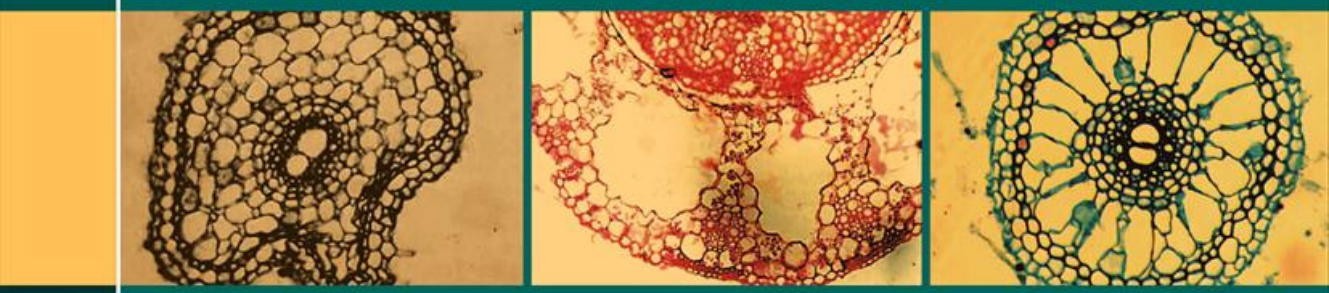
Dinesh K. Maheshwari *Editor*

# Bacteria in Agrobiology:



# Stress Management

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Bacteria in Agrobiology: Stress Management

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Editor

# Bacteria in Agrobiology: Stress Management

Springer

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*Cover illustration*: Optical micrograph showing cross sections of intercellular colonization rice calli and regenerated plantlets by *A. caulinodans*: CS view of root uninoculated control; magnified cross section view of leaf colonized by *A. caulinodans* in regenerated rice plant; possible sites of infection and colonization of rice root (from left to right); see also Fig. 3.1 in "Endophytic Bacteria – Perspectives and Applications in Agricultural Crop Production", Senthilkumar M, R. Anandham, M. Madhaiyan, V. Venkateswaran, T.M. Sa, in "Bacteria in Agrobiology: Crop Ecosystems, Dinesh K. Maheshwari (Ed.)"

*Background*: Positive immunofluorescence micrograph showing reaction between cells of the rhizobial biofertilizer strain E11 and specific anti-E11 antiserum prepared for autecological biogeography studies; see also Fig. 10.6 in "Beneficial Endophytic Rhizobia as Biofertilizer Inoculants for Rice and the Spatial Ecology of this Bacteria-Plant Association", Youssef G. Yanni, Frank B. Dazzo, Mohamed I. Zidan in "Bacteria in Agrobiology: Crop Ecosystems, Dinesh K. Maheshwari (Ed.)"

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# Preface

In general, weather, water availability, pests, and pathogens are the major constraints responsible for affecting the agriculture sector adversely. With the advent of climate change, global agriculture faces a multitude of challenges. The so-called Green Revolution brought more productive wheat, corn, and rice varieties that relied on "high levels" of chemical fertilizers and pesticides; boosted agricultural production and productivity were often accompanied by negative effects on agricultural natural resource base so serious that they jeopardize its productive potential due to various abiotic and biotic stress factors. Abiotic stress influences physiology and ecology and biotic stress mainly affects the ecology of organisms. Long-term alternative technology in the agro-ecosystem is required to ameliorate the ill effects of such stresses.

Prokaryotic organisms including bacteria are well known for their high degree of stress adaptability due to their unique genetic set up and their ability to survive under extreme environmental conditions. It is the vitality in the agro-ecosystems that researchers are investigating for opportunities to enhance agricultural inputs in the form of plant growth promoting Rhizobacteria (PGPR). Such benign biological agents are able to mitigate various abiotic and biotic stress, are in mojo presently.

The book comprises various chapters on the role of the beneficial bacteria (PGPR) in alleviating abiotic stress in general and biotic stress in particular. Their products can be a significant component of management practices to achieve the attainable yield in degraded soil. The success lies in their aggressive root colonization potential around the rhizosphere. Atmospheric threats coupled with edaphic stresses mainly due to anthropogenic activities pose severe challenges to food production. Microbes have devised a sophisticated signaling system for eliciting an adaptive response to stresses. The most dramatic of these behaviors are the purposeful migration or movement of the cells toward favorable conditions. With the aid of ACC deaminase-containing bacteria, such stresses are alleviated because of involvement of their cellular and molecular machinery. An account is provided with respect to ACC deaminase gene transfer into plants used in the phytoremediation of heavy metals to regulate ethylene level under abiotic stress.

Also, such bacteria can be exploited as a successful strategy for protecting the plants against the deleterious effects caused by soil-and seed-borne deleterious plant pathogens. Thus, the PGPR biotechnologies can be exploited as a low-input, sustainable, and environment friendly technology for stress management in plants.

Researchers, teachers, and students of life sciences, especially of microbiology, biotechnology, agricultural sciences, and environmental sciences, will find this book extremely informative and relevant.

I would like to extend my gratitude to all contributors for their authoritative and up to date scientific information organized in a befitting manner. Thanks are due to my students Dr. Abhinav Aeron, Dr. Sandeep Kumar, Mr. Rajat Khillon, and Mr. Narendra K. Maheshwari for assisting me in the compilation of the book. Valuable cooperation extended by Dr. Jutta Lindenborn, Springer, in multifarious ways is gratefully acknowledged.

Last but not the least, I owe thanks to my wife Dr. Sadhana Maheshwari and my son Ashish for taking care of me during this project.

Uttarakhand, India Dinesh K. Maheshwari

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# Chapter 1
# Priming of Plant Defences by PGPR against Fungal and Bacterial Plant Foliar Pathogens

**Alan C. Cassells and Susan M. Rafferty-McArdle**

## 1.1 Introduction

Since the first reports that plants following inoculation with viruses developed local and systemic resistance to a second viral inoculation, it has been shown that local and systemic acquired resistance (SAR) is induced by a range of biotic and abiotic stresses and chemical elicitors (plant "activators") and may confer resistance to plant pests and pathogens (Chester 1933; Ross 1966; Hull 2001). The history of induced resistance has been described by Kuc (2006) and Walters et al. (2007). "Novel" proteins were detected in the inoculated plants and were hypothesised to be expressed uniquely following biotic stress (van Loon et al. 2006). The list of the latter continues to be expanded and includes PRs (pathogenesis-related proteins) and novel isozymes of constitutive proteins (Broekaert et al. 2000). The PR proteins comprise some uncharacterised proteins (PR-1) and characterised proteins including hydrolases (chitinases and β-glucanases), osmotins, proteinase inhibitors, thionins, defensins, ribonucleases and peroxidises whose activities are anti-fungal, antibacterial and pesticidal in nature (Bart et al. 2002; Dickinson 2003; Strange 2003; Thevissen et al. 2003; De Vos et al. 2005; Tuzun and Somanchi 2006). Many PR proteins accumulate in the intercellular spaces and some of them hydrolyse pathogens and pest wall and disrupt their cell membranes. Tuzun and Somanchi (2006) provide a list of PR protein and their functions.

Not all the PR proteins are expressed simultaneously, e.g., SAR induced by virus inoculation is characterised by the presence of the pathogenesis protein PR-1a; and resistance induced by growth-promoting rhizobacteria (PGPR), where no PR-1a is detected, is termed induced systemic resistance (ISR) (Bashan 1998; Glazebrook

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2005; Tuzun 2006). In addition to PR-1, PR-2 (β-glucanase) and PR-5 (thaumatin-like) proteins are associated with SAR, while PR-3 (basic chitinase) and PR-4 (acid hevein-like) proteins are associated with ISR (Anderson et al. 2006).

Subsequent studies on induced resistance showed that some changes may occur in the metabolome where antimicrobial compounds (phytoalexins) also accumulate in induced tissues (van Etten et al. 1994; Mansfield 2000). They accumulate at the site of infection and their rapid accumulation is associated with resistance to pest and pathogens (Glombitza et al. 2004). There is also a group of inhibitory volatile metabolites called the "green leaf volatiles", associated with airborne pest attack and tissue damage (Matsui 2006). The chemical diversity of these metabolites reflects the divergence of secondary metabolic pathways in plant families (Seigler 2007).

Challenge inoculation studies showed that following primary inoculation, the inoculated plants expressed PR proteins more rapidly than uninoculated control plants (see review by Van Loon et al. 2006). It has been hypothesised that the initial inoculation primed the host plant genome, i.e., the genome retained a "memory" of the primary event that allowed more rapid expression of the primed genes on second inoculation (Sarge and Park-Sarge 2005; Bruce et al. 2007; Galis et al. 2009; Dressler 2010).

Early research showed the involvement of salicylic acid (SA) in the signalling of SAR and later, the roles of jasmonic acid (JA) and ethylene (Et) were elucidated in the signalling of ISR (Reymond and Farmer 1998; Turner et al. 2002; Kumar and Klessig 2003; Pozo et al. 2005; Kuc 2006; Vlot et al. 2008). Furthermore, it was shown that there was "cross talk" between the salicylate and ethylene/jasmonate pathways, which resulted in components of one signalling pathway suppressing the other (Pieterse and van Loon 2004). Limitations in the methodology available to the pioneering researchers led, arguably, to a narrow interpretation at the proteomic and metabolomic level of the basis of the phenomena described variously as ISR/immunisation and acquired systemic resistance. What is unclear from the literature is which PR proteins and/or phytoalexins accumulated in each pathogen–host interaction where induced resistance was expressed. Generally, where investigated, the response was characterised as SAR by the presence of PR-1a/its mRNA, or ISR where PR-1a (or its mRNA) was not detected.

With advances in the methodology of genomic, proteomic and metabolomic analysis (Fernie 2003; Sumner et al. 2003; Canovas et al. 2004; Jenner and Young 2005; Lee et al. 2006), it has been shown that induced/acquired resistance involves an array of temporal, qualitative and quantitative genomic, proteomic and metabolomic responses with expression of both host biotic defence proteins (PR proteins) and plant antibiotics (phytoalexins) and also proteins and metabolites associated with host responses to abiotic stresses (Maleck et al. 2001; Cheong et al. 2002; Fofana et al. 2002). There have also been significant advances in our understanding of the detection and recognition by plants of a microbial presence/pathogen attack, of the signalling and signal interpretation involved in the stress response and of the genomic changes which result in "priming" (Conrath et al. 2001, 2006; Ahmad et al. 2010). Priming is the bookmarking or imprinting of the

genome to respond rapidly to a subsequent (similar) pathogen attack (Sarge and Park-Sarge 2005; Dressler 2010). Here, the focus will be on discussion of the application of our current knowledge of plant priming as an approach to the control of diseases caused by fungal and bacterial plant foliar pathogens.

## 1.2 Background

Plants are protected against pathogens by pre-formed barriers and constitutive proteins and metabolites. In addition, plants may respond to pathogen attack by local deposition of cell resistant wall materials and by synthesis locally and systemically of defence proteins and metaboites. Pre-formed and induced respones are described below.

### *1.2.1 Plant Defences Against Foliar Pathogens*

Wild plants are very resistant to pathogens (Chrispeels and Sadava 2003). This resistance is based on constitutive and inducible defences (Strange 2003; Agrios 2005) (Fig. 1.1). The plant's constitutive defences include the presence of cuticles composed of resistant polymers (e.g., callose), and cell walls re-enforced (secondarily thickened) with resistant polymers such as lignin, where "resistant" refers to the low prevalence in environmental microorganisms of enzymes capable of their degradation (Strange 2003). Some fungi use appressoria/physical pressure, sometimes supported by hydrolytic enzymes, to penetrate the plant cuticle; others enter via stomata, natural openings and wounds to circumvent the intractable plant cuticle (Strange 2003; Agrios 2005). The second line of defence is the presence of hydrolytic and inhibitory proteins in the intercellular spaces and in the cells (Strange 2003; Agrios 2005). The cells also contain antimicrobial compounds (phyoanticipins) (van Etten et al. 1994; Dickinson 2003). In support of these preformed defences, there are inducible responses where the constitutive proteins and inhibitory/protective metabolites are upregulated and novel defence proteins, phyoalexins and other protective metabolites are expressed (Dickinson 2003; Strange 2003; Agrios 2005). The inducible defences include callose deposition and lignifications at the cell-wall penetration site (Bird 1988; Dickinson 2003; Strange 2003; Agrios 2005). The expressed defences may include metabolites and enzymes associated with resistance to oxidative stress and water stress (Inze and van Montagu 2002; Taiz and Zeiger 2002).

### *1.2.2 Plant Domestication and Disease Susceptibility*

Before discussing priming and its possible role in plant disease control, it is important to appreciate the current status of pathogen resistance in crop plants

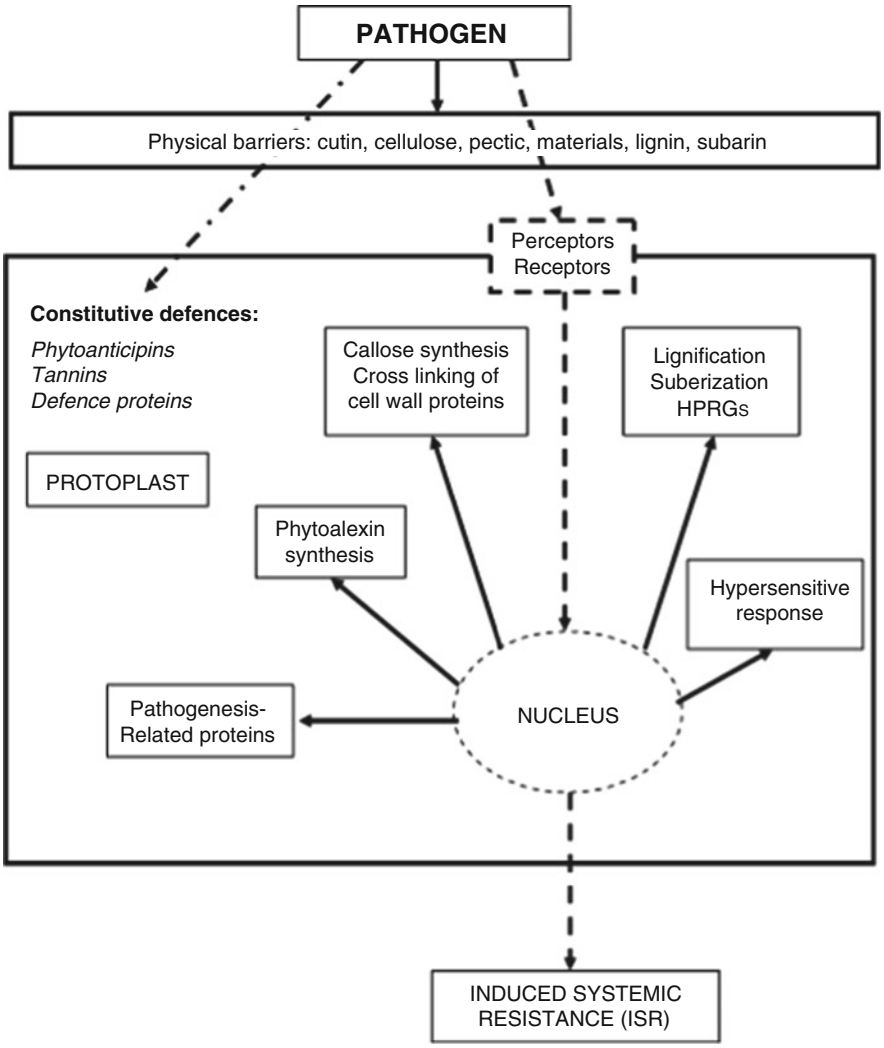



**Fig. 1.1** Constitutive and induced responses of plants to pathogen attack. For further details, see Dickinson (2003), Strange (2003), Agrios (2005); HPRGs - hydroxyproline-rich glycoproteins

(Chrispeels and Sadava 2003; Acquaah 2007). Decreased plant disease resistance is associated with breeding for high yield, organoleptic quality and other commercial traits. The main focus in plant breeding has been on faster growing, fertiliser-dependent, higher-yielding varieties (Chrispeels and Sadava 2003). This is often associated with "soft growth", that is thin-walled, hyper-hydrated tissues more susceptible to wound damage and pathogen attack than in their wild crop ancestors. Another important factor has been breeding for appearance and taste. Chewiness in vegetables has been selected against, as has strong unattractive flavour

(e.g. glucosinolates in brassicas) or toxic metabolites (e.g. solanine in potato) (Lachman et al. 2001), that is, characteristics which are based on thick cell walls and cuticles, and secondary metabolites, have been not selected for/have been bred out (reduced) in some modern varieties. In summary, plant breeding if not deliberately, has subliminally attenuated constitutive resistance resulting in crop plants which are more susceptible to disease at the level of constitutive resistance and may also have reduced the efficacy of inducible resistance. Another factor in the domestication of plants which contributes to disease problems is intensification of cultivation where large-scale plantation of single genotypes provides a selection pressure for virulent pathogen strains (Kaloshian 2004; Cooke et al. 2006).

## 1.3 Inducible Resistance to Plant Foliar Pathogens

A number of stress factors have been outlined that play different roles in inducing resistance to infections, which occur due to foliar pathogens.

### *1.3.1 Early Events in the Plant Stress Response*

An oxidative burst, elevated production of NO (nitric oxide) and calcium ($Ca^{2+}$) signalling are early events in the plant response to biotic and abiotic stresses (Evenas et al. 1998; Bolwell 1999; Bolwell et al. 2002; Arasimowicz and Floryszak-Wieczorek 2007; Reddy 2001). These general responses are seen as preceding or overlapping with the production of specific pathogen stress signalling compounds, including salicylic acid (SA), and resulting in the production of biotic stress proteins and metabolites (Fig. 1.2). Redox homeostasis is hypothesised as a key node in the interface between stress perception and physiological responses (Hippeli et al. 1999; Foyer and Noctor 2005). Perturbation of the cellular redox potential is counterbalanced by activation of antioxidant defences, involving production of regulatory enzymes and metabolites (Gill and Tuteja 2010). High levels of oxidative stress may result in PCD (programmed cell death) in the stressed cells and stress signalling to the surrounding cells and systemically (Horbinski and Chu 2005; Fujita et al. 2006; Potters et al. 2010; Vaahtera and Brosche 2011).

### *1.3.2 ISR and Innate Immunity*

In the 1960s when induced resistance was first elucidated, many workers rejected the hypothesis of plant "immunisation", believing that induced resistance to pathogens and pests, and animal innate immunity, were fundamentally different (Parham 2009). With the emergence of data, especially from the human and

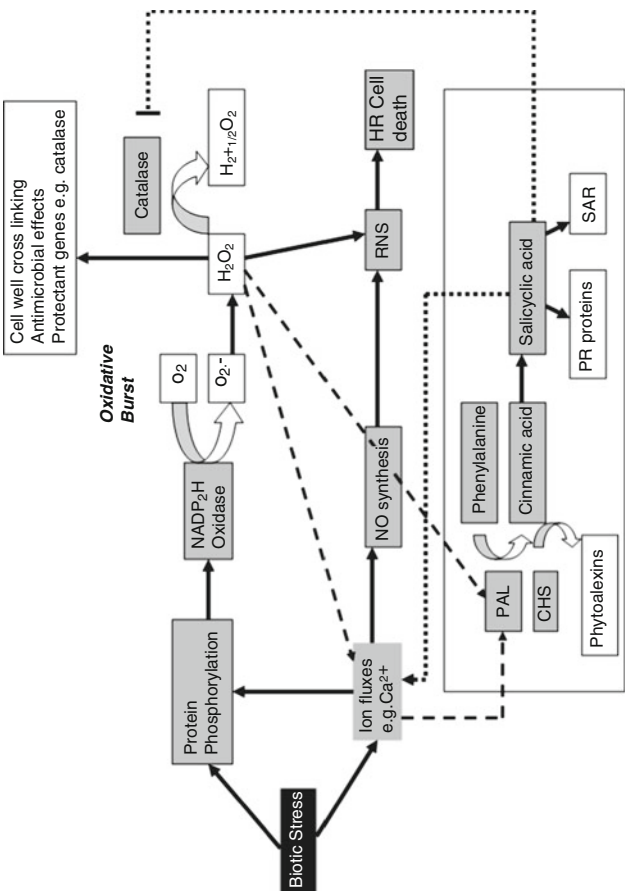



**Fig. 1.2** Early signalling events in the biotic stress response. Key components are $H_2O_2$, $Ca^2$+ ion flux and NO. *RNS* reactive nitrogen species

*Arabidopsis* genome projects (Nishimura and Dangl 2010), this perspective has changed and has led to the hypothesis that innate (non-adaptive) immunity exists in both plants and in animals (Fluhr and Kaplan-Levy 2002; Jones and Takemoto 2004; Chisholm et al. 2006; Jones and Dangl 2006). There is some debate as to whether these systems, or at least elements of innate immune in plants, are the product of divergent or convergent evolution (Parker 2003; Ausubel 2005). A component which is present in the basal innate immune response in plants and animals is the production of defensins produced in JA-ISR (Taylor and Hefle 2001; Bart et al. 2002). Cell surface leucine-rich receptor-like kinases (LRR-RLKs) were discovered, which act as receptors for conserved pathogen molecules (PAMPs – pathogen-related molecular patterns) that recognise both pathogenic and non-pathogenic bacteria and fungi and trigger a first-line innate immune defence response (Fig. 1.3). Subsequent studies indentified the role of some 150 intracellular NB-LRR (nucleotide binding leucine-rich repeat) proteins involved in recognising plant pathogen (and pests) effectors (specific pathogenicity factors) and triggering a defence response. Unlike NB-LRR receptors in animals which recognise PAMPs signals, NB-LRR receptors in plants have evolved the potential to recognise effectors (Caplan et al. 2008; Postel and Kemmerling 2009). The possibility of an adaptive immune response in plants has been raised with the discovery of Toll-interleukin 1 receptor domain proteins in plants, and undoubtedly, there remains much to be elucidated in the plant immune response (Flajnik and Du Pasquier 2004).

The "zig-zag" model was proposed to unify the activity of PAMP and specific effector receptors in the pathogen defence response. In this, PAMP perception triggers innate immunity (PTI – the first "zig"). This may be suppressed by a specific pathogen effector(s): the "zag". If the effector is recognised by the NB-LRR proteins, a stronger defence response results (another "zig") (see review by Nishimura and Dangl 2010; Fig. 1.4). There may be rounds of attack and response ("zig-zags"), where the outcome of the interaction between PTI and the effector-triggered susceptibility (ETS) is ETI (effector-triggered immunity). The outcome of the pathogen attack is qualitatively based on recognition of the effectors and quantitatively expressed as the total of (PTI–ETS) + ETI (Nishimura and Dangl 2010). The effector receptors are related to R-genes in agreement with Flor's gene-for-gene hypothesis (Flor 1947). Consequently, loss of effector receptors in varietal breeding has implications for the potential of induced resistance to combat disease.

It has been hypothesised that under evolutionary pressure, a distinct response to biotrophic pathogens may have evolved in contrast to the earlier (in evolutionary time) defence response against pathogens with a broad host range (Dodds et al. 2006). In the latter regard, it has been proposed that necrotrophs are resisted by JA-ISR and the more advanced biotrophs by SA-ISR (Glazebrook 2005). The implications of this hypothesis have significance for both the understanding of ISR in general and for its commercial exploitation.

As referred to in the Introduction, the term induced systemic resistant syn. "plant immunisation", was first used to describe the phenomenon of resistance induced in plants following inoculation with plant viruses (Kuc 2001). More recently, Tuzun

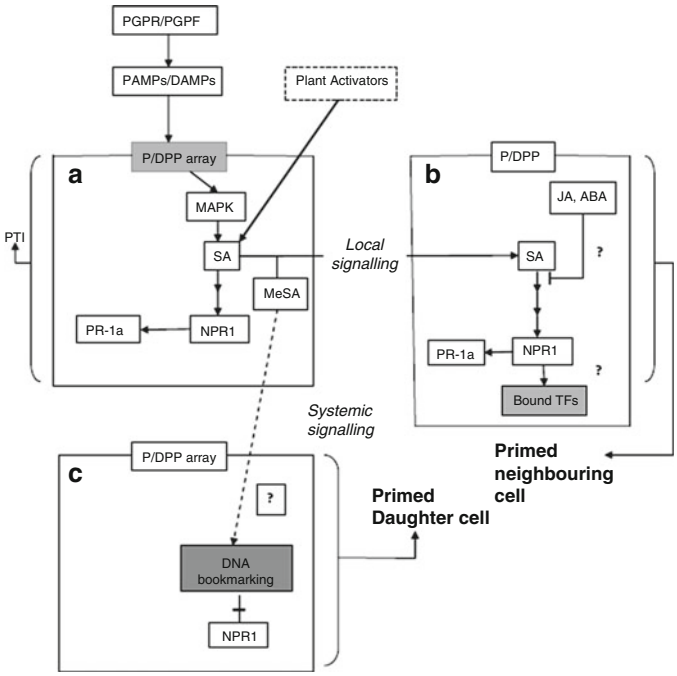



**Fig. 1.3** Simplified outline of the stages in local induction and expression of SA-ISR, systemic expression and priming of SA-ISR and bookmarking (priming) of SA-ISR in daughter cells by PGPR/PGPF. Note other associated events (see Fig. 1.2 and text) are not included here. (**a**) Bacterial and fungal signals produced directly or released by plant extracellular enzymes bind to receptor arrays on the plant membrane. The activated receptor arrays transmit signals via signalling cascades, resulting in the synthesis of appropriate transcription factors culminating in the synthesis of PR-1a and other related PR proteins. SA and analogues act as activators of SA-ISR at the level of the transcription factors (NPR1, etc.). (**b**) SA activates SA-ISR in neighbouring cells, while MeSA activates SA-ISR systemically with expression of PR-1a and other PR proteins. Expression of the PR protein is transient systemically (turns over) but the cells remain primed for a rapid expression of SA-ISR. It is hypothesised that appropriate transcription factors may be present in these primed cells in a bound/inactive form. (**c**) The genome of cells that expressed SA-ISR is bookmarked, possibly based on histone modifications and/or structure changes in the DNA such that in the daughter cells, SA-ISR is expressed rapidly on pathogen attack. While it is hypothesised that bookmarking carries the priming through mitosis, the bookmarked regions may be transcribed and inactive SA-ISR transcription factors may be present in the daughter cells

(2006) has argued that the term ISR be used to describe both induced and acquired resistance and that the term be qualified by the prefixes SA- and JA- referring to the respective signals involved. As alluded to in the previous paragraph, a key as-yet-unanswered question is whether there are only these two basic universal (conserved) primeable mechanisms of ISR involving PR-1a and PR-12 (PDF1.2) and other PR-associated proteins (see Anderson et al. 2006), and whether associated protein and metabolite (including phytoalexins) changes are related to evolved divergent responses in plant families.

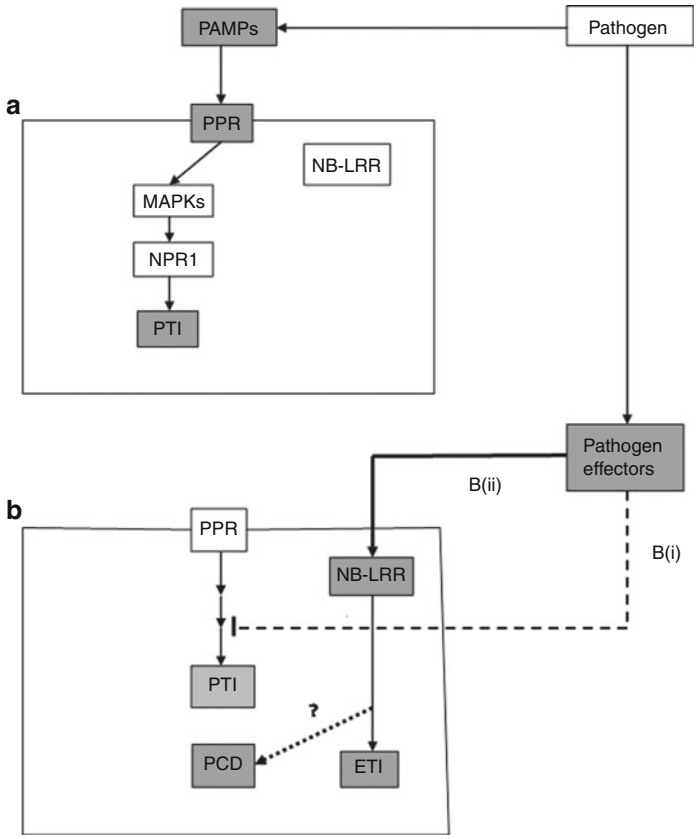



**Fig. 1.4** A simple outline of the key events in pathogenesis. (**a**) Pathogenesis involves PAMPs and PPR arrays (see Fig. 1.3) and results in PTI, which may be expressed as SA-ISR but also as JA + Et-ISR and may involve phytoalexin production. (**b**) Some of the compounds, viz. effector (s), produced by pathogens may inhibit the PRR signal cascade. In the absence of plant receptors, represented here by NB-LLR, disease progresses. Where NB-LLR, or other receptors, are present in the plant programmed cell death may occur in the attacked cell(s) and ETI locally and systemically

### *1.3.3 Pathogen Stress Recognition, Signalling and Interpretation*

Characterised pathogen-associated molecular patterns (PAMPs) include chitin, glycoproteins, lipopolysaccharides and flagellin and microbial fragments released by the action of extracellular plant enzymes (Bianchi 2007; Schwessinger and Zipfel 2008; Nicaise et al. 2009; Zipfel 2009 Ahmad et al. 2010). While PAMPs bind to plant cell-surface receptors and trigger a defence response, other microbial factors may also trigger stress responses by affecting membrane fluidity and by causing oxidative stress and water stress (Inze and van Montagu 2002) (Fig. 1.2). Reactive oxygen species (ROS) also signal stress, resulting in some cases in

hypersensitivity, a form of programmed cell death (Chamnongpol et al. 1998; Greenberg and Yao 2004; Nishimura and Dangl 2010).

Several signalling cascades (Anderson 2000; Choi et al. 2008) have been characterised in plants, of which the mitogen-activated protein kinases (MAPKs) are perhaps the best understood (Garrington and Johnson 1999; Asai et al. 2002; Hall et al. 2002). In studies on the MAP kinase signalling cascade induced by the PAMP flagellin, an $FLS_2$ (a leucine-rich-repeat receptor kinase) signal is passed via $MEKK_1$, $MKK_4/MKK_5$ and $MPK_3/MPK_6$ to transcription factors $WRKY_{22/29}$ (DeYoung and Innes 2006). Activation of this cascade results in resistance to both bacterial and fungal pathogens (Asai et al. 2002). Various receptor signals converge at the MAPK cascade and, possibly with the involvement of cross-talk, are "interpreted" to result in what is an "appropriate" defence response (Boller and Keen 2000; Cherry et al. 2000; Knight and Knight 2001; Kunkel and Brooks 2002; Zhang et al. 2006; Flors et al. 2008). Specificity in the interpretation of signals is hypothesised to be based on the involvement of scaffolding, adaptor and anchoring proteins (Pawson and Scott 1997; Garrington and Johnson 1999; Takashi and Pryciak 2008; Yi et al. 2010). Furthermore, the role of cascades in converting oscillating signals into step-like responses may underlie the deciphering of multiple PAMPs signals of different strengths into one of a few characterised general stress responses involving synthesis of subsets of PR proteins and possibly of other biotic and abiotic proteins and metabolites (Marhl and Grubelnik 2006). It has been hypothesised that plants are primed naturally by rhizosphere microorganisms (Walters 2009). If this is the case, given the diversity of rhizosphere microorganisms, threshold levels of community PAMPs may determine the host response. Where the host roots are inundated with specific inoculants (as in inoculation with PGPRs), the PAMPs and their thresholds involved may be more specific in their induction of a host response. It is unclear whether local induction of a defence response inevitably results in systemic expression of ISR and host priming. The final step in the receptor–signalling–transcription sequence is the activation of the targeted transcription factor, of which the most extensively studied has been NPR1 (Kohler et al. 2002; Pieterse and Van Loon 2004; Shadin Mukhtar et al. 2009). Proteosome regulation of NPRI turnover may be involved in regulating the expression of SA- and JA + Et-ISR (Spoel et al. 2009).

Plant hormones, nitric oxide (NO) and ROS all interact in the stress signalling and cross talk (Genoud and Metraux 1999; Feys and Parker 2000; Gazzarrini and McCourt 2001; Kunkel and Brooks 2002; Bostock 2005; Flors et al. 2008). NO and reactive ROS are involved in the hypersensitive response to biotrophic pathogens (Bolwell 1999; Inze and Van Montagu 2002; Leitner et al. 2009). The perception of PAMPs/DAMPs including oxidative stress, with or without the triggering of PCD, results in the release of SA which signals SA-ISR locally, and methyl salicylate and possibly other molecules signal SA-ISR systemically. Similarly, stress receptors initiate JA-, methyl-JA-, oxylipin- and ethylene signalling of JA-ISR locally and systemically (Guo and Ecker 2003; Glazebrook 2005; Kachroo and Kachroo 2009). Other, as yet uncharacterised signalling molecules may also be involved in both SA- and JA-ISR (Vlot et al. 2008).

The induced proteins and/or phytoalexins exhibit metabolic turnover following ISR induction; however, the extant and daughter cells derived via mitosis in further plant growth are "primed", that is, express ISR more rapidly on challenge inoculation (Conrath et al. 2001, 2006; Goellner and Conrath 2008). The relationship between the initial induced response with regard to the range of proteins and metabolites induced, and the response of primed and daughter cells are unclear, as are the mechanistic differences in the expression of priming in extant and daughter cells (see Sect. 1.3.4). There are specific PAMPs for bacteria and fungi, yet both fungi and bacteria can induce SA-ISR and JA-ISR. This would imply that there are only a few plant responses to the relatively diverse, perceived biotic stresses. One master switch of the stress response may be based on cell redox potential which, at high stress levels, triggers PCD and induces SA-ISR, the outcomes being determined by the range and the intensity of the general stress and host genomic factors (Inze and van Montagu 2002; Anderson et al. 2006). There may also be other generalised defence responses based on phytoalexin production and involving proteomic responses not based on PR-1a or P-12 expression, e.g., based on chitinase and gluconase production (Agrawal et al. 2002; Aziz et al. 2006).

### *1.3.4 Priming of Extant and Daughter Cells*

ISR induction is followed by PR-protein and/or phytoalexin expression, and cells in which expression has occurred remain "on standby" to rapidly re-express SA- or JA + Et-ISR, as initially induced, on challenge inoculation (Bruce et al. 2007). A "memory" of the form of induced ISR is transmitted via mitosis to new (daughter) somatic cells that is, bookmarked in the host genome (Figs. 1.3 and 1.4). The basis of priming of cells existing post-expression of the initial stress response is unclear. There is some evidence that inactive MAP kinases and/or inactive membrane-bound transcription factors may accumulate in primed cells which are activated or released when challenge inoculated, i.e., subjected to a second pathogen attack, resulting in faster and/or stronger expression of the defence response than in unprimed cells (Mou et al. 2003; Beckers and Conrath 2007; Goellner and Conrath 2008; Seo et al. 2008). In the case of daughter cells formed after withdrawal of the priming signals, priming make take the form of preparation of the genome for more rapid transcription of the primed defence genes, that is, be based on bookmarking (Michel 2009; White and Sharrocks 2010). It is believed that in bookmarking, genes that are transcriptionally competent are marked but the mechanism is unknown. The general basis of book marking (epigenetic programming) may be based, inter alia, on the mitosis-dependent modification of histones and/or the persistent binding of transcription factor complexes to gene promoters in mitotic cells where the bookmarked region is more easily accessed by the transcription process (Sarge and Park-Sarge 2005; Dressler 2010). Recently, the role of chromatin structure,

specifically chromatin loops, in epigenetic memory has been reported (Deng and Blobel 2010; Papantonis and Cook 2010; Tollefsbol 2011).

### 1.3.5 Priming of ISR by Chemicals

The agricultural application of priming by chemical activators including inducers of hypersensitivity (programmed cell death) is mentioned here as an alternative or competitive strategy to the priming of plants by PGPRs (Kinnersley 2000; Oostendorp et al. 2001; Walters et al. 2005; Conrath et al. 2006). Many reviewers have reported the widespread effectiveness of priming by numerous chemical activators in many crops and against a wide range of pests and diseases (Anderson et al. 2006). Disease control levels were reported to vary between 20 and 85% (Walters et al. 2005) and were influenced, as in constitutive resistance, by plant nutrition, e.g., by nitrogen supply (Dietrich et al. 2004) and by plant developmental stage (De la Pena et al. 2010; Shiba et al. 2010). In the case of chemical activators, application rate and timing of the application(s) affected the results but treatment as early as the seed stage results in ISR in the growing crop (this supports the hypothesis that bookmarking allows transfer of the primed state through mitosis). Activator induction of ISR can be achieved more rapidly than PGPR-induced resistance but they differ, at least qualitatively, in that induction by PGPRs is primarily via PAMP receptors (see Sect. 1.3.2) rather than stress signal analogues (Table 1.1; Fig. 1.5). However, many PGPRs can produce plant hormones/stress signalling compounds (Lugtenberg and Kamilova 2009).

### 1.3.6 Priming by Biological Inoculants

Induction of ISR by PGPRs and PGPFs (plant growth promoting fungi including mycorrhizal fungi) has been reviewed, e.g., by Vallad and Goodman (2004); Bent (2006); and Shoresh et al. (2010). Historically, PGPRs were considered to induce JA-ISR (evidence based on no PR-1a being detected), as opposed to many chemical activators and plant pathogens which were shown to induce PR-1a (the biomarker for SA-ISR) (Table 1.1; Anderson et al. 2006). Much of the research was carried out using root inoculation of seedlings or of vegetative propagules; more recently, the efficacy of foliar application has been demonstrated (Saravanakumar et al. 2007). In some experiments, dual inoculation was found to yield greater protection than inoculation with either of the inoculants individually (Jetiyanon et al. 2003). In general, the results obtained for biological inoculants are more variable than those obtained for chemical activators, but this may be explained in so far as the chemical activators were of known mode(s) of action, mostly SA analogues, whereas the signals (binding to PAMP or other plant receptors) emitted by most inoculants have not been characterised. Where comparative studies have been carried out, it was

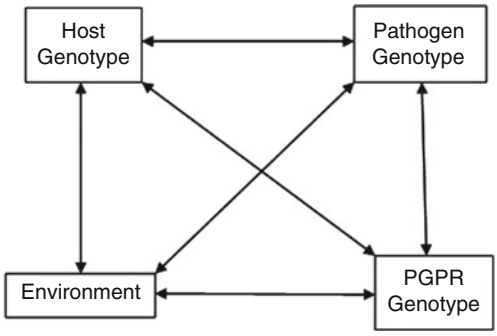
**Table 1.1** Summary of the characteristics of commercial plant activators and biological inoculants reported to induce/prime systemic resistance

| Commercial products | |
|---|---|
| Active component | Mode of action |
| **Chemical inducers** | |
| Phosphorous containing salts | Some reports of local lesions/SA-ISR induction but other reports of enzymes of phytoalexin production without PR protein accumulation (Anderson et al. 2006) |
| BABA (β-aminobutyric acid) | Reports of ROS-mediated responses including SA-ISR, also phytoalexin production and lignifications (Cohen 2002) |
| ROS + SA | Reported activation of SA + JA-ISR (Chamnongpol et al. 1998) |
| BTH (benzo(1,2,3) thiadiazole-7-carboxylic acid) | BTH (a.i. of Bion$^{TM}$) widely reported as inducer of SA-ISR (Oostendorp et al. 2001) |
| Probendazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide) | Resembles pathogen effector in binding to LRR binding protein activating SA-ISR (Sakamoto et al. 1999) |
| **Natural inducers** | |
| Chitin and chitosan | Reported activation of antibiotic activity, phytoalexin synthesis, and JA-ISR (Agrawal et al. 2002; Zhang et al. 2002; Aziz et al. 2006) |
| Harpin | Is a characterised bacterial PAMP which induces SA-ISR (Dong et al. 1999) |
| Strobilurins | Antibiotic activity; potentiates PR-1 production |
| PGPR | Produce SA and PAMPs such as harpins and flagellin; induce SA-ISR and JA-ISR (Bashan 1998; Pieterse et al. 2001; Compant et al. 2005; Glazebrook 2005; Bent 2006) |
| PGPF | Include AMF, many related to fungal pathogens; produce PAMPs and some auxins; commonly induce SA-ISR (Bent 2006) |

shown for example, that the genes induced by the PGPR *Pseudomonas syringae* differed from those induced by the commercial plant activator Bion$^{TM}$ (Schweizer et al. 1999); but even for chemical activators, the response may be plant-genotype dependent (Anderson et al. 2006). That both SA- and JA-ISR can be induced by PGPRs suggests that bacterial PAMPs are all that may be required to induce resistance to both bacterial and fungal pathogens, but with a caution that PGPRs which induce resistance to fungal pathogens may have acquired and expressed some fungal PAMPs (see Cohan 2001, 2002).

## 1.4 Practical Application of Priming

Some proteins and metabolites formed following priming may be allergens or antimetabolites. Priming may be acteived by the use of inoculants or chemical activators. Safety and inducer efficacy are discussed below.




| Factor | Critical components in interaction |
|---|---|
| Host genotype | Reduction of constitutive resistance; loss of PAMPs/DAM PS and effector receptors (major genes) in domestication; presence/absence of pathogen toxin detoxification mechanisms |
| Pathogen genotype | Multiple mechanisms of host penetration; novel virulence effectors; production of novel toxins |
| Environment | Presence of PGPR antagonists in rhizosphere; unfavourable soil temperature, pH and edaphic factors |
| PGPR genotype | Production of broad spectrum antibiotics; production of effective PAMPs; tolerance of environmental factors |

**Fig. 1.5** The disease square, where the conventional disease triangle is expanded to a square when PGPRs are introduced (Agrios 2005). Critical components affecting the relationships are listed in the accompanying table

### *1.4.1 Safety Factors*

Characterisation of PR proteins and phytoalexins has led to attempts, using conventional plant breeding and genetic engineering, to obtain plants expressing components of induced resistance constitutively at elevated levels (Hain et al. 1993; Jach et al. 1995; Cao et al. 1998; Hammond-Kosack and Parker 2003; Punja 2004). However, it is important to recognise that such proteins and metabolites which may persist into the harvested crop may be antigenic or toxic (act as anti-metabolites) for the consumer (Molina and Garcia-Olmedo 1997; Shewry et al. 2001; Taylor and Hefle 2001; Anon 2002; Cassells and Doyle 2003). This is in contrast to their exploitation in plant activation/plant priming where the upregulation of PR proteins and phytoalexins may be transient, not persisting till produce harvest, albeit this would have to be experimentally determined (Novak and Haslberger 2000; Schauzu 2000). Priming by chemical plant activators is subject to safety testing of the activators, as for pesticides. PGPR plant priming products are not currently subject to safety testing but are subject to product labelling restrictions that impede market development (Fravel 2005).

A concern regarding the use of PGPRs as opposed to, e.g., AMF, to prime plants is that the some of the former may be related to human pathogens.

Concern has been expressed, for example, over the use of *Burkholderia cepacia* (*Pseudomonas cepacia*) as a PGPR as human-pathogenic strains of *B. cepacia* posing a risk to cystic-fibrosis sufferers have been isolated from the rhizosphere (Berg et al. 2005). Given the promiscuity of bacteria in exchanging genetic material in the environment, the human health risk should always be borne in mind when screening potential PGPRs for commercial application (Cohan 2001, 2002).

### *1.4.2 Use of Chemical Activators Versus PGPR Inoculants*

A very diverse range of chemical compounds and biological inoculants induces various forms of pathogen resistance in plants (Anderson et al. 2006) (Table 1.1). These range from pathogens and pathogen stress-signalling compounds such as nitric oxide, SA and SA analogues (BTH etc.) to toxins and mineral salts (for a more complete list see Anderson et al. 2006; Kuc 2006; Walters et al. 2007). Mode of action studies would suggest that some of these act directly inducing anti-pathogen defences, e.g., SA and its analogues signal SA-ISR, while others act indirectly through cross talk between stress pathways to confer a degree of resistance, e.g., β-aminobutyric acid (Cohen 2002). The commercial acceptance of Bion$^{TM}$ and other chemical activators (Agrios 2005; Walters et al. 2007) underpins the efficacy of using characterised ISR signalling compounds, but also poses the question as to whether an array of PAMPS/DAMPs signals such as occurs naturally in pathogenesis would confer more effective, broader spectrum protection (Bianchi 2007). The latter approach might result in a "designer PGPR" with characterised elicitor, plant growth promoting and pathogen antagonistic factors. Alternatively, combinations of treatments including PGPRs and plant activators might be employed to achieve broad-spectrum protection against root and foliar pathogens and pests (Vestberg and Cassells 2009).

A practical consideration in using chemical activators versus biological inoculants is that the former can be applied as "quick acting" foliar sprays which induce protection in days as opposed to PGPR inoculants which may take days to weeks (Anderson et al. 2006) (Fig. 1.5). Another concern with root inoculants, especially with PGPRs which are commonly applied to a substrate abundant in microorganisms, is that some of the latter may be antagonistic to the inoculants – a problem which has negatively impacted on the market acceptance of biological inoculants (Cook and Baker 1983). Normally, the density of the inoculum used would be aimed at inundating the root system of the plant. A common strategy to avoid soil antagonism is to isolate potential PGPRs from the crop soil (Jetiyanon et al. 2003). Alternatively, to avoid antagonism, inoculating aseptic microplants, or aseptic seed or seedlings would seem advantageous compared with inoculation of other vegetative propagules (Compant et al. 2005).

### *1.4.3 Market Factors*

Chemical activators and biological inducers of ISR are commercially available (Fravel 2005; Anderson et al. 2006; Walters et al. 2007). The cited advantages of these products include:

- that they are alternatives to fungicides, where resistance to foliar fungal pathogens is a problem (Russell 1995) and where ISR is less likely to be overcome, as it is "polygenic" in character
- where a fungicide is being removed from sale or its application is being restricted by legislation
- that natural ISR inducers are acceptable in biological ("organic") agriculture (Chrispeels and Sadava 2003; Agrios 2005)
- that PGPR- and activator-induced resistance may also be effective against viruses and soil-borne diseases; in the latter case, ISR induction may be supported by PGPR production of additional factors, e.g., antibiotics in the rhizosphere which inhibit pathogen inoculum (Fravel 1988)
- that priming may persist in the cropping cycle, being possibly reprimed repeatedly in the case of PGPRs by colonisation of new roots which potentially reduces the cost of disease control compared with repeated fungicide/activator application, especially for subsistence farmers

The yield penalty of exploiting induced resistance has been discussed, including the risk that plants primed to resist biotrophs may respond more slowly than unprimed plants to necrotrophs and insect attack and vice versa (Heil et al. 2000; Hammerschmidt 2005). Walters and Boyle (2005), however, argue that authors who failed to include challenge inoculation in their experiments underestimated the benefits of induced resistance.

Difficulties exist in convincing growers to adopt new approaches. These are mainly related to product reliability and cost but also include the requirement to train personnel. Of the two approaches, viz., the use of chemical plant activators or biological priming, it is interesting that it is the former, pioneered by the pesticide industry which has been to the fore (Agrios 2005; Fravel 2005). Arguably, this is due to market access, existing client base, ability to transfer technology from the conventional pesticide market to the activator sector and reproducibility of results. Where synthetic activators such as Bion$^{TM}$ are concerned, the manufacturer has the added protection from competition, of patent protection and possibly low manufacturing cost, compared to natural activators of comparable efficacy. The reputation of "biological control" is variable (Cook and Baker 1983) and in regard to ISR, priming has suffered from a lack of knowledge of the specific priming determinants present in the biological inoculants, i.e., what form of priming has been achieved, the influence of environmental factors on inoculants and the problem of antagonism by environmental microorganisms resulting in irreproducibility of results between seasons and sites (Fig. 1.5; Vestberg et al. 2004; Vestberg and Cassells 2009).

A serious barrier to the sale of PGPR inoculants is the restriction on product label claims where specific claims regarding biological control/disease control may not be allowed, depending on the jurisdiction (Fravel 2005). Application of spent PGPR culture medium, a source of PAMPs, to crops to induce resistance would also be subject to product label restrictions, disease control claims and risk assessment (Anon 2005). This is a matter of ongoing discussions between producer organisations and legislators.

## 1.5 Optimisation of Priming by PGPRs

As discussed above, plants respond to pathogens by activating their biotic defences; this may involve activation of the hypersensitive response locally at the site of infection and by induced resistance systemically (Bolwell 1999; Watanabe and Lam 2006). Research has shown that SA-ISR-inducing pathogens (necrotrophs) are best resisted by SA-ISR primed resistance, similarly for JA-ISR and biotrophs (Glazebrook 2005). This may be a simplistic model, as proteomic investigations have shown that ISR may be based on the expression of biomarkers of SA- and /or JA-ISR but also may include biomarkers of enhanced phytoalexin production (e.g., chalcone synthase) and of abiotic stress resistance (e.g., glutathione reductase) (Cheong et al. 2002; Fofana et al. 2002; Glombitza et al. 2004; De Vos et al. 2005). Insects are believed to be resisted most effectively by JA-ISR priming and/or priming of increased green leaf volatile production (Korth and Thompson 2006).

While we know how to induce/prime the JA- and SA-ISR pathways, it is unclear in many cases what we are priming when we use PGPRs and other biological inoculants (Anderson et al. 2006). In general, when using inoculants to prime plants, the pathway(s) primed have only been characterised on the basis of the presence or absence of expression of PR-1a in the inoculated plants. A question then, is to what extent the response to SA results in only a subset of the responses to a PGPR inoculant? The empirical use of PGPRs for growth promotion/disease suppression demonstrates the wide plant-genotype response to different PGPR inoculant strains (Thomma et al. 2001; Zhang et al. 2010). This issue, fundamental to the full exploitation of any differences in the responses elicited, is complicated by likely differences in elicitors/signals/pathogenicity factors between different PGPR inoculants. An experimental priority in this field is to identify the elicitors present in different PGPR isolates and to determine their efficacy in priming effective ISR (Ton et al. 2002). It is estimated that there are approximately 100 PAMP-responsive genes (Schwessinger and Zipfel 2008) but only a subset of these (20–30) are specific to bacterial recognition (Hann et al. 2010). It would be interesting to determine whether dual inoculation with PGPRs and PGPFs, e.g., AMF would confer broader priming that with a PGPR alone (Bent 2006; Bonfante and Anca 2009). There is also the issue of whether priming against the algal-related Chromista (*Phytophthora* and relatives) involves unique PAMPs recognised by specific host plant receptors. There is a significant lack of information also

regarding time course investigations on the biotic stress response in spite of the widespread perception that speed of response is a critical factor in the control of pests and diseases by induced resistance (Mur et al. 2006).

## 1.6 Conclusions

Literature searches for PGPRs reveal a vast number of papers reporting successful experiments using PGPRs to promote plant growth/suppress soil borne and foliar diseases (see reviews by Fravel, 2005; Siddiqui 2005; Anderson et al. 2006; Walters et al. 2007; Maheshwari 2010). It is important to note that many of these experiments were carried out in the laboratory or greenhouse and may not be reproducible under the variable environmental conditions of the field. In these experiments it is not possible generally, to distinguish whether the disease suppression effects of PGPRs were due to antagonism of deleterious rhizosphere microorganisms/pests, plant growth promotion and/or induced resistance, or combinatorial effects of two or more of these mechanisms, as most authors have not confirmed by genomic/proteomic/metabolic analysis the form of ISR, if any, expressed in their trials. Critical elements of the response to a priming agent may be host-genotype dependent. In contrast with SA analogues, PGPR inoculants are subject to strong interactions with the host plant genotype, the environment and soil microorganisms, all of which influence may determine efficacy (Fig. 1.5).

Arguably, the potential for the commercial exploitation of PGPRs for the suppression of disease depends on clarification of the mechanisms involved in priming reproducible broad-spectrum effects in economically important crop species. Commonly, potential PGPR soil isolates are only screened for antibiotic production and demonstration of the efficacy of the antibiotic(s) against the target pathogen(s), and for plant growth promotion (Lugtenberg and Kamilova 2009; Franco-Correa et al. 2010). Screening for the ability to induce/prime host resistance is an altogether more complex and expensive genomic, proteomic and metabolomic screening, albeit the technology is becoming more accessible (Fernie 2003; Sumner et al. 2003; Canovas et al. 2004; Jenner and Young 2005; Qureshi et al. 2007).

We have a superficial understanding of the qualitative basis of PAMP/DAMP signal perception and transduction, of pathogen effector perception and signal transduction but we have a poor understanding of the influence of signal strength on the possibly highly host-genotype dependent signal interpretation and outcomes. This is shown in SA-signalling where ISR may occur with or without a local hypersensitive response, this outcome is likely to be host-genotype dependent (Bolwell 1999; Sharma et al. 2010) .

Fungicides, and plant activators related to known SA- and JA-stress signalling compounds, are both potentially broad spectrum. Interestingly, while some PR proteins, e.g., defensins are widely if not universally present/conserved in plants, some of their induced isozymes, e.g., of chitinases are variable and may differ from those expressed constitutively (Punja and Raharjo 1996). In contrast, the antibiotics

which compose the phytoalexins are specific to families, down to the species/genotype level (Seigler 2007). Given the gene erosion which has occurred in plant domestication (Hammer et al. 2003; Chrispeels and Sadava 2003), it is possible that in some crop varieties both constitutive resistance and induced resistance may have been weakened to the point where they are not effective, in concert, in controlling disease, i.e., the crops are pesticide dependent for disease control (Zhang and Reddy 2001).

In summary, those factors which influence the direction of the market for disease control products – such as the cost of conventional methods, legislation regarding the use of products, consumer demands for reduced pesticide produce – will determine the potential for PGPRs as substitute products for pesticides. In low-input agricultural systems based on varieties with high constitutive resistance, PGPRs arguably are predominantly used as biofertilisers to promote plant growth (Kumar et al. 2009). In high-input agriculture involving crops with lower constitutive resistance, and with higher societal pressure to reduce inputs, PGPRs may have some potential as alternatives to pesticides, e.g., as biofungicides. Currently, their use in high-input agriculture is regarded as unpredictable, with a high dependence on favourable environmental factors for success (Dietrich et al. 2004). Aside from costly proteomic and metabolomic work to elucidate the mechanism(s) of effective PGPR- and PGPF priming (Bonfante and Anca 2009), application of their metabolites (PAMP/DAMP signals) to the plant has been reported, e.g., with harpins (Dong et al. 1999; Anderson et al. 2006). The latter approach rather than root inoculation with live microorganisms may be an option to pursue in relation to priming of disease resistance, but this strategy would face health and safety testing legislation. Finally, even when the interactions between PGPR–PAMPs and specific host genotypes are elucidated, it will remain difficult to control the quantitative elements in the interaction under the influence of different environmental conditions.

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# Chapter 2
# The Management of Soil Quality and Plant Productivity in Stressed Environment with Rhizobacteria

**Dilfuza Egamberdieva**

## 2.1 Introduction

The urgency of feeding the world's growing population while combating soil pollution, salinization, and desertification has given plant and soil productivity research vital importance. Under such circumstances, it requires suitable biotechnology not only to improve crop productivity but also to improve soil health through interactions of plant roots and soil microorganisms (Lugtenberg et al. 2002). Interest in bacterial fertilizers has increased, as it would substantially reduce the use of chemical fertilizers and pesticides which often contribute to the pollution of soil–water ecosystems. Presently, about 20 biocontrol products based on *Pseudomonas*, *Bacillus*, *Streptomyces*, and *Agrobacterium* strains have been commercialized, but there still is a need to improve the efficacy of these biocontrol products (Copping 2004; Chebotar et al. 2000; Lugtenberg and Kamilova 2004). Soil salinity disturbs the plant–microbe interaction, which is a critical ecological factor to help further plant growth in degraded ecosystems (Paul and Nair 2008). Because plants are under saline or water unbalance stress, they become more vulnerable to diseases caused by pathogenic fungi. The use of specific microbe antagonists which stimulate plant growth and/or are natural enemies of pathogens allows a considerable decrease in the use of agrochemicals which are now being used for plant growth stimulation and control of diseases (Lugtenberg et al. 2001). Development of such a stress-tolerant microbial strain associated with the roots of agronomic crops can lead to improved fertility of salt-affected soils (Mayak et al. 2004; Egamberdieva and Kucharova 2009). The use of beneficial microbes in agricultural production systems started about 60 years ago. The effect of plant growth-promoting bacteria on the growth and nutrient uptake of various

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agricultural crops was well addressed by Kloepper et al. (1980), Lifshitz et al. (1987), Kloepper and Beauchamp (1992), Okon et al. (1998), Lugtenberg et al. (2001), and Glick et al. (2007). Biological control of plant disease by rhizobacteria was also previously reported by other authors (Bloemberg and Lugtenberg 2001; Lugtenberg and Kamilova 2004; Adesemoye et al. 2008). There is now increasing evidence that the use of beneficial microbes can enhance plants' resistance to adverse environmental stresses, e.g., drought, salts, nutrient deficiency, and heavy metal contaminations (Glick et al. 2007).

Understanding the integration of bacterial strains in the rhizosphere of plants and the mechanisms of their interactions are widely recognized as a key to improving the level and reliability of plant growth stimulation by plant growth-promoting rhizobacteria. Most PGPR strains do not have a single mechanism which completely accounts for their beneficial effects on the plant (Burdman et al. 2000; Khalid et al. 2004; Khan 2005; Berg et al. 2005). Some mechanisms by which bacteria are able to stimulate plant growth and to prevent damage caused by plant pathogens include mobilization of nutrients (Lifshitz et al. 1987), production of phytohormones (Frankenberger and Arshad 1995), antagonism (Thomashow and Weller 1996), induction of systemic resistance (van Loon and Glick 2004), and competition for nutrients and niches (Kamilova et al. 2005). However, the interactions among these microbes are still not well understood in field applications under different environments. An understanding of the functions and mechanisms by which bacteria can promote plant growth in stressed environments (e.g., arid region) may provide valuable information on plant–microbe interactions to develop new agricultural technologies that may improve soil ecology and plant development. The objectives of this paper are to discuss recent developments and advances in our understanding of the interactions between the plant and plant growth-promoting rhizobacteria and their mechanism of action under fragile and stressed environments.

## 2.2 Ecological Factors' Effect on PGPR Performance

Many factors have been discussed that may affect rhizosphere microbial communities and it is likely different soils, varieties, climatic conditions, etc. will effect PGPR performance (Sorensen 1997; Paul and Clark 1989). The abiotic environment has, however, been recognized as the main criterion determining the efficiency of plant growth-promoting bacteria in the plant root. In our previous studies, we observed that the bacterial strains isolated from loamy sand increased the root, shoot length and dry weight of whole plants of peas, wheat, and maize by 45% compared to the control. They were more effective at 16°C as compared to incubation at 26°C. Plant growth was not stimulated by bacterial strains at 26°C temperature (Egamberdiyeva and Höflich 2001).

The effect of soil type that has different nutrient status on the stimulatory efficiency of bacterial inoculants may be important for successful root inoculation and plant growth stimulation. Latour et al. (1996) indicate that the soil types is the dominating factor responsible for the diversity of the bacterial populations

associated with plant roots. Bacterial strains *P. alcaligenes* PsA15, *B. polymyxa* BcP26, and *B. amyloliquefaciens* BcA12 significantly increased the shoot, root dry weight (20–42%) and N, P, and K uptake of pea, wheat and maize in serozem soil better than in loamy sand soil.

The bacterial strains had a much better effect on growth and nutrient uptake of plants in nutrient-deficient saline serozem soil than in relatively rich loamy sand. According to Paula et al. (1992), the magnitude of the plant response to any microbial inoculation can be greatly affected by the soil condition. Inoculation of plants with bacteria only marginally increased yields when tested under ideal climatic situations. The greatest benefits occurred when crops encountered stressful conditions (Lazarovits and Norwak 1997), while nontreated plants by comparison performed poorly under such conditions where high pH make nutrients less available to them.

Similar results were reported by Defreitas and Germida (1992) that in low fertility soil, *Pseudomonas* significantly enhanced early plant growth. Such inoculation could compensate for nutrient deficiency and improve plant development through the production of plant growth regulators by microbes at the root interface, which stimulated root development and resulted in better absorption of water and nutrients from the soil (Kloepper and Beauchamp 1992; Wu et al. 2005).

PGPR may enhance mineral uptake including N, P, K, and microelements more efficiently from the soil, not only as a consequence of the increase in root surface area, but also by stimulating the ion uptake systems (Burdman et al. 2000).

Defreitas and Germida (1992) showed that several PGPB strains increased root hair size and number, and these tubular extensions of root epidermal cells can be involved in mineral uptake capacity in two ways. First, root hairs represent a large surface available for ion uptake and, second, they are believed to play an important role in nutrient uptake.

## 2.3 Rhizosphere Colonization and Survival

Soil inoculation of beneficial microorganisms will not result in significant effects unless the environment supports growth and survival of the introduced microorganisms (Bull et al. 1991; Devliegher et al. 1995). Survival strategies also depend on the physiological adaptation in the introduced cells, such as adaptation to nutrient-limited conditions and/or other physical chemical conditions, efficient utilization of root-released compounds or specific interactions with plants (Bull et al. 1991; Van Overbeek and Van Elsas 1997; Devliegher et al. 1995). However in the soil, the survival of the inoculated bacteria largely depends on the availability of an empty niche, helping them withstand competition with the often better-adapted native microflora (Rekha et al. 2007).

Earlier reports claim soil salinity has an adverse effect on plant growth-promoting bacterial populations, but members of potentially pathogenic species survive and become enriched in the rhizosphere (Sato and Jiang 1996; Tripathi et al. 2002).

We also observed that bacterial strains isolated directly from the root of wheat grown in saline soil stimulated plant growth but were also human pathogens (Egamberdiyeva et al. 2008). The rhizosphere, which is relatively rich in organic substrates, stimulates microbial growth and also attracts human pathogens which have also evolved to respond to the same signals; thus, the chances to compete effectively with the indigenous microflora in the rhizosphere are high (Roberts et al. 2000; Ji and Wilson 2002; Jablasone et al. 2005).

Continuing with what was reported by Morales et al. (1996) that the survival and colonization of potentially pathogenic human-associated bacteria in the rhizosphere of plants are poor and their persistence and colonization on plants were decreased by coinoculation of pathogens with naturally occurring bacteria (Jablasone et al. 2005), the hypothesis was tested that beneficial bacteria which have been selected as enhanced colonizers (Validov et al. 2006) may be able to outcompete the pathogens, thereby creating a more environmentally friendly agriculture which is healthier for farmers and users.

Efficient colonizers can be obtained after inoculation of seedlings with a mixture of different bacteria followed by growth of the seedling in a gnotobiotic sand system (Simons et al. 1996). Repeated use of this method yields enhanced root-tip-colonizing wild-type bacteria (Lugtenberg et al. 2001). In our previous work, salt-tolerant enhanced root-colonizing rhizobacteria of wheat were isolated after the third cycle of enrichment for possible use in salinated soil (Egamberdieva and Kucharova 2009). They were nonpathogenic strains, since they do not belong to the risk group 2 (Anonymous 1998).

The subsequent survival and establishment of beneficial microorganisms on the roots is also important for continued plant growth promotion or disease control and root-colonizing indigenous microorganisms. Selected enhanced root tip-colonizing bacteria were able to increase culturable bacterial community in the rhizosphere soil of wheat grown in saline soil (Fig. 2.1).

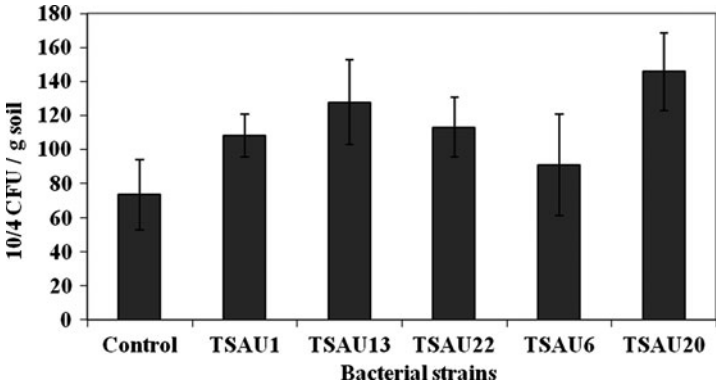


**Fig. 2.1** The effect of root-colonizing bacteria *P. putida* TSAU1, *P. chlororaphis* TSAU13, *P. extremorientalis* TSAU20, *P. extremorientalis* TSAU6, and *P. aureantiaca* TSAU22 on total culturable rhizosphere soil bacterial community of wheat in saline soil ($10^4$ CFU/g soil)

Lugtenberg et al. (2001) have discussed the idea that fast-growing rhizobacteria might out-compete fungal pathogens for carbon and energy sources, and the colonization of large parts of the root system will obviously facilitate biocontrol since colonization can be expected to function as the delivery system for bacterial cells that act as factories of antifungal metabolites. These compounds were shown to be involved in mechanisms through which biocontrol strains can reduce the ability of fungal pathogens to propagate in the soil.

Factors that can influence the survival of microorganisms in soil include soil type, texture, density, pH, temperature, salinity, water potential, organic carbon and inorganic nutrients, as well as the presence of other soil organisms (Van Veen et al. 1997). Information on the effects of different factors on the rhizosphere microflora may help to understand the rhizosphere microbial dynamics in soil. Rifampicin-resistant mutants obtained from bacterial strains *P. chlororaphis* TSAU13 and *P. extremorientalis* TSAU20 were able to survive in the root of wheat and cotton under salt stress. In other experiments, we observed that the total culturable bacteria in the rhizosphere of tomato *P. putida* 1T1, *P. trivialis* 3Re27, *P. extremorientalis* TSAU20 and *S. rhizophila* ep-17 were decreased with increasing salinity level (0–100 mM NaCl) from 5.5 to 5.0 [$\log_{10}$ (CFU/g root)] (Table 2.1).

However, the increase of salinity did not negatively affect root colonization of rifampicin-resistant mutants of *P. putida* 1T1, *P. trivialis* 3Re27, *P. extremorientalis* TSAU20 and *S. rhizophila* ep-17 for tomato. Their numbers were inhibited only at 100 mM NaCl condition (Table 2.2).

Those results showed that in both experiments bacterial strains were able to survive in the root of plants due to their competitiveness and persistence in salinated soil condition (personal communication).

**Table 2.1** The effect of salinity (NaCl) on total bacterial population in the rhizosphere of tomato *P. putida* 1T1, *P. trivialis* 3Re27, *P. extremorientalis* TSAU20 and *S. rhizophila* ep-17 [$\log_{10}$ (CFU/g root)]

| | 0 | 25 | 50 | 75 | 100 |
|---|---|---|---|---|---|
| Treatments | mM NaCl | | | | |
| 1 T1 | 5.3 ± 0.1 | 5.4 ± 0.4 | 5.3 ± 0.3 | 5.1 ± 0.4 | 5.1 ± 0.2 |
| 3Re27 | 5.5 ± 0.2 | 5.4 ± 0.1 | 5.4 ± 0.3 | 5.3 ± 0.3 | 5.2 ± 0.1 |
| TSAU20 | 5.4 ± 0.2 | 5.3 ± 0.3 | 5.3 ± 0.3 | 5.2 ± 0.1 | 5.2 ± 0.1 |
| ep-17 | 5.5 ± 0.2 | 5.4 ± 0.2 | 5.3 ± 0.2 | 5.2 ± 0.2 | 5.0 ± 0.2 |

Plants grown in potting soil for 4 weeks with addition of NaCl of 25, 50, 75, and 100 mM, Rif mutants used for inoculation treatments, $\log_{10}$ (CFU/g root)

**Table 2.2** The effect of salinity (NaCl) on the survival of rifampicin-resistant mutants of *P. putida* 1T1, *P. trivialis* 3Re27, *P. extremorientalis* TSAU20 and *S. rhizophila* ep-17 in the rhizosphere of tomato [$\log_{10}$ (CFU/g root)]

| | 0 | 25 | 50 | 75 | 100 |
|---|---|---|---|---|---|
| Treatments | mM NaCl | | | | |
| 1 T1 | 3.6 ± 0.3 | 3.6 ± 0.3 | 3.6 ± 0.4 | 3.6 ± 0.3 | 3.4 ± 0.3 |
| 3Re27 | 3.9 ± 0.2 | 3.8 ± 0.2 | 3.8 ± 0.2 | 3.8 ± 0.1 | 3.1 ± 0.2 |
| TSAU20 | 3.7 ± 0.1 | 3.7 ± 0.2 | 3.7 ± 0.2 | 3.7 ± 0.1 | 3.2 ± 0.2 |
| ep-17 | 4.0 ± 0.1 | 3.9 ± 0.4 | 3.3 ± 0.5 | 3.2 ± 0.3 | 3.2 ± 0.2 |

Plants grown in potting soil for 4 weeks with addition of NaCl of 25, 50, 75, and 100 mM, Rif mutants used for inoculation treatments, $\log_{10}$ (CFU/g root)

Paul and Nair (2008) reported that the root colonization potential of the salt-tolerant *Pseudomonas* strain was not hampered with higher salinity in soil. As a means of salt tolerance, the strain synthesized the osmolytes Ala, Gly, Glu, Ser, Thr, and Asp in their cytosol.

It is suggested the persisting nature of the introduced bacterial inoculants in nutrient limited habitats is closely related to their ability to resist starvation (Madkour et al. 1990). A direct relationship between starvation resistance and the ability of bacterial survival in soil was reported in earlier studies. Salt-tolerant and temperature-resistant characteristics make bacteria able to adapt under extreme environments (Tripathi et al. 2002).

## 2.4 The Role of Bacterial Phytohormones in Plant Growth Promotion in Saline Soils

Mechanisms by which bacteria can promote plant growth include mobilization of nutrients (Lugtenberg et al. 2002; Lugtenberg and Kamilova 2004) and production of phytohormones (Lifshitz et al. 1987; Frankenberger and Arshad 1995). The microbial synthesis of plant growth regulators is an important factor in soil fertility. Gibberellin and indole acetic acid are secondary metabolites, which are important biotechnological products, obtained commercially from bacteria and fungi for the agricultural and horticultural industry (Patten and Glick 2002).

Indole acetic acid (IAA) is the most common natural auxin found in plants. IAA is involved in physiological processes such as cell elongation and tissue differentiation (Taiz and Zeiger 1991; Frankenberger and Arshad 1995) and has also been associated with the plant growth-promoting effect of numerous rhizospheric microorganisms (Patten and Glick 2002).

It is thought that the repressive effect of salinity on germination could be related to a decline in endogenous levels of plant growth hormones or phytohormones (Zholkevich and Pustovoytova 1993; Jackson 1997; Debez et al. 2001). It has been reported previously that salt stress reduces the supply of cytokinin from root to shoot and also the recovery of diffusible auxin from maize coleoptile tips (Itai et al. 1968). Indeed, the exogenous application of plant growth regulators (PGRs), e.g., gibberellins (Prakash and Parthapasenan 1990; Afzal et al. 2005), auxins (Gul et al. 2000; Khan et al. 2001, 2004), and cytokinins (Dhingra and Varghese 1985; Khan and Weber 1986; Gul et al. 2000) produced some benefit in alleviating the adverse effects of salt stress and they also improve germination, growth, fruit setting, fresh vegetable and seed yields and yield quality (Saimbhi 1993). Other authors also reported that presowing wheat seeds with plant growth regulators such as IAA and gibberellins alleviated the growth-inhibiting effect of salt stress (Singh and Dara 1971; Datta et al. 1998; Sastry and Shekhawa 2001; Afzal et al. 2005).

Sakhabutdinova et al. (2003) also reported that salinity resulted in a progressive decline in the level of IAA in the root system of plants. In this condition, soaking

seeds with plant growth regulators and applying additional natural phytohormones supplied sufficient hormones for normal plant development and growth in saline conditions (Kabar 1987; Afzal et al. 2005).

It is also suggested that root-colonizing bacteria which produce phytohormones, when bound to the seed coat of a developing seedling, may act as a mechanism for plant growth stimulation and these organisms can prevent the deleterious effects of stresses from the environment (Lindberg et al. 1985; Frankenberger and Arshad 1995). Salt-tolerant IAA-producing bacterial strains *P. aureantiaca* TSAU22 and *P. extremorientalis* TSAU20 alleviated quite successfully the reductive effect of salt stress on percentage of germination (up to 79%), probably through their ability to produce IAA (Egamberdieva 2009). They were able to produce indole-3-acetic acid (IAA) in saline conditions as well. Hasnain and Sabri (1996) showed that inoculation of wheat with *Pseudomonas* sp. stimulated plant growth by reducing plant uptake of toxic ions and increasing the auxin content.

