist Hiltner introduced the term of “rhizosphere” to denote a specific soil region which is subject to the influence of plant roots and characterized by greater microbiological activity than the soil away from plant roots. There are innumerable reports in the literature to substantiate the facts that plant roots change soil physicochemical characteristics and create a distinctive habitat for soil microbial communities in that greater diversity and quantity of bacteria, fungi and actinomycetes are present in rhizosphere than in surrounding soil. It has also been known that the rates of metabolic activity of the rhizosphere microbial communities are different from those of the non-rhizosphere soil because plant roots attract soil microorganisms through their exudates (Brimecombe et al. 2001).

Although the exact mechanisms between rhizosphere bacteria and plant growth are not fully understood, it has been well-documented that various soil microorganisms associated with plant roots have an ability to (i) produce or change the concentration of plant growth regulators like indoleacetic acid, gibberellic acid, cytokinins and ethylene (Arshad and Frankenberger 1993; Glick 1995), (ii) manipulate asymbiotic nitrogen fixation (Boddey and Dobereiner 1995), (iii) form antagonism against phytopathogenic microorganisms by production of siderophores (Scher and Baker 1982), antibiotics (Shanahan et al. 1992) and cyanide (Flaishman et al. 1996), and (iv) enhance solubilization of mineral phosphates and other nutrients (Gaur 1990; De Freitas et al. 1997). Today, the bacteria acting roles in these mutual relationships are extensively called as plant growth-promoting rhizobacteria (PGPR) that were first defined by Kloepper and Schroth (1978) as “soil bacteria colonizing the roots of plants following inoculation onto seed and enhancing plant growth” and correspond to 2–5% of rhizospheric bacteria of soil (Antoun and Prévost 2005). The researches conducted over the past few decades revealed that PGPR are a heterogeneous group of bacteria which can improve the extent or quality of plant growth directly or indirectly (Ahmad et al. 2008) and include species of *Achromobacter, Alcaligens, Arthobacter, Azospirillum, Azotobacter, Bacillus Burkholderia, Enterobacter, Klebsiella, Pseudomonas, Serratia* and *Streptomycetes* (Kloepper et al. 1989; Okon and Labandera-Gonzalez 1994; Glick 1995; Gray and Smith 2005).

8.3 Direct and Indirect Effect of PGPR on Plant Growth

The direct plant growth promotion can be simply defined as the enhancement of plant growth by non-pathogenic soilborne microorganisms through several mechanisms such biofertilization, rhizoremediation, phytostimulation and stress controlling.

8.3.1 Biofertilization

Biofertilization is the mechanism in which plant growth promoting substances are directly synthesized or the uptake of certain plant nutrients such as nitrogen (N_2) and phosphorus (P) are facilitated by certain bacterium. Well-known process of biological nitrogen fixation is perhaps the best model for biofertilization. N_2-fixing bacteria such as *Rhizobium* and *Bradyrhizobium* forms nodules on roots of leguminous plants such as soybean, pea, peanut, and alfalfa, in which they convert N_2 into ammonia that can be used by the plant as a nitrogen source (Van Rhijn and Vanderleyden 1995). Some of N_2 fixing bacteria are free-living genera and the nitrogen fixed by these species can become available to plants and fungi when they die and decay. For example, *Azospirillum* can fertilize wheat, sorghum, and maize (Okon et al. 1998).

Low levels of soluble phosphate in soil are also significant factor limiting plant growth. However, certain PGPR solubilize phosphate from either organic or inorganic bound phosphates, thereby facilitating plant growth (Lipton et al. 1987; Vassilev et al. 2006). This process takes places in the synthesis of several enzymes such as phosphatases, phytases, phosphonatases, and C-P lyases, releasing soluble phosphorus from organic compounds in soil. On the other hand, Rodriguez et al. (2006) recently revealed that the release of phosphorus from mineral phosphate was related to the production of organic acids, such as gluconic acid.

8.3.2 Rhizoremediation

The primary metabolisms of some soil bacteria are dependent on degradation of soil pollutants such as trichloroethylene (Walton and Anderson 1990), polycyclic aromatic (Radwan et al. 1995), hydrocarbons and polychlorinated biphenyls (Brazil et al. 1995). Such bacteria have been reported to be effective in *in vitro* conditions but ineffective in pollutant degradation in natural soil (Boopathy 2000; Vidali 2001) possibly due to their low adaptation ability to natural microbial populations and soil conditions. This bottleneck has been minimized by dissociating energy needed for primary metabolism from the energy required for pollutant degradation. Based on this idea, many studies were conducted to explore the contribution of pollutant-degrading rhizobacteria that live on, or are close to, the plant roots so that they can use root exudates as their major nutrient source and degrade polluting agents in the rhizosphere. This mutual relationship is today known as rhizoremediation (Anderson et al. 1993; Schwab et al. 1995) and the first studies concerning rhizosphere degradation of soil pollutants mainly focused on the degradation of pesticides (Hoagland et al. 1994; Jacobsen 1997; Zablotowicz et al. 1994). Recently, detoxification of inorganic pollutants (i.e. heavy metals) in plant-rhizobacteria associations has become a new feature of rhizoremediation process and reviewed well by several authors (Jing et al. 2007; Rajkumar et al. 2009; Kavamura and Esposito 2010).

8.3.3 Phytostimulators

The rhizosphere bacteria have been found to produce specific compounds such as phytohormones (auxins [IAA], cytokinins and gibberelins) and enzymes (1-aminocyclopropane-1-carboxylate [ACC] deaminase) that can stimulate various stages of plant growth. This process has been referred as phytostimulation and IAA has received most of the attention (Brown 1974; Patten and Glick 1996; Garcia de Salamone et al. 2005) since it enhances root development leading to improved mineral and nutrient uptake and root exudation that in turn stimulates bacterial proliferation on the roots (Steenhoudt and Vanderleyden 2000) and prevent detrimental effects of environmental stress factors (Frankenberger and Arshad 1995) such as high salinity (Bianco and Defez 2009). In addition to IAA, cytokinins were also found to stimulate the growth of different crops under both stressed and non-stressed conditions (Ortiz Castro et al. 2008).

8.3.4 Stress Controllers

The discovery of ACC deaminase synthesis by various PGPR (Ghosh et al. 2003; Dey et al. 2004; Hontzeas et al. 2005; Madhaiyan et al. 2006; Yue et al. 2007; Rodriguez et al. 2008) revealed that this enzyme can decrease the level of the phytohormone ethylene in a developing or stressed plant (Glick 1995; Glick et al. 2007a, b) and thereby protect plant against to various stress conditions such as flooding (Farwell et al. 2007); drought (Mayak et al. 2004); high salinity (Saravanakumar and Samiyappan 2006; Cheng et al. 2007); phytopathogens (Wang et al. 2000; Hao et al. 2007) presence of organic pollutants (Reed and Glick 2005; Gurska et al. 2009) and heavy metals (Belimov et al. 2001; Nie et al. 2002; Glick 2003; Reed and Glick 2005; Safranova et al. 2006).

8.3.5 Biological Control of Soil-Borne Plant Disease (Indirect)

The indirect promotion of plant growth occurs when PGPR act as biocontrol agents against to phytopathogenic microorganisms and thereby indirectly stimulate plant growth. These mechanisms include antibiotics production, signal interference, predation-parasitism, induced systemic resistance and competitions for ferric iron and other nutrients as reviewed well by Lugtenberg and Kamilova (2009). Antibiotic producing bacteria kill pathogens using antagonism but they should be able to compete with other organisms for nutrients from the roots to deliver the antibiotic along root system (Chin-A-Woeng et al. 2000).

8.3.6 *Signal Interference*

Signal interference is a biocontrol mechanism which relies on the degradation of the quorum-sensing molecules i.e. homoserine lactones (AHLs) (Lin et al. 2003). AHLs regulate gene expression in response to fluctuations in cell-population density and are also required for the production of the enzymes degrading the cell-walls of the pathogens. For example *B. thuringiensis* was reported to be capable to hydrolyze lactone ring and break the amid link of pathogen AHLs and thus suppress the reproduction of pathogenicity (Lugtenberg and Kamilova 2009).

8.3.7 *Predation-Parasitism*

Predation-parasitism is also another major biocontrol mechanism based on enzymatic destruction of the fungal cell wall and driven by fungal species i.e. *Trichoderma* (Harman et al. 2004). Induced systemic resistance is the phenomenon in which the plants interact with rhizosphere bacteria through their roots can gain resistance to pathogenic bacteria, fungi and viruses. This was discovered by the resistance induced by the rhizobacterium *Pseudomonas sp.* strain WCS417r against *Fusarium* wilt of carnation (Van Peer et al. 1991).

8.3.8 *Ferric Competition*

Some selected rhizobacteria were also found to induce resistance mechanism against to the fungus *Colletotrichum orbiculare* in cucumber (Wei et al. 1991). PGPR can inhibit the growth of fungal pathogens by producing high concentrations of high-affinity siderophores especially when Fe^{3+} concentration is low, e.g., in acid soils in the rhizosphere (Schippers et al. 1987).

8.4 Role and Functions PGPR in Soil-Heavy Metal-Plant Interactions

The direct and indirect stimulation of plant growth by PGPR activities have recently gained much more scientific attention in relation to the removal of heavy metals through plant-PGPR associations because pollution of biosphere by heavy metals has accelerated dramatically due to increasing human activities. Primary sources of heavy metal contamination and pollution in the environment can be listed as industrial (plastic, textiles, microelectronics, wood preservatives) and mining technologies (mine refuse, tailings, smelting), use of agrochemicals (chemical fertilizers, farmyard manure, pesticides) aerosols (pyrometallurgical and automobile exhausts) use of biosolids (sewage sludge, domestic wastes) and fly ashes (coal combustion products) (Leyval et al. 1997; Vivas et al. 2003; Denton 2007; Ma et al. 2011). It has been revealed that PGPR may have beneficial effects on various plants growing in metal contaminated environment (Tokala et al. 2002; Dimkpa et al. 2009a, b) and on the cure of heavy metal contaminated soils during phytoremediation process (Rajkumar et al. 2009; Kavamura and Esposito 2010), which is a novel remediation technology defined as the use of metal accumulating plants to remove, transfer and stabilize these contaminants from soil, sediments and water (Khan 2005).

The researches carried out during the last two decades showed different results on how soil microorganisms are affected from the presence of heavy metals and how they play a part in metal accumulation process in plants. For example, several of early field observations on metal contaminated sites showed that soil microbial diversity was depressed by high concentrations of heavy metals (Jordan and LeChevalier 1975; Brookes and McGrath 1984; Chander and Brookes 1991; Konopka et al. 1999). Experimental studies similarly demonstrated that *Rhizobium* populations were diminished by Cd treatments (Chaudri et al. 1992) and the number of rhizosphere bacteria was also reduced at high concentrations of Co, Cd, Ni, Cu, Zn, Crand Pb (Abou-Shanab et al. 2005). On the other hand, De Souza et al. (1999b) found that saltmarsh bulrush supplied with different rhizosphere bacteria as a single inoculum or a consortium showed increasing metal accumulations in plant roots and shoots. However, some other authors introduced an opposite aspect that some PGPR or mycorrhizal fungi can lessen metal uptake through plant roots and thereby reduce metal toxicity increasing plant biomass (Brown and Wilkins 1985; Dueck et al. 1986; Heggo et al. 1990; Tam 1995).

More recently, rapidly growing viewpoints concerning the role of PGPR in soil-plant-heavy metal relationships are that (i) soil metal pollution can trigger the appearance of heavy metal resistant rhizobacteria in the soil of industrial regions (Aleem et al. 2003; Piotrowska et al. 2005), (ii) high proportions of such bacteria generally appears in the rhizosphere of metal hyperaccumulating *Brassicaceae* spp. such as *Thalaspi caerulescens* (Delorme et al. 2001), *Alyssum bertolonii* (Mengoni et al. 2001) and *Alyssum murale* (Abou-Shanab et al. 2003a) growing in naturally and industrially polluted soils, (iii) several of PGPR have been shown to promote plant growth and health in metal-contaminated soils and thereby play a significant role in accelerating phytoremediation (Grandlic et al. 2008; Kuffner et al. 2008; Kidd et al. 2009; Ma et al. 2009a, b, c; Compant et al. 2010; Dary et al. 2010) and (iv) this unique exploration is related to the positive cooperation between metal accumulating plants and various PGPR populations living in their rhizosphere through metal uptake process (Idris et al. 2004; Mengoni et al. 2004; Barzanti et al. 2007; Jing et al. 2007; Ma et al. 2011). The promoting effect of PGPR on plant growth and metal accumulation process in the presence of heavy metals has been associated with certain specific mechanisms, such as synthesis of siderophore, IAA, ACC deaminase and phosphate solubilization (Jing et al. 2007; Rajkumar et al. 2009; Glick 2010; Ma et al. 2011).

8.4.1 Microbial Fe-Siderophore Complexes

PGPR can improve host plant growth and development in heavy metal polluted soils by using the mechanisms of biofertilization, rhizoremediation, phytostimulation, stress controlling, predation-parasitism as explained previously above or mitigate the effect of heavy metals on plant growth via producing iron-siderophore complexes (Jing et al. 2007). It has been found that insufficient iron resulted in increasing metal toxicity in various metal-accumulating and metal-tolerant plants growing in heavy metal contaminated soils (Römheld and Marschner 1986; Wallace et al. 1992; Ma and Nomoto 1993). These plants can also be chlorotic since chlorophyll biosynthesis and chloroplast development is inhibited by the iron deficiency (Imsande 1998). On the other hand, PGPR synthesize metal-chelating agents called siderophores, which can sequester iron from the soil and provide it to plant cells and thereby serve as an iron source for plants (Reid et al. 1986; Wang et al. 1993). This has also encouraged several workers to investigate if providing plants with siderophore-producing PGPR can prevent them from chlorosis in metal contaminated soils and they noted that PGPR have significantly enhanced plant growth in the presence of heavy metals (Burd et al. 1998, 2000; Carrillo-Castañeda et al. 2003; Barzanti et al. 2007). Siderophores was also found to promote bacterial IAA synthesis by reducing the detrimental effects of heavy metals through chelation reaction (Dimkpa et al. 2009a).

8.4.2 Indole-3-Acetic Acid (IAA) Synthesis

Indole-3-acetic acid (IAA) is one of the significant phytohormones produced by plant-associated bacteria and assists plant germination, growth and reproduction and also protect plants against both biotic and abiotic stress (Dobbelaere et al. 1999; Lambrecht et al. 2000; Taghavi et al. 2009). IAA level can change plant root elongation qualitatively and the concentration of IAA synthesized from PGPR is therefore an important factor modulating plant-microbe interactions (Ma et al. 2011). This has been previously tested by comparing the effects of different IAA production rates of a PGPR (wild and mutant types of *Pseudomonas putida* GR12-2) on canola growth (Xie et al. 1996; Patten and Glick 2002). They found that wild-type *Pseudomonas putida* producing low levels of IAA promoted the root elongation by two- to three-fold while mutant type with high IAA synthesis inhibited canola root elongation which was ascribed to synthesis of ACC due to the interactive effect of high concentrations of IAA with ACC synthase. These results showed that some rhizosphere bacteria can facilitate plant growth by changing plant hormonal balance either direct or indirect through ethylene synthesis (Persello-Cartieaux et al. 2003). PGPR synthesizing IAA were reported to prevent the deleterious effects of environmental stresses (Lindberg et al. 1985; Frankenberger and Arshad 1995). For example, Egamberdieva (2009) noted that

IAA stimulated lengthening of the root and shoot of wheat seedling exposed to high levels of salt. Bianco and Defez (2009) also observed that *Medicago truncatula* showed an increased tolerance against to salt stress when nodulated by the IAA-overproducing strain *Sinorhizobium meliloti* DR-64. Moreover, plants inoculated with this mutant accumulated a high amount of proline, and showed enhanced levels of the antioxidant enzymes superoxide dismutase, peroxidase, glutathione reductase, and ascorbate peroxidase compared with plants inoculated with the parental strain (Glick 2010). IAA production by PGPR was also found to influence plant growth under heavy metal pollution. Kamnev et al. (2005) revealed that IAA synthesizing nonendophytic and facultatively endophytic strains of *Azospirillum brasilense* suppressed Cu^{2+} and Cd^{2+} and affect the plant-growth-stimulating efficiency of associative plant-bacterial symbioses in heavy metal-polluted soils. They also discovered that both Cu^{2+} and Cd^{2+} ions significantly decreased the level of IAA production for *Azospirillum brasilense* strain Sp7, whereas the bacterial growth rate was virtually not affected.

8.4.3 1-Aminocyclopropane-1-Carboxylic Acid (ACC) Production

The plant hormone ethylene have an important role in plant functions such as root initiation and elongation, nodulation, senescence, abscission and ripening as well as in stress signaling (Mattoo and Suttle 1991; Abeles et al. 1992; Arshad and Frankenberger 2002). Ethylene inhibits root elongation, nodulation and auxin transport, induces hypertrophies, speeds aging and promotes senescence and abscission. During periods of environmental stress, plants produce high levels of "stress ethylene" and the growth inhibition occurring as a consequence of an environmental stress is the consequence of the response of the plant to the increased levels of stress ethylene. However, ACC deaminase-containing bacteria can diminish plant ethylene levels in plants and thereby provide some protection against the inhibitory effects of various stresses such as flooding (Grichko and Glick 2001; Farwell et al. 2007) drought (Mayak et al. 2004) high salt (Saravanakumar and Samiyappan 2006; Cheng et al. 2007) phytopathogens (Wang et al. 2000; Hao et al. 2007) the presence of organic toxicants (Glick 2003; Reed and Glick 2005; Gurska et al. 2009) presence of heavy metals (Burd et al. 1998; Belimov et al. 2001, 2005; Nie et al. 2002; Glick 2003; Reed and Glick 2005; Dell'Amico et al. 2005; Farwell et al. 2006; Safranova et al. 2006).

8.4.4 Phosphate Solubilization

Phosphorus is one of fundamental macronutrients for biological growth and development. However its availability is usually limited and only soluble P forms such as $H_2PO_4^{-}$ or $H_2PO_4^{2-}$ can be used for the biomass production (Glass 1989).

Moreover, heavy metals interfere with P uptake and lead to plant growth retardation (Zaidi et al. 2006) in natural ecosystems. One of the major advantages of metal-resistant PGPR is their capability to convert insoluble P into available forms through acidification, chelation, exchange reactions, and release of organic acids (Chung et al. 2005) and mineralize organic phosphates by secreting extracellular phosphates (Gyaneshwar et al. 2002; Van der Heijden et al. 2008). An increase in P availability to plants through the inoculation of phosphate-solubilizing bacteria has been validated both in pot experiments and under field conditions (Pal 1998; Zaida et al. 2003). Abou-Shanab et al. (2005) also investigated the correlation between metal resistance and metal mobilization abilities of rhizobacteria under heavy metals stress and found that the highest biochemical activity and metal resistance were recorded in the order of (i) phosphate solubilizers with Cr, Zn and Pb (92.5%, 82.2% and 68.2%), (ii) siderophore producers with Cr, Zn and Pb (78.5%, 71.02% and 61.6%), and (iii) acid producers with Cr, Zn and Pb (63.5%, 53.3% and 42.9%), respectively.

Overall, reports concerning PGPR-heavy metal-plant growth interactions implies that there is not only one single mechanisms adopted by PGPR towards metals in soil; but that siderophore synthesis and acid productions are involved in metal mobilization processes. This knowledge has been crucial in the recognition of phytoremediation plants and in the understanding of relationships between their hyperaccumulating characteristics and PGPR.

8.5 Role of PGPR in Metal Extraction by Family *Brassicaceae*

Today, use of metal accumulating plants is promising tools for environmental cleanup and a variety of Brassicaceae species have a key role in phytoremediation technology. Worldwide more than 400 plant species are now known to hyperaccumulate various metals (Cd, Co, Cu, Mn, Ni, and Zn), metalloids (As) and non-metals (Se) in their shoots (Freeman et al. 2004). Of these, almost 25% of the documented hyperaccumulating species are members of *Brassicaceae* (Peer et al. 2003). Diverse populations of PGPR have been reported to colonize in the rhizosphere of different metal hyperaccumulating *Brassicaceae* spp. such as *Thalaspi caerulescens* (Delorme et al. 2001), *Alyssum bertolonii* (Mengoni et al. 2001) and *Alyssum murale* (Abou-Shanab et al. 2003a). This information is very important because it may provide researchers distinctive perspectives when considering on different phytoremediation strategies with different plant species and changing soil-environmental characteristics. For example, most of well-known hyperaccumulators including *Brassicaceae* have been reported to exhibit slow grow rates and produce lower biomass when bioavailable metal concentration is very high in the contaminated soil (Puschenreiter et al. 2001; Blaudez et al. 2003; Jing et al. 2007). However, these bottlenecks can be overcome by using a species with a lower metal accumulating capacity but higher growth rates, such as Indian mustard (*Brassica juncea*) (Jing et al. 2007). Another alternative which has received an increasing scientific attention and become an important component of phytoremediation researches is providing such plants with associated PGPR (Wenzel et al. 1999; Glick 2003). For example, the presence of rhizobacteria was reported to increase concentrations of Zn (Whiting et al. 2001), Ni (Abou-Shanab et al. 2003b) and Se (De Souza et al. 1999a) in *Thlaspi caerulescens, Alyssum murale* and *Brassicca juncea*, respectively. Abou-Shanab et al. (2003a) noted that inoculation serpentine soils with *S. macrogoltabidus*, *Microbacterium liquefaciens*, and *M. arabinogalactanolyticum* significantly increased the bioavailable Ni concentration when compared with the uninoculated controls. More interestingly, they observed that the inoculation of Ni-resistant bacteria to surface-sterilized seeds of *A. murale* grown in nonsterile serpentine soil increased Ni uptake into the shoot by 17% (*S. macrogoltabidus*), 24% (*M. liquefaciens*), and 32.4% (*M. arabinogalactanolyticum*) compared with uninoculated controls. This indicates that bacteria facilitated the release of Ni from the nonsoluble phases in the soil and thereby enhanced the availability of Ni to *A. murale* which can be ascribed to acid, siderophore production, and phosphate solubilization (Rajkumar et al. 2009). Similar observations were also reported by Rajkumar and Freitas (2008b), who found that the addition of *Pseudomonas sp*. or *P. jessenii* to surface sterilized root of *R. communis* in autoclaved soil increased the Zn concentrations in shoot and leaf tissues compared with respective non-inoculated controls. Recent examples of PGPR-*Brassicaceae* combinations and their effects on soil-metal-plant growth interactions in various phytoremediation works were simplified from Glick (2010) and Ma et al. (2011) and summarized in Table 8.1.

Serpentine (ultramafic) rocks are formed when the upper mantle penetrated into the top parts of the earth crust due to tectonic movement (Robinson et al. 1996). Weathering of these rocks results in genesis of serpentine soils which are characterized by a moderate pH range (between 6 and 8), low levels of mineral nutrients (N, P, K, Ca and Mg) but potentially high levels of heavy metals such as Co, Cr and Ni (Li et al. 2003). These characteristics render serpentine soils unfavorable for normal terrestrial plants (Nicks and Chambers 1995, 1998), but on the other hand, provide a unique selective inhabitant for the evaluation of endemic serpentine plants (Pichi Sermolli 1948; Wild and Bradshaw 1977; Kruckeberg and Kruckeberg 1990; Prasad and Freitas 1999) which are mainly members of *Brassicaceae* plant family hyperaccumulating heavy metals during their growing on serpentine outcrops (Baker et al. 2000). Serpentine rocks that are hosting *Brassicaceae* plant family are mainly located in Western Australia, New Zealand, South Africa, Japan, Philippines, Brazil, Portugal, Italy, Turkey, Cuba, eastern Canada, and western North America. Although serpentine rocks cover only less than 1% of the earth's surface (Rajkumar et al. 2009), their worldwide distribution attracted many researchers in exploring their distinctive characteristics. This may explain the reason why there is a large body of literature on the ecology and phytoremediation potential of *Brassicaceae* plant family inhabiting in these countries (Minguzzi and Vergnano 1948; Doksopulo 1961; Menezes de Sequeira 1969; Brooks and Radford 1978; Reeves 1988; Vergnano Gambi 1992; Palmer et al. 2001; Mengoni et al. 2003; Reeves and Adiguzel 2004). Another growing

Table 8.1 Examples of PGPR-*Brassicaceae* combinations and their effects on soil-metal-plant growth interactions in various phytoremediation works (Glick 2010; Ma et al. 2011)

Host plant	Bacterial strain	Mechanism/effects	Reference
Indian mustard (Brassica juncea)	*Kluyvera ascorbata* SUD165	Ni immobilization, ACC deaminase/Increasing plant biomass	Burd et al. (1998)
	Sinorhizobium sp. Pb002	ACC deaminase/Increased plant survival and lead uptake	Di Gregorio et al. (2006)
	Variovorax paradoxus, Rhodoccus sp., *Flavobacterium* sp.	IAA, siderophores, ACC deaminase/Increased root length	Belimov et al. (2005)
	Bacterial mixture *(Azotobacter chroococcum* HKN-5 B. megaterium HKP-1 *B. mucilaginosus* HKK-1)	Increased biomass and metal bioavailability	Wu et al. (2006b)
	B. subtilis SJ-101	IAA, phosphate solubilization/Increased nickel uptake;	Zaidi et al. (2006)
	Enterobacter cloacae CAL2	Arsenic immobilization, ACC deaminase/Increasing plant biomass	Nie et al. (2002)
	Pseudomonas sp. PsA, *Bacillus sp*. Ba32 *Azotobacter chroococcum* HKN-5 *Bacillus megaterium* HKP-1, B. m*ucilaginosus* HKK-1	ACCD, siderophore, IAA, P solubilization, N fixation, P solubilization, K solubilization, respectively; metal mobilization/Increasing plant aboveground biomass	Rajkumar et al. (2006) Wu et al. (2006b)
	Bacillus subtilis SJ-101	IAA, P solubilization, Ni bioaccumulation/Increasing shoot length, fresh and dry weights	Zaidi et al. (2006)
	Enterobacter sp. NBRI K28, mutant NBRI K28 SD1	ACCD, siderophore, IAA, P solubilization/Increasing plant biomass, protein and chlorophyll content and increasing phytoextraction of Ni Zn and Cr	Kumar et al. (2008)
	Pseudomonas sp. Ps29C, *Bacillus megaterium* Bm4C	ACCD, siderophore, IAA, P solubilization/Increasing shoot length, plant fresh and dry weight	Rajkumar and Freitas (2008a)
	Pseudomonas aeruginosa KUCd1	Siderophore/Increasing shoot length, root length, wet weight, dry weight, and chlorophyll decreasing Cd uptake in shoots	Sinha and Mukherjee (2008)

(continued)

Table 8.1 (continued)

Host plant	Bacterial strain	Mechanism/effects	Reference
	Enterobacter aerogenes NBRI K24, *Rahnella aquatilis* NBRI K3	ACCD, siderophore, IAA, P solubilization, metal biosorption/Increasing plant height, root length, wet and dry weight, leaf protein and chlorophyll content and increasing Ni and Cr uptake	Kumar et al. (2009)
	*Achromobacter xylosoxidans*Ax10	ACCD, IAA, P solubilization/ Increasing root and shoot length, fresh and dry weight and increasing Cu uptake	Ma et al. (2009a)
	Psychrobacter sp. SRA1 and SRA2, *Bacillus cereus* SRA10	ACCD, siderophore, IAA,P solubilization, Ni mobilization/Increasing root and shoot length, vigour index (roll towel assay), fresh and dry weight	Ma et al. (2009b)
	Pseudomonas sp. SRI2, *Psychrobacter* sp. SRS8 and *Bacillus* sp. SN9	ACCD, siderophore, IAA, P solubilization, Ni Mobilization/Increasing fresh and dry weight and increasing Ni bioavailability and uptake	Ma et al. (2009c)
Canola (Brassica napus)	*Bacillus* sp. RJ16	IAA, Cd mobilization/Root elongation (gnotobiotic conditions), shoot and root dry Weigh and increasing Cd plant uptake	Sheng and Xia (2006)
	Pseudomonas tolaasii ACC23, *P. fluorescens* ACC9, *Mycobacterium* sp. ACC14	ACCD, siderophore, IAA/ Increasing Plant root elongation promotion activity, shoot and root dry biomass and increasing Cd uptake	Dell'Amico et al. (2008)
	Pseudomonas fluorescens G10, *Microbacterium* sp. G16	ACCD, siderophore, IAA, [Pb] mobilization/Increasing root length (root elongation assay), shoot and root dry weight (pot experiment)	Sheng et al. (2008)
Canola (Brassica napus)	*Arthrobacter* sp. MT16, *Microbacterium* sp. JYC17, *Pseudomonas chlororaphis* SZY6, *Azotobacter vinelandii* GZC24, and *Microbacterium lactium* YJ7	ACCD, siderophore, IAA, P solubilization/Increasing root length promotion (Strains MT16, JYC17, SZY6, GZC24, and YJ7 increased root length of Cu-treated and untreated seedlings by 17–38% and 20–41%)	He et al. (2010)

(continued)

Table 8.1 (continued)

Host plant	Bacterial strain	Mechanism/effects	Reference
	Firmicutes sp., *Actinobacteria* sp., *Proteobacteria* sp.	ACCD, siderophore, IAA, arginine decarboxylase production/Increasing root aboveground tissue, dry weight and Cu uptake	Sun et al. (2010)
	P. putida HS-2	Siderophores, IAA, ACC deaminase/Increased seed germination and biomass;	Rodriguez et al. (2008)
	Pseudomonas sp. RJ10, *Bacillus* sp. RJ16	IAA/Increased biomass and metal uptake	Sheng and Xia (2006)
	P. fluorescens, P. putida	Mechanism unknown/Increased seed germination and growth	Ashour et al. (2006)
	P. putida UW4, *P. putida* HS-2	IAA, ACC deaminase/ Increased biomass in the field	Farwell et al. 2006
Alyssum murale	*Microbacterium oxydans* AY509223	Ni mobilization/Increasing Ni uptake in different Ni rich soils	Abou-Shanab et al. (2006)
	Microbacterium arabinogalactanolyticum	Mechanism unknown/Increased nickel uptake;	Abou-Shanab et al. (2003b)
Brassica oxyrrhina	*Psychrobacter* sp. SRA1 and SRA2, *Bacillus cereus* SRA10	ACCD, siderophore, IAA, P solubilization, Ni mobilization/Increasing root and shoot length, vigour index (roll towel assay), fresh and dry weight (pot experiment) and increasing Ni bioavailability and uptake	Ma et al. (2009b)

ACC deaminase 1-aminocyclopropane-1-carboxylate (ACC) deaminase, *IAA* indole-3-acetic acid

interest concerning serpentine soils is their potential for metal resistant PGPR (Mengoni et al. 2004; Barzanti et al. 2007; Rajkumar et al. 2008; Rajkumar and Freitas 2008b; Ma et al. 2009b, c; Turgay et al. 2011). The autochthonous bacteria of naturally heavy metal-contaminated soils such as serpentines may have acquired resistance against heavy metals (Pal et al. 2005) and serpentine areas can be considered an interesting model for the study of the evolution of metal-resistant microorganisms that are completely different from those of artificially contaminated soils (Rajkumar et al. 2008). Therefore, the interactions between serpentine microorganisms and serpentinophytes have attracted the attention of several researchers due to their potential biotechnological applications on bioremediation (Abou-Shanab et al. 2007; Goncalves et al. 2007) and the results showed that new strains and genetic determinants for heavy metal-resistance derived from serpentine soils had high level of tolerance to Ni and other heavy metals (Abou-Shanab et al. 2007; Goncalves et al. 2007).