Also, several workers have demonstrated the production of phytohormones by plant growth-promoting bacteria (Zimmer et al. 1995) and which may have pronounced effects on plant growth and development (Frankenberger and Arshad 1995).

They may supply additional phytohormone to the plants, and thus may help stimulate root growth and reverse the growth inhibiting effect of salt stress to a certain extent in both the shoot and the root (Egamberdieva 2009). Such results suggest recommending root-colonizing bacteria that produce phytohormone to alleviate the salt stress of wheat grown under conditions of soil salinity, without any genetic manipulation of the plant. These organisms should therefore be considered as a seed dressing in field trials to improve growth and yield of wheat in farms where soil salinity is high.

## 2.5 Alleviation of Salt Stress in Plants by PGPR

Inhibition of plant growth by salinity is considered to be due to the toxic effects of NaCl, to the ability of the root system to control entry of ions to the shoot and to slowing down water uptake of plants (Hajibagheri et al. 1989). Jamil et al. (2006) reported that salt stress decreased germination and also delayed the emergence of seeds in four vegetable species.

Plant stress can be reduced by 1-aminocyclopropane-carboxylate (ACC) deaminase-producing bacteria (Glick et al. 1997). The plant hormone ethylene has previously been found to be an inhibitor of plant root elongation (Abeles et al. 1992), and its production in plants is highly dependent on the endogenous levels of ACC. The ACC deaminase enzyme can cleave the ethylene precursor ACC to α-ketobutyrate and ammonium and thereby lower the level of ethylene in developing or stressed plants (Glick 1995; Glick et al. 1998). Thus, plant growth-promoting bacteria contain the enzyme ACC deaminase and colonize the seed coat of a developing seedling, and may decrease the ethylene level so that it does not

become too high to limit root growth (Glick et al. 1998; Hontzeas et al. 2004). Furthermore, by removing ACC, the bacteria reduce the deleterious effect of ethylene, ameliorating plant stress and promoting plant growth (Shah et al. 1997; Glick et al. 2007). These bacteria may enhance the survival of some seedlings, especially during the first few days after the seeds are planted. In our previous study, only strain *P. trivialis* 3Re27 could utilize ACC as the sole N source (Egamberdieva and Kucharova 2009) and showed high stimulatory effect on the growth of plants in saline soils.

Soil salinity particularly disturbs the symbiotic interaction between legumes and rhizobia. Numerous studies have shown that soil salinity causes decrease in nodulation and reduces dramatically $N_2$ fixation and nitrogenase activity of nodulated legumes such as soybean (Singleton and Bohlool 1984), common bean, and faba bean (Rabie et al. 2005).

Other authors reported a decrease of rhizobial colonization and shrinkage of root hairs of soybean, common bean, and chickpea in salt stress (Zahran and Sprent 1986; Singleton and Bohlool 1984; Elsheikh and Wood 1990).

The study of Marcar et al. (1991) indicated that symbiotic properties were more sensitive to salinity than plant growth. Thus, the development of salt-tolerant symbioses is an absolute necessity to enable cultivation of leguminous crops in salt-affected soils (Yousef and Sprent 1988; Lauter et al. 1981; Velagaleti and Marsh 1989).

In the rhizosphere, the synergism between various bacterial genera, such as between *Bacillus, Pseudomonas*, and *Rhizobium* has been demonstrated to promote plant growth and development (Bolton et al. 1990; Halverson et al. 1993). The coinoculation with *Pseudomonas* spp. and *Rhizobium* spp. had enhanced nodulation and nitrogen fixation, plant biomass, and grain yield in various leguminous species including alfalfa (Bolton et al. 1990), soybean (Dashti et al. 1998), chickpea (Goel et al. 2002), and pea (Tilak et al. 2006). We have also observed that root-colonizing *Pseudomonas* strains improve rhizobia–legume interactions. Combined inoculations could be an option to improve plant growth and increase nodule numbers and N content of galega species (personal communications).

Plant growth-promoting bacteria can prevent the deleterious effects of stressors from the environment (Lugtenberg et al. 2001; Egamberdieva 2009). Hasnain and Sabri (1996) reported that inoculation of plants with *Pseudomonas* sp. stimulated plant growth by reduction of toxic ion uptake and increases in auxin contents.

Another explanation for enhancement of nodule formation by the rhizobia in legumes might be the production of hydrolytic enzymes such as cellulases by root-colonizing *Pseudomonas* strains, which could make penetration of rhizobia into root hairs or intercellular spaces of root cells easier, leading to increased numbers of nodules (Sindhu and Dadarwal 2001).

Under drought stress, coinoculation of bean (*Phaseolus vulgaris* L.) with *Rhizobium tropici* and two strains of *P. polymyxa* resulted in augmented plant height, shoot dry weight, and nodule number. Coinoculation of lettuce (*Lactuca sativa* L.) with PGPR *Pseudomonas mendocina* and arbuscular mycorrhizal fungi (*Glomus intraradices* or *G. mosseae*) augmented an antioxidant catalase under severe

drought conditions, suggesting that they can be used in inoculants to alleviate the oxidative damage elicited by drought (Tain et al. 2004; Yang et al. 2009).

## 2.6 Concluding Remarks

As discussed in this review, ecological factors such as temperature and soil nutritional condition affect the performance of plant growth-promoting rhizobacteria. The bacterial inoculation has a much better stimulatory effect on plant growth and nutrient uptake in nutrient-deficient soil than in nutrient-rich soil. The screening and application of the enhanced potential root-colonizing rhizobacteria is essential for developing sound strategies to manage the rhizosphere in such a way that it becomes more difficult for pathogens to colonize the same. Thus, these beneficial bacteria can engineer positive interactions in the rhizosphere and stimulate plant growth, nutrient uptake, and symbiotic performance of *Rhizobium* under saline conditions.

The phytohormone auxin produced by root-colonizing bacteria plays an important role in alleviating salt stress in plants and these organisms should therefore be considered as a seed dressing in field trials to improve growth and yield of crop plants in farms where soil salinity is high. To further understand the highly complex nature of microbial adaptation and their response to alterations in the biological, chemical, and physical environment of the rhizosphere remains a significant challenge. Hopefully, new research will provide farmers with novel control strategies for the development of microbial strains that are more effective and have longer shelf-lives as a "plant growth stimulators" and "biocontrol" to supplement and/or complement chemical fertilizers and pesticides in agriculture.

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# Chapter 3
# Plant Growth Promoting Rhizobacteria as Alleviators for Soil Degradation

**Metin Turan, Ahmet Esitken, and Fikrettin Sahin**

## 3.1 Introduction

The long-term development of global socio-economic systems requires the sustainable use of natural resources. This paradigm is fundamental in the well-established concept of sustainable development defined in the report by Bruntland (1987), which states that sustainable development is development that "meets the needs of the present without compromising the ability of future generations to meet their own needs." The International Institute for Sustainable Development (1996) proposed a Sample Policy Framework which includes a sustainability index that "would give decision makers tools to rate policies and programs against each other." In the past decades, with the progress of the sustainability paradigm, the formulation of metrics and indices of sustainability of systems and sustainable development evolved and produced comprehensive indexing methods (Brown and Ulgiati 1999; Esty et al. 2005). These developments provided a framework to formulate sustainability perception applicable for soil-use and conservation planning.

The sustainable use of soil resources depends on three factors: soil characteristics, environmental conditions, and land use. These factors interact on systems-based principles, where the change in one factor causes alteration in the others. Therefore, the sustainable use of soil resources is a dynamic category. It is important to assess our soil resources from this standpoint and consider soil as the prime object of sustainable use in relation to land management under given natural

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conditions. This approach needs to be an integral part of land-use planning and decision making on different levels, ranging from the local to world scales. Land improvement is an increase in productivity change that reflects both natural- and human-induced processes of degradation and improvement.

Land degradation can be considered in terms of the loss of actual or potential productivity or utility as a result of natural- or human-induced processes acting upon the land; it is the decline in land quality or reduction in its productivity. In the context of productivity, land degradation results from a mismatch between land quality and land use (Beinroth et al. 1994). Mechanisms that initiate land degradation include physical, chemical, and biological processes (Lal 1994). Important among physical processes are a decline in soil structure leading to crusting, compaction, erosion, desertification, anaerobism, environmental pollution, and unsustainable use of natural resources. Significant chemical processes include acidification, leaching, salination, decrease in cation retention capacity, and fertility depletion. Biological processes include reduction in total and biomass carbon and decline in land biodiversity. The latter comprises important concerns related to eutrophication of surface water, contamination of groundwater, and emissions of trace gases ($CO_2$, $CH_4$, $N_2O$, and NOx) from terrestrial/aquatic ecosystems to the atmosphere. Soil structure is the important property that affects all three degradation processes. Thus, land degradation is a biophysical process driven by socioeconomic and political causes.

Farmland degradation can also have important negative effects of the farm, including deposition of eroded soil in streams or behind dams, contamination of drinking water by agrochemicals, and loss of habitat. Existing estimates of the current global extent and severity of the problem should be considered indicative at best. The Global Land Assessment of Degradation (GLASOD), based only on the impressions of experts, estimates that nearly two billion ha worldwide (22% of all cropland, pasture, forest, and woodland) have been degraded since mid-century. Around 3.5% of the two billion totals are estimated to have been degraded so severely that the degradation is reversible only through costly engineering measures, if at all. Just over 10% has been moderately degraded, and this degradation is reversible only through major on-farm investments. Of the nearly 1.5 billion ha in cropland worldwide, about 38% is degraded to some degree.

Various sources suggest that 5–10 million ha are being lost annually due to severe land degradation. If this trend continues, 1.4–2.8% of total cropland, pasture, and forest land will lost by 2020. Declining yields (or increasing input requirements to maintain yields) could be expected over a much larger area. These data are, however, likely to overestimate the problem, as they do not account for the effects of land improvements, which also appear to be widespread.

Many attempts have been made to reduce the drastic effect of stressors on growth and productivity, most focusing on chemical amelioration. Recently, a biological approach using plant growth promoting rhizobacteria (PGPR) inoculation was attempted. PGPRs are free-living microorganisms having beneficial effects on plants by colonizing in their rhizosphere or phyllosphere (Bashan and de Bashan 2005). PGPRs may improve plant growth and yield by indirect and direct

mechanisms. Direct mechanisms may act on the plant itself and effect growth by means of plant growth regulators such as auxin, cytokinins, and gibberellins or lowering of the ethylene in plants, solubilizing of inorganic phosphate and mineralization of organic phosphate and/or their nutrients, asymbiotic fixation of atmospheric nitrogen, and stimulation of disease-resistance mechanisms (systemic acquired or induced resistance) (Zahir et al. 2004; Bashan and de Bashan 2005; Antoun and Prevost 2006; Podile and Kishore 2006). In the indirect mechanism, PGPRs may have an antagonistic effect against phytopathogenic microorganisms and act like biocontrol agents controlling plant disease-causing organisms, stimulating beneficial symbioses, and/or protecting the plant by degrading xenobiotics in contaminated soils (Jacobsen 1997). Additionally, they improve plant tolerance to stresses such as drought, high salinity, metal toxicity, and pesticide load (Bashan and de Bashan 2005).

## 3.2 Modes of Action of PGPRs Under Degraded Soil Conditions

PGPRs are naturally occurring soil microorganisms that colonize roots and stimulate plant growth and yield in a number of ways in many crops under degraded soil conditions. PGPR actions have been divided into two groups: plant growth augmentation by PGPRs that directly benefit the plant growth; and soil bioremediation by PGPRs that indirectly affect plant growth. Amelioration in plant growth can be promoted by PGPR via several diverse mechanisms, including the production of plant hormones such as indole-3-acetic acid, cytokinin, and gibberellins, or degradation of plant ethylene by 1-aminocyclopropane-1-carboxylate (ACC) deaminase and nitric oxide (NO) which is considered a signal molecule under stress conditions in the plant. These stimulate growth of plant organs via cell division and expansion (Taiz and Zeiger 2002) or by improving nutrient availability (Glick 1995; Chabot et al. 1996; Yanni et al. 1997).

In addition to these, the growth stimulation in plants by PGPRs can be a direct effect of the production of secondary metabolites such as phytohormones, riboflavin, and vitamins (Dakora 2003). Enhanced plant nutrition by PGPRs is mainly through increased phosphorous uptake by solubilization of inorganic phosphate or mineralization of organic phosphate. They also release organic acids, which help to make available forms of nutrients (Biswas et al. 2000) and often lead to increased plant growth through uptake of water and mineral nutrients.

### *3.2.1 Ethylene and Bacterial ACC-Deaminase Under Stress Conditions*

Ethylene coordinates and regulates plant growth and functions via several mechanisms. Initially, ethylene was known as a ripening hormone but later

investigations revealed that it merits equal status with the other classes of plant hormones due to its diverse effects and effective role in plant growth and development. In higher plants, the enzyme *S*-adenosyl-L-methionine (SAM) synthetase catalyzes the conversion of methionine to SAM; ACC synthase catalyzes the hydrolysis of SAM to ACC and 5′ methylthioadenosine; and ACC oxidase catalyzes the conversion of ACC to ethylene as well as carbon dioxide and hydrogen cyanide. The recognition of ethylene as a plant growth regulator originated from observations of premature shedding of leaves, geotropism of etiolated pea seedlings when exposed to illuminating gas, early flowering of pineapples treated with smoke, and ripening of oranges exposed to gas from kerosene combustion. The plant hormone ethylene has been known to have an important role in root initiation and elongation, nodulation, senescence, abscission, and ripening in addition to stress signaling. When applied exogenously, it may stimulate adventitious root formation and hairy root initiation, fruit ripening, flower wilting, and leaf senescence. Endogenous ethylene production regulates xylem formation, flowering in some plants, and induces fruit ripening as well as flower wilting. In the case of stress response, ethylene may inhibit root elongation, nodulation and auxin transport, induces hypertrophies, speeds aging, and promotes senescence and abscission. The combination of auxin and ethylene regulates lateral root initiation and exudation of resins and gums (Dugardeyn and van der Straeten 2008).

During periods of environmental stress, plants produce high levels of "stress ethylene." Moreover, much of the growth inhibition that occurs as a consequence of an environmental stress is the result of the response of the plant to the increased levels of stress ethylene which exacerbates the response to the stressor (Glick et al. 2007). In addition, inhibitors of ethylene synthesis can significantly decrease the severity of some environmental stresses. Thus, if ACC deaminase-containing bacteria can lower plant ethylene levels, treatment of plants with these organisms should provide some protection against the inhibitory effects of these stresses (Glick et al. 1998).

The model description of the mode of action of PGPR containing ACC deaminase was precisely elaborated originally by Glick et al. (2007). They comprehensively addressed the question how bacterial ACC deaminase having a low affinity for ACC can effectively compete with the plant enzyme, ACC oxidase, which has a high affinity for the same substrate, resulting in the reduction of plant's endogenous ethylene concentration. They argued that the biological activity of PGPRs relates to the relative amounts of ACC deaminase and ACC oxidase in the system under consideration. For PGPRs to be able to lower plant ethylene levels, the ACC deaminase level should be at least 100- to 1,000-fold greater than the ACC oxidase level. This is likely to be the case, provided that the expression of ACC oxidase has not been induced (Glick et al. 2007).

The role of bacterial ACC deaminase in plant growth promotion was previously explained by the fact that the epiphytic and endophytic ACC deaminase-producing PGPRs synthesize indoleacetic acid (IAA) in response to root exudates including the amino acid tryptophan. Plant cells take up the IAA secreted by PGPRs and synthesize the plant ethylene precursor ACC, which is cleaved by bacterial ACC

deaminase to form ammonia and α-ketobutyrate, both of which are readily metabolized by the bacteria. As a consequence of lowering the level of ACC within a plant, the amount of ethylene that can form is reduced. The result of this interaction is significant increase in plant root and shoot length and biomass, and protection of plants from inhibitory effects of biotic and abiotic stress factors (Glick et al. 2007; Spaepen et al. 2009).

### 3.2.2 Nitric Oxide and Bacterial NO in Plants under Stress Conditions

Nitrogen monoxide or nitric oxide (NO) is an important molecule that acts in many tissues to regulate a diverse range of physiological processes. NO is a highly reactive molecule that rapidly diffuses and permeates cell membranes. It is becoming apparent that NO is also a ubiquitous signal in plants. Different studies have particularly demonstrated NO signaling in the induction of cell death, defense genes, and interaction with reactive oxygen species (ROS) during plant defense against pathogen attack (Besson-Bard et al. 2008; Krasylenko et al. 2010).

Many of previous studies demonstrated that NO radicals have strong affects on plant metabolism and physiology. It has been also known for a long time that plants release NO under normal growing conditions and NO can accumulate in the atmosphere from a variety of sources such as industrial pollution. The effects of NO on plant growth and development were established as being concentration dependent: high concentrations (40–80 pphm) inhibited tomato growth, while low concentrations (0–20 pphm) enhanced it; similar findings were also reported for lettuce and pea plants. NO also increased chlorophyll content in pea leaves, mainly in guard cells, and retarded chlorophyll loss in *Phytophthora infestans*-infected potato leaves. The positive effects of NO on chlorophyll maintenance may reflect NO effects on iron availability. NO has senescence-retarding properties as a result of inhibition of ethylene biosynthesis. On the contrary, some reports revealed that treatment of *Arabidopsis* plants with NO raised the ethylene level and inhibition of NO biosynthesis did not affect the ethylene increment. Fruit ripening is an ethylene-promoted physiological process that can be delayed by NO. During fruit ripening, ethylene formation increases and this occurs together with reduced NO release. Moreover, treatment of fruits with NO also delayed their senescence and prolonged their postharvest period. NO has also been reported as a stimulator of seed germination in different plant species. In summary, NO affects plant growth and differentiation, hypocotyl elongation, senescence, seed germination, root growth, and de-etiolation by interacting with plant growth regulators in different plant species (Palavan-Unsal and Arisan 2009).

NO is mainly formed in actively growing tissue such as embryonic axes and cotyledons and the levels decrease in mature and senesced organs. The small size and high diffusion rate of NO through membranes mean that NO fits the concept

that hormones are easily transported. NO also acts at low concentrations and most of its functions are dependent on its amount; in this framework, NO could be considered to be a plant growth regulator. Recent studies suggested that NO mediates some cytokinin effects. Cytokinin has been demonstrated to induce NO synthesis. NO can imitate some cytokinin effects: NO donors induced betalaine accumulation and NOS inhibitors inhibited cytokinin-induced betalaine accumulation. Nitric oxide is able not only to initiate programmed cell death but also to delay its occurrence. Moreover, NO has been identified as a mediator of guard cell ABA signaling. ABA induces the synthesis of NO in guard cells; NO induces stomatal closure and either scavenging of NO or inhibition of NO synthesis reduces ABA-induced stomata closure. Additionally, NO biosynthesis has also been established to be induced by auxin. NO was needed for root growth and the formation of lateral roots. NO can stimulate cell division and embryogenic cell formation in leaf protoplast-derived cells in the presence of auxin. NO donors sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine delayed GA-induced programmed cell death in aleurone layers. In these cells, death is promoted by ROS, and NO delays death by acting as an antioxidant. In addition, researchers also investigated the relation of NO with the plant stress hormone ethylene. Low concentrations of NO either endogenously produced or exogenously applied in the $10^{-6}$ M range exert significant growth promoting and ethylene inhibiting effects, which are reversed by higher NO concentrations or equimolar applications of the NOS inhibitor N6-methyl-arginine or NO-releasing compounds (Palavan-Unsal and Arisan 2009; Krasylenko et al. 2010).

NO as a key signaling molecule has been involved in mediation of a variety of biotic- and abiotic stress-induced physiological responses in plants. A variety of stresses, for example, drought, low and high temperature, UV and ozone exposure induce the generation of ROS (Neill et al. 2002; Vranova et al. 2002; Arasimovic and Floryszak-Wieczorek 2007). ROS initiate various signaling pathways. Therefore, preservation of suitable ROS levels might correspond to survival response. NO interacts with ROS in different ways and serve as an antioxidant during various stresses.

NO is also a metabolic product of certain bacteria. It is now evident that NO is enzymatically produced for regulatory purposes in plants, fungi, protozoa, and bacteria (Cohen et al. 2006). Nitric oxide (NO) is one of the nitrogen species produced by the nitrate dissimilation pathway in bacteria. It is now known that bacteria, like eukaryotes, can catalyze NO production via nitric oxide synthase (NOS). In a process unrelated to dentrification-linked NO formation of some bacteria, NOS produces NO by oxidizing L-arginine to L-citrulline (Cohen et al. 2010). Most biogenic NO is released as a product of bacterial energy metabolism. Some bacteria also produce NO specifically for cell signaling and, in one group, for nitration of an important secondary metabolite. As a result, NO profoundly affects plant growth, development, and stress physiology. Among the diversity of NO-mediated effects in plants, NO acts downstream of auxins to modulate adventitious and lateral root formation as well as root hair development. NO-ascribed and rhizobacteria-induced effects on root architecture show a great similarity

(Molina-Favero et al. 2007; Cohen et al. 2010). Thus, these bacteria may have beneficial effects on plant growth and development with/without stress conditions.

## 3.3 Plant Growth Amelioration by PGPRs in Degraded Soils

Human activities have often led to degradation of the world's land resources, which are the basis for sustained food security. The global assessment of human-induced soil degradation has shown that damage has occurred on 15% of the world's total land area, mainly resulting from erosion, nutrient decline, salination and chemical deterioration, and physical compaction. These impacts frequently lead to reductions in yields. Land conservation and rehabilitation are essential parts of sustainable agricultural development. While severely degraded soil is found in most regions of the world, the negative economic impact of degraded soil may be most severe in the countries most dependent on agriculture for their incomes.

Salinity is a major abiotic stress reducing the yield of a wide variety of crops all over the world (Ashraf and Foolad 2007). Worldwide, 100 million ha or 5% of the arable land is adversely affected by high salt concentration, which reduces crop growth and yield (Ghassemi et al. 1995). Globally, more than 770,000 $km^2$ of land is salt affected by secondary salination: 20% of irrigated land and about 2% of dry agricultural land (FAO 2000).

The restriction of plant growth and productivity due to salinity is especially acute in arid and semiarid regions around the world (Kuznetsov and Shevyakova 1997). Salinity may occur when there is irregular irrigation, inadequate drainage, and wrong fertilizer application, and it increases extremely particularly in protected cultivation (Wang et al. 2003). Plant survival and growth under saline condition are dependent on adaptations to reestablish ionic balance. Much effort has been devoted toward understanding the adaptive mechanism to salt tolerance (Zhu et al. 1997; Borsani et al. 2001). Salt tolerance in plants depends mainly on the capability of roots for (a) restricted or controlled uptake of $Na^+$ and $Cl^-$ and (b) continued uptake of essential elements, particularly $K^+$ and $NO^{3-}$ (Ashraf et al. 2004).

Strategies for alleviation of salt stress involve developing salt-resistant cultivars, leaching excess soluble salts from upper to lower soil depths, flushing soils that contain salt crusts at the surface, reducing salt by harvesting salt-accumulating aerial plant parts in areas with negligible irrigation water or rainfall for leaching, and amelioration of saline soils under cropping and leaching (Qadir et al. 2000). Breeding for tolerance to salinity in crops has usually been limited by a lack of reliable traits for selection. Multiple genes seem to act in concert to increase salinity tolerance, and certain proteins involved in salinity stress protection have also been recognized (Murillo-Amador et al. 2006).

Salinity induces osmotic stress by limiting absorption of water from soil and ionic stress resulting from high concentration of potentially toxic salt ions within plant cells. Salt stress is also linked to an oxidative stress as a consequence of generation of ROS such as superoxide ion, hydrogen peroxide and hydroxyl

radicals, which are detrimental to plant survival under stress conditions. Salt-stressed plants display a complex oxidative defense strategy, with catalase and peroxidases enzymes being involved in scavenging of the hydrogen peroxide generated in response to oxidative stress. Saline stress is also known to affect many physiological activities related to the accumulation of ions and osmolytes such as proline (Lee et al. 2008). The accumulation of these compounds plays a major role in the process of osmotic adjustment, limiting water loss and ion toxicity. Biochemical alterations in plants due to salt stress may affect the nutritional balance and, consequently, growth and development.

Inorganic phosphate uptake and transport appear to be sensitive to salinity (Ehsanpour and Amini 2003) and this may have severe consequences for the acid phosphatase involved in inorganic phosphate assimilation in plants. The use of PGPRs and symbiotic microorganisms, especially arbuscular mycorrhizal (AM) fungi, may prove useful in developing strategies to facilitate plant growth in saline soils. More specifically, the soil-borne Pseudomonads have received particular attention because of their catabolic versatility, excellent root-colonizing ability, and capacity to produce a wide range of enzymes and metabolites that help the plant withstand varied biotic and abiotic stress conditions (Vessey 2003). Relatively few mechanisms have been demonstrated that explain the increased resistance to environmental stresses of plants treated with bacteria of the genus *Pseudomonas*. PGPRs can facilitate plant growth indirectly by reducing plant pathogens, or directly by facilitating the uptake of nutrients from the environment, by influencing phytohormone production (e.g., auxin, cytokinin, and gibberellins), and by lowering plant ethylene levels and/or production of siderophores (Glick et al. 1997; Kohler et al. 2006). Many studies have demonstrated that inoculation with PGPR improves growth of plants under salt stress (Hamdia et al. 2004; Han and Lee 2004a, b; Mayak et al. 2004; Woitke et al. 2004; Yildirim et al. 2006, 2008a, b; Karlidag et al. 2011).

Soil acidification and aluminium toxicity are probably the major limiting factors to plant growth and crop production in many agricultural areas of the world (Baligar and Fageria 1997; Kamprath 1984). These soils, having high P-fixing capacity, need intensive P fertilization rates for obtaining economic yields and have more than half of their total P as organic P (Sanchez 1976). Widada et al. (2007) have tested for the inoculation effects of arbuscular mycorrhizal fungi (AMF) or/and rhizobacteria (phosphate-solubilizing bacteria, PSB; $N_2$-fixing bacteria, NFB; and siderophore-producing bacteria, SPB) on the growth and nutrients' uptake of sorghum (*Sorghum bicolor*) in acid and low-availability phosphate soil. As a result, the inoculation of either AMF or each rhizobacterium improved the plant dry weight and uptake of nutrients such as N, P, Fe, and Zn. Dual inoculation of AMF and each rhizobacterium yielded higher plant dry weight and nutrients' uptake compared to single inoculation.

Soil alkalinization and lime-induced Fe chlorosis are also major problems in agricultural areas. Lime-induced iron chlorosis is a term often used for chlorosis associated with disturbed Fe metabolism on high Ca-containing soil. In wet calcareous soils, the dynamics of bicarbonate formation is much higher and depends on

high $CO_2$ pressure in the soil and hydrolysis of $CaCO_3$, which requires the presence of water. Thus, lime-induced Fe chlorosis is considered to be a great problem in particular irrigated areas. Ipek et al. (2011) had studied the impact of PGPR inoculants on the growth, yield, and nutrition of strawberry in a greenhouse under high calcareous soil conditions. *Alcaligenes* 637Ca, *Agrobacterium* A18, *Staphylococcus* MFDCa1 and MFDCa2, *Bacillus* M3, and *Pantoea* FF1 inoculated to strawberry root before planting significantly increased fruit yield and mineral nutrition, especially N, P, K, Fe, Mn, and Cu. Contrary to high Ca, calcium deficiency often caused blossom-end rot (BER) in tomato plants. BER can also occur under suboptimal growth conditions such as drought (Nishio and Morita 1991) and high salinity (Ehret and Ho 1986), even when the calcium concentration is sufficient for fruit development under normal conditions. Lee et al. (2010) investigated the effect of *Pseudomonas* sp. LSW25R on tomato growth and BER, because this bacterium stimulates uptake of Ca and promotes growth. They found that root inoculation of LSW25R significantly promoted the fresh weight, height, and dry matter of tomato plants and also reduced blossom-end rot of tomato fruits (up to 61%) as compared to the control.

## 3.4 Bioremediation by PGPRs on Heavy Metal-Polluted Soil

Industrial pollution of surface water and soil has become a serious environmental problem. Soils can be contaminated with heavy metals derived from various sources, including mining and smelting of metals, electroplating, gas exhaust, energy and fuel production, chemical fertilizers, sewage and pesticide application, municipal waste, abandoned mining wastes, improper treatment of industrial wastes, incomplete collection of used batteries, leakage of landfill leachate, accidental spills, highway traffic, and military activities (Kim et al. 2001). Over recent decades, the annual worldwide release of heavy metals reached 22,000 t (metric ton) for cadmium, 939,000 t for copper, 783,000 t for lead and 1,350,000 t for zinc (Singh et al. 2003). Heavy metal contaminants in roadside soils originate from engine and brake pad wear (e.g., Cd, Cu, and Ni) (Viklander 1998; Ozkutlu et al. 2009); lubricants (e.g., Cd, Cu, and Zn) (Birch and Scollen 2003); exhaust emissions, (e.g., Pb) (Sutherland et al. 2003); and tire abrasion (e.g., Zn) (Smolders and Degryse 2002).

Seventeen of the 53 heavy metals are believed to be involved in the biological process and the functioning of organisms and/or ecosystems. Thus, (1) some heavy metals are important as micronutrients (Fe, Mo, and Mn); (2) some toxic heavy metals have roles as trace elements (Zn, Ni, Cu, V, Co, W, and Cr); and (3) there are some heavy metals without any known nutritional functions but are nonetheless toxic for plants and microorganisms (Hg, Ag, Cd, Pb, and U) (Schutzendubel and Polle 2002).

As a result, groundwater may be contaminated by the leaching action of contaminated soils. Heavy metals can be transferred by the intake of vegetation,

and human and animal health can be impacted through ingestion of both water and foods that have been contaminated by the soil (Lo and Yang 1999). Caution should be taken to control the contamination of soils and water.

Toxic heavy metals cause DNA damage, and their carcinogenic effects in animals and humans are probably caused by their mutagenic ability (Knasmuller et al. 1998; Baudouin et al. 2002). Exposure to high levels of these metals has been linked to adverse effects on human health and wildlife. Lead poisoning in children causes neurological damage leading to reduced intelligence, loss of short-term memory, learning disabilities, and coordination problems. The effects of arsenic include cardiovascular problems, skin cancer and other skin effects, peripheral neuropathy (WHO 1997), and kidney damage. Cadmium accumulates in the kidneys and is implicated in a range of kidney diseases (WHO 1997). The principal health risks associated with mercury are damage to the nervous system, with such symptoms as uncontrollable shaking, muscle wasting, partial blindness, and deformities in children exposed in the womb (WHO 1997). Metal-contaminated soil can be remediated by chemical, physical, or biological techniques (McEldowney et al. 1993). Chemical and physical treatments irreversibly affect soil properties, destroy biodiversity, and may render the soil useless as a medium for plant growth.

Until now, methods used for their remediation such as excavation and land fill, thermal treatment, acid leaching, and electroreclamation were not suitable for practical applications, because of their high cost, low efficiency, large destruction of soil structure and fertility, and high dependence on the contaminants of concern, soil properties, site conditions, and so on. Treatments can be done in situ or ex situ, which are both extremely expensive. These include high-temperature treatments, use of solidifying agents, and washing (USDA and NRSC 2000). Thus, the development of phytoremediation strategies for heavy metal-contaminated soils is necessary (Chaney et al. 2000; Cheng et al. 2002; Lasat 2002).

In nature, plants are continuously interacting with a large number of microorganisms. While pathogenic microbes inhibit plant growth, symbiotic microorganisms help plants grow better by providing substances that are, in principle, not synthesized or metabolized by plants themselves. Indeed, farmers have already known through their experience that some soil bacteria are useful to increase crop yields in the fields, implying that these bacteria during evolution have become adapted to soil conditions. The size of plants, especially the roots, is critical for absorbing the underground substances and is known to be positively correlated with the phytoremediation activity of plants (Gleba et al. 1999). In many cases, however, soil contaminants are known to inhibit plant growth likely by activating plant stress responses, causing the dilemma of using plants as soil pollutant removers. Therefore, in the phytoremediation field, many researchers are trying to generate transgenic plants whose tolerance to soil contaminants is increased and whose growing activity under high concentrations of pollutants is retained. In this context, it might be valuable to use some beneficial soil bacteria as stimulants of plant growth to take up more soil pollutants by maintaining plant size.

Phytoremediation is an emerging technology that uses various plants and microorganism to degrade, extract, contain, or immobilize contaminants from soil and water (EPA 2000). The idea of using metal-accumulating plants to remove heavy metals and other compounds was first introduced in 1983, but the concept has actually been implemented for the past 300 years on wastewater discharges (Chaney et al. 1997). Phytoremediation involves the interaction of plant roots and the microorganisms associated with them to remediate soils containing elevated concentrations of organic compounds. These techniques could provide cost-effective methods of remediating soils and groundwater contaminated with metals, radionuclides, and various types of organics, with fewer secondary wastes and less environmental impact than would be generated using traditional methods (Miller 1996).

Some PGPR strains are known to have potential for development in heavy metal contaminated soils as they mitigate the toxic effects of heavy metals on plants (Belimov et al. 2004).

Heavy metals adversely affect bacterial viability (Pennanen et al. 1996), activity (Diaz-Ravina and Baath 1996b), and density (Brookes and McGrath 1984; Fliessbach et al. 1994; Koomen et al. 1990). However, as a consequence of (typically plasmid-encoded) heavy metal resistance, some bacterial populations can adapt to the presence of heavy metals in bulk soil and in the rhizosphere (Diaz-Ravina and Baath 1996a; Malik and Jaiswal 2000; Kozdroj and van Elsas 2000), leading to shifts in microbial community structure (Frostegard et al. 1993, 1996; Gray and Smith 2005; Diaz-Ravina and Baath 1996a). Rhizobacteria have been reported to affect heavy metal availability and accumulation in plants.

Although PGPR was first used for promoting plant growth and for biocontrol of plant diseases, much attention has recently been paid to bioremediation with PGPRs (Huang et al. 2004, 2005; Narasimhan et al. 2003). In contrast with inorganic compounds, microorganisms can degrade and even mineralize organic compounds in association with plants (Saleh et al. 2004). Hence, discovery of effective pathways for degradation and mineralization of organic compounds may play an important role in the future. So far, bacteria capable of degrading a certain kind of organic pollutant, such as polychlorinated biphenyls (PCBs), have been isolated from a range of sites and the pathways encoding genes have also been well studied (Brazil et al. 1995). However, most of these bacteria cannot survive in the near-starvation conditions found in soils, including the rhizosphere (Normander et al. 1999). Recent examples of bioremediation of organic contaminants by PGPRs are shown in Table 3.1. Several effective methods have been developed to improve the degradation efficiency and the tolerance of bacteria to contaminants in soils.

As described above, although rhizobacteria may play an important role in the degradation and mineralization of organic compounds, their metabolic efficiency can be very low. Possible causes may be the small microbial biomass or the low solubility and bioavailability under high toxic pressure (Liste and Alexander 2000). One solution is the employment of plant exudates to promote bacterial degradation. Although PCB-degrading bacteria are found ubiquitously in the environment, the majority of them are still inefficient in degrading PCBs (Donnelly et al. 1994), due

**Table 3.1** Examples of bioremediation of heavy metal by PGPR from polluted land (Yan-de et al. 2007; Zhuang et al. 2007).

| Bacteria | Plant | Heavy metal | Condition | Role of PGPR | Reference |
|---|---|---|---|---|---|
| *Azotobacter chroococcum* HKN-5<br>*Bacillus megaterium* HKP-1<br>*Bacillus mucilaginosus* HKK-1 | *Brassica juncea* | Lead and zinc | Pot experiments in greenhouse | Stimulated plant growth<br>Protected plant from metal toxicity | Wu et al. (2006) |
| *Bacillus subtilis* SJ-101 | *Brassica juncea* | Nickel | Pot experiments in growth chamber | Facilitated Ni accumulation | Zaidi et al. (2006) |
| *Brevundimonas* sp. KR013<br>*Pseudomonas fluorescens* CR3<br>*Pseudomonas* sp. KR017<br>*Rhizobium leguminosarum* bv. *trifolii* NZP561 | None | Cadmium | Culture media | Sequestered Cd directly from solution | Robinson et al. (2001) |
| *Kluyvera ascorbata* SUD165 | *Indian mustard* | Nickel, lead, and zinc | Pot experiments in growth chamber | Both strains decreased some plant growth inhibition by heavy metals | Burd et al. (2000) |
| *Mesorhizobium huakuii* subsp. *rengei* B3 | *Astragalus sinicus* | Cadmium | Hydroponics | Expression of PCSAtgene increased ability of cells to bind $Cd^{2+}$ approximately 9- to 19-fold | Sriprang et al. (2003) |
| *Kluyvera ascorbata* SUD165 and SUD165/26,<br>*Pseudomonas tolaasii* RP23 and *Pseudomonas fluorescens* RS9,<br>*Variovorax paradoxus*,<br>*Rhodococcus* sp. and *Flavobacterium* sp. | Tomato, canola, perennial grasses (Graminaceae), and Indian mustard *(Brassica juncea L. Czern.)* | Cd, Zn, Cu, Ni, Co, Cr, and Pb | Pot and field experiments | Resistant to Cd, Zn, Cu, Ni, Co, Cr, and Pb and stimulation of root elongation of plant seedlings | Burd et al. (1998, 2000), Dell'Amico et al. (2005), Belimov et al. (2005) |

| | | | | | |
|---|---|---|---|---|---|
| *Pseudomonas fluorescens* 2-79 | Wheat | Trichloroethylene (TCE) | Pot experiments in growth chamber | Degraded TCE with toluene *o*-monooxygenase | Yee et al. (1998) |
| *Pseudomonas fluorescens* F113 | Alfalfa | Polychlorinated biphenyls (PCBs) | Pot experiments in growth chamber | More effectively metabolized PCBs with bph gene cloned | Villacieros et al. (2005) |
| *Enterobactor cloacae* CAL2 | Tall fescue | Total petroleum hydrocarbons (TPHs) | Pot experiments in growth chamber | Promoted plant growth in the presence of environmental contaminants such as TPHs | Huang et al. (2005) |

to the rare bacterial population resulting from the lack of sustaining nutrients. Some plants can release structural analogs of PAHs, such as phenols, to promote the growth of hydrocarbon degrading-microbes and their degradation on PAHs (Fletcher and Hedge 1995). The strategy of Narasimhan et al. (2003) for increasing the microbial biomass in rhizosphere is also to use the natural secondary metabolites exuded by wild-type plants. By establishing the *Arabidopsis–Pseudomonas* spp. rhizosphere model, plant secondary metabolites were exuded in sufficient amounts to establish a rhizosphere specific to the rhizobacterial strain capable of metabolizing phenylpropanoids. Also, they indicated that, in cases where the pollutant-degrading microbes are not known to use secondary metabolites, such characteristics could be introduced into them using genetic-engineering methods. Similarly, Chekol et al. (2004) reported reed canarygrass and switchgrass effectively increased microbial dehydrogenase activity to degrade a high level of Aroclor 1248, a kind of PCB.

Several researchers reported inoculation of bacteria into the rhizosphere for the degradation of certain kind of chlorobenzoates and pesticides (Alvey and Crowley 1996; Crowley et al. 1996; Siciliano and Germida 1997); but the mechanisms are not clear. Siciliano and Germida (1999) investigated Dahurian wild rye (*Elymus dauricus*) inoculated with *P. aeruginosa* strain R75 and *P. savastanoi* strain CB35 for the degradation of different kinds of chlorobenzoates such as 2-chlorobenzoic acid (2CBA), 3-chlorobenzoic acid (3CBA), 2,3-dichlorobenzoic acid (23diCBA), and 2,5-dichlorobenzoic acid (25diCBA). They found that inoculants capable of degrading 2CBA can also promote 3CBA degradation but have no effect on 23diCBA and 25diCBA, which suggested the involvement of a different pathway between them. Besides, when these two bacteria were inoculated in a sterile hydroponic plant growth system, no effect on contaminants was detected. So, they hypothesized that inoculants increased degradation of contaminants by affecting the rhizosphere community and the plants provided a suitable habitat for this process.

Over the past 10 years, there has been increasing interest in developing a plant–microorganism-based technology to remediate heavy metal-contaminated soils (Lodewyckx et al. 2001; Cohen et al. 2004; Zhuang et al. 2007; Rajkumar et al. 2009). Phytoremediation of heavy metals includes phytoextraction, rhizofiltration, phytostabilization, and phytovolatilization (Glick 2001). A number of plants which can tolerate and accumulate high concentration of metals were discovered recently and were defined as hyperaccumulators. Ideal hyperaccumulators for bioremediation require the characteristics of rapid growth and a high amount of biomass (Nie et al. 2002). However, in fact, many hyperaccumulators are slow in growth and inhibited in the presence of high concentration of heavy metals. Conversely, the heavy metal contamination has great effects on the microbial communities in soils in several ways (1) it may lead to a reduction of total microbial biomass (Brookes and McGrath 1984; Fliessbach et al. 1994); (2) it decreases numbers of specific populations (Chaudri et al. 1993; Koomen et al. 1990); or (3) it causes shifts in the microbial community structure (Frostegard et al. 1993, 1996; Gray and Smith 2005). Sandaa et al. (1999) suggested that the presence of even small amounts of heavy metals caused a substantial reduction in the total

bacterial diversity. Due to the sensitivity and the sequestration ability of the microbial communities to heavy metals, microbes have been used for bioremediation (Hallberg and Johnson 2005; Kao et al. 2006; Umrania 2006). Although microbial communities in metal-polluted bulk soils have been studied, there is little information on the composition of microbial community in the plant rhizosphere growing in soils that are highly polluted with heavy metals (Dell'Amico et al. 2005). The rhizosphere, with high concentration of nutrients exuded from the roots, attracts more bacteria than in the bulk soils (Penrose and Glick 2001). These bacteria (including PGPR), in reverse, facilitate the growth of the plant. This phytobacteria system is proved to be more effective in removing heavy metals than its ingredients. Soil microorganisms are known to affect the metal mobility and availability to the plant through acidification and redox changes or by producing iron chelators and siderophores for ensuring the iron availability and/or mobilizing the metal phosphates (Abou-Shanab et al. 2003; Burd et al. 2000; Guan et al. 2001). For example, EDTA and EDGA were considered as good chelators to enhance metal availability to plants, whereas these chelators may lead to side effects such as metal leaching and low microbial activity (Ernst 1996; Romkens et al. 2002). Another problem that affects metal uptake is the existing phase of the metal. A large proportion of metal contaminants are unavailable for root uptake by plants, because heavy metals in soils are generally bound to organic and inorganic soil constituents, or present as insoluble precipitates. Hence, the question of how to increase the availability of metals to plants in soils is critical for the success of phytoremediation (Ernst 1996; Kukier et al. 2004). Abou-Shanab et al. (2006) studied the effect of certain rhizobacteria on nickel uptake. They indicated that rhizobacteria facilitated the release of Ni from the nonsoluble phases in the soil, thus enhancing the availability of Ni to *Alyssum murale*. There is a need to improve our understanding of the mechanisms involved in transfer and mobilization of heavy metals by the rhizosphere microbes. The possibility of acid production, and siderophore production and phosphate solubilization cannot be ruled out.

In the case of phytoremediation of metal-contaminated soils and (ground) water, metal availability, uptake and translocation, and phytotoxicity are the main limiting factors (Weyens et al. 2009). Bioaugmentation-assisted phytoextraction represents a promising method (Lebeau et al. 2008). To improve the efficiency of phytoremediation of toxic metal-contaminated soils, plant-associated bacteria can be equipped with pathways for the synthesis of natural metal chelators, such as citric acid, to increase metal availability for plant uptake or, with metal sequestration systems to reduce phytotoxicity and increase metal translocation to aerial plant parts (Sessitsch and Puschenreiter 2008). For instance, after inoculation of *Lupinus luteus* grown on a nickel-enriched substrate with the engineered nickel-resistant bacterium *Burkholderia cepacia* L.S.2.4::nccnre, nickel concentrations in the roots increased approximately 30% (Lodewyckx et al. 2001). Sheng et al. (2008) observed increases in biomass production and total lead uptake in *B. napus* after inoculation with *Microbacterium* sp. G16, a bacterial strain that can produce IAA, ACC deaminase, and siderophores.

Some rhizobacteria can exude a class of secondary metabolite secretion, such as antibiotics (including the antifungals), phosphate solubilization, hydrocyanic acid, IAA, siderophores, and ACC deaminase, which increase bioavailability and facilitate root absorption of heavy metals, such as Fe (Crowley et al. 1991) and Mn (Barber and Lee 1974), as well as nonessential metals, such as Cd (Salt et al. 1995), enhance tolerance of host plants by improving P absorption (Davies et al. 2001; Liu et al. 2000), and promote plant growth (Budzikiewicz 1997; Duffy and Defago 1999; Burd et al. 2000; Ellis et al. 2000; Meyer 2000).

## 3.5 Conclusion

Remediation of degraded soils using biological systems (both microbes and plants) is an emerging area of interest and has shown a substantial progress in situ, which needs to be further consolidated through field trials under different agroclimatic zones of the world. Understanding the mechanistic basis of the physical, chemical, and biological rhizosphere processes and the interactions between resistant plant and PGPRs will be important in modeling better the full impact of remediation in the restoration of derelict lands. Furthermore, the remediation of degraded sites using rhizobacteria is an exciting area of research, since these organisms can easily and inexpensively be mass-produced.

The recent researches of PGPRs on the remediation of degraded soils show a brilliant prospect for the successive studies. Although many successful remediation cases with PGPRs are reported, we still know little about the process mechanism and how PGPRs really interact with plant roots and other bacteria, and how PGPRs can only colonize certain plants in their ecological niche. Some PGPR can increase the tolerance of plants to degraded soil; the PGPR–plant system cannot survive in comparatively extreme environments such as with high concentrations of heavy metals, acidity, and salinity.

Therefore, the molecular engineering of both microbes and plants with desired genes would help immensely to enhance the efficiency of growth-promoting rhizobacteria mediated or plant-based remediation of degraded soils.

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# Chapter 4
# Microbial Products and Soil Stresses

**Mohammad Miransari**

## 4.1 Introduction

Plant can have optimum growth and production under nonstressed conditions. However, there are different soil stresses such as soil salinity, drought, acidity, compaction, heavy metals, and suboptimal root zone temperature adversely affecting plant growth and yield production. Although different methods have been tested on the alleviation of different stresses on plant growth, use of biological methods including soil microbes have been proved to be effective on the alleviation of different stresses (Gamalero et al. 2009; Dimkpa et al. 2009a; Miransari 2010a).

Soil salinity and drought influence plant growth by affecting soil and hence plant osmotic and water potential. In addition, soil salinity can also decrease plant growth due to the presence of ions such as sodium and chloride in extra amounts. Through their competition with some necessary nutrients for plant growth such as potassium and nitrogen and hence disrupting their functioning in the plant, they reduce plant growth and yield production (Munns 2002).

Salinity and drought are common in some parts of the world, especially under arid and semiarid conditions. With respect to their similar effects on water potential in soil and plant, salinity and drought may result in the induction of similar signaling pathways in plants. Such signaling pathways activate the stress genes, which produce proteins such as proline to alleviate the stress. Some of the agricultural soils are also subjected to salinity and drought (Munns 2002).

There are different ways of treating saline soils for crop production including soil leaching and use of tolerant crop plants. In the first method, the rate of leaching fraction is calculated and hence, using extra water, the soil is leached. In the second method plants, which are naturally tolerant to salinity, such as barley, are planted

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under saline conditions, or transgenic plants, which have received the tolerant genes to salinity, are used. Both methods have been proved to be effective in increasing crop yield under salinity (Wong et al. 2008; Schubert et al. 2009).

Under drought, plants which are tolerant to the stress can be planted. There must be some morphological and physiological properties to enable the plant to grow under drought stress. For example, leaf cuticle, root morphology, etc., are among the morphological properties, helping the plant to grow under stress. Plant can produce some proteins such as proline, which increases plant water potential under drought and salinity stress and hence enables the plant to absorb water from the surrounding environment (Chaves et al. 2009).

In addition, plant control over the important process of evapotranspiration is also of especial significance under drought stress. The activity of stomata under drought must be adjusted so that the amount of water evaporated from the plant decreases. Although such a mechanism can also adversely affect the process of photosynthesis by the closure of stomata, it would enhance plant efficiency under drought stress (Wilkinson and Davies 2010).

In humid areas with high rate of precipitation, soil leaching decreases soil pH due to the reduction of basic cations in soil. Decreased pH can adversely affect soil properties including soil structure and the availability of soil nutrients. The adverse effects of soil pH on plant growth under acidic conditions are through the enhanced concentration of ions such as aluminum, iron, and manganese. Such ions can be absorbed at high rates by plant and result in plant malfunctioning due to disrupting the function of some necessary nutrients for plant growth. They can also decrease plant root growth, for example by aluminum (Shaoping et al. 2008).

To alleviate the stress of acidity, application of lime has been extensively used in different parts of the world. Lime or calcium carbonate is able to increase soil pH by producing hydroxyl onions in soil. Lime can also improve soil structure due to presence of calcium in its chemical structure. Compared with sodium, calcium is a divalent cation and hence can bind soil particles, resulting in the establishment of a suitable soil structure (Nicol et al. 2008).

Under compaction, soil bulk density increases and hence root growth in the soil decreases, which reduces the plant water and nutrient uptake and hence plant growth. Different methods have been tested to alleviate the stress on plant growth including the use of a subsoiler and the addition of organic matter to the soil. Such methods have been able to partially alleviate the stress.

The stress of heavy metals in soil is common in, especially, industrial areas. Heavy metals can decrease plant growth through disrupting plant functioning and interfering with the function of necessary nutrients. There have been different methods tested to alleviate the adverse effects of heavy metals on plant growth including using some organic compounds, which can bind heavy metals in soil, and planting hyperaccumulators (Haferburg and Kothe 2010; Miransari 2011).

The stress of suboptimal root zone temperature decreases plant growth and yield production by affecting different plant morphological and physiological properties. Different methods have been used to alleviate the stress, the most important of

which has been the use of breeding techniques to produce plants which can grow faster and complete their growth stages in a shorter time (Farooq et al. 2009).

A wide range of soil microbes which can alleviate soil stresses on plant growth and yield production, including arbuscular mycorrhizal (AM) fungi and plant growth promoting rhizobacteria (PGPR) has been tested. AM fungi are soil fungi developing symbiotic association with most terrestrial plants by building up a very extensive network of hypha and absorbing water and nutrients for the host plant in exchange for carbon (Yang et al. 2009; Dimkpa et al. 2009a). AM fungi also have the following important effects on the soil ecosystem (1) improving soil structure by their extensive hyphal network and production of a protein called glycoprotein, which can bind soil particles, (2) enhancing the plant's ability to absorb higher rates of heavy metals in polluted soils, (3) interacting with the other soil microbes and enhancing their activities, and (4) controlling soil pathogens (Rosendahl 2008; Miransari 2010a, b, 2011a, b).

PGPR can also promote plant growth and yield production by the following interesting mechanisms (1) production of some compounds, for example the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which can alleviate the adverse effects of stress on plant growth by inhibiting ethylene production; production of hydrogen cyanide (HCN), which controls pathogen activities, (2) enhancement of nutrient availability, (3) alleviation of soil stresses on plant growth, (4) production of plant hormones, and (5) fixation of atmospheric N in symbiotic or nonsymbiotic association with their host plant (Berg 2009; Yang et al. 2009).

With respect to the important effects of soil microbes on plant growth under stress and the products that soil microbes produce under stress, some of the most important related findings are presented. Accordingly, the use of soil microbes with their unique abilities can greatly contribute to enhanced ecosystem efficiency under different conditions including stress (Table 4.1). Such abilities are also important in the selection of right microbes for inoculation production (Adesemoye and Kloepper 2009; Miransari 2010b).

## 4.2 Soil Microbes and Salinity Stress

Salinity stress adversely affects plant growth and yield production in different parts of the world, especially in arid and semiarid areas. Use of naturally tolerant plants or genetically modified ones and soil leaching have been among the most common methods of treating saline soil. However, the use of soil microbes and their products has also been proved to be effective in the alleviation of salt stress.

Under different stresses such as saline conditions, the initial stages of symbiosis between rhizobia and their legume host, including the production of signal molecules by the two symbionts are adversely affected. Among the signal molecules, which are produced by the roots of the host plant, is genistein, which is disrupted by the stress. Accordingly, Miransari and Smith (2007, 2008, 2009)

**Table 4.1** Microbial products under stress

| Microbe | Product | Stress | References |
|---|---|---|---|
| *Pseudomonas* spp., *Azospirillum* spp. | ACC deaminase | Salinity, flooding, heavy metal | Glick (2010), Jalili et al. (2009), Zabihi et al. (2011), Arzanesh et al. (2011) |
| *Pseudomonas* spp., *Azospirillum* spp. | Plant hormones | Salinity | Glick (2010), Jalili et al. (2009), Arzanesh et al. (2011) |
| *Pseudomonas* spp. *Azospirillum* spp. | Hydrogen cyanide | Pathogens | Jalili et al. (2009), Abbas-Zadeh et al. (2010), Arzanesh et al. (2011) |
| *Pseudomonas* spp., *Azospirillum* spp. | Phosphatases | Phosphorous deficiency | Jalili et al. (2009), Abbas-Zadeh et al. (2010), Arzanesh et al. (2011) |
| *Pseudomonas* spp., *Azospirillum* spp. | Siderophores | Iron deficiency | Jalili et al. (2009), Abbas-Zadeh et al. (2010), Arzanesh et al. (2011) |
| *Azospirillum* spp. | Polysaccharides | Drought | Sandhya et al. (2009); Belimov et al. (2009) |
| *Bradyrhizobium japonicum* | Genistein | Different stresses | Miransari and Smith (2007, 2008, 2009) |
| AM fungi | Phosphatases | Different stresses | Smith and Read (2008), Miransari et al. (2007, 2008, 2009a, b) |
| AM fungi | Glomalin | Different stresses | Rillig et al. (2003, 2005) |

hypothesized and proved that preincubation of rhizobium with the signal molecule genistein can alleviate the stress of salinity on N-fixation by the bacteria and the host plant. The molecule is able to activate the bacterial genes, and hence partially or completely alleviate the stress of salinity on rhizobium and hence the process of N-fixation.

There is a range of flavonoids produced by legume roots, which make the process of N-symbiosis specific, because each of the flavonoids can only activate one species of rhizobium. Dardanelli et al. (2010) indicated that during the process of symbiosis under salt stress and due to altered production of flavonoids, the PGPR genes may not be activated and hence the necessary lipochitooligosaccharides (LCOs) for root morphogenesis may not be produced by the bacteria. In addition, the presence of bacteria affected the pattern of flavonoid production by soybean roots.

Under salinity stress, the production of ethylene by the host plant increases, which can adversely affect plant growth and yield production. However, PGPRs such as *Pseudomonas* spp. are able to alleviate the stress on plant growth by production of the enzyme ACC deaminase, which can catabolize the precursor of ethylene production ACC into α-ketobutyrate and ammonium (Jalili et al. 2009; Glick 2010). There are also other types of PGPR such as *Azospirillum* spp., which can fix atmospheric N not-symbiotically and also alleviate stresses such as drought on plant growth (Arzanesh et al. 2011).

There are also AM fungi, which can develop nonspecific symbiosis with their host plant and increase its growth by the extensive hyphal network, which significantly increases the uptake of water and nutrients by the host plant (Smith and Read 2008). Such effects can also help the host plant grow under stress conditions. AM

fungi can also accumulate salt ions in their hyphae and prohibit or lower their translocation to the host plant. It has been indicated that use of AM species under field conditions alleviated the stress of salinity on plant growth and yield production. The related reasons were indicated as enhanced nutrient uptake under salinity as well as higher rate of $K^+/Na^+$ in mycorrhizal plants. Various species of AM fungi were different in their ability to alleviate the stress (Daei et al. 2009). Production of enzymes such as phosphatases can also increase phosphorous solubility in soil and hence its uptake by plants. The other likely reasons for the alleviating effects of AM fungi on salinity stress include (1) biochemical alterations (enhanced concentrations of carbohydrates, antioxidants, polyamides, proline, and betaines), (2) physiological alterations (photosynthetic ratios, water properties, production and movement of products across the cellular membrane, production of abscisic acid, and the process of N-fixation), and (3) molecular alterations (gene expression and protein production) (Evelin et al. 2009).

## 4.3 Soil Microbes and Drought Stress

Drought stress can also adversely affect plant growth by reducing the amount of available water for plant use. There has been extensive research work regarding the alleviating effects of AM fungi on plant growth under stress, reviewed by Auge (2001) and Evelin et al. (2009). The extensive hyphal network can significantly increase water and nutrient uptake by the host plant, which is among the most important reasons for the enhanced plant tolerance to the stress.

In their research work, Arzanesh et al. (2011) indicated the most efficient strains of *Azospirillum* spp., which significantly alleviated the stress of drought on wheat growth. The most effective strains had the ability to fix N nonsymbiotically, and produce plant hormones such as auxin. They were also able to produce ACC deaminase, solubilize phosphorous, and produce siderophores. The production of polysaccharides by PGRP is also another effective mechanism by which the bacteria can alleviate the effect of drought stress on plant growth by, for example, enhancing the rate of root colonization by the bacteria. In addition, such polysaccharides can also improves soil structure by binding soil particles and hence alleviate the drought stress (Sandhya et al. 2009; Belimov et al. 2009).

## 4.4 Soil Microbes and Acidity Stress

Under humid condition and due to high amounts of rain, soil cations such as calcium and magnesium are leached, resulting in decreased soil pH and some other adverse effects such as unfavorable structure. The high rate of weathering in humid areas can also modify the 2:1 clay minerals into 1:1 clay minerals and iron and aluminum oxy and hydroxyl minerals and hence decrease the pH. Such effects

together can negatively influence plant growth and yield production. Acid-resistant plant species and lime ($CaCO_3$) have been used as some effective methods to alleviate the stress on plant growth (Shaoping et al. 2008; Nicol et al. 2008).

However, considering the unfavorable effects of stress on soil biological activities can also be useful for the treatment of low pH stress. For example, Miransari and Smith (2007, 2009) found that the process of N-fixation under acidic and salty conditions is prohibited at least in part by the disruption of signal molecule exchange between soybean (*Glycine max* L.) and *Bradyrhizobium japonicum* during the initiation of symbiosis between the two symbionts. Accordingly, they proved that addition of the signal molecule genistein to the bacterial culture before inoculating soybean seeds can partially or completely alleviate the stress. They attributed such a positive effect to the activation of bacterial genes by genistein. Under stress, the production of flavonoids including genistein, which act as secondary metabolites influencing the bacterial genes in a specific manner during the process of symbiosis, is disrupted or decreased to levels much less than necessary for the onset of symbiosis.

On the other hand, under stress, the production of microbial products such as LCOs, which induce morphological alteration in the host plant roots including root hair deformation, curling and bulging, is also disrupted. Accordingly, Miransari et al. (2006) extracted the product by culturing the bacterium under laboratory conditions, while treating with genistein. The produced LCO was then determined qualitatively and quantitatively using HPLC. Then, soybean plant roots were subjected to different pH levels ranging from 4 to 7 and treated with the extracted LCO. Interestingly, addition of LCO resulted in some very similar alteration in soybean root hairs as usually takes place due to the bacteria. Soil microbes may also be able to alleviate the stress of acidic soil pH on plant growth by altering rhizosphere pH (Yang et al. 2009).

## 4.5 Soil Microbes and Compaction Stress

Use of agricultural machinery in the field, especially under high soil moisture, results in the compaction of soil, which particularly at high levels is considered unfavorable for root growth and hence plant growth. Increased soil bulk density makes it difficult for the plant roots to grow into soil pores and absorb water and nutrients. Compacted soils are of unfavorable structure, limiting plant growth and microbial activities. Accordingly, plant growth and yield production decrease under compaction (Miransari et al. 2006).

Miransari et al. (2006, 2007, 2008, 2009a, b) evaluated the effects of soil compaction on corn (*Zea mays* L.) growth and yield production under field (corn) and greenhouse (corn and wheat) conditions using different tractor passing and weights, respectively. They found that, at high compaction levels, plant growth and yield production were adversely affected and signs of pale leaf; reduced height, plant growth, and yield production; and pan cake or cluster growth of plant roots appeared. Accordingly, they hypothesized and proved that using the biological

method of AM fungi utilization may be superior to the use of mechanical methods for the treatment of stress. Both under field (corn) and greenhouse conditions (corn and wheat) after imposing the compaction treatments and at seeding, seeds were inoculated with different AM species of different origin (Iranian and Canadian).

According to the results, AM species were able to alleviate the compaction stress on plant growth and yield production. The following factors may indicate how AM species can alleviate the stress of soil compaction on plant growth and yield production (1) The extensive hyphal network can significantly enhance root uptake of water and nutrients even under compaction (Miransari 2010a, b, 2011a). It is because of the very fine diameter of fungal hypha, compared with even the finest root hairs, enabling the hypha to grow in the smallest soil pores and absorb water and nutrients; (2) AM species are able to produce some enzymes such as phosphatases, which can increase the solubility of nutrients such as phosphorous; (3) production of glomalin by AM fungi can also improve soil structure by binding soil particles; the protein can also be utilized as a source of carbon by other soil microbes; (4) the positive interactions between AM fungi and soil bacteria may increase plant growth under stress (Miransari 2011b); and (5) AM fungi are able to inhibit the adverse effects of pathogens on plant growth. The enhanced growth of mycorrhizal plant roots under compaction, which was mainly due to the increased uptake of P by the plant, was among the most important reasons alleviating the stress of compaction on plant growth and yield conditions both under field and greenhouse conditions.

## 4.6 Soil Microbes and Heavy Metal Stress

Due to industrialization and overuse of some practices such as chemical fertilization, the environment has been polluted with a high rate of pollutants including heavy metals. In addition to the pollution of the environment, heavy metals decrease plant growth and yield production and are considered not beneficial for human health. Accordingly, different methods such as leaching the polluted environment with organic compounds, use of degrading soil microbes, and planting hyperaccumulators have been among the most promising methods of treating soils polluted with heavy metals (Zhang et al. 2009a; Glick 2010; Haferburg and Kothe 2010; Miransari 2011). Hyperaccumulators are plants with the ability to absorb high rates of heavy metals while their growth remain unaffected.

Soil microbes such as AM fungi and PGPR can alleviate the stress of heavy metals by absorbing high rates of heavy metals in their tissues. There are different mechanisms by which soil microbes can detoxify the unfavorable effects of heavy metals in their tissue including intra- and extra-cellular mechanisms. Production of proteins, which absorb heavy metals, and detoxification of metal stress by accumulating them in their vacuoles are among some of the most important mechanisms by which soil microbes can alleviate this stress (Gerhardt et al. 2009; Giller et al. 2009; Haferburg and Kothe 2010).

Hence, molecular methods, which can enhance such abilities, can be used as more efficient ways of treating heavy metal stress. Under heavy metal stress, the production of enzymes, which mobilize nutrients and heavy metals in the rhizosphere, production of plant hormones and siderophores, and interaction between soil microbes can positively affect the process of heavy metal remediation (Zhang et al. 2009b; Abbas-Zadeh et al. 2010; Haferburg and Kothe 2010; Miransari 2011a). The production of microbial siderophores under heavy metal stress alleviated the stress, measured by the following factors: plant chlorophyll and quantification of RNA as well as protein and antioxidant activities (Dimkpa et al. 2009b).

Using microbiological factors such as microbial biomass and physiological parameters, including the activities of total enzymes and hydrolase enzymes at different stages of organic matter mineralization, Li et al. (2009) indicated that it is likely to evaluate the effects of heavy metals on soil microbial parameters. Under stress, usually such biological parameters are adversely affected and hence soil production efficiency decreases.

## 4.7 Soil Microbes and Suboptimal Root Zone Temperature

Suboptimal root zone temperature can adversely affect plant growth and yield production in different parts of the world. This is because the physiological processes in plant including enzymatic activities are adversely affected by the temperature stress. There have been different methods of treating temperature stress, the most important of which is the use of breeding techniques to produce plants tolerant to temperature stress. In such a case, the related genes, which are expressed during stress and produce stress proteins for the alleviation of stress, have been identified and inserted in the genetically modified crop plants. This method has been indicated to be effective under temperature stress (Farooq et al. 2009).

There has also been some other related research work using biological methods. For example, Professor Donald Smith and his research team, from McGill University, Canada, performed a very extensive range of research work regarding the effects of suboptimal root zone temperature on the process of N-fixation by *Bradyrhizobium japonicum* and soybean under both field and greenhouse conditions. They found that similar to the other soil stresses, suboptimal root zone temperature can also inhibit the process of N-fixation by disrupting the process of signal exchange between the two symbionts.

Hence, they preincubated the bacteria with the signal molecule genistein, and then inoculated the seeds both under field and greenhouse conditions. Genistein was able to partially or completely alleviate the effect of temperature stress. In the same range of experiments using aluminum cylinders, undisturbed soil samples with different soil textures (clay, loam, and sand) were collected from the field and tested under greenhouse conditions. The effects of different genistein concentrations (control, 5, 10, and 20 μg/l) and different temperatures (14, 19, and 23°C) imposed by using a compressor on soybean growth were evaluated

(Miransari and Smith 2008). While suboptimal root zone temperature decreased plant growth and nodulation, genistein was able to alleviate the stress. Interestingly, the performance of genistein was also different under different soil textures, as it was more effective on the stress under loam and clay textures. This indicates the importance of soil texture on the process of signaling exchange between the two symbionts.

## 4.8 Conclusion

Some of the most important mechanisms presented by plants or their associative soil microbes have been reviewed. In addition to plants, which can perform some morphological and physiological alterations to alleviate stress, their symbiotic or nonsymbiotic association with soil microbes can also result in some stress-controlling mechanisms. For example, a wide range of different biochemical compounds including different enzymes, plant hormones, proteins, chemical compounds, chelators, and other organic compounds can be produced by soil microbes including AM fungi and PGPRs, which can significantly contribute to the alleviation of soil stresses. Use of biotechnological methods, which can enhance the production of such compounds by soil microbes, can increase plant growth and yield production and hence ecosystem efficiency under different conditions including stress.

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# Chapter 5
# Interactions Between Legumes and Rhizobia Under Stress Conditions

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## 5.1 Introduction

A group of microorganisms fix nitrogen by converting the stable atmospheric nitrogen into a biologically useful form. All organisms which reduce dinitrogen to ammonia belong to the biological group of prokaryotes and they do so with the aid of an enzyme complex, nitrogenase. Nodule bacteria belonging to genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Azorhizobium* can interact with roots of legumes to form nodules, which function as sites for atmospheric nitrogen fixation (Relic et al. 1994). The symbiosis between legumes and rhizobia is a cheaper and usually more effective agronomic practice for ensuring an adequate supply of nitrogen for legume-based crop and pasture production than the application of fertilizer-N.

Successful establishment of the symbiotic interaction involves chemotaxis of the bacteria toward the roots, root colonization, root hair deformation, and rapid division of root cortex cells (van Rhijn and Vanderleyden 1995).

Early events of nodule formation require expression of bacterial nodulation genes, which are induced by plant flavonoids, a large group of structurally related compounds (de Rijke et al. 2006). Rhizobia synthesize lipochitooligosaccharides (Nod factors) that trigger responses in root hairs and induction of early nodulin gene expression. Nod factors allow rhizobia to enter the root and then, the cortical cells

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and induce nodulin gene expression and cell division, leading to nodule primordium formation.

Several environmental conditions are limiting factors to the growth and activity of the $N_2$-fixing system. The most problematic environments for the symbiosis are marginal lands with low rainfall, extremes of temperature, and acidic soils of low nutrient status and poor water-holding capacity (Bottomley 1991). When nitrogen-fixing organisms are attacked by pests or pathogens, the fixation process is usually adversely affected. Also, most cultivated legumes are exposed to chemicals used as plant protectors and fertilizers, which may contain compounds potentially harmful to plant, bacterium, or both.

Conditions of soil stresses may partially or completely inhibit the early steps of symbiosis, including the process of signal exchange between legumes and bacteria, and affect bacteria growth and $N_2$ fixation (Hungria et al. 1991; Brown et al. 2001; Miransari and Smith 2007). A stress condition may interfere with the transcription of nodulation genes in rhizobia by modifying the composition of root exudates or the response of bacteria (Howieson et al. 1992; Andrés et al. 1995, 1998a).

## 5.2 Factors Affecting Legume Nitrogen Fixation

Both biotic and abiotic factors exert their influence on $N_2$ fixation by legumes as discussed below.

### *5.2.1 Biotic Factors*

Among the biotic factors, we must consider primarily the action of pathogenic organisms and the mechanisms of survival of rhizobia in soil. When the participating organisms of the symbiotic interaction are attacked by pathogens, the fixing process is usually unfavorably affected. Plant viruses affect the structure and physiology of host plants and root nodules, and it has been even pointed out that plants attacked by viruses can act selectively on the rhizobial soil through their root exudates, as set would be altered. High levels of virus in nodules can directly affect the activity of the same (Khadir et al. 1984).

Rhizobial species are also susceptible to attack and lysis by bacteriophages usually present in soil. Phages specific against different groups of soil bacteria that can form root and stem nodules of various legumes have been isolated from rhizosphere soil, their roots, and root nodules. They can affect the outcome of legume–rhizobium symbiosis by reducing the population density of susceptible nodule bacterial strains in the soil and provide selective pressure for the evolution of less effective rhizobial variants that generally cause diminutions of efficiency of symbiotic nitrogen fixation (Appunu and Dhar 2008).

Interactions between plant pathogenic fungi and root nodules have also been reported. Tu (1978), described *Rhizobium* species that could colonize the tips of the hyphae of *Phytophthora megasperma*, a fungal pathogen of soybeans and alfalfa, and thus affect the growth of the fungus, providing a protective mechanism that may be of significance for these crops. Rhizobia have been widely used as biocontrol agents against fungal pathogens (Deshwal et al. 2003).

Root nodules can be attacked and used as a source of food for insect larvae. Wolfson (1987) found that some larvae could be strongly attracted to alfalfa and clover nodules and the survival rate of larvae was greater in the presence of root nodules. Moreover, in conditions where nodulation was prevented by the application of nitrogenous fertilizers, the number of larvae and emerging adults was significantly lower. The infection of plants by nematodes causes adverse effects on plant growth, nodule development, nitrogen contents of root and shoot, bacteroids, and nitrogenase activity and it can result in the production of factors that suppress nodulation (Ko et al. 1984; Bhat et al. 2009).

In addition, legumes can produce many toxins from pathogenic organisms. It is thought that an important strategy is the export of fixed nitrogen in nodules in the form of ureides, substances that are not used by herbivorous insects, so that these plants would be at an advantage for which nitrogen export by another mechanism case the path of the amides (Wilson and Stinner 1984). Because the area immediately adjacent to the root system is the leading source of infective rhizobia, it is not surprising that the associated microflora have influenced the development of symbiosis. Occasional failures in the nodulation of crops may be the result of microbial competition that suppresses undesirable microorganisms. Since the 1920s, researchers know that soils containing indigenous rhizobia strains often limit the growth of legumes. Numerous experiences of crop inoculation with $N_2$-fixing bacteria have failed due to the presence of indigenous strains. They occupy the nodules and exclude the inoculated strains, a situation known as the problem of competition between rhizobia.

When you introduce a large number of rhizobia to the soil, such as during planting seasons through seed inoculation, the abundance of these microorganisms in the soil can, however, decrease drastically. This decrease has been correlated with an increase in the density of protozoa, predators capable of feeding avidly on bacteria (Ramírez and Alexander 1980; Wright et al. 1993). Alternatively, members of the microflora can have a positive impact by providing growth factors, degrading toxic metabolites, eliminating pathogenic organisms, immobilizing inorganic nitrogen, and other mechanisms, so that the symbiotic interaction is favored (Derylo and Skorupska 1993; Burdman et al. 1996).

### *5.2.2 Abiotic Factors*

Typical abiotic stresses that can affect the legume–rhizobia symbiosis include extreme temperatures and pH, low water availability, high or toxic concentrations of salt, and the presence of chemicals in the rhizosphere.

#### 5.2.2.1 Temperature

Low and high temperatures affect symbiotic nitrogen fixation severely (Michiels et al. 1994; Hafeez et al. 2000). Temperature regulates the metabolism of the plant and the bacteria, as well as the plant–bacteria association (Young et al. 2006). Sometimes, the sensitivity of the host toward low temperature affects nitrogen fixation severely, leading to an abrupt cut-off at temperatures where the bacterial cells can still grow and metabolize. Root hair infection is much more temperature sensitive than nodule development (Hafeez et al. 2000).

It is generally assumed that rhizobia have to multiply in the rhizosphere to considerable numbers before being able to invade the root hair and reduced multiplication might be involved in the early type of inhibition. It is contrary to findings of Naeem et al. (2008) which indicate that failure in the attachment of rhizobia to the root hair is not because of the low population in the rhizosphere, but is due to the poor development of root hair at different temperatures.

In a study conducted in Australia in 1970, Roughley evaluated the process of nodulation of *Trifolium subterraneum* by *Rhizobium trifolii* strains (now *Rhizobium leguminosarum bv. trifolii*) at different temperatures and in a range between 7°C and 19°C. Low temperatures affected the structure of the nodules and the efficiency of $N_2$ fixation as a greater amount of nodular tissue was formed between 11°C and 15°C. Low temperatures also prevented or did slow down differentiation of bacteroids; and the differentiation zone of the nodule was lower at 7°C and 19°C compared to the surface covered in the range 11–15°C.

Other workers showed that the nodulation process is more sensitive to cold than the nitrogenase activity in itself and that, at low temperatures, a greater amount of the nitrogen fixed is retained by the nodule, suggesting that export to the aerial parts is retarded (Gibson 1969). Naeem et al. (2008) suggested that suboptimun temperatures affect the growth of bacteroids even within the nodule and also affect the cell-to-cell movement of the bacteroids. Reduced nitrogenase activity in such nodules can also be explained on the basis of these effects.

The sensitivity of the binding interaction of $N_2$ at low temperatures in subtropical legumes is a major limitation for the adaptation of this crop in areas of short warm season (Zhang et al. 1995). The addition of inducers was tested as an alternative to relieve the stress condition. The preincubation of bacterial cells with the plant-to-bacteria signal flavonoids and jasmonates compounds could enhance plant growth, photosynthetic rates, and nitrogen fixation and could alleviate low root zone temperature (Lee 2009).

At the other end of the scale, high temperatures can affect the survival and persistence of rhizobia in the soil and their interaction with legumes. This situation is critical for crops in tropical regions, where soil temperature near the surface can reach values between 40°C and 60°C during the summer. High soil temperatures will delay nodulation or restrict it to the subsurface region. Munns et al. (1979) found that alfalfa plant growth in desert environments maintained few nodules in the top 5 cm of soil but were extensively nodulated below this depth. Elevated

temperature and drought reduced plant dry mass and leaf area of alfalfa, especially when both stresses were combined. The inhibitory effect of elevated temperature on plant growth was a consequence of decreased $N_2$ and $CO_2$ fixation rates (Aranjuelo et al. 2007). Moreover, these authors shown that elevated temperature inhibited nodule activity drastically, whereas the inhibitory effect resulting from drought centered on nodule dry mass.

It has been reported that there is considerable variation in the rhizobia strains in terms of survival and nodulation ability at high temperature (Michiels et al. 1994). Karanja and Wood (1988) found that a high percentage of the strains that persisted at 45°C lost their infectiveness. They attributed these losses to plasmid curing.

The protection mechanism of rhizobia to heat is unknown, with some hypotheses ranging from their interaction with clay particles to the presence of plasmids involved in resistance to high temperatures (Barbour and Elkan 1989). Heat shock proteins have been found in rhizobia. Temperature stress promoted the production of a protein with a relative mobility of 65 kDa in four strains of tree legume rhizobia. This protein was not overproduced during salt or osmotic stress, which indicates that it is a specific response to heat stress (Zahran et al. 1994).

#### 5.2.2.2 Water Relations

Drought is a major factor limiting crop production and has a particular negative impact on symbiotic $N_2$ fixation. The nitrogen fixation of legumes is also highly sensitive to soil water deficiency. Many species studied exhibit a reduction in nitrogen fixation when subject to soil moisture deficit (Zahran 1999; Hungria and Vargas 2000; Pimratch et al. 2008).

Root growth and root hairs are strongly influenced by this stress condition, which would be an important factor in the inhibition of nodulation, based in particular on the observation that rehydration of the soil experiences has allowed the resumption of growth and subsequent infection. Declining soil water potential has been correlated with a lower number of threads of infection, even a total inhibition of nodulation (Worrall and Roughley 1976).

Once the infection process starts, a limited supply of water can slow the growth of the nodule and accelerate its senescence. Nitrogenase activity is also decreased significantly, accompanied by the decrease in respiratory activity of the soybean and common bean nodules (Weisz et al. 1985; Gerosa-Ramos et al. 2003). A limitation in metabolic capacity of bacteroids and oxidative damage of cellular components are contributing factors to the inhibition of nitrogenase activity in alfalfa nodules (Naya et al. 2007). In addition, the transport of fixed nitrogen out of the nodule is decreased, possibly due to an insufficient supply of photosynthates in stems and leaves under stress (Huang et al. 1975).

A lower rate of water movement out of the nodule during drought stress may restrict export of $N_2$ fixation products, thus inhibiting nitrogenase activity via a feedback mechanism (Serraj et al. 1999). Drought induces oxidative stress in

nodules. This leads to general decrease of antioxidant activities that are associated with nodule senescence (Hernández-Jiménez et al. 2002; Porcel et al. 2003). It was found that water stress imposed during vegetative growth was more detrimental to nodulation and nitrogen fixation than that imposed during the reproductive stage (Pena-Cabrales and Castellanos 1993).

The mechanisms of desiccation tolerance are not well known. Some suggested mechanisms could be the ability to limit to a minimum the cell metabolism, the increased catalase activity and the presence of specific plasmids for drough tolerance. A considerable amount of information has accumulated about differences in desiccation tolerance of different strains, species, and genera of rhizobia (Al-Rashidi et al. 1982; Aurag and Sasson 1992; Vriezen et al. 2007).

Free-living rhizobia are capable of survival under drought stress or lower water potential (Fuhrmann et al. 1986). However, population densities tend to be lowest under the most desiccated conditions and to increase as the moisture stress is relieved. The survival and activity of microorganisms may depend on their distribution among microhabitats and changes in soil moisture (Orchard and Cook 1983). The migration of rhizobia strains was found to be very limited when water-filled pores in soil became discontinuous as a result of water stress (Hamdi 1970; Wadisirisuk et al. 1989).

Some experiences have shown that working with legumes and rhizobia strains selected for desiccation tolerance, a symbiotic interaction can take place (Soria et al. 1996). At the other end of the scale, many legumes are sensitive to excess water (flooding). Nodule development and function are usually more affected than the infection itself, and some effects such as decreased nitrogenase activity may be even more intense than in the case of water deficit. Reduced to zero, the contribution of $O_2$ to the nodule appears to be the main problem of the effect of waterlogging. The diffusion of $O_2$ within the nodules is in part regulated by a physical barrier located in nodular parenchymal cells.

Under stress conditions, the diffusion resistance increases by identifying a lack of $O_2$ inside the nodule, leading to inhibit its activity (Day and Copeland 1991). The ability of aerobic bacteria to utilize nitrogenous oxides, as terminal electron acceptors, enables them to survive and grow during periods of anoxia. This may be advantageous for the survival of rhizobia in soils (Zablotowicz et al. 1978).

#### 5.2.2.3 Salinity

Salinity is one of the major environmental threats to agriculture and affects approximately 7% of the world's total land area (Türkan and Demiral 2009). Salt stress limits the ability to generate further biomass or to maintain defense mechanisms (Zheng et al. 2009) and, in legumes, also inhibits the process of symbiotic nitrogen fixation. Several hypotheses have been advanced to explain the negative effects of salt on nitrogen fixation in plant legumes, diminished photosynthate supply to the

nodule, reduced supply of respiratory substrates to the bacteroids, and alterations in the oxygen diffusion barrier (Soussi et al. 1998).

The activity of nodules may be affected by the presence of solutes in soil solution in several ways. High concentrations of solutes can inhibit nodulation and, when nodule is formed, the nitrogenase activity can be severely affected. The toxic effect of some ions on plants and microorganisms when they exceed certain levels must be added to the osmotic effect. In a study in soybean, Tu (1981) showed that the interaction of *Bradyrhizobium japonicum* to the root hairs under increasing concentrations of NaCl in the range from 0% to 1.8% in the culture solution was affected. At a concentration of 1%, inhibition was evident in the bending of the hairs, a phenomenon markedly accentuated as rising concentrations. At 0.2% NaCl, growth was slow and at 1.2% nodulation did not occur.

Some experiments carried out with *Vicia faba* (pea) and *Vigna unguiculata* (cowpea) showed that the application of different concentrations of NaCl at the time of formation of root hairs could reduce colonization of roots, curved hairs, structure of the hair and hypodermic cells, the number and weight of nodules, and nitrogenase activity (Zahran and Sprent 1986; Georgiev and Atkins 1993). Arora et al. (2006) reported the effect of different salt concentrations on growth and PHB accumulation of four different *Sinorhizobium* strains.

The negative effects of salinity on the oxygen diffusion barrier, which normally would reduce the $O_2$ flux into the nodule was also demonstrated (Serraj and Drevon 1998). The decline in nitrogen fixation can be attributed to a reduced carbon supply to bacteroids, mainly in the form of malate limitation and likely a result of the salt-induced inhibition of nodule carbon metabolism through the inhibition of sucrose synthase activity (Ben Salah et al. 2009). The accumulation of solutes such as proline, sucrose, and D-pinitol has been described in nodules of some legumes such as alfalfa and cowpea, and they exert an osmoregulatory function in situations of salinity (Irigoyen et al. 1992). The results of our group indicate that the exudate patterns of alfalfa (*Medicago sativa* L.) plant growth on 0.5% NaCl differ from the controls; however, it does not affect the induction capability of *nod* genes (Andrés et al. 1995).

The capacity of root exudates and flavonoid compounds to induce nodulation genes was evaluated using isogenic strains of rhizobia (obtained from Dr. S.A.J. Zaat, Leiden University, the Netherlands), where promoters of the *nod* area were cloned with the structural gene *lacZ* from *Escherichia coli* (Table 5.1). In this system, β-galactosidase production represents induction of *nod* genes. There were

**Table 5.1** Isogenic strains used in the induction bioassay of *nod* genes

| Name[a] | Background | *nodD* source |
|---|---|---|
| RBL5280 | *R. leguminosarum* bv. *trifolii* | *R. leguminosarum* bv. *viciae* |
| RBL5283 | *R. leguminosarum* bv. *trifolii* | *R. leguminosarum* bv. *trifolii* |
| RBL5284 | *R. leguminosarum* bv. *trifolii* | S. *meliloti* |

[a]These strains are derived from *R. leguminosarum* bv. *trifolii*. Plasmid Sym has been removed and the strains carry IncP plasmid with *nodD* genes of different provenance and a plasmid IncQ with *nodABCIJ* promoter cloned before the structural *lacZ* gene

**Table 5.2** Inductive bioassay of *nod* genes by exudates of alfalfa in the presence of NaCl

| Treatments[a] | β-galactosidase units (thousands)[b] | | |
|---|---|---|---|
| | RBL5280 | RBL5283 | RBL5284 |
| Control (no exudates) | 0 | 0 | 0 |
| Alfalfa exudates | 23.2 | 26.5 | 22.1 |
| Alfalfa exudates + NaCl 0.5% | 23 | 26.1 | 22.2 |

[a]Seedlings were grown in a hydroponic system in Jensen solution diluted 10 times and root exudates were collected at 120 h of culture. NaCl was added to the nutrient solution at 0.5%
[b]Data shown are average of three repetitions. Standard error in each column was not higher than 5%

no significant differences between the exudates obtained from the stress situation compared to controls, suggesting that salinity did not affect induction, at least in the tested concentration of 0.5% (Table 5.2).

In common bean (*Phaseolus vulgaris* L.), a negative effect of NaCl on the expression of *nod* genes by *Rhizobium tropici* and *Rhizobium etli* and on nodulation factors' pattern was observed (Dardanelli et al. 2008). The preincubation of *B. japonicum* with the signal molecule genistein, under saline conditions, was described as a method to alleviate the stressful effects of salt on soybean–*B. japonicum* symbiosis (Miransari and Smith 2009).

High salt (NaCl) levels alter the metabolism of rhizobia and also affect nitrogen fixation (Dowling and Broughton 1986). However, rhizobia strains, which are salt tolerant and efficient nitrogen fixers, have been isolated (Rai 1983; Doura et al. 1984; Rosas et al. 1996). The search for strains of rhizobia tolerant to salinity may be an alternative to achieve the establishment of symbiosis in conditions in which crop growth is not altered (Doura et al. 1984; Mohammad et al. 1991; Rosas et al. 1996).

#### 5.2.2.4 pH

Most leguminous plants require neutral or slightly acidic soil for growth, especially when they depend on symbiotic $N_2$ fixation. The nodulation of legumes is generally reduced by low pH conditions on the ground, due to poor growth of rhizobia, besides to an increase in the number of ineffective strains or to the sensitivity of the infection process. Acidity affects several steps in the development of symbiosis, including the exchange of molecular signals between the legume and the microsymbiont (Hungria and Vargas 2000). Initial root colonization by rhizobia is one of the early steps preceding root invasion. Angelini et al. (2003) demonstrated that this process was affected when both acid-tolerant and acid-sensitive (pH 5.0) peanut rhizobia were grown at low pH. This effect seems to be a result of alterations produced by acidic pH on the microsymbiont and not on its host. At acid pH, very low *nodC* gene expression was observed in acid-sensitive isolates, while a change in the flavonoids inducer effectiveness was determined in acid-tolerant isolates.

The availability of some essential nutrients such as calcium, magnesium, phosphorus, and molybdenum is low in acid soils, whereas high levels of aluminum and manganese can become toxic to plants and rhizobia (Coventry and Evans 1989). Soil pH can be altered, depending on the type of plant nitrogen nutrition. Thus, the $N_2$-fixing plants have a net production of protons, which tends to lower the soil pH, a mechanism shown at least in soybean and alfalfa. The buffering capacity of some soils can avoid major changes in pH, but in soils with low cation exchange capacity the problem may be significant. To this must be added that, in general, $N_2$-fixing plants are more sensitive to acidity than plants of the same species that feed on mineral nitrogen (Andrews 1976).

In alfalfa, the number of nodules decreases rapidly when the soil pH is below 4.7. The selection of genotypes tolerant to acidity of both plants and strains of *R. meliloti* (*S. meliloti* today) has had a marked effect on crop productivity in acid soils of Australia (Howieson et al. 1992). The growth and survival of rhizobia are affected at pH below 4.5 in the culture media; however, there is a wide variation in acidity tolerance even among strains of the same species (Hagedorn and Caldwell 1981). Growth in acidified culture media has proved useful for selecting strains with an ability to colonize the rhizosphere and to nodulate their host plant in acid soils (Cooper 1988). Calcium appears to be very important in rhizobia exposed to low pH. This element is involved in many different cell processes in root nodule bacteria: it is essential in maintaining cell wall rigidity (Vincent 1962), stabilizes oligomeric proteins and covalently bound protein peptidoglycan complexes in the outer membrane (de Maagd et al. 1989), as well as having a requirement for chemotaxis (Bowra and Dillworth 1981).

Howieson et al. (1992) reported that calcium improves rhizobial cell growth and survival at low pH, especially in strains such as *S. meliloti* that are acid sensitive. Macció et al. (2002) working with the strain *Bradyrhizobium* sp. *semia* 6144 (peanut symbiont) reported that the bacterial culture showed a growth and viability diminution at low pH (5.0) and at a calcium concentration of 0.05 mM. Increasing concentrations of calcium significantly improved the rhizobial growth under acid stress conditions. Molecules related to plant–bacteria recognition, such as exopolysaccharides and lipopolysaccharides, showed changes at different pH and calcium concentrations. Variations in the cellular permeability were also observed under stress conditions.

Among the mechanisms suggested explaining a greater tolerance for acidity can include extracellular polysaccharide production (Cunningham and Munns 1984). An interesting mechanism is described by O'Hara et al. (1989), whereby the low pH tolerant strains can control their internal environment, which remains alkaline, while susceptible strains cannot maintain an alkaline cytoplasm under conditions of high acidity.

A major problem is to distinguish between the effects of low pH and toxicity of some minerals, especially aluminum. In acidic soils with pH of $>5.0$, where heavy-metal activity is relevant, the presence of available aluminum inhibits nodulation. Rhizobia showed varied responses to aluminum toxicity and several strains that were resistant to aluminum were identified. According to the Arora et al. (2010),

aluminum was found to have a lethal effect on growth, nitrogenase, hydrogenase, nitrate reduction (NR), and nitrite reduction (NiR) of *S. meliloti* RMP5 and *Bradyrhizobium* BMP1.

Legume species vary markedly in their tolerance to aluminum, with some plants being significantly more strongly affected by this ion than rhizobia. Therefore, for acid soils with high aluminum content, improvement is achieved by manipulating the plant rather than the rhizobia (Taylor et al. 1991; Graham 1992).

Highly alkaline soils (pH $>$ 8.0) tend to contain high sodium chloride, bicarbonate, and borate and are often associated with high salinity that reduces nitrogen fixation. The information available on alkalinity is notoriously low compared to that on acidity. Alkaline soils are much less common than acid soils; however, in some places such as northern India, where the soil pH can reach values between pH 8 and 10.5, the situation can be very problematic. Singh et al. (1973) found that the number of nodules that form in alfalfa can be significantly reduced in culture solutions containing 0.1% $Na_2CO_3$ and $NaHCO_3$.

#### 5.2.2.5 Agrochemicals

Agrochemicals are important tools that agriculture currently employs to protect the yield and quality of crops, despite the fact that the information available about the influence that they can have on $N_2$ fixation and nitrogenase activity is incomplete and often inconsistent. Exposure of plants to such substances may be intentional, for instance applying an insecticide to the crop, or unintentional as when there are residues of previous treatments. Most studies on the effects of agrochemicals in legumes have focused on the action on the plant or the bacteria and have studied mainly compounds of the class of herbicides. Several of these compounds are reported for nitrogen fixation, in terms of reduction in number and weight of nodules, activity of nitrogenase, and yields.

A significant amount of research is carried out on soybeans, the first legume grown on a large-scale worldwide; and in many cases the results assessing agrochemicals were contradictory. As an example, the herbicide trifluralin, was reported to inhibitnodulation in soybean (Parker and Dowler 1976). Further, Bollich et al. (1985) reported that trifluralin affect number and weight of nodules and activity of nitrogenase, while Alaa-Eldin et al. (1981) suggested that higher doses of trifluralin than usually employed stimulated nodulation of soybean. Similarly, Curley and Burton (1975) reported that the fungicide pentachlorebenzene (PCNB) reduced soybean nodulation while Mallik and Tesfai (1985) reported that it is safe even at doses ten times higher than usual.

Test laboratory also showed adverse effects of fungicides and pesticides on the survival of microorganisms. However, few studies have taken into account the fact that stage of the symbiotic process is affected by these chemicals originally, which led to the implementation of techniques to determine whether the plant, the microorganism, or both are affected.

Different groups of researchers have found that certain fungicides, employed as chemical seed protectants in legumes, markedly inhibited both nodulation and nitrogen fixation activity (Jakubisiak and Golebiowska 1963; Fisher et al. 1978; Nuti et al. 1984; Kyes-Boahen et al. 2001). Some fungicides were found to not only inhibit bacterial growth, but also proved lethal for these microorganisms (Kekskes and Vincent 1969). The survival rate of *Rhizobium* was reduced when seeds were inoculated with several fungicides. These chemicals also affected plant growth at the seedling stage and during root nodulation (Curley and Burton 1975; Graham et al. 1980; Ruiz Sáinz et al. 1984, 1986; Martensson 1992; Stovold and Evans 2006).

The fungicide thiram, the chemical seed protectant most used worldwide, has been found to be detrimental to *B. japonicum* survival and soybean nodulation (Tu 1980; Rennie and Dubetz 1984). This fungicide affects the viability of microorganisms and is able to cause a number of morphological changes which may affect the bacterial cell wall and thus affect mechanisms involved in plant–microorganism symbiosis (Wrobel 1963; Kekskes and Vincent 1969). Susceptibility for higher concentration of carbaryl was observed on vegetative parameters, root nodulation, and seed germination. Conversely, a lower concentration was found to be stimulatory to plant growth. A decline in nitrogenase activity was observed with increasing concentration of carbaryl (Saraf et al. 1994). Guene et al. (2003) reported varying effects on nodulation and nitrogen fixation of common bean by treatment with dichlorofenothion–thiram, based on the *Rhizobium* strain used. Studies carried out in our laboratories have demonstrated that thiram affects *B. japonicum* viability and causes a significant reduction in the nodulation of soybean. Thiram induces a strong inhibition of nodulation in the crown zone of soybean roots and this effect is maintained until flowering occurs, both under laboratory conditions and in field tests. After flowering, late nodulation takes place in the lower zones of the roots, and although plants recover, there is no improvement in yield because the grain has already been formed (Correa et al. 1989). Thiram concentration beyond 500 μg/ml was observed to be highly toxic with respect to plant growth factors and rhizobial infection to soybean (Bikrol et al. 2005).

Isolation and subsequent inoculation with resistant strains of *Rhizobium* or *Bradyrhizobium* could counteract the negative effects of thiram. However, in spite of the constant search for strains resistant to chemical seed protectants, few studies have had successful results (Golebiowska et al. 1967; Gillberg 1971; Ruiz Sáinz et al. 1984). We obtained *B. japonicum* strains resistant to high doses of thiram (Andrés et al. 1998b). Strains *A1*, *C1*, and *C6* were highly resistant to thiram but had lost their ability to nodulate. Strains *T3B*, *A86*, and *A2* were all highly resistant to thiram and possessed a high capacity to form nodules. These latter strains could be used to inoculate thiram-treated seeds, without a subsequent loss of bacterial viability.

Research on other seed protectants such as captan, tecto, and PCNB showed that they affected bacterium viability during the first stages of nodulation (Gillberg 1971; Graham et al. 1980; Rosas and Carranza 1987). Although bacteria are resistant to a great number of agrochemicals, symbiotic efficiency may be altered by their use (Martensson 1992).

The presence of agrochemicals in the rhizosphere may interfere with the infection process by inhibiting bacteria-induced root hair deformation. Agrochemicals probably affect the plants in a manner that the bacteria-induced root hair deforming factors, similar to Nod Rm-1 and related compounds (Truchet et al. 1991), are incapable to influence morphogenic activity of the plants.

The capacity of alfalfa and soybean exudates and of purified flavonoid compounds to induce nodulation genes in the presence of thiram was evaluated in another study from our group, using the isogenic strains of rhizobia described in the situation of salt stress (Table 5.3). Thiram could alter the composition of root exudates containing flavonoid compounds which are responsible for the "recognition" between plants and microorganisms. Nonreversion of the lack of induction by thiram-treated exudates by addition of flavonoids with tested inductive capacity suggests that some alteration at bacterial wall or membrane level may take place when thiram is present (Andrés et al. 1998a).

**Table 5.3** Inductive bioassay of *nod* genes by alfalfa and soybean exudates in the presence of thiram

| Treatments | β-galactosidase units (thousands) | | |
|---|---|---|---|
| | RBL5280 | RBL5283 | RBL5284 |
| Control (no exudates) | 0 | 0 | 0 |
| Alfalfa exudates | 23 | 27.5 | 23.1 |
| Alfalfa exudates + luteolin | 24.1 | 26 | 27.2 |
| Alfalfa exudates + apigenin | 23.2 | 28.3 | 21 |
| Alfalfa exudates + luteolin + apigenin | 21.6 | 25.4 | 21.9 |
| Alfalfa exudates + thiram | 11 | 5.2 | 3 |
| Alfalfa exudates + thiram + luteolin | 12.6 | 9.2 | 5 |
| Alfalfa exudates + thiram + apigenin | 11.5 | 7.9 | 4.1 |
| Alfalfa exudates + thiram + luteolin + apigenin | 12 | 9.3 | 4 |
| Soybean exudates | 15.7 | 11.2 | 4.6 |
| Soybean exudates + luteolin | 18.2 | 16.3 | 8.8 |
| Soybean exudates + apigenin | 16.1 | 16.3 | 5 |
| Soybean exudates + luteolin + apigenin | 16.1 | 17 | 5.9 |
| Soybean exudates + thiram | 3 | 3 | 2.8 |
| Soybean exudates + thiram + luteolin | 3.5 | 3 | 3 |
| Soybean exudates + thiram + apigenin | 4.2 | 4.1 | 3 |
| Soybean exudates + thiram + luteolin + apigenin | 4 | 3.7 | 3 |
| Luteolin | 18.2 | 20.3 | 13.3 |
| Apigenin | 17.7 | 20.3 | 4.13 |
| Luteolin + apigenin | 18.3 | 20.5 | 11.6 |
| Luteolin + thiram | 0.82 | 0.29 | 0.46 |
| Apigenin + thiram | 0.12 | 0.5 | 1.6 |
| Luteolin + apigenin + thiram | 0.8 | 0.4 | 2.3 |

[a]Seedlings were grown in a hydroponic system in Jensen solution diluted 10 times and root exudates were collected at 120 h of culture. Thiram was added to the nutrient solution at 2 mg/ml. Inductive flavonoids were added to the exudates at a final concentration of 10 μM

[b]Data shown are average of three repetitions. Standard error in each column was not higher than 5%

## 5.3 Conclusion

Biotic and abiotic factors play an important role in the control of legume–rhizobia interactions, limiting the symbiotic process. Since they affect survival and proliferation of the bacteria in soil and rhizosphere, they inhibit the infection process or affect nodule metabolism, altering also legume growth.

Populations of rhizobia species vary in their tolerance to major environment factors; consequently, the selection of resistant strains is an important option. Tolerant strains of rhizobia were isolated for various crop and wild legumes and this appears as a promising possibility that could enlarge the agricultural area in suboptimal regions, therefore increasing the economic prospects of these plants.

The formulations of new inoculants using tolerant strains, some of them including mixtures of microorganisms or factors that enhance the interaction (such as flavonoids or Nod factors) are today an important technological tool. These products can be used to extend the cultivation of native or naturalized legumes.

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# Chapter 6
# Cold-Tolerant PGPRs as Bioinoculants for Stress Management

**Pankaj Kumar Mishra, Shekhar Chandra Bisht, Jaideep Kumar Bisht, and Jagdish Chandra Bhatt**

## 6.1 Introduction

Crop plants of commercial importance are severely restricted by a variety of environmental factors. Among these factors, drought, salt, and low (cold) temperature play a very significant role in reducing agricultural production worldwide (Boyer 1982). Low (cold) temperature is one of the major determinants of agricultural productivity where freezing occurs, even sporadically. In order to produce the adaptive responses to perceive the low temperature, plants require transducer signals to activate the expression of appropriate genes that combat the diverse stresses that impose on living cells at subzero temperature. Low-temperature response has to be integrated with responses to other stresses such as drought and salinity, and there is a commonality in the plant's adaptive mechanism (Smallwood and Bowles 2002). In addition, natural distribution of plant species is determined by their ability to survive freezing events. In plant species from temperate climates, winter survival is greatly influenced by the ability of plants to cold acclimatization, i.e., to increase their chilling/freezing tolerance during a period of exposure to low or nonfreezing temperatures in the autumn (Hincha 2002).

It is not surprising that the impacts of cold stress on plant life have been comprehensively studied and many attempts have been undertaken to improve cold resistance, chilling tolerance, and cold alleviation in important crop plants. However, progress in achieving frost hardiness of plants either by classical breeding or by gene transfer (Jaglo-Ottosen et al. 1998; Kasuga et al. 1999) is difficult, due to the fact that cold resistance is not a quality conferred by the product of a single gene, but has turned out to be a syndrome (Beck et al. 1995; Fowler and Thomashow 2002) comprising many different traits, such as fluidity of the biomembranes, synthesis and accumulation of low- and high-molecular-weight

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cryoprotectants, increase of the potential to cope with oxidative stress and others (Nanjo et al. 1999; Steponkus et al. 1998).

Plant-growth-promoting rhizobacteria (PGPRs) have a high potential in agriculture because they can improve plant growth, especially under limiting or stress conditions (Nabti et al. 2010). To improve cold stress in plant, cold-tolerant PGPRs are recently being used (Barka et al. 2006; Cheng et al. 2007). Certain epiphytic bacteria such as *Erwinia herbicola* and *Pseudomonas syringae* with ice-nucleating activity ($ice^+$ bacteria) served as the ice nuclei that prevent supercooling of plants and increase the amount of frost damage to plants at high subzero temperature (Lindow and Leveau 2002). Increasing population of *P. syringae* ($ice^+$) bacteria resulted in increases in ice-nucleation temperature; preemptive competitive exclusion of $ice^+$ bacteria by naturally occurring but non-ice ($ice^-$)-nucleating bacteria could be an effective and practical means to manage cold/low-temperature (abiotic) stress in plants (Lindow and Leveau 2002). In addition, some of these bacteria, such as epiphytic or endophytic PGPRs, enhance plant growth and productivity while improving their resistance to stress (Barka et al. 2006). PGPRs can directly facilitate the proliferation of their plant host system through the production of the stimulatory phytohormone within the root zone; these hormones stimulate the density and length of root hairs. The increase in root surface area improves the plant uptake potential of water and mineral nutrients from a large volume of soil. The auxin, indole-3-acetic acid (IAA), is an important phytohormone produced by PGPRs and treatment with auxin-producing rhizobacteria has been shown to increase plant growth (Patten and Glick 2002). The IAA-producing capability of microorganisms is useful in their identification and provides a valuable marker when examining the physiological roles or ecological significance of IAA in the establishment and persistence of the organisms (Bric et al. 1991). Besides phytohormone production, plant growth promotion is known to be mediated by a variety of mechanisms including phosphate solubilization (Valverde et al. 2006), siderophore production (Katiyar and Goel 2004), antagonism toward deleterious root microorganisms (Misaghi et al. 1982), deamination of the precursor molecule of the ethylene (Glick et al. 1998) and induction of resistance to biotic and abiotic stresses (Hontzeas et al. 2006; Wang et al. 2000). This chapter encompasses an overview of the current work reported on effects and mechanisms of cold tolerance in plants and management of cold stress by using cold-tolerant PGPR.

## 6.2 Effect of Temperature

The effects of cold temperature range from morphological to molecular levels and are evident at all phonological stages of plant growth. The first and foremost effect of cold is impaired seed germination and poor stand establishment. Cold stress is an important filter for recruitment, survival, productivity and latitudinal and

altitudinal distribution of plants (Sakai and Larcher 1987). Low temperatures and frost set agricultural borders for crop species and in marginal areas can cause severe yield losses. The intensity and occurrence of freezing temperatures, their annual timing and whether they commence episodically, periodically, or regularly (e.g., at night) have led to a variety of low-temperature and frost-survival mechanisms in plants. Plants from both outside the tropics and high tropical mountains are usually frost resistant, i.e., they remain undamaged upon exposure to subzero tissue temperatures. However, the extent of frost damage varies greatly: some species suffer frost damage at just below 0°C, whereas others survive even when dipped in liquid nitrogen at −196°C (Sakai and Larcher 1987). Chilling stress is a direct result of low-temperature effects on cellular macromolecules that cause a slowdown of metabolism, solidification of cell membranes and loss of membrane functions. Freezing stress acts indirectly via extracellular ice crystals that cause freeze dehydration, concentrate cell sap and have major mechanical impacts. There is a consensus that the primary cause of freezing injury in plants is most frequently an irreversible dysfunction of the plasma membrane as a consequence of freeze-induced cellular dehydration (Levitt 1980; Webb et al. 1994; Uemura et al. 1995; Xin and Browse 2000), in contrast to chilling-sensitive plants that show limited potential to acclimate to cold (Sakai and Larcher 1987).

### *6.2.1 Freezing Injury*

Whether or not freezing injury occurs in plants depends on the cold acclimation state. Freezing injury is caused by cellular freeze dehydration and cell contraction and normally involves damage to plasma membrane structure and function (Levitt 1980; Steponkus and Webb 1992; Webb et al. 1994; Uemura et al. 1995; Xin and Browse 2000). In isolated nonacclimated protoplasts, freezing stress and cell dehydration cause the formation of endocytotic plasma membrane vesicles. Under mild injurious stress during thawing, expansion-induced lysis occurs. Under severe stress, the formation of hexagonal II (HII) phase (lamellar-to-nonlamellar phase transition) of the plasma membrane can be observed in regions where the membrane is brought into close apposition with various endomembranes. This is most often the chloroplast envelope and tonoplast. As a result, the protoplasts lose osmotic responsiveness (LOR) during thawing. In cold-acclimated protoplasts, the formation of hexagonal II ($H_{II}$) phase (lamellar-to-nonlamellar phase transition) of plasma membrane is not observed at any injurious temperature, but freeze-induced dehydration results in exocytotic extrusions of the plasma membrane and fracture-jump lesions (FJLs) occur. FJLs are characterized by localized deviations of the fracture plane of the plasma membrane in freeze-fracture electron micrographs and the manifestation of injury by LOR (Uemura et al. 2006).

## 6.3 Cold Acclimation/Adaptive Strategies

Plants differ in their tolerance to chilling (0–15°C) and freezing (<0°C) temperatures. Plants from temperate regions are chilling tolerant, although tolerance to freezing varies but can increase their freezing tolerance by being exposed to chilling, nonfreezing temperatures, a process known as cold acclimation (Levitt 1980), which involves change in gene expression (Seki et al. 2003), membrane lipids, total protein contents, composition of soluble proteins (Saltveit 2000), increase in the sugar, proline, total phenolic contents (Barka et al. 2006), oxygen-scavenging enzymes, low ion leakage from cell membrane, anthocynin accumulation, and altered growth morphology (Saltveit 2000; Briigge et al. 1999). The physiological and metabolic changes that include production of osmoprotectants, such as proline, sucrose, and sugar alcohols, allow osmotic adjustment for continued water uptake (Hasegawa et al. 2000; Kasuga et al. 2004; Zhu 2002). In most plants, natural cold acclimation is induced by exposure to low temperatures. By contrast, plants of tropical and subtropical origins, including many crops, are sensitive to chilling stress and largely lack the capacity for cold acclimation. Factors influencing tolerance of freeze dehydration and membrane stability and factors controlling growth of ice in freezing plants are significant, as is whether extracellular freezing or supercooling occurs. In the cold-acclimated state, reduction or cessation of growth and photosynthesis is often observed, tissue water content is reduced, solutes accumulate (Ulmer 1937; Levitt 1980), cell-wall modifications take place in cereals (Hiilovaara-Teijo and Palva 1999), and abscisic acid levels may transiently increase (Chen and Gusta 1983). Other changes include the modification of lipids and lipid/protein ratios in membranes, the expression of cold-related (COR) proteins and an increase of osmolytes and reactive oxygen species (ROS)-detoxifying substances. Some of the changes induced by cold acclimation are addressed in the following.

### *6.3.1 Changes in Photosynthesis and Growth*

A major effect of cold stress is reduction in photosynthesis, which arises by a decrease in leaf expansion, impaired photosynthetic machinery, premature leaf senescence, and associated reduction in food production. In evergreen leaves, cold acclimation of photosynthesis can overcome the combined effects of low temperature and high irradiation (Adams et al. 2004; Öquist and Huner 2003). In woody plant species, photosynthetic capacity is strongly suppressed during winter (Ottander et al. 1995; Neuner et al. 1999; Savitch et al. 2002), while herbaceous plants show no sustained down regulation of photosynthetic capacity (Öquist and Huner 2003; Hacker and Neuner 2006). Winter cereals continue to grow during cold acclimation and hence, maintain a strong sink capacity, which is in contrast to woody plants that cease to grow and are dormant in winter (Savitch et al. 2002).

This balance between photosynthetic energy absorbance and consumption through metabolism and growth has been termed photostasis (Huner et al. 2003).

### 6.3.2 Membrane Modifications

Cold-stress tolerance of plants involves changes at the whole plant, tissue, physiological, and molecular levels (Fig. 6.1). Cold-induced alternation to the lipid composition of cellular membranes, particularly the degree of nonsaturation of fatty acids, has an important role in chilling tolerance. For that reason, membrane lipids need to be present in the liquid phase. During cold acclimation of frost-resistant species, changes in the proportion of phospholipids increase membrane lipid composition (Senser and Beck 1982; Webb et al. 1994; Uemura et al. 1995, 2006). In many plant species, this is primarily the result of an increase in the proportion of unsaturated molecular species lipids, particularly of phosphatidylcholine and phosphatidylethanolamine and a decrease of cerebrosides (Uemura et al. 2006). Also, chilling tolerance increases with increased fatty acid unsaturation (Nishida and Murata 1996). The melting point of membrane lipids is affected by the degree of unsaturation of fatty acids. The greater the number of double bonds and the shorter the fatty acids, the lower the melting point and the lower the temperature at which solidification occurs. By replacing fatty acids, membranes can adapt to the prevailing temperatures. Genetic manipulation of the chloroplast enzyme glycerol-3-phosphate acyltransferase (GPAT), which is involved in

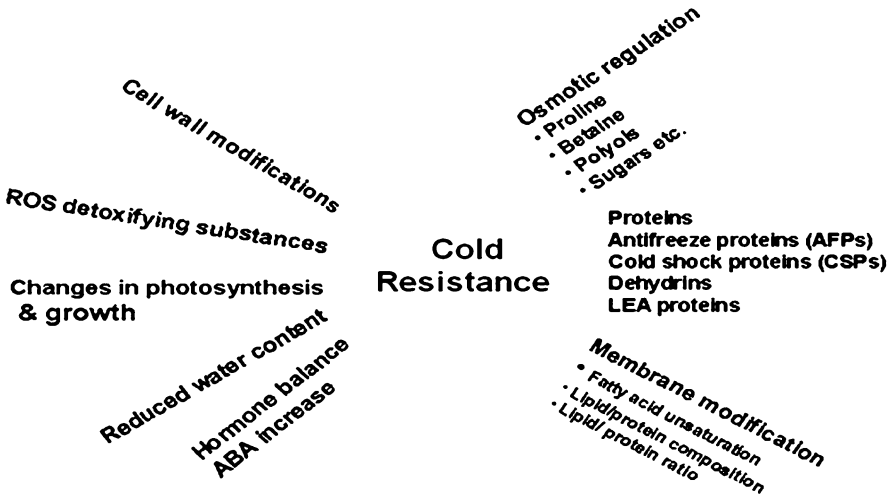


**Fig. 6.1** Most prominent physiological and biochemical changes often accompanying increased cold resistance in plants

phosphatidylglycerol fatty acid unsaturation, changed the chilling tolerance of transgenic tobacco plants to low-temperature exposure (Murata et al. 1992).

Changes in membranes may also include proteins (Uemura et al. 2006). During cold acclimation, some plasma membrane polypeptides have been shown to disappear, decrease, or be substituted by others (Uemura and Yoshida 1984). Membrane proteins are surrounded by less mobile lipids and the zone of less mobile lipids around proteins increases during cooling. A reduction of the protein–lipid ratio has been found to be important in cold acclimation of thylakoid membranes by maintaining adequate membrane fluidity (Schulze et al. 2005).

### 6.3.3 Reactive Oxygen Species-Detoxifying Substances

Exposure of plants to certain environmental stresses quite often leads to the generation of ROS, including superoxide anion radicals ($O_2^-$), hydroxyl radicals ($OH^-$), hydrogen peroxide ($H_2O_2$), alkoxy radicals (RO) and singlet oxygen ($^1O_2$) (Munne-Bosch and Penuelas 2003). Increased antioxidant content (ROS-detoxifying substances) is observed during cold acclimation in plants (Schulze et al. 2005). ROSs are generated in situations with highly energized primary photochemistry but impaired stromal metabolism. They can cause membrane damage by the formation of radicals. ROS-detoxifying substances may be localized in membranes (e.g., tocopherol and xanthophyll in the thylakoid membrane) or found in the cytosol or the stroma of the chloroplast (mainly ascorbate and glutathione, but also flavonoids). Overall, the production of ROS-detoxifying substances is linear with the severity of cold stress (Margesin et al. 2007).

### 6.3.4 Protein Modifications and Cryoprotective Proteins

Several proteins are expressed upon exposure to low temperature and may occur in the cytosol or be secreted to the apoplast. Such factors have various putative functions, including cryoprotection, altered lipid metabolism, protein protection, desiccation tolerance, and sugar metabolism (Hiilovaara-Teijo and Palva 1999). Three types of proteins have been shown to accumulate outside the cells in the apoplast during cold acclimation (a) cell-wall-modifying proteins, (b) a group of pathogenesis-related (PR) proteins that might be a component of the signal transduction pathway triggered during general stress response and (c) antifreeze proteins (AFPs) that interact with extracellular ice (Atici and Nalbantoglu 2003). AFPs have been found in a considerable number of woody and herbaceous plant species (Atici and Nalbantoglu 2003), including an Antarctic plant (Bravo and Griffith 2005). The effect of AFPs in plants on freezing protein (FP) depression appears to be negligible, as it is less than 1°C (Schulze et al. 2005). However, AFPs adsorb onto the surface of ice crystals and modify their shape and growth in a beneficial manner:

instead of one large single ice crystal, more but smaller and slower-growing ones develop. During thawing, AFPs may inhibit recrystallization and formation of larger ice crystals. Larger ice crystals increase the possibility of physical damage within frozen plant tissue (Griffith et al. 1997). Together with ice-nucleating proteins (INPs), AFPs are thought to control extracellular ice formation (Marentes et al. 1993). During cold acclimation, several stress proteins that may function as chaperones and membrane stabilizers during freeze dehydration are expressed in the cytosol (Puhakainen et al. 2004). These proteins have been assigned to the group of late-embryogenesis-abundant (LEA) proteins (Wise and Tunnacliffe 2004). LEA proteins are divided into several different structural groups; one such group (LEA II) consists of dehydrins (Allagulova et al. 2003) that are usually expressed in cells in response to dehydration stress. The proposed functions of dehydrins are considerable. Dehydrins seem to operate as membrane stabilizers, posses cryoprotective function or antifreeze activity, improve enzyme activity under conditions of low water availability, act in osmoregulation and as radical scavengers and as recently shown to have $Ca^{2+}$-binding activity, suggesting action as a $Ca^{2+}$ buffer or $Ca^{2+}$-dependent chaperone (Puhakainen et al. 2004). The expression and activity of many enzymes involved in several different metabolic pathways, such as carbon metabolism, photosynthesis, the detoxifying systems and proline and lignin metabolism, have been shown to change in response to low-temperature exposure (Renaut et al. 2006).

### *6.3.5 Compatible Solutes and Phenolics*

Cold acclimation is associated with the accumulation of a range of low-molecular-weight organic solutes including polyols and soluble sugars as well as several groups of amino acids and ammonium compounds such as betaine (Nomura et al. 1995). These substances can accumulate to osmotically significant levels without disrupting plant metabolism. Solute accumulation in cells has a colligative effect that reduces the cell volumetric collapse at any given subzero temperature (Crowe et al. 1992). Direct solute-specific beneficial actions are thought to be stabilization of macromolecules and membranes and protection of membranes against the deleterious effects of increasingly higher concentrations of electrolytes during freeze dehydration.

Proline is a dominant amino acid that accumulates in many organisms upon exposure to environmental stress and plays multiple roles in plant adaptation to stress (Delauney and Verma 1993; Nanjo et al. 1999; Sung et al. 2003). Proline having cryoprotective function, which accumulates during acclimatization and protection of enzymes from inactivation by chilling, has been demonstrated in *Solanum tubeorsum* culture suspension and bean seedling (Hellegren and Li 1981; Demir and Kocacaliskan 2001). At low-temperature significant correlation between freezing tolerance and an increase of proline concentration was observed in shoot and bud tissue of grapevines (Barka and Audran 1997). Cold hardening was

also correlated to increase in proline amount in barley cultivars and maize mutant callus with high free proline content that survived longer exposure to 4°C as compared to callus with low free proline content (Demir and Kocacaliskan 2001; Abromeit et al. 1992).

Sugar has been considered to be one of the most important factors in freezing tolerance. In woody plants, sugars accumulate from autumn to winter as freezing tolerance increases (Sakai and Yoshida 1968). In some herbaceous plants including wheat, cloudberry, and spinach (Guy et al. 1992), changes in sugar content are correlated with those of freezing tolerance. Accumulation of phenolics is linked to the host defense, which also includes strengthening of cell walls in the exodermises and several cortical cell layers (Compant et al. 2005). The activation of secondary responses associated with the onset of induced resistance including the oxidation and polymerization of preexisting phenols and the synthesis of new phenolics compounds via the activation of the phenylpropanoid pathways.

### 6.3.6 *Lowering Ion Leakage and $Na^+/K^+$ Ratio*

Membrane thermostability has been identified in most modern literature as an indicator of thermo-tolerance. Disruption and damage to cell membranes altered permeability and resulted in a loss of solutes (electrolytes). This damage will cause injury to the plant resulting in cell death. Electrolyte leakage reflects damage to cellular membranes. The amount of electrolyte leakage is a function of membrane permeability. An increase in electrolyte leakage indicates an increase in membrane permeability and reduced cell tolerance to temperature change. Many plants have the ability to reduce membrane permeability and enhance cold resistance. Cold stress leads to dehydration and osmotic imbalances in the plant that form specific lesions (Levitt 1980). As $Na^+$ concentration increases, the normal ratio of $Na^+$ leads to reduced cellular $K^+$ to decreased activities of numerous metabolic enzymes. Stress-specific lesions from exposure to cold and freezing temperature occur as a result of reduction in membrane fluidity and the formation of ice in the intracellular spaces of plant tissue resulting in the physical disruption of the cells and tissue (Schachtman and Liu 1999).

## 6.4 Cold-Tolerant PGPRs as Bioinoculants for Stress Management

Cold-tolerant plant-growth-promoting bacteria (PGPBs) are of great agronomic importance due to the fact that the crop growing cycle in most parts of the world is subject to varying spells of cold temperatures. Indeed, they are metabolically functional at cold condition and produce metabolites such as plant growth

regulators that directly promote growth and facilitate nutrient uptake by plants, while beneficial activities of mesophilic microbes cease at cold temperature. PGPRs enhance plant growth while improving their resistance to stress. PGPRs can stimulate developmental changes in host plants, disrupt phytopathogen organization, induce systemic resistance to pathogens, affect phytohormone production, improve nutrient and water management (Barka et al. 2006).

### *6.4.1 Cold-Tolerant Plant-Growth-Promoting Rhizobacteria*

Soil is a dynamic, living matrix and is a critical resource not only for agriculture production and food security but also toward maintenance of most life processes. Soil is considered a storehouse of microbial activity, although the space occupied by living microorganisms is estimated to be less than 5% of the total space. The volume of soils surrounding roots is influenced chemically, physically and biologically by the plant root, which is commonly referred to as the rhizosphere. This is a highly favorable habitat for the proliferation of microorganisms that exert a potential impact on plant health and soil fertility. PGPRs, which are an important component of the rhizosphere microbial community, were first defined by Kloepper and Schroth (1978). In recent years, the term has been modified to PGPBs to accommodate other strains that are nonrhizospheric in origin (Andrews and Harris 2003). In temperate climates, the growth and activity of such rhizospheric communities are highly dependent on the root zone temperature, since most physiological processes that influence plant growth virtually come to a standstill at suboptimal temperatures. In such a scenario, it is important that the root-colonizing bacteria retain their metabolic versatility at low temperatures, since plant growth promotion is achieved by the action of several metabolic intermediates and end products.

Microorganisms play a major role in sustaining the production and productivity of any agro-ecosystem through a myriad of roles that include nitrogen fixation, nutrient solubilization, mobilization, plant growth promotion and the suppression of harmful pathogens and insects. A unique feature of temperate agro-ecosystems around the world is the short growing periods, which are interspersed by suboptimal temperatures. Under such a scenario, most microbial processes are bound to slow down or worsen and can even come to a standstill, thereby having an adverse effect on the productivity. This effect is most pronounced in the case of nutrient transformations where microbes play an enormous role. In such a scenario where time and temperatures are crucial determinants of both crop growth and microbial growth, cold-tolerant microbes, which retain their activity in suboptimal temperature conditions, play an important role. Since microorganisms are an integral part of any ecosystem, interest has been renewed in the nature and properties of microbes that play a major role in nutrient cycling in cold ecosystems (Haggblom and Margesin 2005). Based on their preference for cold temperatures, microbes are classified as psychrophiles (cold loving) and psychrotolerant (cold tolerant).

Psychrophiles colonize permanently cold habitats, such as Polar regions, high altitudes, the deep sea and grow at temperatures ranging from subzero to 15°C. Environments with periodic, diurnal, or seasonal temperature fluctuations (e.g., areas with continental climates with high summer and low winter temperatures) are favorable for the growth and proliferation of psychrotolerant (also termed cold-tolerant or psychrotolerant) microorganisms, which grow over a wide temperature range from 4 to 42°C and usually grow optimally at temperatures above 20°C (Morita 1975). In high-altitude agro-ecosystems, where cold conditions are usually transitional in nature, psychrotolerant microbes are extremely important since they survive and retain their functionality in cold-temperature conditions, while growing optimally at warmer temperatures. However, unfortunately not many efforts have been undertaken in understanding the nature and properties of these microbes and the quantum of information available on their application in cold agro-ecosystem is very scanty.

One of the major mechanisms of plant growth promotion is the production of the stimulatory phytohormones by PGPRs/PGPBs within the root zone; these hormones stimulate the density and length of root hairs resulting in the enhanced uptake of water and mineral nutrients from soil (Volkmar and Bremer 1998). Apart from phytohormone production, plant growth promotion is known to be mediated by a variety of mechanisms, including siderophore production (Katiyar and Goel 2004), deamination of the precursor molecule of the phytohormone ethylene whose accumulation in root tissue is known to be detrimental to root growth and development (Glick et al. 1998), induction of systemic resistance to plant pathogenic microorganisms (Lavania et al. 2006) and antagonism toward deleterious root microorganisms (Misaghi et al. 1982), of which much has been described recently (Singh et al. 2010).

### *6.4.2 Indole Acetic Acid Producers*

A major mechanism of plant growth promotion is through the production of the stimulatory phytohormone, produced by PGPRs within the root zone; these hormones stimulate the density and length of root hairs. The increase in root surface area improves the plant uptake potential of water and mineral nutrients from a large volume of soil. The IAA-producing capability of microorganisms is useful in their identification and provides a valuable marker when examining the physiological roles or ecological significance of IAA in the establishment and persistence of the organisms (Bric et al. 1991). Auxin production in bacteria is regulated through the proline-linked pentose phosphate pathway (McCue et al. 2000). Selvakumar et al. (2008a, b) reported the occurrence of cold-tolerant PGPB strains *Pantoea dispersa* strain 1A and *Serratia marcescens* strain SRM from the North-Western Indian Himalayas. These strains retained their IAA-producing ability at 4 and 15°C. Seed bacterization with these bacterial strains significantly enhanced plant biomass and nutrient uptake of wheat seedling grown at cold temperatures. Mishra et al.

(2008, 2009a) described the cold tolerance and IAA production by *Pseudomonas* sp. strain PGERs17 and NARs9 at cold temperature and seed bacterization with these strains enhanced the seed germination as well as the root and shoot lengths of wheat seedlings grown at low temperatures. Considering the metabolic versatility of pseudomonads, it is possible to unearth a whole new cold-tolerant PGPB species in the future.

### 6.4.3 ACC-Deaminase Producers

One of the major mechanisms that some PGPBs use to facilitate plant growth involves the functioning of the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme plays a significant role in the regulation of the plant hormone, ethylene, thus influencing the growth and development of plants. Bacterial strains containing ACC deaminase can in part at least alleviate the stress-induced ethylene-mediated negative impact on plants. Like many others abiotic and biotic factors, accelerated ethylene production under high and chilling temperatures has been widely reported by researchers in both plant tissues and microbial species in the rhizosphere. Plants with ACC deaminase expression may cope with this unfavorable situation by lowering ethylene level like that under other environmental stresses (Saleem et al. 2007). A psychrotolerant ACC deaminase-producing bacterium *P. putida* UW4 was found to promote canola plant growth at low temperature under salt stress (Cheng et al. 2007). Considering the role of ethylene in stress physiology, it can be rightly said that much more efforts are needed to decipher the role of ACC deaminase-producing bacterial strains in plant growth promotion under cold-temperature conditions.

### 6.4.4 Siderophore Producers

Iron is an essential micronutrient of plants as it serves as a cofactor of many enzymes with redox activity. A large portion of iron in soils is present in highly insoluble form of ferric hydroxide; thus iron acts as a limiting factor for plant growth even in iron-rich soils. Its availability to the organism is very limited due to the rapid oxidation of ferrous ($Fe^{++}$) to ferric ($Fe^{+++}$) state. Ferric ion is highly insoluble under physiological conditions and makes its acquisition by microorganisms a considerable challenge (Neilands 1995). Microorganisms have evolved specialized mechanisms for the assimilation of iron, including the production of low-molecular-weight iron-chelating compounds known as siderophores, which transport this element into their cells. Siderophores have been implicated for both direct and indirect enhancement of plant growth by rhizospheric microorganisms (Neilands 1981). Siderophores provide an advantage in the survival of both plants and bacteria because they mediate competition that results in

exclusions of fungal pathogens and other microbial competitors in the rhizosphere by a reduction in the availability of iron for their survival (Masalha et al. 2000; Wang et al. 2000). A cold-tolerant mutant of *Pseudomonas fluorescens* with a 17-fold increase in siderophore production and increased rhizosphere colonization was developed by Katiyar and Goel (2004). This mutant strain promoted growth of *Vigna radiata* plants at 25 and 10°C. Studies on siderophore-mediated growth promotion by psychrotolerant bacteria still remains in its infancy and needs to be studied further.

An important facet of the competitiveness of a biocontrol agent is its ability to persist and proliferate. However, it is often difficult to predict the behavior of a particular microbe in the soil, since the soil persistence of a bacterium may be influenced by a number of different factors including soil temperature. Many fungal phytopathogens are most destructive when the soil temperature is low; hence, it is reasonable to expect that the biocontrol agents are also cold tolerant. McBeath (1995) reported the isolation of several strains of *Trichoderma* sp. that acted as biocontrol agents at low temperatures (i.e., 4–10°C) against a range of different pathogenic fungi. Negi et al. (2005) have characterized a group of cold-tolerant *Pseudomonads* from the Garhwal region of the Indian Himalayas. These strains produced siderophores and exhibited plant-growth-promoting activity at temperatures ranging from 4 to 25°C. Seed inoculation with these isolates resulted in the suppression of major root-borne diseases of garden pea. A novel bacterium *Exiguobacterium acetylicum* strain 1P isolated from a high-altitude soil in the north-western Indian Himalayas which has the ability to produce siderophores at 4°C and inhibited the growth and development of *Rhizoctonia solani*, *Sclerotium rolfsii*, *Phythium*, and *Fusarium oxysporium* under in vitro and pot-culture conditions was described by Selvakumar et al. (2009c).

A cold-tolerant *Pseudomonas* sp. strain PGERs17 possessed HCN and siderophore production abilities at 4°C. It exhibited inhibitory activity against several phytopathogenic fungi in three different bioassays. The maximum relative growth inhibition was recorded against *S. rolfsii* and *R. solani* (100%), followed by *Pythium* sp. (73.1%) and *F. oxysporum* (19.7%) in volatile compound assays (Mishra et al. 2008). Malviya et al. (2009) have isolated antagonistic, chitinolytic, psychrotolerant strains of *Streptomyces* from glacial sites of the Indian Himalayas. These strains were found to inhibit the growth of several plant pathogenic fungi. In the present scenario where the demand for pesticide-free food products is on the rise, much more research efforts are required for identifying cold-tolerant strains as biocontrol agents for use in the temperate growing regions.

### 6.4.5 Phosphate Solubilizer

Among the various geochemical cycles mediated by microbes, phosphate solubilization assumes considerable significance due to the indispensability of phosphorous in plant nutrition. Although the soil phosphorus levels are quite sufficient to sustain

plant growth, most forms of phosphorus are present in fixed forms and thereby require transformation. Phosphate solubilization by rhizospheric microflora is one of the most important means of achieving plant growth promotion. Bacterial mineral phosphate solubilization has been mainly attributed to the activity of glucose dehydrogenase – a membrane-bound enzyme that is involved in the direct oxidation of glucose to gluconic acid (Goldstein 1995). Subsequently, gluconic acid is enzymatically converted to 2-ketogluconic acid and 2,5-diketogluconic acid; 2-ketogluconic acid is more effective than gluconic acid in solubilizing phosphate (Kim et al. 2002). Earlier studies on this phenomenon were restricted to mesophilic temperatures (Chung et al. 2005; Chen et al. 2006). The first report of P-solubilization at low temperatures was made by Das et al. (2003) who studied cold-tolerant *Pseudomonas* mutants for their phosphate solubilization activity at low temperature (10°C). They found that all the cold-tolerant mutants were more efficient than their respective wild-type counterparts for phosphate solubilization activity at 10°C as compared to 25°C. P-solubilization by *Pseudomonas* mutants at the psychrotolerant range has also been reported (Katiyar and Goel 2003; Trivedi and Sa 2008). However, considering the environmental stability of mutant strains, for commercial inoculant production it would be prudent to scout pristine environments for naturally occurring psychrotolerant strains. Most progress has been made in this direction, mainly from the Indian Himalayan Region.

Pandey et al. (2006) isolated a cold-tolerant phosphate solubilizing and antagonistic strain of *Pseudomonas putida*, from a subalpine location of Indian central Himalayas. This strain solubilized phosphate in the temperature range of 4–28°C. Recently P-solubilization by a cold-tolerant strain of *Pseudomonas fragi* was reported by Selvakumar et al. (2009a). This is a novel discovery since *P. fragi* is generally associated with spoilage of dairy foods under refrigerated conditions. This strain solubilized phosphate at temperatures ranging from 4 to 30°C, besides increasing the rate of seed germination, plant biomass and nutrient uptake of wheat seedlings under cold-temperature conditions. A rhizosphere-competent phosphate-solubilizing strain of *Acinetobacter rhizosphaerae* was isolated from the cold deserts of the Indian Himalayan region by Gulati et al. (2009). Vyas et al. (2009) screened 19 efficient P-solubilizing fluorescent *Pseudomonas* isolates from the cold deserts of the trans-Himalayas for tolerance against temperature, alkalinity, salinity, calcium salts and desiccation-induced stresses. Phylogenetic analysis based on 16S rRNA gene sequencing placed these bacteria under three groups with 14 strains in group I including *Pseudomonas trivialis* and *P. poae*, two strains in group II together with *Pseudomonas kilonensis* and *P. corrugata* and three strains in group III along with *Pseudomonas jessenii* and *P. moraviensis*. Selvakumar et al. (2009b) reported that the genetic clustering of cold-tolerant phosphate-solubilizing pseudomonads was affected by their geographical origin. Repetitive-element PCR profiles revealed that isolates originating from the warmer southern slopes formed a distinct cluster, while their counterparts from the cooler north facing slopes formed the second cluster. In a recent study, seed bacterization with *Pseudomonas lurida* M2RH3 positively influenced the growth and nutrient uptake parameters of wheat seedlings cv. VL 804 in pot-culture conditions at controlled cold growing

temperature. This is a relatively new species of the genus *Pseudomonas* and opens up a hitherto unknown facet of cold-tolerant bacterium (Selvakumar et al. 2010). The studies that have been mentioned above are mostly of exploratory nature, while the real need of the hour is the development of a commercially viable cold-tolerant PSB inoculant preparation that could be profitably used in temperate agriculture.

### *6.4.6 Nitrogen Fixer*

Nitrogen fixation by symbiotic and asymbiotic bacterial genera is one of the major means by which life is sustained in this planet. However, this process is greatly affected by cold-temperature stress. The effects of low temperature on the activities of rhizobia include depression of nodule competitiveness and nodule functioning. The production of Nod metabolites by *R. leguminosarum* bv. *trifolii* is reduced by lowering the temperature, which in turn may affect the nodulation and yield of host legumes (McKay and Djordjevic 1993). Many studies have shown that suboptimal temperatures affect the competitiveness of rhizobia for nodulation, delay root infection and inhibit nodule function (Lynch and Smith 1994). It has been estimated that under temperate conditions, the establishment of an effective symbiosis a week earlier in the crop-growing season could double the amount of nitrogen fixed and thus increase legume crop productivity (Sprent 1979). Therefore, it is imperative to select cold-adapted/tolerant strains of rhizobia to overcome the cold-induced stress. In a major step in this direction, Prevost et al. (1999) selected cold-adapted rhizobia from Canadian soils with the aim of improving the productivity of legumes that are subjected to cool temperatures during the growing season. For this purpose, they used rhizobia associated with legume species indigenous to arctic and subarctic regions. The candidate rhizobia were *Mesorhizobium* sp. isolated from *Astragalus*, *Oxytropis* spp. and *R. leguminosarum* from *Lathryrus* spp. These rhizobia are considered psychrotrophs due to their ability to grow at 0°C. The advantages of cold-adapted arctic *Mesorhizobium* in improving legume symbiosis were demonstrated with the temperate forage legume sainfoin (*Onobrychis viciifolia*). In laboratory and field studies, arctic rhizobia were found to be more efficient than temperate (commercial) rhizobia in improving the growth of sainfoin and were more competitive in forming nodules. Biochemical studies on cold adaptation revealed higher synthesis of cold shock proteins in the cold-adapted rhizobia than their mesophilic counterparts. Since the arctic *Mesorhizobium* could not nodulate agronomically important legumes, the nodulation genes and the bacterial signals (Nod factors) were characterized as a first step to modify the host specificity of nodulation.

Another approach was to screen for cold-adapted rhizobia naturally associated with agronomic legumes cultivated in temperate areas. It has been shown that the environment from which rhizobia are isolated relates to their ability to enter into symbiosis with legumes under specific environmental conditions. Rhizobia originating from the cooler climes of North America were able to positively

influence the nodulation and nitrogen fixation of soybean compared to their counterparts originating from the warmer southern climes (Zhang et al. 2003). A superior strain of *Sinorhizobium meliloti* adapted for nodulation of alfalfa at low temperatures was selected and found efficient in improving the growth of alfalfa in laboratory and field studies. This strain also performed well in improving the growth of alfalfa after overwintering under cold and anaerobic (ice encasement) stresses, indicating a possible cross-adaptation of selected rhizobia for various abiotic stresses inherent to temperate climates (Prevost et al. 2003). Ideal candidate rhizobia for temperate legumes would therefore require a high degree of nodule competitiveness and nitrogen-fixing abilities combined with cold-tolerant traits. Such rhizobia would retain their membrane fluidity at low temperatures, thereby enabling the synthesis and activity of membrane-associated Nod factors that play a major role in the nodulation and host specificity.

*Azospirillum* is an associative symbiotic PGPB that is predominantly associated with the grasses and cereal crops of the tropics. Tripathi and Klingmuller (1992) proposed that growth, survival, and activity of the bacterium are highly dependent on temperature. Earlier, Kaushik et al. (2001) postulated that a low or nonsignificant effect of *Azospirillum* inoculation in winter crops has discouraged the large-scale use of this bacterium. Kaushik et al. (2000) selected Tn5:lacZ mutants isogenic to wild-type *Azospirillum brasilense* that were capable of growing at cold temperatures. In field studies, two strains of *A. brasilense* were able to influence wheat growth at suboptimal temperatures (Kaushik et al. 2002). Although the temperature regime at which the isolates were evaluated for their plant response was not strictly temperate, this is one of the few studies on field performance of *Azospirillum* under suboptimal temperatures. Considering its agronomic significance, *Azospirillum* is a candidate bacterium with the potential for exploration and development of cold-tolerant isolates.

### *6.4.7 Ice⁻ Bacteria for Frost Management*

Freezing injury in plants is particularly complex because of the nonuniform behavior of different plant parts, e.g., stem, leaf, bud and flowers. Ice nucleation in plants is frequently not endogenous but is induced by catalytic sites present in microbial parasites, which can be found on leaves, fruits, or stems (Lindow 1983). In cold-weather conditions, frost settles on plants and can cause a great deal of damage. The agricultural industry suffers heavy losses every year due to frost-damaged crops. Ice-nucleating activities (INAs) limit supercooling and induce freezing at high subzero temperature by mimicking the structure of an ice crystal surface. They impose an ice-like arrangement on the water molecule in contact with their surface and lower the energy necessary for the initiation of ice formation. The “ice plus” bacteria possess Ina protein (ice nucleation-active protein) found on the outer bacterial wall, which acts as the nucleating center for ice crystals (Lee et al. 1995). This facilitates ice formation at high subzero temperature, while “ice minus” bacteria

do not posses Ina proteins and lower the ice nucleation temperature (Zachariassen and Kristiansen 2000). Researchers hope that spraying ice-minus bacteria on a wide scale of plants may help stave off the annual havoc caused by frosty conditions. Ice-nucleating strains of *P. syringae* increase the frost susceptibility of tomato and soybean when sprayed on leaves prior to low-temperature stress in addition to being a pathogen of these plants (Anderson et al. 1982). Recognition of the gene associated with ice nucleation in *P. syringae* first led to the synthesis of an "ice-minus" mutant, which was found to be inactive in promoting ice nucleation in plants leaves (Xu et al. 1998). Reducing the numbers of ice-nucleating bacteria by different approaches is an effective and environmentally safe method of controlling freezing injury in plants and is considered a classic example of displacement of a bacterial pathogen by a biocontrol agent.

The ice-minus bacterium is a mutant strain of the common wild-type bacteria *P. syringae*. The wild-type *P. syringae* is known as ice-plus bacteria. It contains a surface protein in its outer cell wall that helps with frost formation, hence the name ice-plus. In the case of the mutant *P. syringae*, the frost-facilitating surface protein is missing; hence, bacteria cannot facilitate frost formation and are, therefore, known as ice-minus bacteria. Both ice-minus bacteria and ice-plus bacteria are found in nature. However, the ice-minus bacteria to be used for spraying crops are made on a large scale using rDNA technology.

### 6.4.8 Management of Chilling Resistance/Tolerance by Cold-Tolerant PGPRs

Cold-temperature stress affects the metabolic activity of plants in multiple ways and causes significant yield reduction. To overcome this, several exploratory studies using microbial strains have been carried out. *In vitro* inoculation of *Vitis vinifera* cv. Chardonnay explants with a PGPR *Burkholderia phytofirmans* strain PsJN increased grapevine growth and physiological activity at a low temperature. There was a relationship between endophytic bacterial colonization of the grapevine plantlets and their growth at both ambient (26°C) and low (4°C) temperatures and their sensitivities to chilling. The major benefits of bacterization were observed on root growth (11.8- and 10.7-fold increases at 26°C and 4°C, respectively) and plantlet biomass (6- and 2.2-fold increases at 26°C and 4°C, respectively). The inoculation with PsJN also significantly improved plantlet cold tolerance compared to that of the nonbacterized control. Moreover, relative to the noninoculated controls, bacterized plantlets had significantly increased levels of starch, proline and phenolics. These increases correlated with the enhancement of cold tolerance of the grapevine plantlets (Barka et al. 2006).

Recently, Mishra et al. (2009b) examined the effect of seed inoculation with 12 cold-tolerant PGP *Pseudomonas* strains on wheat growth and physiological changes under greenhouse conditions at 10 ± 2°C. It was observed that bacterization

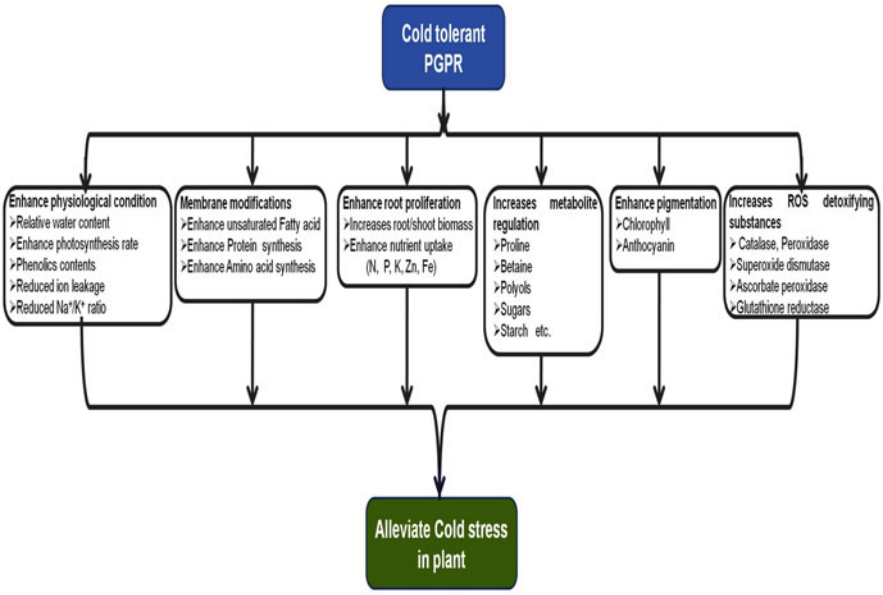


**Fig. 6.2** Alleviation of cold stress by PGPR accompanying increased physiological, biochemical, and plant growth in plants (Adapted from Barka et al. 2006; Mishra et al. 2009b; Bisht et al. 2009)

with Pseudomonads significantly improved root length (27.9–70.5%), shoot length (4.7–26.1%), dry root biomass (1.69–3.19-fold) and dry shoot biomass (1.27–1.66-fold) compared to nonbacterized control; enhanced total chlorophyll, anthocyanin, free proline, total phenolics, and starch content; and decreased the $Na^+/K^+$ ratio, and electrolyte leakage was also observed in bacterized wheat plants indicating the ability of the bacterium to alleviate cold-induced stress in wheat seedlings (Fig. 6.2). These parameters are critical to the plant's ability to tolerate cold-stress conditions. In another study, inoculation of cold-tolerant *Pseudomonas* sp. PPERs23 on wheat in field conditions had significantly ($P < 0.05$) improved root length (41.0%), shoot length (11.9%), dry root biomass (44.4%), dry shoot biomass (53.8%), total chlorophyll (3.1%), total phenolic (37.3%) and 39.4% amino acid content in leaves in comparison with uninoculated control at 60 days of plant growth; enhanced physiologically available iron, protein concentration, anthocyanin, proline, and relative water content; decreased the $Na^+/ K^+$ ratio and electrolyte leakage was also observed in bacterized wheat plants. The bacterium *Pseudomonas* spp. PPERs23 inoculation resulted in a significant increment in grain yield of wheat up to 13.4%, nutrient content in shoot and grain portion as compared to uninoculated control (Maheshwari 2010). Further work is required to observe the effect of cold-tolerant PGPRs/PGPBs on other crops cultivated in temperate regions.

## 6.5 Conclusion

Cold-tolerant PGPRs are widely distributed in the agro-ecosystem and play a variety of roles including plant-growth-promoting biocontrol agent, nitrogen fixation and alleviation of cold stress in plants. Although most research work conducted so far has largely focused on rhizobia, it is a welcome sign that many agriculturally important resourceful microbes are being described from various parts of the world. However, serious attempts are needed to study the mechanism of plant–microbe interaction, especially under stress condition. Another interesting area where research needs to be focused is the identification of cold-active microorganisms that reduce the metal toxicity and harmful waste, since temperature is a major determinant of decomposition and most decomposition processes come to a standstill at suboptimal temperatures. If research efforts succeed in identifying a consortium of potential decomposers with PGPR activity that retain their plant growth potential at lower temperatures, it would be of immense use in agriculture all over world.