8.6 Conclusions and Perspectives

The interfaces between soil microorganisms and plant rhizosphere have a great influence on the decrease of metal toxicity as well as the increase of nutrient uptake. Metal resistant-plant growth promoting rhizobacteria have an exceptional ability to promote the growth of the host plant by various mechanisms, such as production of siderophores, solubilization of phosphate and production of plant growth regulators (hormones). These processes can influence the solubility and the bioavailability of heavy metals to the plant and thus modify their toxic effects. The relationships between PGPR activities, plant and metal uptake process are of practical importance for both metal-contaminated environments and the phytoremediation studies with *Brassicaceae* plant family. The majority of in-vitro observations indicated that *Brassicaceae* spp. had higher biomass and/or better metal accumulations when they were inoculated with metal resistant PGPR isolated from industrially or naturally metal contaminated soils such as serpentines. There is, however, still limited information describing the interactions between plants and microorganisms in metal-contaminated soils and the mechanisms underlying bacteria-assisted phytoremediation in the field. Therefore, further efforts should be given to field-scale researches to understand the diversity and ecology of plant-associated PGPR in multiple metal-contaminated soils.

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Chapter 9
Biochemical and Functional Responses of *Arabidopsis thaliana* Exposed to Cadmium, Copper and Zinc

Adriano Sofo, Antonio Scopa, Tony Remans, Jaco Vangronsveld, and Ann Cuypers

Abstract Phytoremediation has been accepted advantageous over commonly used civil engineering remediation methods in costs, practice and the scale at which the processes operate. Understanding the metabolic answer and the adaptation of plants towards toxic metal exposure opens the way to future phytoremediation of contaminated sites. The majority of these metals get accumulated in plants and may either directly or indirectly find their way into the food chain causing severe secondary consequences. In particular, excess cadmium (Cd), copper (Cu) and zinc (Zn) are known to induce stress effects in all plant species. However, while Cu and Zn are normally present in different soils, and are part of or act as cofactors of many cell macromolecules, plants have no metabolic requirement for Cd. *Arabidopsis thaliana* L. is considered a model plant for many studies as its genomic sequence was completely identified and its mechanisms in genomic, transcriptomic and proteomic regulation are often similar to other plant species. The molecular, biochemical, physiological and morphological characteristics of this species are strongly affected by the exposure to Cd, Cu and Zn. The aim of this work is to give an up-to-date overview on the recent breakthroughs in the area of responses and adaptation of *A. thaliana* to Cd, Cu and Zn, three of the most common metals found in polluted soils, both alone and in combination. This chapter aims to contribute to a better understanding of the fundamental aspects of detoxification of metals and general responses in phytoremediation. The numerous and easily available genetic resources developed in *A. thaliana* should be extended to fast growing plant species of high biomass having significant tolerance to metals and suitable for phytoremediation purposes.

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Abbreviations

Cd	Cadmium
CKs	Cytokinins
Cu	Copper
GSH	Reduced glutathione
IAA	Indole-3-acetic acid
MTs	Metallothioneins
PCS	Phytochelatin synthase
PCs	Phytochelatins
Zn	Zinc

9.1 Introduction

Excess cadmium (Cd), copper (Cu) and zinc (Zn) are known to induce stress effects in all plant species. However, while Cu and Zn are normally present in different soils, and are part of or act as cofactors of many cell macromolecules (e.g., Cu/Zn-proteins and cytochromes), plants have no metabolic requirement for Cd. Cadmium levels in soils are generally low (an average of 0.3 μM) and, for this reason, plants usually do not experience this metal under normal environmental conditions. Nevertheless, Cd is efficiently absorbed by plant roots, translocated *via* xylem, and compartmentalized in vacuoles, and it influences the transcription of several genes (Vangronsveld et al. 2009; Cuypers et al. 2010).

Elements, such as Cu, Zn and Cd belong to the group of so-called ‘heavy metals’. In small amounts, most of these elements are indispensable for many organisms, but their increased doses induce acute or chronic poisoning. Some environments, such as the serpentine soils, have naturally high concentrations of metals. Moreover, mining and industrialization have also led to soils with increased metal contents. In recent years, phytoremediation techniques have been applied on Cd and other metals as well as on organics (Vangronsveld et al. 2009). Many studies have been focusing on plant metal uptake and transport, their entry into regular metabolic channels of the plant, and plant metal chelation and sequestration. Furthermore, important responses induced by Cu, Zn and Cd, such as the synthesis of phytochelatins (PCs) and metallothioneins (MTs), were observed in many plant species (Kvesitadze et al. 2006).

Metals enter the plant cells from air, soil and water, but plants absorb contaminants primarily through their roots and leaves, whose absorption differ essentially from each other. Due to their positive charge, Cd, Zn and Cu are present mainly as soil-bound cations or complexed in organic molecules (e.g., humic/fulvic acids, extracellular enzymes, aliphatic and aromatic hydrocarbons, alcohols, phenols, amines, etc.), and so they enter the roots dissolved in water, like nutrients, and they move towards the transport tissue (xylem) mainly along the apoplast. To a lesser extent, Cd, Zn and Cu are bound to negatively-charged dust particles, so plants interact with these metals during their precipitation from the atmosphere when they penetrate into leaves via the cuticle (Kvesitadze et al. 2006). Once absorbed by roots and leaves, environmental contaminants are translocated to different plant organs by the same physiological processes transporting nutrients.

The plant's abilities to absorb, conjugate, compartmentalize and accumulate metals within its cells determines the ecological detoxification potential of a species. High biomass production, well-developed roots, and a strong defense system are the most important overall criteria for plants to be successfully applied in phytoremediation processes of metal contaminated soils (Kabata-Pendias and Mukherjee 2007). Some plant species are able to accumulate high amounts of metals in their roots and subsequently translocate them to the above-ground organs (the so-called "phytoextraction"), eliminating in this way contaminants from the soils, sediments and sludges. There are several plant species that not only tolerate large quantities of metals but hyperaccumulate them. Hyperaccumulators are defined as plants that can accumulate 10,000 $\mu g\ g^{-1}$ dry weight Zn or Mn, 1,000 $\mu g\ g^{-1}$ dry weight Ni, Co, As, Se, or Cu, or 100 $\mu g\ g^{-1}$ dry weight Cd, and they have gained great interest as potential sources of genes for developing plants for phytoremediation (Hassinen et al. 2007). As hyperaccumulating plants have extraordinary capabilities of uptake and metal tolerance, they are the best sources of genes to be used for phytoremediation purposes. Despite the recent exploitation of high-throughput methodologies, such as cDNA analysis and microarrays, the overall picture of plant metal tolerance, accumulation, and translocation is far from being complete (Hassinen et al. 2007). It is also noteworthy that other techniques are used to remediate metal-contaminated soils, such as phytostabilization.

Arabidopsis thaliana L. ("*Arabidopsis*" throughout the text) is considered a model plant for many studies as its genomic sequence was completely identified and its mechanisms in genomic, transcriptomic and proteomic regulation are often similar to other plant species. The molecular, biochemical, physiological and morphological characteristics of *Arabidopsis* are strongly affected by the exposure to Cd, Cu and Zn (Tan-Kristanto et al. 2003; Van Belleghem et al. 2007; Remans et al. 2008; Smeets et al. 2009; Semane et al. 2010; Watanabe et al. 2010; Cuypers et al. 2010) (Fig. 9.1). Furthermore, a cumulative role in toxic metal accumulation and tolerance in this species was observed (Verbruggen et al. 2009). *Arabidopsis* offers many advantages in the search for novel genes and for this reason, many studies have been carried out in this species regarding the tolerance and accumulation of metals other than Cd/Cu/Zn, such as iron (Duy et al. 2007; Stacey et al. 2008), lead (Kim et al. 2006; Liu et al. 2009), caesium and strontium (Kanter et al. 2010), aluminum (Goodwin and Sutter 2009), selenium (Zhang et al. 2007; Dutilleul et al. 2008), uranium (Vanhoudt et al. 2008), and arsenic (Li et al. 2006; Singh and Ma 2007), and mercurial derivatives (Bizily et al. 1999; Li et al. 2006).

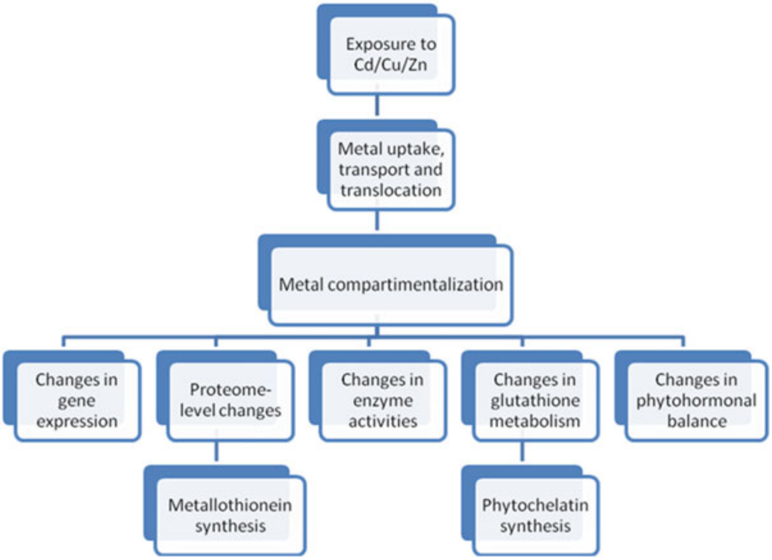

Fig. 9.1 Effects of cadmium, copper and zinc on *Arabidopsis thaliana* (Data from Sanità di Toppi et al. 2003; Tan-Kristanto et al. 2003; Van Belleghem et al. 2007; Remans et al. 2008; Smeets et al. 2009; Semane et al. 2010; Watanabe et al. 2010; Cuypers et al. 2010)

The great importance of the studies on *Arabidopsis* responses to metals is because of the fact that it belongs to the family of Brassicaceae (Cruciferae), whose cultivated species are known to be good accumulators of toxic metals, allocating large amounts of most toxic metals to above-ground organs. Nowadays, about 400 plant species are known to hyperaccumulate metals, and an important part of them belong to the family of Brassicaceae (Pence et al. 2000; Wójcik et al. 2005a, b; van de Mortel et al. 2006; Courbot et al. 2007; Gasic and Korban 2007; Hassinen et al. 2007; Mijovilovich et al. 2009; Vangronsveld et al. 2009). Furthermore, many genera of Brassicaceae (e.g., *Brassica*, *Alyssum*, *Arabis*, *Arabidopsis*, *Berkheya, Bornmuellera, Cardamine*, *Cochlearia, Peltaria, Pseudosempervivum, Stanleya*, *Streptanthus*, and *Thlaspi*) are well known in terms of their utility in toxic metals-remediation strategies. In particular, the oleiferous genus *Brassica* includes good Cd/Cu/Zn-accumulating species and it also has a great agronomic importance, being the third most important source of vegetable oil in the world after palm and soybean oil. Finally, the basic biology, ecology, population genetics and molecular evolution are often similar among different plant species. Therefore, the wealth of functional and genomic tools of *Arabidopsis* could be applied to gain insights into adaptive evolution of ecologically important traits and genome-wide processes at the basis of metal uptake and compartmentalization among other plant species (Clauss and Koch 2006; Przedpełska and Wierzbicka 2007).

On this basis, the aim of this work is to give an up-to-date overview on the recent breakthroughs in the area of responses and adaptation of *Arabidopsis* to Cd, Cu and Zn, three of the most common metals found in increased concentrations in contaminated soils, both alone and in combination. As *Arabidopsis* is a model plant for many genomic, transcriptomic and proteomic studies, this chapter could be important for a better understanding of the fundamental aspects of detoxification of metals (in terms of tolerance and accumulation potential) and for their use in phytoremediation approaches.

9.2 The Environmental Impact of Cadmium, Copper and Zinc

9.2.1 Cadmium

Cadmium is a major environmental contaminant that enters human food via accumulation in crop plants (Kabata-Pendias and Mukherjee 2007), and is considered as being one of the most ecotoxic metals that exhibits adverse effects on all biological processes in humans, animals, and plants (Cuypers et al. 2010). It is produced mainly as a by-product in mining and refining of Zn, with uses in the production of batteries, pigments, coatings and stabilizers. In uncontaminated soils, its content is highly governed by soil texture and ranges from 0.22 to 0.51 mg kg^{-1} dry soil. In contaminated soils (>1 mg kg^{-1}), the major sources of pollution are atmospheric deposition and P-fertilizers. Contents of Cd in plants vary in the range of 5–400 μg kg^{-1} dry matter, and are higher in roots than in shoots. The behavior of Cd in plants is closely related to Zn as both metals are bivalent cations with a high affinity for sulfur, particularly sulphydryl groups. Some researchers have reported on the association of Cd with other elements in plants (e.g., Zn, Cu, Se, P, Cl) and have highlighted the key role of pH, carbonates, and organic carbon in Cd soil bioavailability (Kabata-Pendias and Mukherjee 2007). The accumulation or exclusion of Cd by plants differ at the family levels, but *Brassicaceae* and *Fabaceae* species show the highest and the lowest tolerance to Cd, respectively (Kabata-Pendias and Mukherjee 2007).

9.2.2 Copper

Copper is used for the production of conductor materials and in the manufacturing of motors and electrical equipment, and for the fabrication of household articles, coins, art objects and ammunition (Kabata-Pendias and Mukherjee 2007). Copper is also widely used in agriculture (fertilizers, pesticides etc.) and, due to its bacteriostatic properties, it is also used as a feed additive in livestock and poultry nutrition. The general values for the average total Cu contents in soils of different types are reported to range between 20 and 30 mg kg^{-1}. The phytoavailability of Cu is influenced by its chemical form, and is not a function of its total concentration but of several soil variables, such as pH, oxidation and reduction potential, organic matter, soil texture, mineral composition, temperature, and water regime. Contaminated soils contain up to 5,000 mg kg^{-1} of Cu, whereas Cu content in plants usually ranges from 3 to 15 mg kg^{-1}. Plants growing on Cu-polluted sites tend to accumulate increased amounts of this metal (up to 1,000–10,000 mg kg^{-1} in some plants of the Labiatae family), especially near industrial areas, and in soils treated with Cu-bearing herbicides. Copper is only slightly mobile in plants, as it is strongly bound by nitrogen and proteins.

9.2.3 Zinc

Zinc is an important component of various alloys and is a catalyst in different chemical productions (e.g., rubber, pigments, plastic, lubricants, and pesticides). It is used in the industry of batteries, automotive equipment, medical applications, and pipes and household devices (Kabata-Pendias and Mukherjee 2007). This metal, compared to Cd, has a relatively low toxicity for humans, but the ingestion or inhalation of larger doses of Zn, especially in forms of inorganic compounds, can be harmful to individuals. The micronutrient Zn has an essential role in physiological and metabolic processes in plants as a cofactor or as a structural element in 300 catalytic and noncatalytic proteins, but it is very toxic when available in elevated amounts (van de Mortel et al. 2006). In uncontaminated soils, Zn contents averages from 10 to 300 mg kg^{-1}, and silicates, carbonates, phosphates, oxides and organic matter may contribute to Zn retention. In strongly contaminated soils, where Zn can reach levels from 1,000 to 10,000 mg kg^{-1}, the metal derives from atmospheric deposition, fertilizers, pesticides, sewage sludge, leaching from galvanized materials, manure, waste, slag and ashes. Contents of Zn in food plants varies in the range of 18–47 mg kg^{-1}, and it is higher in roots than in foliage. Sensitive terrestrial plants die when soil Zn concentration exceeds 100–200 mg kg^{-1} soil, but some species are known to hyperaccumulate Zn, as for example *Thlaspi* species that can contain above 10,000 mg Zn kg^{-1} and were applied for the phytoremediation of contaminated soil (Sarret et al. 2002; McGrath et al. 2006). The mobility of Zn within plants highly varies depending on species and plants' nutritional status.

9.3 Metal Uptake, Transport and Translocation

9.3.1 Occurrence of Metal Ions Throughout the Plant

As leaf Cd concentrations in excess of 5–10 μg g^{-1} dry matter are toxic to most plants, they have evolved mechanisms to limit Cd translocation to the shoot (Sanità di Toppi et al. 2003). Generally, the presence of Cd in the rhizosphere inhibits root elongation and influences root anatomy, but apoplastic movement of Cd to the xylem can be restricted by the development of the exodermis, endodermis, and other extracellular barriers (Lux et al. 2011).

Making use of nuclear microscopy techniques (NMP), Ager et al. (2002, 2003) studied leaves of wild type and transformed lines of *Arabidopsis* overexpressing the *Atcys-3A* gene (Domínguez-Solís et al. 2001) grown in a Cd-enriched Hoagland medium. They proved that Cd is preferentially sequestered in the central region of epidermal trichomes, where it is likely complexed to phosphate and sulfur. The fact that *Arabidopsis* trichomes participate in metal detoxification has also been suggested by Wienkoop et al. (2004), who identified proteins involved in sulfur metabolism and detoxification in these cells by specific cell sampling and shotgun peptide sequencing (nano LC/MS/MS). The preference for Cd localization in the peripheral parts of leaf blades was confirmed by Wójcik and Tukiendorf (2004).

Van Belleghem et al. (2007) examined the subcellular Cd localization in roots and leaves of *Arabidopsis* exposed to different Cd levels (from 0 to 50 μM) by means of energy-dispersive X-ray microanalysis (EDXMA). They found that in the root cortex Cd is associated with phosphorus (Cd/P) in the apoplast, and sulfur (Cd/S) in the symplast, and that the transport route of Cd through the cortex is mainly apoplastic. In the endodermis, where Cd transport is forced through symplast, sequestration of Cd/S was present in cells as granular deposits. In the central cylinder, Cd transport occurred mainly in the apoplast. Furthermore, large amounts of precipitated Cd in the phloem suggest that Cd re-translocation from the shoot occurs. In leaves, Cd was detected in tracheids but not in the mesophyll tissue. Extensive symplastic and apoplastic sequestration in the root parenchyma combined with re-translocation back to the roots via the phloem confirms the Cd-excluder strategy of *Arabidopsis*. In *Arabidopsis* plants grown on Cd-containing hydroponics (5–100 μM), examined by Wójcik and Tukiendorf (2004) using EDXMA, Cd was not detectable in the cytoplasm, vacuoles and organelles within roots or cell walls of tissues other than the pericycle, so confirming the preference of the apoplastic route for Cd transport (Van Belleghem et al. 2007).

The situation in the hyperaccumulator species *Thlaspi caerulescens*, related to *Arabidopsis*, is quite different, as Cd in roots is mainly located in cortex parenchyma cells, endodermis, parenchyma cells of the central cylinder and xylem vessels, whereas in leaves it accumulates in the vacuoles of cells lying on the way of water migration from the vascular cylinder to epidermal cells (Wójcik et al. 2005a, b). In this species, Cd is passively transported by the transpiration stream and the mechanisms of Cd detoxification in roots seem to be both apoplastic and symplastic.

Sarret et al. (2002) determined the chemical forms of Zn in the Zn-tolerant and hyperaccumulator *Arabidopsis halleri* and in the non-tolerant and non-accumulator *Arabidopsis lyrata* by combining chemical analyses and X-ray analyses. Plants were grown with various Zn concentrations (100 and 250 μM $ZnSO_4$). In aerial parts of *A. halleri*, Zn was predominantly octahedrally coordinated and complexed to malate. A secondary organic species was identified in the bases of the trichomes, which contained elevated Zn concentrations, and in which Zn was tetrahedrally coordinated and complexed to carboxyl and/or hydroxyl functional groups.

Kashem et al. (2010) used *A. halleri* grown for 3 weeks in $ZnSO_4$ levels ranging from 0.2 to 2,000 μM. Plants proportionally absorbed Zn excess by roots on the basis of the Zn concentration applied, but they did not show reductions in shoot and root dry weight. The percentage of Zn translocation in shoot varied from 69% to 90% of the total Zn, suggesting that the shoot was the major sink of Zn accumulation in this species. The concentration of Zn found in shoots indicated that *A. halleri* has an extraordinary ability to tolerate and accumulate Zn. In the roots of *A. halleri,* Zn phosphate, Zn malate and Zn citrate were the three Zn species detected. Zinc phosphate was mainly found in both the roots and aerial part of *A. lyrata* – a non-accumulator species.

9.3.2 Metal Transporters in Uptake and Translocation

To absorb and translocate metal ions, plants utilize a large number of membrane transporters (Wintz et al. 2003). P-type H^+-pumps, the organellar proton pumps and many ATP-binding cassette (ABC) proteins are primary active transporters (Ludewig and Frommer 2002; Cobbett et al. 2003a, b) involved in metal transport.

P_{1B}-type ATPases transport metal ions (Cu^+, Cu^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, etc.) across biological membranes in plants (Cobbett et al. 2003a, b). *Arabidopsis* is remarkable for its large number of type 1B metal transporting ATPases if compared to other organisms. It is likely that these transporters are involved in the metabolism of Cu and Zn and possibly a third metal (Cobbett et al. 2003a, b). Among *Arabidopsis* P-type pumps, HMA2 (Heavy Metal Associated 2 protein) is responsible for Zn^{2+} efflux from the cells and therefore is required for maintaining low cytoplasmic Zn levels and normal Zn homeostasis (Eren and Argüello 2004). Observations indicate a primary role for HMA2 and HMA4, whose expression is predominantly in the vascular tissues of roots, stems, and leaves, in essential Zn translocation (Hussain et al. 2004; Talke et al. 2006; Courbot et al. 2007). HMA2, has N- and C-terminal domains that can bind Zn ions with high affinity (metal-binding domains, MBDs), but only the N-terminal domain seems to be essential for functioning *in planta* while the C-terminal domain may contain a signal important for the subcellular localization of the protein (Wong et al. 2009; Zimmermann et al. 2009). Besides being essential in Zn translocation, a nearly complete abolition of root-to-shoot Cd translocation resulting from the loss of function of HMA2 and HMA4 in the phytochelatin (PC) -deficient *cad1-3 Arabidopsis* mutant indicates their importance in Cd translocation in *Arabidopsis* (Wong and Cobbett 2009). Both *hma2* and *hma4* mutations also confer increased sensitivity to Cd in PC-deficient mutants of *Arabidopsis*, suggesting that they may also influence Cd detoxification (Hussain et al. 2004). Another protein of this family, HMA7, is involved in Cu^+ transport. While the MBD of HMA7 features a CxxC sequence motif characteristic for

Cu^+ binding sites, those of HMA2 and HMA4 contain a CCxxE motif, unique for plant Zn^{2+}-ATPases (Zimmermann et al. 2009). HMA3, belonging to the P_{1B-2} subgroup, likely plays a role in the detoxification of biological (Zn) and non-biological (Cd, Co and Pb) metals by participating in their vacuolar sequestration (Morel et al. 2009). Abdel-Ghany et al. (2005) identified two previously uncharacterized genes (*PAA1* and *PAA2*) coding for P-type ATPase in *Arabidopsis*, that are located in the chloroplast and are required for sequential Cu transport over the envelope and thylakoid membrane, respectively in order to foresee an efficient photosynthetic electron transport. Another P-type ATPase gene of *Arabidopsis* is *RAN1*, involved in Cu homeostasis (Hirayama et al. 1999). The *ran1* mutants have a non-functional ethylene response phenotype because the ethylene receptors are Cu-dependent proteins.

ABC proteins mediate the transport of substances by coupling the release of chemical energy stored in ATP to substrate translocation. An important ABC protein involved in Cd transport, accumulation and tolerance is *Arabidopsis* MRP7, localized both in the tonoplast and in the plasma membrane. The over-expression of this transporter increases Cd-tolerance and results in enhanced Cd root-to-shoot transport and accumulation in leaf vacuoles, indicating more efficient detoxification (Wojas et al. 2009).