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# Chapter 7
# Hormonal Signaling by PGPR Improves Plant Health Under Stress Conditions

**Chaitanya Kumar Jha and Meenu Saraf**

## 7.1 Introduction

Plant growth and development involve a tight coordination of the spatial and temporal organization of cell division, cell expansion, and cell differentiation. Orchestration of these events requires the exchange of signaling molecules between the root and shoot, which can be affected by both biotic and abiotic factors. The interactions that occur between plants and their associated microorganisms have long been of interest, as knowledge of these processes could lead to the development of novel agricultural applications. Plants produce a wide range of organic compounds including sugars, organic acids, and vitamins, which can be used as nutrients or signals by microbial populations. On the other hand, microorganisms release phytohormones, small molecules, or volatile compounds, which may act directly or indirectly to activate plant immunity or regulate plant growth and morphogenesis. Several chemical changes in soil are associated with plant-growth-promoting rhizobacteria (PGPRs). Some bacterial strains directly regulate plant physiology by mimicking synthesis of plant hormones, whereas others increase mineral and nitrogen availability in the soil as a way to augment growth. Identification of bacterial chemical messengers that trigger growth promotion has been limited in part by the understanding of how plants respond to external stimuli. PGPRs are used as inoculants for biofertilization, phytoestimulation, and biocontrol. The general effect of PGPRs is an increased growth and productivity of plants. Their contribution can be exerted through different mechanisms including root system architecture modulation and increased shoot growth by production of phytohormones such as auxins and cytokinins. Other indirect mechanisms include the effects of products such as antibiotics and hydrogen cyanide, which promote

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plant growth by inhibiting the growth of deleterious microorganisms in the rhizosphere. PGPRs can induce defense programs such as systemic acquired resistance (SAR) and induced systemic resistance (ISR), thus reducing phytotoxic microbial communities. They also can elicit induced systemic tolerance (IST) to abiotic stress. Proposed signal molecules for plant-growth promotion by PGPRs include bacterial synthesis of the plant hormones indole-3-acetic acid (Loper and Schroth 1986), cytokinin (Timmusk et al. 1999), and gibberellin (MacDonald et al. 1986); breakdown of plant-produced ethylene by bacterial production of 1-aminocyclopropane-1-carboxylate deaminase (Glick 1999); and increased mineral and N availability in the soil (Lin et al. 1983), although low-molecular-weight plant volatiles such as terpenes, jasmonates, and green leaf components have been identified as potential signal molecules for plants and organisms of other trophic levels (Farmer 2001; Farag and Pare 2002). Extensive communication occurs between plants and microorganisms during the different stages of plant development in which signaling molecules from the two partners play an important role. Fungal and bacterial species are able to detect the plant host and initiate their colonization strategies in the rhizosphere by producing canonical plant-growth-regulating substances such as auxins or cytokinins. On the other hand, plants are able to recognize microbe-derived compounds and adjust their defense and growth responses according to the type of microorganism encountered. This molecular dialog will determine the final outcome of the relationship, ranging from pathogenesis to symbiosis, usually through highly coordinated cellular processes (Bais et al. 2004). Bacterial phytopathogens are not restricted to infecting aerial or root tissues exclusively; as such, communication between the shoot and root can confer a survival advantage to the plant and potentially limit or prevent diseases. For instance, beneficial soil bacteria can confer immunity against a wide range of foliar diseases by activating plant defenses, thereby reducing a plant's susceptibility to pathogen attack (Van Loon et al. 1998). For many years, this was considered the basis by which beneficial microorganisms could increase plant yield when inoculated in crops; however, it is increasingly appreciated that classic and novel microbial signals may also directly participate in plant morphogenesis.

## 7.2 Hormonal Signals Involved in Plant Growth and Development

Most commonly proposed signal molecules for plant-growth promotion by PGPRs include bacterial synthesis of the plant hormones auxin, cytokinin, and gibberellin and breakdown of plant-produced ethylene by bacterial production of 1-aminocyclopropane-1-carboxylate deaminase. In the last 5 years, additional signals from microbes have been found to play a role in plant morphogenetic processes, including the *N*-acyl-L-homoserine lactones (AHLs) and volatile organic compounds (VOCs). AHLs belong to a class of bacterial quorum-sensing signals

from Gram-negative bacteria such as *Pseudomonas*. These compounds enable bacterial cells to regulate gene expression, depending on population density. Very recently, it was found that AHLs can be recognized by plants, alter gene expression in roots and shoots, and modulate defense and cell growth responses. In a similar way, bacterial volatiles such as acetoin and 2,3-butanediol produced by certain PGPRs can be used for plant–bacteria communication and as a plant-growth promotion triggers.

### *7.2.1 Auxins*

Diverse bacterial species produce auxins as part of their metabolism, including indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), or their precursors (Fig. 7.1). Evidence indicating that IAA is a positive regulator of plant growth comes from the analysis of mutants that overproduce it, such as *super root* and *yucca*, which have long hypocotyls and increased numbers of lateral roots and root hairs and the positive effect of IAA application on the growth of excised stems and hypocotyls and of auxin analogs in intact *Arabidopsis* seedlings. The signaling mechanisms by which *Trichoderma virens* promote growth and development were further investigated in *Arabidopsis thaliana* by Contreras-Cornejo et al. (2009). It was found that mutations in genes involved in auxin transport or signaling, *AUX1*, *BIG*, *EIR1*, and *AXR1*, reduced the growth-promoting and root developmental effects of Trichoderma inoculation. When grown under axenic conditions, *T. virens* produced IAA and the IAA-related substances indole-3-acetaldehyde (IAAld) and indole-3-ethanol (IEt). Interestingly, the application of all three compounds to *Arabidopsis* seedlings showed a dose-dependent effect on biomass production, increasing yield in small amounts (nanomolar range) but repressing growth at higher concentrations (millimolar range). Auxins derive from tryptophan metabolism, and their effects depend on the concentration, the organ affected, and the physiological status of the plant. Auxins synthesized by the plant and the microorganisms differ only in the biosynthetic pathway, depending on the plant and/or microorganism. More than 80% of soil bacteria in the rhizosphere are capable of producing auxins; thus, the potential of these microorganisms to affect the endogenous levels of this regulator and, therefore, its effects on plant growth are remarkable. Auxins principally affect plant roots (Salisbury 1994). Those released by rhizobacteria mainly affect the root system, increasing its size and weight, branching number, and the surface area in contact with soil. All these changes lead to an increase in its ability to probe the soil for nutrient exchange, thereby improving plant nutrition and growth capacity (Gutiérrez Mañero et al. 1996). Another important result of inoculation with auxin-producing bacteria is the formation of adventitious roots, which derive from the stem. The auxins induce the stem tissues to redifferentiate as root tissue. All the above effects can vary considerably depending on the auxin concentration that reaches the root system, including an excess that could be inhibitory.

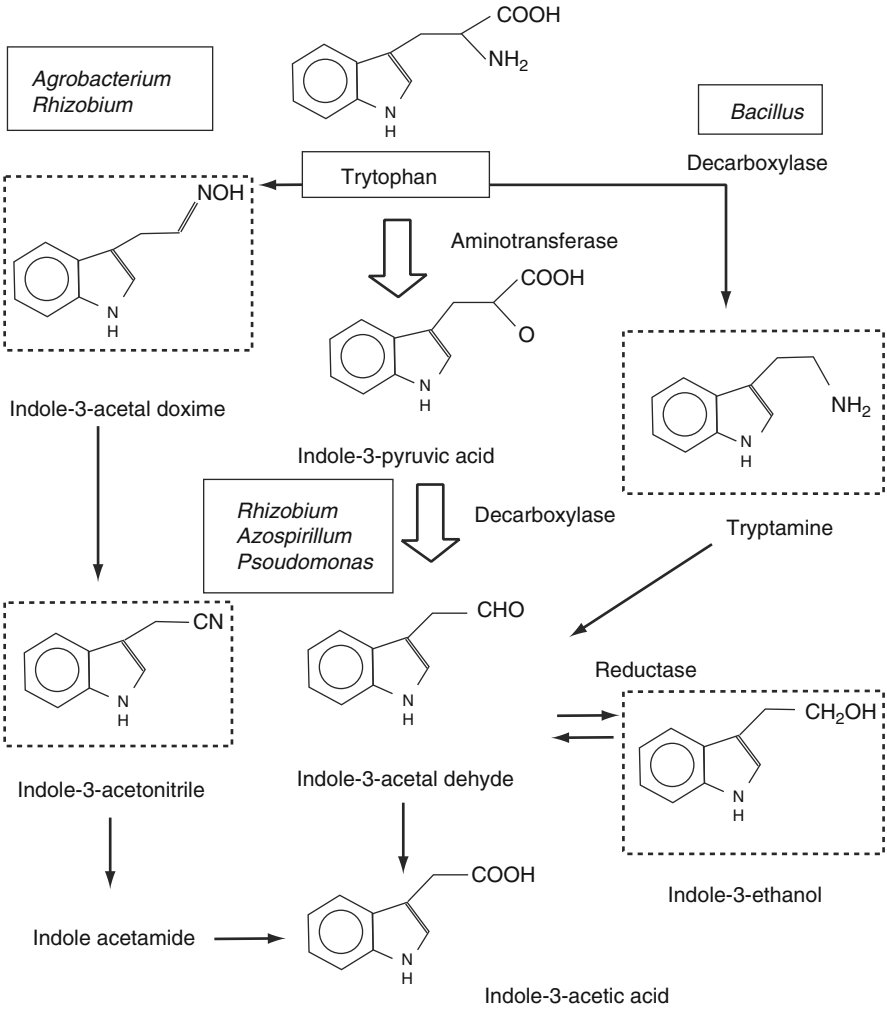


**Fig. 7.1** Tryptophan-dependent auxin biosynthetic pathways in plants and microorganisms (adapted from Solano et al. 2008)

### *7.2.2 Cytokinins*

Cytokinins are purine derivatives that promote and maintain plant cell division in cultures and are also involved in various differentiation processes, including shoot formation, primary root growth, and callus formation. Plants continuously use cytokinins to maintain the pools of totipotent stem cells in their shoot and root meristems (Howell et al. 2003; Leibfried et al. 2005). Endogenous cytokinin overproduction in transgenic plants causes pleiotropic phenotypic alterations, including cytokinin-auxotrophic growth of calli in vitro (Howell et al. 2003).

Analysis of cytokinin-overproducing and cytokinin-deficient mutants has confirmed a stimulatory role for these compounds in the regulation of cell division activity in the shoot meristem and young leaves (Rupp et al. 1999; Frank et al. 2002). Recent data indicate regulatory interactions between cytokinins and alkamides; these latter compounds belong to a novel class of plant signals reported to affect both shoot and root system architecture in plants (López-Bucio et al. 2007). Auxins and cytokinins interact in the control of many important developmental processes in plants, particularly in apical dominance, and root and shoot development. The balance between auxin and cytokinin is a key regulator of in vitro organogenesis. Exposing callus cultures to a high auxin to cytokinin ratio results in root formation, whereas a low ratio of these hormones promotes shoot development. Many experiments have demonstrated the existence of synergistic, antagonistic, or additive interactions between auxins and cytokinins, suggesting complex signal interactions involved in the modulation of root and shoot architecture.

### *7.2.3 Gibberellins*

There is little information regarding microorganisms that produce gibberellins, although it is known that symbiotic bacteria existing within nodules in leguminous plants to fix nitrogen (rhizobia) are able to produce gibberellins, auxins, and cytokinins in very low concentrations when the plant is forming the nodule and there is a high cell duplication rate (Atzorn et al. 1988). However, the production of gibberellins by PGPRs is rare, with only two strains being documented that produce gibberellins, *Bacillus pumilus* and *Bacillus licheniformis* (Gutiérrez Mañero et al. 2001). These bacteria were isolated from the rhizosphere of *A. glutinosa* and have shown a capacity to produce large quantities of gibberellins GA1, GA3, GA4, and GA20 in vitro. These types of hormones are the largest group of plant regulators, including more than 100 different molecules with various degrees of biological activity. The common structure of these diterpenic growth regulators is a skeleton of 19–20 carbon atoms, and there is a clear relationship between structure and biological effect. The reason for the pronounced effect of gibberellins is that these hormones can be translocated from the roots to the aerial parts of the plant. The effects in the aerial part are notable and more so when the bacteria also produce auxins that stimulate the root system, enhancing the nutrient supply to the sink generated in the aerial part. The gibberellin characterization in bacteria using physico-chemical methods was first reported by Atzorn et al. (1988), who demonstrated the presence of GA1, GA4, GA9, and GA20 in gnotobiotic cultures of *Rhizobium meliloti*. Apart from *Azospirillum* sp. and *Rhizobium* sp., production of gibberellin-like substances has also been claimed in numerous bacterial genera, although the techniques used (TLC, bioassays, and HPLC-UV) are of poor resolution and/or reliability. Using unequivocal physico-chemical methods, such as GC-MS, production of gibberellins has been confirmed in *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae* (Bastián et al. 1998) and *Bacillus* sp.

(Gutiérrez Mañero et al. 2001) in addition to *Azospirillum* sp. (see above). In fungi, the general pathway (Fig. 7.2) is similar to that of higher plants, although the genes and enzymes involved differ. Recently, Tudzynski et al. (2003) completed the cloning of six genes of the gibberellin biosynthesis gene cluster in *Gibberella fujikuroi* and determined the functions of these genes, thus defining the complete gibberellin biosynthetic pathway in this fungus. Notably, all the enzymes involved are membrane-related cytochrome P450 monooxygenases and none are soluble dioxygenases. These enzymes comprise the gibberellin-specific GGPP synthase (GGS2), ent-K synthase (CPS/KS), and four cytochrome P450 monooxygenase genes (P450-1–P450-4) closely linked in a gene cluster (Mende et al. 1997; Linnemannstöns et al. 1999). P450-4 encodes Kox, catalyzing the three oxidation steps between ent-K and ent-KA (Tudzynski et al. 2001), while P450-1 encodes a highly multifunctional monooxygenase, which catalyzes four steps involving oxidation at two carbon atoms, in the main pathway from ent-KA to GA14 via GA12-aldehyde. P450-2 was shown to encode a GA20ox, which converts GA14 to GA4 by removal of C-20 (Tudzynski et al. 2002). P450-3 encodes the 13-hydroxylase that converts GA7 to the end product, GA3, while another gene, orf3, encodes the desaturase that converts GA4 to GA7 (Tudzynski et al. 2003). Thus, although information on the enzymes and their genetic control involved in the metabolism of gibberellins in higher plants and fungi is abundant, evidence for bacterial biosynthesis is scarce. Tully et al. (1998) sequenced a cluster of three complete P450 genes (CYP112, CYP114, and CYP117) in *Bradyrhizobium japonicum*, plus a partial P450 gene fragment (CYP115P) previously shown to encode a cytochrome P450. Although the biochemical functions of the products of these genes are uncertain, similarities in structure with other genes suggest an operon involved in terpenoid synthesis bearing some resemblance to plant and Gibberella genes for ent-KS.

### *7.2.4 Abscisic Acid*

Abscisic acid (ABA) is a stress signal, which moves in the xylem from the roots to the aerial parts of the plant, where it regulates stomatal movement and the activity of shoot meristems. Root-growth-promoting microorganisms in the rhizosphere, lateral ABA flows in the root cortex across apoplastic barriers, ABA redistribution in the stem, leaf apoplastic pH values, and the action of β-glucosidases, in both the apoplast and the cytosol of the mesophyll, play an important role in the regulation of signal intensity. The significance of ABA glucose ester as a long-distance stress signal is discussed. Recently, it has been shown that growth-promoting rhizobacteria have an impact on ABA flows in plants. Arkhipova et al. (2005) detected substantially increased amounts of ABA in the shoots of lettuce that were treated with the cytokinin-producing bacterium *Bacillus subtilis*. The authors concluded that locally high cytokinin concentrations induced ABA biosynthesis in the roots. The newly formed ABA would then be loaded quickly to the xylem vessels without a significant deposition in the root tissues, a situation that resembles that of

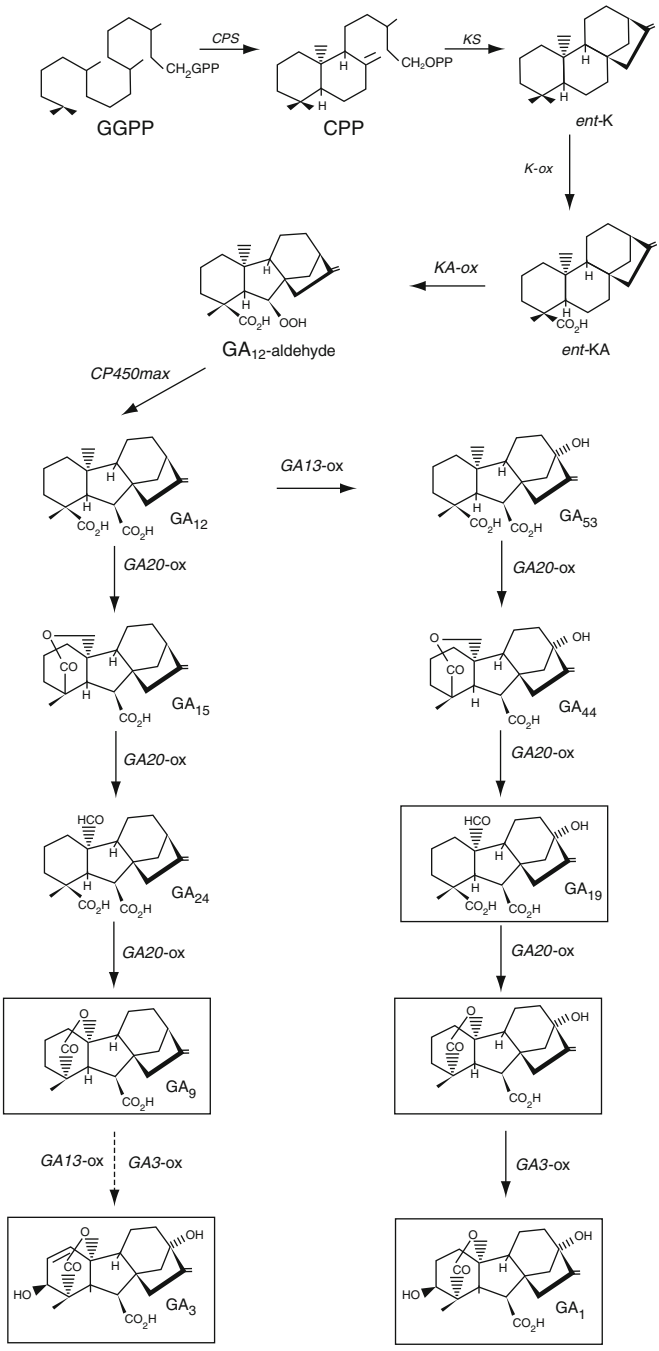


**Fig. 7.2** Pathway for gibberellins in *Azospirillum* sp. based on well-established steps in vascular plants and fungi, and data available on gibberellin metabolism studies with this bacterium. Gibberellins already characterized by GC-MS as produced by *Azospirillum* sp are boxed. The conversion of GA9 to GA3 has been demonstrated in vitro with gnotobiotic cultures of the

phosphorus and potassium-deficient castor bean plants. The intensity of the root-to-shoot ABA signal is regulated on four different anatomical levels (1) the rhizosphere, (2) the root cortex, (3) the stem, and (4) the leaves. Accordingly, four processes have to be considered (1) interactions between growth-promoting rhizobacteria and the root; (2) anatomical features of the root cortex (Casparian bands); (3) apoplastic pH values in the stem and the leaf; and (4) the action of apoplastic and cytosolic β-glucosidases in the leaves. The conjugate ABA glucose ester (ABA-GE) is a perfect long-distance signal because it is translocated without loss to the surrounding tissues (Fig. 7.3). Virgin areas of research are predominantly the impact of soil conditions and rhizospheric microorganisms on ABA signaling and the physiology and biochemistry of transporters that allow the passage of ABA-GE across biomembranes into the apoplast or microsomes (Jiang and Hartung 2007).

## 7.3 Ethylene Signaling

The implication of ethylene signaling in the responses of plants to biotic interactions is well recognized. It is undoubtedly an important piece in the plant's armory against pathogen attacks as well as response to beneficial bacteria such as nitrogen-fixing rhizobacteria (e.g., ethylene transduction pathway represses nodule formation in legumes; Asamizu et al. 2008; Penmetsa et al. 2008). How these bacteria can affect the plant ethylene signaling pathway (Fig. 7.4)? Most of them are proteobacteria that harbor in their genome a gene (*AcdS*) coding for an ACC deaminase. During the plant–bacteria interaction, ACDS is thought to metabolize the ethylene precursor ACC. As such, it is generally admitted that much of the ACC produced by the plant ACC synthase (ACS) activity in roots may be exuded in the rhizosphere, where it will be taken up by the rhizobacteria and subsequently hydrolyzed by AcdS (Glick et al. 1998). This should decrease ACC content in root and predictably ethylene biosynthesis, thereby partially releasing the negative regulation exerted by this gaseous hormone on root development and ultimately plant growth (Glick et al. 1998). It should also diminish defense mechanisms, thus favoring the interaction of bacteria with plants. This is an attractive hypothesis that started to gain some momentum since either introducing an *AcdS* gene or removing it, which stimulates or respectively affects the interaction with the plant. This suggests that PGPR-triggered root hair elongation is independent of ethylene biosynthesis or signaling pathway. In addition, it does indicate that AcdS activity alters local regulatory processes, but not systemic regulations such as those that control root architecture. Our work also indicates that root hair elongation induced by PGPR inoculation is probably an auxin-independent mechanism. These findings were unexpected since genetic screens for abnormal root hair development mutants

**Fig. 7.2** (continued) bacterium, while the metabolism of GA20 to GA1 has been established both in vitro and in vivo (i.e., in association with dy mutants of rice). Adapted from Bottini et al. (2004)

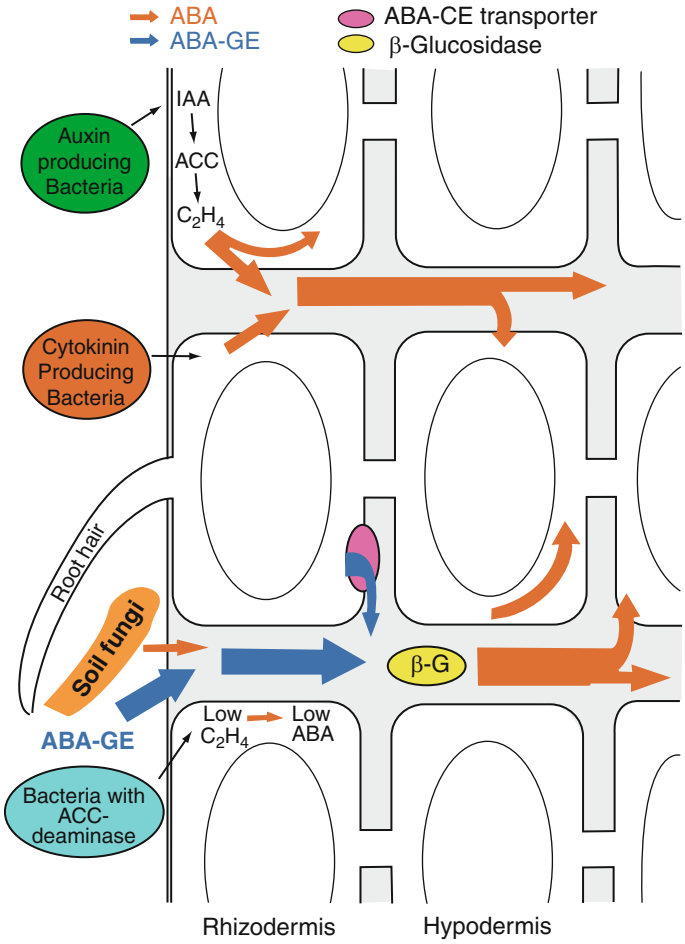


**Fig. 7.3** Schematic presentation of the origin and the flows of ABA (*red arrows*) and ABA-GE (*blue arrows*) in the root cortex. A possible role of auxin (IAA) and cytokinin-producing rhizobacteria and of bacteria exhibiting 1-aminocyclopropane-1-carboxylate deaminase is shown. Soil fungi produce and release large amounts of ABA. The *width of the arrows* symbolizes the intensity of the flows. *Arrows* originating in the cytosol symbolize the cytosolic ABA biosynthesis in the root cortex. Adapted from Jiang and Hartung (2007)

led to the isolation of ethylene and auxin mutants. Ethylene induces resistance when applied before infection but, when generated during infection or applied after symptoms have become manifest, stimulates disease progress. In part, these differing effects might be related to the dual action of ethylene in that it sometimes acts as a virulence factor of the pathogen but, other times, the activity of other pathogens is affected negatively. Furthermore, the speed with which the pathogen is able to colonize infected tissues and the mechanisms that the pathogen use to overcome the

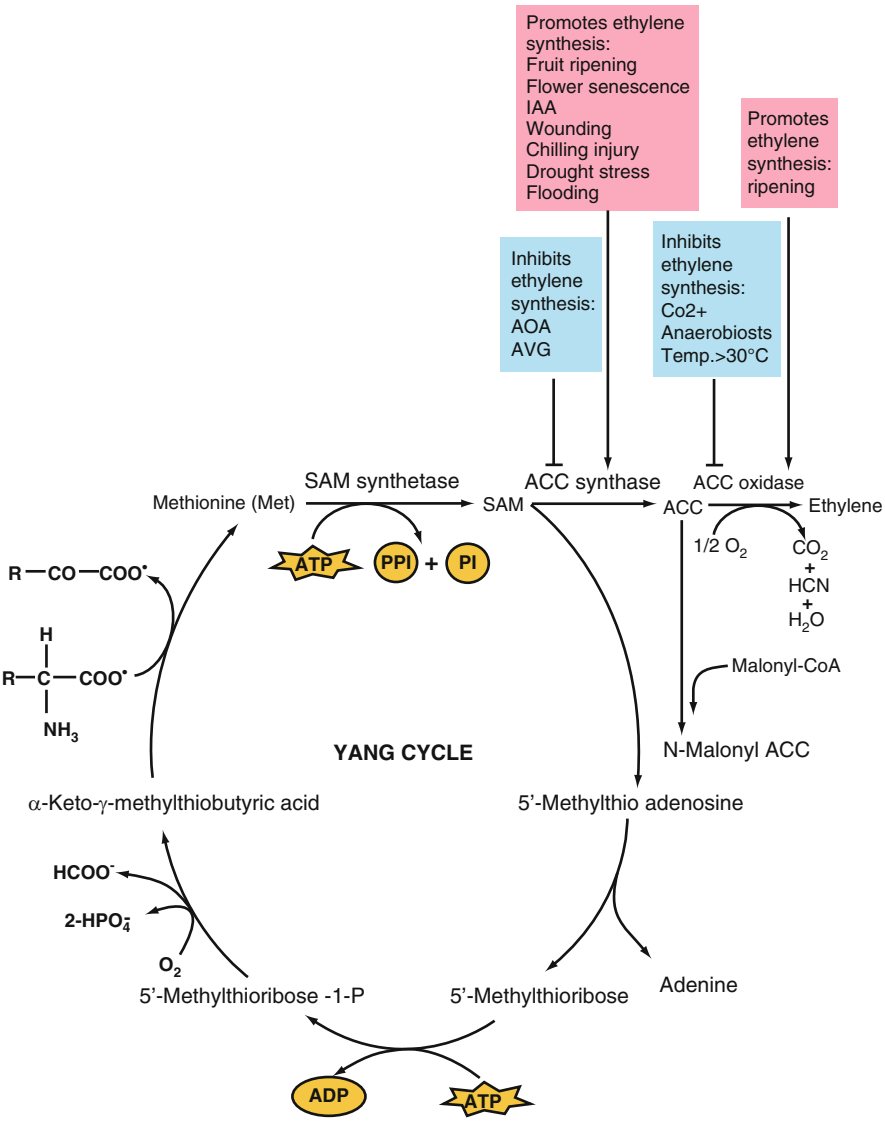


**Fig. 7.4** Ethylene biosynthetic pathway and the Yang cycle (Taiz and Zeiger 2006) *ACC* 1-aminocyclopropane-1-carboxylic acid; *SAM* *S*-Adenosyl-methionine *AOA* aminooxyacetic acid; *AVG* aminoethoxy-vinylglycine

effects of ethylene action might play a role. Cells in front of an advancing pathogen are likely to react differently from cells in the process of succumbing, and possibilities for discriminating in time and space between the effects of ethylene in cells at different stages of infection would be highly desirable.

## 7.4 Airborne Signaling by Volatile Organic Compounds

VOCs are defined as compounds that have high-enough vapor pressures under normal conditions to significantly vaporize and enter the atmosphere. This class of chemicals includes compounds of low molecular weight ($<300$ g $mol^{-1}$), such as alcohols, aldehydes, ketones, and hydrocarbons (Vespermann et al. 2007; Dunkel et al. 2009). Analysis of the volatiles emitted from two of the most potent growth-promoting strains, *B. subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a, revealed that two compounds, 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, were shared by both bacterial strains, whereas other PGPR strains that did not trigger enhanced growth via volatile emissions also did not share this same subset of volatile components (Ryu et al. 2004). Acetoin synthesis in *B. subtilis* involves the enzymes acetolactate synthase (pyruvate to acetolactate) and acetolactate decarboxylase (acetolactate to acetoin), which are encoded by *alsS* and *alsD*, respectively. The first two genes in this pathway are organized in a previously characterized operon, whereas the gene that converts acetoin to 2,3-butanediol (the acetoin-reductase step) is presently unknown (Ramos et al. 2000). Most interestingly, insertional inactivation of the *als* operon (strain BSIP1173) has only a small effect on the growth behavior of *B. subtilis* under a variety of conditions tested (Ramos et al. 2000), whereas the mutation totally abolished 2,3-butanediol production (Ramos et al. 2000) and negated all growth-promotion effects triggered by airborne signals. Confirmation that this four-carbon volatile alcohol indeed does trigger plant-growth promotion was provided by the exogenous application of 2,3-butanediol to *A. thaliana* seedlings that resulted in a dose-dependent growth promotion. These volatile alcohols are products of an alternative reductive pathway originating from pyruvate that provides an alternative source of NAD under anaerobic conditions (Fig. 7.5). Indeed, with 3-hydroxy-2-butanone and 2,3-butanediol, the qualitative and quantitative composition of volatile blends emitted by the growth-promoting strains differed significantly from nongrowth-promoting bacteria or medium alone. In the mechanisms that PGPRs use to interact with plants, VOC emission has a crucial part to play. The role of VOCs on antibiosis and the biocontrol of plant pathogens is the mechanism that has received most attention in the last decade, as the finding that certain volatiles having antifungal properties determine to a large extent the biocontrol performance of many rhizobacteria (Whipps 2001; Trivedi and Pandey 2008). There are numerous reports showing that volatiles produced by bacteria, such as ammonia, butyrolactonas, HCN, phenazine-1-carboxylic acid, and alcohols, may have activity in vivo in different fungal species (Whipps 2001; Trivedi and Pandey 2008). The effects of these volatiles on fungi range from mycelium growth inhibition and promotion to the stimulation or reduction of sporulation. Therefore, volatiles can be used for communication between bacteria and their eukaryotic neighbors. It was shown that the volatiles from any one bacterial strain do not cause the same effect or the same

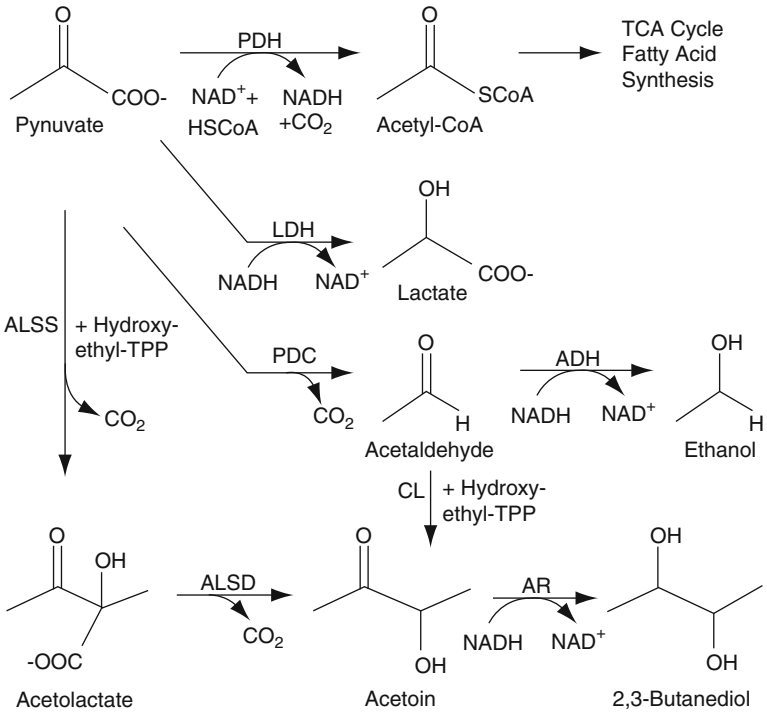


**Fig. 7.5** Proposed pathways for airborne signaling (anaerobic fermentation) in *B. subtilis* (modified Ramos et al. 2000). Enzymes with known coding genes include pyruvate dehydrogenase (PDH), lactate dehydrogenase (LDH), pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), acetolactate synthase (ALSS), acetolactate decarboxylase (ALSD), and acetoin reductase (AR)

degree of response in all fungi; rather, the responses depend on the specific fungi–bacteria combination (Kai et al. 2009).

## 7.5 Quorum Sensing

Bacterial cell-to-cell communication utilizes small diffusible signals, which the bacteria both produce and perceive. The bacteria couple gene expression to population density by eliciting a response only when the signaling reaches a critical threshold. The population as a whole is thus able to modify its behavior as a single unit. Several classes of microbially derived signaling molecules have now been identified. Broadly, these can be divided into two main categories (1) amino acids and short peptide derivatives, commonly utilized by Gram-positive bacteria (Shapiro 1998), and (2) fatty acid derivatives, called homoserine lactones (HSLs), frequently utilized by Gram-negative members (Dunny and Dunny 1999; Whitehead

et al. 2001). Irrespective of the nature of the signal molecule, the whole network functions by its reentry into the cell via either diffusion or an active transport (Whitehead et al. 2001). The signaling mechanism involves subsequent interaction of the signal with an intracellular effector that will induce the pathway for the concerned phenotype. In Gram-negative bacteria, the most commonly used quorum-sensing signals are AHLs. It is now apparent that AHLs are used for regulating diverse behaviors in rhizosphere-inhabiting bacteria and that plants may produce their own metabolites, which may interfere with quorum-sensing signaling (Fig. 7.6). AHLs are composed of a homoserine lactone residue linked to an acyl side chain. The specificity derives from the length of the acyl chain (4–18 carbon atoms), substitution at the C3 position, and saturation level within the acyl chain (Zhu et al. 1998; Raffa et al. 2004). AHLs can be broadly classified as long, medium, or short chained depending on whether their acyl moiety consists of more than eight, between 8 and 12, or less than 12 carbon atoms, respectively (Ortíz-Castro et al. 2008; Scott et al. 2006). These molecules are freely diffused through the bacterial membrane and distribute within the rhizosphere (Steidle et al. 2001; Schuhegger et al. 2006). There are three ways to interfere with QS mediated by

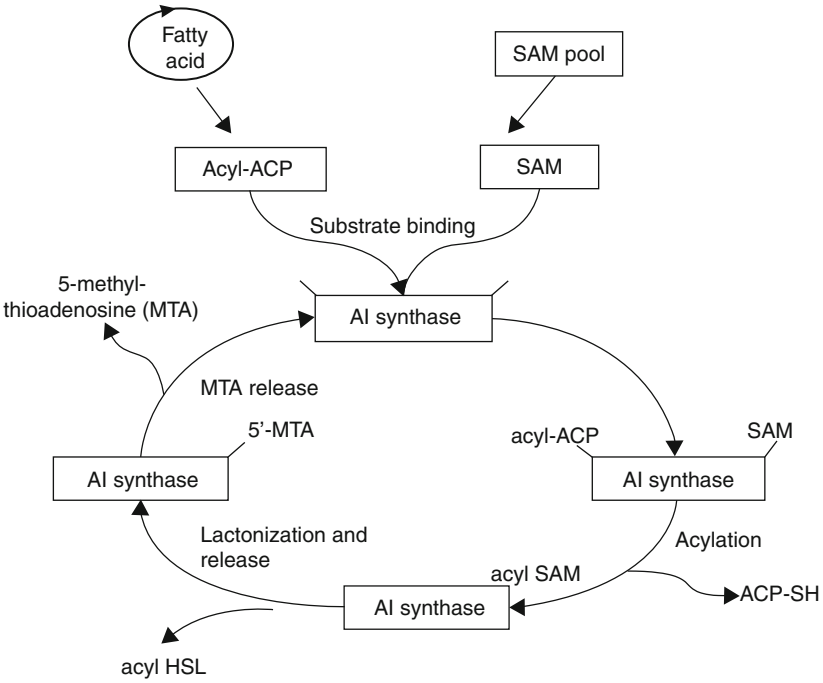


**Fig. 7.6** Putative biosynthetic scheme for acyl–HSL synthesis by LuxI-type protein (adapted from Charu Gera and Srivastava 2006). SAM binds to the active site on LuxI, and the hexanoyl group is transferred from the appropriately charged ACP. The hexanoyl group forms an amide bond with the amino group of SAM, 5′-methylthioadenosine is released, and a lactonization reaction results in the synthesis of acyl HSL

AHLs (1) blocking binding of AHLs with its receptor, (2) competitive inhibition, and (3) degradation of AHL. There are three major groups of AHL biosynthetic enzymes (1) LUX 1 type, which appears to be most common, (2) AHL biosynthetic enzyme LUXMtype, which has no significant homology with LUX 1, and (3) a third class of AHL synthase Hdts has been identified from the biocontrol strain (*P. fluorescens* F113). It is presumed that possession of different AHL synthases may afford some protection from competitors or host species, developing inhibitory molecules that target the synthase.

The presence of AHL-producing bacteria in the rhizosphere of tomato induced the salicylic acid and ethylene-dependent defense responses, which play an important role in the activation of systemic resistance in plants and conferred protection against the fungal pathogen *Alternaria alternate* (Schuhegger et al. 2006). Furthermore, AHLs were found to be taken up by plants in a process dependent on the length of the acyl chain (Von Rad et al. 2008; Götz et al. 2007). Application of a homoserine lactone, a breakdown product of AHL by means of soil bacteria, to bean roots leads to an increase in stomatal conductance and transpiration in shoots. This in turn is beneficial for both the plant and the bacteria through an increase in mineral nutrient uptake (Joseph and Phillips 2003). A number of Gram-positive bacteria are also known to employ quorum-sensing systems. The nature of the signal molecules used in these systems differs from that of Gram-negative organisms (Dunny and Leonard 1997). Quorum sensing (QS) is used to regulate the development of bacterial competence in *B. subtilis* and *Streptococcus pneumoniae*, conjugation in *Enterococcus faecalis*, and virulence in *Staphylococcus aureus* (Dunny and Leonard 1997).

### 7.5.1 *Quorum Quenching: Manipulating Quorum Sensing for Disease Control*

The promising outcomes of the above-described proof-of-concept approaches represent a considerable advance in bacterial disease control. It has been clearly established now that quorum quenching is a feasible approach to control bacterial infections. However, we should also be aware that our understanding about quorum-sensing regulation of bacterial virulence is still fragmentary, with most information coming from in vitro experiments. Host and environmental factors could also play significant roles in the modulation of bacterial quorum-sensing systems. Good examples are plants as well as other bacterial species that could produce AHL mimic compounds that activate bacterial quorum-sensing systems (Pierson et al. 1998; Teplitski et al. 2000). Further investigation on bacterial quorum-sensing systems, especially in the context of host–pathogen interaction, would be essential to maximize the potential of the quorum-quenching strategy in our fight against bacterial plagues. Host plant resistance has been used extensively for disease control in diverse crop species. It is governed in many cases by the "gene-for-gene" system,

i.e., the specific recognition between pathogen *avr* (avirulence) gene and its cognate plant disease resistance (R) gene. A plant displays a resistance phenotype when corresponding *avr* and R genes are present in the pathogen and the plant, respectively, or becomes susceptible if either is absent or inactive (Dangl and Jones 2001). However, in many cases, host plant resistance is not durable as a result of constant genetic evolution in pathogens, in particular, loss of avirulence genes (Leach et al. 2001).

## 7.6 Induced Resistance

Induced resistance is a physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli, whereby the plant's innate defenses are potentates against subsequent biotic challenges. This enhanced state of resistance is effective against a broad range of pathogens and parasites (Van Loon 2000). The two most clearly defined forms of induced resistance are (1) SAR and (2) ISR, which can be differentiated on the basis of the nature of the elicitor (Fig. 7.7). SAR is induced either upon infection by an avirulent pathogen or upon restricted infection by a virulent pathogen. SAR depends on the synthesis of salicylic acid (SA) by the host and is effective against pathogens that are restricted by salicylic acid-dependent basal resistance responses, such as tobacco mosaic virus in tobacco. This type of induced resistance is marked by the local and systemic accumulation of newly induced pathogenesis-related proteins (PRs) that might, or might not, be effective against the pathogen involved. ISR is triggered by selected strains of nonpathogenic rhizobacteria and does not require salicylic acid but does depend on the responsiveness of the plant to jasmonic acid (JA) and ethylene. ISR is effective against pathogens that are restricted by jasmonic acid- and ethylene-dependent

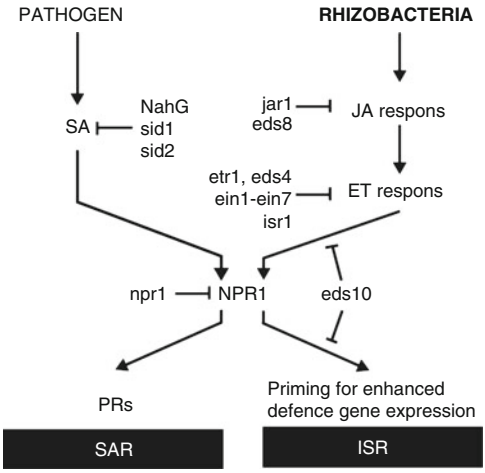


**Fig. 7.7** Signal transduction pathways of SAR and ISR [adapted from Pierson et al. (1998)]. *NPR1* nonexpressor of PR genes

basal resistance mechanisms, such as the fungus *Alternaria brassicicola* in *Arabidopsis*. This type of induced resistance is not associated with the induction of PRs, even though ISR, like SAR, requires the presence of a functional NPR1 protein. Several pathogens of *Arabidopsis* have been shown to be resisted by a combination of salicylic acid-dependent and jasmonic acid- and ethylene-dependent defenses. Several rhizobacteria trigger the salicylic acid (SA)-dependent SAR pathway by producing SA at the root surface, whereas other rhizobacteria trigger different signaling pathway independent of SA. The existence of SA-independent ISR pathway has been studied in *A. thaliana*, which is dependent on jasmonic acid (JA)/ET and ethylene signaling. SA, JA, and ET are the main molecules signaling the activation of defense genes and the role of SA is well known in mediating resistance to biotrophic pathogens, whereas JA/ET is mainly associated with necrotrophic pathogens (Thomma et al. 2001).

### *7.6.1 SA-Dependent Signaling*

Several studies have demonstrated that SA is required for SAR and it is associated with the accumulation of PR proteins, contributing to resistance (Durrant and Dong 2004). Nonexpresser of PR genes1 (NPR1) was activated by SA and moves to the cellular nucleus to interact with TGA transcription factors for further activating transcription of downstream defense genes leading to SAR (Durrant and Dong 2004). TGA factors are a class of bZIP TFs and they have the ability to bind to the SA, JA, and auxin-inducible *activating sequence-1* (*as-1*) element found in the cauliflower mosaic virus 35S (CaMV 35S) promoter or the related *ocs* element in the octopine synthase promoter (Jakoby et al. 2002). Some of the TGA factors have been identified in a recent study of *Arabidopsis* TGA TFs, which revealed that three related genes (*TGA2*, *TGA5*, and *TGA6*) were required for *PR* gene expression and disease resistance (Zhang et al. 2003).

### *7.6.2 JA-Dependent Signaling*

JA and MeJA are signaling molecules and they are important for initiating and/or maintaining developmental processes and defense responses in various plants (Wasternack and Hause 2002). The JA-responsive genes and genes involved in JA biosynthesis. In addition, plant defensin 1.2 (*PDF1.2*) is also induced by JA and is often used as a marker for JA signaling in *Arabidopsis* even though it is known to be induced by ET as well (Chen and Bleecker 1995; Penninckx et al. 1996).

### 7.6.3 ET-Dependent Signaling

The role of ET in plant defense is well known; however, ET plays a dual role in the interaction between plants and pathogens and the complex roles played by this hormone have been reviewed (Van Loon et al. 2006). From the plant's perspective, increased production of ET, as an early, active reaction of plants to the presence of pathogens, is coupled to the initiation of defense responses (Boller 1991). From the perspective of the pathogen, ET serves as a virulence factor to improve the colonization of plant tissue (Arshad and Frankenberger 1992). In addition, the timing of exposure to ET might decide whether resistance is induced or inhibited; the mechanisms used by the pathogen to overcome the influence of ET and the speed of the pathogen to colonize infected tissues may contribute to the dual roles of ET (Van Loon et al. 2006).

### 7.6.4 Induced Systemic Tolerance

The term "induced systemic tolerance" (IST) was recently proposed for PGPR-induced physical and chemical changes in plants that result in enhanced tolerance to abiotic stress (Mantelin and Touraine 2004). The role of VOCs emitted from *B. subtilis* GB03 on IST to salt stress (100 mM NaCl) in *Arabidopsis* was evaluated by Zhang et al. (2008). The authors observed that VOCs concurrently downregulated *HKT1* (*High-affinity K + transporter1*) expression in roots, but upregulated it in shoots, resulting in lower Na + accumulation throughout the plant.

## 7.7 Coinoculation of Different Rhizobacterial Strains

Direct interactions occurring between members of different microbial types often result in the promotion of key processes benefiting plant growth and health. It has been suggested that the development of plant-growth-promoting consortium (PGPC) could be a feasible strategy for increased activity and better viability of PGPRs. When these strains are made into an inoculum consortium, each of the constituent strains of the consortium not only competes with the others for rhizospheric establishments but also complements functionally for plant-growth promotion (Shenoy and Kalagudi 2003). Gupta et al. (2002) reported results for the management of fungal diseases by using microbial combination involving *S. fredii* KCC5 and *P. fluorescens* LPK2 reduced wilt disease and proved the most effective in reducing disease incidence. Research on the mechanisms by which PGPRs enhance nodule formation implicates their production of plant hormones among the coinoculation benefits. For example, Chebotar et al. (2001) demonstrated that some *Pseudomonas* strains, but not all, increased nodule number and acetylene

reduction in soybean plants inoculated with *B. japonicum*. The use of *gus-A* marked rhizobacteria allowed the authors to demonstrate that the bacteria colonized the root. Azcón-Aguilar and Barea (1978), using both cell-free supernatants of PGPR cultures and pure chemicals, first demonstrated that plant-growth-regulating substances produced by PGPRs affected nodulation and nitrogen fixation. Recently, Mañero et al. (2003) extended these observations. The possibility that metabolites other than phytohormones, such as siderophores, phytoalexins, and flavonoids, might enhance nodule formation has also been proposed (Lucas-Garcia et al. 2004), but this hypothesis has not been verified. Inoculation of phosphate-solubilizing bacteria (PSB) enhanced nodulation and $N_2$-fixation ($^{15}N$) by alfalfa plants, in parallel with an increase in the P content of plant tissues (Toro et al. 1998). It is, therefore, thought that an improvement in P nutrition of the plant resulting from the presence of PSB was responsible for increased nodulation and $N_2$-fixation, as it is well known that these processes are P dependent (Barea et al. 2005).

## 7.8 Conclusions and Future Trends

The highly sophisticated field of bioscience, comprising the interactions of microorganisms with their hosts (higher organisms), has been increasingly attracting attention during the past decade both in basic research and in applied fields, particularly those related to agricultural and environmental biotechnology. Although current efforts are directed toward laboratory-based assays of molecules involved in QS systems, their in situ operation in the rhizosphere appears imminent. Such information will permit not only the delivery of more appropriate and effective bioinoculants for plant and soil health but also the cell-density-dependent control of in situ biological equilibrium, a feature of consequence in minimizing competition with indigenous microorganisms for the limited resources available in this unique ecosystem. In this review, we have considered four major classes of signals that participate in the interactions that occur between plants and beneficial microorganisms: hormonal, airborne, ethylene, and quorum. It can be generally appreciated that plants are able to sense and respond to rhizosphere-inhabiting bacterial and fungal populations and their products. Apart from the discovery of different types of chemical communication for interkingdom signaling, a challenge for the future is to begin to address the possibility that there is a significant specific communication. A further challenge is to determine the role played by the consortia and other secondary metabolites produced by their combinations.

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# Chapter 8
# Microbial ACC-Deaminase Biotechnology: Perspectives and Applications in Stress Agriculture

**Sajid Mahmood Nadeem, Maqshoof Ahmad, Zahir Ahmad Zahir, and Muhammad Ashraf**

## 8.1 Introduction

In soil environment, a healthy plant faces a number of environmental stresses, both biotic and abiotic, which have a negative impact on growth and development. Among various environmental stresses, some of the important abiotic stresses include flooding, salinity, heavy metals, drought, chilling, soil compaction, mechanical impedance, and nutrient deficiency (Abeles et al. 1992; Glick et al. 2007; Saleem et al. 2007). During stress conditions, certain physiological disorders such as enhanced ethylene production, nutritional, and hormonal imbalance may occur in plants (Table 8.1) that affect normal plant processes (Ashraf 1994; Marschner 1995; Glick et al. 1997; Sairam and Tyagi 2004). Accelerated ethylene production under stress conditions is a major cause of reduced plant growth and development (Sarquis et al. 1991; Morgan and Drew 1997; Bernardo et al. 2000a, b; Sobeih et al. 2004; Saleem et al. 2007). Ethylene is considered to be a stress hormone that is released as a response when plants are subjected to environmental stresses, including edaphic and adaphic ones (Tank and Saraf 2010). Although a low concentration of ethylene is essential for normal plant growth, at high concentrations, it has a negative impact on plant growth and development (Arshad and Frankenberger 2002). An elevated level of ethylene concentration inhibits root growth, which ultimately affects overall plant growth and development

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**Table 8.1** Plant responses to stressed conditions

| Plant | Type of stress | Plant response | References |
|---|---|---|---|
| *Vicia faba* | Salinity stress | High ethylene production Inhibition of growth and enhanced leaf and flower abscission and senescence | El-Beltagy and Hall (1974) |
| Cucumber | Salinity stress | Decrease in photosynthesis and ultimately plant growth | Drew et al. (1990) |
| Eucalyptus | Salinity stress | Damage to morphology, anatomy, ultra-structure and metabolism of plant species | Prat and Fathi-Ettai (1990) |
| Citrus | Salinity stress | High ethylene production and enhanced leaf abscission | Gomez-Cadenas et al. (1998) |
| | Salinity stress | Increase in ethylene production, enhanced leaf abscission leading to death of the plant | Storey and Walker (1999) |
| | Salinity stress | Accumulation of high amounts of chloride, increased ethylene production, and induction leaf abscission in stressed plants | Gómez-Cadenas et al. (2003) |
| | Salinity stress | Increased accumulation of chloride ions, reduction in $CO_2$ assimilation, increased ethylene production and triggered abscission of the injured leaves | Arbona et al. (2005) |
| | Salinity stress | Enhanced ethylene production which caused leaf abscission | Arbona et al. (2006) |
| Lettuce | Salinity stress | More ethylene evolution in salt tolerant than salt sensitive plants | Zapata et al. (2003) |
| Sunflower | Salinity stress | Enhanced ethylene production, reduction in plant growth leading to senescence of the plant | Alvarez et al. (2003) |
| Spinach, lettuce, melon, pepper, broccoli, beetroot, tomato | Salinity stress | No significant correlation between ethylene production and germination percentage | Zapata et al. (2004) |
| Spinach, lettuce, melon, pepper, broccoli, beetroot, bean, tomato | Salinity stress | Highest reduction in fresh weight plants | Zapata et al. (2008) |
| Rice | Salinity stress | Increased ethylene biosynthesis, reduction in plant growth | Lutts et al. (1996) |
| Wheat | Salinity stress | Decreased root, shoot growth and yield | Abbaspoor et al. (2009) |

(continued)

**Table 8.1** (continued)

| Plant | Type of stress | Plant response | References |
|---|---|---|---|
| Chickpea | Salinity stress | Enhanced ethylene production, membrane injury, $Na^+/K^+$ ratio and $Cl^-$ content | Kukreja et al. (2005) |
| Jojoba | Salinity stress | Increased ethylene production leading to a significant negative relationship with plant growth | Roussos et al. (2006) |
| | Salinity stress | Ethylene production caused epinasty; it was negatively correlated with the level of salt tolerance | Jones and El-Abd (1989) |
| | Salinity stress | Increased ethylene production leading to epinasty in plants | El-Iklil et al. (2000) |
| | Salinity stress | Enhanced ethylene production | Mayak et al. (2004a) |
| | Salinity stress | Increased ethylene concentration, electrolyte leakage, total sugars, and epinasty and membrane injury, Decrease in root number, shoot fresh weight and shoot height | Shibli et al. (2007) |
| | Salinity stress | Reduction and transport of inorganic phosphate | Ehsanpour and Amini (2003) |
| Barley, sunflower, rice and finger millet | Salt stress | Reduction in soluble proteins | Ashraf and Waheed (1993), Amini and Ehsanpour (2005), Parida and Das (2005) |
| *Vigna radiata* | Salinity stress | Reduction in number and fresh weight of pods per plant | Elahi et al. (2004) |
| Maize | Drought stress | Increase in soluble sugar contents and proline, decrease in starch content | Mohammadkhani and Heidari (2008) |
| Maize | Drought stress | Increased concentration of $Na^+$ and $Cl^{-+}$ | Tester and Davenport (2003) |
| Common bean (*Phaseolus vulgaris* L.) | Drought stress | Increased abscisic acid (ABA) content in the leaves, reduction in endogenous cytokinin levels and closing of stomata | Figueiredo et al. (2008) |
| Soybean (*Glycine max*) | Salinity stress | Increased Malondialdehyde (MDA), ascorbate peroxidase (APX), glutathione reductase (GR), proline, and glucose and glycine betaine | Han and Lee (2005) |
| Tomato | Flooding stress | Enhanced ethylene caused abnormal growth | Jackson (1997) |

(continued)

**Table 8.1** (continued)

| Plant | Type of stress | Plant response | References |
|---|---|---|---|
| *Arabidopsis thaliana* | Heavy metal stress | Enhanced ethylene production | Arteca and Arteca (2007) |
| *Brassica napus* | Heavy metal stress | Decreased root, shoot growth and chlorophyll content | Dell'Amico et al. (2008) |
| Maize | Nutrient stress | Reduced root hydraulic activity of root | Fan et al. (2007) |
| *Medicago falcate* L. | Nutrient stress | Reduced root hydraulic activity and enhanced ethylene production | Li et al. (2009) |
| Maize | Heat stress | Significant decrease in shoot dry mass, relative growth rate | Ashraf and Hafeez (2004) |
| Sugarcane | Heat stress | Declines in net assimilation rate | Wahid (2007) |
| Grapes | Heat stress | Damaged the mesophyll cells and increased plasma membrane permeability | Zhang et al. (2005) |

(Mattoo and Suttle 1991; Frankenberger and Arshad 1995; Sobeih et al. 2004). Any check on this enhanced synthesis of ethylene is essential for improving plant growth under stress conditions (Mayak et al. 2004a; Glick 2005; Glick et al. 2007; Saleem et al. 2007). This can be achieved by treating the plants with chemical substances inhibiting ethylene (Sharp and LeNoble 2002; Glick 2005; Dodd et al. 2004) such as aminoethoxyvinyle glycine (AVG), 1-methylcyclopropene (1-MCP), cobalt ion ($Co^{2+}$) and silver thiosulfate (Yang and Hoffman 1984; Mckeon et al. 1995; Mibus et al. 2007) or biological inhibitors such as 1-aminocylopropane-1-carboxylic acid (ACC)-deaminase enzyme (Mayak et al. 1999). Although chemical inhibitors are also effective in regulating the ethylene concentration (Abeles et al. 1992; Sisler and Serek 1997), there are environmental concerns about the toxicity and longevity of these chemicals (Ahmadi et al. 2009). Under these circumstances, one possible solution is the use of plant growth promoting rhizobacteria (PGPR) containing ACC-deaminase to protect the plants from harmful effects of environmental stresses including flooding, salinity, drought, heavy metal, and pathogens (Glick et al. 1997; Lugtenberg et al. 2001; Mayak et al. 2004b; Nadeem et al. 2007, 2009; Belimov et al. 2009b; Wenzel 2009; Lugtenberg et al. 2001).

PGPR are beneficial free-living organisms present in the rhizosphere in association with the roots of different plants (Kloepper et al. 1989). Due to the presence of root exudates as a source of their nutrition, these PGPR are generally present more or less near the root surface (Whipps 1990) and promote plant growth in a number of ways (Patten and Glick 1996; Lucy et al. 2004; Kohler et al. 2006). Application of these bacteria to seeds or crops promotes plant growth or protect the plants from harmful effects of salinity (Nadeem et al. 2009, 2010), drought (Zahir et al. 2008), heavy metals (Belimov et al. 2009a), and soil-borne pathogens (Kloepper et al. 1980).

Therefore, the concept of using these bacteria for enhancing growth is gaining ground. PGPR bind to soil and are present in high concentration around the roots due to the presence of a large number of nutrients exuded by plant roots (Whipps 1990; Glick et al. 1999). Some of these microorganisms belong to important genera which include *Pseudomonas*, *Bacillus*, *Serratia*, *Azospirillum*, *Klebsiella*, *Azotobacter*, *Enterobacter*, *Burkholderia*, *Paenibacillus*, *Herbaspirillum*, and *Arthrobacter* (Kloepper et al. 1988, 1989; Belimov et al. 2001, 2005; Selosse et al. 2004; Pandey et al. 2005; Blaha et al. 2006; Spaepen et al. 2009). These PGPR promote plant growth by a number of direct and indirect mechanisms such as nitrogen fixation, phosphorus solubilization, siderophores synthesis, phytohormone production, and protection against soil-borne pathogens (Kloepper et al. 1989; Glick and Bashan 1997; Glick et al. 1995, 1999; Patten and Glick 1996; Kohler et al. 2006; Cartieaux et al. 2003; Van Loon 2007).

As discussed earlier, plant growth is inhibited under stress environments and this inhibition in growth generally occurs due to elevated levels of ethylene that are produced under stress conditions (Maas and Hoffman 1977; Singleton and Bohlool 1984; Gindin et al. 1989; Abeles et al. 1992; Frankenberger and Arshad 1995; Huang et al. 1998; Arshad and Frankenberger 2002; Arteca and Arteca 2007). The major mechanism used by PGPR containing ACC-deaminase for promoting plant growth under stress conditions are the lowering of ethylene level in the plant by hydrolyzing ACC, an immediate precursor of ethylene biosynthetic pathway (Glick et al. 1998). These PGPR use ACC as a source of their nitrogen that results in low ACC levels in the plant root, which ultimately decreases the ethylene level in plants. The alleviation of harmful effects of high ethylene results in plant growth promotion under stress conditions (Mayak et al. 2004a, b; Nadeem et al. 2007; Zahir et al. 2009).

In this chapter, the impact of ethylene on plant growth, the role of PGPR containing ACC-deaminase in stress agriculture, with special reference to ethylene production under stress conditions, the basic mechanisms used by PGPR containing ACC-deaminase for alleviating this stress-imposed effects, and the effectiveness of inoculation with these strains under different stress environments are reviewed and discussed in detail. The basic objective of this review is to understand a useful and environment-friendly practical approach to facilitate plant growth and development under stress environment.

The stress-induced ethylene and its impact on plant growth, the inhibition of stress induced ethylene, and the effectiveness of inoculation under various stresses are also discussed critically in the following sections.

## 8.2 Stress-Induced Ethylene Production in Plants

A number of plant mechanisms, such as seed germination, senescence, root elongation, and fruit ripening, are regulated by ethylene (Bleecker and Kende 2000; Belimov et al. 2002). The low level of ethylene initiates plant growth, while an

elevated level of ethylene is inhibitory to plant growth (Holguin and Glick 2001; Arshad and Frankenberger 2002). Stress is any external factor that causes a harmful effect on plant growth and development. It can alter various steps of ethylene biosynthesis and it has been noted that a significant amount of ethylene is produced under stress conditions (Lutts et al. 1996; Gomez-Cadenas et al. 1998; Zapata et al. 2004; Arteca and Arteca 2007; Shibli et al. 2007). This synthesis of ethylene has been proposed as a potential stress indicator (O'Donnell et al. 1996; Blumwald 2000; Kukreja et al. 2005; Glick et al. 2007; Belimov et al. 2009a) and causes the senescence of plant parts (Abeles et al. 1992) that may result in physiological disorders in plant cells (Mattoo and Suttle 1991; Bernardo et al. 2000b).

Ethylene is produced in response to a number of environmental factors, such as salinity (Abdel-Ghaffar et al. 1982), high temperature and drought (Marshall 1964; Maas and Hoffman 1977), physical impendence (Kays et al. 1974; Huang et al. 1998), wounding (Lulai and Suttle 2004), flooding (Gindin et al. 1989), metal stress (Arteca and Arteca 2007; Belimov et al. 2009b), and disease (Lulai 2001). When plants are subjected to environmental stresses such as drought, salinity, heating, chilling, mechanical, and nutritional stress, at initial stages, the concentration of ACC increases (Gomez-Cadenas et al. 1998) and causes a marked increase in ethylene synthesis (Abeles et al. 1992; Stearns and Glick 2003; Mayak et al. 2004b; Glick et al. 2007; Belimov et al. 2009a). This ethylene concentration depends upon the strength of stress and also on the growth stages of the plant (Botella et al. 2000). For example, increasing salinity levels result in elevated levels of ACC and ethylene in plants (Lutts et al. 1996; Bar et al. 1998; Botella et al. 2000; El-Iklil et al. 2000; Arbona et al. 2005). Salinity stress reduces the germination and growth rate of plants while the rate of ethylene synthesis increases (Zapata et al. 2003). With increasing salinity, a corresponding increase in ethylene production, due to increase in ACC, was noticed by Kukreja et al. (2005).

The production of ethylene under stress conditions also depends upon crop variety, and ethylene evolution is more in salt-sensitive cultivar than that in the salt tolerant one (El-Shintinawy 2000). Ethylene production also varies with crop species as different plant species, i.e., lettuce, melon, spinach, broccoli, pepper, tomato, and beetroot species responded differently with respect to ethylene production rate when studied during germination under salinity stress (Zapata et al. 2004). Maximum ethylene production was observed in pepper followed by tomato, broccoli, and bean plants (Zapata et al. 2008). Jojoba is considered a salt-tolerant plant and ethylene evolution was also observed in this plant by Roussos et al. (2006), and ethylene production increases with salinity. When stress ethylene concentration increases, it results in a senescence that may result in physiological and biochemical disorders in plants (Shibli et al. 2007). Ethylene production also increases significantly in response to mechanical impendence (Kays et al. 1974; Huang et al. 1998). The root growth is inhibited in compacted soil which might be due to enhanced ethylene synthesis (Mattoo and Suttle 1991; Morgan et al. 1993; Hussain et al. 1999).

Flooding promotes ethylene biosynthesis, as enhanced ethylene production was recorded in tomato, when aerated tomato roots growing in solution culture were

made anaerobic. Further, initiation of two parallel root systems by splitting the stems of these plants showed an increase in ethylene levels for those leaves which were connected to anaerobic roots (Jackson and Campbell 1976; Bradford and Dilley 1978). It may be that such environment is considered more conducive for ethylene accumulation due to its entrapment in water (Drew 1992). The increase in ethylene concentration under flooded conditions is generally due to increase in the activity of ACC-synthase and ACC-oxidase in root and shoot, respectively (Olson et al. 1995). It is also evident from the work of English et al. (1995) that soil flooding increased ACC oxidase activity in tomato plants and resulted in enhanced ethylene synthesis. Under anaerobic conditions, ACC is synthesized in plant roots and transported to shoot, where it is oxidized to produce ethylene and causes abnormal growth like leaf epinasty (Else et al. 1995; Jackson 1997).

Most plants are sensitive to temperature fluctuations. Temperature fluctuation leads to changes in plant hormonal imbalances and ultimately affects plant growth and development (Cheikh and Jones 1994). Temperature stress, both chilling and high temperature, also enhances the ethylene concentration (Wang 1987) and there exists a positive correlation between temperature and ethylene concentration (Otani and Ae 1993; Antunes and Sfakiotakis 2000).

In soil environment, plants are also subject to pathogen attack, which is a major threat to crop production. This also results in enhanced ethylene concentration that causes disease symptoms and reduces plant resistance against diseases (Frankenberger and Arshad 1995; Boller 1991; Lund et al. 1998). Similarly, ethylene synthesis is also stimulated by wounding (Lulai and Suttle 2004), most likely by the induction of ACC synthase activity (Kende 1993; Watanabe et al. 2001).

Drought stress is one of the major abiotic stresses that limit crop yields, both at cellular and molecular levels (Ingram and Bartels 1996; Vinocur and Altman 2005; Saleem et al. 2007). It has been estimated that about one-half of the earth is vulnerable to drought every year (Kogan 1997). Like other stresses, plant water deficit has been broadly related with an enhanced level of ethylene (Adato and Gazit 1974; Apelbaum and Yang 1981; Graves and Gladon 1985; Sharp 2002; Balota et al. 2004; Mayak et al. 2004a), which leads to abnormal growth (Mattoo and Suttle 1991). The enhanced ethylene synthesis under water stress is of interest because the elevated levels of ethylene could be responsible for inhibiting root and shoot growth, abscission, and premature senescence of plants parts (Morgan et al. 1990; Sharp 2002). Water deficit causes soil drying, and plant growth is inhibited as a result of enhanced ethylene production (Sobeih et al. 2004). Increased ethylene concentration under water stress also causes alteration in plasma membrane integrity, leaf dry weight, and pigment content (Beltrano et al. 1999; El-Shintinawy 2000; Balota et al. 2004).

The level of heavy metals in soil is continuously increasing due to their addition through agricultural and industrial activities such as deposition of sewage sludge, use of agrochemicals, water disposal, and vehicle exhausts (Dell'Amico et al. 2008). The high concentration of these contaminants affects plant growth and inhibition in root growth in contaminated soils might be due to enhanced ethylene

synthesis in response to metal stress (Rodecap and Tingey 1981; Abeles et al. 1992; Safronova et al. 2006). High ethylene production under heavy metal stress not only inhibits root growth but also causes the depletion of nutrients such as iron, and decreases $CO_2$ fixation (Prasad and Strazalka 2000; Glick et al. 2007). The stress induced by contaminants present in air also causes enhanced ethylene synthesis in plants (Moeder et al. 2002; Vahala et al. 2003).

Phytopathogenic organisms are a major threat for sustainable crop production worldwide. Pathogenic stress increases the ethylene production in plants, which inhibits plant growth (Lulai 2001).

The above discussion indicates that under stress conditions, elevated levels of ethylene affect plant growth. It is also evident from this discussion that ethylene is produced both under biotic and abiotic stresses. The extent of ethylene production depends upon the strength of stress and also crop species. However, almost all the plant species produce elevated levels of ethylene when subjected to a variety of environmental stresses.

## 8.3 Impact of Ethylene on Plant Growth

Earlier, it was considered as a ripening hormone; however, later investigations by several workers showed that it plays a diverse and effective role in plant growth (Arshad and Frankenberger 2002; Belimov et al. 2002). In plants, several physiological processes such as seed germination, tissue differentiation, root elongation, flowering initiation, leaf abscission, flower opening, organ senescence, and leaf and fruit abscission are regulated by endogenous level of ethylene (McGarvey et al. 1990; Abeles et al. 1992; Frankenberger and Arshad 1995; Johnson and Ecker 1998; Bleecker and Kende 2000; Chae and Kieber 2005; Glick et al. 2007). The production of ethylene may be inhibitory or stimulatory, depending upon its concentration (Arshad and Frankenberger 2002), the nature of physiological process (Johnson and Ecker 1998), and the growth phase of plant (Abeles et al. 1992). When ethylene is present at high levels ($>25$ μg $L^{-1}$), it can be damaging for plants and leads to epinasty, shorter roots, and premature senescence (Holguin and Glick 2001). Normal plant processes take place at normal concentration (10–25 μg $L^{-1}$) of ethylene. However, an overproduction of ethylene under stress conditions can cause a negative impact on plant growth and development (Mattoo and Suttle 1991; O'Donnell et al. 1996; Blumwald 2000; Penrose et al. 2001; Sobeih et al. 2004; Saleem et al. 2007; Glick et al. 2007; Belimov et al. 2009a). Some of the major plant processes that are affected by high ethylene concentration have been discussed in the following subsections.

### 8.3.1 Root Inhibition

Ethylene, in the beginning, induces seed germination (Esashi 1991). The physiological processes which are affected by ethylene include release of dormancy, shoot and root growth differentiation, adventitious root formation, induction of flowering and increased femaleness in dioecious plants, fruit ripening, leaf senescence, and leaf and fruit abscission (McGarvey et al. 1990; Abeles et al. 1992; Johnson and Ecker 1998). At low concentrations of ethylene, root growth promotion occurs, whereas high concentrations of ethylene cause a negative impact on root growth (Jackson 1991; Frankenberger and Arshad 1995; Belimov et al. 2002; Saleem et al. 2007; Visser and Ronald 2007; Glick et al. 2007). Ethylene production in plant roots is accelerated in response to both biotic and abiotic stresses (Abeles et al. 1992; Frankenberger and Arshad 1995: Arshad and Frankenberger 2002). When plants are exposed to environmental stresses, ethylene concentration increases in the roots (Abeles et al. 1992; Glick et al. 2007), which inhibits seed germination and root growth (Sarquis et al. 1991; Bernardo et al. 2000a; Smalle and van Der Straeten 1997; Ma et al. 2003). Root growth is equally inhibited by ethylene under growth pouches as well as in Petri plates on saturated filter paper (Locke et al. 2000; Penrose et al. 2001; Belimov et al. 2002).

Legumes bearing nodules crops are well known for their ability to fix nitrogen from the atmosphere. Enhanced ethylene production decreases nodulation due to its negative impact on root growth (Goodlass and Smith 1979; Frankenberger and Arshad 1995; Sun et al. 2007). The increased aluminum concentration enhances ethylene production and the response of ethylene to root inhibition is similar: either it is aluminum-induced or it evolves in response to the application of ACC and ethephon. However, this negative impact of aluminum is reduced when plants are treated with ethylene inhibitors. The stress-induced inhibitory effect of ethylene is alleviated by the application of ethylene inhibitors such as AVG or silver ions (Kim and Mulkey 1997; Nukui et al. 2000). Root elongation is also inhibited after the application of ACC and this negative impact may be alleviated by the application of ethylene inhibitors.

The light-induced production of ethylene inhibits root growth as it was observed that the application of ACC into the medium resulted in ethylene production that inhibited root elongation in a way similar to the illumination of roots with light (Eliasson and Bollmark 1988). Root inhibition is also caused by other sources of stress-induced ethylene. For example, IAA-induced ethylene production (Chadwick and Burg 1967; Kim and Mulkey 1997) and ethylene production induced due to oxygen deficiency in flooded soils (Visser and Ronald 2007). Thus, increased accumulation of ethylene causes inhibition of root growth and development (Alacon et al. 2009).

Enhanced ethylene synthesis leading to reduced root hydraulic conductivity decreases water transport to plant parts under phosphorus-deficient environment (Carvajal et al. 1996; Borch et al. 1999; Fan et al. 2007; Li et al. 2009). In this case,

increased ethylene concentration may be reduced by the application of ethylene inhibitors, i.e., $CoCl_2$ and AVG.

### 8.3.2 Impact on Nodulation

Soil bacteria belonging to the genus Rhizobium have the ability to fix atmospheric nitrogen and form nodules on roots of leguminous crops (Bai et al. 2002; Kannaiyan 2002). These root nodules can be invaded by bacteria leading to intercellular bacterial inclusions in which nitrogen fixation takes place (Spaink 1996). Rhizobium–legume symbiosis is a very precise mutual association (Sinharoy et al. 2009) and several bacteria and plant factors affect nodulation and infection (Caetano-Anollés and Gresshoff 1991; Spaink 1996). Some of important factors include host specificity, presence of flavonoids, and phytohormones (Antoun et al. 1978; Rolfe and Gresshoff 1988; Tisdale et al. 1990; Sinharoy et al. 2009).

Nodulation is a complex process that involves a number of steps such as root hair curling, formation of infection thread and nodule meristem, development of bacteriods, and fixation of nitrogen (Sinharoy et al. 2009). Ethylene plays a classical role in legume–rhizobia association and inhibition of nodulation process occurred in various spp. such as *Lotus japonicus*, *Medicago truncatula*, *Medicago sativa*, and *Pisum sativum*, by the application of ethylene or its precursor, ACC (Lee and Larue 1992; Nukui et al. 2000; Oldroyd et al. 2001). In fact, nodulation is affected by ethylene at both low as well as high concentration (Gobbelaar et al. 1971).

Ethylene acts as a negative regulator of nodulation (Hirsch and Fang 1994) which regulates the processes of nodulation directly or indirectly (Guinel and Geil 2002); however, nodulation is promoted by inhibition of ethylene synthesis (Zaat et al. 1989; Ligero et al. 1999; Guinel and Sloetjes 2000). For example, elevated levels of ethylene could cause a negative effect on infection threads, and up to 99% abortion in the infection thread of alfalfa was observed (Vasse et al. 1993). Nodulation is controlled by ethylene signaling pathway in common bean (Tamimi and Timko 2003).

Ethylene acts as an autoregulator to control the nodulation process (Arshad and Frankenberger 2002). Rhizobia affect fundamental processes by synthesizing lipochitooligosaccharides (LCOs), also called nodulation factors (Nod factors) (Spaink 1996). Nod factors facilitate the entry of rhizobia into the root and affect various plant physiological processes, such as induction of root hair deformation, nodulin gene expression, formation of infection thread, and nodule primordial development (Spaink 1996; Heidstra et al. 1997; Schulaman et al. 1997; D'Haeze and Holsters 2002; Radutoiu et al. 2003; Cooper 2007). The activity of Nod factor is affected by increased endogenous ethylene (Oldroyd et al. 2001); thus, ethylene could modulate the activation of Nod factor pathway.

### 8.3.3 Physiological Aspects

Although ethylene is involved in breaking seed dormancy, its elevated level has a negative impact on seed germination (Mayer and Poljakoff-Mayber 1989; Smalle and Van Der Straeten 1997). High ethylene concentration not only inhibits root elongation but also induces a number of physiological and biochemical disorders in plants, including senescence, epinasty, chlorophyll destruction, and leaf abscission (Shibli et al. 2007). The enhanced ethylene synthesis could be responsible for inhibiting root and shoot growth, abscission, senescence, alteration in plasma membrane integrity, and pigment content (Morgan et al. 1990; Beltrano et al. 1999; Sharp 2002; Balota et al. 2004). It can also induce physiological changes like stomatal closure, increase in respiratory enzymes, regulation of the synthesis of compounds such as chlorophyll, and phenolic compounds (Arshad and Frankenberger 2002). Ethylene has a negative impact on plant growth by causing the senescence of plant parts (El-Beltagy and Hall 1974). It causes considerable reduction in growth and enhanced leaf and flower senescence. Premature senescence of plant parts under stress conditions also occurs due to alternation of ethylene concentration (Abeles et al. 1992). The petals of *Tradescantia* flower showed senescence when subjected to ethylene. However, ethylene production was inhibited by the application of ethylene inhibitor AVG (Suttle and Kende 1978). Similarly, application of ethylene inhibitor 1-methylcyclopropane (1-MCP) checked the senescence of younger leaves of *Arabidopsis thaliana* and inhibition of ethylene synthesis decreases the senescence of leaves (Alexieva et al. 2004).

Ethylene is also a primary signal induction of epinasty in plants. Epinasty is the downward curvature of leaf and occurs under stress conditions due to the production of ethylene. Ethylene production is stimulated in response to salt stress in plants and this accelerated ethylene causes epinasty (Jones and El-Abd 1989; El-Iklil et al. 2000). However, $Co^{2+}$, i.e., ethylene inhibitor, blocks the synthesis of ethylene and decreases the extent of epinasty (Jones and El-Abd 1989). The application of ethylene inhibitor ($Co^{2+}$) also alleviates the mecocorp (an auxin analog)-induced epinasty (Coupland and Jackson 1991). Potato plants showed an epinastic response to elevated ethylene concentration. This response did occur when ethylene concentration was low (Tonneijck et al. 2004). Epinasty is also affected by light and it is more during the night as compared to during the day time (Tonneijck et al. 2004). It may be because ethylene concentration increases during night time.

Leaf abscission is another physiological disorder and the extent of leaf abscission increases with the application of ethylene (Beyer 1975) and also with the degree of water stress (Jordon et al. 1972). Leaf abscission of cotton depends upon the leaf position as it was observed that ethylene caused abscission of the still expanding third leaf, but had no effect on fully expanded first leaf (Suttle and Hultstrand 1991).

The elevated levels of ethylene also cause a negative impact on chlorophyll content and its loss occurs with the passage of time (Knee 1991; Adachi et al. 1998).

The chlorophyll destruction may be slowed down by the application of norbornadiene, i.e., an inhibitor of ethylene. Stress conditions exhibit a severe loss of chlorophyll and, thus, decrease the rate of photosynthesis (Sergi et al. 2002). The chlorophyll destruction is also affected by temperature and its destruction increases corresponding to an increase in temperature (Knee et al. 2009).

From the above discussion, it is evident that although low level of ethylene is essential for regulation of different processes, at high concentration, it causes a negative impact on plant growth and development. It not only inhibits the process of root elongation and nodulation, but also causes a number of physiological disorders in plants, including epinasty, leaf abscission, chlorophyll destruction, and senescence. It is also revealed from the literature that a check on stress ethylene will be helpful for normal plant growth under unfavorable environmental conditions. The accelerated ethylene synthesis can be blocked by the application of ethylene inhibitors which might be helpful for maintaining normal growth.

## 8.4 Inhibition of Stress-Induced Ethylene Biosynthesis

As it has been discussed in the previous sections, ethylene is produced under stressed conditions and this elevated level of ethylene causes drastic effects on normal plant processes. The accelerated level of ethylene can be controlled by using ethylene inhibitors, including chemical as well as biological inhibitors. These inhibitors are helpful for protecting plants from the negative effects of ethylene and, therefore, may be used effectively in agriculture (Sisler and Serek 2003; Feng et al. 2004).

These inhibitors include chemical compounds and biological inhibitors such as PGPR (Coupland and Jackson 1991; Kim and Mulkey 1997; Mckeon et al. 1995; Mayak et al. 2004a, b; Glick et al. 2007). The use of abscisic acid (ABA) is also effective for controlling ethylene synthesis under water stressed environment. It was observed that in an ABA-deficient environment, root growth was enhanced by maintaining ethylene concentration using ethylene inhibitors (Sharp and LeNoble 2002). Similarly, leaf growth of tomato plants improved by antisense suppression of ethylene (Dodd et al. 2004).

As discussed, plants can be protected from the elevated level of stress-induced ethylene by chemical and biological inhibitors. The effectiveness of these inhibitors has been discussed in the following sections.

### *8.4.1 Chemical Inhibition*

In higher plants, ethylene is synthesized via methionine-ACC pathway (Yang and Hoffman 1984). During this pathway, first the conversion of methionine (MET) to S-adenosylmethionine (SAM) takes place. Then, SAM is converted to ACC and in

the final step, the conversion of ACC into ethylene occurs. Two enzymes, ACC synthase and ACC oxidase, regulate the rate of ethylene synthesis (Yang and Hoffman 1984; Kende 1993; Prescott and John 1996) and the ability of plant tissues to produce ethylene depends on the presence of ACC (Yu and Yang 1980).

Ethylene biosynthesis can be inhibited by the use of chemical inhibitors. The chemical approaches include the use of silver ion ($Ag^{+}$), AVG, aminooxyacetic acid (AOA), 1-MCP (Kim and Mulkey 1997; Guinel and Sloetjes 2000), and $CuSO_4$ (Axelrood-McCarthy and Linderman 1981; Achilea et al. 1985). These chemicals have been successfully used to lower ethylene level in plants or to alter a plant's sensitivity to ethylene, especially during fruit ripening and flower wilting (Abeles et al. 1992; Sisler and Serek 1997). For example, ethylene production by *Azospirillum falciforme* was inhibited by the application of >1 μM $Co^{2+}$ (Arshad and Frankenberger 1989). Similarly, $Co^{2+}$, when applied in the range of 10–100 μM, inhibited the conversion of ACC in to ethylene (Mckeon et al. 1995). Aminoethoxyvalineglycine is a very potent and effective inhibitor of ACC synthase generally used to check the synthesis of ethylene under stress conditions (Suttle and Kende 1978; Kim and Mulkey 1997). In addition to these, some other chemicals like cyclopropanes and silver thiosulfate are also used as ethylene inhibitors. The cyclopropanes are also useful for increasing the life of cut-flowers and potted plants (Yang and Hoffman 1984; Sisler and Serek 1997). Similarly, 1-MCP not only inhibits the ethylene synthesis but is also effective for improving display qualities of substances (Mibus et al. 2007).

### *8.4.2 Biological Inhibition*

To eliminate environment hazards, a sustainable and environment-friendly approach is required, which would be helpful for maintaining plant growth under unfavorable environmental conditions.

Due to the toxic environmental effects of chemical inhibitors of ethylene, there is a need to use biological inhibitors, such as PGPR. It has been mentioned earlier that the amount of ethylene in the plant root depends upon the concentration of ACC in the roots. In soil environment, there are a number of microorganisms that contain an enzyme ACC-deaminase (Salah and Glick 2001; Hontzeas et al. 2005; Sessitsch et al. 2005; Blaha et al. 2006; Belimov et al. 2007; Zahir et al. 2009; Nadeem et al. 2010). This microorganism plays a well-understood role in the regulation of the plant hormone ethylene and, thus, growth and development of plants are modified (Arshad and Frankenberger 2002; Glick 2005). Some PGPR have the ability to hydrolyze ACC into ammonia and α-ketobutyrate by the activity of ACC-deaminase (Shah et al. 1998; Glick et al. 1999). Bacterial strains containing ACC deaminase can eliminate or at least alleviate the stress induced ethylene-mediated negative impact on plants (Glick 2005; Safronova et al. 2006).

PGPR containing ACC-deminase stimulate plant growth by lowering ethylene concentration. This can be explained by using the model proposed by Glick et al.

(1998). According to this model, PGPR attach to the surface of seed or root in response to root exudates (Whipps 1990). The rhizobacteria synthesize and secrete indole acetic acid (IAA) (Patten and Glick 1996). This IAA is also taken up by the plant and along with the endogenous plant; IAA stimulates the activity of ACC-synthase to convert SAM to ACC (Kende 1993). Much of this ACC is exuded from seeds or plant roots along with other small molecules (Penrose and Glick 2001) and taken up by the bacteria and subsequently hydrolyzed to ammonia and α-ketobutyrate. This lowering of ACC reduces ethylene concentration within the plant, thereby alleviating the inhibitory effect of high ethylene concentration on root elongation (Patten and Glick 1996).

Consistent with this model, inoculation of plants with PGPR having ACC-deaminase enzyme causes marked improvement in root growth and biomass production, particularly under stressed conditions (Belimov et al. 2001, 2005; Safronova et al. 2006; Zahir et al. 2009; Nadeem et al. 2007, 2010). Improved plant growth and development under stressed conditions have also been observed in transgenic plants having ACC-deaminase gene (Grichko and Glick 2001b; Sergeeva et al. 2006). It was also observed that PGPR containing ACC-deaminase mitigate the ACC-imposed effect in the similar way as did the chemical inhibitor $Co^{2+}$ (Shaharoona et al. 2007; Nadeem et al. 2010). The decreased ACC level results in low endogenous ethylene concentration, which reduces the harmful effects of stress-induced ethylene; thus, the plant may develop a better root system (Glick et al. 1999).

Moreover, the use of PGPR containing ACC-deaminase activity is advantageous because ACC deaminase trait is common among a number of PGPR species, which are native to the rhizosphere and, consequently, possess a vast array of survival potential in the rhizosphere and rhizoplane. In addition to this mechanism, PGPR also facilitate plant growth and development in a number of ways and protect the plant from deleterious effects of environmental stresses. PGPR containing ACC-deaminase boost plant growth particularly under stressed conditions by the regulation of accelerated ethylene production in response to a multitude of abiotic and biotic stresses like salinity (Abdel-Ghaffar et al. 1982), high temperature, and drought (Marshall 1964; Maas and Hoffman 1977), water logging (Gindin et al. 1989), pathogenicity (Lulai 2001), and contaminants (Arteca and Arteca 2007).

Plants inoculated with PGPR containing ACC-deaminase are better able to thrive under stressed conditions while demonstrating a normal growth pattern. An adequate supply of nutrients is necessary for normal plant growth. In some cases, although nutrients are present in the soil, due to unfavorable environmental conditions, their uptake is restricted and results in more drastic effect on plant growth. It has also been observed that inoculation with these PGPR enhances the uptake of essential nutrients and promotes plant growth under stressed conditions (Nadeem et al. 2007; Yue et al. 2007; Kohler et al. 2009), and the detrimental effects of stress on plant growth can be alleviated by enhancing the uptake of essential nutrients (Giri et al. 2007). Certain PGPR strains can solubilize phosphorus and make it available for plant (Cattelan et al. 1999; Yao 2004). Similarly, an enhanced uptake of $K^+$ and restricted uptake of $Na^+$ has been observed under salt-stressed conditions (Zahir et al. 2009; Nadeem et al. 2010).

Indole-3-acetic acid has a positive effect on plant growth (Vessey 2003); however, at high concentration, it inhibits root growth (Xie et al. 1996) because it stimulates ACC-synthase (an enzyme) to convert SAM (S-adenosylmethionine) to ACC, i.e., precursor of ethylene (Kende 1993). For example, inoculation with *Pseudomonas putida* significantly enhances the root elongation of tomato seedlings subjected to elevated levels of IAA, i.e., 0–10 μg $mL^{-1}$ (Gravel et al. 2007). This may be attributed to microbial degradation of IAA and/or ACC-deaminase activity that results in low ACC concentration leading to reduce ethylene production.

The rhizobacterial species *Variovorax paradoxus* effectively colonize plant roots under stress conditions and, hence, can be used efficiently for attenuating the growth inhibition of plants under stress conditions (Belimov et al. 2009a). These PGPR-containing ACC-deaminase decreased the stress induced endogenous ethylene level (Mayak et al. 2004b) and ACC concentration in xylem of plants (Belimov et al. 2009b). The inoculation with *A. brasilense* increases relative water content in leaf which may be due to the production of ABA by the bacterial strains (Cohen et al. 2008), and thus reduce the ACC level in plants.

The nodulation process involves signal exchange between the host and the bacterium, i.e., rhizobium. PGPR, including rhizobia, develop a strong interaction during the process of root colonization and some PGPR have the ability to improve nodulation and $N_2$ fixation process (Table 8.2) (Zhang et al. 1996; Lucas-Garcia et al. 2004). The PGPR produce plant growth hormones which enhance the nodulation process. It has been reported that some *Pseudomonas* strains increased nodule number and acetylene reduction in soybean plants inoculated with *Bradyrhizobium japonicum* but others have no effect on these parameters (Chebotar et al. 2001). This may be attributed to the enhanced surface area for infection through colonizing the plant roots, thus enhancing nodulation. Similarly, Coinoculation under sterile jar conditions with *Pseudomonas* strains and an effective *Mesorhizobium* sp. is also equally effective for enhancing nodulation. This might be due to the reason that coinoculation with *Pseudomonas* increases the surface area of roots for the attachment of rhizobia or enhances the production and release of flavonoid-like compounds that induce the transcription of rhizobial nodulation genes (Goel et al. 2001).

In addition to decreasing ethylene level, these PGPR may also be beneficial for regulating nutrition and hormonal balance in the plants in the presence of environmental stresses.

## 8.5 Perspectives of Inoculation with PGPR Containing ACC-Deaminase Under Stressful Environments

In stress environment, in addition to nutritional and hormonal imbalances, plant growth is particularly affected by the enhanced ethylene biosynthesis. It is highly likely that reducing ethylene concentration in plants could be useful for alleviating the negative impact of high ethylene on plant growth and development.

**Table 8.2** Coinoculation with PGPR and Rhizobia for plant growth promotion

| Growth conditions | Crop | Rhizobia | PGPR | Effect | References |
|---|---|---|---|---|---|
| Normal conditions | *Cicer arietinum* | *Rhizobium* | *Enterobactor* | Coinoculation enhanced the growth and nodulation and was more efficient than separate inoculation | Mirza et al. (2007) |
| Normal conditions | Pigeon pea | *Rhizobium* spp. | *Bacillus megaterium* | Coinoculation was more effective for improving the growth and nodulation of pigeon pea and siderophores production was more with coinoculation | Rajendran et al. (2008) |
| Normal conditions | *Glycine max* | *Bradyrhizobium japonicum* | *Bacillus* spp. | Coinoculation enhanced growth and nodulation in *Glycine max* | Bai et al. (2002) |
| Normal conditions | *Cajanus cajan* | *Rhizobium* spp. | *E. coli* | More plant growth, more utilization of ferric citrate by *Rhizobium* spp. | Geetha et al. (2007) |
| Normal conditions | *Vigna radiata* | *Bradyrhizobium* spp. | *Bacillus subtilis* | Increased dry matter yield, chlorophyll content and P uptake | Zaidi and Khan (2006) |
| Drought stress | Common bean (*Phaseolus vulgaris* L.) | *Rhizobium tropici* | *P. polymyxa* | Augmented plant height, shoot dry weight and nodule number | Figueiredo et al. (2008) |
| Salinity stress | Soybean (*Glycine max*) | *Bradyrhizobium japonicum* USDA 110 | *Bacillus subtilis, Bacillus megaterium* | Decreased Malondialdehyde, ascorbate peroxidase (APX), glutathione reductase (GR), proline, glucose, glycine betaine, etc. increased root shoot growth | Han and Lee (2005) |

It is hypothesized that the inoculation with PGPR containing ACC-deaminase can facilitate plant growth under stress conditions by regulating the ethylene level in the plants. The inoculated plants might be able to withstand stress conditions and continue to maintain their growth and development (Table 8.3). Some examples of improved plant growth in stress environment due to inoculation with PGPR containing ACC-deaminase are discussed below.

### *8.5.1 Salinity Stress*

Soil salinity is one of the major abiotic stresses that hamper agricultural productivity worldwide and reduce plant growth and yield in arid and semiarid regions (Greenway and Munns 1980; Cicek and Cakirlar 2002). It has been estimated that more than 900 million ha world land is salt affected and have negative impacts on agricultural production (Flowers 2004). The irrigated areas with water scarcity, high temperature, and evapotranspiration, using poor quality irrigation water, and adopting poor irrigation management practices are also affected by salinity (Cano et al. 1998; Abed Alrahman et al. 2005). Salt stress is a complex abiotic stress and plant growth is affected by complex interaction of specific ion effect, osmotic effects, hormonal and nutritional imbalances (Parakash 1982; Gorham et al. 1985; Munns 1993; Ruiz et al. 1997; Arbona et al. 2005).

Cereals are the most important crops all over the world; however, salinity is a major constraint for sustainable crop production. Although wheat is generally considered to tolerate salt concentration to some extent (Maas and Hoffman 1977), salinity causes a significant decrease in growth and grain yield of wheat. Therefore, proper management will be helpful for enhancing plant growth in the presence of salinity. This can be attained by the inoculation of these crops with suitable strains of PGPR. For example, *Pseudomonas* sp. enhanced the wheat growth under stress conditions by auxin production and reducing the uptake of toxic ions and formation of stress-specific proteins (Hasnain and Sabri 1996). Similarly, *Pseudomonas* spp. containing ACC-deaminase effectively improved the growth of maize at low and high salinity concentration (Kausar and Shahzad 2006).

It is evident from literature that ethylene evolution is high at elevated level of salt concentration (Youssef et al. 2000; Roussos et al. 2006), which is harmful for plant growth, particularly roots. The performance of PGPR having ACC-deaminase activity has been evaluated for improving maize growth under salinity stress. Although salinity depresses the growth of plant seedlings, PGPR inoculation reduces this depressive effect (Nadeem et al. 2007). Inoculation also improves the relative water content and $K^+/Na^+$ ratio. This growth promotion ability varies among different strains of PGPR (Nadeem et al. 2009).

PGPR containing ACC-deaminase have variable potential to protect the plant from the deleterious effects of salinity. This might be due to the differential ability of these strains to hydrolyze ACC and also their root colonization (Nadeem et al. 2010). For example, *Pseudomonas putida* have a better ability to mitigate negative

**Table 8.3** Alleviation of impact of abiotic stresses on plant by PGPR containing ACC-deaminase

| Crops | Bacteria/transgenic plants with ACC-deaminase | Plant Response | References |
|---|---|---|---|
| PGPR inoculation under salt stress | | | |
| Tomato | *Achromobacter piechaudii* ARV8 | Inoculation increased the fresh and dry weight of tomato seedlings in the presence of 172 mM NaCl. Bacteria also increased the water use efficiency and reduced the production of ethylene by tomato seedlings | Mayak et al. (2004a) |
| | *P. fluorescens*<br>*P. aeruginosa*<br>*P. stutzeri* | Enhanced root/shoot length and biomass production | Tank and Saraf (2010) |
| Transgenic canola | ACC-deaminase gene from *Agrobacterium rhizogenes* | Salt tolerance in plants increased and inoculation gave significant increase in fresh and dry weight, leaf protein and chlorophyll contents over noninoculated control | Sergeeva et al. (2006) |
| | Gene from *P. Putida* UW4 | Plants demonstrated relatively more vigorous growth than nontransgenic plants | Cheng et al. (2007) |
| Cotton | *Klebsiella oxytoca* | Plant height and dry weight of cotton increased by 14.9 and 26.9%, respectively. Inoculation also enhanced the absorption of N, P, K and Ca, and decreased the absorption of Na under salinity stress | Yue et al. (2007) |
| Groundnut | *P. fluorescens* PF1,<br>*P. fluorescens* PF2 and<br>*P. fluorescens* RMD1 | Salt tolerance increased manifold. *Pseudomonas fluorescens* TDK1 showed greater performance for improving growth of groundnut | Saravanakumar and Samiyappan (2007) |
| Maize | *P. syringae*, *P. chlororaphis*,<br>*P. bathycetes*,<br>*E. aerogenes*,<br>*F. ferrugineum*,<br>*P. fluorescenc* | Inoculation improved growth under high levels of salinity. Relative water content, chlorophyll content and $K^+$/$Na^+$ ratio were enhanced by inoculation over control | Nadeem et al. (2007) |
| | PGPR having ACC-deaminase | Significant increase in root–shoot length, root–shoot fresh and dry weight, and chlorophyll pigments was observed at EC 12 $dSm^{-1}$ compared to noninoculated control | Nadeem et al. (2006) |

(continued)

**Table 8.3** (continued)

| Crops | Bacteria/transgenic plants with ACC-deaminase | Plant Response | References |
|---|---|---|---|
| | *P. putida* biotype A, *P. fluorescens* biotype A | Plants demonstrated good root–shoot length and seedling growth against salinity stress under gnotobiotic conditions | Kausar and Shahzad (2006) |
| Wheat | *P. putida, P. aeruginosa* and S. *Proteamaculans* | Increased plant height, root length, grain yield, 100-grain weight and straw yield under high salinity, and also enhanced chlorophyll content and $K^+/Na^+$ ratio compared to control | Zahir et al. (2009) |
| | *Pseudomonas fluorescens* 153, 169, *Pseudomonas putida* 108, 4 | Increased root, shoot growth and grain yield | Abbaspoor et al. (2009) |
| | *P. putida, E. cloacae*, *S. ficaria, P. fluorescens* | Inoculation improved the growth and yield of wheat and enhanced the uptake of $K^+$ and relative water content. Inoculation also decreased the intensity of classical triple response | Nadeem et al. (2010) |
| Rice | *P. fluorescens* MSP-393 | High salinity stress did not affect the colonization efficiency of the strain. The bacteria maintained root colonization potential by osmotolerance mechanisms | Paul and Nair (2008) |
| Canola | *P. fluorescens*, *P. putida* | Inoculation enhanced germination of seeds and seedling growth | Jalili et al. (2009) |
| Cucumber | *P. putida* | The parameters like root shoot weight and leaf number were more in plants inoculated with *P. putida* than its mutant lacking ACC-deaminase | Gamalero et al. (2009) |
| Ryegrass | *Pseudomonas* sp., *Citrobacter* sp., *Enterobacter* sp., *Klebsiella* sp. | Alleviated salt stress and significantly promoted seedlings growth in gnotobiotic pouch assay. Inoculation significantly increased root and shoot dry weight in soil pot trial | Ji and Huang (2008) |
| PGPR inoculation under flooding/drought stress | | | |
| Transgenic tomato | Heinz 902 expressing the bacterial gene | | |

(continued)

**Table 8.3** (continued)

| Crops | Bacteria/transgenic plants with ACC-deaminase | Plant Response | References |
|---|---|---|---|
| | | Plant demonstrated good growth against flooding stress | Grichko and Glick (2001b, c) |
| Tomato, pepper | *P. putida* GR12-2, *Achromobacter piechaudii* ARV8 | Inoculation significantly increased fresh and dry weight of tomato and pepper seedlings. Bacteria reduced the production of ethylene by tomato seedlings under water stress | Mayak et al. (2004b) |
| Transgenic canola | ACC-deaminase gene from *Agrobacterium rhizogenes* / inoculation with *P. Putida* UW4 | Transgenic plants and/or plant treated with *P. putida* showed more prolific growth | Farwell et al. (2007) |
| Pea | *V. paradoxus* 5C-2 | Inoculation improved growth, yield and water use efficiency of drought-stressed peas | Belimov et al. (2009a) |
| | *P. fluorescens* biotype G, *P. fluorescens*, *P. putida* biotype A | Plant showed an increased tolerance against drought stress by improving fresh–dry weight, root–shoot length and water use efficiency | Zahir et al. (2008) |
| | *P. putida*, *P. fluorescens* | Inoculation significantly decreased the drought stress-imposed effects on the growth and yield of peas | Arshad et al. (2008) |
| *Catharanthus roseus* | *P. fluorescens* | Enhanced the growth parameters under drought stress and ameliorated the drought induced growth inhibition by increasing fresh and dry weight | Jaleel et al. (2007) |
| Potato | *Variovorax paradoxus* 5C-2 *Achromobacter xylosoxidans* Cm4 | Inoculation increased tuber yield and number and improve water use efficiency of potato | Belimov et al. (2009b) |
| PGPR inoculation under heavy metal stress | | | |
| Indian mustard | *Kluyvera ascorbata* SUD165, *K. ascorbata* SUD165/26 | Toxic effect of heavy metals was not pronounced in inoculated plants | Burd et al. (2000) |
| | *P. brassicacearum*, *P. marginalis*, *P. oryzihabitans*, *P. putida*, *Alcaligenes* sp., *V. paradoxus*, *B. pumilus*, *Rhodococcu* sp. | Inoculation stimulated root elongation in the presence of 300 μM $CdCl_2$ solution | Belimov et al. (2001) |
| | *Rhodococcus* sp., *Variovorax paradoxus* sp. | The bacteria was tolerant to Cd toxicity and capable of | Belimov et al. (2005) |

(continued)

**Table 8.3** (continued)

| Crops | Bacteria/transgenic plants with ACC-deaminase | Plant Response | References |
|---|---|---|---|
| | | stimulating root elongation in the presence and absence of toxic Cd concentration and useful for phytoremediation of polluted soil | |
| | *Enterobacter* sp. | Inoculation stimulated plant biomass and enhanced phytoextraction of Ni, Zn and Cr | Kumar et al. (2008a, b) |
| Transgenic Canola | *Enterobacter cloacae* CAL2 | Plant showed a great tolerance against arsenate | Nie et al. (2002) |
| | ACC-deaminase gene from *Agrobacterium rhizogenes* | Significantly increased tolerance to nickel (Ni) compared to nontransformed plant | Stearns et al. (2005) |
| Common reed | *P. asplenii* | Plant showed normal growth under high level of $Cu^{+2}$ | Reed et al. (2005) |
| Transgenic tomato | Gene from *E. cloacae* | Inoculation enhanced plant growth under the stress of heavy metal like $Cd^{+2}$, $Co^{+2}$, $Cu^{+2}$ $Ni^{+2}$, $Pb^{+2}$ and $Zn^{+2}$ | Grichko et al. (2000) |
| Pea | *P. brassicacearum* Am3, P. marginalis Dp1 | The bacteria alleviated the Cd-induced inhibition of nutrient by root | Safronova et al. (2006) |
| Tomato | *Methylobacterium oryzae* CCBMB20, *Burkholderia* sp. CBMB40 | Inoculation reduced the ethylene emission and increased the tolerance against Ni and Cd | Madhaiyan et al. (2007) |
| Tomato | *Bacillus subtilis* | Improved plant growth due to more production of growth hormones | Woitke et al. (2004) |
| Canola | *Kluyvera ascorbata* SUD165 | Plant showed normal growth under heavy metals (Ni, Pb, Zn, Cr) stress | Burd et al. (1998) |
| | *Pseudomonas tolaasii* ACC23, *Pseudomonas fluorescens* ACC9, *Alcaligenes sp*. ZN4 and *Mycobacterium sp*. ACC14 | Improved plant growth. Increased the plant biomass and consequently the total cadmium accumulation | Dell'Amico et al. (2008) |
| Brassica juncea | *Variovorax papadoxus* 5C-2 | Enhanced tolerance against Cd | Belimov and Wenzel (2009) |
| PGPR inoculation under pathogenic stress | | | |
| Cucumber | *Pseudomonas putida*, strain 89B-27 | Strains induced systemic resistance against Fusarium wilt | Liu et al. (1995a) |

(continued)

**Table 8.3** (continued)

| Crops | Bacteria/transgenic plants with ACC-deaminase | Plant Response | References |
|---|---|---|---|
| | *Serratia marcescens* strain 90-166 | | |
| | *Pseudomonas putida*, strain 89B-27<br>*Serratia marcescens* strain 90-166 | Strains induced systemic resistance against bacterial angular leaf spot | Liu et al. (1995b) |
| | *Pseudomonas putida*, strain 89B-27<br>*Serrattia marcescens* strain 90-166 | Strains induced systemic resistance against Fusarium wilt and bacterial angular leaf spot | Liu et al. (1995c) |
| Cucumber and tomato | *P. putida*, *S. marcescens* | Inoculation induced systemic resistance against Cucumber mosaic cucumovirus | Raupach et al. (1996) |
| Canola | *P. fluorescens* CHA0 | Protected the cucumber against *Pythium* damping off and potato tuber against Erwinia soft rot compared to strain lacking of ACC-deaminase | Wang et al. (2000) |
| Transgenic tomato | ACC-deaminase gene from *E. cloacae* UW4 | Inoculation reduced the symptoms of Verticillium wilt and increased disease tolerance in plant | Robison et al. (2001a) |
| Maize | *Klebsiella oxytoca* MKR7, *Pseudomonas* sp. 4MKS8 | Enhanced the growth by providing relief against parasitic infestation of *Striga hermonthica* | Babalola et al. (2003) |
| Tomato | *Pseudomonas brassicacearum* AM3 | Inoculation increased the root elongation and biomass of tomato by masking the phytopathogenic properties of bacteria | Belimov et al. (2007) |
| Low temperature stress | | | |
| Soybean | *Serratia proteamaculans* | Enhanced nodulation and yield | Dashti et al. (2000) |
| Grapevine plantlets | *Burkholderia phytofirmans* | Enhanced chilling resistance by improving plant growth and physiological activity | Ait Barka et al. (2006) |
| Canola | *Pseudomonas putida* | Promoted plant growth at low temperature in the presence of salinity | Cheng et al. (2007) |

impact of salinity than *Serratia proteamaculans* for improving wheat growth under salt stressed conditions (Zahir et al. 2009). Similarly, *Pseudomonas stutzeri*, among three *Pseudomonas* spp., proved to be better for enhancing the growth of tomato plants, and this might be attributed to low uptake of $Na^+$ by this strain compared to others (Tank and Saraf 2010). The inoculation with *P. putida* UW4 having ACC-deaminase enzyme caused a 100% increase in shoot weight of canola under salinity stress (Cheng et al. 2007). Similarly, *P. fluorescens* was also effective for enhancing seed germination and seedling growth of canola (*Brassica napus* L.), (Jalili et al. 2009). The better performance of *Pseudomonas* spp. for improving wheat growth in the presence of salts has also been reported by Egamberdieva and Kucharova (2009). In addition to *Pseudomonas* spp., *Achromobacter piechaudii* also significantly enhanced the growth of tomato under salinity stress. Salt stress-induced ethylene evolution is also reduced by the ACC-deaminase activity of this bacterium (Mayak et al. 2004a).

Legume crops are well known due to their economic importance and nitrogen fixation. The site of nitrogen fixation is nodules and better nodulation is a sign of improved plant growth. It has been observed that nodulation is inhibited by the application of ACC or exogenous ethylene (Nukui et al. 2000; Oldroyd et al. 2001); however, the application of ethylene inhibitors enhanced the nodulation (Lee and LaRue 1992; Yuhashi et al. 2000).

A number of PGPR strains containing ACC-deaminase have been proven better for enhancing growth of nonlegumes under stress environment; however, *P. flourescens* has received much attention owing to its ACC-deaminase activity, better root colonization, and production of enzymes and metabolites that enhance the plant's ability to tolerate environmental stresses (Mayak et al. 2004b; Nadeem et al. 2007, 2009, 2010; Zahir et al. 2009). Most of these studies were conducted in lab or green house and little is known about the effectiveness of this strain in legumes, particularly under salt affected field conditions.

Ethylene is produced due to a number of environmental factors such as salinity (Abdel-Ghaffar et al. 1982), high temperature, and drought (Marshall 1964; Maas and Hoffman 1977). The growth of Rhizobia is either completely inhibited or reduced under salinity (Burton 1967), which may affect nodulation and nitrogen fixation. Plant growth promoting bacteria containing ACC deaminase boost plant growth, particularly under stressed conditions, by the regulation of accelerated ethylene production. Beneficial responses due to interaction of PGPR with rhizobia on legumes have been reported by a number of scientists (Azcon-Aguilar and Barea 1981; Hicks and Loynachan 1989; Saxena and Tilak 1994; Lata and Tilak 2000; Deshwal et al. 2003).

As stated, PGPR containing ACC-deaminase are not only effective for reducing stress-induced ethylene, but are also helpful for the absorption of nutrients required for better growth. Inoculation also enhances the absorption of major nutrients such as N, P, K and Ca and decreases the uptake of the $Na^+$ (Yue et al. 2007). The *Klebsiella oxytoca* (Rs-5) containing ACC-deaminase mitigates the negative effects of salt stress and promotes plant growth.

To confirm the effectiveness of PGPR containing ACC-deaminase for improving plant growth under stresses, it is necessary to evaluate its role by comparing it with its mutant. Cheng et al. (2007) evaluated the role of PGPR containing ACC-deaminase and its mutant (without ACC-deaminase) for improving growth of canola under salinity stress conditions. The growth of canola plants inoculated with *P. putida* UW4 containing ACC-deaminase and its mutant (no ACC-deaminase) was monitored at 10 and 20°C in the presence of salinity. Although, salinity inhibited the plant growth however, *P. putida* UW4 alleviated this inhibitory effect of salinity. It was further observed that the mutant strain did not improve plant growth in the presence of salinity.

The growth enhancement occurred as a result of inoculation with rhizobacteria containing ACC-deaminase motivates the scientists to construct transgenic plants with the expression of ACC-deaminase gene. These transgenic plants produce less ethylene and have effects on the physiological processes of plant (Klee et al. 1991; Klee and Kishmore 1992; Stearns et al. 2005). For example, transgenic canola plants with the expression of ACC-deaminase gene have the ability to thrive better under salt-stressed conditions (Sergeeva et al. 2006).

### *8.5.2 Heavy Metals Stress*

Several reports are available in the literature indicating the presence of high concentration of heavy metals particularly, in industrial areas, and these high concentrations are harmful for the ecosystem (Singh and Steinnes 1994). Although some metals are beneficial for plant growth, if these metals are present in high concentration, they impart toxicity to plants (Ernst 1998). These metals also enhance the ethylene synthesis that ultimately affects the plant growth in a number of ways (Prasad and Strazalka 2000; Safronova et al. 2006; Arshad et al. 2008). For example, arsenic causes a negative impact on seed germination and root elongation (Fargasova 1994), and cadmium also inhibits root growth and affects nutrient uptake and homeostasis (Sanita di Toppi and Gabrielli 1999). In addition to increase in ethylene concentration, heavy metals also cause iron deficiency and evolution of active oxygen species (Burd et al. 1998; Buchanan 2000).

PGPR with ACC deaminase protect plants from the deleterious effects of heavy metals by lowering ethylene concentration that is produced during heavy metals stress; and therefore facilitate the plants in establishing a better root system (Burd et al. 2000; Arshad et al. 2008). Inoculation enhanced plant growth in cadmium (Cd) contaminated soil (Belimov et al. 2001) and these strains have variable efficacy to reduce the deleterious effect of heavy metals (Belimov et al. 2005; Dell'Amico et al. 2008). This growth promotion may be attributed to ACC-deaminase activity and the production of siderophores and IAA by PGPR.

The transgenic plants with the expression of ACC-deaminase gene also enhance the survival of plants when they are exposed to heavy metals. For example, the expression of ACC-deaminase gene in a transgenic tomato plant protects the plant

from the harmful effects of heavy metals (Grichko et al. 2000). Similarly, transgenic plants having ACC-deaminase gene have greater ability to withstand heavy metals (Cd and Ni) stress (Grichko and Glick 2001c; Nie et al. 2002; Stearns et al. 2005).

Although fly ash soils have a high concentration of essential plant nutrients, these soils also contain heavy metals such as Ni, Pb, Cr, and Ba, that cause surface and ground water contamination (Kalra et al. 1998; Gupta et al. 2002a). These metals, when present at super optimal concentration, affect plant growth and development (Sinha and Gupta 2005). Inoculation with PGPR containing ACC-deaminase has the ability to enhance plant growth in Cd contaminated soil (Belimov et al. 2001), thus reducing the negative impact of heavy metals on plant growth (Kumar et al. 2008b). These PGPR have the ability to improve plant growth due to the utilization of ACC (Jacobson et al. 1994; Gupta et al. 2002b). Microbial strains have variable efficacy to remediate the metal contaminated soils in different environments and soil conditions (Gupta et al. 2002c; Belimov and Wenzel 2009).

PGPR also promote nodulation in contaminated soils. The PGPR strain isolated from Cd-contaminated soil increased the nodulation in clover plants (Vivas et al. 2005) due to the fact that the PGPR accumulated Cd in their cells and, thus, reduced the available Cd concentrations in the solution, thereby reducing its Cd uptake by plants and rhizobia, which eliminated Cd toxicity and increased nodulation. The other aspect of this increased nodulation may be because of the enhanced enzymatic activity in the soil (phosphatase, dehydrogenase etc.) and auxin production around PGPR inoculated roots.

### *8.5.3 Drought Stress*

Drought is one of the major abiotic stresses and affects almost all the climatic regions of the world (Wilhite 2000). The changes in environmental scenario result in increasing aridity due to the decrease in annual rainfall that causes agriculture to come under sustained pressure to feed an ever increasing population (IPCC 2001). Plants respond to water stress at both cellular and molecular level and are highly sensitive to even mild dehydration stress (Bray 1997; Liu et al. 1998; Kasuga et al. 1999).

Like other environmental stresses, when plants are subjected to drought stress, an accelerated level of ethylene is produced (Apelbaum and Yang 1981; Hoffman et al. 1983; Mayak et al. 2004a), which leads to the abnormal growth of a plant (Morgan et al. 1990; Mattoo and Suttle 1991). Under drought, plant growth can be enhanced by promoting root growth that allows uptake of water from the deeper parts of the soil profile (Reid and Renquist 1997). This can be achieved by the inoculation of plants with PGPR containing ACC-deaminase (Glick et al. 1998; Ghosh et al. 2003; Shaharoona et al. 2006), which promote the growth of plants under drought-stressed conditions by reducing the production of ethylene

(Mayak et al. 2004a). During water stress, bacterium does not influence the plant water content; however, its presence enhances plant recovery when rewatering the plant (Mayak et al. 2004b). These rhizobacteria containing ACC-deaminase stimulate the root biomass of plants growing under dry condition, as evidenced by their higher growth rate (Dodd et al. 2004). For example, maize plants in which stress-induced ethylene production was suppressed by an antisense gene for one isoenzyme of ACC synthase showed delayed leaf senescence under drought (Young et al. 2004).

In medicinal plants, the content of economically important metabolite is more important than the yield as it determines the cost of its extraction (Levy 1982). *Catharranthus roseus* is a medicinal plant that contains antihypertension alkaloid ajmalicine. The inoculation with *P. fluorescens* partially ameliorated the stress induced inhibition of plant growth by enhancing growth parameters. Inoculation with *P. fluorescens* also increased the content of ajmalcine (Jaleel et al. 2007). It is a well established fact that some strains of *P. fluorescens* contain an enzyme ACC-deaminase (Glick et al. 1995; Saravanakumar and Samiyappan 2007; Zahir et al. 2008, 2009). Therefore, this strain may be employed to protect the plant from the deleterious effects of drought stress. These PGPR also have some other growth-promoting characteristics, such as siderophore production, auxin production, and root colonization, in addition to ACC-deaminase activity (as also observed by Nadeem et al. 2007; Ahmed and Hasnain 2008). The inoculation mitigates the drought stress effect with variable efficacy at different moisture levels. This might be attributed to the fact that inoculation reduced the ethylene synthesis and resulted in better plant growth under drought stress (Arshad et al. 2008). Inoculation with *V. paradoxus* also improves growth, yield, and water use efficiency of plants under drought conditions compared to its mutant (Belimov et al. 2009a). These PGPR enhance vegetative growth, flowering, and yield; however, they have limited impact on leaf water relations (Belimov et al. 2009b).

The leguminous crops and rhizobia are very sensitive to drought stress (Sinclair et al. 2001). The nitrogen accumulation and yield potential of legumes decreases with decreasing water potential in the drying soil due to lower efficacy of $N_2$ fixation process under water deficit conditions (Serraj et al. 1999, 2001; Sinclair et al. 2007). Coinoculation can enhance plant growth by altering the morphological and physiological processes in the root system (Bashan and Levanony 1990; Sarig et al. 1992), such as increase in the number of lateral roots and root hairs, thus, increasing the surface area for more nutrient and water uptake. Thus, coinoculation of PGPR containing ACC-deaminase and rhizobia could be an effective approach for sustainable agro-ecosystem under stressed environment.

### *8.5.4 Flooding Stress*

For normal plant growth, proper aeration is necessary. Oxygen supply to plant roots is reduced under flooding conditions (Jackson et al. 1985). Flooding causes reduced

root permeability, water absorption, and mineral uptake that leads to the closing of stomata, ultimately resulting in reduced photosynthesis and inhibition of stem and root growth (Grichko and Glick 2001a). Flooding can occur several times in a growing season and the root environment becomes anaerobic and causes an induction in the expression of ACC synthase, which results in the accumulation of ACC in the root tissues (Else and Jackson 1998; Glick et al. 2007). Hypoxic roots show high activity of ACC synthase and high concentration of ACC compared to aerated roots (Morgan and Drew 1997). The ACC synthase activity in roots results in ethylene production (Machckova et al. 1997). However, ACC does not change to ethylene in root due to anaerobic conditions under flooding, whereas ACC oxidase which catalyses this reaction requires oxygen (Glick et al. 2007). Therefore, ACC is transported to shoot under aerobic environment; ethylene is produced (Bradford and Yang 1980). Ethylene concentration increases under flooding conditions due to an increase in ACC synthase in submerged roots and ACC oxidase in shoots (Else et al. 1995). In plants, IAA induces ethylene production and inhibitory effects of high IAA on root growth are also mediated by ethylene (Grichko and Glick 2001a). The ethylene production can be decreased by suppressing some of the enzymes of ethylene synthesis; however, as these enzymes are also involved in some other plant processes, the PGPR containing ACC-deaminase may be used to convert ACC (immediate precursor of ethylene) into α-ketobutyrate and ammonia (Grichko and Glick 2001a). The adverse effect of ethylene produced under flooding can be eliminated by inoculating the plant with PGPR containing ACC-deaminase. The inoculated plants produce low ethylene, reducing the damage of elevated ethylene concentration (Grichko and Glick 2001b).

The transgenic plants expressing ACC deaminase show increased tolerance to flooding stress. The negative effects of root hypoxia on plant growth can also be reduced in transformed (transgenic) plants. Plants that have ACC-deaminase gene under the control of the rolD promoter are protected to the greatest extent (Grichko and Glick 2001b).

### *8.5.5 Miscellaneous*

In the previous sections, we discussed the role of PGPR containing ACC-deaminase in enhancing plant growth and development in the presence of a number of environmental stresses including salinity, heavy metals, drought, and flooding. These microorganisms also play a key role in facilitating plant growth in other unfavorable environmental conditions such as pathogenicity, nutritional stress, temperature, wilting etc. In this section the role of PGPR containing ACC-deaminase to mitigate the depressing effects of some of such unfavorable constrains are discussed.

Phytopathogenic organisms are a major threat to successful crop production. The ethylene synthesis in plant is enhanced with severity of pathogenic infection. The PGPR have antagonistic effects against pathogens and stimulated plant growth

(Dobbelaere et al. 2003; Donate-Correa et al. 2005; Pandey et al. 2005; Domenech et al. 2006). Some pathogenic bacteria contain ACC-deaminase enzyme (Joardar et al. 2005; Blaha et al. 2006) that helps the plant to withstand under stress by cleaving the ACC into ammonia and α-ketobutyrate. However, it is not clear whether the presence of this enzyme in pathogenic bacteria protects the plants from pathogenic effects or not. The work of Belimov et al. (2007) provides some information about this mechanism. They concluded that the pathogenic bacteria *P. brassicacearum* Am3 containing ACC deaminase promoted tomato growth. Rhizobacteria containing ACC-deaminase are more effective in controlling the disease, compared to that of rhizobacteria without this enzyme (Wang et al. 2000). The transgenic plants with the expression of ACC deaminase produce low stress ethylene and protect the plant from the damage of various phytopathogens (Robison et al. 2001b). Such evidences indicates that bacterial ACC deaminase could play a prominent role for increasing disease resistance in plants

Ethylene is also synthesized in response to wounding and pathogen attack (Abeles et al. 1992; Boller 1991; Kim and Hwang 2000). The inhibition of ethylene results in reduced symptoms in susceptible disease interactions (Elad 1988; Bashan 1994; Cooper et al. 1998; Lund et al. 1998). Thus, plants can be protected effectively by preventing the disease-related ethylene production by ACC-deaminase activity.

Plants are also sensitive to fluctuation in temperature and heat stress (Mendelsohn et al. 1994; Robertson et al. 1998). Like other environmental stresses, temperature stress also affects plant growth, which might be due to the production of elevated level of ethylene (Cheikh and Jones 1994). Reports are available in literature showing that ACC-deaminase also protects the plant from temperature stress (Bensalim et al. 1998; Ait Barka et al. 2006).

Ethylene and its precursor ACC also have a potential role in flower senescence, leaf abscission, and wilting of flowering species (Reid and Wu 1992; Woltering and van Doorn 1988). Ethylene production decreases the shelf life of flowers that is a major impediment in the success of flowering business (Saleem et al. 2007). PGPR containing ACC-deaminase can also enhance the shelf life of flowers (Nayani et al. 1998).

In addition to all above discussed stresses, plants are also affected by some parasitic weeds in the soil environment that cause a negative impact on plant growth. Microorganisms can be used as biological agent to control these parasitic weeds (Abbasher and Sauerborn 1992; Marley et al. 1999; Berner et al. 1999). Bacterial inoculation increases plant growth, but with variable efficacy (Babalola et al. 2003). They demonstrated that the variable difference in growth promotion may be due to competition among the isolates because of different genetic potential of the isolates.

It is evident that plant growth under unfavorable environment can be promoted by the use of PGPR containing ACC-deaminase enzyme. These PGPR not only protect the plant from the negative impact of high ethylene concentration but also enhance plant growth and development by a number of other mechanisms, such as

facilitating nutrient uptake, production of phytohormones, solubilization of nutrients etc.

## 8.6 Conclusions and Future Prospects

The above discussion epitomizes that environmental stresses such as salinity, drought, flooding, heavy metals, pathogens etc. have a negative impact on plant growth and development. In the presence of unfavorable environmental conditions, ethylene level of the plant increases and causes drastic effects on plant growth. The basis of increasing this elevated level of ethylene is the increase in ACC, an immediate precursor of ethylene biosynthetic pathway. This increase in ethylene concentration can be checked by the use of chemical or biological inhibitors of ethylene. As there are environmental concerns about the chemical inhibitors of ethylene, the use of PGPR containing ACC-deaminase is one of the newly emerging approaches that may effectively be used for protecting plants from harmful effects of undesirable environmental conditions. These PGPR not only protect the plant from negative effects of elevated ethylene level but also facilitate the plant growth by a number of ways, such as by producing plant growth regulators, solubilization of nutrients, siderophore production etc. Therefore, inoculation of seed/plants with PGPR containing ACC-deaminase enzyme or expression of bacterial ACC-deaminase gene into plants could be very efficient in facilitating plant growth in stress environment.

The work of different scientists discussed in previous sections indicates that performance of these rhizobacteria is generally evaluated under controlled conditions. Although, a few studies indicated their growth promoting activities under natural conditions, field investigations are still needed to demonstrate the efficacy of this approach in stress environment. The wide scale use of this technology may decrease the use of chemical inhibitors of ethylene and that will be an added advantage to the environment. Bio-fertilizers using such strains may not only have a positive impact on soil environment but may also reduce the dependence on chemical fertilizers.

Although, PGPR containing ACC-deaminase strains as well as the transgenic plants with the expression of ACC-deaminase gene protect the plant from drastic effects of stress environment; yet, some aspects still need further investigations. These include the interaction of PGPR with other microbial population, such as fungi and rhizobium, survival of PGPR under stress environment, survival of transgenic plants under natural environment and, also, the minimum population of these strains that will be beneficial for enhancing growth under stress environment. The well focused approach, keeping in view the molecular and physiological aspect of stress tolerance, is required to facilitate crop production on problem soils.

Therefore, future research is required to explore (1) the performance of these strains under natural stress environment, (2) the positive aspects of these strains with rhizobia and fungi in the presence of biotic and abiotic stresses, (3)

identification of target gene for developing transgenic plants and also the effectiveness of these transgenic plants under field conditions, (4) the extent to which soil physical and chemical properties influence the activity of PGPR strains, (5) minimum population size that will be effective for reducing ACC concentration in plant roots, and (6) selection of suitable strains having multiple characteristics for biofertilizers preparation and also different aspects related to biofertilizers.

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# Chapter 9
# Rhizobacterial ACC Deaminase in Plant Growth and Stress Amelioration

**D. Saravanakumar**

## 9.1 Introduction

Natural and agricultural ecosystems harbor a wide variety of microorganisms that play an integral role in plant health, crop production, and the preservation of multiple ecosystem functions. The estimated number of prokaryotic cells in our planet's soil is $2.6 \times 10^{29}$, providing an enormous capacity for genetic diversity and a great potential for exploitation. Bacteria are by far the most abundant organisms in soil and they play a key role in nutrient cycling and soil fertility. Bacteria that provide some benefit to plants are of two general types (1) those that form a symbiotic relationship, which involves formation of specialized structures or nodules on host roots, and (2) those that are free living in the soil and often found near, on, or even within plant tissues. The beneficial free living soil bacteria near the rhizosphere region are generally referred to as plant growth promoting rhizobacteria (PGPR) and are found in close association with the roots of many different plants. Although numerous free living soil bacteria are considered to be PGPR, not all bacterial strains of a particular genus and species have identical metabolic capabilities and interactions with plants. They survive in seed or soil, multiply in the spermosphere in response to seed exudates rich in carbohydrates and amino acids, attach to the root surface and become endophytic by colonizing the root cortex region. The root collar region where the root joins the main stem is a site of intense exudation and is more strongly colonized by bacteria than the root tip.

PGPR includes bacteria belonging to the genera *Azotobacter*, *Azospirillum*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Klebsiella*, *Pseudomonas* and *Serratia* (Vessey 2003). More specifically, the soil-borne fluorescent pseudomonads and Bacillus have received particular attention in agriculture throughout the global

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science because of their catabolic versatility, excellent root colonizing abilities, and their capacity to produce a wide range of antifungal metabolites. They have been studied as plant growth promoters for increasing agricultural production and as biocontrol agents against plant diseases (Schroth and Hancock 1982; Burris 1998; Chen et al. 1996; Kloepper and Beauchamp 1992; Liu et al. 1995). Their interaction with plants exhibit several benefits in agricultural crops, such as growth promotion and alleviation of biotic and abiotic stresses.

## 9.2 Benefits of Plant–PGPR Interaction

PGPR exhibits multiple mechanisms to promote plant growth and to serve as a potential agent for nullifying the effects of biotic and abiotic stresses. Growth promotion by PGPR is complex and appears to comprise both changes in the microbial balance in the rhizosphere and alteration in the host plant physiology (Glick et al. 1999). PGPR promotes plant growth and enhances biotic and abiotic stress resistance through (1) fixing atmospheric nitrogen and supplying to plants, (2) synthesizing various phytohormones including auxins and cytokinins, (3) providing mechanisms for solubilization of minerals, (4) out-competing phytopathogens for nutrients and niches on the root surface (Kloepper et al. 1988; O'Sullivan and O'Gara 1992; Loper et al. 1997), (5) antibiotic synthesis (Haas and Defago 2005), (6) secretion of iron binding siderophores to obtain soluble iron from the soil and provide it to the plant and thereby deprive the harmful organisms in the vicinity of soluble iron (Dowling et al. 1996), (7) production of low molecular weight metabolites such as hydrogen cyanide with antifungal activity (Dowling and O'Gara 1994), (8) production of oxidative stress enzymes, such as catalases, superoxide dismutases and peroxidases, for scavenging active oxygen species, and (9) lowering the production of stress ethylene in plants with enzyme 1-Aminocyclopropane-1-carboxylate (ACC) deaminase (Glick et al. 1998; Penrose et al. 2001).

Although PGPR have multiple mechanisms for growth promotion and stress resistance in agriculture, it is established from the earlier demonstration by several research workers that PGPR strains that possess ACC deaminase activity have selective advantage over other bacteria during biotic (Wang et al. 2000) and abiotic stress (Glick et al. 1994; Mayak et al. 2004a, b; Saravanakumar and Samiyappan 2007) conditions. Thus, in this chapter, the role of ACC deaminase activity, especially against abiotic stresses such as water stress, flooding, salt stress, cold and high temperature, and heavy metal stress that cause growth retardation in agricultural settings will be discussed.

## 9.3 Plant Growth Promotion by ACC Deaminase

PGPR has a significant impact on plant growth and development in two different ways viz., indirectly or directly. On the one hand, the indirect promotion of plant growth occurs when bacteria decrease or prevent some of the deleterious effects of a phytopathogenic organism by one or more mechanisms. On the other hand, the direct promotion of plant growth by PGPR generally entails providing the plant with a compound that is synthesized by the bacterium, or facilitating the uptake of nutrients from the environment (Glick 1995; Glick et al. 1999). Plant growth benefits due to the rhizobacteria include increases in germination rates, root growth, yield including grain, leaf area, chlorophyll content, magnesium, nitrogen and protein content, hydraulic activity, tolerance to drought and salt stress, shoot and root weights, and delayed leaf senescence (Lucy et al. 2004). Direct stimulation of plant growth include the enzyme ACC deaminase secreted by PGPR that can lower plant ethylene levels, which is produced during biotic and abiotic stress (Glick et al. 1999).

In general, ethylene is required for seed germination by many plant species and the rate of ethylene production increases during germination and seedling growth (Abeles et al. 1992). Low levels of ethylene appear to enhance root initiation and growth, and promote root extension; higher levels of ethylene, produced by fast growing roots, can lead to inhibition of root elongation (Mattoo and Suttle 1991; Ma et al. 1998). In addition, ethylene is synthesized in plant tissues from the precursor ACC during abiotic stress conditions, which, in turn, retard root growth and cause senescence in crop plants (Sheehy et al. 1991; Ma et al. 2003a). Interestingly, the introduction of a PGPR possessing ACC deaminase activity can cleave the plant ethylene precursor ACC and thereby lower the level of ethylene in a developing seedling or stressed plant (Jacobson et al. 1994; Glick et al. 1997; Shah et al. 1998; Mayak et al. 2004b). By facilitating the formation of longer roots through the action of ACC deaminase, these growth-promoting bacteria enhance the survival of plant seedlings under various abiotic stresses (Wang et al. 2000; Grichko and Glick 2001).

In addition, plants that are treated with ACC deaminase-containing PGPR are dramatically more resistant to the deleterious effects of stress ethylene that is synthesized as a consequence of stressful conditions such as flooding (Grichko and Glick 2001), heavy metals (Grichko et al. 2000), the presence of phytopathogens (Wang et al. 2000), and drought, and high salt (Mayak et al. 2004a, b). In each of these cases, the ACC deaminase-containing PGPR markedly lowered the level of ACC in the stressed plants, thereby limiting the amount of stress ethylene synthesis and, hence, the damage to the plant. These bacteria are beneficial to plant growth as in the optimal environmental conditions.

### 9.3.1 *Rhizobacterial ACC Deaminase on Nutrient Fixation*

Nitrogen is one of the most common nutrients required for plant growth and productivity as it forms an integral part of proteins, nucleic acids, and other essential biomolecules (Bockman et al. 1997). More than 80% of nitrogen is present in the atmosphere but is unavailable to plants. It needs to be converted into ammonia, a form available to plants and other eukaryotes. Glick et al. (1999) reported the fixation of nitrogen in nonleguminous plants by rhizobacterial strains. This phenomenon by PGPR is correlated with the activity of ACC deaminase. Similarly, PGPR strain utilizing ACC as sole nitrogen source increased the fixation of biological nitrogen, nodulation, and growth of *Lupinus albus* I. cv. *multolupa* (Garcia et al. 2004).

ACC deaminase-producing rhizobia can also enhance nodulation and, hence, the nitrogen fixation efficiency. Ma et al. (2003a, b) reviewed the existence of ACC deaminase in a number of rhizobial strains and found that the inhibitory effect of ethylene on plant root nodulation can be reduced by the activity of ACC deaminase. For example, the ACC deaminase-producing *Sinorhizobium meliloti* showed 35–40% greater efficiency in nodulating alfalfa, likely by lowering ethylene production in the host plants (Ma et al. 2004). Similarly, *Rhizobium leguminosarum* bv. *viciae* 128C53K enhanced the nodulation of pea, and the minus ACC deaminse mutants showed lower nodulation efficiency (Ma et al. 2003a). Recently, it was demonstrated that ACC deaminase producing *Klebsiella oxytoca* Rs5 promoted cotton seedlings' height and dry weight by 14.9% and 26.9%, respectively. Nutrient analysis has exhibited the bacterium's ability to increase cotton's absorption of N, P, K, and Ca (Yue et al. 2007). These studies clearly revealed the beneficial effects of ACC deaminase in promoting plant growth by helping the plants to absorb nutrients from various environmental sources.

## 9.4 Concept Model for ACC Deaminase

Ethylene biosynthesis starts with the S-adenosylation of methionine to give S-adenosylmethionine in plants. This step is followed by the closing of cyclopropane ring to form ACC, which is then oxidatively cleaved to give ethylene (Adams and Yang 1979). In this context, the introduction of ACC deaminase through PGPR degrades a cyclopropanoid amino acid ACC to α-ketobutyrate and ammonia (Honma and Shimomura 1978) and plays a key intermediate in the biosynthesis of plant hormone ethylene (Burroughs 1957). The introduction of ACC Deaminase in higher plants by recombinant technology reduces the production level of ethylene and delays the ripening progression of fruits (Klee et al. 1991; Reed et al. 1996). Thus, this enzyme provides a way to regulate ethylene biosynthesis and fruit ripening.

Later, it was demonstrated that PGPR containing ACC Deaminase gene can stimulate plant growth even under stress stipulated conditions through the activity of the enzyme ACC deaminase (Jacobson et al. 1994; Glick et al. 1995, 1998; Saravanakumar and Samiyappan 2007). The model for the role of ACC Deaminase in the stimulation of plant root growth and their role in reducing severity of stress conditions has been proposed by many research workers (Glick et al. 1994; Hall et al. 1996; Holguin and Glick 2001). All the concept models have suggested that PGPR can lower ethylene levels against stress situations and, thus, stimulate plant growth as illustrated in Fig. 9.1.

Glick and his coworkers have carried out much research on ACC deaminase at University of Waterloo, Canada since the 1990s, and their model on ACC deaminase has elucidated that PGPR bind to the surface of seeds in response to tryptophan and other amino acids exuded from the germinating seeds, synthesize, and secrete indole acetic acid (IAA) (Glick et al. 1998). The IAA taken up by the seed and together with endogenous IAA, stimulate plant cell proliferation, cell elongation, or induce the synthesis of ACC synthase, the enzyme that catalyzes the conversion of *S*-adenosyl-L-methionine (SAM) to ACC, the immediate precursor of ethylene (Yang and Hoffman 1984). It is quite likely that much of the ACC produced by this reaction is exuded by the germinating seeds, is taken up by the bacterium, and subsequently hydrolyzed to α-ketobutyrate and ammonia by ACC deaminase (Fig. 9.1) (Glick et al. 1998; Penrose 2000). The model predicts that the uptake

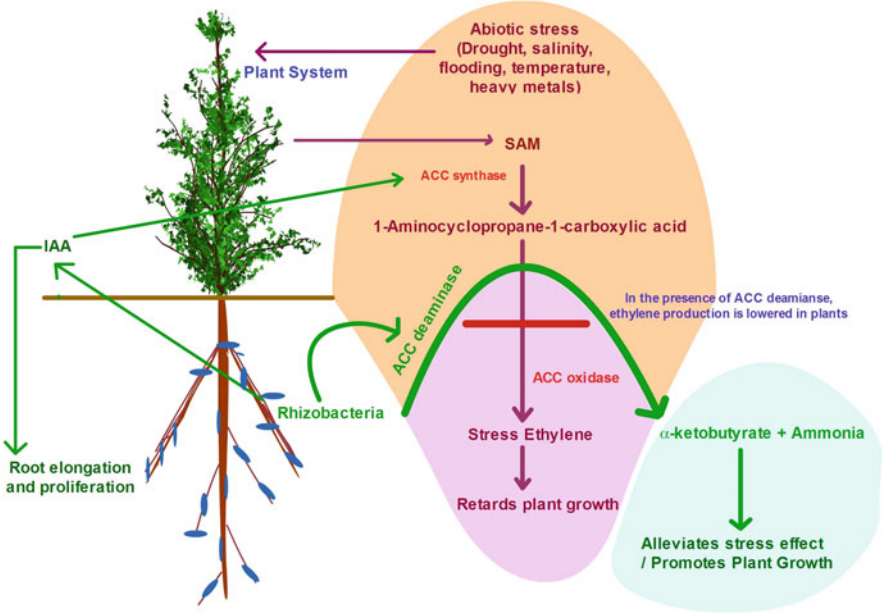


**Fig. 9.1** Schematic diagram showing the activity of rhizobacteria ACC deamianse in ethylene mediation during abiotic stress conditions

and cleavage of ACC by ACC Deaminase decreases the amount of ACC, as well as ethylene, outside the germinating seed, thereby acting as a sink for ACC.

## 9.5 The Origin and Nature of ACC Deaminase

ACC deaminase found in soil microorganisms is a pyridoxal-5-phosphate-dependent enzyme that catalyzes the cleavage of ACC, which is the immediate precursor of ethylene (Honma and Shimomura 1978). It was originally found in a soil bacterium *Pseudomonas* sp strain ACP (Sheehy et al. 1991). Since its initial discovery, ACC deaminase has been found in soil bacteria, yeast, and fungi; however, ACC deaminase has not been found in plants (Glick et al. 1999). Genes encoding ACC deaminases have been isolated from various bacterial organisms including *Pseudomonas* sp. strain ACP (Sheehy et al. 1991), *P. chloroaphis* 6G5 and *Pseudomonas* sp. strain 3F2 (Klee et al. 1991), *P. fluorescens* strain TDK1 (Saravanakumar and Samiyappan 2007), and *Enterobacter cloacae* UW4 and CAL2 (Glick et al. 1998). The amino acid sequences of the bacterial genes are highly homologous, each containing an open-reading frame (ORF) of 1,017 bp (encoding 338 amino acid residues) ranged between 36,500 and 41,800 Da (Honma 1985; Jacobson et al. 1994).

## 9.6 Rhizobacterial ACC Deaminase in Plant Stress Resistance

Plants that are constantly exposed to a variety of environmental stresses such as drought, temperature, salinity, air pollution, heavy metals, pesticides, and soil pH limit the plant productivity. The deleterious consequences of abiotic stresses include increase of root ethylene synthesis, ionic imbalance, and hyperosmotic shock in plant (Niu et al. 1995; Zhu et al. 1997; Mayak et al. 2004a, b). In this context, breeding for stress resistance has been practiced over several years for the development of resistant crop varieties. However, the breakdown of resistance after the release of a crop variety resulted in the loss of intensive work carried out during the development of resistant varieties. This emphasizes the need for the development of suitable alternative technology that can cope up with different environmental stress. In this regard, an engineering of plants that are resistant to a various abiotic stresses could be a better choice as the plant science advances the fields of molecular biology and biotechnology. However, it is impractical to attempt to engineer plants against all of kinds of stresses that they might encounter in the environment, as these can vary from one locale to another and from one season to the next. Thus, one can think of either selecting or engineering a rhizobacteria that protect plants against a range of different abiotic stresses. A more general strategy might include inoculation of plant seeds or roots with plant growth-promoting or biocontrol bacteria that contain ACC deaminase for amelioration of various abiotic stress effects as indicated in Table 9.1.

### 9.6.1 Salinity

Soil salinity is a major worldwide problem for agriculture, especially for crops that are grown under irrigation. This is because saline conditions are inhibitory to the growth of many plants. Moreover, salt stress has been previously reported to cause increased production of ethylene in some plants (Feng and Barker 1992). In many instances, removing or blocking the effect of stress induced ethylene production alleviates the stress effect (Glick et al. 2007). In addition to the use of traditional breeding and plant genetic engineering with the production of transgenic plants, the use of PGPR containing ACC deaminase could be useful in developing strategies to facilitate plant growth in saline soils. ACC deaminase- producing bacterium, *Achromobacter piechaudii* ARV8, isolated from the rhizosphere of a *Lycium shawii* plant growing in a dry riverbed in the Arava region of Israel, dramatically lowered the level of ethylene and prevented inhibition of plant growth in tomato plants grown in the presence of high concentrations of salt (Mayak et al. 2004b). The same bacterial strain lowered the ethylene level and significantly decreased the growth inhibition of peppers and tomatoes from drought stress (Mayak et al. 2004a). Similarly, recent findings from Sergeeva et al. (2006) established the dual role of ACC deaminase in enhancing growth promotion and greater tolerance in transgenic canola plants against high salt stress.

It has been shown that the bacterium *Pseudomonas putida* GR12-2, exhibiting ACC Deaminase activity, stimulates the root elongation of canola, lettuce, tomato, and wheat seedlings (Hall et al. 1996). At the same time, ACC Deaminase deficient mutants do not promote root elongation (Glick et al. 1994; Li et al. 2000). Inoculations of canola with *P. putida* GR12–2 increased the root and shoot biomass of seedlings under optimum growth conditions as well as in the presence of excess salt or cold temperature stress (Glick et al. 1997). Recently, Saravanakumar and Samiyappan (2007) demonstrated that *P. fluorescens* strain TDK1 possessing ACC deaminase activity enhanced the saline resistance in groundnut plants, which in turn resulted in increased yield when compared with the groundnuts treated with *Pseudomonas* strains not having ACC deaminase activity.

Similarly, Cheng et al. (2007) found that *P. putida* UW4 significantly improved canola shoot dry weight by fivefold at 20°C, whereas a mutant strain of UW4 lacking ACC deaminase activity (UW4/AcdS$^-$) did not promote plant growth. These results are consistent with the proposed model that the bacterial ACC deaminase activity of PGPR can lower the plant ethylene levels and hence promote plant growth. It should be noted that Cheng et al. (2007) found that the Na concentrations in canola shoots were also increased by inoculation of *P. putida* UW4 by three- to sixfold. Similarly, *P. putida* strain N21 isolated from salt affected areas significantly increased the wheat plant height, root length, grain yield, 100 grain weight and straw yield up to 52, 60, 76, 19 and 67%, respectively, over noninoculated control. It is highly likely that under salinity stress, ACC deaminase activity of these microbial strains might have caused reduction in the synthesis of stress induced inhibitory levels of ethylene. The results suggested that these strains

**Table 9.1** ACC deaminase producing rhizobacteria in mediation of abiotic stress

| Rhizobaceria | Inoculated plants | Type of stress | Results of the study | Reference |
|---|---|---|---|---|
| *Pseudomonas putida* N21<br>*Pseudomonas aeruginosa* N39<br>*Serratia proteamaculans* M35 | Wheat (*Triticum aestivum*) | Salt | Enhanced plant height, root length, grain yield, 100-grain weight and straw yield<br>Increased chlorophyll content and K+/Na + of leaves | Zahir et al. (2009) |
| *Pseudomonas putida* UW4 | Canola (Brassica napus) | Salt | Plant growth is enhanced in addition to higher uptake of sodium by canola | Cheng et al. (2007) |
| *Pseudomonas fluorescens* TDK1 | Groundnut (*Arachis hypogaea*) | Salt | Enhanced plant growth and pod yield under saline field conditions | Saravanakumar and Samiyappan (2007) |
| *Pseudomonas syringae* S5<br>*Pseudomonas fluorescens* S20<br>*Enterobacter aerogenes* S14 | Maize (*Zea mays*) | Salt | Increased plant height, root length, total biomass, cob mass, and grain yield under pot conditions<br>Higher K+/Na + ratios in combination with high relative water, chlorophyll and low proline contents | Nadeem et al. (2007) |
| *Achromobacter piechaudii* ARV8 | Tomato (*Lycopersicon esculentum*) | Salt | Enhanced the plant growth of tomato seedlings | Mayak et al. (2004b) |
| *Achromobacter piechaudii* ARV8 | Tomato (*L. esculentum*), pepper (*Capsicum annuum*) | Drought | Increased the fresh and dry weights of both tomato and pepper seedlings exposed to transient water stress<br>Continued plant growth during both the water stress and after watering was resumed | Mayak et al. (2004a) |
| *Pseudomonas fluorescens* TDK1 | Mung bean (*Vigna mungo*) | Drought | Increased plant growth and yield under pot culture conditions.<br>Showed enhanced resistance against root rot pathogen that is closely associated with drought conditions. | Vineetha (2007) |
| *Enterobacter cloacae* UW4, *P. putida* UW4 | Tomato (*L. esculentum*) | Flooding | Plant showed a substantial tolerance to flooding stress.<br>Leaf chlorophyll content and shoot growth enhanced | Grichko and Glick (2001) |

| | | | | |
|---|---|---|---|---|
| *Burkholderia phytofirmans* PsJN | Potato (*Solanum tuberosum*) | Temperature | Maintain stem length, shoot and root biomass | Bensalim et al. (1998) |
| *Pseudomonas putida* UW4 | Canola (*Brassica napus*) | Temperature | Plant growth is enhanced in addition to higher uptake of sodium by canola | Cheng et al. (2007) |
| *Kluyvera ascorbata* SUD165 | Canola (*Brassica napus*) and Tomato (*L. esculentum)* | Nickel toxicity | Increased seedling root and shoot length was recorded under gnotobiotic conditions | Burd et al. (1998) |
| *Pseudomonas asplenii* AC | Canola (*Brassica napus*) and Reed (Phragmites australis) | Heavy metals | Promote plant growth both under copper and creosote contaminated soils | Reed and Glick (2005), Reed et al. (2005) |
| *Rhizobium leguminosarum*, *Mesorhizobium roti*, *Bradyrhizobium japonicum* | Pea (*Pisum sativum*) and leguminous plants | Ethylene stress during nodulation | Enhances the nodulation of pea plants likely by modulating ethylene levels in the plant roots during the early stages of nodule development | Ma et al. (2003a, b), Okazaki et al. (2004) |

could be employed for salinity tolerance in wheat (Zahir et al. 2009; Nadeem et al. 2010).

Recently, Sgroy et al. (2009) isolated ACC deaminase producing endophytes from the *Prosopis strombulifera* grown under extreme salinity conditions. This revealed the existence of endophytic bacteria that produce ACC deaminase under saline conditions. It is also interesting to note in the recent study that bacterial ACC deaminase, in combination with arbuscular mycorrhizal fungus, ameliorates plant growth under stressful conditions (Gamalero et al. 2010). It was demonstrated that under optimal growth conditions, *P. putida* UW4/AcdS$^+$ increases root colonization by *Gigaspora rosea*, resulting in synergistic effects on cucumber growth. Interestingly, ACC deaminase is mainly involved in the bacteria–fungus interactions in optimal conditions, while under stressful conditions, this enzyme plays a role in plant–bacterium interactions. Similarly, the inoculation of pepper with *Bacillus* sp. TW4 led to relief from osmotic stress, which is often manifested as salinity stress. In these plants, genes linked with ethylene metabolism under abiotic stress such as *caACCO* (encoding ACC oxidase) and *caLTPI* (an abiotic stress-inducible gene encoding a lipid transfer protein) were down-regulated (Sziderics et al. 2007). This clearly revealed the activity of ACC deaminase by *Bacillus* sp. TW4 in lowering stress ethylene.