NRAMPs (natural resistance-associated macrophage proteins) have been characterized in animals and plants as divalent transition metal transporters involved in metal metabolism and host resistance to certain pathogens. In *Arabidopsis*, over-expression of *AtNramp3* and *AtNramp4* results in Cd hypersensitivity and in Cd and Fe accumulation. These genes are normally expressed in both roots and aerial parts, and disruption of the *AtNramp3* gene leads to slightly enhanced Cd resistance of root growth (Thomine et al. 2000). For this reason, *AtNramp* genes likely encode metal transporters of both the metal nutrient Fe and Cd. Furthermore, members of the ZIP family, in particular, *ZIP2* and *ZIP4*, are involved in Cu transport, whereas *AtOPT3* participates in the transport of various cations (Cu, Mn, Fe) (Wintz et al. 2003). Recently, Verbruggen et al. (2009) described and summarized exhaustively the strategy that non-hyperaccumulator species like *Arabidopsis* use for Cd uptake and accumulation. In *Arabidopsis*, the ZIP transporter IRT1 seems to be a main entry for Cd.

Two members of the *Arabidopsis* Yellow Stripe-Like (YSL) family, *AtYSL1* and *AtYSL3* codify for two oligopeptide transporter families, predicted to be integral membrane proteins involved in delivery of metal micronutrients to and from vascular tissues (Waters et al. 2006). Indeed, leaf Fe concentrations are decreased in the double mutant, whereas Mn, Zn, and especially Cu concentrations are elevated, whereas in seeds of double-mutant plants, the concentrations of Fe, Zn, and Cu are low. Haydon and Cobbett (2007) have identified Zn-sensitive *Arabidopsis* mutants for the gene *ZINC-INDUCED FACILITATOR 1* (*ZIF1*) that encodes a member of the major facilitator superfamily (MFS) of membrane proteins, one of the two largest families of membrane transporters found on earth. Shoots of *zif1* mutants showed increased accumulation of Zn but not of other metal ions, and overexpression of *ZIF1* confers increased Zn tolerance and interveinal leaf chlorosis, suggesting that ZIF1 is involved in a novel mechanism of Zn sequestration, possibly by transport of Zn-ligand complexes into vacuoles. van der Zaal et al. (1999) isolated a Zn transporter gene, *ZAT1*, and observed that transgenic *Arabidopsis* plants in which *ZAT1* was over-expressed exhibited enhanced Zn accumulation in roots and increased Zn-tolerance. However, transgenic plants expressing an anti-sense construct showed no altered phenotype. Finally, among further implicated proteins are IRT3 and ZIP10, which have been proposed to contribute to cytoplasmic Zn influx in *Arabidopsis* (Talke et al. 2006).

9.4 Metal Compartmentalization

9.4.1 Proteins and Transporters Involved in Metal Homeostasis

Different proteins and transporters are involved in Cd/Cu/Zn detoxification/homeostasis in *Arabidopsis*. A complete inventory of *Arabidopsis* metallochaperone-like proteins containing a predicted HMA domain revealed a large family of 67 proteins. In a recent study, Tehseen et al. (2010) found that 45 proteins, the HIPPs, have a predicted isoprenylation site while 22 proteins, the HPPs, do not. Sequence comparisons divided the proteins into seven major clusters (I–VII). Cluster IV is notable for the presence of a conserved Asp residue before the CysXXCys, metal binding motif. Promoter-GUS reporter expression analysis indicated variable spatial expression of these HIPPs, but it is probable that HIPPs have a role in Cd-detoxification, possibly by binding Cd.

Regarding Zn, AtMTP1 has been demonstrated to be a Zn transporter localized in the vacuolar membrane and mediates Zn detoxification and storage by vacuolar sequestration of Zn (Desbrosses-Fonrouge et al. 2005). AtMTP1 is not produced throughout the plant, but primarily in the subpopulation of dividing, differentiating and expanding cells, and contributes to cellular metal accumulation and to basal metal tolerance in cells of growing tissues.

The main detoxification pathway of Cd in roots relies on phytochelatin (PC) complexation (cfr. next section) and vacuolar transport of Cd–PCs complexes of low molecular weight (LMW). In the vacuole, high molecular-weight complexes (HMW) that contain sulphides (S^{2-}) may be formed, but the stability of those complexes is still not well understood. Cadmium can also be transported to the vacuole by the activity of different transporters (cation exchangers, HMA3) or as Cd-GS_2 complexes by an unidentified ABC transporter, and part of the vacuolar Cd(II) pool could be refluxed back into the cytosol by NRAMP activity. Metallothioneins (MTs), a particular class of proteins later discussed in detail, can also act as potential Cd ligands in the cytosol.

9.4.2 Phytochelatins and Metallothioneins

Phytochelatins (PCs; general formula (γ-Glu-Cys)nGly)), are polymerized chains of glutamic acid and cysteine residues synthesised from reduced glutathione (GSH) (γ-Glu-Cys-Gly) in a reaction mediated by phytochelatin synthase (PCS) (Cobbett 2000; Sanità di Toppi et al. 2003). They are a class of metal-binding peptides playing an important role in metal homeostasis and detoxification, and they represent a major detoxifying pathway for metals in plants and many other organisms (Sanità di Toppi and Gabbrielli 1999; Wójcik and Tukiendorf 2004). Moreover, Cd application induced the biosynthesis of PCs in root and shoot tissues of *Arabidopsis* (Wójcik and Tukiendorf 2004; Połeć-Pawlak et al. 2005). On the other hand, no PC accumulation was detected in Cd-treated *Thlaspi caerulescens* plants (hyperaccumulator strategy), suggesting that naturally selected tolerance in this species is not associated with enhanced PC synthesis (Wójcik et al. 2005a, b; Verbruggen et al. 2009).

In *Arabidopsis*, Cd movement through the root symplast is restricted by the production of PCs and the sequestration of Cd-chelates in vacuoles, and PC deficiency resulted in an increase in shoot Cd concentrations (Wong and Cobbett 2009). Whether long-distance transport of PCs occurs during metal detoxification remains unknown. The findings of Gong et al. (2003) and Chen et al. (2006) showed that transgenic expression of *TaPCS1* (a phytochelatin synthase gene from wheat) in *Arabidopsis* suppresses the metal sensitivity of PC-deficient Arabidopsis mutants *cad1-3*. The same authors demonstrated that PCs can be translocated from roots to shoots, and that the transgenic expression of the *TaPCS1* gene increases long-distance root-to-shoot Cd transport and reduces Cd accumulation in roots.

The *Arabidopsis CAD1* (= *AtPCS1*) gene encodes a PCS, and *cad1* mutants are phytochelatin deficient and Cd hypersensitive (Vatamaniuk et al. 1999; Peterson and Olivier 2006). In *Arabidopsis*, the PCS1 activity is positively related to GSH levels of up to 60 mM (depending on Cd levels), and it is enhanced if the concentration of Cd(II) increases (with maximum activity at approximately 60 μM Cd, depending on GSH levels). *Arabidopsis* PCS1 possesses a Cd(II) binding site where Cd (II) binds to activate the enzyme, but it likely has also a second Cd(II) binding site where Cd(II) binds to induce an inhibitory effect, as demonstrated by mathematical models (Ogawa et al. 2011). Noteworthy, the *Arabidopsis* genome also contains a highly homologous gene *AtPCS2* that encodes a functional PCS apparently non-redundant with *AtPCS1* (Cazalé and Clemens 2001). Localization studies of both *Arabidopsis* PCS forms, whose action is likely cytosolic, revealed a ubiquitous presence of AtPCS1 in *Arabidopsis* seedlings, while AtPCS2 was only detected in the root tip (Blum et al. 2010).

With an over-expression of *Arabidopsis* PC synthase gene (*AtPCS1*), a hypersensitivity reaction due to the very high levels of PCs occurred in plants subjected to Cd (50 or 85 μM $CdCl_2$, and this hypersensitivity was also observed for Zn but not for Cu (Lee et al. 2003a, b). The toxicity could be due to the depletion of GSH to produce PCs or to the increasing energy request (ATP) due to the high PC chelation and storage in the vacuole. This indicates that PC homeostasis is of key importance for metal chelation in *Arabidopsis*. The overexpression of the same *Arabidopsis* gene in tobacco plants enhances Cd^{2+} tolerance and accumulation but only when GSH is added to the culture medium (Pomponi et al. 2006), highlighting the importance of GSH/PCs balance in the cells. In another study (Lee et al. 2003a, b), transgenic *Arabidopsis* lines were generated following transformation with a construct containing the *AtPCS1* cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter (*35::AtPCS1*). These plants exhibited ~14-fold increase in the level of the *AtPCS1* transcript. After being exposed to 85 μM $CdCl_2$ for a 3-day period, ~30% increase in the level of PC production occurred in transgenic lines if compared to wild-type, but transgenic lines showed higher sensitivity to Cd than wild-type seedlings. In the same work, *Arabidopsis* plants of other transgenic lines exhibited approximately two-fold increase in Cd tolerance compared to wild-type plants. Surprisingly, these six Cd-tolerant lines, showing only ~15% increase in PC content, presented increased Cd accumulation in their shoots. In Cd-tolerant lines, the positive effect of PC may be higher than its negative effect; while, in Cd-hypersensitive transgenic lines the reverse may be true. Lee et al. (2003a, b) concluded that PC has a positive effect in chelating non-essential metals, while it has an unknown negative effect (e.g. chelating essential metals or disrupting disulfide bond). The authors presume that the unknown toxic effect of PCs may be similar to the toxic effects of both Cys and GSH, both components of PCs, as these inhibit plant growth at supra-optimal concentrations.

Tennstedt et al. (2009) indicated a contribution of PCS expression to Zn^{2+} sequestration, using a known PC-deficient *Arabidopsis* mutants (*cad1-3* and *cad1-6*) with respect to Zn homeostasis. They found that PC-deficient mutants show pronounced Zn^{2+} hypersensitivity and significant reduction in root Zn accumulation. Plants grown under control conditions consistently showed PC2 accumulation. Moreover, in wild type plants, Zn^{2+}-elicited (20 μM for 5 days) PC2 accumulation in roots reached about 30% of the level of Cd^{2+}-elicited (0.5 μM for 5 days) PC2 accumulation, suggesting that PC formation is essential for Zn^{2+} tolerance and provides a driving force for the accumulation of Zn. This function might also help explain the occurrence of PCS genes throughout the plant kingdom and in a wide range of other organisms.

Wójcik et al. (2009) found that Cu (ranging from 5 to 50 μM) did not induce phytochelatin accumulation nor significantly affected the GSH level in *Arabidopsis* plants, but it caused changes in the root structure and leaf chloroplasts ultrastructure, suggesting that GSH is not directly involved in Cu detoxification and tolerance in this species.

Metallothioneins (MTs) are proteins found in various eukaryotes having a low molecular weight (less than 10 kD), a large fraction of cysteine residues, and a high metal content with coordination of metal ions in metal-thiolate clusters (Cobbett and Meagher 2002). In the *Arabidopsis* genome, seven functional MT genes have been reported. However, exhaustive analysis of the genome sequence suggests the presence of at least four additional MT genes (Cobbett and Meagher 2002). It was also observed that MT gene expression in *Arabidopsis* could be induced by Cu and, to a lesser degree, by Zn and Cd. In a comparative study of different *Arabidopsis* genotypes (Murphy and Taiz 1995), variation in Cu tolerance among genotypes, measured by root growth inhibition, was highly correlated with the expression of MT2a in Cu-treated plants. It seems that MTs in *Arabidopsis* are able to play a role in metal tolerance, and their primary role may be in metal homeostasis (Cobbett and Meagher 2002). In the study of Guo et al. (2008), six Arabidopsis MTs (MT1a, MT2a, MT2b, MT3, MT4a, and MT4b) were expressed in Cu- and Zn-sensitive yeast mutants. All four types of MTs provided similar levels of Cu tolerance and accumulation to the yeast mutants, and the type-4 MTs (MT4a and MT4b) conferred greater Zn tolerance and higher accumulation of Zn than other MTs to the mutants. To examine the functions of MTs in plants, the authors studied *Arabidopsis* plants that lack MT1a and MT2b, demonstrating that the lack of MT1a, but not MT2b, led to a 30% decrease in Cu accumulation in roots of plants exposed to 30 μM $CuSO_4$. Furthermore, when MT deficiency was combined with PC deficiency, growth of the *mt1a-2 mt2b-1 cad1-3* triple mutant was more sensitive to Cu and Cd if compared to the *cad1-3* mutant, suggesting that MTs (and MT1a in particular) are important for plant metal homeostasis, and that they function cooperatively with PCs to protect plants from Cu and Cd toxicity. Zhigang et al. (2006) analysed the protective function of a plant type-2 MT after its expression in *Arabidopsis* seedlings. When BjMT2 cDNA was expressed in *Arabidopsis* under the regulation of the 35S promoter, seedlings exhibited an increased tolerance against Cu^{2+} and Cd^{2+} based on shoot growth and chlorophyll content. Analysis of transiently transformed cells of *Arabidopsis* leaves by confocal laser scanning microscopy (CLSM) revealed exclusive cytosolic localization of a BjMT2::EGFP (enhanced green fluorescent protein) fusion protein in control and metal-exposed plant cells. Remarkably, ectopic expression of BjMT2 reduced root growth in the absence of metal exposure, whereas in the presence of 50 or 100 μM Cu^{2+} root growth in control and transgenic lines was identical.

9.5 Effects of the Cd, Cu and Zn at Different Biological Organization Levels

9.5.1 Gene Expression

Different genomic and transcriptomic approaches have been used to investigate the effects of metals on genomic and transcriptomic level (Hassinen et al. 2007). Furthermore, data on genetic markers, analysis of quantitative trait loci and microarray data are publicly available via TAIR (http://www.arabidopsis.org/), where some of the datasets are related to metals. From a combination of these data, it appears that a higher rate of cysteine biosynthesis is required in *Arabidopsis* under Cd stress for a better plant protection or adaptation mechanism. In fact, the transcription of some key genes for cysteine biosynthesis, such as *Atcys-3A* (cytosolic *O*-acetylserine(thiol)lyase) and *SAT* (serine acetyltransferase) gene family, is significantly induced by exposure of *Arabidopsis* plants to Cd stress, and this is accompanied by increases in cysteine and glutathione (GSH) levels (Barroso et al. 1999; Dominguez-Solís et al. 2001; Howarth et al. 2003). As the employment of cysteine-rich chelating compounds such as PCs is one of the defense systems against toxic metals in plants, Harada et al. (2002) studied Cd stressed ($CdCl_2$ up to 200 μM for 4 h) *Arabidopsis* plants to investigate the response of the genes involved in GSH biosynthesis. They found significant increases in transcripts for ATP sulfurylase, APS reductase and sulfite reductase, all of which are involved in cysteine synthesis, and in total thiols (mainly GSH, PCs and cysteine), suggesting that under Cd stress, *Arabidopsis* activates the sulfur assimilation pathway to provide an enhanced supply of GSH for PC biosynthesis. AtATM3, an ATP-binding cassette transporter of *Arabidopsis*, is a mitochondrial protein involved in the biogenesis of iron-sulfur clusters and iron homeostasis, and its gene is upregulated in roots of plants treated with Cd^{2+} or Pb^{2+} (100 μM $CdCl_2$ or 1 mM $Pb(NO_3)_2$ for 24 h) (Kim et al. 2006). In addition, the authors found that that *AtATM3*-overexpressing or *AtATM3*-constitutively-expressing plants are more tolerant to Cd, whereas *AtATM3* mutant plants were more sensitive to Cd than their wild-type controls. Since non-protein thiols, such as GSH and PCs, are positively correlated with metal resistance and the closest homolog of AtATM3 in fission yeast (*Schizosaccharomyces pombe*), HMT1, is a vacuolar membrane-localized phytochelatin-Cd transporter, Kim et al. (2006) hypothesized that GSH-Cd(II) complexes formed in the mitochondria are exported by AtATM3, hence contributing to Cd resistance in *Arabidopsis*. Transcriptional regulation in response to Cd treatment was also investigated in both roots and leaves of *Arabidopsis* treated with low (5 μM) or high (50 μM) Cd concentrations for 2, 6, and 30 h, using a genome microarray (Herbette et al. 2006). One of the main responses observed in roots was the induction of genes involved in sulfur assimilation-reduction and GSH metabolism. In addition, HPLC analysis of GSH and PC content showed a transient decrease of GSH after 2 and 6 h of metal exposure in roots correlated with an increase of PC contents. Altogether, the results suggested that plants subjected to Cd activate the sulfur assimilation pathway by increasing transcription of related genes to provide an enhanced supply of GSH for PC biosynthesis.

To understand the mechanisms of Cd-induced NO synthesis in roots and leaves of *Arabidopsis*, a microarray analysis was performed by Besson-Bard et al. (2009) in Cd-exposed plants (30 μM $CdCl_2$ for 24 h). The authors found that NO contributes to Cd toxicity by favoring Cd^{2+} versus Ca^{2+} root uptake and by initiating a cellular pathway resembling those activated upon iron deprivation. They identified 43 genes encoding proteins related to iron homeostasis, proteolysis, nitrogen assimilation/metabolism, root growth, and transporters such as *IRT1*, that encodes for a Cd, Mn, Zn and Co cation transporter (Korshunova et al. 1999; Rogers et al. 2000).

To investigate the cellular responses of *Arabidopsis* to Zn, van de Mortel et al. (2006) examined in detail the transcription profiles of roots of *Arabidopsis* plants grown with 0, 2 and 25 μM of Zn. A total of 608 Zn-responsive genes with at least a three-fold difference in expression level were detected in response to changes in Zn supply. A large fraction of these genes are of yet unknown function, but many of them appear to be involved in metal homeostasis, abiotic stress response, and lignin biosynthesis. In Cu-exposed *Arabidopsis* plants (1 mM $CuSO_4$ for 30 min), Mira et al. (2002) described two Cu-induced messengers encoding a vegetative storage protein (VSP2) that could act as a temporary storage of amino acids during processes in which these are mobilized, as it happens when plants are subjected to severe oxidative stress.

To confirm transcript data obtained from e.g. microarray analysis or to study the transcript levels of specific genes under metal stress, it is essential to perform quantitative RT-PCR measurements. In this regard, Remans et al. (2008) used a strategy for accurate normalisation of the measured gene expression using a minimum of three reference genes [*AT5G15710* (F-box protein), *AT2G28390* (SAND family protein) and *AT5G08290* (mitosis protein YLS8)] in roots and leaves of *Arabidopsis* exposed to Cd (2–10 μM) and Cu (0.5–2 μM) during 24 h. That metals induce oxidative stress in plants was illustrated by the influence of Cd and/or Cu on ROS (reactive oxygen species) producing and antioxidative defence mechanisms in *Arabidopsis*, addressed by either induction or reduction of specific pro- and antioxidant gene transcripts (Smeets et al. 2008, 2009; Remans et al. 2010; Cuypers et al. 2010).

9.5.2 Proteins

The effects of metals at the proteome level are less investigated, and extensive proteomic studies to unravel the mechanisms of metal uptake and tolerance in plants are yet to be completed. A significant proportion of the *Arabidopsis* genome encodes membrane proteins, especially transport proteins and putative sensors that cope with these conditions (Ludewig and Frommer 2002). Thus, the necessity to regulate uptake of nutrient metals, many of which can be cytotoxic at high concentrations, is particularly important.

Roth et al. (2006) found alterations in the root proteome of hydroponically grown *Arabidopsis* plants exposed to 10 μM Cd^{2+} for 24 h, a condition that triggers PC synthesis. Two dimensional gel electrophoresis and western analysis indicated significant changes in protein abundance upon Cd^{2+} treatment. Most of the identified proteins belong to four different classes: (1) metabolic enzymes such as ATP sulfurylase, glycine hydroxymethyltransferase, and trehalose-6-phosphate phosphatase; (2) glutathione *S*-transferases; (3) latex allergen-like proteins; and (4) unknown proteins. Their results showed a selective enrichment of the protein family glutathione *S*-transferases, suggesting the generation of internal sinks for reduced sulfur after exposure of plants to Cd. On the other hand, Semane et al. (2010) studied the leaf proteome of 3-week-old *Arabidopsis* seedlings exposed for 1 week to 1 μM Cd. Their data indicated that plants adapted their metabolism to cope with the Cd exposure and only moderate protein changes were observed, whereas at higher levels (10 μM Cd) growth reduction, chlorosis of rosette leaves, lipid peroxidation and enhanced peroxidase activity occurred. In particular, 21 proteins were up-regulated in response to Cd, functionally grouped into five classes: (1) proteins involved in oxidative stress response and GSH/PC metabolism, (2) photosynthesis and energy production, (3) protein metabolism, (4) gene expression, and (5) proteins with various or unknown function.

Kung et al. (2006) screened for copper-interacting proteins in *Arabidopsis* roots *via* copper-immobilized metal affinity chromatography (Cu-IMAC). They identified 35 proteins involved in redox/hydrolytic reactions, amino acid metabolism, glutathione metabolism, phosphorylation, translation machinery, membrane-associated proteins, and vegetative storage proteins. Finally, they predicted and scored six potential Cu- interacting motifs present in Cu-IMAC-isolated proteins with higher frequency than in the whole *Arabidopsis* proteome. To understand better Zn-responsive proteins, Fukao et al. (2009) investigated Zn-treated (300 μM $ZnSO_4$), *Arabidopsis* roots. Ten up-regulated and 17 down-regulated proteins were identified, 15 of which showed a significant correlation with previously reported transcriptomic data.

Besides an open screening for changes in protein abundance under metal stress, also studies on specific proteins are conducted. Li et al. (2010) suggested that NRT1.8 functions in the removal of nitrate from xylem vessels. Indeed, long-distance transport of nitrate requires xylem loading and unloading, a successive process that determines nitrate distribution and subsequent assimilation efficiency. Interestingly, *NRT1.8* appeared to be the only nitrate assimilatory pathway gene that was observed to be strongly up-regulated by Cd^{2+} in roots, and the *nrt1.8-1* mutant showed a nitrate-dependent Cd^{2+}-sensitive phenotype. Further analyses showed that Cd^{2+} stress increases the proportion of nitrate allocated to wild-type roots compared with the *nrt1.8-1* mutant. The NRT1.8 transporter likely plays an important role in protecting the plant against Cd toxicity, and possibly against a wide range of biotic and abiotic stresses (Gojon and Gaymard 2010).

9.5.3 Phytohormones

Arabidopsis plants exposed to stress caused by metals often resemble, in terms of the redistribution of growth, plants altered in phytohormone metabolism (Pasternak et al. 2005; Kai et al. 2007).

Indole-3-acetic acid (IAA) and its metabolites are the most widely auxins in plants (Hansen and Halkier 2007; Kai et al. 2007; Ludwig-Müller 2007). Key components of the cell cycle and signal-transduction pathways that promote and attenuate auxin-dependent lateral roots initiation have been identified in *Arabidopsis* (Casimiro et al. 2003). Pasternak et al. (2005) observed that in *Arabidopsis* Cu-exposed plants (30 up to 100 μM $CuSO_4$) root hair density was significantly increased and an acceleration of the emergence of lateral roots occurred. These authors observed that phenotypes of *Arabidopsis* plants exposed to $CuSO_4$ resemble plants altered in auxin metabolism.

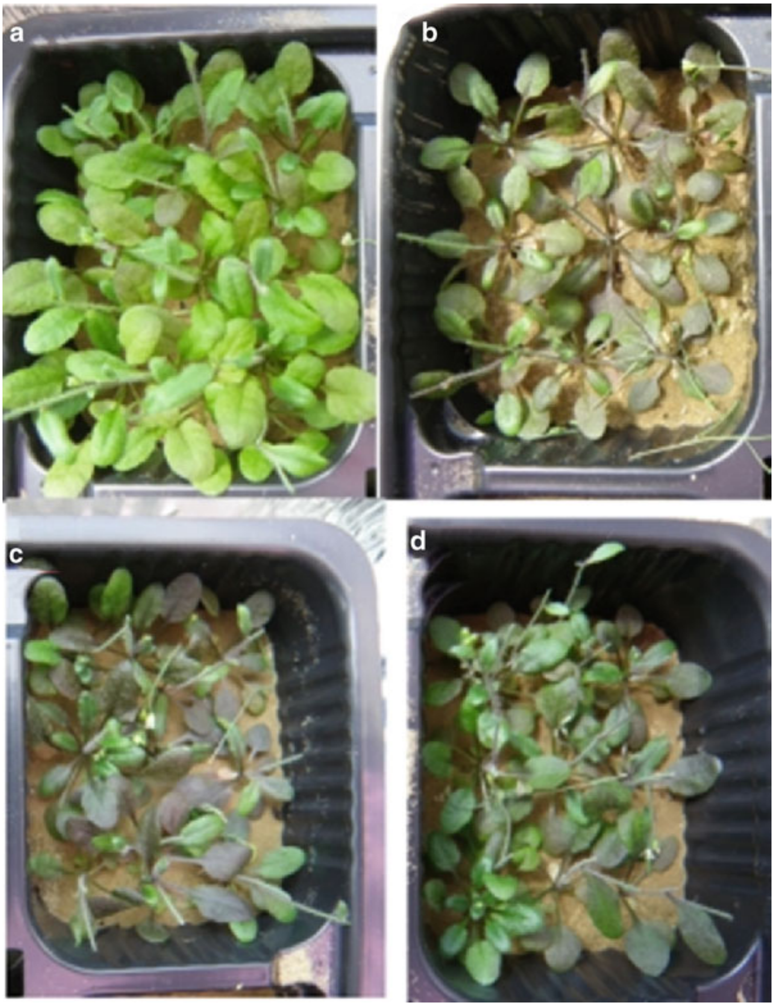

Fig. 9.2 Seedlings of *Arabidopsis thaliana* not exposed to metals (control (**a**), and exposed for 12 days to 10 μM Cd (**b**), 5 μM Cu (**c**) and 150 μM Zn (**d**)) (Source: A. Sofo)

Significant morphological changes (degree of root branching and number of root hairs) together with increased IAA root levels were found by Sofo et al. (unpublished data) in roots of *Arabidopsis* plants exposed to Cd, Cu and Zn, applied separately or in different combinations (Fig. 9.2). Map-based cloning was used to find the *ILR2* gene that affects IAA-leucine resistance in the *Arabidopsis ilr2-1* mutant (Magidin et al. 2003). This gene encodes a protein that is polymorphic among *Arabidopsis* accessions and it was found to modulate a metal transporter, thus providing a link between auxin-conjugate metabolism and metal homeostasis. Considerable effort has also been directed at clarifying the processes and factors contributing to IAA homeostasis during metal exposure, but the entire picture remains to be elucidated, as IAA synthesis is regulated in response to different and complex signaling pathways (Hansen and Halkier 2007).

Another class of hormones, cytokinins (CKs) are implied in meristem activity, *de novo* bud formation, release from apical dominance, leaf expansion, reproductive development, and senescence. In many plant species, moderately high Cd levels (5–50 μM) and longer exposures to Cd (3 weeks) significantly decreases cytokinin export from the root tips to the shoot (Prasad 1995). In *Arabidopsis* plants grown in the presence of Cd, Cu and Zn, applied separately or in different combinations, reduced CKs levels in shoots, associated to CKs increases in roots, were observed by Sofo et al. (unpublished data) (Fig. 9.2). Since in *Arabidopsis* the genes encoding ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases (*ipt*) and/or the activities of the corresponding enzymes (IPTs) are of basic importance in CK biosynthesis (Miyawaki et al. 2006), the application of metals likely could up-regulate the transcription of these genes.

In *Arabidopsis*, Cd-induced inhibitory effects were reported to be concomitant with an increase in endogenous abscisic acid (ABA) levels in plant tissues indicating the possibility of this phytohormone mediating a part of the metal-imposed phytotoxicity (Sharma and Kumar 2002). Furthermore, it was observed that Cu or Cd applied on *Arabidopsis* seedlings, causes a rapid increase of jasmonic acid, followed by a rapid decrease observed during 7 successive hours (Maksymiec et al. 2005), so indicating that jasmonic acid is connected with the mechanism of toxic action of both metals in plants. Interestingly, jasmonic acid applied on *Arabidopsis* seedlings exposed to Cu or Cd (100 μM up to 144 h) enhances their sensitivity to these metals, in terms of reductions of membrane peroxidation and quantum yield of PSII, so demonstrating an important role of jasmonic acid in metal stress signaling (Maksymiec et al. 2007). Indeed, salicylhydroxamate and propyl gallate, two inhibitors of jasmonic acid synthesis, are able to prevent some deleterious inhibitory effect of Cu and Cd in *Arabidopsis* (Maksymiec and Krupa 2002).