### *9.6.2 Drought*

Availability of water is one of the most important factors, which determine geographical distribution and productivity of plants (Bartels 2001). Water stress is perceived as water deficit and can occur with different severity and more than one-half of the earth is susceptible to drought every year (Ramanjulu and Bartels 2002). A continuation of a mild water deficit leads to drought and even desiccation (loss of most of the protoplasmic free or bulk water). The response and adaptation of plants to such conditions are very complex and highly variable. Of these, accumulation of stress ethylene is considered to play a significant role in retarding plant growth during water stress conditions. *A. piechaudii*, a PGPR having ACC Deaminase activity, significantly increases the fresh and dry weight of both tomato and pepper seedlings exposed to transient water stress. In addition, the bacterium reduced the production of ethylene in tomato seedlings following water stress. During water deprivation, the bacterium did not influence the reduction in relative water content, but significantly improved the recovery of plants when watering was resumed (Mayak et al. 2004a). Further, it is a known fact that severe drought stress decreased the levels of chlorophyll *a*, *b*, and total chlorophyll (Reddy and Rao 1968). Interestingly, in case of transformed canola seedlings with a bacterial ACC, deaminase expressed high leaf chlorophyll content than that of nontransformed plants.

Recently, Vineetha (2007) has studied that *P. fluorescens* strain TDK1 strain possessing ACC deaminase activity enhanced drought tolerance in water stress imposed plants as well as plants challenged with root rot pathogen (*Macrophomina*

*phaseolina*) that is closely associated with drought conditions. The same author has evidenced the added advantage of *P. fluorescens* strain TDK1 containing ACC deaminase over other strains, to cope up with *M. phaseolina*, drought, and drought associated *M. phaseolina*, without compromising the growth and yield attributing characters when applied to groundnut plants. This also revealed the usefulness of rhizobacterial ACC deaminase activity in dicotyledonous plants like ground nuts which are more susceptible to effects of ethylene (Hall et al. 1996) under water stress conditions.

### 9.6.3 Flooding

In addition to saline and water stresses, it has been shown that PGPR strains are able to counteract flooding problems by reducing the negative effect of ethylene accumulation during stress conditions. Generally flooding enhances the biosynthesis of ethylene in roots and stem of plants. Initially, ACC is synthesized in roots and transported to plant shoots where it is converted to ethylene by ACC oxidase (Bradford and Yang 1980; Else et al. 1995). The molecular basis clearly explained that the increase in ethylene production observed in shoots of flooded tomato plants is due to an increase in the activity of both ACC synthase in the submerged roots and ACC oxidase in the shoots (Olson et al. 1995). The accelerated production of ethylene in the shoots of flooded tomato plants is responsible for abnormal growth under flooding conditions (Jackson 1997). The transgenic tomato plants expressing ACC deaminase under the control of the rolD promoter exhibited flooding tolerance by maintaining the leaf chlorophyll content to a statistically significant level compared to nontransformed plants (Grichko and Glick 2001). The same authors in 2005 had studied the effects of inoculation with ACC deaminase PGPR on tomato exposed to flooding situation. Seeds of wild-type tomato plants were inoculated either with *P. putida* UW4, *E. cloacae* CAL2, *P. putida* (ATCC17399/ pRKACC), or *P. putida* (ATCC17399/ pRK415); the first three of these bacterial strains carried and expressed the gene for ACC deaminase. Interestingly, tomato plants inoculated with ACC deaminase PGPR showed substantial tolerance to flooding stress, entailing that bacterial ACC deaminase reduced the effects of stress induced ethylene.

### 9.6.4 Cold and High Temperature Stress

Similar to other abiotic stresses, plants are sensitive to temperature changes and respond to seasonal variations and diurnal changes in the season. The change in temperature (either at increasing or decreasing mode) due to global warming pose a serious threat to world agriculture. A fluctuation in temperature leads to hormonal imbalances in plants and, thus, their growth is significantly influenced. Like many

other abiotic stresses, increased ethylene production under high and chilling temperatures has been reported by several researchers, both in plant tissues and microbial species in the rhizosphere (Wang 1987; Strzelczyk et al. 1994). Thus, an introduction of rhizobacteria containing ACC deaminase into the plant ecosystem could ease an unfavorable situation by lowering ethylene level that takes place under other environmental stress conditions. For example, a rhizobacterium possessing ACC deaminase activity, *Burkholderia phytofirmans* PsJN inoculated potato clones grown under two different temperatures (20/15°C; 33/25°C day night temperature), which, even at high temperature, were able to maintain their stem length, shoot and root biomass (Bensalim et al. 1998). Tuberization was also enhanced by as much as 63% in bacteria-treated clones. The same bacterium enhanced plant growth and physiological activity of grapevine explants under in vitro at both ambient (26°C) and low (4°C) temperatures. The bacterium also significantly improved cold tolerance of plantlet, compared to that of the nonbacterized control, which was more sensitive to exposure to low temperatures. Similarly, *P. putida* strain UW4 possessing ACC deaminase promoted canola plant growth at low temperature conditions (Cheng et al. 2007). These studies clearly indicated the potential of ACC deaminase in normalizing plant growth through lowering stress ethylene induced by extreme temperature conditions.

### *9.6.5 Heavy Metal Stress*

Similar to other stresses, the presence of heavy metals such as $Ni^{2+}$, $Pb^{2+}$, $Zn^{2+}$, and $CrO_4$ can also affect the plant growth by inducing stress ethylene. This could be ameliorated by ACC deaminase producing bacterium under natural environment conditions. It was demonstrated in the earlier studies that a plant growth promoting bacterium, *Kluyvera ascorbata* SUD165, displayed ACC deaminase activity resistant to the toxic effects of $Ni^{2+}$, $Pb^{2+}$, $Zn^{2+}$, and $CrO_4$. Canola seeds inoculated with this bacterium and then grown under gnotobiotic conditions in the presence of high concentrations of nickel chloride were partially protected against nickel toxicity. Further, it is proposed by the authors that plant growth-promoting effect of bacterium in the presence of nickel was due to its ability to lower the level of stress ethylene induced by the nickel (Burd et al. 1998, 2000). Similarly, the presence of plant growth promoting bacteria *A. piechaudii* ARV8 that produces ACC deaminase and IAA, increases plant resistance to organic and inorganic contaminants (Reed and Glick 2005; Reed et al. 2005; Belimov et al. 2005). The laboratory studies also demonstrated that transgenic plants expressing the gene for the enzyme ACC deaminase were more resistant to growth inhibition in metal contaminated soil (Stearns et al. 2005; Nie et al. 2002).

## 9.7 Genetically Modified Bacteria with ACC Deaminase

It is well known that rhizobacteria exhibits multiple mechanisms to interact with plant tissues and to enhance plant growth under stress conditions. However, it was demonstrated in several studies that the rhizobacterium having ACC deaminase activity had advantage over other strains under varying environmental conditions. Thus, it is quite necessary to genetically engineer the bacterium with ACC deaminase gene *acdS* and their regulatory regions into the bacterium that lacks this activity. This could enhance the beneficial effect of bacterium on plant growth under different environment conditions. For example, *E. coli* and *Pseudomonas* strains that lack ACC deaminase but have been transformed to express a *Pseudomonas acdS* gene are able to promote the elongation of canola roots in growth pouches (Shah et al. 1998). Similarly, the efficacy of some biocontrol pseudomonads was also significantly enhanced following the introduction of a *Pseudomonas acdS* gene (Wang et al. 2000). However, it is important that the complex transcriptional regulatory system that controls the expression of many *acdS* genes should work in all bacteria. When *Azospirillum* strains lacking ACC deaminase were transformed with a *Pseudomonas acdS* gene under the control of the regulatory *acdR* gene, ACC deaminase was not expressed (Holguin and Glick 2001). But, when the native regulatory region of the *Pseudomonas acdS* gene was replaced by either the *E. coli lac* promoter or the *tet* promoter, ACC deaminase was expressed at a high level and the growth promoting activity of the transformed *Azospirillum* strain was significantly improved (Holguin and Glick 2001). Similarly, *Sinorhizobium meliloti* strain transformed with *acdS* gene from *R. leguminosarum* enables the transformed bacterium to nodulate alfalfa plants and stimulates their growth by 35–40% more than the nontransformed strain (Ma et al. 2004). These studies clearly indicate the possibility of improving the efficacy of different rhizobacterial strains with the genetic engineering of ACC deaminase gene.

## 9.8 Plants Expressing ACC Deaminase Gene

Similar to the manipulation of ACC deaminase gene in different microorganisms, several transgenic plants including tomato, canola, and tobacco that express ACC deaminase have been engineered. These transgenic plants have been reported to be tolerant of metals (Grichko et al. 2000; Stearns et al. 2005), high salt (Sergeeva et al. 2006), certain pathogens (Lund et al. 1998; Robison et al. 2001), and flooding (Grichko and Glick 2001). Interestingly, in all these cases, transgenic plants that express the enzyme ACC deaminase responded similarly to nontransformed plants treated with ACC deaminase-containing plant growth-promoting bacteria. This demonstrated the lower advantage of transgenic plants as compared to plants treated with plant growth-promoting bacteria. The bacteria inoculated plants

could perform well when compared to transgenic plants due to multiple mechanisms other than ACC deaminase activity. Thus, it is opined that the use of plant growth-promoting bacteria appears, in many cases, to present a superior alternative to the use of transgenic plants.

## 9.9 Conclusion

In each of the abiotic stresses, it was demonstrated that ACC Deaminase containing PGPR markedly lowered the level of ACC in the stressed plants and limits the amount of stress ethylene synthesis and hence the reduction in damage to the plant. These rhizobial bacteria are beneficial to plant growth, since plants are often subjected to ethylene producing stresses in the natural environment. Thus, it is proposed that the activity of ACC deaminase will be useful in both agricultural and horticultural settings. Besides, the ecology of bacterium and physiology of the plant may also interact with plant system to increase resistance to all kinds of stresses. Thus, it is necessary to take up the studies on genomics and proteomics of plant–rhizobacteria mediated stress resistance in agricultural plants. In addition, the consistency of these bacteria has to be tested at varying environmental conditions and should be promoted worldwide for achieving sustainable agriculture. Further, the commercial formulation of these versatile (native) or universal beneficial bacteria is to be evolved and made available to the farming community in all the developed and developing countries in order to promote agricultural production under varying environmental conditions after concrete and comprehensive studies. Thus, the future research on rhizobacterial ACC deaminase should be focused in this line for betterment of basic and applied aspects of beneficial bacteria that can enhance the global agricultural production.

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# Chapter 10
# Bacterial Mediated Alleviation of Abiotic Stress in Crops

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## 10.1 Introduction

Today, the most daunting task ahead of farmers, scientists, and policy makers the world over is the ability to feed the world's ever growing population from a shrinking land resource. Since the demand for food is expected to rise by 3–5 times, the current food production has to increase by 60% in order to meet the demands of the future (Wild 2003). This task gets compounded by the immediate threat of climate change that looms large over the entire globe, with several developing nations already bearing the brunt of this phenomenon. Global warming and changes in precipitation patterns, leading to untimely drought, are already affecting crop production in developing countries (Pandey et al. 2007; Barrios et al. 2008). Climate change models have predicted that warmer temperatures and increases in the frequency and duration of drought during the twenty-first century will have net negative effects on agricultural productivity (St Clair and Lynch 2010). Global warming and its associated effects are expected to impose abiotic stresses, such as extremes of temperatures, drought and flooding, that are bound to have adverse effects on food production.

Crop production also faces a battery of abiotic stresses that arise as a result of the inherent edaphic factors and anthropogenic activity. These include stresses induced by soil salinity, heavy metal contamination, and organic pollutants. In developing countries, it has been estimated that on an average, nearly two thirds of the soils are prone to edaphic constraints that significantly reduce crop yields (Lal 2000). Edaphic stresses affect crop growth and microbial activity in cultivated lands and reduce the productivity of the eco-system as a whole. Therefore in this race against time to produce more food, the mitigation of abiotic stresses (of both

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climate and edaphic origin) becomes imperative in order to achieve the designated goals. Several agronomic strategies have been proposed to mitigate climate related abiotic stresses in cropping systems. These include the selection of resistant cultivars, change in sowing dates, water saving land modifications, etc. Recommended strategies for mitigating edaphic stresses include choice of resistant cultivars and root stocks, use of hyperaccumulator plants, etc. But, a lesser explored and utilized option is the use of the omnipresent and omnipotent microbes for alleviation of abiotic stresses in plants. Though a fair degree of success has been achieved in the utilization of microbes in the alleviation of edaphic stresses, especially those caused by heavy metals (Glick 2010), their utility in the alleviation of climate related abiotic stresses is still in the nascent stages. We shall therefore attempt to throw more light on the mechanisms that operate in microbial species that posses stress alleviating potential and their potential utility in sustainable agriculture. Though the ambit of this review would ideally include the Arbuscular Mycorrhizal Fungi (AMF) and other saprophytic fungi, we would restrict our discussion mainly to bacterial species that play a major role in abiotic stress alleviation.

## 10.2 Abiotic Stresses in Crops

Crop production environments and their associated factors often impose varying levels of abiotic stresses on crops and thereby prevent the full realization of the genetic potential of the plant in terms of yield and quality. The most commonly encountered abiotic stress factors and their effects on plant growth and development are discussed in the following section.

### *10.2.1 Types of Abiotic Stresses and Their Importance in Crop Production*

Depending on the agro-ecological situation, including cultivation practices, crops encounter a wide variety of abiotic stresses, which can be classified broadly as those of atmospheric origin and of edaphic origin. Abiotic stresses of atmospheric origin include extremes of temperature (both hot and cold), drought, and flooding due to excessive precipitation. Abiotic stresses associated with edaphic factors include soil related constraints like low fertility status, soil salinity, acidity, and the presence of toxic levels of heavy metals/organic pollutants in the rhizospheric region. Besides chemical factors, several physical soil constraints viz., poor soil texture, rockiness, compaction (Lal 1987), and slope steepness (Lal 1998), also affect crop production. Put together, all these characteristics determine the water holding capacity of the soil, the degree of root contact with the soil matrix, and cation exchange capacity, and therefore influence plant nutrient acquisition and yields (Marchner 1995).

Abiotic stresses of atmospheric origin are gaining importance since Asia, Africa, and South America experienced a 0.7–1.0°C increase in temperature during the twentieth century. Climate prediction models suggest that by the end of the twenty-first century, temperature averages on those continents would increase by at least another 2–4°C (IPCC 2007a). It has been projected that variability in precipitation patterns would increase with longer periods of droughts interspersed with more intense rainfall events (Sun et al. 2007). These changes in temperature and precipitation are expected to have net negative effects on global agriculture (IPCC 2007b).

Certain abiotic stresses of edaphic origin arise due to anthropogenic activity, while excess irrigation and use of saline water for irrigation have contributed to soil salinity. The use of sewage water for irrigation and sewage sludge as manure for crop production have caused the accumulation and build up of toxic levels of heavy metals in several parts of the world. This problem has been compounded by improper toxic waste disposal methods, which have rendered vast tracts of land unfit for crop production. A ubiquitous problem is the indiscriminate use of organic pollutants and crop protection chemicals that have prolonged residual effects and disturb the delicate soil equilibrium.

### *10.2.2 Effects of Abiotic Stresses on Plant Growth and Development*

Soil moisture is the master environmental variable because its availability integrates climate and soil conditions, while drought is an important selection force on biological organisms and can drastically alter plant community structure and function (Holmgren et al. 2006). One of the important innovations in human agriculture was irrigation which mitigated the negative impacts of water deficit on crop growth. In the context of climate change, the irrigation requirement of arid and semiarid agro-ecosystems are estimated to increase by 10% with every 1°C rise in temperature (Grover et al. 2010). Considering the dwindling quality and quantity of freshwater resources, and poor irrigation infrastructure, adaptation to drought in developing countries appears to be limited. Soil moisture deficit not only directly impacts crop productivity, but also reduces yields through its influence on the availability and transport of soil nutrients. Drought stress affects plant hormone balance by reducing the endogenous cytokinin level and increasing the levels of abscisic acid (ABA) content in the leaves, thereby eliciting stomatal closure. The cytokinin–ABA antagonism might be the result of metabolic interactions because they share a common biosynthetic origin (Figueiredo et al. 2008). Because nutrients are carried to the roots by water, soil moisture deficit therefore decreases nutrient diffusion over short distances and the mass flow of water-soluble nutrients such as nitrate, sulfate, Ca, Mg, and Si over longer distances (Barber 1995).

Drought reduces the availability of $CO_2$ for photosynthesis, which can lead to the formation of reactive oxygen species (ROS). ROS, such as superoxide radicals,

hydrogen peroxide and hydroxyl radicals, cause lipid peroxidation of membranes (Sgherri et al. 2000). Active oxygen species can act on unsaturated fatty acids and loosen the membranes, and finally affect the DNA. Drought stress may cause damage to cells either directly or indirectly, through the formation of ROS such as superoxide radicals and $H_2O_2$ (Mittler and Zilinskas 1994). Drought stress is also known to affect many biochemical activities such as nitrate reductase, which catalyses the rate-limiting step in the nitrate assimilation pathway. Under drought conditions, NR activity decreases in plants due to the lower uptake of nitrate from the soil by the roots (Caravaca et al. 2005). Drought increases the vulnerability to nutrient losses from the rooting zone through erosion (Gupta 1993). Under drought situations, roots are known to extend their length, increase their surface area, and alter their architecture in an effort to capture less mobile nutrients such as phosphorus (Lynch and Brown 2001). Drought also disrupts root–microbe associations that play a major role in plant nutrient acquisition.

An aberration in the temperature above or below the normal recorded range in a region is bound to have a cascading effect on plant growth and development. This occurs because temperature affects the photosynthetic rate, plant water relations, flowering and fruit, set in both tropical and temperate crops (Abrol and Ingram 1996). Baker and Allen (1993) reported increased water requirements and decreased yields in rice, soy bean, and citrus under higher maximum and minimum temperatures. The unprecedented heat wave in Northern India during the year 2004 resulted in a production loss of 4.4 million tons of wheat crop (Samra and Singh 2004). In the coming years, we will have to grapple with such instances of production loss as a result of heat waves during critical crop growth stages.

Soil salinity is a major edaphic stress and causes severe stress on plant growth and development. Increased salinization of arable land is expected to result in 30% land loss within the next 25 years and up to 50% by the middle of the twenty-first century (Wang et al. 2003). Soil salinity affects plants in three ways. The primary effect of increased salt concentration in the soil is the inability of plants to draw water from the soil, even though the soil appears quite moist. In effect, the plant suffers from a form of drought which can result in retarded growth and reduced yield. Secondly, some salts, such as Na and Cl enter the plant system through water and affect the plant's physiological processes often resulting in reduced growth, leaf burn and even plant death. Thirdly, high amounts of ions such as Na and Cl affect the availability of other essential plant nutrients like K, Mg, N, or P which are extremely important for plant growth (Tester and Davenport 2003). As vast expanses of cultivable lands continue to be affected by soil salinity, there is an urgent need to address this issue, in the best interests of global food production.

In metal contaminated soil, poor plant growth and root development are major limiting factors for successful crop production. The commonly encountered effects include reduced rates of photosynthesis, and increased uptake of heavy metals that have a cascading effect on most physiological traits. Table 10.1 lists out a few recorded effects of altered plant metabolism as a result of heavy metal contamination.

**Table 10.1** Effect of heavy metals on plant metabolism

| Heavy metals | Crop | Effect | References |
|---|---|---|---|
| Hg, Cd | Tomato | Growth and photosynthetic pigments are affected | Oancea et al. (2005) |
| Cd | French bean | The biomass reduction by Cd toxicity could be the direct consequence of the inhibition of chlorophyll synthesis and photosynthesis | Padmaja et al. (1990) |
| Cd | Pea | Decreased uptake of nutrient elements, inhibition of various enzyme activities, induction of oxidative stress | Sandalio et al. (2001) |
| Cd, Cu, Mo | *Cucumis sativus L* *Thlaspi ochroleucum* | Heavy metals accumulation in the tissue resulted in a decrease of biomass and the chlorophyll concentration in the leaves/stems | Burzynski and Buczek (1989), Ouzounidou et al. (1992), Abdel-Basset et al. (1995) |

## 10.3 Bacterial Mediated Abiotic Stress Alleviation Mechanisms

Bacteria that help plants overcome the ill effects of abiotic stress, are endowed with certain specialized functional traits. Any bio prospecting program for bacterial stress alleviation should ideally target isolates that express high levels of these traits. The abiotic stress alleviation traits and their associated mechanism are described in detail in the following section.

### *10.3.1 Role of Ethylene and ACC Deaminase in Abiotic Stress Alleviation*

Ethylene is a gaseous plant growth hormone produced endogenously by almost all plants. At concentrations as low as 0.05 ppm, it plays an extremely crucial role in plant growth regulation (Abeles et al. 1992), such as in growth promotion of vegetative plant parts, nodulation of legumes by rhizobia and rooting of cuttings. It is involved in transduction of a signal between the recognition of salt stress and the response in terms of physiological processes. The accumulation of ethylene in stressed plants is well documented (Jackson 1997). Under stress conditions, the endogenous production of ethylene is accelerated substantially, which adversely affects the root growth and consequently the growth of the plant as a whole. The term "stress ethylene" is used to denote the elevated levels of ethylene formed in response to both biotic and abiotic stress. Besides ethylene also induces the plant defense responses which help to enhance the survival of the plant under adverse conditions (Abeles 1973). The relation between this gaseous hormone and microbial mediated abiotic stress alleviation is provided by the enzyme 1-aminocyclopropane-1-carboxylate

(ACC) deaminase, which cleaves ACC, the precursor molecule of ethylene and therefore lowers the level of ethylene in a stressed plant.

ACC deaminase is a multimeric enzyme with a monomeric subunit molecular mass of approximately 35–42 kDa. It is a sulfhydryl enzyme that utilizes pyridoxal 5-phosphate as an essential cofactor. ACC deaminase has been found in a wide range of Gram-negative bacteria (Wang et al. 2001; Saleem et al. 2007 and references contained therein), Gram-positive bacteria (Belimov et al. 2001), rhizobia (Ma et al. 2003), endophytes (Pandey et al. 2005), and fungi (Jia et al. 1999). In addition, based on sequence similarity, many microorganisms have putative ACC deaminase genes (Glick 2005). ACC deaminase catalyzes a cleavage of ACC that includes cyclopropane ring fragmentation, and deamination of ACC to form α-ketobutyrate and ammonia (Honma and Shimomura 1978; Honma 1985). Though its substrate, ACC is of plant origin, ACC deaminase is localized within the cytoplasm of the microorganism that produces it (Jacobson et al. 1994). In such a scenario, ACC is exuded by plant tissues (Hontzeas et al. 2004) and is then taken up by the ACC deaminase producing microbe (Glick et al. 1998).

The mode of action of ACC deaminase in association with plants was postulated originally by Glick et al. (1998). They proposed that in order to overcome the competition from plant ACC oxidase which has a very strong affinity for ACC, microbial ACC deaminase levels need to be 100–1,000-fold higher. Such a scenario is likely only when the expression of ACC oxidase has not been induced in the plant system. In the normal course, PGPR in the vicinity of the root or seeds synthesize and secrete indole-3-acetic acid (IAA) from tryptophan and other small molecules present in seed or root exudates. This IAA gets adsorbed on the seed or root surface of the plants (Fallik et al. 1994). Apart from promoting plant growth, IAA stimulates the activity of the enzyme ACC synthase to convert S-adenosyl methionine (SAM) into ACC (Kende 1993). A significant portion of ACC is exuded from plant roots or seeds, taken up by the soil microbes and converted into ammonia and α-ketobutyrate, thereby reducing the levels of precursor molecule that can be converted into ethylene. Furthermore, the equilibrium between the internal and the external ACC levels is maintained through exudation of more ACC into the rhizosphere. ACC deaminase producing soil microbial communities are postulated to stimulate plants to biosynthesize more ACC, while simultaneously providing microorganisms with a unique source of nitrogen. This mutual cooperation between the plant and microbial partners plays a significant role in the alleviation of abiotic stress.

### *10.3.2 Rhizobacteria Mediated Induction of Systemic Tolerance in Plants*

Some plant growth promoting rhizospheric bacteria are endowed with certain unique abiotic stress alleviation traits, apart from the regular plant growth promotional traits. These strains thereby enable plants to overcome the ill effects of various abiotic stresses. The term Induced Systemic Tolerance (IST) has been

recently coined to accommodate the microbial induced physical and chemical changes in plants which result in enhanced tolerance to abiotic stresses (Yang et al. 2009). Though reduction in plant ethylene levels as a result of ACC deaminase production by rhizobacteria falls under this category, we have dealt with it separately, considering its importance and widespread practical utility. IST is conceptually different from Induced Systemic Resistance (ISR) which falls in the domain of biotic stresses. In one of the earliest studies in this direction, Timmusk and Wagner (1999) reported that inoculation with the plant growth promoting rhizobacterium (PGPR) *Paenibacillus polymyxa* enhanced the drought tolerance of *Arabidopsis thaliana*. By using RNA display, they concluded that mRNA transcriptions of a drought-response gene, *EARLY RESPONSE TO DEHYDRATION 15 (ERD15)*, were augmented in inoculated plants compared to uninoculated controls.

Since much of the injury on plants under abiotic stress is due to the oxidative damage at the cellular level, antioxidant enzymes such as peroxidase and catalase that have the ability to remove free radicals and prevent damage to the membranes and DNA acquire much importance in abiotic stress management (Bowler et al. 1992; Scandalios 1994). Certain rhizobacterial species have the ability to elevate the levels of such enzymes in the plant system. Kohler et al. (2008) demonstrated the higher activity of antioxidant catalase in lettuce plants under severe drought conditions when inoculated with PGPR *Pseudomonas mendocina* and AMF (*Glomus intraradices* or *G. mosseae*) and postulated that they can be used in inoculants to alleviate the oxidative damage elicited by drought. Saravanakumar et al. (2010) reported the ability of *P. fluorescens* Pf1 to increase the activity of catalase and peroxidase in water stressed green gram plants when compared to untreated plants. The bacterized plants were found to tolerate stress better than the uninoculated controls. The ability of the rhizobacterial strain *Pseudomonas putida* GAP-P45 to improve the plant biomass, relative water content , leaf water potential, proline sugars, and free amino acids of maize plants exposed to drought stress was recently reported by Sandhya et al. (2010). An interesting feature of this study was the decreased levels of the antioxidant enzymes such as ascorbate peroxidase, catalase, glutathione peroxidase in inoculated plants under drought stress prompting the authors to conclude that the inoculated plants felt less stress compared to their uninoculated counterparts. This conclusion throws up an interesting paradigm on the role of antioxidant enzymes in plant stress alleviation.

Ryu (2004) showed that *Bacillus subtilis* GB 03 induces systemic tolerance against salinity stress in *Arabidopsis* plants. Transcriptional validation of the HIGH-AFFINITY $K^+$ TRANSPORTER 1 (HKT1) which adjusts the $Na^+$ and $K^+$ levels differentially depending on the plant tissue, revealed that bacterial volatile organic compounds (VOC) down regulated HKT1 expression in roots, but up regulated it in shoot tissues, thereby resulting in lower $Na^+$ levels in the whole plant. Exposure of an *athkt1* mutant to bacterial VOCs not only resulted in typical salt-stress phenotypes, such as stunting, but also led to the inhibition of seedling growth.

### *10.3.3 Exopolysaccharide Production by Rhizospheric Bacteria and Promotion of Rhizospheric Soil Aggregation*

Maintenance of soil structure is an important feature of sustainable agriculture because it impacts a range of processes influencing crop yield. The soil structure gets altered by stresses such as drought that render the physico-chemical and biological properties of soil unsuitable for soil microbial activity and crop growth. In such a scenario, the role of exopolysaccharide (EPS) production by microbes is very important. The EPS produced by microbes protects the microbes against inhospitable conditions and enables their survival. Hartel and Alexander (1986) observed a significant correlation between the amount of EPS produced by cowpea *Bradyrhizobium* strains and their desiccation tolerance. Similarly, the capsular material of *Azospirillum brasilense* Sp245 was found to contain high molecular weight carbohydrate complexes (lipopolysaccharide–protein (LP) complex and polysaccharide–lipid (PL) complex) and is held responsible for protection under extreme conditions, like desiccation. Addition of these complexes to a suspension of decapsulated cells of *A. brasilense* Sp245 significantly enhanced survival under drought stress (Konnova et al. 2001). EPS also help microorganisms to irreversibly attach and colonize the roots due to involvement of a network of fibrillar material that permanently connects the bacteria to the root surface (Sandhya et al. 2009).

EPS producing microorganisms also promote the formation of microaggregates (<250 μm diameter) and macroaggregates (>250 μm diameter) (Edwards and Bremmer 1967; Tisdall and Oades 1982; Oades 1993). Plant roots and fungal hyphae fit in the pores between micro aggregates and thus stabilize macro aggregates (Oades and Waters 1991). The EPS released into soil as capsular and slime materials by soil microbes can be adsorbed by clay surfaces due to cation bridges, hydrogen bonding, Van der Waals forces, and anion adsorption mechanisms, thus forming a protective capsule around soil aggregates (Tisdall and Oades 1982). Plants treated with EPS-producing bacteria display increased resistance to water stress (Bensalim et al. 1998). It has been postulated that the EPS provides a microenvironment that holds water and dries up more slowly than the surrounding environment thus protecting the bacteria and plant roots against desiccation (Hepper 1975). Production of EPS by bacteria has been shown to improve permeability by increasing soil aggregation and maintaining higher water potential around the roots; in this way, there was an increase in the uptake of nutrients by plant, with an increase in plant growth apart from protection from drought stress (Miller and Wood 1996; Alami et al. 2000).

Another interesting feature of drought stress alleviation by microbes is the improvement in the Root Adhering Soil/Root Tissue (RAS/RT) ratio as a result of inoculation with EPS producing bacteria. It has been postulated that higher EPS content and better aggregation of RAS could help the plants to take up a higher volume of water and nutrients from rhizospheric soil (Miller and Wood 1996), resulting in a better growth of plants; besides, this phenomena has also been known to counteract the negative effects of drought stress (Munns 2002).

Alami et al. (2000) observed a significant increase in (RAS/RT) in sunflower rhizosphere inoculated with the EPS-producing rhizobial strain YAS34 under drought conditions. Similar results were obtained with wheat plantlets inoculated with *P. polymyxa* (Gouzou et al. 1993) and *Pantoea agglomerans* (Amellal et al. 1998) under salt stress. But, studies in this direction have been far and few and require renewed interest in order to unravel the potential of microbes in abiotic stress alleviation.

## 10.4 Microbes as Stress Alleviating Agents Under Various Stress Situations

Among the various classes of soil microbes, rhizobacteria that live in close proximity with the plant roots are known to play an important role in alleviation of various abiotic stresses. In the following section, we shall discuss specific reports of abiotic stress alleviation by rhizobacteria.

### *10.4.1 Drought Stress*

Alleviation of drought stress by rhizobacterial species has received much attention in the past. Mayak et al. (2004a) reported that, under transient water stress, the ACC deaminase producing PGPR *Achromobacter piechaudii* ARV8 significantly increased the fresh and dry weights of both tomato and pepper seedlings and reduced the ethylene production under transient drought stress. They hypothesized that those bacteria populating sites, where water is limited and repeated dry periods occur frequently, are likely to be able to better promote plant growth compared to bacteria isolated from sites where water sources are abundant. This hypothesis found support in their finding that *A. piechaudii* ARV8, a bacterial strain isolated from an arid site, performed better than *P. putida* GR12-2, that was originally isolated from the rhizosphere of grasses in the High Canadian Arctic (Lifshitz et al. 1986), where water is abundant. Dodd et al. (2005) observed that the effects of inoculation of pea plants with the ACC deaminase producing bacterium *Variovorax paradoxus* 5C-2 were more pronounced and consistent under controlled soil drying. In long-term experiments, plants inoculated with ACC deaminase producing bacterium gave more seed yield (25–41%), seed number and seed nitrogen accumulation than non inoculated plants, while nodulation, which was depressed in water stress conditions, was restored in inoculated plants. Arshad et al. (2008) also observed similar results with ACC deaminase producing bacteria that partially eliminated the effects of water stress on growth, yield, and ripening pea in both pot and field trials. Marulanada et al. (2009) reported that three indigenous bacterial strains viz., *Pseudomonas putida*, *Pseudomonas* sp., and *Bacillus megaterium* isolated from water stressed soil could

**Table 10.2** Drought stress alleviation by bacterial species in various crops

| Crop | Bacterial species | Effect | References |
|---|---|---|---|
| Lettuce | *Bacillus* sp | Increased AM fungal colonization in roots and enhanced photosynthesis | Vivas et al. (2003) |
| *Catharanthasus roseus* | *Pseudomonas fluorescens* | Improved plant growth | Jaleel et al. (2007) |
| Bean | *Paenibacillus polymyma* and *Rhizobium tropici* | Altered hormonal balance and stomatal conductance | Figueiredo et al. (2008) |
| Lettuce | *Pseudomonas mendonica* | Increased phosphatase activity in roots and proline accumulation in leaves | Kohler et al. (2008) |

stimulate plant growth under dry conditions. Recent evidence in this direction was provided by Sandhya et al. (2009), who reported that inoculation of *Pseudomonas* sp. strain GAP-P45 was found to increase the survival, plant biomass, and RAS/RT of sunflower seedlings subjected to drought stress. The inoculated bacteria could efficiently colonize the root adhering soil, rhizoplane, and increase the percentage of stable soil aggregates. In a separate study, they observed that *Pseudomonas* strain GAP 45, increased compatible solutes and antioxidant status of maize plants under water stressed conditions (Sandhya et al. 2010). Some of the recorded instances of drought stress alleviation by different crops are presented in Table 10.2.

### *10.4.2 High/Low Temperature Stress*

In the context of climate change, microbial mediated alleviation of high/low temperature stress are receiving recent attention. Though the ability of some fungal species to confer heat resistance on plant species has been known (Mc Lellan et al. 2007), we would restrict our discussion to bacterial species that confer heat tolerance on plant species. Srivastava et al. (2008) isolated a thermotolerant *P. putida* strain NBR10987 from drought stressed rhizosphere of chickpea. The thermotolerance of the strain was attributed to the over expression of stress sigma factor σs and enhanced biofilm formation at high temperatures. Ali et al. (2009) reported the ability of a thermo tolerant strain *Pseudomonas* AKM-P6 to alleviate the heat stress in sorghum seedlings. Inoculation induced the biosynthesis of high-molecular weight proteins in leaves under elevated temperature, reduced membrane injury, and improved the levels of cellular metabolites like proline, chlorophyll, sugars, amino acids, and proteins. The protein profiles of inoculated and uninoculated sorghum seedlings exposed to ambient and elevated temperature revealed the presence of three additional polypeptides (~70.4–60.8 and ~55.1 KDa) in the

seedlings exposed to elevated temperature, indicating a possible role of inducible proteins in microbial mediated heat tolerance mechanisms.

In one of the earliest studies on conferment of cold tolerance by microbes, Barka et al. (2006) observed that in vitro inoculation of grapes (*Vitis vinifera* cv. chardonnay) explants with a PGPR, *Burkholderia phytofirmans* strain PsJN, increased grapevine root growth and plantlet biomass, and physiological activity at a low temperature. They reported that bacterized plantlets had significantly increased levels of starch, proline, and phenolics compared to the uninoculated plantlets. These increases correlated with the enhancement of cold tolerance of the grapevine plantlets. A psychrotolerant ACC deaminase producing bacterium *P. putida* UW4 was found to promote canola plant growth at low temperature under salt stress (Cheng et al. 2007). In a study with wheat seedlings inoculated with cold tolerant Pseudomonads, Mishra et al. (2009a), observed changes in parameters critical to the plant's ability to tolerate cold stress conditions. Bacterization significantly enhanced total chlorophyll, anthocyanin, free proline, total phenolics, and starch contents; while a decrease in $Na^+/K^+$ ratio and electrolyte leakage was observed in bacterized seedlings. In another study, they observed that inoculation with cold tolerant bacterium *Pseudomonas* spp. strain PPERs23 significantly improved root length, shoot length, dry root biomass, dry shoot biomass, total chlorophyll, total phenolics, and amino acid contents of wheat seedlings. The bacterium also enhanced the physiologically available iron, protein concentration, anthocynine, proline, relative water content; and decreased the $Na^+/K^+$ ratio and electrolyte leakage, thereby enhancing the cold tolerance of wheat plants (Mishra et al. 2009b). The potential of novel cold tolerant plant growth promoting bacterial species viz., *Pantoea dispersa*, *Serratia marcescens*, *Pseudomonas fragi*, *Exiguobacterium acetylicum* and *Pseudomonas lurida* in promoting plant growth at cold temperatures was demonstrated by Selvakumar et al. (2008a, b, 2009, 2010a, b). They attributed the observed effects mainly due to auxin production and phosphate solubilization by the bacterial species. Under temperate conditions, such bacteria can sustain their metabolic processes and aid in plant growth promotion.

Frost injury as a result of cold temperatures is one of the most commonly encountered problems in temperate agriculture. Freezing injury in plants as a result of ice nucleation is frequently not endogenous, but is induced by catalytic sites present in microbial parasites, which can be found on leaves, fruits or stems (Lindow 1983). Ice nucleating strains of *P. syringae* are known to increase the frost susceptibility of tomato and soybean when sprayed on leaves prior to low temperature stress, in addition to being a pathogen of these plants (Anderson et al. 1982). Recognition of the gene associated with ice nucleation in *P. syringae* first led to the synthesis of an "ice-minus" mutant, which was found to be inactive in promoting ice nucleation in plants leaves (Xu et al. 1998). The recent development in this direction is the identification of naturally occurring phyllospheric bacteria with low ice nucleating activity, so that they can be sprayed on leaves to overcome frost damage.

### *10.4.3 Soil Salinity*

Soil salinity has a profound effect on seed germination, which is the most vital aspect of successful crop production. Under such situations, it is essential to stimulate seed germination and seedling growth (Lambers 2003). The most appropriate solution in such situation is to use salt tolerant bacterial inoculants that produce auxins, gibberellins, and promote plant growth under salinity conditions (Mayak et al. 2004b). Giri and Mukerji (2004) reported that, in saline soil, higher absorption of P in inoculated plants may improve their growth rate and salt tolerance and suppress the adverse effect of salinity stress. A related approach is the use of phosphate solubilizing bacteria to increase the P availability to plants, since improved P nutrition enhances biological nitrogen fixation and the availability of other nutrients because these bacteria are also known to produce plant growth-promoting substances (Gyaneshwar et al. 2002). ACC deaminase producing bacteria can also reduce salinity stress by reducing stress ethylene levels (Glick et al. 1998). Plants mitigate salt stress by the accumulation of protein and proline in leaves. The accumulation of proteins in leaves under stress is an adaptation mechanism as they bind to membranes, regulating membrane water permeability in cells and influencing water movement among tissue and organ (Ashraf et al. 2003); in addition, they can prevent and reduce the denaturation of other cellular micromolecules under dehydrative conditions (Campbell and Close 1997). Studies on the ability of bacterial species to induce the production of protective proteins and solute molecules require intensification. Some examples of salinity stress alleviation by bacteria are presented in Table 10.3.

**Table 10.3** Bacterial mediated alleviation of salinity stress in crops

| Crop | Bacterial species | Effect | References |
|---|---|---|---|
| Tomato | *Achromobacter piechaudii* | Reduced levels of ethylene and improved plant growth | Mayak et al. (2004b) |
| Canola | *Pseudomonas putida* UW 4 | The bacterium promoted plant growth at 1 mol/L and 150 mol/L at 10 and 20°C respectively | Cheng et al. (2007) |
| Groundnut | *Pseudomonas fluorescens* | Enhanced ACC deaminase activity | Saravanakumar and Samiyappan (2007) |
| Maize | *Rhizobium*, *Pseudomonas* | Decreased electrolyte leakage and, increase in proline production, maintenance of relative water content of leaves, and selective uptake of K ions | Bano and Fatima (2009) |
| Cotton | *Pseudomonas putida* Rs-198 | Increase the absorption of the $Mg^{2+}$, $K^{2+}$ and $Ca^{2+}$ and decrease the uptake of the $Na^{2+}$ from the soil | Yao et al. (2010) |

### 10.4.4 Flooding

With the threat of nonseasonal floods looming large over various developing nations as a result of global climate change, water logging of cropped fields is a reality that we need to accept in the future. Water logging causes the ACC, which is synthesized in roots, to be transported to plant shoots, where it is converted to ethylene by ACC oxidase (Bradford and Yang 1980). The increased ethylene levels observed in shoots of waterlogged plants corresponds with enhanced activities of ACC synthase in the submerged roots and ACC oxidase in the shoots (Chao et al. 1997; Olson et al. 1995). Grichko and Glick (2001) observed significant tolerance to flooding in 55 day old tomato plants inoculated with ACC deaminase producing strains of *Pseudomonas* and *Enterobacter*. Much more efforts are required to unravel the potential of rhizobacterial species in alleviating stress water logging stress.

### 10.4.5 Heavy Metals

In metal contaminated soil, the poor performance of plant growth and root development are major limiting factors for phytoaccumulation of metals. To overcome these problems, improvement of the microbial activity in rhizosphere in addition of organic amendments is necessary (Park et al. 2011). Under heavy metal contamination conditions, it has been hypothesized that plant growth is mainly achieved by IAA production (Patten and Glick 2002), ACC deaminase activity (Penrose and Glick 2001), and siderophores which help plants to acquire sufficient Fe in the presence of overwhelming amounts of other metals (Burd et al. 2000). Table 10.4 presents some published instances of heavy metal stress alleviation by bacterial species.

### 10.4.6 Organic Pollutants

The presence of organic pollutants above permissible levels can have adverse effects on plant growth and development. This has been majorly attributed to the production of ethylene by plant roots in the presence of toxic levels of organic pollutants (Coupland and Jackson 1991; de Prado et al. 1999). Therefore, the focus in this direction has been the utilization of ACC deaminase producing bacterial species for stress alleviation (Reed and Glick 2005). Considering the variety of organic pollutants and the extent of cultivable land that are prone to stress as a result of toxic levels of organic pollutants, it is imperative to intensify research in this direction.

**Table 10.4** Heavy metal stress alleviation by bacterial species

| Crop | Bacteria | Effect | References |
|---|---|---|---|
| Tomato | *Kluyvera ascorbata* | Toxic effects of $Ni^{2+}$, $Pb^{2+}$ and $Zn^{2+}$ not pronounced on plants | Burd et al. (2000) |
| Tomato | *Methylobacterium oryzae* and *Burkholderia* sp. | Reduced uptake and translocation of nickel and cadmium | Madhaiyan et al. (2007) |
| Pea | *Pseudomonas brassicacearum* Am3, *Pseudomonas marginalis* Dp1 and *Rhodococcus* sp. Fp2 | Stimulation of root growth and enhanced nutrient uptake | Safronova et al. (2006) |
| Pea | *Rhizobium* sp. RP5 | Inoculation enhanced the plant growth parameters in presence of 290 mg Ni $kg^{-1}$ and 4890 mg Zn $kg^{-1}$ of soil | Wani et al. (2008) |

### *10.4.7 Nutrient Deficiency Associated Stresses*

Many rhizospheric bacteria enhance the uptake of plant nutrients from the soil by facilitating improved root growth under low input crop production situations (Cummings and Orr 2010 and references contained therein). A recent development in this direction has been the discovery that a *Pseudomonas* strain reduced the incidence of blossom end rot, a physiological malady that arises due to calcium deficiency in tomato fruits grown under hydroponic conditions (Lee et al. 2010). Considering the myriad of complex relationships that exist between different essential plant nutrients, a novel attempt in this direction would be the discovery of bacterial species that affect macro and micro nutrient uptake by plant species under different deficient and toxic situations.

## 10.5 Conclusion

In view of the urgency in which planners and policy makers the world over are engaged in addressing the threats of climate change, no effort, however small it may seem, can be ignored. With the gamut of information available on the abiotic stress alleviating potential of microbes, it is worthwhile to intensify research efforts so that their potential can be unearthed to the maximum and the power of these tiny creatures be utilized for the benefit of mankind. While most research findings are at the preliminary level, the need of the hour is the development of ready to use formulations that can be under different stress situations. This requires much more effort in terms of identification of the right kind of microbes and addressing the issue of delivery systems, both of which require concerted interdisciplinary efforts from microbiologists, plant physiologists, and agronomists. Besides alleviating

abiotic stress in crops, these microbes can serve as interesting models for studying the stress tolerance mechanisms in prokaryotic forms of life. Another possibility would be the transfer of beneficial genes from such microbes to cultivated plants, similar to the development of transgenic plants that express the ACC deaminase gene (Grichko et al. 2000). But considering the regulatory issues involved in the development of transgenic crops, it would be a much more viable and cost effective option to develop easy to handle formulations of abiotic stress alleviating microbial formulations. Another exciting area of research and development is deciphering the role of bacterial species in the alleviation of other abiotic stresses caused by atmospheric gaseous pollutants.

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# Chapter 11
# Rhizobacteria Mediated Induced Systemic Tolerance in Plants: Prospects for Abiotic Stress Management

**Birinchi Kumar Sarma, Sudheer Kumar Yadav, Dhananjaya Pratap Singh, and Harikesh Bahadur Singh**

## 11.1 Introduction

Plants including agricultural crops are facing continuous environmental threats from different biotic and abiotic factors, which have increased over time due to change in global climate pattern as well as human interference. These environmental stresses limit plant productivity and thereby pose an overall threat to food security. Apart from conserving phytodiversity in their ecological habitats, the need has also arisen to increase the productivity of crop plants to feed the ever-burgeoning human population on the blue planet – the earth. Tailoring genotypes for desirable performance under abiotic stress conditions is essential for the sustainability of crop production in view of climate change (Battisti and Naylor 2009; Lobell et al. 2008). Conventional breeding has been able to meet the problem so far (Cooper et al. 2009), but the time has come to search for alternative means to increase the pace of crop productivity to meet the projected demand for agricultural products. Improvement in the genetic base of the crop plants for better adaptability to the abiotic stresses such as heat, salt, drought, and cold, is a time-consuming process and improving all crop plants to face the challenge of abiotic stress equally at the same time is very unlikely. In order to meet the increasing demand of food supply, better adaptation of the crop plants to abiotic stresses as well as geographical expansion of agricultural lands by making unusable lands usable for crop production are a necessity. In this regard, useful soil microbes may become very handy and provide a quick-fix solution to the problem.

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Plant roots attract several kinds of soil microflora and become quickly colonized by soil-borne bacteria and fungi that may have either beneficial or deleterious effects on the plant (Tardieu and Tuberosa 2010). Among the best examples are, symbiotic microorganisms such as mycorrhizal fungi that aid in the uptake of water and minerals, notably phosphate (Egerton-Warburton et al. 2008), and root-nodulating *Rhizobium* bacteria that fix atmospheric nitrogen for the plant (Spaink 2000). Several other types of beneficial soil-borne microbes, such as plant-growth-promoting bacteria and fungi, can stimulate plant growth indirectly by suppressing plant diseases (Kloepper et al. 2004; Singh et al. 2002; Waller et al. 2005). Rhizospheric bacteria capable of promoting plant growth under various situations are known as plant-growth-promoting rhizobacteria (PGPR). They colonize the rhizosphere of many plant species and impart benefit to the plants by increasing plant growth and reducing disease development (Kloepper et al. 2004; Singh et al. 2003). Recent investigations have also shown that some strains of PGPR also elicit tolerance to abiotic stresses, such as drought, salt, and nutrient excess or deficiency (Yang et al. 2009). Special attention is being given to research directed to the development of PGPR-based bioinoculants for stimulation of the plant's defensive metabolism, which will allow crop cultivation in soils imposing abiotic stresses, including salinity and drought, on plants (Barriuso et al. 2008).

## 11.2 Induced Systemic Resistance and Induced Systemic Tolerance

A range of activities has been found to be associated with PGPR and these activities can affect plant growth either directly or indirectly. Direct promotion of plant growth takes place mostly due to phytohormone(s) released as well as nutrient mobilization by the PGPR strains in the rhizosphere, which are subsequently taken up by the host plants, thereby influencing their growth. Indirect promotion of plant growth occurs when PGPR antagonize or prevent the effects of harmful microbes (Glick 1994; Kloepper et al. 2004). Interestingly, some PGPR can also suppress plant diseases caused by a range of pathogens through elicitation of physical and chemical changes related to plant defense (Sarma et al. 2002) and the process is known as "induced systemic resistance" (ISR) (van Loon et al. 1998). Recently, reports are also becoming available about the role of PGPR in eliciting plants to tolerate abiotic stresses such as drought, salt, and high and low temperature. Yang et al. (2009) termed this phenomenon as "induced systemic tolerance" (IST) for PGPR-induced physical and chemical changes in plants that result in enhanced tolerance to abiotic stresses. Microbe-associated molecular pattern (MAMP) analogs cell surface components such as flagellin and lipopolysaccharides (LPS) of beneficial *Pseudomonas* spp. are found to be potent inducers of the host immune response. However, flagellin and LPS mutants of rhizobacterial strains were nevertheless often as effective as the wild-type strains, suggesting that multiple MAMPs are involved in the activation of the plant's immune response (Bakker et al. 2007).

Similarly, low-molecular-weight $Fe^{3+}$-specific chelators (siderophores) under low iron availability condition (Leeman et al. 1996), as well as antibiotics such as 2,4-diacetyl phloroglucinol (2,4-DAPG) which are produced by some fluorescent *Pseudomonas* spp., can also function as MAMPs in triggering the immune response (Weller et al. 2002). Recently, the biosurfactant massetolide A from *P. fluorescens* SS101 (Tran et al. 2007), a surfactin lipoprotein produced by *Bacillus subtilis* (Ongena et al. 2007), other rhizobacterially produced compounds such as *N*-alkylated benzylamine (Ongena et al. 2005), and *N*-acyl-L-homoserine lactone (Schuhegger et al. 2006) as well as the volatile organic compound (VOC) 2,3-butanediol produced by *Bacillus* spp. (Ryu et al. 2004) were shown to induce resistance in several host plants against their pathogens. The systemic defense responses that are triggered by beneficial microorganisms are controlled by a signaling network in which the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play important roles (Glazebrook 2005). Host plants fine-tune their defense response through cross-communication of SA, JA, and ET pathways, depending on the invader encountered (Koornneef and Pieterse 2008). Several of these phenomena are also found to be associated with IST (Yang et al. 2009). Timmusk and Wagner (1999) showed a possible connection between biotic and abiotic stress responses induced by PGPR *Paenibacillus polymyxa* in *Arabidopsis thaliana*. Similarly, Barriuso et al. (2008) also showed that the PGPR strains that protected *A. thaliana* plants from the foliar pathogen *P. syringae* also protected the plants from salt stress and ISR was mediated by expression of *PR1* gene whose expression involves activation of the SA-dependent pathway. Despite several common linkages between ISR and IST pathways, several mechanistic differences also exist (Xiong and Yang 2003).

## 11.3 PGPR-Mediated Salt Tolerance

Soil salinity has become a serious threat to crop yield and food security, as significant areas of cultivated lands are getting affected by salinity reported from all over the world (Rengasamy 2006). According to Frommer et al. (1999), approximately 20% of once-irrigated land worldwide presently has become salt contaminated. In Pakistan alone, every year nearly 100 acres of land are becoming salt stressed (Rozema 1999). Salt tolerance in plants, especially to NaCl stress, depends on variations in tissue tolerance to $Na^+$. *Arabidopsis* employs a multitude of mechanisms to achieve tolerance to $Na^+$ accumulation in the leaf but the relationship between $Na^+$ accumulation in the leaf and whole-plant $Na^+$ tolerance is not clearly understood (Møller and Tester 2007). At the cellular level, differential $Na^+$ tissue tolerance could be achieved by differences in the expression or activity of proteins involved in vacuolization of cells within the leaf structure (Glenn et al. 1999; Niu et al. 1996). Guo et al. (2001) demonstrated that a constitutively activated Salt Overly Sensitive (SOS)2 kinase can be achieved by deletion of the

autoinhibitory domain or by site-specific modifications of the catalytic domain of the protein kinase that offers an approach to regulate stress signaling that controls ion homeostasis. Furthermore, overexpression of *AtNHX1* enhances plant salt tolerance, presumably by vacuolar $Na^+$ compartmentalization that minimizes the toxic accumulation of the ion in the cytosol and facilitates growth in the saline environment (Apse et al. 1999; Zhang and Blumwald 2001).

PGPRs with the ability to elicit ISR in plants also have the potentiality to induce IST as the expression of some genes involved in the defensive response, e.g., genes involved in ET, JA, and abscisic acid (ABA) pathways, are common with the genes expressed in salt stress situations (Cheong et al. 2002; Timmusk and Wagner 1999). Glick et al. (1997) showed that the PGPR strain *Pseudomonas putida* GR12-2 capable of stimulating plant growth and that produces the enzyme 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase which hydrolyzes ACC can also promote early development of canola seedlings under salt stress. Increased amount of soil $Na^+$ content has a negative impact on plant growth and, thereby, agricultural productivity. HKT1 originally identified from wheat roots is a high-affinity $K^+$ ion transporter and controls $Na^+$ import in roots. Zhang et al. (2008) showed that the soil bacterium *B. subtilis* GB03 confers salt tolerance in *A. thaliana* by tissue-specific regulation of *HKT1*. They showed that GB03 under salt stress concurrently downregulates *HKT1* expression in roots and upregulates its expression in shoots, resulting in lower $Na^+$ accumulation throughout the plant compared with controls. Further, they showed that GB03 fails to rescue salt-stressed *athkt1* mutants from stunted foliar growth and elevated total $Na^+$. Barriuso et al. (2008) used four strains of PGPR to induce systemic resistance on *A. thaliana* Col 0 against salt stress and found that *Bacillus* sp. strain L81 and *Arthrobacter oxidans* strain BB1 reduced plant mortality due to salt stress significantly. They also showed that the SA-dependent pathway was involved in the L81- and BB1-induced defense responses. As evidence, they showed that the expression of *PR1* was induced, which is a gene associated to the SA-dependent pathway.

Salt stress also confers oxidative stress upon the plants as a consequence of the generation of reactive oxygen species (ROS), which are detrimental to plant survival under salt stress. PGPR *P. mendocina* alone and in combination with an arbuscular mycorrhizal fungus *Glomus intraradices* or *G. mosseae* increased the biomass of *Lactuca sativa* cv. *Tafalla*, along with antioxidant enzymes and proline content in foliage, in the plants exposed to salt stress in salt-sensitive plants (Kohler et al. 2009). In another experiment, Han and Lee (2005) demonstrated that inoculation with two PGPR strains, *Serratia proteamaculans* ATCC 35475 and *Rhizobium leguminosarum* bv. *viciae* 128C56G alleviated the salinity effects on the antioxidant enzymes, photosynthesis, mineral content, and growth of *Lactuca sativa* grown on saline soils. Similarly, Bano and Fatima (2009) had studied the effect of coinoculation of the PGPR strains *Pseudomonas* sp. 54RB and *Rhizobium* sp. Thal-8 on two varieties of maize under salt stress. While salt stress had a detrimental effect on growth and development of the plants, coinoculation of the PGPR strains resulted in some positive adaptive responses of maize plants under salinity. The maize cv. Agaiti 2002 was more responsive to inoculation and was relatively

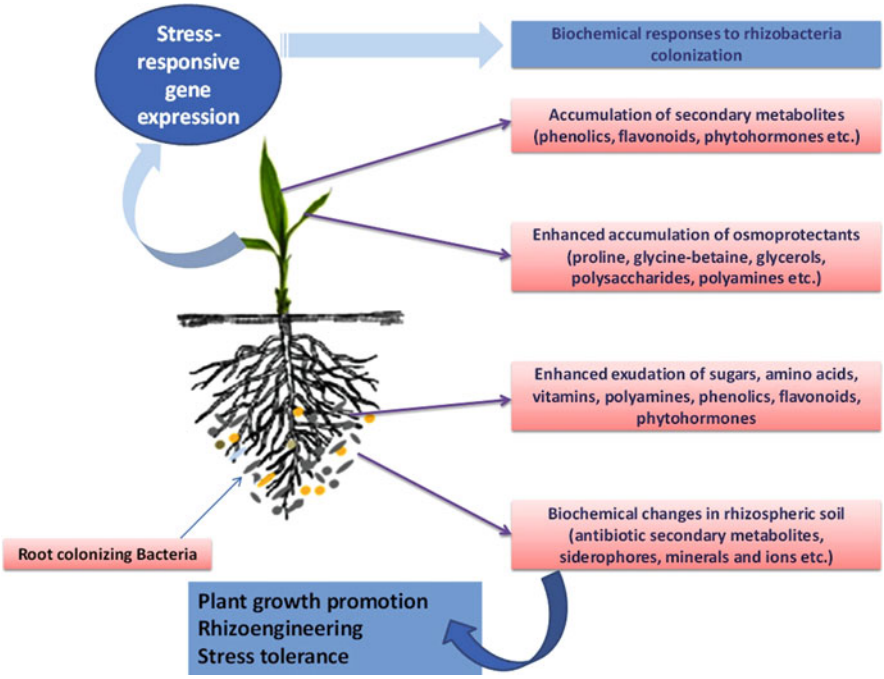


**Fig. 11.1** Rhizobacteria mediated metabolic responses in plants

less tolerant to salt. They concluded that the salt tolerance from beneficial bacterial inoculation was mediated through decrease in electrolyte leakage and in osmotic potential as well as increase in the production of the osmoregulant proline, maintenance of relative water content of leaves, and selective uptake of K ions (Fig. 11.1).

## 11.4 PGPR-Mediated Temperature Tolerance

Elevated temperature, a consequence of global climate change, also has an adverse effect on crop productivity (Fischer et al. 2002; Drigo et al. 2008). Heat stress negatively influences photosynthetic rate, plant water relations, flowering, and fruit set in both tropical and temperate crops (Abrol and Ingram 1996). Increased water requirements and decreased yield in rice were reported due to shift in maximum and minimum temperatures (Baker and Allen 1993). The activation of genes responsive to heat stress is mediated by heat stress transcription factors (Hsfs). Plant Hsfs have a highly complex gene family comprising of more than 20 members and the existence of hs-induced Hsf genes are thought to modulate transcription during long-term hs response (Baniwal et al. 2004; Nover et al. 2001). In tomato, HsfA1a

acts as a master regulator for induced thermotolerance (Mishra 2002) and cannot be replaced by any other Hsf. Interestingly, class B and class C Hsfs, in contrast to class A Hsfs, do not have any transcription activators on their own (Kotak et al. 2004; Czarnecka-Verner et al. 2000, 2004). However, the Hs-induced HsfB1 of tomato represents a coactivator cooperating with HsfA1. Similarly, tomato and *Arabidopsis* group A4 Hsfs act as potent activators of hs gene expression, whereas group A5 Hsfs inhibit HsfA4 activity but not other members of class A Hsfs.

Breeding for heat-tolerant cultivars or development of transgenics is time consuming and not cost-effective (Vanaja et al. 2007). Again, microbes may become useful in this regard. Thermotolerance of *P. putida* NBRI0987 according to Srivastava et al. (2008) is due to overexpression of stress sigma factor $\sigma^S$ and enhanced biofilm formation at high temperature. Gouestbet et al. (2002) demonstrated earlier that heat shock proteins that stabilize the membrane are induced under stress conditions and confer thermotolerance to elevated temperature. Ali et al. (2009) identified a thermotolerant strain of *Pseudomonas* sp. AKM-P6 possessing PGPR activities from the rhizosphere of pigeon pea grown under semiarid conditions in India. The strain helped sorghum seedlings to withstand heat stress through induction of biosynthesis of high-molecular-weight proteins in leaves, reduced membrane injury, and increased the contents of cellular metabolites such as proline, chlorophyll, sugars, amino acids, and proteins. The thermotolerance conferred by the strain is predicted to be due to the production of exopolysaccharides. In a unique study, thermotolerance in *Dichanthelium lanuginosum* plants by a symbiotic *Curvularia* sp. was reported by Redman et al. (2002). Interestingly, when the fungus and plant were grown separately, neither could tolerate the heat regime. While understanding the ability of the symbiotic fungus to confer heat tolerance to its host plant, Marquez et al. (2007) concluded that the ability of the fungus to confer thermotolerance was due to a thermotolerant virus that infects the symbiotic fungus. Similarly, McLellan et al. (2007) showed that the rhizosphere fungus *Paraphaeosphaeria quadriseptata* also enhanced thermotolerance to *A. thaliana* through induction of HSP101 and HSP70 proteins.

## 11.5 PGPR-Mediated Cold Tolerance

Abiotic stresses generate very complex stimuli that possess many different yet altered attributes and every single stimulus may provide the plant cell with quite different information. As an example, chilling stress may immediately result in mechanical constraints, changes in activities of macromolecules, and reduced osmotic potential in the cell. Cold stress affects the growth and development of plants adversely and thereby prevents the expression of the full genetic potential of plants by either limiting metabolic reactions or chilling induced inhibition of water uptake. Plant cells can sense cold stress through membrane rigidification caused due to reduced fluidity of the cellular membranes (Chinnusamy et al. 2007). *COR* (*COLD RESPONSIVE*) genes are induced by this membrane rigidification process

and confer cold acclimation in crops such as alfalfa and *Brassica napus* (Orvar et al. 2000; Sangwan et al. 2001). Expression of *COR* genes initiates when ectopic expression of CBFs (C-repeat binding factors) takes place following cold stress and binds to *cis*-elements in the promoters of *COR* genes (Stockinger et al. 1997; Liu et al. 1998). ICE1 (Inducer of CBF Expression1), a transcription factor, binds to MYC recognition elements in the CBF promoters and activation of expression of CBF3, CBF2, and COR genes during cold acclimation and concluded that cold-stressed induced posttranslational modification is required for ICE1 to activate downstream genes (Chinnusamy et al. 2003).

It is predicted that a single sensor might only regulate branches of the signaling cascade that are initiated by one aspect of the stress condition rather than perceiving the stress condition and controlling all subsequent signaling (Xiong et al. 2002). For example, due to changes in membrane fluidity caused by low temperature (Murata and Los 1997), a signaling cascade responsive to membrane fluidity would initiate following detection of this change by a sensor but would not necessarily control signaling initiated by an intracellular protein whose conformation/activity is directly altered by low temperature. Following this, secondary signals including hormones can initiate another cascade of signaling events different from the primary signaling. The expression of the wheat gene *TaARD* responsible for aci-reductone-dioxygenase activity is inhibited by wounding and environmental stimuli including low temperature; its signal transduction is known to be carried out by ET and therefore correlates the ET signaling in response to abiotic stress including low temperature stress. Similarly, the mitogen-activated protein kinase (MAPK) cascade plays a crucial role in various abiotic stresses (Table 11.1).

**Table 11.1** Some important plant defense genes induced under abiotic stress

| Stress | Host gene expressed under abiotic stress | References |
|---|---|---|
| Salt | *HKT1* | Zhang et al. (2008) |
| | *PR1* | Barriuso et al. (2008) |
| | *RD29A* | Shinozaki and Yamaguchi-Shinozaki (2000) |
| | *AtNHX1* | Apse et al. (1999) |
| Heat | *HSP101* and *HSP70* | |
| | *HsfA1a*, *HsfA4*, and *HsfB1* | von Koskull-Döring et al. (2007) |
| Cold | *OsMAPK5* | Xiong and Yang (2003) |
| | *OSISAP1* | Mukhopadhyay et al. (2004) |
| | *RD29A* | Shinozaki and Yamaguchi-Shinozaki (2000) |
| | *COR genes* | Chinnusamy et al. (2007) |
| Drought | *RD29A* | Shinozaki and Yamaguchi-Shinozaki (2000) |
| | *ERD15* | Timmusk and Wagner (1999) |
| | *UVR8*, *CP5*, *CDPK*, *STKL*, *MSBP*, *SPDS*, *NADP-ME*, *PDH*, *CSMO*, *APP*, *ADOR*, *ALDH*, *GST*, *SPDS*, *HSP17.8*, and *DHN3* | Guo et al. (2009) |

To understand the role of MAPKs in modulating the interaction of defense pathways activated by biotic and abiotic factors, Xiong and Yang (2003) overexpressed the rice *OsMAPK5* gene and found that it enhanced abiotic stress tolerance including cold tolerance. Interestingly, they also observed that silencing the same gene enhanced the host resistance toward the pathogen *Magnaporthe grisea* but reduced cold tolerance. Similarly, another gene from rice, which is intronless (*OSISAP1*) and encodes a zinc-finger protein, is induced by abiotic stresses including cold stress (Mukhopadhyay et al. 2004). On overexpression of the gene in transgenic tobacco conferred tolerance to cold.

PGPRs can also play a significant role in helping plants to withstand cold tolerance, as several genes that confer resistance to host plants against abiotic stresses are also induced due to PGPR activities. Barka et al. (2006) showed that in vitro inoculation of *Vitis vinifera* explants with a PGPR strain *Burkholderia phytofermans* strain PsJN increased growth and physiological activity of grapevine at a low temperature. They observed a positive correlation between the endophytic bacterial colonization in the grapevine plantlets and their growth at low and chilling temperatures.

## 11.6 PGPR-Mediated Drought Tolerance

Dehydration represents a common stress challenge to plant cells under drought, salt, and cold conditions. Plants adopt elegant survival systems to protect themselves against environmental stresses including drought. Stomatal closure effected by ABA to minimize water loss through transpiration is one of the common mechanisms operative under such condition (Schroeder et al. 2001; Leung and Giraudat 1998). A large array of genes is also activated during these stress conditions, which are often referred to as "stress genes." A significant overlap has been identified in the activation of these genes by each of these conditions (Cheong et al. 2003). Many drought-responsive genes also are responsive to salt or cold (Shinozaki and Yamaguchi-Shinozaki 2000; Xiong et al. 2002). For example, the gene *RD29A* is one such common stress-inducible gene (Shinozaki and Yamaguchi-Shinozaki 2000). Apart from that, the stress hormone ABA level also increases in both drought and salt conditions, which also activates *RD29A*. Drought tolerance in barley is governed by two sets of genes: the genes that act as regulators in signal transduction (transcription factors UVR8 and CP5; protein kinases CDPK and STKL; and signaling regulators MSBP and SPDS) and the functional genes that directly enhance drought-stress tolerance (carbon metabolism for stomatal behavior NADP-ME and PDH); biosynthesis and translocation of glycine–betaine for osmoprotection (CSMO and an APP); scavenging ROS for detoxification (ADOR, ALDH, GST, and SPDS) and stability of proteins and membranes for protecting the cell from injury under drought stress (HSP17.8 and DHN3). In barley, thus, it is thought that drought tolerance is probably achieved through the reestablishment of cellular homeostasis, the enhancement of functional and structural protections of proteins and membranes, and the adjustment of stomata (Guo et al. 2009).

PGPRs are known to impart various benefits to the host plants including the host's ability to withstand drought stress (Yang et al. 2009). Mayak et al. (2004) evaluated the potentiality of bacteria from arid and salty environments for conferring resistance in tomato and pepper plants to water stress. They found that the plant-growth-promoting bacteria *Achromobacter piechaudii* ARV8, having ACC deaminase activity, significantly increased the fresh and dry weights of both the plants exposed to transient water stress. In addition, the bacterium reduced the production of ET by tomato seedlings. Similarly, Cho et al. (2008) reported that root colonization of plants with rhizobacteria *Pseudomonas chlororaphis* 06 induces tolerance to drought stress. Tolerance to drought conferred by the bacteria was correlated with reduced water loss caused by high percentage of stomatal closure. They demonstrated that a volatile metabolite 2R, 3R-butanediol produced by *P. chlororaphis* 06 mediated the stomatal closure and subsequently drought resistance. They observed that the induced drought tolerance required SA, as free SA content is increased following treatment with 2R, 3R-butanediol to the drought-stressed *Arabidopsis* plants, and hence induction of resistance to drought in *Arabidopsis* by *P. chlororaphis* 06 is through a SA-dependent mechanism. Similarly, Figueiredo et al. (2008) used two strains of *P. polymyxa* along with *Rhizobium tropici* singly and in combination in common bean plants under drought stress. They found that coinoculation of bean with all the three bacterial strains resulted in increase in plant growth, nitrogen content, and nodulation and mitigates the phytohormone imbalances caused by drought stress. In another study, Timmusk and Wagner (1999) saw that *A. thaliana* plants, under drought stress, when inoculated with a PGPR strain of *P. polymyxa* became resistant to drought stress as well as the biotic stress generated by the pathogen *Erwinia carotovora*. Further, they observed that mRNA transcriptions of a drought-responsive gene, early responsive dehydration 15 (*ERD15*), was also augmented and they concluded that the gene and/or gene classes associated with plant defenses against biotic and abiotic stress may be coregulated. Similarly, Kohler et al. (2008) showed that inoculation with the PGPR *P. mendocina* alone or in combination with an arbuscular mycorrhizal (AM) fungus, *G. intraradices* or *G. mosseae*, on *L. sativa* L. cv. Tafalla conferred drought resistance to the plants affected by water stress. They found that inoculation with the PGPR and AM fungi also stimulated nitrate reductase and phosphatase activities in lettuce roots and proline accumulation in leaves significantly. Total peroxidase (POX) and catalase (CAT) activities were also increased in response to drought. Their results support the potential use of PGPR as an inoculant to alleviate the oxidative damage produced under water stress. Saravanakumar et al. (2010) recently also showed that the plant-growth-promoting bacterial strain *P. fluorescens* Pf1 increased the vigor index, fresh weight, and dry weight of green gram (*Vigna radiata*) seedlings in vitro apart from conferring resistance to water stress. Quantitative and qualitative analyses of stress-related enzymes indicated the greater activity of CAT and POX as well as greater accumulation of proline in Pf1-treated green gram plants compared to untreated plants.

## 11.7 Concluding Remarks and Future Perspectives

Abiotic stresses are posing a serious threat to crop production under the global climate change scenario. Agricultural experts are endeavoring to find out quicker solution as every year crop production is being affected by a higher degree from abiotic stresses. Expanding geographical area, breeding for abiotic stress tolerance, and microbial-mediated alleviation of abiotic stresses are the prime areas being focused upon at the moment. Among all, microbe-mediated abiotic stress management has gained popularity recently, as it has the potentiality to serve the purpose in an inexpensive manner with high rapidity and efficiency. However, thorough screening and testing of the microbes are needed to be carried out in both in vitro and in vivo before they are released to tackle the problem. Moreover, indigenous microbes should be given priority for their use locally for successfully achieving the goal, as they have the better acclimation ability over an imported strain.

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# Chapter 12
# PGPR for Protection of Plant Health Under Saline Conditions

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## 12.1 Introduction

For centuries, agriculture in arid and semiarid environments has faced an increase in soil salinity. Salinity is one of the major abiotic stress factor limiting plant growth and productivity (Khan and Panda 2008). The total salt-affected land worldwide is estimated to be 900 million ha, 6% of the total global land mass (Flowers 2004). According to the Food and Agricultural Organization (FAO), if corrective measures are not taken, salinization of arable land will result in 30% land loss in the next 25 years and up to 50% by the year 2050 (Munns 2002). Salinity prevents plants from taking up water, exposing them to drought stress. These stresses have an adverse effect on plants, hampering their growth and finally production. Soil salinity is defined as the concentration of dissolvable salts extracted from soil by water (Richards 1954). Natural boundaries imposed by soil salinity also limit the caloric and the nutritional potential of agricultural production. These constraints are most acute in the areas devoted to agriculture; therefore, the urgent need of biological agents (biopreparations) is accepted worldwide. Interest in the use of such biopreparations that replenish the soil, add value, and enhance production and yield in saline conditions is the primary recommendation. Plant growth-promoting

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rhizobacteria (PGPR) are a group of bacteria that can actively colonize plant roots and increase plant growth (Kloepper and Schroth 1978). PGPR may improve plant growth and yield by direct and indirect mechanisms. Indirect mechanisms have been observed with most PGPR strains. Direct mechanisms may act on the plant itself and affect growth by means of plant growth regulators, solubilization of minerals, and fixation of atmospheric nitrogen. The intimate relationship of PGPR with plants is a long-established theory, but the recent issue is to apply these bacteria in agronomy to mitigate salt stress, for which the information is meager. To date, many bacterial genera, such as *Alcaligenes*, *Azospirillum*, *Bacillus*, *Clostridium*, *Klebsiella*, *Pseudomonas*, *Rhizobium*, *Thiobacillus*, *Serratia*, and *Streptomyces*, which are plant growth promoters under saline conditions are being used and tested (Whipps 2001). Various researchers aim to develop salt-tolerant crops to overcome the effect of salinization. This is not an easy and economical approach for sustainable agriculture, whereas microbial inoculation to alleviate salt stress is a better option (Hartmond et al. 1987). The use of PGPR as inoculums in agriculture to alleviate salt stress is the most promising approach to enhance production and yield in salinity-affected regions. The great opportunity for salinity tolerance research now is its ability to be combined with PGPR. The aim of the present review is to point out the status of salinization, its constrains, and to draw the focus on future research strategies for the development of better plant growth promoting inoculants in saline-affected regions, particularly in developing countries, using bioformulations. Bioformulations offer an environmentally sustainable approach to increase crop production and health, contributing substantially in making the twenty-first century the age of biotechnology. Apart from bioformulation, reclamation and improving fertility of stressed sites is another aim to be focused on. The promising approach toward tackling the problem of soil salinity utilizing beneficial microorganism(s) including PGPR will make the greatest contribution to the agricultural economy, if inexpensive and easy to use stress-tolerant strain formulation(s) could be developed.

## 12.2 Salinization and its Constraints

Most of the salty land has arisen from natural causes from the accumulation of salts over long periods of time in arid and semiarid zones (Rengasam 2002). Sodium chloride is the most soluble and abundant salt released; apart from natural salinity, a significant proportion of recently cultivated agricultural land has become saline owing to human activities such as unsuitable agricultural functions (Abolfazl et al. 2009). Repeated use of external inputs destroys the soil biota and reduces the nutritive value of soil, resulting in salinization which causes various stresses in agricultural plants. Soil salinity prevents plant growth and development with adverse effects such as osmotic stress, $Na^+$ and $Cl^-$ toxicity, ethylene production, plasmolysis, nutrient imbalance and interference with photosynthesis, decrease of photosynthetic capacity due to the osmotic stress, and partial closure of stomata (Drew et al. 1990). Plants suffering saline stress present alterations in their homeostasis, mainly because of a reduction in the osmotic potential and an inadequate ionic distribution, which causes a nutritional imbalance.

## 12.3 Deleterious Effect of Salinity on Agricultural Crops

Salinity inhibits plant growth, production and ultimately results in hampering of the agricultural economy of developing countries. Various oxidative and ionic damages in plants are the result of salinization. Absorption of sunlight leads to reactive oxygen species (ROS) formation mainly in the chloroplast either via photoreduction of $O_2$ to form superoxide (the Mehler reaction) or through the interaction of triplet-excited chlorophyll to form singlet-excited oxygen (Asada 2000). ROS are highly reactive and can cause widespread damage to membranes, proteins, and deoxyribonucleic acid (DNA). Although the role of salt stress in inducing oxidative damage has been widely studied (Stepien and Klobus 2005), the extent to which regulatory processes are induced under such conditions and the extent to which variation in their capacity determines the degree of damage incurred by plants exposed to salt have not been widely investigated. The most common effects of salinity on glycophytes are loss of turgor, reduction in growth, resulting in smaller leaves, shorter stature, early senescence, decreased photosynthesis, respiratory changes, loss of cellular integrity, tissue necrosis, and even death of the plant (Cheeseman 1988). The major reason for the detrimental effects of low to moderate salt concentrations is the negative osmotic pressure caused by the salts in the root zone. Depending upon the composition of the saline solution, ion toxicities or nutritional deficiencies may also arise because of the predominance of a specific ion or competitions among cations or anions (Bernstein et al. 1974). The accumulation of high concentrations of $Na^+$ or $Cl^-$ in the leaves generally results in the formation of burn-like lesions that injure cells in transpiring leaves; nutritional deficiencies may be manifested similar to those that occur in the absence of salinity (Vijayan et al. 2008). Calcium deficiency symptoms are common when the Na/Cl ratio is high in soil water. Salt concentration in the old leaves makes them die early (Munns 2002), resulting in early senescence of older leaves and retardation of growth by increasing the production of abscisic acid (ABA) and ethylene (Kefu et al. 1991). An adverse effect of salinity on the rate of photosynthesis was reported in mulberry and in many other woody plants (Tattini et al. 1995). Optimum or threshold range of salinity toleration by most of the agricultural crops has been found to be 40 mM (for glycophytes), but this tolerance could be enhanced by the use of biological approaches such as the use of salt-tolerant PGPRs.

## 12.4 PGPR(s) Conflicting Stress and Ameliorating Salinization

When farmers see their agricultural crops declining in yield and production due to salinity, they often expect a dramatic treatment to make them lush, green, and healthy again so that productivity increases. As a result, they start using chemicals and fertilizers disregarding their future effects. The extensive use of certain synthetic organic chemicals in the past decades has led to a number of long-term environmental problems. One of the recent focuses of research involves implication of PGPR to

combat salt stress. The development of biological products based on beneficial microorganisms can extend the range of options for maintaining the healthy yield of crops in saline habitat. In recent years, a new approach has been developed to alleviate salt stress in plants, by treating crop seeds and seedlings with PGPR. *Achromobacter piechaudii* was reported to be capable of increasing the fresh and dry weights of tomato seedlings grown in the presence of 172 mM NaCl (Mayak et al. 2004). To successfully persist in a changing environment, a microorganism must sense such change and react appropriately. *Chryseobacterium balustinum*, a PGP bacterium, has been reported to promote germination and increase root surface, total nitrogen content, and biological nitrogen fixation (BNF) in *Lupinus albus* seedlings in saline conditions (Gutierrez-Manero et al. 2003). Coinoculation of *C. balustinum* and the *Sinorhizobium fredii* led to a significant increase in the number of nodules and root growth of Osumi soybean plants under saline conditions (Estevez et al. 2009). Moreover, *C. balustinum* can trigger an induced systemic response (ISR) and protect *Arabidopsis* and soybean plants against salt stress. To alleviate the negative effect of soil salinity on soybean, *Bradyrhizobium japonicum* with two PGPR strains, *Bacillus subtilis* and *Sinorhizobium proteamaculans* were inoculated. Total dry weight in all treatments containing PGPR strains increased by about 10% under salinity stress. It also increased by 3.5–4.5% compared to the control treatment (Estevez et al. 2009). A similar result was reported by Vivas et al. (2003), who showed that inoculation with *Bacillus* sp. and its coinoculation with *Glomus* sp. both increased stomatal conductance of lettuce (Han and Lee 2005a) in the saline state. Ashraf et al. (2004) reported that root inoculation of wheat plants with exopolysaccharide-producing bacterial isolates (*Aeromonas hydrophila*, *Bacillus insolitus*, and *Bacillus* sp) native to the salt-affected soils, through increasing extent of soil aggregation around roots, provided a "blanket salt-tolerant cover" to the roots. In consequence, a combination of biotic and abiotic factors (Jonathan et al. 1998), through regulating activities of the stress phytohormones promoted and controlled growth of roots of the inoculated wheat plants grown under salt stress conditions. Many rhizobia isolated from *Acacia*, such as *Sinorhizobium arboris*, turned out to be moderately salt tolerant, capable of growing in 0.3–0.5 M (2–3%) NaCl (Zahran et al. 1994). Various PGPRs including *Rhizobium*, *Pseudomonas*, *Acetobacter*, *Bacillus*, and *Flavobacterium* and several *Azospirillum* can maintain their PGP ability even at high saline conditions.

## 12.5 Mechanisms of Stress-Tolerating PGPR to Promote Plant Growth in Saline Conditions

Any living organisms under stressful condition opt for either of the two strategies: fight or flight. Since plants are sessile, they cannot run away from adverse conditions, so they fight back; their tolerance capacity, growth, and production can be increased with the help of several mechanistic actions of PGPRs as shown in Fig. 12.1.

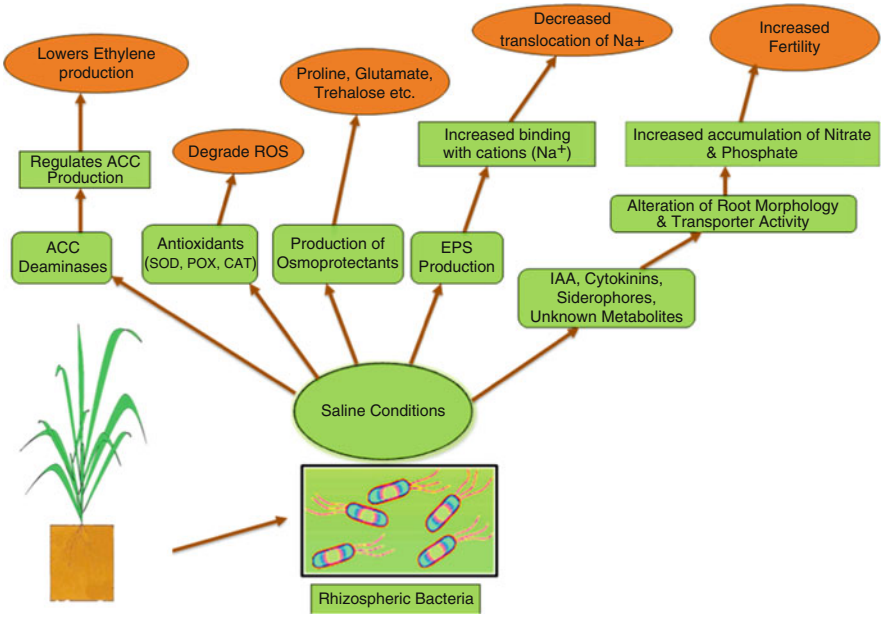


**Fig. 12.1** Increase in salt tolerance and survival of plant in saline habitat elicited by plant growth-promoting rhizobacteria

### *12.5.1 Osmoprotectants or Osmotolerance*

The cytoplasmic membrane of bacteria is permeable to water but forms an effective barrier for most solutes in the medium and metabolites in the cytoplasm. To survive under osmotic stresses, the cells need to adapt by accumulating specific solutes under hyperosmotic conditions and releasing them under hypoosmotic conditions. Such solutes include $K^+$, amino acids (e.g., glutamate and proline), amino acid derivatives (peptides and N-acetylated amino acids), quaternary amines (e.g., glycine, betaine, and carnitine), sugars (e.g., sucrose and trehalose), and tetrahydropyrimidines (ectoines) (Galinski and Truper 1994). These solutes are often referred to as compatible solutes because they can be accumulated to high levels by *de novo* synthesis or transported without interference with vital cellular processes. In fact, many compatible solutes proved to be effective stabilizers of enzymes, providing protection not only against high salt but also against high temperature, freeze–thawing, and drying (Yancey et al. 1982). The bacterial cells may synthesize (some of the) compatible solutes following an osmotic upshock and degrade them following an osmotic downshift, but the initial response is much more rapid if compatible solutes can be taken up from the medium and/or released into the medium via semiconstitutive transport systems. The transport systems involved in osmoregulation can be subdivided into specific uptake systems, stretch-activated channels, specific efflux systems, and aquaporins. After a sudden decrease in

osmolarity or cell decay, accumulated compatible compounds may be liberated into the surrounding environment and subsequently taken up, via an active transport process, by other organisms (plants) under osmotic stress (Miller and Wood 1996). Such organic compounds, taken up and accumulated by organisms (plants) unable to synthesize them *de novo* and able to improve growth under inhibitory osmolarities, are called osmoprotectants. Hence, in natural environments, the concept of osmoprotectant supposes an ecological cycle in which compatible solutes are shuttled from producers to consumers injured by a sudden change in the osmotic strength of their medium. Glycine betaine (GB) provides substantial evidence supporting this concept. GB is synthesized by only a few microorganisms and is actively transported and accumulated as an osmoprotectant by a large variety of cells (Csonka and Hanson 1991). It is generally accepted that an osmoprotectant must be accumulated durably within the cell to be effective. This concept was established on the basis of studies of members of the family *Enterobacteriaceae* and several Gram-positive and Gram-negative bacteria, including *Sinorhizobium meliloti*, which catabolizes most of the osmoprotectants known to date, including GB and ectoine (Talibart et al. 1997). Indeed, ectoine is a compatible solute that is synthesized *de novo* by many halotolerant bacteria. It acts as a potent osmoprotectant for both *Escherichia coli* and *S. meliloti* but displays highly contrasting behaviors in these two bacteria: ectoine is accumulated at high intracellular concentrations in enteric bacteria but is never accumulated by stressed cells of *S. meliloti* (Talibart et al. 1997). This intriguing observation led to the hypothesis that ectoine may belong to a new class of nonaccumulated osmoprotectants that are not detectable by the usual methods, such as natural-abundance $^{13}C$ nuclear magnetic resonance (NMR) spectroscopy. Several studies provide evidence that sucrose acts as a powerful osmoprotectant for *S. meliloti* in media of inhibitory osmolarity. Bacterial osmolytes are accumulated either by uptake from the environment (exogenous osmolytes) or by *de novo* biosynthesis (endogenous osmolytes). Several organic osmolytes found in the environment also function as so-called bacterial osmoprotectants or osmoprotective compounds. These operational definitions usually refer to exogenous solutes that strongly stimulate bacterial growth in hyperosmotic environments (Kempf and Bremer 1998). For example, GB (*N*,*N*,*N*-trimethylglycine) and 3-dimethylsulfoniopropionate (DMSP) are common algal and plant osmolytes that function as exogenous osmoprotectants in numerous bacterial species, including the model organisms *E. coli* and *B. subtilis* (Pichereau et al. 1998). Likewise, proline and ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) also function as powerful osmoprotectants for many bacterial species (Bernard et al. 1993). GB, proline, and ectoine are highly effective osmoprotectants because many bacteria can rapidly accumulate large amounts of these compounds via specific osmoporters that are either induced or activated in hyperosmotic environments (Kempf and Bremer 1998). Various species of *Azospirillum*, *Pseudomonas*, *Bacillus*, and *Rhizobium* have the ability of osmoprotection in saline habitats. Arora et al. (2006), reported accumulation of poly-β-hydroxyl butyrate (PHB) in rhizobial cells as one of the protective measures taken during high saline conditions.

### 12.5.2 Aminocyclopropane-1-Carboxylate Deaminase

The overproduction of ethylene in response to abiotic and biotic stresses leads to inhibition of root growth and, consequently, growth of the plant as a whole. PGPR containing Aminocyclopropane-1-Carboxylate (ACC) deaminase regulates and lowers the levels of ethylene by metabolizing ACC, a precursor of plant-produced ethylene. ACC deaminase has been widely reported in numerous microbial species of Gram-negative bacteria, Gram-positive bacteria, rhizobia, endophytes, and fungi (Jia et al. 1999). ACC deaminase PGPR boosts plant growth particularly under stressed conditions by the regulation of accelerated ethylene production in response to a multitude of abiotic and biotic stresses such as salinity, drought, water logging, and temperature. Mayak et al. (2004) reported that *A. piechaudii* having ACC deaminase activity significantly increased the fresh and dry weights of tomato seedlings grown in the presence of NaCl (up to 172 mM). The bacterium reduced the production of ethylene in tomato seedlings, which was otherwise stimulated when seedlings were challenged with increasing salt concentrations. However, the sodium content of the plant was not decreased whereas the uptake of phosphorous and potassium were slightly increased, which might have contributed in part, to the activation of processes involved in the alleviation of the adverse effect of salt on plant growth. The bacterium also increased the water use efficiency (WUE) in saline environment and helped in alleviating the salt suppression of photosynthesis. Recently, Saravanakumar and Samiyappan (2007) reported that *Pseudomonas xuorescens* containing ACC deaminase activity enhanced the saline resistance in groundnut plants and increased yield as compared with that inoculated with *Pseudomonas* strains lacking ACC deaminase activity. Cheng et al. (2007) have also confirmed that ACC deaminase bacteria conferred salt tolerance onto plants by lowering the synthesis of salt-induced stress ethylene and promoted the growth of canola in saline environment.

### 12.5.3 Auxin Production Stimulating ACC Synthetase

Some PGPR synthesize and secrete the plant growth regulator IAA (indole-3-acetic acid), which can enter plant cells and stimulate root growth. Primary roots treated with wild-type strain *Pseudomonas putida* were on average 35–50% longer than the roots of canola seeds treated with an IAA-deficient PGPR strain (Patten and Glick 2002). In addition to stimulating plant growth as plant growth regulator, IAA can also stimulate ACC synthase to produce more ACC, which can be transformed into ethylene by ACC oxidase (Mayak et al. 2004). Conversely, the simultaneously produced ACC deaminase can hydrolyze ACC and inhibit ethylene production. As a consequence, the final effect on ethylene production or root growth depends on the balance of the IAA and the ACC deaminase produced in concert by *P. putida*. In a recent study, Gravel et al. (2007) used *P. putida* to alleviate the detrimental effect of excess exogenous IAA on tomato seedlings, possibly through repressed ethylene production resulting from microbial

degradation of IAA in the rhizosphere (and the resultant decrease in ACC)/or by ACC deaminase activity present in both microorganisms. In conclusion, plant responses to IAA exuded from ACC deaminase-producing PGPR vary with plant species, root growth rates, and its balance with ACC deaminase activity. The decrease in ethylene levels by ACC deaminase not only downregulates the plant stress responses but also relieves the ethylene-repressed auxin responses factor (ARF) synthesis, leading to plant growth promotion resulting from both stress alleviation and growth stimulation. However, with the increase in ARF synthesis, ACC synthase is also stimulated to produce more ACC and ethylene, which represses the ARF synthesis. In this way, ethylene limits its own production. PGPR synthesize and secrete IAA, which gets adsorbed on the seed or the root surface of the plants (Fallik et al. 1994), from tryptophan and other small molecules present in seed or root exudates (Whipps 1990). Some of the newly synthesized IAA is taken up by the plant and, in conjunction with the endogenous plant IAA, can further stimulate plant cell proliferation and elongation. In the meanwhile, IAA stimulates the activity of the enzyme ACC synthetase to produce ACC. According to Glick et al. (1998), a significant portion of ACC may be exuded from plant roots or seeds and taken up by the soil microbes or hydrolyzed by the vital microbial enzyme ACC deaminase to yield ammonia and α-ketobutyrate. The uptake and subsequent hydrolysis of ACC by microbes decreases the amount of ACC outside the plant. Furthermore, the equilibrium between the internal and the external ACC levels is maintained through exudation of more ACC into the rhizosphere. Soil microbial communities containing ACC deaminase activity cause plants to biosynthesize more ACC than they would otherwise need and stimulate ACC exudation from plant roots, while providing microorganisms with a unique source of nitrogen (ACC), and consequently, the growth of microorganisms containing ACC deaminase is accelerated in the close vicinities of plant roots as compared to the other soil microorganisms.

### *12.5.4 Phosphate Solubilization*

Although phosphate-solubilizing microorganisms are abundant in the soil and in the rhizosphere of most plants, their application in saline soils as a biofertilizer or bioconverter as a means of solubilizing fixed phosphorus has not yet been successfully practiced in full-scale agricultural applications. Some soil microorganisms have been shown to be effective in releasing phosphate from bound inorganic soil phosphate through solubilization and mineralization. Microorganisms that convert insoluble phosphates into soluble forms are termed phosphate-solubilizing microorganisms (PSMs). This is achieved through the acidification, chelation, ion exchange reactions, and production of low molecular weight organic acids such as gluconic acids. These processes have the potential to decrease the use of phosphate fertilizer by mobilizing the fertilizer constituents present in the soil and at the same time reducing costs and improving crop yields (Chaiharn and Lumyong 2009). As the establishment and performance of PSMs are highly likely to be affected by

environmental factors, especially under stressful conditions (Nautiyal 1999), it is essential to isolate microorganisms from the relevant microbial niches to maximize their chances for use as a means of improved phosphate-solubilizing ability in the field. The general principle of stress adaptation can be brought about when a bacterium which is exposed to sublethal stress can become more resistant to subsequent applications of the same stress (or in some instances, to a different stress). Strains isolated from acid soils have the potential to solubilize phosphate under stressful conditions (high salt and pH). The stress-tolerance potential of phosphate-solubilizing strains isolated from acidic soils has been reported earlier (Thakuria et al. 2004). Baldani et al. (2000) inoculated phosphate-solubilizing bacteria, *Herbaspirillum seropedicae* and *Burkholderia* sp., to the soil and showed that these bacteria increased the weight of crop 1.5–21% over uninoculated controls under saline conditions. Bashan and Holguin (1997) showed the beneficial effect of *Azospirillum* on plants increasing growth, yield and also showed improved solubilization of phosphorus and other inorganic nutrients. *Bacillus* sp. and *Azospirillum* sp. have similarly been used as a biofertilizer for various crops. Phosphate solubilization by *Pseudomonas* strains isolated from the cold deserts of the Himalayas at high levels of alkalinity, salinity, calcium salts, and desiccation indicates that these strains have evolved the ability to solubilize phosphate under stressful milieu and that some fluorescent *Pseudomonas* strains with the potential to solubilize phosphates under high stress conditions are well adapted to the cold desert environment.

### *12.5.5 Biological Nitrogen Fixation*

BNF in deserts is mediated mainly by some heterotrophic bacteria, associative bacteria, cyanobacteria, actinorhizal plants, and legumes. The $N_2$ fixed by heterotrophic free-living bacteria is of minor importance as a mechanism for N input in arid soils (Zahran et al. 1995). Associative dinitrogen-fixing bacteria may be potentially important in supplying N to associated grasses in arid lands (Wullstein 1989). An allied concern with regard to soil moisture in the reclamation of environmentally impacted sites, e.g., arid lands, can be the effect of salinity on the survival of rhizobia in soil systems. Salinity affects the survival and distribution of rhizobia in soil. Some of the rhizobia are tolerant to higher levels of salts, up to 1.8 M NaCl. These salt-tolerant rhizobia underwent morphological and metabolic changes, as well as structural modifications, to cope with and adapt to salt stress. Recent reports support the finding that some rhizobia have the potential to form a successful symbiosis with legumes under salt stress. Most of the work done emphasizes the previous understanding about the sensitivity of the host legumes to salt stress. Therefore, salt-tolerant legume genotypes should be selected. In the past few years, rhizobia which form successful $N_2$-fixing *Rhizobium*–legume symbioses under salt stress (up to 120–150 mM NaCl) have been selected. In a recent study reported by Mashhady et al. (1998), *R. meliloti* formed a successful symbiosis with *Medicago sativa* under saline conditions (100 mM NaCl). Also, recent reports point

out that those rhizobia from naturally growing tree legumes in the deserts are prominent and effective salt-tolerant rhizobia. In fact, *Rhizobium*–legume symbioses are currently the most important nitrogen-fixing systems, which may have the potential to increase N input in arid lands. The leguminous plants include species or varieties which are extremely well adapted to the drastic conditions of arid lands. The role of BNF as a nonpolluting and more cost-effective way to improve soil fertility compared to other ways, such as fertilizers and sewage sludge with their high levels of toxic metals, is well established. The *Rhizobium*–legume symbiosis is superior to other $N_2$-fixing systems with respect to $N_2$-fixing potential and adaptation to severe conditions. Several symbiotic systems of legumes, which are tolerant to extreme conditions of salinity, alkalinity, acidity, drought, and fertilizer and metal toxicity, have been identified. These associations might have sufficient traits necessary to establish successful growth and $N_2$ fixation under the conditions prevailing in arid regions. In fact, the existence of *Rhizobium*–tree legume symbioses, which are able to fix appreciable amount of $N_2$ under arid conditions, is fascinating. These symbioses represent the best source of the ideal fertilizer in arid regions and therefore command great interest as the focus of replenishment and rehabilitation of arid regions (Zahran 1999). Kumar et al. (1999) reported isolation of salt-tolerant strains of *Mesorhizobium loti* from *Acacia* showing efficient nitrogenase activity in saline condition.

### *12.5.6 Exopolysaccharides*

The PGPR strains can produce bacterial exopolysaccharides (EPSs) which bind cations including $Na^+$ and decrease the content of $Na^+$ available for plant uptake, thus helping alleviate salt stress in plants (Ashraf et al. 2004). Thus, PGPR strains markedly increased the tolerance of maize plant by lowering the sodium concentration and consequently the $Na^+/K^+$ ratio (Han and Lee 2005a). It was observed that the $Na^+$ content of soybean grown under saline conditions decreased due to inoculation of EPS-producing strains. Inoculation not only reduced the $Na^+$ and $Cl^-$ concentration in maize but also induced a marked and progressive increase in N, P, and K concentration under salinity stress. Vivas et al. (2003) reported that N, P, and K concentration in lettuce inoculated by *Bacillus* sp. under stress conditions were increased by about 5, 70, and 50%, respectively, over control. Bacterial EPS(s) in the soil ecology system plays a significant role in soil aggregation and soil adhesion. PGPR strains, especially EPS-producing bacteria, can induce soil salinity tolerance and growth promotion in soybean seedlings (Bezzate et al. 2000). EPS-producing bacteria under soil salinity conditions have been found to restrict $Na^+$ uptake by wheat roots (Ashraf et al. 2004). PGPR strains, especially EPS-producing bacteria, can induce soil salinity tolerance and growth promotion in soybean seedlings under greenhouse conditions. It may be postulated that increasing the population density of EPS-producing bacteria in the root zone could decrease the content of Na available for plant uptake, thus helping to alleviate salt stress in plants. It is

supposed that bacterial EPS protects bacteria from desiccation by altering their microenvironment (Roberson and Firestone 1992). Inoculation of plant roots with an EPS-producing *Rhizobium* strain was reported to improve soil structure (Ross et al. 2000). Altogether, an additional explanation for the improved survival of the drought-stressed seedlings with compatible solutes could be that the increased rhizobial population having enhanced EPS production and/or other metabolic activities colonizes roots and rhizosphere, subsequently protecting roots from the drought stress.

### *12.5.7 Antioxidative Activity*

During saline conditions, generation of ROS such as the superoxide radical ($O_2^-$), hydroxyl radical ($OH^-$), and hydrogen peroxide ($H_2O_2$) alter antioxidant activity. ROS causes oxidative damage to biomolecules such as lipids and proteins and eventually leads to plant death (Del Rio et al. 2003). PGPR such as *S. proteamaculans* and *Rhizobium leguminosarum* produce antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT) and nonenzymatic antioxidants such as ascorbate, glutathione, and tocopherol. Ascorbate peroxidase (APX) is part of the scavenging cycle and catalyzes the reaction of ascorbic acid with $H_2O_2$, while glutathione reductase (GR) catalyzes the regeneration of ascorbic acid (Han and Lee 2005b). To understand the protective action of antioxidants against salinity stress, lettuce plants were treated with PGPR strains followed by measurement of the level of antioxidant activity. Inoculation with PGPR strains under salinity stress decreased enzyme activity with increasing salinity stress. Ruiz-Lozano et al. (2001) reported that mycorrhizal lettuce plants showed increased SOD activity under drought stress and this was correlated to plant protection against drought. Stress resistance in plants has been related to more effective antioxidant systems (Bor et al. 2003). Detoxification of cellular $H_2O_2$ through the activity of the Asada–Halliwell scavenging cycle is an important element of plant defense mechanisms against ROS (Lee and Lee 2000). The above-mentioned mechanisms are monitored in PGPR and those strains showing tolerance in high salinity conditions are screened and its biopreparations as bioformulation are applied in the field.

## 12.6 Bioremediation by Bioformulation

Bioformulations are best defined as biologically active products containing one or more beneficial microbial strains in easy to use and economical carrier materials. Usually, the term bioformulation refers to preparations of microorganism(s) that may be a partial or complete substitute for chemical fertilizers/pesticides (Arora et al. 2010). By the end of the nineteenth century, the practice of mixing "naturally inoculated" soil with seeds became a recommended method of legume inoculation in the USA (Smith 1992). A decade later, the patent for first rhizobial biofertilizer

"Nitragin" was registered in USA (Nobbe and Hiltner 1896). The erratic performances of bioinoculants under field conditions have raised concerns about the practical potential offered by microbial releases into soil (Arora et al. 2007). Although much is known about the survival of bacteria within the protective environment of an inoculant carrier, little is known about the stresses that bacteria must endure upon transfer to the competitive and often harsh soil environment (Heijnen et al. 1992). Inoculants have to be designed to provide a dependable source of beneficial bacteria that survive in the soil and become available to the plant. Mixed bacterial inoculants surviving in stress condition have to be developed so that these formulations encapsulate the living cells, protect the microorganisms against many environmental stresses, release them to the soil, and ultimately enhance crop yield. Use of stress-tolerating strains of PGPR biopreparations either as aqueous suspensions or in bioformulations of sawdust, rice husk, tea waste, and talc-based bioformulants promoted growth in agricultural crops even under saline conditions (Ross et al. 2000). A successful PGP agent must be an aggressive colonizer with better competence and storage conditions in its formulation and use. Encapsulation enables slow and controlled cell release from the immobilization matrix of the alginate gel bead upon inoculation into soil, facilitates in establishing the stable PGPR population, and minimizes the possibilities of decline in population over time. The versatile nature of humic acid in the soil environment also extends the prospects of this encapsulation technique to the bioremediation of contaminated soil. The development of novel formulations is a challenging task but, regardless of whether the product is new or improved, the product must be stable during storage and transportation, easy to handle and apply, enhance the activity of the organism in the field, and be cost-effective and practical (Young et al. 2006). Biotech Consortium India Limited (BCIL) is seeking companies interested in licensing the bacterial strains and formulation of biofertilizer for effective application to saline soil. These strains have phosphate-solubilizing and nitrogen-fixing properties for effective application in a saline soil environment. Research has been constantly focusing on exploring the microbial diversity of the saline coastal regions and isolating efficient saline-tolerant plant growth promoters that are well suited for the saline soils of the coasts. A formulation with individual strains of salt-tolerant *Phosphobacteria*, *Azospirillum*, *Rhizobium*, etc. has been developed and tested through field trials. The field trials showed increased crop productivity between 15 and 20% at a salinity level of up to 4 $dSm^{-1}$. Saline-tolerant bioformulations have potential applications for sustainable agriculture in the regions where the soil electrical conductivity is higher than the normal (Paul et al. 2006).

## 12.7 Molecular Approaches

Transgenic plants have been developed from a single plant cell. In view of a report by the International Service for the Acquisition of Agro-Biotech Applications (ISAAA) the top five countries growing transgenic crops in 2005 were USA,

Argentina, Brazil, Canada and China (ISAAA 2006). However, transgenic approach is being effectively pursued by plant scientists these days not only to improve quality and yield but also to increase tolerance to biotic and abiotic stresses in number of crops. Instead of improving salt-tolerance traits in various crops through genetic engineering plant biologists have focused much on genes that encode: ion transport proteins, compatible solutes, antioxidants, heat shock and late embryogenesis abundant proteins and transcription factors for gene regulation (Ashraf et al. 2008). Engineering plants for over expression of genes for different types of antiporters has recently gained a ground as an effective plant salt tolerance. In transgenic lines of different crops with engineered genes for different types of transports and osmoprotectants are listed (Table 12.1). Microbial genes have often been used to engineer traits implicated in stress tolerance, transgenic tobacco plants carrying bacterial gene coding for mannitol-1-phosphatedehydrogenase (*mt1D*) accumulated mannitol in their cytoplasm and were with enhanced biomass growth under salt stress (Tarczynski et al. 1992). Transgenic plants transformed with *imt1* gene coding for myo-inositol-omethyltransferas enzyme taking part in the biosynthesis of ononitol were more drought and salt tolerant than the wild-type plants (Sheveleva et al. 1997). Trehalose-6-phosphate synthase is a key enzyme for trehalose biosynthesis in yeast, encoded by the structural gene *TPS1*. The gene of *Saccharomyces cerevisiae* was constitutively expressed in transgenic potato plants. The *TPS1* transgenic potato plants showed significantly increased drought resistance. Abiotic stress tolerance was successfully manipulated in rice by overexpression of *E. coli* trehalose biosynthetic genes (*otsA* and *otsB*) (Garg et al. 2002). Recently trehalose synthase (*TSase*) gene of the edible wood fungi *Grifola frondosa* was reported to be expressed in tobacco. The transformants accumulated higher levels of products of trehalose compared to many other known transgenic plants (400-fold higher than tobacco cotransformed with *E. coli TPS* and *TPP*, twofold higher than rice transformed with a bifunctional fusion gene (*TPSP*) of the trehalose-6-phosphate (T-6-P) synthase (TPS) and T-6-P phosphatase (TPP) of *E. coli*, and 12-fold higher than tobacco transformed with yeast *TPS1* gene). Tobacco and potato were first transformed with the gene of the bacteria *B. subtilis*, coding for the levansucrase enzyme. The same *SacB* gene of *B. subtilis* was used for sugar beet transformation (Smith Pilon et al. 1999). Continued efforts in the identification and description of stress-tolerant taxa and physiological and molecular studies to understand their tolerance mechanisms are therefore justified. Identification of regulatory genes and transcription factors involved in stress-inducible expression of osmoprotectant biosynthetic pathways are also of great interest. Tools such as vectors for multiple gene transfer (von Bodman et al. 1995), stress-inducible promoters and efficient selectable markers will also need to be developed and evaluated. The gene products involved in ion homeostasis have been identified by the use of yeast model systems and by analyzing mutants altered for salt sensitivity (Liu et al. 2000).

There are large numbers of specific proteins reported in various genera of PGPR that showed increase in their level of expression upon adverse conditions such as salt. In a postgenomic era, proteomics is one of the best strategies used to reveal the

**Table 12.1** Transgenic lines of different crops with engineered genes for different types of transports and osmoprotectants

| Gene engineered | Transgenic host | Sources | Traits improved | Growth conditions for testing | Growth improvement in transgenic lines under salt stress |
|---|---|---|---|---|---|
| *EhCaBP* for calcium binding protein | Tobacco (*Nicotiana tabacum* L) | *Entamoeba histolytica* | Enhanced rate of germination and growth | Growth room | About 20–37% increase in dry weight |
| Plasma membrane $Na^+/H^+$ antiporter sodium 2 (*SOD2*) | Rice (*Oryza sativa*) | Yeast (*Saccharomyces pombe*) | Accumulated higher $K^+$, $Ca^{2+}$, $Mg^{2+}$ and lower $Na^+$ contents and enhanced net photosynthetic rate (100%) and decreased MDA level | Greenhouse | 32.5% increase in shoot fresh weight at 150 mM and 57.1% at 300 mM NaCl |
| Trehalose-6- phosphate synthase (TPS1) | Tomato (*Lycopersicon esculentum* L) | Yeast (*Saccharomyces cerevisiae*) | Higher trehalose content (0.15 mg/g), improved plant growth and higher chlorophyll and starch content in transgenic plant at 100 Mm NaCl as compared to wild types | Greenhouse | Improved seed dry weight above 23.2% |
| Choline dehyrogenase (*bet A*) | Cabbage (*Brassica oleracea*) | *Escherichia coli* | Improvement in plant biomass, chlorophyll content and relative water content and less negative water potential and osmotic potential of transgenic plant compared to wild type at 150 and 300 mM | Greenhouse | Improved plant biomass about 21.3% at 150 mM and 20% at 300 mM NaCl |
| bet gene clusture i.e., *betA* (choline dehydrogenase), *betB* (betaine aldehyde dehydrgenase), *bet I* (putative regulatory protein) *and bet T* (choline transport system) | Freshwater cyanobacterium (*Synechococcus* sp. PCC7942) | *E. coli* | Transgenic plants showed improves cell growth even at 0.4 M NaCl and exhibited higher activities of PSl and PSll at 200 Mm NaCl than those in wild plants | Culture medium | Cell number increased 125.5% at 0.4 M NaCl |

| | | | | | |
|---|---|---|---|---|---|
| Mannitol-1-phosphate dehydrogenase (*mtID*) | *A. thalina* L | *E. coli* | Mannitol was synthesized in transgenic plant but not in wild type plant. Germination percentage, fructose, inositol and proline content of transgenic plant was increased under 200 mM NaCl | Growth room | Transgenic seeds containing mannitol germinated upto 400 mM NaCl while in wild plants germination ceased at 100 mM |
| L-2,4- diaminobutyric acid acetyltransferase (*ectA*), L-2,4-diaminobutyric acid transaminase (*ectB*) and L-ectoine synthase (*ectC*) | Tobacco (*N. tabacum* L.) | *Halomonas elongate* | Improved accumulation of ectoine, increased tolerance to hyperosmotic stress and better growth in transgenic plants | Tissue culture room | Transgenic plants had normal growth pattern at 500 mM NaCl while in wild type plants it was inhibited |
| Myo–Inositol O-methyltransferase (ImtI) | Tobacco (*N. tabacum* L.) | *Mesembryanthemum crystallinum* | Improvement in methylated inositol D-ononitol content, photosynthetic $CO_2$ fixation and stable accumulation in transgenic plants at 250 mM NaCl | Hydrophonics in greenhouse | Improved growth at 250 mM NaCl |

dynamic expressions of whole proteins in cells and their interactions. Due to its high-resolution, two-dimensional PAGE, combined with high-throughput mass spectrometry and bioinformatics, it is widely used for protein separation and identification, which is considered sufficiently discriminating to allow the unique identification of unknown proteins (Shen et al. 2002). Identification of differently displayed proteins could be used to ascertain the genes responding to relative physiological actions and clarify the functions of genes (Paul et al. 2006).

## 12.8 Future Prospects and Challenges

In many cases, the convincing molecular evidences for stable integration of the genes coincide with very low concentrations of the relevant compounds which are difficult to be related to the occurring stress tolerance. The relatively minor impact of the organic osmolytes on cellular water relations and the controversy about osmotic adjustment of the transgenic plants leads to the presumption that these substances mainly participate in stabilization and protection. Thus, it appears that the discussion is not about the efficiency of the gene transfer but more for the reasonable explanation of the positive results obtained. In this respect, stable transgenic lines resulting from well-established commercial cultivars provide further opportunities for elucidation of the complex role of osmoprotectants in abiotic stress tolerance and the practical breeding. The bottom line of every inoculation technology is its successful application under agricultural and industrial conditions. The microbial formulation and application technology are crucial for the development of commercial salt-tolerant bioformulation effective under saline conditions. Microbial mixtures as multitasking inoculants and stress protecting bioformulations are one alternative to overcome inconsistent in vivo effects. The study by Adesemoye et al. (2008), showed that inoculation with mixed strains were more consistent than single strain inoculations. Another future possibility for efficient inoculation, valid for plants propagated from tissue culture is to inoculate the salt-tolerating PGPR into the plant cell suspension and regenerate embryos and eventually stress tolerating plants. These plants will probably be inoculated from their onset as this can be done in a tissue culture laboratory accustomed to sterile and precise work, this will result in even inoculation of the plants (Alcaraz-Melendez, Center for Biological Research of the Northwest, La Paz, Personal communication). A potentially promising future application could be the enhancement of drought tolerance or salt tolerance of transgenic plants by identification of enzymes and genes involved in the synthesis of novel osmoprotectants found in stress-tolerant microorganisms that can be expected to provide more such opportunities for stress tolerance engineering in agricultural crops (Apse and Blumwald 2002). Further work should be done to highlight the mechanisms of stress tolerance by PGPR in salinized conditions and to uplift the status of stress tolerance mechanisms in plant.

## 12.9 Conclusion

Soil salinization is the combined result of anthropogenic environmental impact and climatic characteristics limiting natural vegetation processes, soil and plant functioning. Understanding the mechanisms of salt tolerance in PGPR is expected to contribute to the long-term goal of plant–microbe interactions for exploitation of salinity-affected regions for crop productivity. The present review recognizes and suggests the role of salt-tolerating plant-growth promoting microorganisms ST-PGPM and their biopreparations or bioformulations as a nonpolluting and more cost-effective way to improve production in a human-deteriorated environment. In general, they could contribute to reduce the burden of soil nutrient loss in arable lands, to counteract part of the negative effects of saline stresses on plant growth, and help plants to avoid or minimize contaminants uptake. These results encourage new investigations on their application in agriculture.

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# Chapter 13
# PGPR as Inoculants in Management of Lands Contaminated with Trace Elements

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## 13.1 Introduction

The rhizosphere is a special area in the immediate vicinity of the root, where a great intensity of organic molecules exchange is observed due to root exudate-driven metabolic activities of soil microbial populations. As an important factor in this zone, PGPR possesses many abilities, some considered as tools for solving environmental problems. They are beneficial soil bacteria, which may facilitate plant growth and development directly and indirectly (Glick 1995). Direct stimulation may include benefits to the plants such as fixed nitrogen, phytohormones, sequestered iron by bacterial siderophores, and soluble phosphate, while indirect plant stimulation is attributed to biocontrol (antagonistic interrelations with soil-borne phytopathogens) (Glick and Bashan 1997). The inoculation of bacterial strains producing exopolysaccharides enabled plants to withstand the initial effects of salts and the osmotic stresses and it also benefited the inoculated plants in terms of a better exploitation of the soil nutrients and through providing an increased extent of rhizodeposits in the soil for gearing up of the soil microbial activities (Ashraf et al. 2006). The aim of this chapter is to provide an overview of the role of PGPR inoculations in soils contaminated with trace elements.

The presence of trace elements in arable soils is normal. Some of them do not have any nutritional value, while others are of vital indispensability for living

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beings. By definition, trace elements are chemical components that naturally occur in soil, plants, and wildlife in minimal concentrations. Also known as trace minerals, trace elements are necessary for the optimal development and metabolic functioning of all living beings. In terms of humans, proper cell metabolism, effective immune function, and healthy reproduction are dependent on a total of 72 trace elements. Unfortunately, even though the agricultural industry takes measures to ensure adequate nutrients content in soil, the concentration of essential elements in some crops has diminished. In fact, the only natural food source that still represents all of these essential trace elements is seafood.

Several trace elements are essential for energy generation, hormone regulation, and neurotransmissions in the brain. These include copper, iodine, and iron. Other trace elements, such as selenium and magnesium, are required to utilize nutrients such as calcium and vitamin C. Zinc, in particular, may be considered the workhorse of the essential trace elements as it is involved in more than 200 enzymatic functions in the human body, plants, microbes, etc. A less familiar member of this group, molybdenum, has been linked to the body's ability to eliminate toxins.

A problem occurs when their concentration increases many times and becomes dangerous for living beings. At the same time, the accumulation of heavy metals, such as Cd and Pb, and metalloids, such as, **As** in edible parts of the plants, are very dangerous because of the health problems that they originate in humans and animals, as final depots of the food chain. Phytoremediation is an environmentally friendly technology that could solve this contamination problem using different strategies, depending on the type of the problem: phytostabilization, phytoimmobilization, phytoextraction, phytovolatilization, etc. Microorganisms in general and bacteria in particular interact with other living beings in soil and especially with plants and pathogens, and of course, with the soil properties, including trace elements. They are of vital importance in nutrient cycles, are widespread in all types of soils and ecosystems, and depending on the conditions, may influence one or another pathway of a compound or element conversion; thus, soil rhizobacteria play a very important role in each one of the above-mentioned strategies.

Elevated concentrations of trace elements in soils affect microbial growth and development, community density and diversity, and activities and processes (Bååth 1989; Giller et al. 1998). To survive, microorganisms in general and PGPRs in particular, have to adapt to the abiotic stress conditions. One prerequisite for further development and survival in those habitats is the resistance to trace elements, which often is a multielemental resistance. Various authors discuss the effects of trace elements on microorganisms, their community structure, and mechanisms of toxicity and resistance (Giller et al. 1998; Bååth 1989; Nies and Silver 1995; Ernst 1998). Generally, those effects are expressed as reduction of density and biomass of microorganisms (Bååth 1989; He et al. 2005), and reduction of activity (soil respiration and enzymes) (Akerblom et al. 2007; Doelman and Haanstra 1979; He et al. 2005) and community structure (assessed using denaturized gradient gel electrophoresis (DGGE) or r- and K-strategists) (Blagodatskaya et al. 2007). In addition, high trace-element concentrations in soils also have been reported

to cause increase of ethylene production (Rodecap et al. 1981; Safronova et al. 2006), thus decreasing root growth and worsening the negative effects of toxicity.

Microorganisms are responsible for more than 80% of soil biological activity and transformation of at least one-third of the elements in the periodic table. These transformations consist of either valence change (redox processes) or state changes (solid, liquid, or gaseous) and play a basic role in many biochemical cycles. In this sense, a good example is the conversion of atmospheric $N_2$ in organic form (ammonium) by nitrogen-fixing bacteria. It is known that more than 40 elements are affected by the activity of microorganisms including macronutrients (C, N, S, O, H, and P), oligoelements (Mg, Fe, Ca, and K), and trace elements (Mn, Mo, Cu, Ni, Se, and Zn). All of them interact in diverse chemical processes, structures, and cell functions. Their transformation could be a result of processes of assimilation or incorporation in cell biomass, of dissimilation to obtain energy, or detoxification (Stolz and Oremland 1999).

During the past years, there has been seen an increasing interest in the role of PGPR in phytoremediation of trace element-contaminated soil, especially by inoculation of these bacteria (bioaugmentation) (Fig. 13.1). Recently, a number of reviews have been published treating the role of PGPR in phytoremediation of these soils (Kidd et al. 2009; Wenzel 2009; Lebeau et al. 2008; Karami and Shamsuddin 2010; Jing et al. 2007). The particular interest regarding this is related to the ultimate findings that rhizobacteria tolerant to metals play an important role in the alleviation of stress to the plants, interacting with them in the stress environment, thus stimulating root and shoot growth, suppressing soil-borne plant pathogens, and influencing trace element behavior in such soils (Belimov et al. 2007; Di Gregorio et al. 2006; Egamberdieva 2009; Penrose and Glick 2001). Later phenomenon, depending on the conditions, may be understood as immobilization or mobilization of trace elements as very promising strategies.

## 13.2 Abilities that Characterize the Rhizobacteria as Plant Growth Promoters

Free-living rhizobacteria that are beneficial to the plants are often named plant growth promoters (Kloepper et al. 1989). Different genera such as *Pseudomonas*, *Brukholderia*, *Azotobacter*, *Kluyvera*, *Enterobacter*, etc. (Glick 1995; Honma and Shimomura 1978; Burd et al. 1998; Belimov et al. 2002) are included in this group of bacteria. PGPR may affect plant growth and tolerance directly or indirectly in several ways: nitrogen fixation, providing plant with mineral N; sequestration of iron through siderophores and leaving it to the plant; phytohormones synthesis, mainly indoleacetic acid (IAA), which stimulates plant growth; solubilization of elements, such as phosphorus, and its use by the plant metabolism; and synthesis of enzymes, which regulate plant growth and development (Kloepper et al. 1989; Glick et al. 1994; Davison 1988). All these abilities may be a key factor in abiotic stress conditions such as those produced by heavy metals.

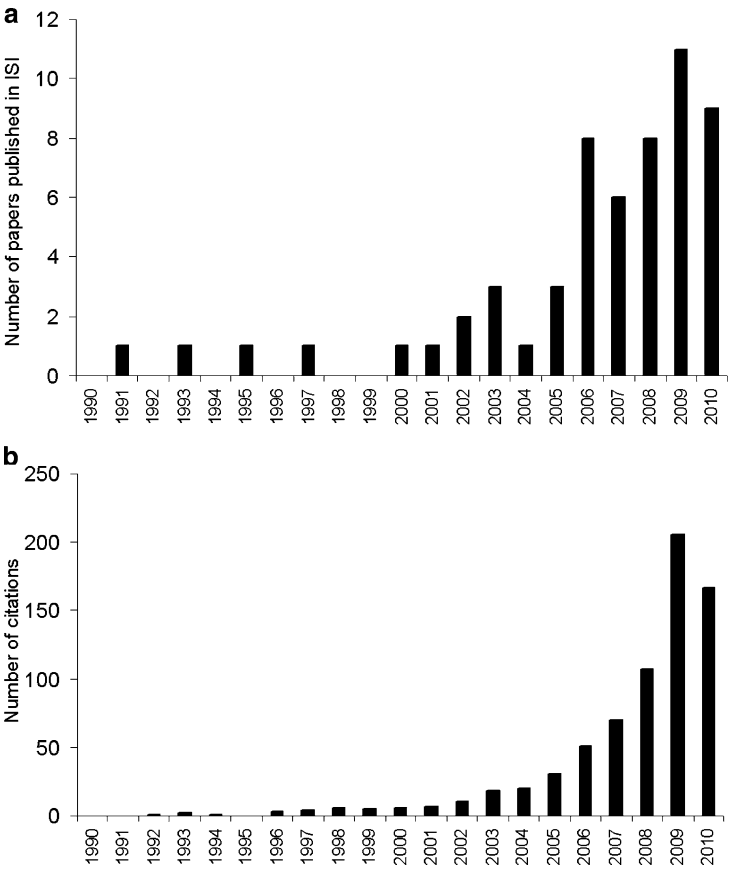


**Fig. 13.1** Publications featuring the use of PGPR in the phytoremediation of trace element-contaminated soils by inoculation (**a**) and their correspondent citations (**b**). Source: ISI Web of Science; search parameters: ("PGPR" or "rhizobacteria") and ("metal*" or "element*") and ("inoculat*" or "bioaugmentation") and ("soil*")

### *13.2.1 Possession of ACC Deaminase*

Stress factors are widespread in agriculture. One of the most common is the stress produced by heavy metal contamination. In such conditions, plants, mainly dicotyledons, respond with elevated production of endogenous phytohormone ethylene, inhibiting root growth and seed germination (Jackson 1991; Lynch and Brown 1997). Artificially, the symptoms could be overcome by application of aminoethoxyvinylglycine (AVG), which an inhibitor of ethylene biosynthesis (Hall et al. 1996). Thus, plant root continues growing in stress conditions.

Some PGPR contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the immediate precursor of the plant hormone ethylene (Glick et al. 1995; Burd et al. 1998; Honma and Shimomura 1978),

stimulating root growth. In the proposed model (Glick et al. 1998), important quantities of ACC are exuded by the roots and subjected to reduction producing ammonia and α-ketobutyrate. Thus, the ACC concentration in root surroundings is decreased and the plant tries to maintain the equilibrium by exuding more ACC in the rhizosphere, lowering the internal levels. ACC exudation is stimulated by the ACC deaminase-containing bacteria, which can utilize the compound as a unique source of nitrogen. Continuous exudation leads to acceleration of growth of the population of bacteria containing ACC deaminase in the immediate vicinity to the roots. The main result is that the internal ethylene biosynthesis level is reduced as a consequence of lower concentrations of ACC (Glick et al. 1998).

### *13.2.2 Production of Indole-3-Acetic Acid*

The production of phytohormones in plant rhizosphere by PGPR stimulates increase in density and elongation of root hairs. According to Vessey (2003), PGPR strains express their promotion effect due to phytohormone production. Auxin or IAA is an important phytohormone produced by PGPR. Commonly, IAA is believed to increase root growth and length, enhancing root surface area, thus enabling greater access to nutrients by the plant, enhancing tolerance and probably, the accumulation during phytoextraction.

### *13.2.3 Siderophores Production*

Microorganisms produce and secrete compounds named siderophores to sequester iron in the environment. They are small molecules (most of them are less than 1,000 Da). Siderophores consisted of lateral chains and functional groups that possess ligands with strong affinity to bind to the ferric ion (Neilands 1995). They are classified as catecholates, hydroxamates, and α-carboxylates, depending on the nature and binding sites with the iron (Winkelmann 2002). In spite of this, siderophores produced by *Pseudomonas* species (typically PGPR) are classified as "mixed," e.g., pyoverdins contain hydroxamate and catecholate functional groups (Meyer and Stintzi 1998). The siderophores are produced as free ligands that become complexed with iron as released into the extracellular environment. A ferric complex is then transported into the cell via specific transport receptor proteins. Inside the cell, the siderophore is freed from the transport receptor and again released outside as free ligand and can repeat the cycle (Kuhad et al. 2004). The secretion of siderophores may be assayed easily by a simple and universal method that is a modification of the method of Schwyn and Neilands (1987) by Pérez-Miranda et al. (2007).

### 13.2.4 Interactions of Rhizobacteria and Plant Pathogens in Soil

Although plant growth in agricultural soils is influenced by both abiotic and biotic factors, physical and chemical approaches are predominantly used to manage the soil environment and increase crop yields. The application of microbial products for this purpose is less common despite the enormous attention centered on their role in reducing plant diseases. Significant control of plant pathogens and enhancement of plant development have been demonstrated by PGPR in the laboratory and in the greenhouse conditions. Rhizospheric bacteria can influence plant growth by direct mechanisms such as production of phytohormones (Tsavkelova et al. 2006) and improving nutrient uptake and indirect mechanisms such as an antagonistic activity against harmful insects (Antoun and Prevost 2005), plant pathogenic bacteria, fungi, and nematodes (Oostendorp and Sikora 1989, 1990; Hasky-Günter et al. 1998; Frankenberger and Arshad 1995; Kim et al. 1998; Kumar et al. 2009). PGPRs that indirectly enhance plant growth via suppression of phytopathogens use different mechanisms, as well. The effects of these rhizobacteria have been attributed to their ability to produce various compounds including iron-chelating siderophores (Neilands 1986; Carson et al. 1994) that make iron unavailable to pathogens and hydrogen cyanide, which suppresses the growth of fungal pathogens (Hassanein et al. 2009). They are able to synthesize antifungal antibiotics and fungal cell wall-lysing enzymes or to compete with other soil microorganisms during root colonization for an ecological niche or a substrate. Rhizobacteria are capable to induce systemic resistance to pathogens (Compant et al. 2005; Haas et al. 2000) and abiotic stresses in host plants (Mayak et al. 2004; Nowak and Shulaev 2003). Despite their different ecological niches, free-living rhizobacteria and endophytic bacteria use some of these mechanisms to promote plant growth and to control phytopathogens (Bloemberg and Lugtenberg 2001; Hallman et al. 1997; Lodewyckx et al. 2002; Maheshwari 2010). Direct mechanisms of plant growth promotion can be demonstrated in the absence of rhizosphere microorganisms including plant pathogens. Indirect mechanisms involve the ability of rhizospheric microorganisms to reduce the deleterious effects of plant pathogens on crop yield. Even in simplified model laboratory systems, the study of biocontrol involves interactions among a minimum of three organisms. Therefore, despite its potential in agricultural applications, biocontrol is one of the most poorly understood areas of plant–microbe interactions and using bacterial species as biocontrol agents have not been extensively explored.

Antibiosis is commonly considered as one of the main characteristics of PGPR. One of the reasons may be that antibiotic production is one of the criteria for screening organisms for a study; antibiotic production has recently been recognized as an important feature in the biological control of plant diseases by rhizospheric bacteria. There are numerous reports of the production and importance of antimicrobial metabolites. For instance, it was found that oomycin A is responsible for 70% of the ability of *Pseudomonas* to reduce *Pythium* root infection in cotton and 50% of its ability to increase cotton seed emergence (Howie and Suslow 1991). To demonstrate the role of antibiosis in biological control, mutants lacking antibiotics

production ability have been used. A mutant strain of *Erwinia herbicola* Eh1087 (Ant2) could grow at the same rate as wild-type strain Eh1087 but did not suppress development of the disease caused by *E. amylovora* (Whipps 2001). Many other microbial metabolites have been studded for their antimicrobial activity, range, and mode of action. Many of them have a broad-spectrum activity. For example, the broad-spectrum activity of pyrrolnitrin, produced by *Pseudomonas* and *Burkholderia* species, has shown activity against a wide range of *Basidiomycetes*, *Deuteromycetes*, and *Ascomycetes*, including several economically important pathogens and against several Gram-positive bacteria and in particular *Streptomyces* species (Raaijmakers et al. 2002). However, the classic and commercially successful biocontrol, based on antibiotic producing strains is the application of nonpathogenic *Agrobacterium* against *Agrobacterium tumefaciens* (Whipps 2001).

Another widely studied microbial metabolites with low molecular weight (<1,000 Da) are the siderophores. Although some siderophores are known to chelate other ions, their specificity to iron is the most consistent feature (Chincholkar et al. 2007). Several evidences indicate that siderophore production when iron is limited is responsible for the antagonism of some strains of *P. aeruginosa* against *Pythium* spp. (Antoun and Prevost 2005). Also, HCN expression and production by *Pseudomonas* was strongly dependent on iron availability (Keel et al. 1989) and may act synergistically with siderophores. Siderophores produced by rhizosphere microorganisms has been considered not only to improve rhizosphere colonization of the producer strain but to also play an important role in iron nutrition of the plant (Vansuyt et al. 2007).

PGPRs compete with communities of other microorganisms associated with the host plants, growing in the rhizosphere or on and in the host tissues (Compant et al. 2005). This competition in the rhizosphere is important when microorganisms compete for scarce nutrient resources. Even if nutrients are limiting, the region around the root is relatively rich in nutrients due to the loss of as much as 40% of plant photosynthates from the roots. Also, the establishment of beneficial organisms on the roots limits the infection by pathogenic organism in later stages. It is competitiveness related plant defense. Thus, high populations of PGPR may affect colonization not only by plant pathogens, but also inhibit the slightly parasitic or nonparasitic, but toxigenic microorganisms; this is a significant advantage of the bioaugmentation method. Although there is a difference between laboratory experiments and field application of PGPR and their full potential has not been reached yet, the results are very promising and may offer growers an effective control of serious plant diseases.

## 13.3 Phytoremediation Management Strategies Where PGPR Takes Part

Phytoremediation is a relatively new technology based on the use of green plants and soil microorganisms to overcome environmental pollution problems. Whereas organic pollutants are degraded partially or to full mineralization, the heavy metals

are not biodegradable. Their state could be changed, immobilized, or volatilized, etc., so they may be taken up into the plants or in soil biota. In all interaction with trace elements, the role of soil microorganisms, and especially rhizobacteria, is crucial. They are in the immediate vicinity and constant contact with the root surface and soil properties, including trace elements, exchanging substances and compounds, influencing soil pH, and interacting with all living organisms. Generally, among all types of phytoremediation, of particular interest is the role of PGPR in phytoextraction and phytoimmobilization/stabilization of trace element-contaminated lands.

## 13.4 Rhizobacteria Inoculants-Induced Metal Mobilization in Phytoextraction

During the past years, the attention paid to the PGPR in abiotic stress environment has increased (Fig. 13.1). The main expectation has been directed to the mobilization of contaminants for further uptake by plants. The bioavailability of elements is the main prerequisite for phytoextraction. Presence of the element in soil solution permits uptake from plant root. Generally, the low amount of extracted metals is often associated to the low availability that is due to the nature of trace element and the type of soil or soil properties such as pH, CEC, and organic matter (Kayser et al. 2001).

A number of studies showed that soil inoculants (bioaugmentation) resulted in increment in available soil fraction of the metals (Lebeau et al. 2008). Ordinarily, metal bioavailability is divided into three different pools, although the way of assessment differs. Cao et al. (2007) used next extractants: water-soluble metals, exchangeable metal (with $KNO_3$), and bound or absorbed metals (with EDTA). On the other hand, McGrath and Cegarra (1992) used other procedures to extract sequentially heavy metals from soil (1) 0.1 M $CaCl_2$ (1:10, w/v) for 16 h; metals in soil solution and in exchangeable forms; (2) 0.5 M NaOH (1:10, w/v) for 16 h followed by aqua regia digestion; metals associated with organic matter; (3) 0.05 M $Na_2H_2EDTA$ (1:10, w/v) 1 h; metals mainly in the carbonate fraction; and (4) digestion with aqua regia; residual metals. More extraction procedures have been studied in order to determine the quantity of trace elements in soil that can be taken up by the plant roots. In this sense, Maiz et al. (2000) assessed the convenience of two sequential extraction procedures: procedure A consisted of step A1, mobile fraction, and step A2, mobilizable fraction, and procedure B followed the method proposed by Tessier et al. (1979). These and many other studies are the base that helps to assess the effect of inoculation of PGPR in rhizosphere on trace elements' bioavailability in soils. For instance, Braud et al. (2006) have shown that Pb concentration in exchangeable fraction is increased by more than 100% due to inoculation of *Pseudomonas aeruginosa* and *P. fluorescens* followed by biding to carbonates, while the other fractions (bound to Fe–Mn oxides, organic matter, and

residual) remain unchanged. Abou-Shanab et al. (2006) have shown a 15-fold increase of extractable Ni after introduction of *Microbacterium arabinogalactanolyticum*. In other study, Shilev et al. (2003) found that the inoculation of *P. fluorescens* in contaminated soils resulted in increment of xylem fluxes from roots to the shoots of sunflower. This higher translocation could be associated to the bacteria induced increase of water transport; thus inoculated plants, independently of the presence or not of arsenic, showed higher level of exudation. In another study with the same strain and the strain *P. fluorescens* CECT 378 in salinity stress conditions (100 mM), Shilev et al. (2011) found that both bacterial populations decreased considerably the accumulated $Na^+$ in sunflower plants grown in peat, while $K^+$ content and plant biomass increased. The authors related this effect mainly with the IAA and siderophores production, because ACC deaminase activity was not tested yet. Mayak et al. (2004) reported that *Achromobacter piechaudii* possessing ACC deaminase activity increase notably the biomass of tomato in presence of up to 170 mM of salt, which was attributed to the reduction of ethylene concentration originated by the salt stress. In this case, the accumulation of sodium did not decrease, while the bacteria increased water-use efficiency.

The capacity of PGPR to increment plant growth, biomass, and nutritional status for increasing plant tolerance to the contaminants is another issue that attracts the attention of researchers. An important mechanism is the utilization of ACC as a unique source of carbon and nitrogen through the enzyme ACC deaminase, discussed in Sect. 13.2.1. Thus, in many cases elongation of plant roots was obtained that facilitated increment of biomass (Glick et al. 1994; Shilev et al. 2001; Belimov et al. 2002, 2005; Dell'Amico et al. 2008; Safronova et al. 2006). The fact that in many cases PGPR improves the rate of shoot biomass is a prerequisite for successful phytoextraction. In one of their valuable studies, Belimov et al. (2005) found a positive correlation between the root elongation in *Brassica juncea* plants and in vitro ACC deaminase activity of Cd-tolerant PGPR. The authors suggested that the utilization of ACC is an important bacterial trait determining root growth promotion. The isolated bacteria offer promise as inoculants to improve growth of the metal-accumulating plant *B. juncea* in the presence of toxic Cd concentrations and for the development of plant inoculant systems useful for phytoremediation of polluted soils. In another study, Belimov et al. (2002) found that nutritional status plays an important role in promoting effect of rhizobacteria. Phosphorus availability, in particular, is a key factor, which affected the response to inoculation with rhizobacteria that possess ACC deaminase. In their work, bacteria stimulated the root elongation of P-sufficient plants only with increased biosynthesis of ethylene. Associating the stimulation of plant growth promotion with reduction of content of nutrient elements in plant, the authors suggested that low P availability and high ammonium concentrations could limit the positive effect of PGPRs possessing ACC deaminase. Dell'Amico et al. (2008) describe the effect of two rhizobacterial strains of genus *Pseudomonas*, isolated from Cd-contaminated soil, that possess ACC deaminase and actively

produce IAA and siderophores in presence of Cd. As a result, plant biomass and extracted Cd increased. Burd et al. (1998) reported the important potential of the bacterium *Kluyvera ascorbata* SUD165 tolerant to Ni, Pb, Zn, and Cr. The population of this strain protects canola and tomato seeds from nickel toxicity by the utilization of ACC. A few years ago, Wu et al. (2006a) showed that inoculation with *Pseudomonas putida* 06909 decreased significantly cadmium toxicity, increasing the accumulation of this metal in sunflower plants, although no investigation of ACC deaminase activity of the stain was made.

Microbial siderophores are produced to sequester iron, enhancing the mobility of Fe III and other cations (Diels et al. 2003). Braud et al. (2006) demonstrated that there are close positive relationships between siderophore production and the concentration of Cr and Pb in the exchangeable soil fraction. Nitrogen-fixing, phosphorus- and potassium-solubilizing bacteria (Wu et al. 2006b) may decrease soil pH by excreting low-molecular-weight organic acids, thus enhancing the bioavailability of Cd, Pb, and Zn (Chen et al. 2005). Braud and coworkers demonstrated that metal concentration of easily extractable fractions increase several days after bioaugmentation and continue higher for more than 6 months. As one of the most well-known producers of siderophores, *Pseudomonas* may change metal speciation in soils. Also, hydroxamate siderophores, such as desferrioxamine B, can complex Pb potentially helping Pb uptake by plants (Dubbin and Ander 2003). A classic example is the phytoremediation of Se by Indian mustard (*B. juncea*), because of its rapid growth, high biomass, and accumulation and volatilization of Se (Terry and Zayed 1998). Earlier, De Souza et al. (1999) reported that the role of rhizobacteria is crucial to achieve the best rates of accumulation and volatilization of Se.

Fässler et al. (2010) demonstrated the importance of IAA in stress alleviation of sunflower. In soils artificially polluted with Pb and Zn, they applied IAA concentrations between $10^{-9}$ and $10^{-12}$ M and without IAA. The experiments showed that the addition of $10^{-10}$ M IAA reduced the negative metal effects on root and shoot weight, root length, and root volume and surface area. Most of the positive effects of IAA and other hormones (gibberellins and cytokinins) in conditions of abiotic stress produced by trace elements are attributed to the improved plant growth and tolerance to the stress factor, and also to other mechanisms (Kloepper et al. 1989; Patten and Glick 2002). The root elongation of *B. napus* was shown to be stimulated by IAA produced by PGPR (Sheng and Xia 2006), while in many cases it depends on the quantity of produced IAA: 4 μg $ml^{-1}$ of culture media of *Brevibacillus* B-I (Vivas et al. 2006), 55 μg $ml^{-1}$ of *Bacillus subtilis* SJ-101 (Zaidi et al. 2006), 0.6–14.5 μg $mg^{-1}$ cell DW of isolate *P. fluorescens* biotype F, and 1–7.2 μg $mg^{-1}$ cell DW of collection strain *P. fluorescens* CECT 378 (Shilev et al. 2011) About 159.5 μg/ml IAA was observed in a two-species microbial consortium, of *Burkholderia* sp. MSSP and *Sinorhizobium meliloti* PP3 (Pandey and Maheshwari 2007).

### 13.4.1 Rhizobacteria Inoculants-Induced Metal Immobilization in Phytostabilization

Phytostabilization is the immobilization of a contaminant (element) in soil through absorption and accumulation by roots, adsorption onto roots, or precipitation within the root zone of plants, and the use of plants and plant roots to prevent contaminant migration via wind and water erosion, leaching, and soil dispersion. The immobilization process is very important and it is strongly connected to the toxicity of contaminant due to its availability (accessibility) to living organisms. Also, an improved metal tolerance to contaminant will allow the establishment of strong vegetation cover for consequent phytostabilization. As a part of the biotechnological method, phytoremediation, it uses a natural way for immobilization. One important kind of natural immobilizing agent are the microorganisms, instead of chemicals. It has been well recognized that some microorganisms have a high affinity for heavy metals and can absorb or precipitate them (Ledin et al. 1999; Diels et al. 2003; Lu et al. 2006). Metal biosorption by microbial inoculants is of particular interest from the point of view of phytostabilization because of the development of complex mechanisms for metal tolerance/resistance: exclusion by intra- or extracellular sequestration; sorption by cell envelopes' structural components (metal-binding functional groups); efflux transport systems, which excrete toxic elements out of the cell; and enzymatic detoxification (Nies 2003; Bruins et al. 2000). In that sense, Jézéquel and Lebeau (2008) compared *Bacillus* sp. and *Streptomyces* sp. as regards the inoculation technique, i.e., inoculum size and free or immobilized cells. After 3 weeks of a batch incubation, the potentially phytoavailable Cd was reduced, at the maximum, to a factor 14.1 and 4.3 with *Bacillus* sp. and *Streptomyces* sp., respectively. In another study, coinoculation of lupines with a consortium of metal-resistant PGPRs in multiple metal contaminated soil (including *Bradyrhizobium* sp., *Pseudomonas* sp., and *Ochrobactrum cytisi*) produced an additional improvement of plant biomass. At the same time, a decrease in metal accumulation was observed, both in shoots and in roots, which could be due to a protective effect exerted on the plant rhizosphere (Dary et al. 2010).

Pollutant toxicity, hostile soil conditions (lack of organic matter content, soil structure, etc.), and nutrient deficiency are typical problems challenging the establishment of vegetation on contaminated sites. Apart from the selection of pollutant-tolerant plants, rhizosphere processes and their proper management may be crucial for the success of phytostabilization (Wenzel 2009).

With the purpose of ameliorating nutrient deficiencies, advantage can be taken of rhizosphere processes together with cocropping of legumes and inoculation with iron-solubilizing and nitrogen-fixing bacteria. Bioaugmentation with metal-tolerant or -resistant PGPR can support the establishment and improve the state of phytostabilized crops, and detoxification mechanisms in the rhizosphere may be enhanced by microbial rhizosphere inoculants. The main challenge for the design of phytostabilization systems relates to combining different approaches to ameliorate multiple constraints (nutrient and water deficiency and toxicity due to mixed

contamination) and to control their efficiency in field conditions (Wenzel 2009). In some cases, resistance of PGPR to metal toxicity in soil could be a significant problem. Attempts to develop heavy-metal-resistant bacteria have been made. Several biotechnology methods to increase the frequency of spontaneous mutation have been used, such as ultraviolet irradiation and $HNO_2$ induction to improve resistance. The UV-irradiation mutation technique has been widely used for the production of industrially valuable compounds synthesized by microbial mutant cells. In the past decade, several researches have been focused on applying this technique to improve the evolution of microbial strains to acquire more resistibility and affinity for heavy metals (Zhao et al. 2005; Marc and Vistor 2002). Jiang et al. (2009) investigated the immobilization of Cd in soil using bioaugmentation with a UV-mutated *B. subtilis* 38 together with compost. Mutated strain improved the capability to bioaccumulate Cd four times over the wild type; thus, bioaugmentation with *B. subtilis* 38 coupled with the compost amendment exhibited the best capacity for immobilizing Cd in soil and allowed only 8.7% of the total Cd as extractable fraction under the optimum conditions.

## 13.5 Inoculation Methods Used in Stress Environment

Generally, metal concentration in a microorganisms-assisted plant is higher than in the controls (nonbioaugmented soil). Bacteria, mainly PGPR and fungi, of the group AMF, are used as pure cultures or as cocultures. Whether the soil is bioaugmented or not, metal concentrations in roots are generally higher than in shoots, by a factor exceeding 10 for most experiments. When the concentration of metals accumulated by plants increases with bioaugmentation, this increase is proportionally higher in roots than in shoots, most of the time. However, some experiments have shown the contrary with bacteria (De Souza et al. 1999; Shilev et al. 2003) and fungi (Malcova et al. 2003; Leung et al. 2006).

Many times, experiments demonstrating plant root elongation–promotion activity in laboratory conditions were carried out inoculating one strain alone or several strains as cocultures by soaking seed of different species, with or without trace elements (Belimov et al. 2005; Patten and Glick 2002). Regarding the effectiveness of the method of inoculation, Jiang et al. (2009) reported that single application of *B. subtilis* 38 to Cd-contaminated soil could not reduce significantly the bioavailable fraction of the metal compared to the control soil. In soils inoculated by watering with 3 mg cells $g^{-1}$ soil, the bioavailable Cd began to decrease rapidly after 15 days.

In order to acquire a better understanding of the effects of the different delivery modes of bacterial inoculants on plant growth and on the community structure of rhizosphere bacterial populations, Ciccillo et al. (2002) inoculated *Burkholderia ambifaria* MCI 7 into the rhizosphere of maize plants by either seed adhesion or incorporation into soil. The results revealed that when applied as a maize seed treatment, *B. ambifaria* MCI 7 promoted plant growth significantly; on the contrary,

when incorporated into soil, the same strain reduced plant growth markedly. As far as the bacterial community structure is concerned, *B. ambifaria* MCI 7 affected the indigenous microflora of treated plants according to the application method: seed treatment brought about an abrupt decrease in bacterial diversity, whereas incorporation into soil increased bacterial diversity. Moreover, changes in bacterial diversity were limited to r-strategist bacteria. So, the method of application can be an essential element in determining the effects of the inoculant on plant growth. In an experiment that studied the elongation and promotion effects of bacterial inoculation on spring rape roots together with phosphorus status of the plant, Belimov et al. (2002) prepared and applied a suspension of three *Pseudomonas* strains and *Alcaligenes xylosoxidans* Cm4 in concentration $5 \times 10^8$ cells/ml used in the same day by adding in a nutrient solution for watering in a concentration of $10^6$ cells/ml to obtain good growth of *B. napus*.