Finally, Cu and Cd, but not Zn, were found to elicit the greatest amount of ethylene produced by *Arabidopsis* plants (Arteca and Arteca 2007). These authors found that inflorescence stalks and root tips produced the greatest amount of ethylene in response to $CuSO_4$ or $CdSO_4$ over a range of concentrations from 0 to 800 μM, whereas all other plant parts tested released significantly lower levels. It was found that increasing leaf age, light and high temperatures caused a dramatic decrease in Cu/Cd-induced ethylene production in both inflorescence stalks and leaves of *Arabidopsis*.

9.6 The Multi-pollution Context

Monometallic exposure is very unusual in real-world situations, so it is very important to study both the metal-specific effects and the mechanisms induced when the plants are exposed to more metals simultaneously (Smeets et al. 2009). In fact, the combined exposure to more metals could enhance some of the effects that are induced with only one metal. Moreover, it can happen that plants able to hyperaccumulate some metals without showing any physiological damage, can be strongly susceptible to other ones (Mijovilovich et al. 2009). For instance, seed is a developmental stage that is highly protected against external stresses in the plant life cycle. However the toxicity of Cu, Zn and particularly Cd, alone or in combination, on seed germination in *Arabidopsis* was proven (Li et al. 2005). The same authors highlighted that Cu and Zn are significantly less effective on reducing seedling growth when compared to Cd.

The studies on *Arabidopsis* plants subjected simultaneously to different metals are quite scarce. In one of these studies from Maksymiec and Krupa (2006), a considerable increase of hydrogen peroxide accumulation and superoxide radicals was observed during the first hours of exposure of plants to excess Cu and Cd (100 μM $CuSO_4$ and $CdSO_4$). Furthermore, excess Cd, in contrast to Cu, increased the SOD activity. The metal-induced oxidative stress was also confirmed by other studies. Smeets et al. (2009) observed a specific Cd-related induction of NADPH oxidases and metal-specific patterns of superoxide dismutases in 3-week-old *Arabidopsis* seedlings exposed to Cu and/or Cd (10 μM $CdSO_4$ and $CuSO_4$) for 24 h. Both metals induced gene expression of several H_2O_2-quenching enzymes and lipid peroxidation possibly by the activation of lipoxygenases. Skórzyńska-Polit et al. (2006) studied the activity and cellular localization of lipoxygenases (LOX) in *Arabidopsis* plants grown under excess Cd and Cu (both at 0, 5, and 50 μM) in solution cultures for 7 days. LOX was localized mainly in the cytoplasm as well as inside the chloroplasts and its activity was significantly higher in metal-exposed plants than in control plants. Moreover, the authors observed that the changes in ultra-structure of the leaf parenchyma cells were more evident in plants treated with Cd than those exposed to Cu. LOXs are particularly important during oxidative-stress responses, as they catalyse the dioxygenation of polyunsaturated fatty acids containing a *cis, cis*-1,4-pentadiene backbone, producing hydroperoxy fatty acids, which are highly reactive compounds that are toxic to cells, but are also precursors of oxylipin signalling molecules like jasmonates.

9.7 Conclusions and Perspectives

Understanding the metabolic responses and the adaptation of plants towards metal exposure opens the way to future phytoremediation of contaminated soils. The majority of these metals accumulate in plants and may either directly or indirectly find their way into the food chain causing severe secondary consequences. For this reason, phytoremediation has been accepted advantageous over commonly used physical remediation methods in costs, practice and the scale at which the processes operate. Usually, fast growing plants of high biomass are considered potential candidates for toxic metal accumulation but may be hampered with significant tolerance capacity, limiting their use for phytoremediation purposes. Identifying the particular gene(s) underlying a specific adaptation to metals is a major challenge in modern biology (Roosens et al. 2008). Therefore, the study of naturally occurring variation in *Arabidopsis* provides a bridge between functional genetics and evolutionary analyses. Nevertheless, the use of this species to study adaptation is limited to those traits in common with other plant species used for phytoremediation. Therefore, in order to fully understand the genetics of adaptation of plants to metals, the numerous and easily available genetic resources developed in *Arabidopsis thaliana* should be extended to other plant species.

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Chapter 10
Brassicas in Turkey and Their Potential Role for Degraded Habitats' Remediation

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Abstract A survey undertaken in Turkey revealed that there are six species of *Brassica* genus distributed in the wild in Turkey. *Brassica elongata, B. nigra*, *B. tournefortii*, *B. cretica*, *B. deflexa* and *B. campestris*. Cultivated forms found in Turkey are kale, cauliflower, cabbage, brussel sprouts, kohlrabi and broccoli. Mediterranean region is most probably concerned with their domestication and cultivated forms of the genus were probably introduced into Asia during ancient times. Studies conducted on the germination and growth behaviour of some of these species revealed that the seeds of *B. nigra* show a dormancy period of 6 months, whereas other species germinate immediately. Growth regulators not only stimulate the germination under salinity stress, but also overcome the delaying effects of salt. Ethephon sprayed on plants using different solutions showed that at higher concentrations there is a stimulation in vegetative and reproductive growth. Molecular studies of the genomes of species of the Brassicaceae such as *B. napus* has revealed that there is an extensive genome duplication, indicative of multiple polyploidy events during evolution. Extensive genetic and molecular analysis has been done on six cultivated *Brassica* species. The four closely related crop species *B. rapa*, *B. juncea*, *B. napus*, and *B. carinata* provide about 12% of the worldwide edible oil supply. The other two species *B. nigra* and *B. oleracea* provide many vegetables for healthy human diet having a valuable source of dietary

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fiber, vitamin C and other anticancer compounds. *B. nigra* has the second smallest genome size among the six cultivated species of *Brassica*.

Brassica species are well known as metal accumulators and some of them are being used for phytoremediation in contaminated soils. Approximately 25% of the documented metal hyper accumulating species are members of the *Brassicaceae*. Because of their slow growth and low biomass, other fast-growing and high biomass *Brassica* crop plants, for example *B. juncea* and *B. nigra* have been evaluated for their ability to hyper accumulate metals from contaminated soils. The Diyarbakir ecotype of *B. nigra* distributed in the southeastern part of Turkey is a good hyperaccumulator of Cu. Microarray analysis undertaken during the comparative transcriptome analysis in order to find out the expression level of metal induced genes and transcriptome changes both in low and high Cu treated plants showed that some of the genes were highly expressed (several hundred fold) with Cu treated plants compared to control. Microarray data using Affymetrix GeneChip Arabidopsis Genome Array (ATH1-121501 Genechip) indicated that possibly several genes including the genes in glutathione pathway, metal ATPase and ABC transporters are involved in metal tolerances in this ecotype.

Keywords Brassicas • Degraded habitats • Hyperaccumulator • Microarray • Remediation

10.1 Introduction

Turkey has on record one of the oldest evidences of the out of the deltas agriculture (Catalhoyuk-Konya) (Ozturk et al. 1983). It has most probably served as the starting point for the flow of some materials and cultures into Asia. The five geographical regions in the country show very interesting phytogeographical characteristics, being meeting place of three floristic regions namely; Euro-Siberian, Irano-Turanian and Mediterranean, with differing ecological as well as climatic characteristics. We find in general some useful information concerning the species of *Brassica* and many other Crucifers in the recent comprehensive study on the flora of Turkey (Davis 1965). The mustard family includes about 375 genera and 3,200 species, which are widely distributed in the temperate and cold parts of the world. The species of this family have served both mankind and his livestock, since ancient times. These species have been utilised in a variety of forms such as; vegetables, fodder plants, medicinal plants, dye plants, condiments, oil crops and ornamentals. The genus *Brassica* contains about 100 species, including rapeseed, cabbage, cauliflower, broccoli, Brussels sprouts, turnip, various mustards and weeds (Warwick and Black 1991). *Brassica* oilseed species now hold the third position among oilseed crops and are an important source of vegetable oil. The ten most common Brassica oil-seed crops grown for commercial purposes are rape seeds, (*B. campestris* L. and *B. napus* L.) and mustards (*B. juncea* (L.) Czern. & Coss. and *B. carinata* A. Br.). Others such as *B. nigra* (L.) Koch and *B. tournefortii* Gouan are grown on

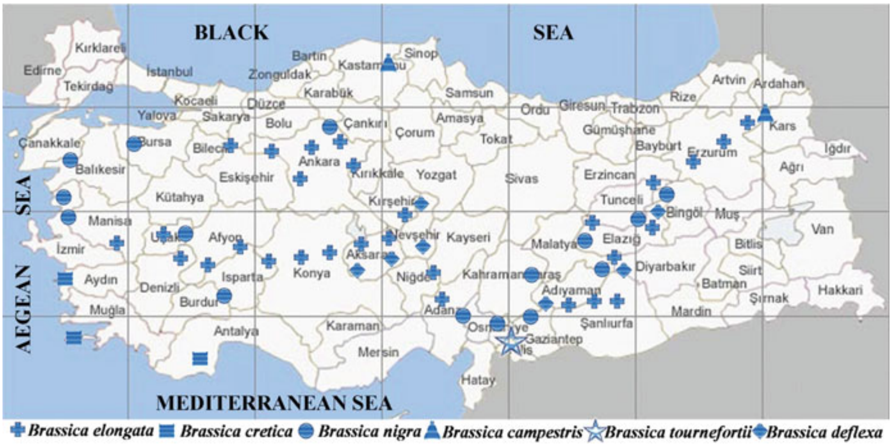

Fig. 10.1 Distribution map of naturally growing six species of Brassica in Turkey

a very small scale. *B. napus, B. juncea* and *B. carinata* are amphidiploids, whereas *B. campestris* and *B. nigra* are diploid (Ashraf et al. 2001). The Mediterranean region was most probably concerned with their domestication. There are six species of this genus distributed in the wild in Turkey namely; *Brassica elongata, B. nigra*, *B. tournefortii*, *B. cretica*, *B. deflexa* and *B. campestris* (Fig. 10.1).

B. elongata Ehrh. is widely distributed species within the Irano-Turanian region of Turkey, both in Central as well as Eastern Anatolian parts. It grows as weed in wheat, barley and gram fields and also as a ruderal. It is the species with highest chromosome numbers within the monogenomic species (Gomez-Campo and Hinata 1980; Ozturk et al. 1983). *B. deflexa* Boiss. is found abundantly between Konya and Nevsehir (Sultanhani, Aksaray) as well as Gaziantep, Malatya, Diyarbakir and Elazig. The plants are distributed in wheat fields and as a ruderal. It possesses a lower number of chromosomes in the genus *Brassica*. The distributional pattern of *B. elongata* and *B. deflexa* in Turkey emphasize the importance of this area in the evolutionary diversification of the genus *Brassica*, because these two species-which do not grow in Western Europe-have unique chromosome numbers (n = 11 and n = 7 respectively). *B. nigra* (L.) Koch is found throughout the Mediterranean. It shows wide distribution along the channels and irrigated field sides in Turkey except for very cool regions and forms big colonies of very vigorous plants around Adana. The moisture conditions of the soil regulate the development of this species. *B. cretica* is distributed on the south-west facing limestone cliffs of Priene ruins at an altitude of 100–400 m, on the rocks along a 4 km path of Kanyon Deresi in Kusadasi National Park (40–120 msl), Resadiye Peninsula (Knidos) on the west facing cliff (2–20 msl), Antalya highway along the coastal cliffs from Karagoz to Adrasan, as well as in the Kirlangic Islands and Sulu Ada, Akyarlar tunnel on the way to Antalya; on the cliffs along the old road and over the tunnel (20–50 msl). *B. tournefortii Gouan* grows along the dried stream sides in the vicinity of Karatas (Adana). This thermophyll coastal habitat exactly matches the behaviour of this species

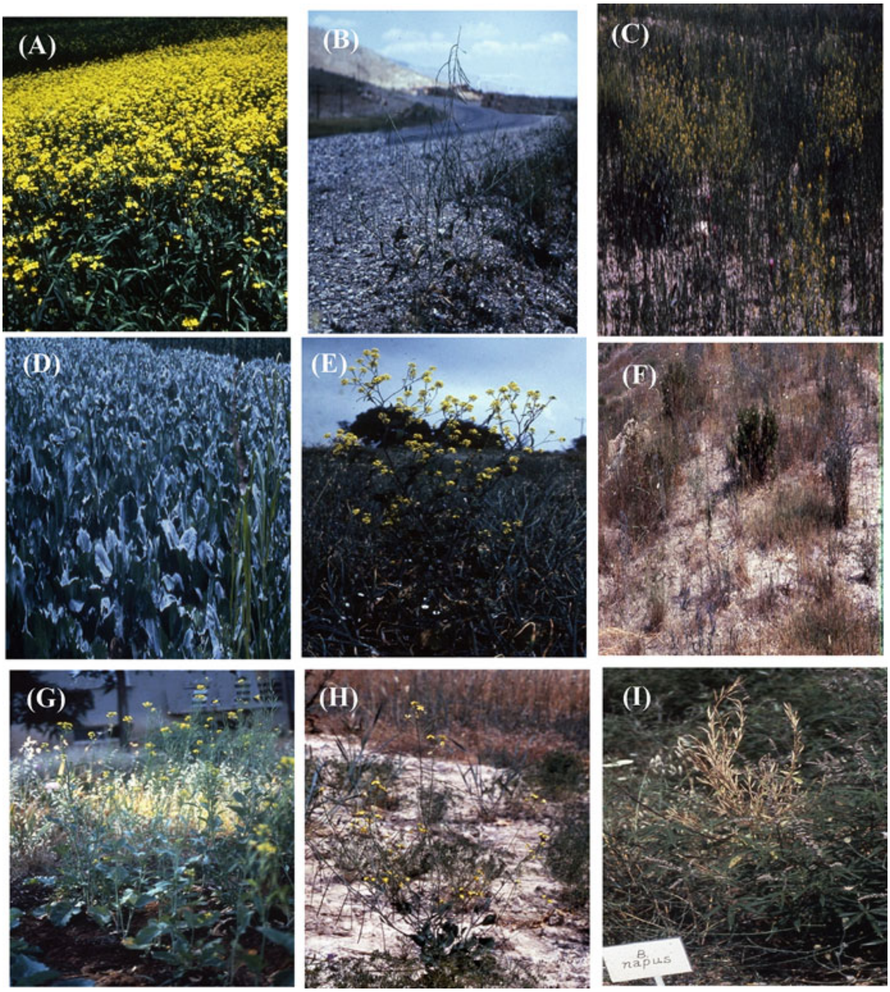

Fig. 10.2 *Brassica campestris* (**a**), *Brassica deflexa* (**b**), *Brassica nigra* (**c**) *Brassica oleracea* (**d**), *Brassica elongata* (**e**), *Brassica tournefortii* (**f**), *Brassica juncea* (**g**), *Brassica deflexa* (**h**), *Brassica napus* (**i**)

observed in other localities along its extensive distributional area, from Spain upto Iran. *B. campestris* L. (including *B. rapa* L.) and *B. juncea* (L.) Czern. (an allotetraploid between *B. campestris* and *B. nigra*) are widely cultivated in Asia; particularly, Japan, China and India. The wild *B. campestris* is also found in some parts of the Mediterranean region. It is believed that, the cultivated forms of this species were introduced into Asia during ancient times. Mizushima and Tsunoda (1967) have described *B. campestris* as a weed of wheat fields in the Ankara region, but this habitat exists no longer now and it seems that it has been destroyed by the application of herbicides and by extensive plowing (Fig. 10.2).

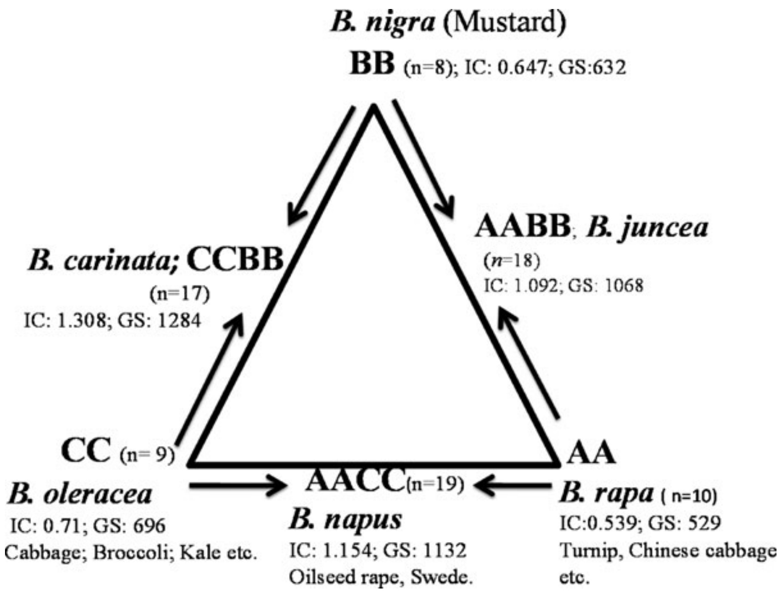

Fig. 10.3 The "Triangle of U" representing the genomic relationships among different diploid and amphidiploids Brassica species. *1C* 1C nuclear DNA content (pg), *GS* genome size (Mbp) (UN 1935; Johnston et al. 2005)

B. oleracea is cultivated all over the world and we come across its wild relatives quite a lot, along the coasts and in the islands of the Mediterranean region. *B. oleracea* var. *acephala* locally known as "Kara Lahana" is cultivated throughout the Black Sea coast. *B. napus* is found to grow in the wheat fields near Ayancik locally known as Kara Pancar (600 msl), Cicek Yayla (Kastamonu), roadside near Akcapinar on the way to Gaziantep. However, their exact nature at the infra-specific level has not been clarified yet.

The cultivated *Brassica* species are the group of crops most closely related to *Arabidopsis thaliana*. Chromosome numbers in the *Brassicaceae* vary from $2n = 8$ to $2n = 256$ (Lysak et al. 2005). The species typically termed the "diploid" *Brassica* species, *B. rapa* ($n = 10$), *B. nigra* ($n = 8$) and *B. oleracea* ($n = 9$) contain the A, B and C genomes, respectively. Each pairwise combination has hybridized spontaneously to form the three allotetraploid species (UN 1935), *B. napus* ($n = 19$, comprising A and C genomes), *B. juncea* ($n = 18$, comprising A and B genomes) and *B. carinata* ($n = 17$, comprising B and C genomes). The genome of *B. rapa* is the smallest, at *ca*. 500 Mb (Arumuganathan and Earle 1991), and a genome sequencing project is under way, with both sequences and sequence annotations in the public domain http://brassica.bbsrc.ac.uk/ (Fig. 10.3). The relationships among the cultivated species as presented in Fig. 10.1 were first clarified by Morinaga (1934) and verified by UN (1935) (Fig. 10.3). The four most widely cultivated species *B. juncea*, *B. napus*, *B. oleracea*, and *B. rapa* are all highly polymorphic and include oilseed crops, root crops, and vegetables crops such as Chinese cabbage, broccoli, and Brussels sprouts (Johnston et al. 2005). Because the *Brassica* amphidiploid species can be generated synthetically with the help of embryo rescue techniques, this complex of three diploid species and their corresponding polyploids (Fig. 10.3) is today one of the most useful model systems for investigations of polyploidy in crop plants (Song et al. 1995; Lukens et al. 2006; Snowdon 2007).

10.2 Uptake of Solutions, Germination and Salinity Interactions in *Brassica* Taxa

Water uptake of *B. nigra* and *B. juncea cv. obatakana* seeds at different temperatures shows that former imbibes maximum amount at 25°C, minimum at 35°C whereas in the latter these lie at 15°C and 25°C respectively. In both species very little imbibition occurs at 5°C. Water uptake from 0.1% to 0.5% NaCl solutions is better than at higher percentages. Same is true for sugar concentrations of 0.1% and 0.5%. Uptake of various salt and sugar solutions by *Brassica* taxa varies with change in species and temperature (Ozdemir et al.1994a).

B. oleracea var. *acephala* shows optimum germination at 25°C and prefers light conditions more than darkness. Salt solutions inhibit germination even at lower levels (1%) but this effect is successfully alleviated by GA_3, KIN and IAA. These regulators cannot overcome the inhibition at higher salt concentrations (2%, 3%). In *B. oleracea* optimum germination takes place at 25°C, but seeds are indifferent to light. Seeds of *B. rapa* show maximum germination at 30°C but it decreases under continuous dark conditions and increases under continuous light. The seeds of *B. nigra* show a dormancy period of 6 months, whereas other species germinated immediately. The seeds generally not sensitive to photoperiod, however, exposure to extended dark period reduced seed germination. *B. nigra* and *B. juncea* show medium tolerance up to 1% salinity level but at 1.5% level tolerance decreases by 32%. An application of IAA and GA_3 alleviates the salinity stress up to 1.5% level and to some extent at 2% level. Alleviation of salt stress occurred more in *B. juncea* than *B. nigra*. In *B. oleracea* var. *botrytis* and *B. oleracea* var. oleracea the application of growth regulators improves germination and seedling growth. The effects of giberellic acid (GA_3), indole acetic acid (IAA), kinetin (KIN) and combinations of these (GA_3 + KIN; GA_3 + IAA; GA_3 + KIN + IAA) on the germination of *B. campestris* seeds, collected from the wild populations growing in the wheat fields at Kars in the east Anatolian part of Turkey were studied in a medium with salt (NaCl: 0.5, 1, 1.5, 2, 3, 4, 5%) and without salt. Two series of experiments were set up. In the first series a low concentration of growth regulators (10 ppm) was used throughout the experiment, whereas in the second series seeds were soaked for 2 h in 50 and 100 ppm concentrations of growth regulators and then left for germination in the salt mediums. Seeds of *B. campestris* showed a 100% germination at 20°C in distilled water. The germination was, however, delayed or inhibited by NaCl, depending upon concentration. The growth regulators, on the other hand, not only stimulated the germination of these seeds under a salinity stress, but also overcame the delaying effects of salt in this connection. GA_3 proved to be more effective than KIN and IAA in the alleviation of salinity stress. Synergistic effects of regulator combinations were evident (Ozdemir et al. 1994b; Ozturk et al. 1992, 1994a, b, 1997, 2006; Dogu et al. 2002).

There are contrasting reports regarding the response of these species to salinity at different plant developmental stages, but in most of them it is evident that they maintain their degree of salt tolerance consistently throughout the plant ontogeny. Most of the *Brassica* species have been categorized as moderately salt tolerant, with the amphidiploid species being the relatively salt tolerant in comparison with the diploid species. Due to the higher salt tolerance of the amphidiploids, it has been suggested that their salt tolerance has been acquired from the A (*B. campestris*) and C (*B. oleracea*) genomes. However, significant inter- and intraspecific variation for salt tolerance exists within brassicas, which can be exploited through selection and breeding for enhancing salt tolerance of the crops (Ashraf and McNeilly 2004).

The pattern of uptake and accumulation of toxic ions (Na^+ and Cl^-), in tissues of plants subjected to saline conditions appears to be mostly due to mechanism of partial ion exclusion (exclusion of Na^+ and/or Cl^-) in most of the species, although ion inclusion in some cases at intraspecific levels has also been observed. Maintenance of high tissue K^+/Na^+ and Ca^{2+}/Na^+ ratios has been suggested as an important selection criterion for salt-tolerance in *Brassica* species. Osmotic adjustment has also been reported in *Brassica* plants subjected to saline conditions, but particularly to a large extent in salt-tolerant species or cultivars. In canola (*B. napus*), no positive relationship has been observed between salt tolerance and erucic acid content of seed oil in different cultivars. If four different combinations of nitrate to ammonium 0:100, 25:75, 50:50 and 75:25 are applied to the canola plants under 0 and 200 mM NaCl the highest wet and dry weight, leaf area, relative water content, photosynthesis ratio, transpiration ratio and leaf potassium content is obtained from those plants which are treated with nitrate and ammonium in 50:50 ratio under non saline condition (Ashraf 2001). Salinity significantly decreases wet and dry weight, leaf area, relative water content, photosynthesis and respiration ratio and leaf potassium content. On the other hand fatty acids content increases due to salinity stress, highest content is obtained from 25:75 ratios i.e., high ammonium treatments (Ashraf and McNeilly 2004).

The relative response of *B. campestris, B. carinata* A.Br., *B. juncea* , and *B. napus* to different salts at the germination and seeding state using solution and sand culture techniques has revealed that NaCl has greater inhibitory effect on percentage germination. No consistent correlation has been observed between data for germination and seedling biomass production except in *B.napus* which has high germination. *B. napus* is relatively tolerant and *B. campestris* sensitive to salinity (Ashraf et al. 1989; Ashraf and Mcneilly 1990).

Studies on the waterlogging tolerance of *B. campestris, B. carinata, B. juncea* and *B. napus* has shown that shoot fresh and dry biomass is highest in *B. juncea* and lowest in *B. napus*. This treatment causes a marked reduction in chlorophyll content in all four species, which also reflects the reduction in photosynthetic activity

(Ashraf 2001). A marked increase in soluble protein content of *B. juncea* and a significant increase in total amino acids in *B. carinata* has been observed under these conditions. The inhibition in N uptake in plants and subsequent redistribution of N within shoot from old leaves to young leaves under waterlogged conditions contribute to lower the total plant N content and in turn to inhibit plant growth (Ashraf and Mehmood 1990a, b). This evidence can be easily related to the inhibition in both soluble proteins and free amino acids in waterlogging sensitive *B. napus*. However, an increase in the soluble proteins in *B. juncea* and amino acids in *B. carinata* plays some role in sustaining growth under such conditions. An increase in iron content in both shoots and roots has been observed in all four species. *B. juncea* accumulates lower amount of iron in both shoots and roots as compared to the other species, whereas *B. carinata* has also lower iron in the roots (Ashraf and Naqvi 1992a, b). The results for iron content in waterlogged soil show that soils supporting the four species have greater exchangeable iron and manganese compared with the control except in the soil supporting *B. carinata*, where no significant increase in exchangeable manganese content has been observed. It is now well evident that plants adapted to waterlogged soils have a specific tolerance mechanism for Fe^{2+} and Mn^{2+}, as these elements in excessive amounts are toxic to plant growth. The lower uptake of both iron and manganese in shoots of *B. juncea* reflects its resistance to take toxic elements. These results support the fact that waterlogging tolerant species are capable of controlling the uptake of these elements either by using an internal mechanism for tolerance of these elements and/or by the root exclusion system due to immobilization of elements in or on the roots *i.e.* higher contents of these elements in the roots (Ashraf and Nazqvi 1992b).

A low amount of Ca^{2+} in the salt affected soils causes growth inhibition in plants. The effects of varying Ca^{2+} concentrations in saline solution medium on the growth and physiological responses of different *Brassica* species reveals that *B. carinata* has significantly greater plant dry biomass at varying Na/Ca ratios. *B. campestris* is the lowest in plant dry matter. There is no effect of increasing Na/Ca ratios of the growth medium either on water relations parameters except that turgor potential increases in *B. napus*. Chlorophyll b and total chlorophyll decrease significantly in *B. campestris* and *B. napus* at the highest Na/Ca ratio, whereas these variables remains unaffected in other species at all Na/Ca ratios. Stomatal diffusive resistance remains unchanged at varying Na/Ca ratios of growth medium. Total shoot sugars of the species except *B. carinata* increases at the highest Na/Ca ratio, whereas the root weight, sugar content increases with the increase in external Na/Ca ratios in all species except *B. campetris*. Soluble proteins and free amino acids increase only in *B. napus* at the highest Na/Ca ratio, whereas these osmotica remain unaffected in the remaining species. However, proline increases considerably at the highest Na/Ca ratio in all species except some, where it increases at Na/Ca ratios, 30, and 60. No definite relationship is found between water relations parameters, different osmotica and growth in different *Brassica* species (Ashraf and Naqvi 1992a).

There is no significant effect of additional amount of Ca^{2+} in the saline medium (150 mM NaCl) on the shoot biomass and seed yield of *B. carinata* and

B. campestris. By contrast, shoot dry matter of *B. napus* and *B. juncea* increases significantly with increase in Ca^{2+} concentration of the growth medium, but seed yield remains unaffected. Decrease in Ca^{2+} concentration of the saline growth medium reduces percent oil content in *B. carinata, B. juncea*, and *B. campestris*. Increase in Na/K ratio of the external medium affects ion uptake differently in different species. Root K^+ and Ca^{2+} of the species decreases with a decrease in Ca^{2+} supply. In *B. juncea* high Ca^{2+} concentration in the salt treatment reduces the shoot and root Ca concentrations and promotes the K^+ uptake (Ashraf and Naqvi 1992a, b). Salinity decreases the shoot biomass production and NR activity in different lines of canola. NRA is found to be positively correlated with biomass accumulation under salt stress. The results show that three amphidiploids, *B. napus, B. carinata,* and *B. juncea* are salt-tolerant compared with their putative diploid relatives, *B. campestris, B. oleracea,* and *B. nigra*. The three parental diploid species differ in shoot biomass and seed yield when grown in saline solutions, comparing *B. campestris* (A genome), *B. oleracea* (C genome), and *B. nigra* (B genome) (Ashraf 2001).

The species *B. napus* and *B. carinata* produced significantly greater shoot fresh and dry matters than their parents under saline conditions. A close association was found between growth, and assimilation rate for all species differing in degree of salt tolerance. Stomatal conductance (*gs*) was reduced due to salt stress in all species but this variable had no significant correlation with assimilation rate (A). However, the amphidiploid salt tolerant species, *B. napus* and *B. carinata* had significantly greater photosynthetic rate, water use efficiency (A:E), intrinsic water use efficiency (A:*gs*) than those of their diploid parents Nyla et al. 2001). In conclusion, high salt tolerance of the two amphidiploid species, *B. napus* and *B. carinata* was associated with a high assimilation rate, water use efficiency and intrinsic water use efficiency but there was little association of the tolerance of these species with stomatal conductance, leaf water potential or transpiration rate (E) (Pettigrew and Meredith 1994; Faville et al. 1999). Higher stomatal conductance in plants is known to increase CO_2 diffusion into leaves thereby favouring higher photosynthetic rates. Higher net assimilation rates could in turn favor a higher biomass and higher crop yields (Taiz and Zeiger 1998). But the results for photosynthetic rate and stomatal conductance presented here for six Brassica species do not show any significant relationship, though these two variables declined consistently in all six species with increase in salt concentration of the growth medium.

The relative intercellular CO_2 concentration in all *Brassica* species except *B. juncea* the substomatal concentration decreased consistently with increase in external salt concentration. High salt tolerance of the two amphidiploid species, *B. napus* and *B. carinata* was found to be associated with their high assimilation rates, water use efficiency and intrinsic water use efficiency but there was little association of the tolerance of these species with respect to stomatal conductance, leaf water potential, or transpiration rate.

Salinity tolerance during germination and early seedling growth was evaluated for Brassica species such as *B. oleracea capitata*, *B. oleracea botrytis* and *B. napus* in four treatments of salinity showed that different treatments of salinity had considerable effect on the germination, germination rate, shoot and root length, shoot and root fresh weight, leaf area and number of leaves of canola, cabbage and cauliflower. Germination percentage in all three species showed considerable decrease with increasing salinity. This reduction was more in cauliflower as compared to cabbage and canola. The required time for germination also increased with increasing levels of salinity. The seedling growth of three species was significantly inhibited by all salinity levels. Shoot growth of all three plant species were more affected as compared to root growth at all salinity levels. Fresh shoot and root weight, leaf area and number of leaves were also severely affected at all salinity treatments (Ashraf et al. 1994; Jamil et al. 2005a, b).

If different combinations of nitrogen salts are applied to the canola plants under different salinity conditions the highest wet and dry weight, leaf area, relative water content, photosynthesis ratio, transpiration ratio and leaf potassium content is obtained from those plants which are treated with equal amount of the salts under non saline condition. Salinity significantly decreases wet and dry weight, leaf area, relative water content, photosynthesis and respiration ratio and leaf potassium content. On the other hand fatty acids content increases due to salinity stress, highest content is obtained from 25:75 ratios *i.e.*, high ammonium treatments (Qasim et al. 2002; Bybordi et al. 2009, 2010a, b, c; Bybordi and Tabatabaei 2009; Bybordi 2010a, b).

10.3 Comparative Genome Studies

Genomic-assisted breeding approaches have considerably advanced with increasing availability of genome and transcriptome sequence data for several model plants and crop species. Complete and/or draft genome sequences have become available for many plant species such as *Arabidopsis thaliana*, rice, poplar, grape, papaya, sorghum, *Medicago truncatula* and soybean (Feuillet et al. 2011). Whole genome sequencing is either sequenced but is not yet publicly available or is in progress for several other crops such as maize, wheat, rapeseed, Chinese cabbage and some other crops (Feuillet et al. 2011).

The genome of *A. thaliana* was the first of any plant to be sequenced (*Arabidopsis* Genome Initiative 2000) and it is one of the smallest known nuclear genomes in higher plants. This complete sequence of *Arabidopsis* genome has developed into the most important resource for gene isolation and characterization in *Brassica* crops and has also served as an important reference genome for other members of the *Brassicaceae*. The data available from the *Arabidopsis* Information Resource (TAIR) includes the complete genome sequence along with gene structure, gene product information, metabolism, gene expression, DNA and seed stocks, genome maps, genetic and physical markers, and information about the Arabidopsis research community. Gene product function data is updated every 2 weeks from the latest published research literature and community data submissions. Gene structures are updated 1–2 times per year using computational and manual methods as well as community submissions of new and updated genes (Lu and Last 2008) (http://www.arabidopsis.org/).

Comparative genomics is a powerful tool for genome analysis and annotation. The biology of *Arabidopsis* and *Brassica* are very similar and the comparison of genetic mapping between species of *Brassicaceae* revealed collinear blocks even though the species differed with respect to genome size, base chromosome number, and ploidy. The *Arabidopsis* genome may act as an anchor genome, and markers positioned on it can be utilized for reciprocal localization of markers in *Brassica* species (Lagercrantz and Lydiate 1996; Lagercrantz 1998; Sillito et al. 2000). Knowledge of the position of genes controlling qualitative traits as well as quantitative trait loci (QTLs) in *A. thaliana* can be used to predict the location of homologous genes in *Brassica* species.

Physical genome maps and sequence data from *A. thaliana* together with comparative analysis of its syntenic relationships to *Brassica* genomes provide potentially powerful tools for genome analysis and gene discovery in rapeseed (*Brassica napus* L.), the closest major crop relative to the model plant. Generally 80–90% homology is found between the exons of putative orthologous genes in *Arabidopsis* and *Brassica* (Schmidt and Bancroft 2011), meaning that knowledge from *Arabidopsis* is highly relevant for gene isolation and characterization in *Brassica* crops.

An effective method to use functional PCR markers for physical mapping of *A. thaliana* gene loci in *B. napus* has been described (Fourmann et al. 2002; Snowdon and Friedt 2004). Several genetic linkage maps based on a range of marker types, including Restriction Fragment Length Polymorphism (RFLPs), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs) and Amplified Fragment Length Polymorphisms (AFLPs), have been produced for *B. rapa* (Kim et al. 2006; Kapoor et al. 2009). PCR based markers have been widely used in developing genetic linkage maps for *B. oleracea* (Kaczmarek et al. 2009), *B. nigra* (Truco and Quiros 1994), *B. juncea* (Pradhan et al. 2003) and *B. napus* (Lombard and Delourme 2001).

Because of the high economic value of *Brassica* species throughout the world and their potential to be models for the study of polyploidization, genome sequencing projects for *Brassica* species, especially *B. rapa* and *B. oleracea*, have been initiated (http://www.brassica.info) (Hong et al. 2008). The genome of *B. rapa* is expected to be completely sequenced within the near future (for progress see http://www.brassica.info), and current technological developments in the field of ultra-fast DNA sequencing are beginning to revolutionize the fields of polymorphism discovery, genome analysis and molecular breeding. The number of expressed sequence tag (EST) sequences available for *Brassica* species has skyrocketed in the past few years as sequencing costs have diminished, enabling DNA sequence mining to become extremely useful for the identification and development of single nucleotide polymorphism (SNP) markers in oilseed rape. In the near future it can be expected that high-density *B. napus* SNP arrays will play an important role in development of dense genetic maps for oilseed rape.

Next-generation sequencing technologies are also set to rapidly accelerate SNP discovery, so that ultra-high density SNP maps will probably become available in the relatively near future. High throughout SNP screening methods will also be a valuable resource for whole-genome allele-trait association studies, which can potentially play a major role in the identification of genes contributing to complex traits.

Microsatellites or simple sequence repeat (SSR) markers are an important genomic resource and have gained increasing importance for determining genetic relationship among closely related species. The SSRs are present and distributed in the genomes of all eukaryotes. Because of the abundance and specificity of SSRs, these are considered as important DNA markers for genetic mapping and population studies. The important features of SSR markers coupled with their ease of detection have made them useful molecular marker in different crops (Snowdon 2007). Therefore, detection of SSRs in the unigenes and ESTs of *Brassicaceae* species may help in designing a new set of DNA markers and may provide more insight in the genetic diversity among these species. This will also provide an excellent opportunity to breeders to find some agriculturally important genes, to clone and use them in Brassica breeding programs. In one study, 131,286 ESTs of five *Brassicaceae* species were assembled into unigene contigs and compared with *Arabidopsis* gene indices. Almost all the unigenes of *Brassicaceae* species showed high similarities with *Arabidopsis* genes except those of *B. napus,* where 90% of unigenes were found similar. A total of 9,699 SSRs were identified in the unigenes. Functional annotation of unigenes showed that the majority of the genes are present in metabolism and energy functional classes. The DNA markers developed in this study can be used for mapping, tagging, and cloning of important genes in *Brassicaceae* (Bhati et al. 2010).

The B genome of *B. nigra* is considered to be an important source of useful genes in *Brassica* breeding, including drought tolerance, disease resistance, and oil seed quality (Pradhan et al. 2011). Desirable traits can be transferred or combined through interspecific hybridization. The B genome of *B. nigra* is a major donor as a source of resistance to several blackleg diseases (*Leptosphaeria maculans*, *Phoma lingam*) (Roy 1984). This trait has been successfully transferred to *B. napus*, a major oilseed crop in the Europe and North America.

PCR based IP (Intron polymorphism) markers were used to analyze genome wide synteny between *Brassica juncea* (AABB genome) and *Arabidopsis thaliana*. The arrangement of 24 genomic block segments in the A, B and C *Brassica* genomes were analyzed in order to understand the karyotypic variations in three diploid *Brassica* genomes. Comparative genomics between the three *Brassica* lineages established the major rearrangements, translocations and fusions pivotal to karyotype diversification between the A, B and C genomes of *Brassica* species (Panjabi et al. 2008). This inter-relationship between the *Brassica* lineages vis-à-vis *Arabidopsis* would facilitate the identification and isolation of candidate genes contributing to traits of agronomic value in crop Brassicas and the development of unified tools for *Brassica* genomics.

The genomes of *Brassica* species, although 4–10 times larger than that of *A. thaliana*, are still of a tractable size for genomic technologies. Physical maps are being constructed for the *Brassica* A genome in Korea and for both the A and C genomes in the UK. Partial physical mapping of the genome of *B. napus* is being conducted in Canada and in the EU. Although such physical maps will be of great value for the identification of specific regions of the genomes of these important crops, they will not permit the detailed analysis of the entire *Brassica* genome, the preparation of microarrays to analyse the transcriptome, or the efficient design of markers associated with the sequences of specific genes for use in breeding programmes. To achieve these things, the complete sequence of at least one of the *Brassica* genomes will be required. It is necessary to sequence only one *Brassica* genome initially at both the macrostucture (chromosome level) and the microstructure (gene-by-gene level) level. The international Brassica research community is working together to establish communal genomic resources (http://www.brassica.info). A steering group has been formed for the Multinational *Brassica* Genome Project. This steering group represents the international *Brassica* research community and has the roles of promoting international cooperation and helping to defining the strategic goals of the community in the area of *Brassica* genomics. The steering group recently agreed that these goals should include the genetic anchoring of the BAC-based physical maps being constructed for *Brassica* genomes, and that the 500 Mb *Brassica* A genome should be sequenced by an international consortium, with a some target completion date (http://www.brassica/resource/dna-sequences).

10.4 Microarray Resource for Transcriptome Profiling in *Brassica* Species

Complementary to genome sequencing is the wide spread application of transcriptome sampling strategies, which has resulted in large collections of expressed sequences tags (ESTs) for nearly all economically important plant species (see in above section) (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). Additionally, advances in sequencing technologies (the next generation sequencing technologies) have reduced the sequencing cost and increased the sequence capacity at an unprecedented rate, making whole genome sequencing possible for each important crop species. As a result, genomics-assisted breeding have gained momentum, with the capacity for significant improvements in the accuracy and efficiency for predicting phenotypes from genotypes.

A genome wide view of gene expression programs is required to understand the underlying mechanisms of the coordination of genes at the molecular level. As described above, the completion of the full genome sequence for *Arabidopsis* has led to the development of several genomics resources for high throughput gene expression studies, including cDNA and oligonucleotide-based microarrays (Hennig et al. 2003; Zimmermann et al. 2004). DNA microarray technology has made a revolutionary transformation in studying differential gene expression and can be used to study the collaboration of multigene for one trait and meanwhile discovering and locating the target genes. This technology has been widely used to study different traits (Price et al. 2004; Yamakawa et al. 2007) since its appearance, including transcriptional regulation in response to drought, salinity and heavy metal treatments (Yamaguchi and Blumwald 2005; Herbette et al. 2006; Weber et al. 2006).

A microarray resource for use by the Brassica research community has been recently developed (Trick et al. 2009). A 60-mer oligo microarray comprising 94,558 probes was developed using the unigene sequences. Gene expression was analyzed in reciprocal resynthesised *B. napus* lines and the *B. oleracea* and *B. rapa* lines used to produce them. The analysis showed that significant expression could consistently be detected in leaf tissue for 35,386 unigenes. Expression was detected across all four genotypes for 27,355 unigenes, genome-specific expression patterns were observed for 7,851 unigenes and 180 unigenes displayed other classes of expression pattern. Principal component analysis (PCA) clearly resolved the individual microarray datasets for *B. rapa*, *B. oleracea* and resynthesised *B. napus*. Quantitative differences in expression were observed between the resynthesised *B. napus* lines for 98 unigenes, most of which could be classified into non-additive expression patterns, including 17 that showed cytoplasm-specific patterns.

Additionally, the Affymetrix GeneChip® Brassica Exon 1.0 ST Array has been developed and it is a 5 μM 49-7875 format array, containing 2.4 million 25-base oligonucleotide probes representing 135,201 gene models, with 15 probes per gene distributed among exons. Discrimination of the gene models was based on an E-value cut-off of $1E^{-5}$, with <98% sequence identity. The 135 k Brassica Exon Array was validated by quantifying transcriptome differences between leaf and root tissue from a reference Brassica rapa line (R-o-18), and categorization by Gene ontologies (GO) based on gene orthology with *Arabidopsis thaliana*. Technical validation involved comparison of the exon array with a 60-mer array platform using the same starting RNA samples. The 135 k Brassica Exon Array is a robust platform and is accessible as a track within the public BrassEnsembl genome browser at http://www.brassica.info/BrassEnsembl/index.html (Love et al. 2010).

10.4.1 Transcriptional Regulation in Response to Cu Treatments to Brassica nigra

Arabidopsis thaliana has become a model molecular genetics system because of its extensive genetic characterization, compact genome, known genomic sequence and compact growth habit, and the availability of a wide variety of tools for its molecular genetic manipulation. However, it does not accumulate metal. Interestingly, approx. 25% of the documented metal hyperaccumulating species are the members of the *Brassicaceae* (Peer et al. 2006).

The heavy metal accumulating species *Brassica nigra* and *B. juncea* have received attention due to its possible use for phytoremediation of heavy metal-polluted soils (Muthukumar et al. 2007; Memon et al. 2008). A strong Cd accumulation has been demonstrated for the trichomes covering the leaf surface of *Brassica juncea* (Salt et al. 1995). The ability to efficiently translocate heavy metal ions from the root to the shoot has led to the proposal that *B. juncea* could be used to decontaminate heavy metal polluted soils (Salt et al. 1995). Though *B. juncea* is not reported to be a hyper accumulator of metals but its relative high biomass, rapid growth, high economic value as industrial crop and relatedness to metal hyperaccumulating plants makes it promising plant for both use in phytoremediation and for generation of biofuel.

Tolerance to metals is based on multiple mechanisms such as cell wall binding, active transport of ions into the vacuole and formation of complexes with organic acids or peptides (Memon and Schroder 2009). Here, one of the most important mechanisms for metal detoxification in plants appears to be chelation of metals by low molecular weight proteins such as metallothioneins and a family of peptide ligands, the phytochelatins. For example, glutathione (GSH), a precursor of phytochelatin synthesis, plays a key role not only in metal detoxification but also in protecting plant cells from other environmental stresses including oxidative stress. The enzymes in this pathway have been well characterized in *Brassica juncea* (Schafer et al. 1997).

In the last decade, the tremendous developments in molecular biology and the success of genomics have highly encouraged studies in molecular genetics, mainly transcriptomics, for the identification of the functional genes implied in metal tolerance in plants (Hammond et al. 2006; Muthukumar et al. 2007). These studies have already succeeded in the identification of hundreds of genes that largely belong to the metal-homeostasis network (Memon and Schroder 2009). To understand the genetics of metal accumulation and adaptation, the vast arsenal of resources developed in *A. thaliana* could be extended to one of its closest relatives that display the highest level of adaptation to high metal environments such as *A. halleri* and *T. caerulescens*. Further studies could also be carried out with fast growing, high biomass producing and economically important *Brassica* sps which can be used for both for phytoremediation and for biofuel and/or industrial oil production.

In this section some recent advances in understanding the genetic and molecular basis of the metal induced gene expression in plants including the gene expression work which is being carried out in authors laboratory on some metal accumulating plant species in *Brassicaceae* family will be discussed in detail.

While surveying the flora of Cu mining areas of Southeastern Anatolia, we discovered several endemic metal accumulator plants and interestingly a *Brassica nigra* ecoptype found from Diyarbakir site contained a very high amount of Cu in their shoots (around 700 ppm Cu in their leaves) (Fig. 10.4) (Memon et al. 2006). When plants from this ecotype were regenerated from callus culture and grown in soil culture containing 200 ppm Cu, the shoots accumulated x3 more Cu (700 µg/g D.W.) than roots (Yildizhan and Memon, unpublished data).

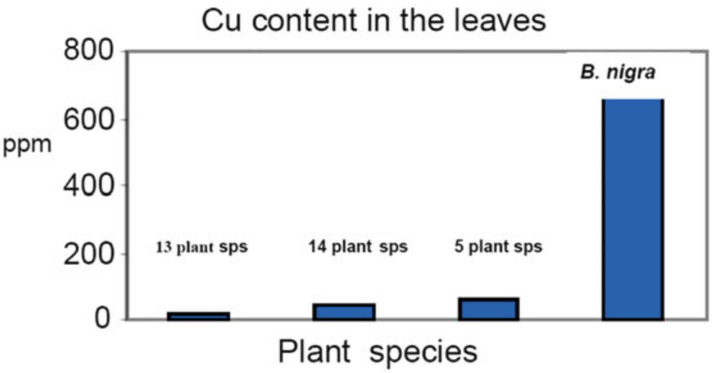

Fig. 10.4 A histogram showing the Cu content in the leaves of plant species collected from Southeastern part of Turkey (Memon et al. 2008)

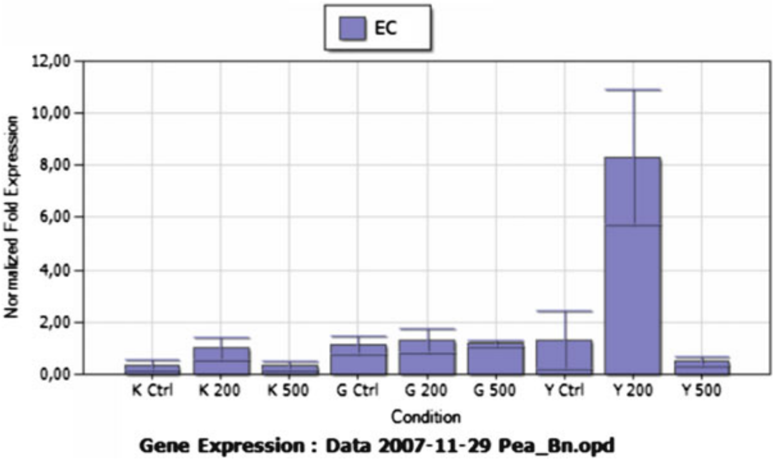

Fig. 10.5 R-T (real time) PCR experiments showing γ-EC expression in roots (*K*), stems (*G*) and leaves (*Y*) of Brassica nigra grown in 0 (control), 200 and 500 μM Cu (Memon et al. 2008)

This ecotype could be considered as a good candidate for Cu phytoremdiation (Memon et al. 2001, 2006). Our data showed that ~20,000 μg Cu g^{-1} DW was accumulated in the shoots of *B. nigra* when grown at 500 μM Cu. The expression γ-ECs and PCS was also increased several times in shoots when plants were subjected to high Cu concentration (Memon et al. 2009). Specially the expression of key regulatory enzyme in glutathione pathway (γ-EC) was increased around ninefold in the leaves when plants were subjected to 200 μM Cu treatment (Fig. 10.5).

Comparative transcriptome analysis was carried in order to find out the expression level of metal induced genes and transcriptome changes both in low and high Cu treated plants. Microarray analysis showed that some of the genes were up regulated (several hundred folds) and some were down regulated when plants were exposed to high Cu (Memon et al. 2009). The results of up regulated genes are shown in Table 10.1. The Cu accumulation capacity of Diyarbekir ecotype was determined and compared with other *Brassica nigra* ecotypes 6619, 6620 and 6630 obtained from different sites of Western Europe. In these comparative studies *Brassica nigra* Diyarbekir ecotype was found to be a super accumulator of Cu compare to other European ecotypes (Memon et al. 2009).

Microarray analysis showed several hundred folds up regulation of metal related genes including the genes involved in glutathione pathway, metal ATPase and ABC transporters in *B. nigra* when treated with 500 μM Cu. Currently we are carrying out metabolomic studies with metal treated accumulator and non accumulator ecotypes of *B. nigra* by using HPLC-MS-MS inorder to identify metabolomic pattern in accumulator and non-accumulator ecotypes. Our aim is to identify specific metabolites which are upregulated and/or down regulated with Cu treatment in both ecotypes.

10.5 Conclusions and Perspectives

To understand fully the genetics of metal accumulation, the vast genetic resources developed in *A. thaliana* must be extended to other metal accumulator species that display traits absent in this model species. *A. thaliana* microarray chips could be used to identify differentially expressed genes in metal accumulator plants in *Brassicaceae*. The integration of resources obtained from model and wild species of the *Brassicaceae* family will be of utmost importance, bringing most of the diverse fields of plant biology together such as functional genomics, population genetics, phylogenetics, and ecology. Like in present work with *Brassica nigra*, *Arabidopsis* array have been previously used to compare *Arabidopsis* transcriptome to other related *Brassicaceae* species such as *Arabidopsis halleri* (Becher et al. 2004; Weber et al. 2004), *Thlaspi caerulescens* (Hammond et al. 2006), *Thellungiella halophila* (Taji et al. 2004; Gong et al. 2005), *Brassica oleracea* (Hammond et al. 2006) and *B. napus* (Liet al. 2005). It will be interesting to use recently developed Affymetric GeneChip® Brassica Exon 1.0 ST Array to identify gene expression profile in *Brassica nigra* and other *Brassica* sps subjected to high and low metal concentrations.

Further development of phytoremediation technology requires an integrated multidisciplinary research effort that combines plant biology, genetic engineering, soil chemistry, soil microbiology, as well as agricultural and environmental engineering.

Table 10.1 Summary of microarray on *A. thaliana* Genechips (Arabidopsis ATH 1-14501 Genome array) hybridized with cDNA from *Brassica nigra* grown at 0 (control) and 500 μM Cu

Enterez gene	Uni gene ID	Annotation	Fold expression
814671	At4290	Homeobox-leucine zippi protein 17 (HB-17)	542.66
841606	At37745	Leucine-rich repeat protein kinase	305.303
839766	At18928	Lipase	222.9
826933	At51233	Protein kinase family protein	988.867
843799	At34864	Glutathione 5-transferase	4,453.22
817206	At68413	AKT1 potassium channel protein	268.304
828055	At54106	Small lipase related protein	944.145
832383	At8800	Cytochrome P450 family protein	853.668
826797	At63693	S-locus Lectin Protein kinase family protein	257.197
840050	At62375	MHD-box family protein	627.242
829490	At48932	Metal transporting P-type ATPase (PAA1)	298.124
824253	At35444	Zinc finger homeobox protein ZF-HD homeobox	274.521
843519	At23741	Sucrose transporter/sucrose-proton symporter (SUC1)	131.859
823644	At1043	2-phosphoglycerate kinase related	747.391
835115	At62438	CCAAT-box binding transcription factor Itap5a putative	542.568
829483	At31581	ABC transporter family protein	374.546
835775	At27567	Histone acetyl transferase family protein	2,464.59
821403	At38505	F-box family protein	392.733
839706	At23008	Auxin responsive GH3 family protein	636.692
831284	At10905	myb family transcription factor (MYB40)	240.056
838207	At41885	Transporter-related	4,437.08
832081	At54918	Sec7 domain-containing protein	409.803
835989	At50537	wound-responsive protein related	349.045
821739	At70382	E3 ubiquitin ligase SCF complex submit SKP1/ASK1 (At9)	360.018
827187	At33200	ABC transporter family protein	767.817
821369	At53370	No apical meristem (NAM) family protein	64,755.1
840117	At51872	Calmodulin (putative)	395.849
818315	At37430	Heavy metal associated domain containing protein	962.659
829867	At31273	Hydroxyproline-rich glycoprotein family protein	167.632
818856	At36994	LEH domain containing protein	957.017
840311	At10999	Inositol phospholi 5-phosphatase I IP5PI	60.167
816596	At39660	Glycosylhydrolase family 5 protein/cellulase family protein	266,678
839166	At17257	Proline-rich extensin, putative	14,276.4
843088	At52423	lysine and histidine specific transporter putative	755.624
839416	At51465	Epsin N terminal homology (ENTH) domain-containing protein/Clathrin assembly protein	53,631.3
828430	At54490	Receptor-like protein kinase putative	108.975

(continued)

Table 10.1 (continued)

Enterez gene	Uni gene ID	Annotation	Fold expression
828480	At2572	High mobility group (HMG1/2) family protein	280.771
838269	At41859	α-Trehalose-phosphate synthase, UDP-forming putative/Trehalose-6-phosphate	452.523
830681	At54772	SNAP 25 like	169.613
818520	At70170	ABC transporter	907.673
833815	At55216	Plant defensin-fusion protein putative	845.084
821552	At50206	Cytochrome P450 family protein	1,546.55
834610	At43916	Heatshock transcription factor	223.442
827857	At32663	UV-damaged DNA binding protein putative	1,472.9
841241	At20838	Serine/threonine protein kinase putative	835.259
816759	At39463	Oxidoreductase 20G-Fe(II) oxygenase family protein	366,027
829309	At31713	WRKY family transcription factor	169.063

The results show the fold (x) expression of genes in 500 μM Cu treated plants compared to control (Memon et al. 2009)

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Chapter 11
Chelate Assisted Phytoextraction Using Oilseed *Brassicas*

Firdaus-e-Bareen

Abstract Members of the family Brassicaceae have a special ability to absorb such large amounts of metals as are often beyond the tolerance range of other plants. Among the oilseed *Brassicas,* work on phytoextraction has been centered on *Brassica juncea*, a well known metal hyperaccumulating species. The oilseed *Brassicas* mainly include *B. carinata* (Ethiopian mustard), *B. elongata* (elongated mustard), *B. juncea* (Indian mustard), *B. napus* (oilseed rape/canola), *B. narinosa* (broad-beaked mustard), *B. nigra* (black mustard) and *B. rapa* (turnip mustard). Although, there has been a considerable research on the phytoextraction abilities of these plants from heavy metal contaminated soil, lesser work has been done with reference to chelate-assisted phytoextraction. Research on chelate assisted phytoextraction has mainly been centred on *B. juncea* and *B. napus* on account of a better performance of these plants in metal uptake. Chelating agents like EDTA are capable of improving translocation of metals from roots to shoots and then into leaves. Higher bioaccumulation factors have been observed in stems and leaves of plants under the influence of chelating agents. As many of these oilseed crops yield edible oil, the high heavy metal content translocated to the oil bearing seeds is important. Research shows evidence that the seeds contain a considerable amount of hazardous toxic metals if grown on metal contaminated sites. However, the translocation into seeds is checked under lower doses of chelating agents like EDTA. Among the heavy metals, most of the research work on chelate assisted phytoextraction has been on Pb contaminated soil and application of chelating agents like EDTA and EDDS have shown significantly higher metal uptake in plants. Work has also been done on heavy metals like Cd, Cu, Cr and Zn. Chelate assisted phytoextraction has two main drawbacks. Firstly, the phytotoxic effect of the chelate itself with a potentially long residence time in soil and secondly, the

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leaching hazard of biolabile heavy metals to cause ground water pollution. Several measures have been suggested to overcome these hazards.

Keywords Oilseed *Brassicas* • Phytoextraction • Chelating agents • *B. juncea*

Abbreviations

CDTA	trans-1,2 Cyclohexylene dinitrilo tetraacetic acid
DTPA	Diethylene triamino pentaacetic acid
EDDS	S,S-ethylene diamine disuccinic acid
EDTA	Ethylene diamine tetraacetic acid
EDTA	Ethylene diaminetetraacetic acid
EGTA	Ethylene diaminesuccinic acid
GEDTA	Bis (2-aminoethyl) ethyleneglycol tetraacetic acid
HEDTA	Hydroxylethylene diamine tetraacetic acid
LMWOA	Low molecular weight organic acids
NTA	Nitrilotriacetic acid
PGPR	Plant growth promoting rhizobacteria

11.1 Introduction

The genus *Brassica* in the tribe Brassicae is the economically most important genus, among the 51 existing genera (Gomez-Compo 1980). Many crop species within this genus provide edible roots, leaves, stems, buds, flowers and oilseeds. The important vegetable crop species include *Brassica oleracea* L. (cabbage, broccoli and cauliflower) and *B. rapa* L. (Chinese cabbage, turnip mustard). Others constitute the oilseed crops like *B. napus* L. (rapeseed, canola), *B. juncea* (L.) Czern & Coss, *B. carinata* A. Braun, *B. nigra* (L.) Koch and varieties of *B. rapa*. *Brassica napus* is the most productive oilseed *Brassica* species under cultivation and is grown in many countries of the world whereas *B. rapa* and *B. juncea* are cultivated as an oilseed in India (Rakow 2004). *Brassica* oilseed production has increased worldwide over the last 45 years and has become the most important world sources of vegetable oil after soybean and palmolive. India is the largest producer of *Brassica* oilseeds followed by China and Canada.

Contamination of soil by heavy metals is a global environmental issue due to intensive agricultural and industrial development in many parts of the world. Of a greater concern is the food chain contamination reaching the human population and causing a severe threat of metal specific diseases.

Due to the inherent capability of a number of *Brassicas* to accumulate metals, many of them have been employed in phytoremediation strategies. Among all, *Brassica juncea* has gained world fame in bioaccumulation of metals. Phytoextraction is one strategy in which plants are used to extract toxic metals from soil and render them within permissible limits. The plants used for phytoextraction of toxic metals from soil are expected to have two important traits, a high biomass production and a high metal uptake capability (Römkens et al. 2002). This is the reason that fast growing, deep rooting, high biomass producing plants have been focused by researchers for metal phytoextraction. Many of the *Brassica* spp. constitute an important source of oilseeds and at the same time have been used in phytoextraction from heavy metal polluted soils. Furthermore, synthetic chelators have been used to artificially enhance the solubility of heavy metals from the soil solid phase in soil solution and to increase the heavy metals phytoavailability (Nowack 2002; Kos and Leštan 2004). A number of synthetic chelating agents like DTPA, EDTA, natural chelating agents like EDDS, NTA and organic acids like citrate have been employed to solubilize the metals and enhance their uptake in plants.

11.1.1 Historical Background of Chelate Assisted Phytoextraction

A chelating agent is a substance whose molecules can form several bonds to a single metal ion for example EDTA can form four to six bonds with metal ions. Thus the chelating agents act as multi dentate ligands (Alkorta et al. 2004). Historically chelating agents have been used to solubilize micronutrients in hydroponics and as soil extracts as well as a source for micronutrient fertilizers.

A chelate is a chemical compound composed of a metal chemically bound to a chelating agent. Metal EDTA chelates include chelates of Cu, Fe, Pb and Zn with high stability constants which degrade very slowly. Those with low-stability constants include chelates of Ca, Mg and Mn with shorter degradation time (Stroutalinov et al. 2000). EDTA is a non-selective metal chelating agent which forms complexes with a variety of metals including the alkaline earth metals like Al, Ca, Fe and Mg (Zeng et al. 2005) and heavy metals like Cd, Pb, Mn, Ni and Zn (Skoog et al. 1996). Due to its non-selective nature, the co-dissolution of major elements along with trace metal ions is caused and the mass balance between the reagent and cations in any EDTA-soil medium is strongly controlled by major metal extraction. Therefore all the extractable metals present in the concerned sample must be considered in any phytoextraction strategy (Manouchehri et al. 2006).

Chelating agents have been used in agriculture as additives in micronutrient fertilizers since the 1950s (Foth and Ellis 1988). Such substances are capable of forming complexes with metal ions, thereby increasing the bioavailability of heavy metals in soils (Hsiao et al. 2007). The main property of the chelator is that it can bind to heavy metal ions and render them uncharged. An uncharged ion is much easy to get through cellular membranes due to its high mobility (Wu et al. 2010). Translocation of metal-EDTA complex into shoots requires entry into the vessels which is thought to follow the symplastic (cell to cell) or apoplastic (through breaks in the endodermis) pathway (Collins et al. 2002; Jarvis and Leung 2002). Jarvis and Leung (2002) have shown that HEDTA chelated Pb followed an apoplastic pathway while EDTA chelated Pb followed a symplastic pathway of movement in plants of *Pinus radiata*.

Synthetic chelators have been used conventionally to facilitate micronutrient uptake in plants in soil as well as hydroponics. In the late 1990s, EDTA has been suggested as a chelating agent to enhance the phytoextraction process (Blaylock et al. 1997; Huang et al. 1997; Vassil et al. 1998; Epstein et al. 1999). However, the amounts of the various competing cations and the nature of calcareous or non-calcareous soil determine to which extent it will complex with the target heavy metals (Manouchehri et al. 2006). Selectivity for complexation with Cd and Pb has been observed by EDTA, for Cd by EGTA and both Cu and Cd by NTA (Hong and Pintauro 1996a, b). A better mobilization of Pb by EDTA has been observed in comparison to Cd, Cu, Ni and Zn by Blaylock et al. (1997) and subsequent uptake and translocation to shoots of *Brassica juncea*. Hsiao et al. (2007) have observed more total uptake of Ni over Cr in *B. juncea* using EDTA. Komárek et al. (2010) observed that there is more competition of ions like Al, Cu and Fe for EDTA and thus leaching of some beneficial or essential metals cannot be overlooked. Bareen and Tahira (2010) have also observed a better mobilization and translocation of Cr in *B. campestris, B. juncea* and *Spinacea oleracea* as compared to Na with EDTA. Similarly, Duo et al. (2010) observed EDTA to be more effective for phytoextraction of Mn, Ni, Cd, Pb than for Cu and Zn by *Lolium perenne* in the field.

Synthetic chelating agents, the most important of which is EDTA, have been used for enhancement of the phytoextraction process as early as 1990. There have been diverse results for metal uptake in various plant species, dependent upon the metal content of the soil, the type of soil itself and the amount of EDTA applied. EDTA has been used to increase the availability of metals for phytoextraction by accumulating plants (Huang et al. 1997; Wenzel et al. 2003). To increase the bioavailability of heavy metals in soils, synthetic chelating agents have been used in the process of phytoextraction (Alkorta et al. 2004). The chelating agent EDTA has become one of the most tested mobilizing amendments for less mobile/available metals, such as Pb (Saifullah et al. 2009). Among the chelators, the readily biodegradable EDDS or more slowly biodegradable EDTA have been used in some studies (Jaworska et al. 1999; Luo et al. 2006; Yip et al. 2009).

11.2 Hyperaccumulator *Brassicas* Used in Phytoextraction

Over 400 vascular plants have been identified as metal hyperaccumulating species. However, a number of them are unsuitable for phytoextraction as they show limited growth and low biomass production (Japenga et al. 2007; Koopmans et al. 2007). Hyperaccumulating species are considered those which accumulate more than 100 mg kg^{-1} of Cd, 1,000 mg kg^{-1} of Cu, Cr, Pb and Ni or 10,000 mg kg^{-1} of Zn in the above ground parts of plants in dry weight of tissues (Brooks 1998; Baker et al. 1998; Schmidt 2003).

Keller et al. (2003) reported that root size, density and spatial distribution can heavily affect metal movement in soil and uptake efficiency by plants including hyperaccumulators. Since most known hyperaccumulators have a low annual biomass production, considerable research has been focused on methods that enhance the availability of heavy metals in soils and increase phytoextraction efficiency of potential accumulators (Baker et al. 1998). Many hyperaccumulators are not able to accumulate some metals from multi-metal contaminated soils during phytoextraction (Lombi et al. 2001; Do Nascimento et al. 2006).

Research on phytoextraction has shifted toward rapidly developing high biomass plant species as it was recognized that chelating agents could be used to enhance their metal uptake as reviewed by several researchers (Schmidt 2003; Alkorta et al. 2004; Evangelou et al. 2007; Meers et al. 2008; Saifullah et al. 2009). There are about 12 important species of *Brassica* with a number of economically important varieties among which *B. juncea* has long been employed in such studies. *Brassica juncea*, is a dry land species which is known to accumulate high levels of heavy metals including Cd, Cr, Cu, Ni, Pb, and Zn under some conditions that particularly enhance the solubility of the metal cations (Kumar et al. 1995; Salt et al. 1995; Ebbs et al. 1997; Liphadzi and Kirkham 2005). Using such a plant with high biomass yields, and a chemically enhanced method of phytoextraction has been proposed as a viable strategy for removing heavy metals from soils over a reasonable time frame (Blaylock et al. 1997; Huang et al. 1997).

Among the oilseed *Brassicas, B. juncea* has been most widely employed in chelate assisted phytoextraction experiments as shown in Table 11.1. *Brassica carinata, B. napus, B. narinosa* and *B. napus* have also been used to some extent. As oilseed *Brassicas* constitute important pot herbs in addition to oil yielding crops, whenever they are used for phytoextraction, the plants need either to be dried and incinerated to ash or the metals need to be recycled and purified. It is also important to determine if the metals are translocated and accumulated in the seeds. Using seven different cultivated plants, Bareen and Tahira (2010) have observed quite a high bioaccumulation of Cr in seeds of *B. campestris* and *B. juncea* under the effect of high doses of EDTA. As both are oilseed crops, proper disposal along with seeds is therefore suggested for such crops by these authors. Thus, care should be taken about the determination of heavy metal status of oilseeds before extraction into edible oil.

Early experiments regarding chelate assisted phytoextraction began in the late 1990, when high biomass producing species like *Brassica juncea* were used in pot experiments (Blaylock et al. 1997; Ebbs and Kochian 1998; Baker et al. 1998). A variety of chelating agents was used by Blaylock et al. (1997) like citrate, CDTA, DTPA, EDTA, EGTA and a much greater efficiency of EDTA was observed in solubilizing Pb and enhancing its uptake in *B. juncea* as compared to other metals. In general, EDTA formed chelates with all metals. Vassil et al. (1998) observed that EDTA chelates Pb outside of the plant and soluble Pb-EDTA complex is transported through the plant and accumulates in the leaves of *B. juncea*. Huang et al. (1998a) observed citrate to effectively enhance U hyperaccumulation in

Table 11.1 Oilseed *Brassicas* used for phytoextraction of metals using various chelating agents

Oil seed *Brassica* used in phytoextraction	Metal extracted	Chelating agent used	Dose used per kg soil	Effect on plants	Reference
B. juncea Grown in growth chamber in pots	Cd, Cu, Pb, Ni, Zn	Citrate, CDTA, DTPA, EDTA, EGTA	5, 10 mmol	Biomass reduction	Blaylock et al. (1997)
B. juncea and two others Grown in hydroponically and then in pots	Zn	EDTA	2.5 g	No biomass reduction, slight chlorosis	Ebbs and Kochian (1998)
B. chinensis, *B. juncea*, *B. narinosa* Grown in pots	U	Acetate, Citrate, Malate	5, 10, 15, 20 mmol	No toxicity symptoms	Huang et al. (1998a, b)
B. juncea Grown hydroponically	Pb	EDTA	0.1–2.5 mmol	Drying of tissues, necrotic lesions (>0.5 mmol of EDTA)	Vassil et al. (1998)
B. juncea Grown in pots	Pb	EDTA	5 mmol 10 mmol	Biomass reduction	Epstein et al. (1999)
B. juncea and seven others Grown in the field	Cd, Cu, Zn	NTA	4.2, 8.4 mmol	No toxicity symptom	Kayser et al. (2000)
B. juncea and one other Grown in growth chamber in pots	Cd, Pb	Citrate, EDTA	0.05%, 0.02%	No toxicity symptoms	Bricker et al. (2001)
B. rapa Grown in soil columns	Cd, Pb, Zn	EDTA	5 mmol 10 mmol	Biomass reduction	Grčman et al. (2001)
B. rapa and two others Grown in pots	Pb	Citrate, DTPA, EDTA, HEDTA, NTA	1, 1.5, 3, 5 mmol	No toxicity symptoms	Shen et al. (2002)
B. rapa Grown in soil columns	Cd, Pb, Zn	EDDS, EDTA	5, 10 mmol	Biomass reduction Necrotic lesions on leaves	Grčman et al. (2003)

B. juncea Grown in pots	Cd	EDTA	1.78 mmol per L	Biomass reduction	Jiang et al. (2003)
B. napus, *B. rapa* and 12 others Grown in soil columns	Cd, Pb, Zn	EDDS, EDTA	5 mmol EDTA, 10 mmol EDDS	Necrotic lesions on leaves, rapid senescence	Kos et al. (2003)
B. napus Grown in pots/field lysimeters	Cu, Pb, Zn	EDTA	0.25, 0.5, 1, 2, 0.21, 0.41, 0.83, 1.65 g	No toxicity symptoms	Wenzel et al. (2003)
B. juncea, *B. rapa* and ten others Grown in pots	Cd, Cu, Pb, Zn	EDTA	2.5, 5 mmol	Biomass reduction, leaf curling, chlorosis, necrosis, stunting, rapid senescence and drying	Chen et al. (2004)
B. rapa Grown in soil columns	Cu	Citrate, DTPA, EDDS, EDTA	5 mmol	No biomass reduction, necrotic lesions on leaves (exclusive in EDTA)	Kos and Lestan (2004)
B. juncea Grown in pots	Cd, Cu, Pb, Zn	Citrate, EDTA, Oxalate, Malate	3 mmol	Necrotic lesions on leaves, chlorosis, senescing	Wu et al. (2004)
B. rapa and three others Grown in pots	Cd, Cu, Pb, Ni, Zn	EDDS, EDTA	1, 3, 5 mmol	No biomass reduction	Meers et al. (2005)
B. juncea Grown in pots	Cd	Citrate, NTA	10, 20 mmol	Slight biomass reduction	Quartacci et al. (2005)
B. juncea Grown in pots	Pb	EDTA	2 mmol	Biomass reduction	Di Gregorio et al. (2006)
B. juncea Grown in pots	Cd, Cu, Pb, Ni, Zn	Citrate, EDTA, Gallate, Oxalate, Vanillate	10 mmol	Biomass reduction (in case of EDTA only), chlorosis and necrotic lesions on leaves	Do Nascimento et al. (2006)
B. juncea Grown in pots	Cu, Fe, Pb, Mn, Ni, Zn	DTPA could prove a good extractant for metals	–	–	Gupta and Sinha (2006)

(continued)

Table 11.1 (continued)

Oil seed *Brassica* used in phytoextraction	Metal extracted	Chelating agent used	Dose used per kg soil	Effect on plants	Reference
B campestris, B. chinensis, B. juncea, B. pekinensis and 14 others Grown in pots	Cd, Cu, Pb, Zn	EDDS, EDTA	3 mmol	Biomass reduction	Luo et al. (2006)
B. juncea	Cd, Co, Cu, Cr, Fe, Pb, Mn, Ni, K, Na, Zn	EDTA could prove a good extractant for metals	–	–	Gupta and Sinha (2007)
B. juncea Grown in pots	Cr, Ni	Citrate, DTPA, EDTA, Oxalate	0.05, 0.10 mmol	Biomass reduction, slight chlorosis	Hsiao et al. (2007)
B. carinata Grown hydroponically/in pots	As, Cd, Cu, Pb, Zn	EDDS, NTA	5 mmol	No visual toxicity symptoms	Quartacci et al. (2007)
B. juncea and B. napus Grown in growth chamber in pots	Cd, Cu, Pb, Zn	EDTA	3, 6, 10 mmol	Biomass reduction	Turan and Esringü (2007)
B. juncea Grown hydroponically	Cd	EDTA	10, 20, 40, 80, 160, 500 µmol	Biomass increase upto 60 µmol, biomass reduction above this dose	Seth et al. (2008)
B. juncea and one other Grown hydroponically	Ra, U	Citrate, EDTA	20 mmol Citrate, 4 mmol EDTA	Phytotoxicity beyond 5 mmol EDTA	Tomé et al. (2009)
B. napus Grown in pots	Cd	CDTA, DTPA, EDTA, EGTA	33.4 mg CDTA, 37.9 mg DTPA, 35.9 mg EDTA, 36.7 mg EGTA	Biomass reduction, leaf necrosis, stunting	Van Engelin (2009)
B. campestris, B. juncea and five others Grown in pots	Cr, Na	EDTA	1, 5, 10 mmol	Biomass reduction, slight chlorosis	Bareen and Tahira (2010)

B. carinata Grown in pots	Cu	EDDS, CuEDDS	30, 150 µmol	Not mentioned	Cestone et al. (2010)
B. napus Grown in pots	Pb	EDTA	0.5, 2 g	Biomass reduction, chlorosis and necrosis	Sinegani and Khalilikhah (2010)
B. napus Grown in the field	Pb, Mn, Zn	EDTA	3 g	Significant amelioration of biomass	Zaier et al. (2010a)
B. juncea Grown in hydroponics	Pb	FeEDTA	10 µmol per L	Biomass reduction, chlorosis	Zaier et al. (2010b)
B. napus Grown in pots	Cu	EDDS, EDTA	2, 4, 8 mmol	Growth suppression	Zeremski-škorić et al. (2010)

B. juncea. Negative effect of chelators in the form of biomass reduction was observed in plants by Blaylock et al. (1997) and Epstein et al. (1999).

The work on chelate assisted phytoextraction after the year 2000, has concentrated more on toxic metals like Cd, Cu, Pb, Zn and other than *Brassica juncea*, crops like *B. carinata*, *B. napus* and *B. rapa have* also been used. A comparison of citrate and GEDTA was made to enhance phytoextraction of yellow mustard along with two other species and citrate was found ineffective (Japenga and Römkens 2000). Kayser et al. (2000) used NTA to enhance phytoextraction potential of *B. juncea* and seven other plants for metal uptake in field studies. The results were not found as effective as in greenhouse studies, although, Cd, Cu and Zn were solubilized without change in soil pH. Bricker et al. (2001) compared the phytoextraction potential of *B. juncea* and *Zea mays* using soil amendments in the form of citrate, EDTA and composted sewage sludge. Higher citrate and EDTA were successful in removing Cd by both plants and Pb by *Zea mays*. Grčman et al. (2001) observed EDTA to effectively enhance Cd, Pb and Zn uptake in *B. rapa* but it also caused leaching of metals in soil columns. Shen et al. (2002) observed that among the five chelating agents, citrate, DTPA, EDTA, HEDTA and NTA, when applied to *B. rapa* to enhance metal uptake, EDTA was found to be the most effective and citrate the least. In a later study, Grčman et al. (2003) observed EDDS and EDTA to be equally effective in enhancing Pb uptake in *B. rapa* while at the same time EDDS was less phytotoxic. They insisted on the development of new techniques in order to increase the metal bioavailability and their translocation into harvestable parts. Jiang et al. (2003) used *B. juncea* for removal of Cd in a pot experiment using EDTA. Although EDTA application did not enhance Cd uptake but seemed to stimulate root to shoot translocation of Cd. They emphasized that EDTA led to substantial increase of soluble Cd in soil and its application may prove to be a potential environmental risk. Kos et al. (2003) observed the effect of different doses of EDDS and EDTA on uptake of Cd, Pb and Zn in *B. napus* and *B. rapa* along with 12 other crops and found EDDS to be less effective than EDTA. They found more metal uptake with increase in doses of the chelators from 5 mmol to 10 mmol kg^{-1} of soil.

Wenzel et al. (2003) used *B. napus* grown in pots and field lysimeters for phytoextraction of Cu, Pb and Zn in the presence of EDTA. Very little phytoextraction efficiency was observed by *B. napus* in a moderately polluted soil. Chen et al. (2004) compared the phytoextraction efficiency of *B. juncea* and *B. rapa* along with eight others for Pb using EDTA and suggested that high biomass and deep rooted plants should improve the phytoextraction process provided new irrigation strategies are utilized to minimize leaching. Kos and Leštan (2004) compared the application of citrate, DTPA, EDDS and EDTA on Cu uptake by *B. rapa* and EDTA was found to be the most effective among all. Wu et al. (2004) observed the efficiency of *B. juncea* in removing Cd, Cu, Pb and Zn after application of four chelating agents. As compared to EDTA, citrate, malate and oxalate had virtually no effect on enhancement of metal uptake. Meers et al. (2005) compared the potential of *B. rapa* and three other plants for phytoextraction of heavy metals using EDDS and EDTA. Both treatments were not effective and enough phytoextraction efficiency was not shown by any plant. Quartacci et al. (2005) studied the effect of citrate and NTA application on Cd uptake in *B. juncea*. NTA was observed to be much more effective than citrate in enhancing Cd uptake and appeared to be a stronger complexing agent. They observed that citrate application may be beneficial in a more highly polluted soil because of less leaching tendency.

The research on chelate assisted phytoextraction is still going on and the recent work in the past 5 years has shown new trends with certain other perspectives. Di Gregorio et al. (2006) studied the effect of EDTA application on Pb uptake in *Brassicajuncea* in the presence of an autochthonous strain of PGPR (*Sinorhizobium* sp.) and a surfactant. A much better performance of *B. juncea* was observed to tolerate stress and a higher biomass production was observed for phytoextraction. Do Nascimento et al. (2006) compared the efficiency of synthetic chelating agents DTPA and EDTA with organic acids citrate, gallate, oxalate and vanillate in enhancement of metal uptake by *B. juncea* and removal of Cd, Cu, Ni and Zn from a multi-metal contaminated soil. They were of the view that biodegradable organic acids be used in phytoextraction due to two reasons, to reduce the risk of leaching of metals and also because they offer lower phytotoxicity. Hsiao et al. (2007) observed the effect of citrate, DTPA, EDTA and oxalate for enhancing Cr and Ni phytoextraction from serpentine-mine tailings using *B. juncea*. EDTA and DTPA were found to be efficient chelating agents as compared to organic acids. But as EDTA and DTPA caused low biomass production in plants they recommended preference for organic acids over synthetic chelators.

Gupta and Sinha (2006, 2007) observed metal phytoextraction by *B. juncea* in two different studies and came to conclude that DTPA and EDTA could prove to be efficient extractors for metals from soil. Luo et al. (2006) compared the effect of EDDS and EDTA on 17 plant species including *B. campestris*, two varieties of *B. chinensis* and *B. pekinensis,* EDDS was found effective in mobilizing metals. However, none of the species was found effective for phytoextraction. Quartacci et al. (2007) observed *B. carinata* to be the best hyperaccumulator of multimetals. They observed that EDDS was more effective than NTA in phytoextraction of Cu Pb & Zn but not of As and Cd. Turan and Esringü (2007) studied the effect of EDTA on enhancement in uptake of Cd, Cu, Pb and Zn in *B. juncea* and *B. napus*. The latter appeared to be a more efficient extractor of metal with a higher production of biomass. Seth et al. (2008) found *B. juncea* to be a good hyperaccumulation of Cd which may be used for phytoremediation. They found that it has the capacity to combat high oxidative stress while enhanced level of PCs can detoxify metal via chelation or sequestration in vacuoles. Tomé et al. (2009) observed that presence of citrate and EDTA enhanced recovery of uranium in *B. juncea*. van Engelen et al. (2007) found EDTA to be the most effective chelant causing greater hyperaccumulation of Cd in *B. juncea*.

Bareen and Tahira (2010) showed that upto 5 mmol dose of EDTA was enough to solubilize metals and even 1 mmol was effective for increased uptake of Cr in two species of *Brassica* and *Spinacea oleracea* as compared to higher doses causing groundwater contamination. Cestone et al. (2010) studied the effect of EDDS and Cu EDDS on Cu uptake in *B. carinata* and found that the pathway of the two compounds into the plants and their translocation were different. Sinegani and Khalilikkah (2010) observed that the best application time for EDTA is 30 days after sowing for *B. napus* causing higher lead phytoextraction and not affecting much biomass. Zaier et al. (2010a) observed that EDTA enhances decontamination by *B. napus* without affecting plant growth. Zaier et al. (2010b) made a comparison of *Sesuvium portulacastrum,* a native halophyte and *B. juncea* for Pb phytoextraction from salt affected soil. *Sesuvium portulacastrum* was found much more effective. Zeremski-škorić et al. (2010) compared the effect of EDDS and EDTA application on Cu uptake in *B. napus* and observed EDDS to be much more effective in Cu uptake. However, they observed increased amount of EDDS not only limited the Cu uptake but also caused serious growth suppression. They recommended an amount of 8 mmol kg^{-1} of EDDS for maximum uptake of Cu from soil.

11.3 Efficiency of Chelating Agents in Translocation of Metals

Members of the Brassicaceae have the ability to take up heavy metals from contaminated soils and transport them to the shoots (Kumar et al. 1995). A number of researchers have observed an improved translocation of metals from roots to shoots in chelate assisted phytoextraction. The use of several chelators, including EDTA, increased the solubility of metals in the soil facilitating their easy availability, uptake and translocation from root to shoot in the vascular plant (Blaylock et al. 1997; Bricker et al. 2001; Wu et al. 2004).

Vassil et al. (1998) observed that EDTA chelates Pb outside of the plant and the soluble Pb-EDTA complex is transported through the plant thus accumulating in the leaves of plant as shown in case of *B. juncea*. Chen and Cutright (2001) reported effective translocation of Cd and Ni in *Helianthus annuus* after addition of EDTA but for Cr no translocation was observed. A better translocation of Pb in cabbage, murgbean and wheat has been shown by Shen et al. (2002) with the application of EDTA.

Hsiao et al. (2007) observed that citrate and oxalate enhanced root to shoot translocation in *B. juncea* in a much better way while similar translocation is unfavorable using EDTA and DTPA. Citrate and oxalate caused significant increases in soluble Cr in soil and translocation of Cr and Ni was enhanced in plants. However, a greater translocation within the plant was observed for Cr than Ni in lower doses. Seth et al. (2008) observed that root to shoot translocation of Cd can be improved with EDTA in *B. juncea*. Tomé et al. (2009) observed a better translocation of Ra in *B. juncea* after application of EDTA. Duo et al. (2010) observed that EDTA caused greater root to shoot translocation of metals like Mn, Ni, Cd, Pb in *Lolium perenne* in the field. Bareen and Tahira (2010) EDTA showed better translocation of Cr from root to shoot in *Brassica* spp. and *Spinacea oleracea* but not as much for Na. Sinegani and Khalilikhah (2010) also observed a higher translocation factor (TF) of Pb with increasing level of EDTA in *B. napus*.

11.4 Types of Chelates and Their Phytotoxicity

There are a number of synthetic aminocarboxylic acids that have been used to make micronutrients available to plants. The most popular is EDTA, while others include CDTA, DTPA, EGTA, HEDTA etc. The second group is of natural and biodegradable chelating agents like EDDS and NTA. The third group consists of low molecular weight organic acids like citrate, malate, oxalate etc.

Taking the toxicity, unselectivity and inefficacy of EDTA into account, a careful consideration concerning ecology, economy and human health is imperative before chelators are put to practical use (Bizily et al. 2000). It has also been observed that too much EDTA can result in a reduced biomass that overwhelms the advantages of increased translocation and leads to a decrease in the total amount of target metal extracted (Chen and Cutright 2001, 2002; Turan and Angin 2004; Lesage et al. 2005; January et al. 2008). The overall toxicity symptoms are generally observed in the form of biomass reduction, chlorosis of leaves, appearance of necrotic lesions on leaves and stunting. This concern has led to a developing consensus that familiar chelating agents such as EDTA may not be suitable for field work (Meers et al. 2008; Saifullah et al. 2009) When the chelating agent is applied to soils, metal-chelant complexes are formed and taken up by the plant, mostly through a passive apoplastic pathway (Nowack et al. 2006).

EDTA is probably the most efficient chelating agent at increasing the solubility of heavy metals in soil solutions from the soil solid phase, thus increasing the concentration of heavy metals in plant shoot tissues (Blaylock et al.1997; Huang et al. 1997; Ebbs et al. 1997). Complexation of heavy metals with various chelating agents typically follows the order EDTA and related synthetic chelates > NTA > citrate > oxalate > acetate as shown by several experiments (Hong and Pintauro 1996b; Krishnamurti et al. 1998). Synthetic chelators such as EDTA and DTPA, typically form metal complexes with high stability constants that are degraded slowly and have been demonstrated as relatively biologically stable, even under conditions favorable to biodegradation (Hong et al. 1999; Bucheli-Witschel and Egli 2001). EDTA is poorly photo-, chemo- and biodegradable in soils and is relatively biologically stable under conditions favorable to biodegradation (Hong et al. 1999; Nörtemann 1999). EDTA has shown different behavior than other extractants like DTPA in metal bioavailability to *B. juncea* (Gupta and Sinha 2007).

At higher concentrations, synthetic chelating agents like EDTA can also be phytotoxic to plants and may disrupt plant root membranes (Vassil et al. 1998). Generally, the Pb concentration in shoots is directly proportional to the amount of EDTA or related chelating agents added to soil. Reduction of plant biomass by addition of chelating agents may result in higher metal concentrations in plant tissue but less total mass of metal removed from soil (Chen and Cutright 2001).

One method of overcoming toxicity of EDTA is to allow plants to germinate and have an established root system prior to adding the chelating agent. Elevated amount of heavy metals in soluble complexes may persist for weeks or months in the field after application of EDTA (Lombi et al. 2001). By applying the chelating agent only to mature plants, the chelate-poisoning concern may be reduced. A few soil-based experiments have already shown that the timing and manner of chelator application can be significant (Barocsi et al. 2003; Liphadzi et al. 2003; Lesage et al. 2005; Meers et al. 2005). Wang et al. (2007) found EDDS to be efficient in the release of Cu from stable soil complexes. Luo et al. (2006) while studying the residual effects of EDDS and EDTA on incoming crops, found EDDS to have less phytotoxic effect (Luo et al. 2006). Meers et al. (2008) showed that degradability of EDDS in soils varied distinctly, but it was completely degraded within a period of 4–5 days. It has an estimated half life of only 2.5 days in natural soils (Leštan and Grčman 2002). NTA is also observed to decompose under aerobic conditions having a half life of 3–7 days (Bucheli-Witschel and Egli 2001; Egli 2001).

EDDS has been proposed as a substitute of EDTA in enhancing soil remediation, due to environmental concerns about EDTA (Yip et al. 2009). EDDS was found to be less phytotoxic than EDTA to plants of *B. rapa* (Grcman et al. 2001). EDDS is an easily biodegradable chelating substance in soils, with fewer residual effects on the environment (Jaworska et al. 1999; Luo et al. 2006). However, EDTA has been found to be more efficient than EDDS in desorption of Pb from soils (Komárek et al. 2007). Zaier et al. (2010a) observed on the other hand that sludge ameliorates biomass production in *B. napus* by application of EDTA without affecting plant growth provided the chelate application is made to 2 month old plants in the form of 3 g kg^{-1} dose. It is important to reduce the phytotoxicity of chelating agents by applying them at a certain stage of maturity in order to minimize their effect and to get the maximum amount of metal phytoextracted from the soil.

11.5 Persistance of Chelates and Associated Leaching Hazards

The practical use of EDTA for enhancement of metal removal in phytoremediation strategies may require careful site-specific evaluation to minimize the risk of secondary environmental contamination (Seth et al. 2008). Most synthetic chelators are thought to form chemically and microbiologically stable complexes with heavy metals that are suspected to contaminate groundwater (Hong et al. 1999; Bucheli-Wistchel and Egli 2001). In metal contaminated soils treated with EDTA, metal EDTA complexes leached into soil pore water persisted for several weeks (Lombi et al. 2001).

Grčman et al. (2001) have shown a high solubility of Pb, Zn and Cd in soil columns treated with 10 mmol kg^{-1} EDTA. EDTA enhanced the uptake of metals in *B. rapa* but also caused leaching of heavy metals due to heavy metal-chelate complex formation. Thus, they emphasized on developing methods to control leaching of heavy metals. EDTA has been shown to enhance phytoextraction of heavy metals from contaminated soil but due to high solubility of EDTA-metal complexes and leaching to groundwater, there is a potential leaching risk for extended periods of time (Sun et al. 2001). Madrid et al. (2003) have shown through analysis of drainage water from columns that EDTA solubilized metals like Cd, Fe, Mn and Pb and their amount exceeded the permissible limits of drinking water.

Similarly, excess amounts of Cd (Jiang et al. 2003) and Cu (Jiang et al. 2004) were observed to become bioavailable by addition of EDTA. Wenzel et al. (2003) observed very little phytoextraction efficiency of *B. napus* in a moderately polluted soil but on the other hand reported that metal leaching may persist for extended periods. After chelate application a major problem is metal-laden seepage towards groundwater. According to Wu et al. (2004), although EDTA appeared to be more effective in increasing Cu and Pb concentration in shoots but the overall efficiency of chelate assisted phytoextraction appeared to be low due to risk of groundwater pollution as well as leaching of useful ions like Fe especially during the rainy season. Application of both EDTA and DTPA resulted in significant increases in soluble Cr in soil (Hsiao et al. 2007). According to van Engelen et al. (2007) EDTA and EGTA pose a severe threat to leaching of Cd. Seth et al. (2008) have shown that EDTA and EGTA pose more severe threat to leaching of Cd. Several measures have been suggested in controlling the leaching hazard associated with the application of chelating agents.

11.5.1 Use of Natural Chelating Agents

Among the natural aminocarboxylic acids, extensive work has been carried out with EDDS and to limited extent on NTA. Natural chelating agents such as EDDS and NTA have been proposed as an alternate to EDTA on account of a shorter degradation period and lesser toxicity as compared to synthetic chelants like EDTA (Tandy et al. 2006; Quartacci et al. 2006, 2007; Luo et al. 2006; Meers et al. 2008). Environmentally safe EDDS has also been used in phytoextraction studies by Grčman et al. (2003), Kos et al. (2003), Kos and Leštan (2004), Meers et al. (2005), Cestone et al. (2010) and Zeremski-škorić et al. (2010). According to Quartacci et al. (2007) EDDS degradates rapidly reducing associated leaching risk as compared to NTA. Still, it has been observed that the solubility of metals in chelate-assisted phytoextraction increases by quite a high factor (Grčman et al. 2003; Kos and Leštan 2004; Quartacci et al. 2007).

NTA has been used by Kayser et al. (2000) in Field studies and compared with other chelators by Shen et al. (2002) and Quartacci et al. (2005, 2007). The main difference in the synthetic and natural chelators lies in the degradation time. Natural aminocarboxylic acids are thought to be biodegraded at a faster rate thus showing lesser threat of toxicity to plants as well as leaching hazard.

11.5.2 Use of Organic Acids

Natural LMWOA are capable of solubilizing mineral soil components like heavy metals (Wasay et al. 1998). They have been used and compared with other chelating agents by many researchers like Blaylock et al. (1997), Japenga and Römkens

(2000), Bricker et al. (2001), Shen et al. (2002), Kos and Leštan (2004), Wu et al. (2004), Quartacci et al. (2005, 2006), Do Nascimento et al. (2006), Hsiao et al. (2007) and Tomé et al. (2009).

Organic acids like citrate and oxalate have also been used as a chelating agent by Gramss et al. (2004) in *Brassica chinensis* and by Huang et al. (1998a) in *B. chinensis, B.* juncea and *B. narinosa*. The main advantage of using LMWOA is their high degree of biodegradability and less leaching hazard (Meers et al. 2008). Shen et al. (2002) observed the efficiency of Pb chelation and subsequent uptake in cabbage roots and observed them in the order of EDTA<HEDTA<DTPA<citric acid. (Huang et al. 1998a, b) have stated that the use of citric acid is beneficial as it has a rapid biodegradation rate changing them to CO_2 and H_2O. According to Hsiao et al. (2007) organic acids like citrate and oxalate should replace synthetic chelating agents like DTPA and EDTA to minimize the risk of leaching of metals.

11.5.3 Using Dual Pipe System

Collection of metal enriched drainage water by a dual pipe sub-irrigation system which can be recycled for further phytoremediation as an effective way to reduce leaching of metals has been suggested by Madrid et al. (2003). However its feasibility in field experiments with larger areas seems doubtful.

11.5.4 Dose Management

The increasing concentration of EDTA does not have a significant effect on the amount of metal chelates (Manouchehri et al. 2006). It has been mostly observed that increasing the dose of chelating agent increases the mobility of metal (Bareen and Tahira 2010; Zhao et al. 2011) so a proper dose which is enough to mobilize the metal should be selected to reduce the risk of leaching. Zhao et al. (2011) have suggested a dose of 5 mmol kg^{-1} of soil to minimize leaching, whereas Bareen and Tahira (2011) have suggested a dose of only 1 mmol kg^{-1} of soil to minimize leaching hazard, provided effective and high biomass producing indigenous plants like *Suaeda fruticosa* are used for phytoextraction.

11.5.4.1 Splitting Up Doses of the Chelating Agents

Splitting up the application of chelant into separate doses has been suggested by Kayser et al. (2000) and Shen et al. (2002). However, Grčman et al. (2001) have given a negative feedback because splitting the dose reduced the effectivity of metal uptake by the chelating agent.

11.5.5 Using Permeable Barriers for Metals

Permeable barriers with reactive metals, nutrient sawdust, soyameal enriched vermiculite, peat or agricultural hydrogel and apatite have been used and successfully tested by Kos and Leštan (2004) in reducing the leaching of metals. Kos and Leštan (2004) compared the application of citrate, DTPA, EDDS and EDTA on Cu uptake by *Brassica rapa* and at the same time, effectively controlled leaching by permeable barriers made up of nutrient enriched sawdust and apatite. Similarly, Zhao et al. (2011) have shown that permeable barriers made of soya meal and apatite effectively reduced leaching of Cu, Zn and Pb from compost but did not fully prevent their leaching. In addition a competitive plant like turfgrass (*Lolium perenne*) can further absorb the metals at a fast rate and minimize leaching. The choked permeable barrier could be excavated and deposited after the soil remediation process has been completed.

11.6 Current Research Directions and Future Perspectives

In order to determine the actual efficiency of chelate assisted phytoremediation, the assessment needs to be made in the field. Field studies are necessary to observe the uptake efficiency of plants under natural conditions (Evangelou et al. 2007). Field studies with chelates have been carried out by Kayser et al. (2000), Grčman et al. (2001), Liphadzi et al. (2003), Anderson et al. (2005), Clemente et al. (2005), Xu et al. (2007), Nengschwandtner et al. (2008), Zaier et al. (2010b) and Bareen and Tahira (2011).

Observations and experiences of different researchers widely differ from each other. Some like Kayser et al. (2000) observed better phytoextraction of metals like Cd, Cu and Zn under greenhouse conditions as compared to field using seven different plants including *Brassica juncea*. Clemente et al. (2005) also observed a better uptake of Cu, Pb and Zn in greenhouse trials with *B. juncea*. Later on Neugschwantner et al. (2008) also observed better uptake of Cd and Pb in *Zea mays* under greenhouse conditions.

On the other hand Liphadzi et al. (2003) observed better phytoextraction by sunflower of metals like Cd, Pb and Ni in the field. Better phytoextraction of gold in the field by *Z. mays* and *B. juncea* and better phytoextraction of Pb by three plants in the field has been observed by Anderson et al. (2005) and Zhuang et al. (2005) respectively. Practical use of EDTA for enhancing phytoextraction requires careful site specific evaluation to minimize environmental hazard (Seth et al. 2008). Komárek et al. (2010) observed that chelant-assisted phytoextraction has reached a dead end. EDDS enhanced mobility of Cu but overall efficiency of process was slow. Flushing of Cu-contaminated soils with EDDS is suitable for removing this metal. Bareen and Tahira (2011) have observed a greater uptake of Cr and Na by a native halophyte *Suaeda fruticosa* in the field on account of greater biomass production by using only 1 mmol kg^{-1} dose of EDTA.

Evangelou et al. (2007) suggested using a combination of natural chelators and a plant with a high biomass and adequate metal tolerance to increase phytoextraction efficiency. As the process of chelant-enhanced phytoextraction is slow, it should be applied to low-contaminated soils, in order to reduce metal concentrations in a reasonable time frame. This highlights the fact that research focused on chelant-enhanced phytoextraction of metals from contaminated soils has probably reached a dead-end (Komárek et al. 2010). Recent reviews by Evangelou et al. (2007), Meers et al. (2008) and Saifullah et al. (2009) have suggested that massive solubilization of heavy metals by field applications are not feasible or sustainable approaches for phytoextraction due to the environmental persistence and leaching hazards of synthetic chelators. According to Evangelou et al. (2007) chelant-assisted phytoextraction should be abandoned in favor of alternative options. From the 12 years of research on specifically more effective EDTA and similar biodegradable compounds like EDDS and NTA, it has to be decided if this field has any potential for valuable research. In most studies, including the most recent studies, EDTA has still been suggested as the most effective chelating agent both in greenhouse studies and field studies. Thus its role in enhancing metal uptake in phytoextraction studies cannot be denied.

In view of the fact that oilseed *Brassicas* constitute an important high biomass group of plants with good phytoextraction potential, this resource should be put to use to decontaminate metal polluted soil. Keeping in view the research undertaken in this field, it can be concluded that the right combination of plant and chelating agent, as well as the dose of the chelant and time of application should be carefully selected to get the maximum output. Using lower doses in the field conditions would not only minimize the phytotoxic effect but would also control unnecessary mobilization and leaching of metals to groundwater. EDTA could be preferred in the field with low doses and single applications with appropriate species of *Brassica*.

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Chapter 12
Roles of Rhizospheric Processes and Plant Physiology in Applied Phytoremediation of Contaminated Soils Using *Brassica* Oilseeds

Sarah Neilson and Nishanta Rajakaruna

Abstract The current chapter reviews in detail significant physiological mechanisms of metal accumulating *Brassica* species and discusses rhizospheric processes and soil management, including the role of soil amendments such as chelators in enhancing the uptake of toxic metals, focusing on their roles in phytoremediation of contaminated sites worldwide, in addition to presenting an overview of the field of phytoremediation, including its merits and shortcomings. Recent progress towards the use of oilseed *Brassica* species in field-based studies is also discussed.

Keywords *Brassica* oilseeds • Contaminated soils • Phytoremediation • Rhizospheric processes

12.1 Introduction

Heavy metal contamination of soil and water is a major threat to human and ecosystem health, making the cleanup of metal-contaminated sites a high priority (Ensley 2000). The persistent nature of heavy metal contaminants in the environment has meant that the most commonly used methods of cleanup of contaminated soil are excavation and removal, incineration, and chemical treatment (Pilon-Smits 2005). These methods are not only damaging to the environment, but are also expensive, making it largely impossible for developing nations to adopt such methods (Rajakaruna et al. 2006). The United States spends \$6–8 billion per year on environmental cleanup, and globally the cost is \$25–50 billion a year (Tsao 2003; Pilon-Smits 2005).

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Recently, much research has been conducted on the use of hyperaccumulator plants in the cleanup of metal-contaminated sites (Cunningham and Ow 1996; Pilon-Smits and Freeman 2006). Hyperaccumulators take up high concentrations of heavy metals from the soil and translocate them into above-ground biomass at concentrations exceeding, in most cases, 0.1% total dry leaf tissue mass (>1,000 μg metal g^{-1} dry leaf tissue) (Baker et al. 2000). The threshold values set for hyperaccumulation are element-dependent: Mn and Zn hyperaccumulators accumulate >10,000 μg g^{-1} dry mass, hyperaccumulators of As, Co, Cu, Ni, Se and Pb accumulate >1,000 μg g^{-1}, and hyperaccumulators of Cd accumulate >100 μg g^{-1} (Reeves and Baker 2000). Hyperaccumulators of Al (Jansen et al. 2002), B (Babaoglu et al. 2004) and Fe (Rodríguez et al. 2005) have also been identified. The “elemental defense” hypothesis (Boyd 2004, 2009) speculates that hyperaccumulating species utilize the metals for defense against natural enemies, such as herbivores and pathogens (Fones et al. 2010; Quinn et al. 2010; Rascio and Navari-Izzo 2011; Strauss and Boyd 2011). Hyperaccumulators are often restricted to metal-enriched soils such as those found on serpentinite outcrops and other metalliferous rocks (Reeves 2002) and are worthy of careful study and conservation (Whiting et al. 2004; Rajakaruna and Boyd 2008; Harrison and Rajakaruna 2011).

While some plants can tolerate metalliferous soils by excluding metals (*i.e.* metal excluders) through binding them to exuded organic acids or sequestering them within the roots, hyperaccumulators are distinguished from such excluders by their high rate of metal uptake and translocation and their ability to accumulate and detoxify these extraordinarily high levels of metals in their shoots, especially leaves (Rascio and Navari-Izzo 2011). Recent research shows that the process of hyperaccumulation results from the overexpression of genes which encode trans-membrane transporters (Rascio and Navari-Izzo 2011). In this chapter, we will explore the processes of hyperaccumulation from the perspectives of internal plant physiological processes and soil-plant relations, and their relevance to developing sound metal cleanup technologies.

Although the currently documented >450 metal hyperaccumulators occur in over 34 different families, 25% of hyperaccumulating species are in the family Brassicaceae (Rascio and Navari-Izzo 2011), the best known of which are in the genera *Alyssum* and *Noccaea* (formerly, *Thlaspi*) (Verbruggen et al. 2009). *Arabidopsis thaliana*, also from Brassicaceae, has been used extensively as a model organism for plant-based research (Bevan and Walsh 2005), including metal tolerance and accumulation (Cho et al. 2003). Given *A. thaliana*’s genome is mapped and there is a high level of sequence homology between it and its metal accumulating relatives, including *A. halleri* (Becher et al. 2004; Weber et al. 2004), *A. thaliana* has provided excellent means to explore the genetic basis for both metal tolerance and accumulation in related taxa, making Brassicaceae a model family for the study of metal tolerance and hyperaccumulation.

The use of hyperaccumulators to clean up toxic sites, *i.e.* phytoremediation, is fast gaining global recognition as a viable green technology to clean up metal contaminated sites (Pilon-Smits and Freeman 2006). Phytoremediation is based on the premise that plants which remove heavy metals from the soil and translocate them to their above-ground biomass can then be harvested and disposed of through incineration or metal recovery, known as phytomining (Brooks et al. 1998). Phytoremediation is a much more environmentally friendly and, importantly, a less expensive alternative to traditional excavation-based methods of cleanup. However, there are several limitations to wide-scale implementation of this technology (Angle et al. 2001; Pilon-Smits 2005), including the length of time the cleanup might take and the fact that soil environments are complicated and can be volatile, and therefore the extent of uptake and cleanup harder to predict. Hence, more research, especially field experiments, needs to be done in order to advance this new technology toward widespread application.

12.2 Internal Plant Physiological Mechanisms

To avoid metal toxicity, plants employ internal physiological mechanisms of metal detoxification. Much research has been done in this area (see Kramer 2010), but much remains to be done. Generally, detoxification involves chelation of the metal cation by ligands or organic acids, or vacuolar or cell wall sequestration away from metabolic sites in the cytoplasm, usually within localized areas in the shoot (Baker et al. 2000; Lasat and Kochian 2000; Salt et al. 2000; Broadhurst et al. 2004; Chaney et al. 2007; Memon and Schroder 2009). The breadth and scope of these mechanisms are detailed and complex.

12.2.1 Chelation, Ion Uptake and Metal Loading

Both apoplastic and symplastic pathways are involved in the transport of inorganic ions. The three main steps in inorganic ion transport in the symplastic pathways are active transport of metals across root membranes; entry of metals into symplast during translocation from root to shoot; and chelation and sequestration of metals into specific compartments in the leaves (Maestri et al. 2010). Chelation of metals within the plant allows for xylem loading and transport, as well as for sequestration. These mechanisms involve many metal-specific chelators—*i.e.* ligands and organic acids—many of which have only begun to be studied or have not yet been characterized. Research on chelators and transporters mostly focuses on their genetic basis in order to determine how they work and how genetic engineering might help in producing more commercially desirable hyperaccumulators for phytoremediation (Pilon-Smits and LeDuc 2009). The purposes of the proceeding sections are not to delve into the genetic basis of metal tolerance and hyperaccumulation (see Pollard et al. 2002; Yang et al. 2005a, b for detailed reviews), but discuss general trends with respect to internal ion transport and detoxification of heavy metals in hyperaccumulators.

The role of chelators in hyperaccumulation is to form complexes with heavy metal ions. This can serve the function of aiding in transport, or it can be the terminus of the ion, leading to sequestration in the shoot of the plant. Metallothioneins and phytochelatins are two classes of chelators involved in metal accumulation (Cobbett and Goldsbrough 2002). These are sulfur-donor ligands which are better electron donors than oxygen and therefore form high-stability complexes with first-row transition metals (Baker et al. 2000). The role of genes in transporting metals across electrochemical gradients and in translocation in general is clearly important. The overexpression of certain genes in hyperaccumulators leads to greater amounts of chelators and transporters within the plant (Verbruggen et al. 2009). Enhanced root uptake of Zn, for example, seems to be driven by overexpression of members of the ZIP family—Zn- and Fe-regulated transporter proteins (Verbruggen et al. 2009; Rascio and Navari-Izzo 2011). In Se hyperaccumulators like *Stanleya pinnata* (Brassicaceae; Zhu et al. 2009), uptake seems to be correlated with the presence of a high-affinity $SO4^{2-}$ transporter in the plant, which is more expressed in *S. pinnata* than in non-hyperaccumulating congeners (Rascio and Navari-Izzo 2011). Further, in non-hyperaccumulators, many members of the ZIP family are expressed only under conditions of Zn deficiency, whereas they seem to be expressed independent of Zn supply in hyperaccumulators (Verbruggen et al. 2009). Further, reduced vacuolar sequestration of Zn in the root cells of *Thlaspi caerulescens* (now *Noccaea caerulescens,* see Koch and Mummenhoff 2001 and Koch and Al-Shehbaz 2004 for the taxonomic revisions; however, we maintain previous nomenclature in the chapter) and enhanced activity of the gene HMA4 (P-type metal ATPase) facilitated active xylem loading (Verbruggen et al. 2009).

Among the hyperaccumulation-related chelators resulting from overexpressed genes are metallothioneins (Clemens 2001), which are cysteine-rich, low-molecular-weight, metal-binding proteins that can bond with different metals; however, they are primarily implicated in metal homeostasis in mammals (Maestri et al. 2010) and their role in plant metal uptake and sequestration is still largely unclear. There are several families of metal transporters that have been implicated in metal uptake and translocation: Plasma membrane and tonoplast transporters involved in uptake, tonoplast transporters for remobilization from vacuoles, transporters for xylem loading, and endomembrane transporters (Maestri et al. 2010). Different organic acids and ligands have been found to be associated with various metals in distinct parts of different plants; for example, in *T. caerulescens*, most Zn in roots was associated with histidine, while in shoots it was associated with organic acids (Verbruggen et al. 2009). Also in *T. caerulescens*, Cd in the leaves was found to be bound with sulfur ligands (Verbruggen et al. 2009). In *Arabidopsis halleri*, Zn was mostly stored in the vacuoles of mesophyll, while in *T. caerulescens* it was in the vacuoles of the epidermal cells (Verbruggen et al. 2009). In both cases, vacuolar sequestration of Zn in the leaves was thought to be the main mechanism of detoxification and this is a distinguishing trait between hyperaccumulators and non-hyperaccumulators. In general, the inside of the vacuole is an environment conducive to formation of metal-organic acid complexes because it is acidic; in fact it was observed that within the vacuoles of *T. caerulescens* and *A. halleri*, a large pool of malate led to the formation of Zn-malate complexes, in mesophyll vacuoles of *A. halleri* and in the epidermal cell vacuoles in *T. caerulescens* (Verbruggen et al. 2009).

Several studies have pointed to the amino acid histidine as playing an important role in hyperaccumulation (Baker et al. 2000; Verbruggen et al. 2009; Richau et al. 2009; Kramer 2010). Baker et al. (2000) found that the Ni as a histidine complex has a higher stability than Ni complexed with any other amino or organic acid. Histidine forms stable complexes with Ni, Zn, and Cd (Verbruggen et al. 2009). Dose-dependent increases in histidine occurred in the xylem sap of hyperaccumulator *Allysum lesbiacum* but not in the congeneric, non-hyperaccumulator *A. montanum* (Verbruggen et al. 2009). Histidine also plays a key role as a chelator in the tolerance as well as the high rate of translocation of Ni in *A. lesbiacum* (Kramer 2010). However, Chaney et al. (2007) reported that *Thlaspi* taxa with histidine-producing genes did not increase Ni accumulation, and that increased histidine did not make normally non-accumulating plants hyperaccumulate Ni. Therefore, the role and importance of histidine in hyperaccumulation may vary depending on other factors than simply the presence of histidine alone. Another organic acid which is associated with Ni in aqueous extracts of leaf material is malate (Baker et al. 2000). Malate has also been implicated in Zn tolerance, binding to Zn upon uptake, carrying ions to the vacuole where they are complexed by terminal acceptors, perhaps S-containing mustard oils in *T. caerulescens*, after which the malate is released to transport more Zn (Baker et al. 2000). Many Ni and Zn hyperaccumulators have high basal levels of malate and citrate, which may increase with increasing levels of Ni and Zn in the shoot, implying their role in detoxification (Chaney et al. 2007). *Thlaspi caerulescens* has also been shown to possess an association between malate and Cd (Chaney et al. 2007).

12.2.2 Translocation

Salt et al. (2000) reported most cellular Ni in the hyperaccumulator *Thlaspi goesingense* is associated with organic acids. Most organic acids are vacuolar; therefore this supports the hypothesis of vacuolar localization of Ni in hyperaccumulators. Storage of Zn in leaf vacuoles has also been documented (Lasat and Kochian 2000); leaf cells in *T. caerulescens* were bathed in xylem solution with high levels of Zn, suggesting that reabsorption of Zn from xylem into leaf cell walls is an important component of hyperaccumulation in this species As pointed out earlier, the major difference between hyperaccumulators and metal excluders seems to be that the hyperaccumulators translocate a significantly greater percentage of accumulated heavy metal ions to their shoots for sequestration in the leaves, whereas metal excluders, while still being able to contain large amounts of heavy metals, do so in the roots (Lasat and Kochian 2000; Broadhurst et al. 2004; Memon and Schroder 2009; Richau et al. 2009; Salt et al. 2000; Verbruggen et al. 2009). Salt et al. (2000) report that in hydroponic solution, root Ni concentration in non-hyperaccumulator *T. arvense* was higher than in hyperaccumulator *T. goesingense*, while the reverse was true of shoot Ni concentration. *Thlaspi arvense* also displayed lower shoot biomass than *T. goesingense* upon Ni exposure. However, both plants translocated Ni to the shoot at the same rate when exposed to nontoxic Ni levels, highlighting the different mechanisms by which hyperaccumulators and non-hyperaccumulators cope with high levels of heavy metals (Salt et al. 2000).

12.2.3 Root Anatomy and Physiology

The first part of a plant to come into contact with the soil is the root. The question as to whether the root of a hyperaccumulator is structurally different than that of a non-accumulator may account for some of the differences in uptake of heavy metals. Mench et al. (2009) found that *Thlaspi caerulescens* develops a periendodermal layer, a zone in the roots with thickened inner tangential cell walls which form a continuous layer, externally attached to the endodermis. They are located near the root tip, which may indicate a role in ion transport (Mench et al. 2009). Ion transport in the root was elaborated upon by Lasat and Kochian (2000), reporting that in hyperaccumulator *T. caerulscens* and excluder *T. arvense*, transport of Zn across root cell membranes is mediated by proteins with similar Zn affinities. However there might be a higher expression of Zn transporters in *T. caerulescens*, which could account for the deployment of more transporters to the root cell membrane in that species than in *T. arvense*. Richau et al. (2009) also compared Ni translocation patterns of *T. caerulescens* and *T. arvense*. They found that the high rate of Ni translocation in *T. caerulescens* compared to *T. arvense* seems to be dependent on two factors: greatly enhanced concentration of root histidine in *T. caerulescens* and a strongly decreased ability to accumulate histidine-bound Ni in root cell vacuoles in *T. caerulescens*. The concentration of free histidine in *T. caerulescens* was ten-fold higher than that in *T. arvense*, but only slightly higher in leaves, regardless of Ni exposure. Xylem loading of Ni was enhanced by exogenous histidine in *T. caerulscens* but not in *T. arvense*. These results suggest that root-to-shoot transport is constitutive at the species level for *T. caerulescens*, rather than simply enhanced Ni uptake (Richau et al. 2009). Shoot-derived tonoplast vesicles in *T. caerulescens* accumulate more Ni than root-derived ones, while the opposite is true in *T. arvense*. It is implied that chelation of Ni by histidine in the cytoplasm inhibits root vacuolar sequestration in *T. caerulescens* but not in *T. arvense*. Lower vacuolar sequestration of Zn in hyperaccumulator roots is also implicated in higher Zn accumulation in leaves. In addition, it was reported that in three hyperaccumulating *Alyssum* species, dose-dependent Ni exposure induced increases in histidine in xylem sap not seen in non-hyperaccumulators of Brassicaceae (Richau et al. 2009). However, for some non-hyperaccumulators (*A. montanum* and *Brassica juncea*) exogenously supplied histidine increased Ni tolerance and Ni xylem loading (Richau et al. 2009). The same was not true for *A. lesbiacum*, likely because of its five-fold higher (compared to *B. juncea*) root histidine concentration (Richau et al. 2009). Further, the formation of Ni-histidine complex inhibited the retention of Ni in root cell vacuoles of *T. caerulescens*

(Verbruggen et al. 2009). These findings suggest there are generally higher levels of free histidine in the roots of hyperaccumulators compared with those of non-hyperaccumulators and that greater levels of translocation occur in the hyperaccumulators as a result.

12.2.4 Metals Localization and Sequestration

Salt et al. (2000) evaluated Ni concentrations in hyperaccumulator *T. goesingense* compared with the non-accumulating *T. arvense*. Protoplasts were isolated from both species, and it was found that the ones from *T. goesingense* were more Ni-tolerant than those of *T. arvense*, suggesting a cellular mechanism of Ni tolerance in the leaves of the hyperaccumulator. It appears that Ni was localized within the vacuole of *T. goesingense,* as has also been shown for Cd and Zn (Salt et al. 2000).

Broadhurst et al. (2004) found that the majority of hyperaccumulated Ni in five *Alyssum* hyperaccumulator species grown in Ni-enriched soils is stored in either the leaf epidermal cell vacuoles or in the basal portions of the numerous stellate trichomes. In fact, the metal concentration in these trichome basal compartments was ~15–20% of dry weight, which is one of the highest concentrations to be reported in living plant tissue (Broadhurst et al. 2004). The authors also found that there was simultaneous and region-specific localization of high levels of Ni, Ca, and Mn within the trichomes, although the soils were only Ni-enriched and not Ca- or Mn-enriched. The region-specific localization of hyperaccumulated Ni, Ca, and Mn was found in all five *Alyssum* taxa, over a range of Ni concentrations (Broadhurst et al. 2004).

In another study, Ghasemi et al. (2009) measured Ni accumulation in the trichomes of the serpentine-endemic Ni-hyperaccumulator *Alyssum inflatum*. Using elemental analysis of the plants in their native habitat in Iran, they did not find that the trichomes accumulated any more Ni than the rest of the shoot. However, after treating plants with different concentrations of Ni in the growth medium and staining them with dimethylglyoxime (a stain specific for Ni), it was found that staining of the trichomes increased as the external dose of Ni increased. At lower Ni concentrations, the metal accumulated at the base of the trichomes; at higher Ni levels, accumulation extended to the rays and cell walls. Therefore, high levels of Ni can be accumulated in the trichomes of *A. inflatum*, and Ni accumulation as a whole can reflect Ni concentration in the shoot.

Galeas et al. (2006) looked at seasonal differences in the uptake of Se and S (chemically similar elements) in two hyperaccumulators, including *Stanleya pinnata*, over two growing seasons. The authors found that there were several stages of accumulation which depended on the time of year. In both hyperaccumulators tested, it was found that Se traveled from root to young leaves in the spring, then moved from aging leaves to flowers and seeds in the summer, and finally back to roots in autumn. These findings on seasonal translocation and allocation of metals could have significant impacts on the application of phytoremediation methods.

In the preceding section we touched upon the depth and breadth of internal plant physiology of hyperaccumulators which is crucial for developing better practices of phytoremediation. The importance of the rhizosphere in uptake of metals in hyperaccumulators and its potential roles in the advancement of phytoremediation technologies are discussed in the following section.

12.3 Soil Environment-Confined Processes

12.3.1 Chelation in the Rhizosphere

The contrasts in root vs. shoot chelation and sequestration of heavy metals between hyperaccumulators and excluders presented in the previous section are not exclusive to internal plant physiology. Indeed, roots live in the soil, and therefore interact with soil and its myriad of components, including biota. In the previous section, we reported on studies which found that metal-tolerant excluders or non-hyperaccumulators tend to sequester heavy metals in their roots, while hyperaccumulators express much higher levels of translocation rates of these metals which are subsequently stored in the leaves. The same players in the processes of sequestration and transport—*i.e.* chelators such as histidine—also play a role in the initial uptake of metals from the soil environment. One hypothesis posits that roots of hyperaccumulators, which have a high 'requirement' for certain heavy metals, exude chelators into the rhizosphere in order to aid in uptake. Several studies have approached this hypothesis with varying results. Wenzel et al. (2003) conducted an experiment on the hyperaccumulator, *T. goesingense*, and two excluders, *Silene vulgaris* (Caryophyllaceae) and *Rumex acetosella* (Polygonaceae), growing on serpentine soil and found that organic ligand exudation may contribute to enhanced solubility and replenishment of metals in the rhizosphere of hyperaccumulators. They reported higher dissolved oxygen content (DOC) and Ni concentrations in water extracts of the rhizosphere of *T. goesingense* as well as decreased exchangeable Ni, which they attributed to excessive Ni uptake. They concluded that enhanced Ni solubility in the rhizosphere of *T. goesingense* was driven by the formation of Ni-organic acid complexes and ligand-induced dissolution of Ni-bearing minerals.

Salt et al. (2000), however, concluded that the speculation that Ni-specific chelators exuded by roots were responsible for greater uptake of Ni in *T. goesingense* was not supported because they were unable to identify any such high-affinity Ni-chelating compounds in the root exudate. In contrast, it was found that the root exudate of non-hyperaccumulator *T. arvense* actually contained higher levels of known Ni-chelators, histidine and citrate, than the root exudates of *T. goesingense* (at a Ni exposure rate of 25 mM over 48 h). Furthermore, the levels of these Ni chelators in the root exudate of *T. arvense* increased upon Ni exposure, while the same compounds in the *T. goesingense* exudates remained at steady levels. Therefore, it was concluded that root-exuded histidine does not play a significant role in enhanced foliar accumulation of Ni by *T. goesingense*. These findings, however, suggest that the increased release of histidine by the non-accumulator *T. arvense* roots may be part of a Ni-detoxification strategy by chelating Ni in the rhizosphere, thereby reducing the activity of the Ni in the soil solution and decreasing its toxicity (Salt et al. 2000). Nevertheless, it was found that at elevated Ni concentrations, this mechanism was quickly overwhelmed, leading to plant toxicity. Puschenreiter et al. (2003) also concluded that increased DOC in the rhizosphere of the excluder species *T. arvense* alleviated phytotoxicity by formation of metal-organic compounds.

12.3.2 Importance of pH

The existing soil chemical and physical features of a site are vital in plant-rhizosphere interactions, and pH is likely the most important factor characterizing the soil environment of a plant (Rajakaruna and Boyd 2008). It affects a myriad of processes, some well-understood and some yet undiscovered; however understanding how pH affects hyperaccumulation potential is a critical step in developing sound phytoremediation technologies. pH has been shown to affect the metal extraction capacity of *T. caerulescens* for both Cd and Zn (Rascio and Navari-Izzo 2011; Wang et al. 2006). Wang et al. (2006) studied *T. caerulescens* in two soils with varying levels of Cd and Zn at six pH levels, and concluded that lowering the pH in both soils not only increased the soluble forms of both metals, but that *T. caerulescens* had the highest shoot biomass at the lowest pH (4.74) and the highest shoot metal concentration at the second-lowest pH (5.27). Kukier et al. (2004) conducted an experiment on the effect of pH on uptake of Ni and Co by hyperaccumulators *Alyssum corsicum* and *A. murale*. The plants were tested using three types of soil. Two of the soils—Quarry muck (Terric Haplohemist) and Wellend (Typic Epiaquoll)—came from a site in Port Colborne, Ontario, Canada that had been contaminated by a Ni refinery. The third soil, Brockman (Typic Xerochrepts), was a serpentine soil from Oregon, USA. Soils were treated with limestone to adjust the pH from highly acidic to mildly alkaline. Both the Quarry muck and the Wellend soils had a relatively low initial pH (5.66 and 5.24, respectively), while the Brockman serpentine had a slightly higher initial pH (6.30). Both *Alyssum* species grown in the industrially contaminated soils showed increased Ni shoot concentrations with increased soil pH; the highest fraction of total soil Ni extracted was 6.3% from the Quarry muck, followed by Wellend (4.7%) and Brockman (0.84%) at pH 7.3, 7.7, and 6.4, respectively (Kukier et al. 2004). Interestingly, increased Ni uptake occurred despite a decrease in water-soluble Ni in the soil, which is contrary to what is generally seen with agricultural crops (Kukier et al. 2004). There was a slight decrease in shoot Ni concentration with increased pH in the serpentine soil. Cobalt concentrations increased with increased pH in the Quarry muck, yet decreased in the Wellend and Brockman soils. The differences in uptake of Ni and Co from the different soils with varying pH by the *Alyssum* taxa was thought to be related to differences in percent organic matter and Fe content of soils (Kukier et al. 2004). These conclusions, not surprisingly, point to other important aspects of the soil environment, not directly related to pH, which can also affect plant uptake of heavy metals. However, the study shows a general trend toward what might be expected, which is that increased pH generally leads to increased uptake of heavy metals by hyperaccumulators. In fact, Maestri et al. (2010) suggest that root exudates such as organic acids and phytosiderophores may play a role in metal mobilization and uptake because of their ability to acidify, not necessarily to chelate metals. However, root exudates from *T. caerulescens* did not increase Cd and Zn mobility compared with those of non-accumulators (Maestri et al. 2010). It is clear that the chemical environment of the rhizosphere plays an important role in metal bioavailability and mobility in the soil. For example, Cd and Zn uptake by *T. caerulescens* has been shown to be affected by the chemical form of N in the soil (Maestri et al. 2010). However, there are other aspects to the soil environment than just inorganic and organic chemicals; namely dominant biota, including rhizobacteria and mycorrhizae.

12.3.3 Rhizobacteria and Mycorrhizae

The importance and potential role of rhizobacteria and mycorrhizae in the uptake of heavy metals from the soil is a vast and largely unexplored area of research, but it could greatly aid in the development of phytoremediation technologies. Rhizobacteria and mycorrhyzae, through metabolic activities, affect the soil environment, including bioavailability and mobility of elements (Mench et al. 2009). Abou-Shanab et al. (2003) found that rhizobacteria play an important role in increasing availability of Ni in soil, thereby enhancing Ni accumulation by *A. murale*. They isolated three bacteria species (*Sphingomonas macrogoltabidus, Microbacterium liquefaciens*, and *M. arabinogalactanolyticum*) from the rhizosphere of *A. murale* and added them to sterile and non-sterile Ni-rich serpentine soils, to examine their ability to solubilize Ni in soil and their effect on Ni uptake by *Alyssum*. The authors observed that *S. macrogoltabidus* significantly reduced Ni extraction from soil; *M. arabinogalactanolyticum* significantly increased Ni extraction from soil; and *M. liquefaciens* had no effect. They also found that inoculation with these bacteria did not significantly impact extractability of other metals. However, it was reported that Ni uptake into the shoot of *A. murale* increased by 17%, 24%, and 32.4% for *S. macrogoltabidus, M. liquefaciens,* and *M. arabinogalactanolyticum*, respectively. Whiting et al. (2001) conducted a study on rhizospheric bacteria and their role in Zn uptake and concluded that microbes play an important role in Zn uptake of *T. caerulescens*. Addition of randomly selected strains of bacteria (*M. saperdae, Pseudomona monteilly,* and *Enterbacter* *cancerogenes*) to surface-sterilized seeds of *T. caerulescens* increased Zn shoot concentration two-fold compared to the sterile control and total Zn accumulation increased four-fold along with shoot biomass, although not with root biomass (Whiting et al. 2001). Interestingly, the same experiment conducted with non-hyperaccumulator *T. arvense* had no effect. The authors concluded that bacteria increased availability of water-soluble Zn; liquid media that supported bacterial growth mobilized 1.2- to 1.8-fold more Zn from soil. Therefore, the overall conclusion of the study was that bacteria facilitate solubilization of nonlabile forms of Zn (*i.e.* increase availability of water-soluble Zn) in the rhizosphere of hyperaccumulator *T. caerulescens*.

Of course, when heavy metals are present at toxic levels, all soil biota are affected, which means that the plant-soil relationship will also be determined by the level and type of contamination. Zarei et al. (2010) conducted a study on the impact of metal contamination on arbuscular mycorrhizal fungi (AMF). These are common soil organisms that have been repeatedly found in heavy metal contaminated soils (Del Val et al. 1999; Khan 2005; Giasson et al. 2006). AMF have widespread symbiotic relationships with plants, but the specific nature of these relationships varies with specific AMF isolates, host plants, and soil properties (Zarei et al. 2010). AMF were examined at an open pit mine in Iran, in soils ranging from non-polluted to Pb- and Zn-polluted, and involving several dominant plant species. With increasing Pb and Zn concentrations, the number of AMF sequence types decreased, suggesting a negative correlation between heavy metal contamination and AMF diversity and abundance; however, one sequence type was found only in the highly contaminated area, indicating a locally adapted strain of AMF (Zarei et al. 2010). The authors also found that $CaCO_3$ and available P were important in AMF distribution, highlighting the need to take the edaphic environment into careful consideration when planning phytoremediation trails.

12.4 Implications for Phytoremediation and Next Steps

The widespread application of phytoremediation will have many challenges beyond those encountered in the laboratory (Angle and Linacre 2005). There are myriad of site-specific factors which will influence the success of any phytoremediation effort. Practical application of phytoremediation technologies will necessarily be informed by laboratory experiments.

The success of any phytoremediation effort is determined by several key factors. Many hyperaccumulators are metal-specific, can only be used in their native habitats, have slow growth rates and shallow root systems as well as low biomass, and not every metal of interest has a corresponding hyperaccumulator (Schat et al. 2000; Pilon-Smits 2005; Rascio and Navari-Izzo 2011). There is also almost no research to date on agronomics and disease potential, and only limited testing of genetics and breeding potential (Rascio and Navari-Izzo 2011). Additionally, the key roles of the rhizosphere in metal tolerance and hyperaccumulation should be further investigated (see Alford et al. 2010).

According to Chaney et al. (2007), development of phytoremediation technologies rely on two major criteria: domestication of hyperaccumulators and cloning of all relevant genes. Domestication of hyperaccumulator species has already been shown effective for hyperaccumulators of Ni, Cd, Se, and As (Chaney et al. 2007) and there are many ongoing efforts exploring the genetic basis and cloning of relevant genes (Yang et al. 2005a, b; Pilon-Smits and LeDuc 2009; Wright and von Wettberg 2009).

Domestication of plants implies cultivation. When plants are cultivated in the field, it is necessary to think about length of season, climate, level of contamination, method of seed-sowing (spacing, etc.), and plant-soil interactions not only at the level of metal-availability, but also in terms of fertility, so that optimum above-ground biomass can be achieved. For example, Baker et al. (2000) found that *Thlaspi* and *Alyssum* species were responsive to soil fertilization and that growth potential can be enhanced by fertilization. This, however, raises the question of the types of fertilizer being used—a commercial chemical fertilizer, compost, seaweed, or some other organic treatment. All provide nutrients to the plant, but chemical fertilizers do not necessarily enhance the quality of the soil. One experiment to assess the feasibility of Ni phytomining tested *A. bertolonii* in the field with applications of N-P-K fertilizer over 2 years, resulting in increasing biomass three-fold- or about 13,500 kg ha^{-1} without diluting the shoot Ni concentration of the plants (Rascio and Navari-Izzo 2011). A similar field experiment with the South African Ni-hyperaccumulator *Berheya coddii* (Asteraceae) reported a fertilized yield of 22,000 kg ha^{-1} with an estimated amount of 100 kg Ni ha^{-1} being achievable at most sites worldwide (Rascio and Navari-Izzo 2011). However, further research is needed to determine if and when fertilizer should be used, the degree to which it increases growth potential, and what after-effects it might have of the remediated site, positive or negative. It is also important to keep pH in mind, and optimum pH for phytoextraction may need to be determined for individual soils as well as plant species to be utilized (Chaney et al. 2007).

Wang et al. (2006) found that *T. caerulescens*, when soil pH was optimized, accumulated 40% of total Cd in highly contaminated soil and 36% in low-contamination soils, with just one planting. This suggests that finding optimum pH that is site- and plant-specific could prove very useful in maximizing phytoremediation potential. Further, *T. caerulescens* hyperaccumulates Cd and Zn, and yields a maximum of 2 tons ha^{-1} of shoot dry matter (Rascio and Navari-Izzo 2011). However, pot and field studies have shown that *T. caerulescens* can, with selective breeding, achieve the equivalent of a 5 ton ha^{-1} growth rate with a desirable combination of yield and shoot metal concentration (Rascio and Navari-Izzo 2011).

Another important factor to consider in applied phytoremediation is biomass potential. The greater the above-ground biomass, the greater the concentration of metal to be removed. Salt et al. (2000) found that the non-accumulator *T. arvense* displayed lower shoot biomass than its hyperaccumulating congener, *T. goesingense*, after Ni exposure. However, both plants translocated Ni to the shoot at the same rate when exposed to nontoxic Ni levels (Salt et al. 2000). Wenzel et al. (2003) showed that mean shoot biomass of *T. goesingense* exceeded excluders' biomass (*S. vulgaris* and *R. acetosella*), and furthermore that root biomass decreased in the order of *S. vulgaris* > *T. goesingense* > *R. acetosella*. This gives credence to the previously discussed phenomenon of excluders having greater root biomass than hyperaccumulators.

In the context with the problem of contamination by multiple metals, several *Thlaspi* species can accumulate more than one metal (Pongrac et al. 2009), notably *T. caerulescens*, which in addition to Zn and Cd can also accumulate Ni and Pb, and could remove up to 60 kg Zn ha^{-1} and 8.4 kg Cd ha^{-1} (Rascio and Navari-Izzo 2011). However, a major sticking point remains the time frame, which can be decades. It is estimated that nine croppings of *T. caerulescens* would be required to remediate soil from 440 to 300 mg Zn kg^{-1}, and that to use this plant on soils containing 2,100 mg Zn kg^{-1} would take 28 years (Rascio and Navari-Izzo 2011). Therefore, the level of contamination always needs to be taken into account. Further, levels of hyperaccumulation vary among species and among populations and ecotypes of the same species. For example, among ecotypes of *T. caerulescens*, a southern French ecotype accumulates more Cd than Zn (Rascio and Navari-Izzo 2011) most effectively at an acidic pH and, importantly, in useful amounts (Chaney et al. 2007). Thus, extensive field explorations are required to locate and characterize population-level differences in the metal-accumulating potential of known metal accumulating species as well discover new accumulators, especially from the Brassicaceae, a family with a predisposition for metal tolerance and accumulation.

Chaney et al. (2008) also point out that serpentine soils, because of their origins from peridotite and related parental rocks, tend to have low Ca/Mg quotients. Soil Ca has been shown to reduce Ni uptake (and phytotoxicity) in crop plants, so plants native to serpentine soils may have limited tolerance for soils used for commercial phytomining. The authors compared two serpentine endemic species, *A. murale* and *A. pintodasilvae*, with cabbage (*Brassica oleracea*) in nutrient solutions that mimicked serpentine as well as normal soils, with varying levels of Ca and Mg. The *Alyssum* taxa showed much greater tolerance to high Ni and Mg and low Ca than cabbage. A non-linear correlation was found between Ni translocation and shoot concentration, shoot yield, and shoot Ca levels; all increased with increasing Ca up to 2 mM, then decreased at the highest Ca levels. The results suggest that shoot biomass and yield potential when phytomining with *Alyssum* taxa in high-Mg soils would benefit from Ca application (Chaney et al. 2008).

Many laboratory experiments rely heavily on the application of synthetic chelators, such as EDTA, to make metals more available to plants. However, as Chaney et al. (2007) argue, this approach is impractical, and indeed counterproductive, in the field. EDTA and similar synthetic chelators contaminate the soil themselves, thus negating the intended purpose of soil cleanup. EDTA increases metal uptake in plants by injuring the roots of plants, thus decreasing the barriers to uptake. Further, only a fraction of the metal "freed up" by EDTA is taken up into the plant shoot, leaving the rest to be leached out of the topsoil. Add to the mix that

EDTA is also expensive, especially when used in field trials, and you have a strong case against the practical use of synthetic chelators (Chaney et al. 2007). According to Chaney et al. (2007), too much research has focused on trying to develop Pb extraction technologies with *B. juncea* (Podar et al. 2004; Lai et al. 2008; Hanen et al. 2010; Zarei et al. 2010), whereas in reality, even if there was enough phosphate present in contaminated soils to give good crop yields, most Pb remains in the soil or the roots and, without soil amendments, *B. juncea* has little ability to absorb Pb from contaminated soils (Chaney et al. 2007).

There are, however, promising examples of phytoremediation and phytomining in the field. Chaney et al. (2007) point out that *Alyssum* biomass is one of the richest known Ni bio-ores, and that *Alyssum* species can be effectively developed into commercial phytomining practices. Broadhurst et al. (2004) report that *A. murale* and *A. corsicum*, two species that are endemic to serpentine soils in Mediterranean Europe, have been employed in commercial phytomining of Ni because, unlike many other serpentine endemic species, they can hyperaccumulate Ni from other types of soil, including limestone, organic, and loam. *Alyssum bertolonii* and *Berkheya coddii* are fast-growing Ni hyperaccumulators that have been shown in small-scale field experiments to be suitable for phytoremediation, especially as they have high-biomass, high-Ni concentration, and are easy-to-propagate; further, *B. coddii* is cold-tolerant making it useable even in temperate regions (Rascio and Navari-Izzo 2011). Experiments with gene transfer have also shown that transgenic *B. juncea* takes up more Se and has greater Se tolerance than the wild species when grown under both soil and hydroponic conditions (Rascio and Navari-Izzo 2011). Somatic hybrids have been produced between *T. caerulescens* and *Brassica napus* which accumulated levels of Zn that would have been toxic to *B. napus* at a greater biomass than *T. caerulescens* (Rascio and Navari-Izzo 2011). Similar hybrids have been made between *T. caerulescens* and *B. juncea* which removed large amounts of Pb from soil (Rascio and Navari-Izzo 2011). A transgenic *B. juncea* plant was tested by Banuelos et al. (2005) as well as Bennett et al. (2003) under field conditions and found to have greater biomass and Se (Banuelos et al. 2005) and Cd (Bennett et al. 2003) accumulation potential than the wild type. These examples make a case for continuing research into hybridization, selective breeding, and gene transfer and controlled field trials of ‘engineered’ taxa prior to widespread use (Eapen and D’Souza 2005).

One caveat when considering phytoremediation or phytomining is the environmental impact of introducing hyperaccumulating species to a non-native habitat. As in a recent case in O’Brien, Oregon, USA, *A. murale* and *A. corsicum* from Mediterranean Europe, appear to have naturalized and become invasive in nearby serpentine outcrops, potentially threatening native plants (http://www.oregon.gov/ODA/PLANT/WEEDS/edrr.shtml). It is unclear how invasive these plants would be in the long-term or their impact beyond the immediate vicinity; however, it is vital to understand the biology and ecology of the plant to be used, in as much detail as possible before undertaking field-based phytoremediation or phytomining operations.

12.5 Conclusions

In this chapter, we have discussed the major components of internal plant physiology and plant-rhizosphere interactions critical for understanding phytoremediation. Much of this knowledge is based on studies conducted on members of the Brassicaceae. The *Brassica* oilseeds and related species (notably, *Alyssum* and *Thlaspi*) will continue to lead the way for developing effective phytoremediation technologies. Additionally, the serpentine endemic species *Streptanthus polygaloides* (Brassicaceae) has also shown promise as a viable candidate for phytoremediation and phytomining of Ni (Boyd and Davis 2001; Li et al. 2003). We have also discussed areas of plant and soil properties that require further research as phytoremediation becomes a more viable and desirable method of environmental cleanup, as well as findings from recent applied phytoremediation studies. *Brassica* oilseeds represent a large proportion of heavy metal hyperaccumulating plants and, with an ever-growing understanding of the mechanisms by which they hyperaccumulate metals and the optimum conditions under which they do so, comes an ever-increasing commercial expansion of phytoremediation—a much less expensive and more environment-friendly form of soil-contaminant cleanup than traditional methods of excavation and chemical treatment.

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