## 13.6 Transgenic Bacteria and Plants with Expression of Bacterial ACC Deaminase

Genetic engineering tools and biotechnological methods are highly effective in the genetic manipulation of many characteristics into a single organism for achieving goals and objectives. To reach the goal of successful bioremediation, scientists introduce specific genes from one organism into another to accelerate the key processes of phytoremediation. In this direction, the introduction of genes responsible for expression of ACC deaminase in plants has received more attention than in microorganisms.

Reed and Glick (2005) reported that both native and transformed *Pseudomonas asplenii* AC were equally useful in the promotion of seed germination and root elongation under stress conditions caused by copper in contaminated soils. This could be because the efficiency of transgenic inoculated strains is determined by several biotic and abiotic factors such as soil pH, temperature, and moisture content and their competition with native soil microflora and microfauna. As described by many scientists, the ACC deaminase trait has also been found in endophytes. Therefore, the selection of endophytes having both ACC deaminase and specific degradation genes could also be a useful approach for developing a successful phytoremediation strategy. Grichko et al. (2005) expressed bacterial ACC deaminase in tomato (*Lycopersicum esculentum*) cv. Heinz 902 under the transcriptional control of either two, tandem 35S cauliflower mosaic virus promoters (constitutive expression), the rolD promoter from *Agrobacterium rhizogenes* (root-specific expression), or the pathogenesis-related prb-1b promoter from tobacco. The growth of transgenic tomato plants in the presence of Cd, Cu, Co, Mg, Ni, Pb, or Zn was studied. They tested metal accumulation and ACC deaminase activity in both shoots and roots, root and shoot development, and leaf chlorophyll content. Transgenic tomato plants expressing ACC deaminase particularly controlled by the

prb-1b promoter accumulated larger amounts of metals within the plant tissues. Nie et al. (2002) expressed ACC deaminase genes in canola plants and tested their potential to grow in the presence of high levels of arsenate in the soil for metal accumulation. They also tested the ability of the plant growth-promoting bacterium *E. cloacae* CAL2 to facilitate the growth of both nontransformed and ACC deaminase-expressing canola plants. In all cases, transgenic canola-expressing ACC deaminase genes accumulated larger amounts of arsenate from the contaminated soil than nontransformed canola plants. More recently, Stearns et al. (2005) reported similar results in the case of phytoremediation of nickel-contaminated soils. They observed that the growth of transgenic plants constructed through rolD promoters demonstrated more growth under high nickel concentration compared to nontransgenic and other transgenic plants expressing ACC deaminase genes.

Farwell et al. (2006) reported the successful utilization of transgenic canola and *P. putida* and *P. putida* in in-situ phytoremediation of a nickel-contaminated soil. The results related to enhanced plant growth of both transgenic and nontransformed canola by ACC deaminase bacteria compared with plants that were not inoculated. Another study by Farwell et al. (2007) compared the growth of transgenic canola expressing ACC deaminase with nontransformed canola inoculated with *P. putida* containing ACC deaminase activity under multiple stresses in situ. They reported that flooding reduced the growth of canola, but nickel accumulation in transgenic and nontransgenic plant tissues was increased.

All these results showed the very promising future of transgenic plants expressing ACC deaminase genes and inoculated with PGPR containing ACC deaminase genes under abiotic stress by trace elements.

## 13.7 Conclusions

The role of PGPR in the management of trace element-contaminated lands is not well understood. Studies were carried out in laboratory, greenhouse, and field conditions, showing the effectiveness of inoculation on plant tolerance, root development, or accumulation of elements in a stress environment. Under such conditions, it is very important to take into account all factors that may influence the goal of the biotechnology used. The capabilities of PGPR have to be considered in their totality together with plant-based mechanisms of contaminants' accumulation/immobilization and detoxification and with the site-specific characteristics. Although phytoremediation technologies are being commercially used, it is clear that the complexity of interactions in the plant root–microbe–contaminant system require further efforts to improve the knowledge of involved rhizosphere processes, in this way improving the management of such sites.

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# Chapter 14
# The Use of ACC Deaminase to Increase the Tolerance of Plants to Various Phytopathogens

**Leonid Chernin and Bernard R. Glick**

## 14.1 Introduction

Plant pathogens affect crops worldwide and are a major obstacle to the production of food, fiber, and ornamental crops. Losses due to plant diseases may be as high as $30–50 billion in cultivated and stored crops and represents approximately 20% of total global food production annually. The use of chemicals is still the main strategy to prevent plant diseases (Hall and Mann 1999). The most efficient way of preventing crop diseases caused by these pathogens is to use chemicals to disinfect the soil against pathogens and pests. However, pesticides common in agricultural practice create a health hazard for many nontarget organisms. In fact, their use has become more limited every year due to legislators' concern for human and animal health, as well as for the quality of life and the environment. In addition, excessive application of pesticides has led to an increased proportion of pathogens that are resistant to these chemicals. Thus, each year, plant diseases cause millions of dollars worth of crop damage, despite extensive use of pesticides. Therefore, the development of safer, environmentally friendly control alternatives which promote sustainable agroecosystems is urgently required and has become a research priority in developed countries. Agricultural biotechnology offers a new approach to the problem: the development of alternative, efficient, and ecologically safer biological methods of plant disease control. One of the most promising is biological control, which is based on natural antagonistic interactions among microorganisms and offers a practical means to control plant diseases while avoiding problems caused by the misuse or overuse of synthetic pesticides. Biological control of plant diseases

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can be achieved directly, through introduction of specific microbial antagonists into the soil or plant material, or indirectly, by changing the conditions prevailing in the plant's environment, thereby modifying the microbiological equilibrium of the plant's ecosystem. In some cases, a combination of indirect and direct approaches leads to the best biocontrol results. All known biocontrol agents (BCAs) utilize one or more of these general indirect or direct mechanisms. At the product level, this includes the production of antibiotics, siderophores, and cell-wall-lytic enzymes, and the production of substances that promote plant growth. Additionally, successful colonization of the root surface is considered a key property of prospective antagonists. The best BCAs may use multiple mechanisms of action against one or more pathogens. Antagonists are generally naturally occurring, mostly soil microorganisms that have some trait or characteristic that enables them to interfere with pathogen or pest growth, survival, infection, or plant attack. Usually, they have little effect on other soil organisms, leaving the natural biology of the ecosystem more balanced and intact than would a broad-spectrum chemical pesticide. Plant genes also play a role in biocontrol efficiency of disease-suppressive microflora (reviewed by Whipps 2001; Chet and Chernin 2002; Compant et al. 2005; van Loon 2007; Weller et al. 2007; Berg 2009; Lugtenberg and Kamilova 2009; Peter et al. 2009).

Several plant diseases caused by soil-borne plant pathogens have been shown to be inhibited by certain soils. Soil suppression is considered a rather complex phenomenon in which the antagonists establish themselves in the soil and interfere with the growth or survival of the pathogenic microorganism. Studies of why some soils can naturally suppress plant diseases, while others cannot have provided the basis of much of the research on BCAs. Soil suppressiveness may be constitutive, an inherent property of the soil regardless of its cropping history, or adaptive, whereby soil suppression is only achieved after a specific cultural practice – such as monocropping – has been adopted. For example, soil suppressiveness of *Fusarium* wilt of melons (Alabouvette and Lemanceau 1999) and of *Gaeumannomyces graminis* var. *tritici* (*Ggt*)-take-all of wheat (Weller et al. 2002) are examples of constitutive and adaptive soil suppression, respectively.

Many plant growth-promoting bacteria (PGPB), and specifically plant growth-promoting rhizobacteria (PGPR) that inhabit the plant rhizosphere, have been shown to improve plant health and increase yield (Maheshwari 2010) and have been used for the biocontrol of plant disease (Kloepper et al. 1989). The bacteria appear to protect plants against a wide range of pathogens and the potential for commercial utilization is promising. PGPB are the main reservoir of agents for the control of phytopathogens, and representatives of many genera have been used to control plant diseases (Reed and Glick 2004). PGPB bind to the surface of either the seeds or the roots of a developing plant. Successful strains colonize roots and suppress pathogens by mechanisms including niche exclusion and competition, direct antagonism of pathogens by antibiosis and parasitism or predation, and by triggering systemic host plant defense responses. Some PGPBs also stimulate plant growth directly, by synthesizing phytohormones and siderophores, fixing nitrogen, or solubilizing minerals (Lugtenberg and Kamilova 2009; Spaepen et al. 2009).

Among the most intensively studied PGPRs are strains of *Pseudomonas fluorescens* from soils. Most pseudomonads with biocontrol activity produce one or more potent antifungal metabolites that often are major determinants of direct biocontrol mechanisms. The best characterized are simple compounds such as the antibiotics phenazines, 2, 4-diacetylphloroglucinol (DAPG), pyrrolnitrin (Prn), pyoluteorin (Plt), and others (Ligon et al. 2000; Raaijmakers et al. 2002; Weller et al. 2007; Mavrodi et al. 2010). Some other PGPBs, e.g., strains of *Serratia plymuthica* besides some antibiotics, including Prn, produce one or more enzymes capable of achieving the complete hydrolysis of the chitin that is present in many fungal cell walls; these enzymes include *endo*-chitinases, chitobiosidases, and chitobiases, and *N*-acetyl-β-glucosaminidases (Chernin and Chet 2002; De Vleesschauwer and Hofte 2007; Berg 2009).

In this review, we focus on another enzymatic mechanism of biocontrol activity of many rhizospheric bacterial antagonists: the ability to produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCD) [EC 4.1.99.4] which can cleave ACC, the immediate precursor of the plant hormone ethylene (ET) in plants, to α-ketobutyrate and ammonia (Honma and Shimomura 1978) and thereby lower plant ET levels. Lower ET levels typically result in longer roots and less inhibition of ET-sensitive plant growth, following environmental or pathogen-induced stress. This enzyme has subsequently gene which has been isolated and the biochemical reaction mechanisms involved in ACCD activity were characterized (reviewed by Hontzeas et al. 2006; Glick et al. 2007a). PGPB strains producing this enzyme may have a competitive edge over other microorganisms in the rhizosphere because they can use ACC as a source of nitrogen (Glick and Bashan 1997) and they help plants to overcome many of the detrimental effects of biotic and abiotic stresses (Glick et al. 2007b; Saleem et al. 2007).

## 14.2 Participation of Ethylene in Plant Defense Against Pathogens

Plant hormones such as ET, auxin, cytokinins, and gibberellins that influence plant development are among the main regulatory mechanisms of plant growth promotion. Promotion of lateral root formation is a typical auxin effect, while gibberellins and cytokinins both stimulate shoot development (Tanimoto 2005). The effect of ET is in some sense contradictory. At high levels it enhances senescence and organ abscission, at moderate levels it inhibits both root and shoot elongation (Abeles et al. 1992), while at low levels it can promote growth in several plant species, including *Arabidopsis* (Pierik et al. 2006) and induce the transcription of a plant's stress-defensive proteins (Czarny et al. 2007).

In response to virulent pathogen infections, the host plant activates diverse defense mechanisms in an attempt to limit the infection and spread by the pathogen. Plants respond to pathogen infection by increased production of the ET biosynthetic

enzymes, ACC synthase and ACC oxidase, followed by a peak of ET which in turn accelerates various pathogenicity symptoms associated with disease development (Abeles et al. 1992; van Loon and Glick 2004). Several components of the ET signal transduction pathway, including those encoding by *ETR1* and *CTR1* genes of *Arabidopsis*, have been now identified (Clark et al. 1998; Hua and Meyerowitz 1998). The *ETR1* gene is related to the superfamily of catalytic receptors in eukaryotes referred to as two-compound regulators (Chang et al. 1993). The ETR1 protein is an ET receptor with histidine protein kinase activity. The CTR1 protein belongs to serine/threonine protein kinases that initiate the mitogen-activated protein kinases cascades. Both ETR1 and CTR1 are negative regulators of ethylene signaling (Huang et al. 2003). The gene encoding phenylalanine ammonia lyase (PAL) is believed to be activated by the Jasmonic acid (JA)/ET signaling pathway in the context of induced plant defenses (Diallinas and Kanellis 1994; Kato et al. 2000). PAL1 is the first enzyme in the phenylpropanoid biosynthesis pathway, which provides precursors for lignin and phenols, as well as for salycilic acid (van Loon et al. 1998). Other enzymes of the phenylpropanoid pathway, including peroxidases, are also induced in resistant reactions (Yedidia et al. 2000). Peroxidases are also known for their role in the production of phytoalexins and reactive oxygen species and formation of structural barriers (Hiraga et al. 2001). Pathogenesis-related (PR) proteins such as β-1,3-glucanase (PR2) and chitinase (PR3), known to disrupt the fungal mycelial wall, can be induced by SA as well as by pathogenic attack; however, some chitinases are induced by JA and not SA (Salzer et al. 2000).

ET and some signal molecules, including SA and JA, are critical components of both local and systemic responses of a plant to pathogen invasion (Pieterse et al. 2007, 2009; van Loon 2007). An increase of ET formation in pathogen-attacked plants has been related both to defense responses leading to resistance as well as to symptom development during pathogenesis. ET is an important factor in the regulation of plant reaction to pathogens (Knoester et al. 1998, 1999). ET production is known to cause necrosis in plant tissues in response to numerous environmental and developmental signals (Abeles et al. 1992). In addition to phytohormones and signal molecules, such as ET, SA, and JA mentioned above, plant resistance to pathogens also requires production of a number of enzymes shown to be activated in response to pathogen attack. Among the defense enzymes are hydrolases such as chitinases and glucanases considered along with osmotin-like proteins, as newly formed PR proteins with antimicrobial potential; and peroxidases, which play a key role in the plant-resistance process, being involved in phenolic compounds' (e.g., phytoalexins) synthesis and reinforcement of the cell walls (e.g., the deposition of structural polymers, such as callose and lignin) for structural barrier formation. ET is an inducer of several pathogen defense-related enzymes, e.g., peroxidase, glucanase, and chitinase, providing support for the regulative role of ET in resistance responses (reviewed by van Loon et al. 1998; van Loon 2007; Pieterse et al. 2009).

Considerable data indicate the key role of ET in systemic acquired resistance (SAR) and in induced systemic resistance (ISR) (Pieterse et al. 2000, 2001). SAR includes local resistance and broad-spectrum defenses developed by a plant after

primary infection, for example, with a necrotizing pathogen which switched on a signal transduction pathway (Durrant and Dong 2004). SAR can provide long-term resistance throughout the plant to subsequent infection by different pathogens. As mentioned above, the SAR response correlates with the activation of pathogenesis-related (PR) genes. This process generally requires the involvement of SA as a signal molecule which has been shown to increase in both infected and uninfected tissues (van Loon et al. 1998; van Loon 2007). SA has been shown to be necessary for the generation of SAR, elicits PR protein patterns similar to those induced by pathogens, and the treated plants develop resistance to viral, bacterial, and fungal pathogens. SAR results in the development of a broad-spectrum defense which inhibits pathogen growth; however, it is not effective against, or induced by, all pathogens (Govrin and Levine 2002). Thus, cooperative interactions between JA, ET, and SA in the susceptible response of tomato to *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) was demonstrated, indicating that the functions of these hormones may be species specific (O'Donnell et al. 2003). In *Arabidopsis*, the evidence supports a model in which ET and JA coordinately mediate one defense response, while SA mediates a distinct and antagonistic response (McDowell and Dangl 2000; Genger et al. 2008). SAR induced by low ET levels increased plant damage brought on by high ET levels (van Loon and Glick 2004).

Certain rhizosphere bacteria confer another form of disease resistance called rhizobacteria-mediated induced ISR (van Loon et al. 1998). Contact with pathogenic and nonpathogenic microorganisms triggers a wide range of defense mechanisms in plants that protect them against invasion. Specific strains of PGPRs, especially fluorescent pseudomonads colonize roots and develop a phenotypically similar mode of defense. Several studies have shown that ISR is not an SA-dependent phenomenon but rather requires components of the JA signaling pathway followed by the ET signaling pathway (van Loon et al. 1998; van Loon 2007; Pieterse et al. 2007, 2009). The first enzyme in the biosynthesis pathway of JA is lipoxygenase (LOX) which utilizes linoleic and linolenic acids for the production of JA and controls a feed-forward loop in jasmonate synthesis (Reymond and Farmer 1998). However, the role of ET in these processes appears to be contradictory and depends on the pathogen used (van Loon and Glick 2004). Studies of ET receptors have suggested that these receptors are negative regulators of the ET response. A mutation in the ET-binding domain creates a plant that is insensitive to ET and has lost the ability to bind ET. This type of mutation, described in Never-ripe (Nr) tomato plants, results in ET insensitivity so that ripening of homozygous (*Nr*/*Nr*) fruit is delayed and incomplete (Tieman et al. 2000). The ET insensitivity conferred by the *Nr* mutation arises from a single base substitution in the N-terminal coding region of the gene *Le-ETR3* (Wilkinson et al. 1995). *Le-ETR3* encodes an ET receptor because normal ET responses are suppressed or blocked in *Nr* plants (Lanahan et al. 1994).

Lund et al. (1998) have shown that a tomato *Never ripe* mutant impaired in ethylene perception exhibited a significant reduction in disease symptoms in comparison to the wild-type plants after inoculation with virulent bacterial (*X. campestris* pv *vesicatoria* and *Pseudomonas syringae* pv *tomato*) and fungal

(*Fusarium oxysporum* f. sp. *lycopersici*) pathogens that cause widespread necrosis in susceptible tomato cultivars. Bacterial spot disease symptoms were also reduced in tomato genotypes impaired in ethylene synthesis and perception, thereby corroborating a reducing effect for ethylene insensitivity on foliar disease development. The authors considered the wild-type ET sensitivity to be a deleterious agronomic trait in the compatible plant–microbe interactions. Therefore, engineering plants to ET insensitivity can potentially provide them with tolerance to a broad spectrum of virulent pathogens. This conclusion has a practical value which was confirmed by employment of bacterial ACCD genes and the ACCD-producing PGPB to protect plants against several bacterial and fungal diseases.

In addition to the above-mentioned biotrophic pathogens, ET was shown to play an essential role in the pathogenicity of tumorigenic *Agrobacterium*. The genus *Agrobacterium* belongs to the *Rhizobiaceae* family of α-Proteobacteria and includes plant pathogens that cause crown gall and hairy root diseases. Thus, the soil-borne bacterium *Agrobacterium tumefaciens* induces tumors (crown galls) in more than 1,000 different species of dicots as well as some species of monocots, including many economically important plants. Strains of *A. vitis* are the predominant cause of crown gall tumors of grape in various grape-growing regions worldwide (Tzfira and Citovsky 2008). The pathogenicity of *Agrobacterium* strains is due to the presence of Ti (tumor inducing) plasmids, a specific segment (T-DNA) of which is transferred and integrated into the plant host cell genome. The T-DNA possesses oncogenic (*onc*) genes which are involved in phytohormone synthesis following their expression in transformed plants. The delivery, insertion, and subsequent expression of these *onc* genes into the plant genomic DNA leads to a phytohormonal imbalance in the infected plants, altering the normal rate of cell division and resulting in tumorous growth (Tzfira and Citovsky 2008).

In addition to the well-known roles of auxin and cytokinin in crown gall formation and morphogenesis, the plant hormone ET that functions as a regulator in many aspects of plant life also plays an important role in this process. *A. tumefaciens*-induced galls produce very high ET concentrations suggesting that tumor-induced ET is a controlling factor in gall development (Aloni and Ullrich 2008). Up to 140 times higher ET levels are produced in crown galls than in wounded, but not infected control stems of tomato, reaching a maximum at 5 weeks after infection (Aloni et al. 1998; Wächter et al. 1999). It was also suggested that the high auxin levels induced by the T-DNA-encoded oncogenes stimulate ET production (Aloni and Ullrich 2008), as IAA is well known to activate the transcription of the enzyme ACC synthase. The vigorous ET synthesis in galls is enhanced by high levels of auxin and cytokinin (Wächter et al. 1999). These results demonstrate that there is a critical role for ET in determining crown gall development and morphogenesis. Thus, the T-DNA-encoded oncogenes, namely *iaaH*, *iaaM*, and *ipt*, trigger a cascade of the following phytohormones: auxin, cytokinin, ET, abcisic acid, and JA, which together with gene *6b* expression-dependent flavonoid accumulation promote crown gall growth (Veselov et al. 2003; Gális et al. 2004).

## 14.3 Role of Enzyme 1-Aminocyclopropane-1-Carboxylate Deaminase in the Beneficial Characteristics of PGPB

A number of PGPB belonging to various taxonomic groups including *Pseudomonas* contain ACCD, the enzyme which cleaves ACC, the immediate precursor of ET (Honma and Shimomura 1978; Glick et al. 1998; Glick 2004; Belimov et al. 2001, 2005). These bacteria, typically occurring on the root surface – but also present as endophytes (Sessitsch et al. 2005; Sun et al. 2009) – degrade ACC to ammonium and α-ketobutyrate for use as carbon and nitrogen sources. The presence of ACCD is relatively common among soil microorganisms (Glick et al. 2007a). Using ACC as a sole nitrogen source, a number of PGPRs were quickly selected from different soil samples (Penrose and Glick 2003). ACCD has been widely reported in numerous species of PGPB such as *Agrobacterium genomovars*, *Azospirillum lipoferum*, *Alcaligenes*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Methylobacterium fujisawaense*, *Pseudomonas, Ralstonia solanacearum*, *Rhizobium*, *Rhodococcus*, *Sinorhizobium meliloti*, and *Variovorax paradoxus* (e.g., Belimov et al. 2001; Blaha et al. 2006; Rasche et al. 2006; Duan et al. 2009). Most *Pseudomonas* strains exhibiting biocontrol activity isolated from locations worldwide contained ACCD (Wang et al. 2001). Moreover, ACCD activity has been reported in some plant pathogenic bacteria (Belimov et al. 2001, 2007; Blaha et al. 2006; Safronova et al. 2006); however, it is not clear whether the presence of ACCD in these bacteria could mask their pathogenic effects and promote plant growth. The ability to produce ACCD was also demonstrated in the biocontrol fungus *Trichoderma asperellum*. RNAi silencing of the ACCD gene in *T. asperellum* showed decreased ability of the mutants to promote root elongation of canola seedlings in pouch assays. These data suggest a role for ACCD in the plant root growth promotion effect by *T. asperellum* as well as in various PGPB (Viterbo et al. 2010).

Glick et al. (1998) proposed a model in which a PGPB-containing ACCD attached to plant tissue provides a sink for ACC from the plant tissue and, thereby, reduces ET synthesis, promotes root elongation, and reduces the plant's stress symptoms. This activity of ET is related to the effects of another plant growth hormone, indoleacetic acid (IAA). Bacteria synthesize and secrete IAA, which is taken up by the plant in response to tryptophan and other compounds that are found in seed or root exudates. Bacterial IAA, together with endogenous plant IAA, can stimulate plant cell proliferation or plant cell elongation, or it can induce the synthesis of the plant enzyme ACC synthase that converts *S*-adenosyl methionine to ACC. This ACC may be exuded from seeds or plant roots and taken up by the bacteria and subsequently converted by ACC deaminase. Since a dynamic equilibrium of ACC concentration exists between root, rhizosphere, and bacterium, bacterial uptake of rhizospheric ACC stimulates plant ACC efflux, decreases root ACC concentration and root ET evolution, and can increase root growth (Glick et al. 1998). Accordingly, rhizosphere inoculation with ACCD-containing bacteria decreases root ACC levels and ET evolution (Penrose et al. 2001; Belimov et al. 2002; Mayak et al. 2004a). The enzyme ACCD, when present in the PGPB that are

found in the rhizosphere of many plants, can lower the stress perceived by the plant and also derepress the expression of auxin response genes in the shoots (Glick et al. 2007b). In addition, bacteria that contain ACCD can suppress the expression or functioning of other plant signaling molecules such as jasmonic acid and giberellin (Czarny et al. 2006; Cheng et al. 2010). This is, of course, a consequence of the lowering of plant ET levels by the action of the ACCD.

Inoculation of plants with PGPRs containing ACCD may lead to various subsequent physiological changes in plants (reviewed by Glick et al. 2007a, b; Saleem et al. 2007). The ability of ACC-utilizing PGPR to ameliorate plant growth inhibition caused by ET through a decrease in ACC content and ET production (Penrose et al. 2001; Belimov et al. 2002; Mayak et al. 2004b) has been demonstrated. Stimulation of root elongation and biomass production of different plant species by inoculations with PGPR having ACCD activity has been repeatedly documented, particularly when the plants were subjected to stressful growth conditions (Hall et al. 1996; Burd et al. 1998; Glick et al. 1998; Belimov et al. 2001, 2005; Safronova et al. 2006). The mutant of strain *Pseudomonas putida* UW4 deficient in ACCD activity simultaneously lost the ability to elongate roots in infected canola plants (Li et al. 2000). The evolutionary importance of ACCD activity for interaction of PGPBs with plants was proved by data showing horizontal transfer of ACCD genes between a broad range of plant-associated bacteria (Hontzeas et al. 2005).

Since ET has been found to be required for the induction in plants of systemic resistance elicited by rhizobacteria (see above), it might naively be assumed that treating plants with ET-lowering bacteria would prevent this induction. However, lowering of ET levels by bacterial ACCD does not appear to be incompatible with the induction of systemic resistance. Indeed, some bacterial strains possessing ACCD also induce systemic resistance (van Loon and Glick 2004). This may reflect the fact that there is an initial very small peak of ET close in time to the onset of a stress and then a second much larger peak some time later (Glick et al. 2007a). The first peak is thought to initiate a defensive response by the plant (systemic resistance), while the second ET peak is so large that processes inhibitory to plant growth are initiated. Immediately following an environmental stress, the pool of ACC in a plant is low as is the level of ACCD in the associated bacterium and only some of the ACC will be cleaved by the bacterial enzyme, with the remainder being converted into the first small ET peak.

The effect of PGPB on plant gene expression has been assessed using differential display PCR, randomly amplified PCR, microarrays, or a proteomic approach, in each case following growth under specific conditions. Thus, Hontzeas et al. (2004a, b) showed that in the presence of ACCD, some plant stress response genes are no longer turned on by PGPB. The authors compared a wild-type ACCD-containing PGPB with an ACCD-minus mutant of this strain and found that the wild-type bacterium lowered the stress level in the plant so that the plant no longer perceived a mild stress. Microarray studies of plant responses to PGPB revealed that many changes in gene expression occur within genes related to the stress response, wounding and pathogenesis signaling, auxin responses, cellular metabolism, and the ET response (Cartieaux et al. 2003; Czarny et al. 2007; Verhagen et al. 2004; Wang et al. 2005).

### 14.3.1 Involvement of Bacterial ACC Deaminase in Biocontrol of Plant Pathogens

Rhizobacteria-containing ACCD activity are free-living, soil-borne bacteria that can affect plant growth directly through various ways such as nitrogen fixation, solubilization of phosphorus, and increasing growth by regulating endogenous level of plant hormones or indirectly by increasing the natural resistance of the host against pathogens (Glick 2004; Lugtenberg and Kamilova 2009; Spaepen et al. 2009). Protection against pathogens by PGPB could be a part of consequences related with the ability of these bacteria to defend plants against environmental stresses. Plant ET synthesis is enhanced with the severity of pathogenic infection, while some ET synthesis inhibitors are known to decrease the severity of pathogen infections in plants significantly (Saleem et al. 2007). Bacterial strains bound to roots and having ACCD activity can significantly decrease ACC levels in plants. Thereby, the amount of stress ET production decreases and subsequent damage to the plant, which might occur as a consequence of that ET decrease, could also be minimum (Grichko and Glick 2001). Considerable evidences have demonstrated the beneficial role of bacterial ACCD in decreasing stress reactions in plant growth under different stresses, including salinity, flooding, drought, toxicity of high concentrations of heavy metals present in pollutant soils, and the presence of toxic organic compounds (Glick et al. 2007b; Arshad et al. 2007).

In addition, there are several reports which support the hypothesis that ACCD-producing rhizobacteria have antagonistic effects against microbial pathogens. A number of reports demonstrate the ability of ACCD-producing bacteria isolated from plants to suppress the growth of several fungal plant pathogens mainly in vitro (Wang et al. 2001; Donate-Correa et al. 2005; Pandey et al. 2005; Rasche et al. 2006). Plants treated with ACCD-containing bacteria were shown less susceptible to a range of pathogenic agents (reviewed by Arshad and Frankenberger 2002; Arshad et al. 2007; Saleem et al. 2007). However, in all these reports, except for Wang et al. (2001), no direct evidence was provided that the observed effects were a consequence of ACCD activity and not of other compounds (such as antibiotics, siderophores, and lytic enzymes) operating in the same bacterial cells along with ACCD.

More substantive proof of the biocontrol activity of ACCD was acquired with oncogenic strains of *Agrobacterium*. It was shown that *A. tumefaciens* strain C58 partially lost most of its ability to induce crown gall tumors on tomato plants upon either being transformed with an ACCD gene, *acdS,* from the PGPB strain *P. putida* UW4, or being coinoculated with this natural ACCD-producing strain (i.e., *P. putida* UW4). In both types of experiments, it was observed that the presence of ACCD was inhibitory to tumor development on both tomato and castor bean plants (Hao et al. 2007). This observation was extended by demonstrating that soaking the roots of tomato (*Solanum lycopersicum*) seedlings in a suspension of the ACCD-producing strains of *P. putida* UW4, *Burkholderia phytofirmans* PsJN, or *Azospirillum brasilense* Cd1843, transformed by plasmid pRKTACC carrying the

ACCD-encoding gene *acdS* from strain UW4, significantly reduced the development of tumors on tomato plants infected 4–5 days later with pathogenic *Agrobacterium* strains injected into a wound on the plant stem. The fresh mass of tumors formed by plants pretreated with ACCD-producing strains was typically 4–5-fold less than that of tumors formed on control plants inoculated only by a pathogenic *Agrobacterium* strain. Significantly, less reduction of the tumors mass was observed when roots were soaked with ACCD-nonproducing strains, i.e., *P. putida* UW4*acdS*$^{-}$, *B. phytofirmans* PsJN*acdS*$^{-}$, or *A. brasilense* Cd1843. Simultaneously, the level of ET per gram of tumor mass formed on plants pretreated with ACCD-producing bacteria decreased 4–8 times in comparison to ET evolution from tumors formed on control plants or plants pretreated with bacteria deficient in ACCD production. These results strongly support the idea that ET is a crucial factor in *Agrobacterium* tumor formation and that ACCD-produced rhizosphere bacteria may protect plants infected by pathogenic agrobacteria from crown gall disease (Toklikishvili et al. 2010). The ACCD-producing *B. phytofirmans* strain PsJN used in this work was shown to establish rhizosphere and endophytic populations associated with various plants, including tomato and grapevines, where it stimulates plant growth (Barka et al. 2002; Sessitsch et al. 2005; Sun et al. 2009), while the strains *P. putida* UW4 and *A. brasilense* Cd1843 are not endophytes (B.R. Glick unpublished). Thus, these results indicate that along with PGPR, endophytes are also effective at controlling infection by agrobacteria, this in addition to many plant-beneficial traits provided by these bacteria (Hardoim et al. 2008). Furthermore, these results show the prophylactic potential of ACCD-producing bacteria against crown-gall formation, since the effect was observed when the treatment of plants with protecting and pathogenic bacterial strains was separated both spatially and temporally. From a practical point of view, it is important that the ACCD-producing strains demonstrated their ability to suppress crown galls induced by the related pathogen *A. vitis* (as well as *A. tumefaciens*), known to be the main causative agent of crown-gall disease on grape, which is still a problem to control (Otten et al. 2008). The ACCD strains exhibit a comparable level of protection against *A. tumefaciens* and *A. vitis*; therefore, the possibility of using ACCD-producing rhizosphere bacteria to specifically protect sensitive plants from *A. vitis*-induced crown galls is potentially of great importance to grape growers.

## 14.4 Transgenic Bacteria and Plants Producing ACC Deaminase

ACCD-containing bacteria may be viewed as general agents toward various plant pathogens. It is generally quite straightforward to employ ACCD genes for enhancement of plant growth promoting, and biocontrol activity of PGPB that lack this activity and for a better understanding of the mechanisms responsible for the induction of tolerance in plants inoculated with ACCD-producing bacteria against both biotic and abiotic stresses (Glick and Bashan 1997; Glick et al. 2007b).

ACCD-producing rhizosphere bacteria have been used for protection of plants against various kinds of biotic and abiotic stresses caused by pathogens, salinity, drought, flooding, heavy metals, etc., as well as for phytoremediation of contaminated soil environment (reviewed by Arshad and Frankenberger 2002; Glick et al. 2007a, b; Saleem et al. 2007). The genes encoding ACCD have been isolated from a number of soil bacteria and fungi (Klee et al. 1991; Sheehy et al. 1991; Klee and Kishore 1992; Shah et al. 1998; Viterbo et al. 2010). Some of these genes have been used to transform plants as an alternative to the use of ACCD-containing bacteria and as an appropriate approach to understand the role of ET in the regulation of many physiological processes in plants.

### *14.4.1 Biocontrol of Plant Pathogens by PRPB Genetically Engineered to Express ACC Deaminase*

Transformation of bacterial strains lacking ACCD activity with isolated *acdS* genes and their regulatory regions has been shown to improve their usefulness. For example, *E. coli* and *Pseudomonas* strains that lack ACCD but have been transformed to express a *Pseudomonas acdS* gene are able to promote the elongation of canola roots in growth pouches (Shah et al. 1998). In other studies, genetic modification of PGPR expressing ACCD genes helped in altering nodulation in legumes and biological control of plant disease. Transformation of a strain of *Sinorhizobium meliloti* with an *acdS* gene from *Rhizobium leguminosarum* enables the transformed bacterium to nodulate alfalfa plants and stimulate their growth by 35–40% more than the native (nontransformed) strain of *S. meliloti* (Ma et al. 2004). The effectiveness of some biocontrol pseudomonads was also significantly enhanced following the introduction of a *Pseudomonas acdS* gene. Thus, the *P. putida* strain UW4 gene *acdS,* encoding ACCD, was exploited to enhance plant growth-promoting ability in a number of biocontrol bacteria. A derivative of *P. fluorescens* CHA0 expressing *acdS* from *P. putida* UW4 under its own promoter increased root length in canola seedlings and provided improved protection to cucumber against *Pythium ultimum* damping-off disease, demonstrating the involvement of plant ET in the plant–pathogen interaction (Wang et al. 2000). However, the efficiency of expression of introduced *acdS* genes is host specific. Thus, when a *Pseudomonas acdS* gene was cloned under the control of the regulatory *acdR* gene and introduced into *Azospirillum* strains lacking ACCD, the *acdS* gene was not expressed in these bacteria (Holguin and Glick 2001). Conversely, when the native regulatory region of the *Pseudomonas acdS* gene was replaced by either the *E. coli lac* promoter or the tetracycline (*tet*) promoter, ACCD was expressed at a high level and the growth-promoting activity of the transformed *Azospirillum* strain was significantly improved (Holguin and Glick 2003). The *P. putida* UW4 gene *acdS* was exploited to further enhance the activity of biocontrol strains of *S. plymuthica* and *P. fluorescens* against the fungal pathogen

*Rhizoctonia solani*. The transconjugants obtained showed high ACCD activity similar to that observed in strain UW4 and provided improved protection of beans against *R. solani* rot root disease under greenhouse conditions (Kim and Chernin unpublished). These results are consistent with the potential of using an *acdS* gene as a tool to enhance PGPR biocontrol activity.

### 14.4.2 Transgenic Plants Expressing Bacterial ACC Deaminase Are More Resistant to Pathogens

Several transgenic plants (tomato, canola, and tobacco) that express ACCD have been engineered (Table 14.1). Modifying the amount of ET produced under ripening or stress conditions is the goal of a wide array of transgenic strategies (Stearns and Glick 2003). Enzymes that degrade SAM or ACC, the precursors of ET, have been shown to effectively reduce ET levels without drastically altering the physiology of the plant (Klee et al. 1991; Robison et al. 2001a). Expression of sense or antisense versions of enzymes from the ET biosynthesis pathway should also allow for genetic control of ET levels (Hamilton et al. 1990).

The other approach to achieve a similar goal is to construct a plant with decreased ET level due to expression of a heterologous ACCD gene. By inserting the gene for ACCD into tomato plants under the control of the CaMV 35S promoter, fruit with delayed ripening were produced. No significant phenotypic differences were reported between plants with high levels of ACCD and control plants, except for the difference in fruit ripening (Klee et al. 1991). Other transgenic tomato lines have been produced using the same strategy and with similar results (Reed et al. 1995). In addition, the transgenic tomato plants that expressed ACCD were protected from flooding, heavy metals, and arsenic inhibition (Grichko and Glick 2001; Grichko et al. 2000; Nie et al. 2002).

Moreover, the transgenic tomato plants that expressed ACCD and had a lowered level of stress ET show significant higher tolerance to various pathogens. Thus, transgenic tomato plants expressing a bacterial ACCD gene from *P. putida* UW4 were significantly more resistant to *Verticillium dahliae* wilt disease compared to nontransformed tomato plants (Robison et al. 2001b; Tamot et al. 2003). Three promoters were used to express ACCD in the plant (1) CaMV 35S (constitutive expression); (2) *rol*D (limits expression specifically to the site of *Verticillium* infection, i.e., the roots); and (3) *prb*-1b (limits expression to certain environmental cues, e.g., disease infection). Significant reductions in the symptoms of *Verticillium* wilt were obtained for *rol*D- and *prb*-1b- but not for 35S-transformants. However, with a root-specific promoter *rol*D, which mimics the effect of adding ACCD-producing PGPB to the plant roots, the most prominent results were obtained. The pathogen was detected in stem sections of plants with reduced symptoms, suggesting that reduced ethylene synthesis results in increased disease tolerance. The obtained effective control of *Verticillium* wilt, the disease known as rather difficult to manage, indicates a way to prevent this disease by decrease of ET production in infected plants via the

**Table 14.1** Characteristics of transgenic plants expressing bacterial genes encoding ACC-deaminase

| Plant species | Source of ACC-deaminase gene | Promoter | Relevant characteristics | References |
|---|---|---|---|---|
| *Lycopersicum esculentum* L. | *Pseudomonas* sp. | (CaMV) 35*S* | Delay of fruit ripening; suppression of *Agrobacterium* crown galls | Klee et al 1991; Toklikishvili et al 2010 |
| *Petunia hybrida*, *Nicotiana tabacum*, *Lycopersicon esculentum* | *P. chlororaphis* | the figwort mosaic virus (FMV)35S and fruit specific tomato promoters, e.g., E8 | Delay of senescence of flowers and fruit ripening | Klee and Kishore 1992 |
| *Lycopersicum esculentum* L. | *P. chlororaphis* | (CaMV) 35*S* | Delay of fruit ripening | Reed et al 1995 |
| *Lycopersicum esculentum* L. | *P. putida* | (CaMV)35*S*, *rol*D and *prb*-1b | Resistance to stress caused by heavy metals | Grichko et al 2000 |
| *Lycopersicum esculentum* | *P. putida* | (CaMV) 35*S*, *rol*D and *prb*-1b | Increased tolerance against flooding stress | Grichko and Glick 2001 |
| *Lycopersicum esculentum* | *P. putida* | (CaMV) 35*S*, *rol*D and *prb*-1b | Increased tolerance against Verticillium wilt | Robison et al 2001b |
| *Brassica napus* | *P. putida* | (CaMV) 35*S* | Better prolific growth | Nie et al 2002 |
| *Lycopersicon esculentum* | *P. putida* | *prb*-1b | Better vigorous growth | Tamot et al 2003 |
| *Lycopersicon esculentum* | *P. putida* | (CaMV) 35*S*, *rol*D and *prb*-1b | Increase of plant growth, leaf fluorescence, chlorophyll content, fruit weight, lycopene and β-carotene contents in fruits | Grichko et al 2005 |
| *Brassica napus* | *P. putida* | (CaMV) 35*S* and root specific *rolD* promoter from *Ag. rhizogenes* | Higher tolerance toward $Ni^{2+}$ toxicity. | Stearns et al 2005 |
| *Brassica napus* | *P. putida* | (CaMV) 35*S*, *rol*D and *prb*-1b | Higher salt tolerance | Glick et al 2007a |

tissue-specific expression of ACCD. The conclusion from this interesting work was that ET evolved during compatible or susceptible disease interactions may hasten and/or worsen disease symptom development; if so, the prevention of disease-response ethylene should reduce disease symptoms.

Recently, transgenic tomato plants expressing a bacterial ACCD (Klee et al. 1991) were found to be highly resistant to crown-gall formation in comparison to the parental, nontransformed tomato plants (Toklikishvili et al. 2010). These results additionally support the idea that ET is a crucial factor in crown-gall formation. Aloni et al. (1998) using the ET-insensitive tomato mutant *Never ripe* (*Nr*) observed that although tumors caused enhanced ET production in both *Nr* and control plants, since the *Nr* plants did not perceive ET, tumor formation was inhibited. Insensitivity of the *Nr* mutant to ET action, in some sense, mimics the effect of decreased ET levels in galls formed by plants treated with ACC deaminase-producing bacteria or transgenic tomato-expressing bacterial ACCD. Moreover, it is likely that such a plant would be damaged to a lesser extent by most pathogens, including *Agrobacterium*, than the wild-type version. This conclusion is supported by the previously mentioned results (Lund et al. 1998) demonstrating significant reduction of symptoms of vascular wilt on leaves caused by *various* foliar bacterial pathogens in *Nr* mutant lines.

It is worth noting, however, that despite the fact that transgenic plants expressing bacterial ACCD showed enhanced resistance to certain biotic and abiotic stresses, the use of such transgenic plants does not provide any essential advantage compared to treating plants with ACCD-producing PGPB. Moreover, in view of the limitations involved in the practical application of transgenic plants due to various public concerns, it can be concluded that bacteria-treated plants might be preferred for sustainable agriculture compared with transgenic plants, also reflecting the fact that PGPB do more for plants than simply lower their ethylene levels.

## 14.5 Conclusion

Considerable evidence has been presented in this review to substantiate the idea that bacterial ACCD which can cleave ACC, the immediate precursor of ET in plants, could play a key role in inducing disease tolerance in plants. However, additional research is needed to further understand the details of how ET and other plant hormones produced by ACCD-producing PGPBs protect plants against pathogens. This additional work should answer many questions regarding the commercial use of these bacteria as a novel class of BCAs, including their application in combination with already commercialized biocontrol compounds and/or as an alternative, ecologically safe part of the integrated pest-management strategy to protect crops against various diseases. Additionally, the use of ACCD-producing PGPBs could be considered a superior alternative to the use of ACCD-producing transgenic plants. The application of PGPBs, whose main biocontrol strategy is based on ACCD and not on antibiotic production, provides an attractive

commercial possibility as a part of safer "green" strategies for plant pathogen management. Specifically, ACCD-producing root-associated bacteria affords a greater benefit to the plant, reflecting the fact that in addition to lowering ethylene levels, the bacteria also offer a variety of other benefits to the plant. One of the major advantages in employment of ACCD-producing PGPBs in sustainable agriculture practice is that, in addition to their biocontrol characteristics, these bacteria may help the crops to survive a range of growth inhibitory environmental conditions. Thus, ACCD-producing PGPRs can help plants to defend against different biotic and abiotic stresses, thereby avoiding public concerns regarding the introduction and commercialization of transgenic plants having more or less comparable level of tolerance to the same detrimental effects.

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# Chapter 15
# Nutrient Availability and Management in the Rhizosphere by Microorganisms

**Dinesh K. Maheshwari, Sandeep Kumar, Narendra K. Maheshwari, Dhara Patel, and Meenu Saraf**

## 15.1 Introduction

Soil microorganisms constitute a large dynamic source and sink of nutrients in all ecosystems and play a major role in nutrient cycling (Cambardella and Eliott 1992; Collins et al. 1992), soil structure (Lynch and Bragg 1985), reduction in phytopathogens, and other alteration in soil properties influencing plant growth and development. Differences in microbial characteristics and their properties are restricted to general ecological enumerations which are limited in their ability to perform adequately in a stressed ecosystem. Certain groups of soil-inhabiting bacteria including plant-growth-promoting rhizobacteria (PGPR) are one of the most sensitive biological markers available that has been found quite beneficial for most classifying disturbed and contaminated ecosystems. A multiplicity of microorganisms and their functioning is required to create soils and maintain fertility through complex cycles and interactions. In fact, the smallest organisms are responsible for cycling nutrients such as N, P, K, and S, and making these minerals available to plants (Table 15.1). A gram of fertile agricultural soil may contain 2.5 billion bacteria besides other organisms. They play diverse roles in soil. Direct functional attributes including nitrogen fixation, phosphate solubilization, iron acquisition, zinc solubilization, potassium mobilization, and phytohormone production besides other attributes of significance in growth promotion of plants make them suitable natural resources for stress management. Plants can be subject to different types of stresses while in field. Crop plants face a number of hostile

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**Table 15.1** List of microbial genera affecting micronutrient availability

| Microorganisms | Plant growth promoting traits |
|---|---|
| *Pseudomonas fluorescens* | Induced systemic resistances and antifungal activity |
| *Rhizobium leguminosarum* | Siderophores, IAA, HCN, Ammonia, and exopolysaccharide |
| *Mesorhizobium ciceri* | Siderophores and IAA |
| *Proteus vulgaris* | Siderophores |
| *Pseudomonas* spp., *Bacillus* spp. | Phosphate solubilization, siderophores, and IAA |
| *Azospirillium amazonense* | Nitrogenase activity |
| *Gluconacetobacter diazotropicus* | Zinc solubilization |
| *Brevibacillus* spp. | Zinc resistance and IAA |
| *Pseudomonas putida* | Siderophores, Pb, and Cd resistance |
| *Kluyvera ascorbata* | ACC deaminase, siderophores, and metal resistances |
| *Bacillus subtilis* | Antifungal activity |
| *Enterobacter aerogens*, *Enterobacter cloacae* | Phosphate solubilization |
| *Pseudomonas putida*, *Pseudomonas mendocina* | Phytase production |
| *Pseudomonas* spp. | Manganese reducers |
| *Arthrobacter* spp., *Gaeumannomyces* spp. | Manganese oxidizers |

Source: Ahemad and Khan (2011), Rengel and Marschner (2005)

conditions right from seed germination till harvest. A majority of PGPR genera can alleviate and alter stress conditions so as to protect the plants under abiotic and biotic stresses.

The root system plays an important role in plant productivity because roots explore the soil for uptake of essential nutrients. The mechanistic mathematical models based on ion uptake, soil nutrient supply, and root morphology had consistently indicated the importance of root morphology parameters in the uptake of a variety of nutrients, especially N, P, and K (Barber and Cushman 1981; Barber and Silverbush 1984). Root growth is not only sensitive to external concentration of nutrients but also regulated by plant-growth-regulating substances such as auxins and ethylene (Arshad and Frankenberger 2002). Ethylene is a versatile plant hormone that participates in the regulation of many physiological responses (Mattoo and Suttle 1991). Ethylene production in roots is also influenced by nutrient status around the roots (Abeles et al. 1992), and it could be involved directly and indirectly in plant responses to nutrient deficiency and toxicities (Corey and Barker 1989; Barker and Corey 1990). Abscisic acid (ABA) similar to ethylene is involved in plant responses to abiotic and biotic stress conditions. Its alteration inhibits seed germination, initiation of flowering, sex expression of flowers, etc. It is involved in protection against drought, salinity, and heavy toxic materials. ABA is produced by several bacteria namely *Azotobacter brasilens*, *Bradyrhizobium japonicum* (Boiero et al. 2007), etc. Available literature revealed the limited number of genera containing ABA. It was speculated that ABA induces growth by inhibiting the

synthesis of kinetins, thus decreasing its pool, resulting in an increase in root/shoot and plant growth by alleviating stress (Spaepen et al. 2009).

Microbial inoculants as a source of biofertilizers have become a hope for most countries regarding the economical and environmental point of view. Plant–microbe interactions in the rhizosphere influenced crop yield significantly. The region around the root, i.e., rhizosphere, is relatively rich in nutrients because of plant photosynthates, to the extent of 40%, lost from the root system. Consequently, the rhizosphere supports large microbial populations capable of exerting beneficial, neutral, or detrimental effects on plant growth.

Nutrients are important for growth and development of plants and also microorganisms and they are important factors in disease control (Agrios 2005). All the essential nutrients can affect disease severity (Huber and Graham 1999). However, there is no general rule, as a particular nutrient can decrease the severity of the disease but can also increase the severity of the disease, incidence of other diseases, or have a completely opposite effect in a different environment (Marschner 1995; Graham and Webb 1991; Huber 1980) (Table 15.2).

Although plant disease resistance and tolerance are genetically controlled (Agrios 2005), they are affected by the environment and especially by nutrient deficiencies and toxicities (Marschner 1995; Krauss 1999). The physiological functions of plant nutrients are generally well understood, but there are still unanswered questions regarding the dynamic interaction between nutrients and the plant–pathogen system (Huber 1996a). It is important to manage nutrient availability through fertilizers or change the soil environment to influence nutrient availability and in that way to control plant disease in an integrated pest-management system (Huber and Graham 1999; Graham and Webb 1991). The use of fertilizers produces a more direct means of using nutrients to reduce the severity of many diseases and together with cultural practices can affect the control of diseases (Marschner 1995; Atkinson and McKinlay 1997; Oborn et al. 2003).

There are pathogens that can immobilize nutrients in the rhizosphere, the soil surrounding plants roots, or in infected tissues such as roots, while others interfere

**Table 15.2** Effect of N and K level on disease severity of several diseases

| Pathogens or disease | Low K | High K | Low N | High N |
|---|---|---|---|---|
| *Puccinia graminae* | Increase | Decrease | Decrease | Increase |
| *Xanthomonas oryzae* | Increase | Decrease | – | – |
| *Xanthomonas vesicatoria* | – | – | Increase | Decrease |
| *Tobacco mosaic virus* | Increase | Decrease | Decrease | Increase |
| *Alternaria solani* | Increase | Decrease | Increase | Decrease |
| *Plasmodiophora brassicae* | – | – | Decrease | Increase |
| *Fusarium oxysporum* | Increase | Decrease | Increase | Decrease |
| *Pyrenophora tritici-repentis* | Increase | Decrease | – | – |
| *Erysiphe graminis* | Increase | Decrease | Decrease | Increase |
| *Oidium lycopersicum* | – | – | Decrease | Increase |
| *Pseudomonas syringae* | – | – | Decrease | Increase |

Source: Dordas (2007)

with translocation or utilization efficiency and can cause nutrient deficiency or hyperaccumulation and nutrient toxicity (Huber and Graham 1999). Also, other organisms can utilize a significant amount of nutrient for their growth, causing a reduction in the availability of nutrients for the plant and an increase in its susceptibility due to nutrient deficiency (Timonin 1965). Recently, the emerging role of PGPR in agrobiology has been described by Aeron et al. (2011).

## 15.2 Root Exudates

Compared with the soil organic matter, root exudates represent an easily degradable nutrient source for microorganisms, allowing some microbial species to proliferate rapidly in the rhizosphere. Plants grown with deficient versus sufficient nutrient supply often have differential microbial communities in the rhizosphere (Marschner et al. 2004). Nutrient deficiency can influence rhizosphere microorganisms either directly (by affecting their nutrition) or indirectly (by altering root morphology and exudation). *Lupinus albus* produces cluster roots under P deficiency; cluster roots of different ages have different microbial community compositions, as influenced by root exudation (Marschner et al. 2002). In general, PGPR establish to reach root surfaces by active motility facilitated by bacterial flagella guided by chemotactic responses, as observed by a number of workers. Conversely, chemotaxis of rhizosphere-competent *Azospirillum* occurred due to accumulation of sugars, amino acids, and organic acids; however, the degree of chemotactic responses to each of these compounds differed or varied according to strains.

The chemical fertilizers, N, P, K, and S and many more micronutrients may also act as chemoattractants to various genera of PGPR. The chemotactic response was known as metabolism-dependent chemotaxis and observed in several bacterial strains including *E. coli* and *Azospirillum brasilens*. The nitrogen source proline and the carbon source glycerol were reported to elicit a chemotactic response in *E. coli*. Recently, Singh et al. (2008) observed that the growth-supporting ability of root exudates of chir-pine root favor the establishment and the maintenance of *B. subtilis* BN1 in its rhizosphere. In fact, bacterial communities residing in the rhizosphere respond, in particular, with respect to density, composition, and activity to the plethora and diversity of organic exudates, resulting in plant-species-specific microflora that may eventually vary with the stage of plant growth (Wieland et al. 2001). The root exudation is believed to be plant specific and this specificity may reflect the evolution or specific physiological adaptation to conditions of a particular soil habitat. The type of root exudates is crucial for the ecosystem distribution and niche specificity of certain plants (Table 15.3). The composition of root exudates was shown to vary with plant species and stage of plant growth (Jaegar et al. 1999). Under certain conditions, many compounds present in the root exudates stimulate a positive chemotactic response in bacteria (Somers et al. 2004). Being a major driving force for microbial root colonization, root exudation could be engineered precisely to stimulate specific microbial colonization on the

**Table 15.3** Possible role of different types of root secretions

| Role | Action |
|---|---|
| Acquisition of nutrients | Fetchers: seek and fetch (e.g., phytosiderophores)<br>Modifiers: modification of the rhizosphere soil with protons and reductants (lower pH)<br>Ectoenzymes: convert unusable organic forms into usable ones (e.g., phosphatases) |
| Acquisition of water | Modification of the rhizosphere and soil with mucilage |
| Protection against physical stress | Response to high soil strength through modification of interface by lubrication and amelioration of rhizosphere soil |
| Protection against pathogens | Defensive response to invasion via production of phytoalexins, microbial response, or production of antibiotics |
| Protection against toxic elements | Response to a toxic nutrient such as complexation of aluminum or sequestering of sodium |
| Protection against competition | Modifications of rhizosphere soil with phytoactive compounds such as allelochemicals |
| Establishment of symbiotic relationships with microbes<br>*Rhizobium*<br>Mycorrhiza (VAM)<br>*Azotobacter/Azosprillum*<br>Others | Chemotactic response, nitrogen fixation, P and mineral uptake, production of rooting agents, many important known and unknown functions |

Source: http://www.biogro.com

roots. Oger et al. (1997) earlier said that genetically engineered plants produce opines that alter the rhizosphere community. In fact, due to a high diversity of chemical influences in the rhizosphere of different plants, roots drive the specific selection of microbes from the indefinite pool of soil microbial diversity.

## 15.3 Interface Between Soil and Plant Root

The rhizosphere is an interface between the soil and the plant root and plays a very significant role in plant growth and development. This property is due to root exudation of certain organic metabolites of high nutrient content that form a unique microenvironment in the terrestrial ecosystem. PGPR supply macro- and micronutrients, as exemplified by the well-established association between rhizobia–legume symbiotic relationships (Fig. 15.1).

Greater exudation not only supports higher population of microbial cells, but the average bacterial cell colonizing a particular seed also becomes metabolically active; but this applies to a particular crop such as pea. These observations suggest profoundly different influential effects of the microenvironment that vary by nutrient status on the expression of bacterial traits. Further, plant exudation

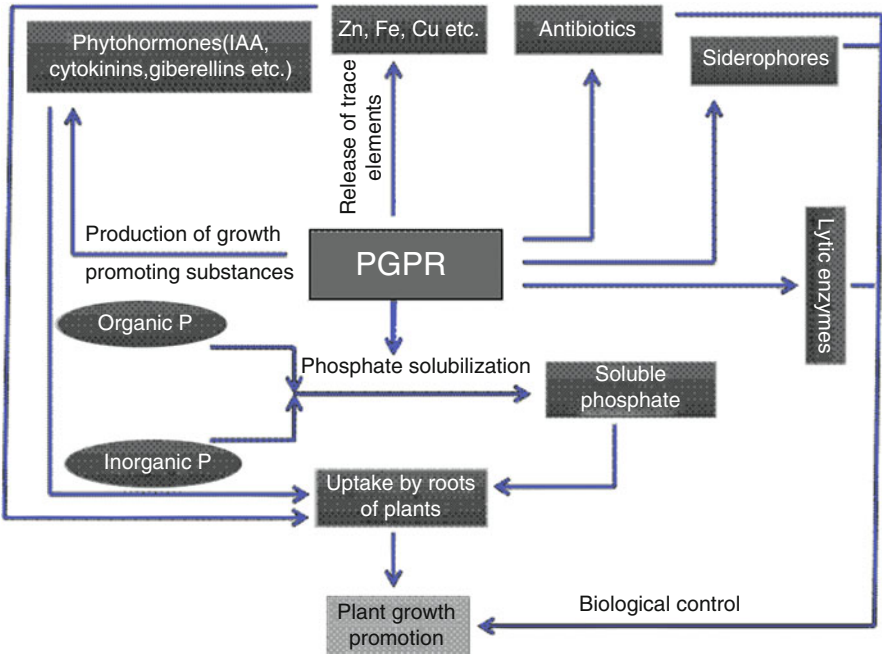


**Fig. 15.1** Mechanism involved in plant growth promotion by rhizobacteria. Modified and adapted from Khan et al. (2010)

(quality and quantity) influences the behavior of the bacterial cell. The individual components of the exudates have been shown to differentially influence root colonization and metabolic activity of PGPR. However, the influence by both the type and the concentration of exudates' components may be completely different in taxonomically unrelated bacteria, such as fluorescent pseudomonads which are partial to organic acids over sugars as the preferred carbon source for growth.

Adoption of efficient strains in the form of microbial inoculants is a requisite. It can manage nutrient dynamics as well as pathogenesis to more environmental biosafety, while improving crop production and lowering inputs of expensive and hazardous agrochemicals. The establishment of a multinational network of international experts created a broad-based understanding of the physiology of PGPR and the plant–microbe ecology with special reference to rhizobia–rice association for the reduction of the requirement of additional nitrogenous fertilizer inputs. Such advancement led to assist low-income group farmers who produce rice on marginally fertile soils deficient in nitrogen and many other nutrients. In another case, an increase in the cereal yield by about 15–20% was obtained using the full recommended amount of chemical fertilizer input to obtain similar results.

## 15.4 Flooding Stress

Oxygen deprivation to plant roots is the main consequence of flooding (Jackson 1985); when oxygen level falls below 50 nmol $m^{-3}$ (normal 230 m mol $m^{-3}$), hypoxia occurs. In a period of 1 h of flooding, the partial pressure of oxygen declines from 20.8 to 7.9 kPa, and further decreases to 1 kPa after 1 day of flooding. In such a short span of onset of flooding, soil microorganisms consume all the available oxygen, and different toxic compounds begin to accumulate in the soil. The plants show different symptoms, which arise due to reduced root permeability, water absorption, and mineral uptake; closure of stomata followed by a decrease in photosynthesis; alteration in hormone level; inhibition of stem and root growth; hypertrophy or cell enlargement of lenticels; and premature fruit drop (Vertapelian and Jackson 1977).

Else et al. (1995) reported the release of high amount of ACC into the soil during the state of flooding. ACC deaminase-containing bacteria degrade ACC and establish their niche in the root zone. Such a process may be an effective noninvasive approach to ACC amelioration level of ACC decreases. In PGPR, the expression of an ACC deaminase gene, which is increased under anaerobic conditions, helps to lower the level of endogenous ACC and hence the concentration of ethylene in a plant (Grichko and Glick 2001). In flooded soil, other microbes are suppressed and bacteria predominate. Further, bacteria which proliferate due to oxygen deprivation, and utilize ACC excluded by plant roots, are likely to be selectively enriched. In tomato, seed bacterization with ACC deaminase-containing bacteria tolerates flooding stress, and plants are protected due to deleterious effects of root hypoxia (Grichko and Glick 2001).

## 15.5 Nutrient Stress

Plants face some abiotic stress conditions including soil nutrient of different macro- and micronutrients. The important stresses due to deprivation of nutrients are given below.

### *15.5.1 Nitrogen*

Nitrogen (N)'s most recognized function in the plant is its presence in the protein molecules. Besides, some important molecules such as purines, pyrimidines, and the coenzymes also contain N. Nitrogen deficiency in plants causes yellowing or chlorosis of leaves in the more mature leaves. In severe deficiency, the lowermost leaves on a plant become dry and yellow. N deficiency is found in many plants in the production of pigments other than chlorophyll when N is lacking. It is an

essential macronutrient. Its supply is met by both biological and chemical fertilizers. Biofertilization by PGPR accounts for the increased N supply by approximately 65%. The most efficient N fixers are symbiotic bacterial strains belonging to different rhizobial genera and species. These bacteria form a host-specific symbiosis with different genera of legumes. There are about 750 genera with 18,000–19,000 species of plants belonging to the family Leguminaceae and only 15–20% of the genera have been studied for their nodulation.

Additionally, some free-living nitrogen fixers such as *Azospirillum*, *Herbaspirillum*, *Acetobacter*, *Azotobacter*, and *Azorcus* are also able to fix atmospheric N. Except *Azospirillum*, other bacterial genera are predominantly found as endophytes inside the tissue of roots, stems, and leaves. A practical challenge is to widen the plant host range of symbiosis toward other nonleguminous crops such as wheat, corn, and rice. In fact, it is now clear that rhizobia contain genes on the *symplasmid* as well as on chromosomal symbiotics lands that are homogeneous to those encoding type 3 secretion systems used by pathogenic bacteria to deliver virulence factors into host cells. Actually, rhizobial type 3 systems secrete specific proteins which are involved in the symbiosis. Similar genes encoding type 3 secretion systems have also been identified in PGP *Pseudomonas fluorescens*. Many PGPR genera are known to increase effective nodulation in leguminous crop and these genera facilitate symbiosis of applied rhizobia. The effect of N is quite variable in the literature mainly due to the different response, depending on the type of the pathogen. Regarding the obligate parasites, e.g., *Puccinia graminis* and *Erysiphe graminis*, when there is high N supply there is an increase in severity of the infection; however, when the disease is caused by facultative parasites, e.g., *Alternaria*, *Fusarium*, and *Xanthomonas* spp., high N supply decreases the severity of the infection.

The difference between the obligate and facultative parasites is due to the nutritional requirements of these two types. Obligate parasites require assimilates supplied directly from living cells. In contrast, facultative parasites are semi saprophytes which prefer senescing tissue or which release toxins in order to damage or kill the host plant cells. Therefore, all factors which support the metabolic activities of host cells and which delay senescence of the host plant can increase resistance or tolerance to facultative parasites (Agrios 2005; Vidhyasekaran 2004). The main factor for the increased susceptibility to obligate parasites at high N rates is the various anatomical and biochemical changes together with the increase in the content of the low-molecular-weight organic nitrogen compounds which are used as substrates for parasites. It is believed that plants grown under conditions of low N availability are better defended against pathogens because there is an increase in the synthesis of defense-related compounds (Bryant et al. 1983; Herms and Mattson 1992; Hoffland et al. 1999, 2000; Wilkens et al. 1996).

At high $NO_3$, disease is decreased in the case of *Fusarium oxysporum*, *Botrytis cinerea*, *Rhizoctonia solani*, and *Pythium* spp. In contrast, at high $NH_4$, disease is decreased in the case of *Pyricularia oxyzae*, *Thielaviopsis basicola*, *Sclerotium rolfsii*, and *Gibberella zeae*. The form of N can affect the pH of the soil and also the

availability of other nutrients such as Mn. Also, the level of N can affect the phenolics content of plants, which are precursors of lignin. In addition, at high levels of N, there is a decrease in Si content, which can affect disease tolerance.

### *15.5.2 Phosphorous Stress*

Phosphorus (P) is the second most commonly applied nutrient in most crops and is part of many organic molecules of the cell (deoxyribonucleic acid (DNA), ribonucleic acid (RNA), adenosine triphosphate (ATP), and phospholipids) and is also involved in many metabolic processes in the plant. P has been shown to be most beneficial when it is applied to control seedlings and fungal diseases, where vigorous root development permits plants to escape disease (Huber and Graham 1999). Phosphate fertilization of wheat can have a significant effect and almost eliminate economic losses from *Pythium* root rot (Huber 1980). Foliar application of P can induce local and systemic protection against powdery mildew in cucumber, roses, wine grapes, mango, and nectarines (Reuveni and Reuveni 1998). To separate the direct and the indirect effects of P availability on rhizosphere microorganisms, *Hordeum vulgare* and *Cucumis sativus* were grown in a P-deficient soil supplemented with P via soil or foliar application (Marschner et al. 2004) (Table 15.4). In the foliar treatment, the effect was indirect (mediated by the plant) because the rhizosphere community composition was different between P-deficient and P-supplied plants.

Although the total amount of P in the soil may be high, it is mainly (>80%) present in forms unavailable to plants because of adsorption, precipitation, or conversion to organic forms. In acidic soils, P forms iron/aluminium (Fe/Al) phosphates and gets adsorbed to Fe/Al oxides or humic substances. In alkaline calcareous soils, P is often precipitated as calcium (Ca)–P. Organic P (mainly phytate) may represent more than 50% of total P in many soils (Osborne and Rengel 2002). Generally, three broad categories of P-efficiency mechanisms exist in plants to increase availability and uptake of P under deficiency conditions (1) alteration of the geometry or architecture of the root system, (2) secretion or exudation of chemical compounds into the rhizosphere, and (3) association with microorganisms. Plants growing in P-deficient soil allocate a greater proportion of assimilates to root growth and tend to have fine roots of a small diameter and

**Table 15.4** List of microbial genera affecting phosphorus (P) and manganese (Mn) availability

| | |
|---|---|
| P solubilizers | *Bradyrhizobium*, *Rhizobium*, *Gordonia*, *Enterobacter*, *Rahelia*, *Panthoea*, *Pseudomonas* |
| Phytase producers | *Pseudomonas* |
| Mn reducers | *Pseudomonas* |
| Mn oxidizers | *Arthrobacter*, *Gaeumannomyces* |

Source: Rengel and Marschner (2005)

therefore, a large surface area. Fine roots, and especially root hairs (Gahoonia et al. 2001; Nigussie et al. 2003), are effective in scavenging P from the soil environment because of a large surface area of contact with the soil.

Large amounts of phosphoric fertilizers added to soil enhance agro productivity on a regular basis but most of these remain immobilized in large quantity and hence is not available to plants. Organic compounds containing phosphorus are decomposed and mineralized by bacteria and other groups of microorganisms. Fluorescent pseudomonads, rhizobia, and few others showed phosphate-solubilizing activity that has helped in the enhancement of growth and productivity of oilseed crops, such as sunflower, peanut, and mustard. The main mechanism involved is the solubilization of inorganic phosphate by the action of mineral and organic acid production (Deshwal et al. 2003). The precipitated inorganic phosphate potential of PGPR in agricultural innovations is discussed by Kaymak (2011).

Overexpression of P transporters may result in excessive accumulation of P in cells and plant death from P toxicity (Smith 2002), showing the complexity of the nutrient-deficiency response. Hence, we need to understand the fine regulation of the whole sequence that comprises signal perception, transmission, and response to nutrient-deficiency stress before successfully transforming plants to upregulate mechanisms responsible for increasing nutrient availability as well as the capacity to take up nutrients. Characterizing promoters specific to a nutrient-deficiency response (Schunmann et al. 2004), in addition to cloning major genes involved in the plant response to nutrient deficiency is, therefore, crucial for successful production of nutrient-efficient plant genotypes that modulate their response according to nutrient availability. Promoters and major genes from one plant species could be used to transform other species. At least in the case of P nutrition, this approach could be successful because the regulation of P-deficiency-induced genes appears to be conserved in different plant species, for example, in *L. albus* and *Medicago sativa* (Liu et al. 2005).

#### 15.5.2.1 Exudation of Carboxylates

Exuded organic acid anions may have a role both in solubilization of mineral nutrients and as growth substrates for microorganisms. Typical carboxylates (organic acid anions) found in root exudates include citrate, malate, malonate, acetate, fumarate, succinate, lactate, and oxalate (Rengel 2002). Malate and citrate are the major root exudate components for some plant species, such as cluster root-forming *L. albus* (Neumann and Römheld 1999). Because carboxylates are excellent substrates for microbial growth, high concentrations of carboxylates may occur temporarily and only at rapidly growing root apices not yet densely colonized by microorganisms.

Carboxylates may be exuded by P-deficient roots at appreciable rates [an average rate of 0.57 nmol citrate $cm^{-1}$ root $h^{-1}$ for *Brassica napus* (Hoffland et al. 1989) or 200–400 nmol oxalate $g^{-1}$ soil $h^{-1}$ for *Cassia spectabilis*, with rhizosphere soil

containing at least 29 μmol oxalate $g^{-1}$ soil (Radersma and Grierson 2004)]. Exuded carboxylates may solubilize various P complexes (Hinsinger 2001).

#### 15.5.2.2 Phosphatases and Phytase

Plants and microorganisms increase exudation of P-hydrolyzing enzymes under P deficiency. These enzymes break down organic P, thus making P available for uptake. Phosphatases are not effective in mineralizing phytate (inositol hexaphosphate), the major form of organic P in many soils. However, phytase specifically catalyzes the breakdown of phytate. Roots excrete little, if any, phytase, whereas microorganisms (e.g., *Aspergillus niger*) exude large amounts (Richardson et al. 2001), indirectly enabling plants to utilize phytate (Osborne and Rengel 2002). Genetic modification of plants to excrete microbial phytase (George et al. 2005) may allow plants to increase P uptake, but the effectiveness of phytase is limited by the low phytate availability in soil and binding of phytase to soil particles.

### *15.5.3 Potassium Stress*

Potassium (K) is abundant (0.5–2.5%) in most soils but a small fraction of it is available to plants. Contrary to nitrogen and phosphorus, in most tropical soils, need for potassium arises because it becomes deficient due to leaching and/or fixation. The unavailable form of K in soil is about 90–98% of the total K in soil. About 0.1–2% of this amount is readily available. Further, unlike nitrogen and phosphorus, potassium forms no coordinated compounds; rather, it exists solely as $K^+$ either in soil solution or bound to negative charges on tissue surfaces through radicals.

When potassium is limiting, the deficiency symptoms appear on the leaf surfaces in the form of white spots on their edges. Sometimes, chlorosis and necrosis of the leaf edges are also seen. The symptoms arise first on the lower leaves progressing toward the top as the severity of the deficiency increases. Potassium deficiency may also occur in young leaves at the top of high-yielding fast-maturing crops such as cotton and wheat. Lodging in small grains and stalk breakage in corn are also reported. Such plants become more prone to bacterial and fungal diseases. Lack of K in wetland rice greatly increases the severity of foliar diseases such as stem rot and sheath blight. Potassium chloride is by far the most widely used chemical fertilizer for plant nutrition. It directs application to the soil, readily dissolves in soil water. Other forms are potassium sulfate, potassium magnesium sulfate, muriate of potash, etc. There are considerable populations of K-solubilizing bacterial genera present in soil and plant rhizosphere (Sperberg 1958). Some soil microbes solubilize unavailable forms of K-bearing minerals by excreting organic acids which either directly dissolve the rock potassium or chelate silicon ions to bring the potassium for plant uptake. Increasing the availability of minerals in soils using phosphorus, potassium, and zinc-solubilizing microorganisms along with

raw sources of rock minerals such as feldspar or rock phosphate have been reported (Lin et al. 2002; Sahin et al. 2004; Giris et al. 2008).

Potassium decreases the susceptibility of host plants up to the optimal level for growth: beyond this point, there is no further increase in resistance which can be achieved by increasing the supply of K and its contents in plants (Huber and Graham 1999). The high susceptibility of the K-deficient plant to parasitic disease is due to the metabolic functions of K in plant physiology. Under K deficiency, synthesis of high-molecular-weight compounds (proteins, starch, and cellulose) is impaired and there is accumulation of low-molecular-weight organic compounds. Also, K may promote the development of thicker outer walls in epidermal cells, thus preventing disease attack. K can also influence plant metabolism, as K-deficient plants have impaired protein synthesis and accumulate simple N compounds such as amides which are used by invading plant pathogens. Application of K can decrease *Helminthosporium* leaf blight severity and increase grain yields in wheat (Sharma and Duveiller 2004; Sharma et al. 2005). It has been shown that K fertilization can reduce the intensity of several infectious diseases of obligate and facultative parasites (Table 15.2).

Potassium application also increases plant growth but most studies indicate that its application also increases nematode reproduction. Application of *B. japonicum* and *Glomus mosseae* with potassium fertilizer gave best results in improving plant growth besides reducing nematode population.

### *15.5.4 Sulfur Stress*

In Indian soil, sulfur (S) is abundant in fine-texture soil than in coarse soil because the latter contains a greater amount of organic matter. S is the fourth major nutrient besides nitrogen, phosphorus, and potassium because its availability to growing plants allows certain amino acids (cysteine and methionine) to be synthesized in optimum amounts. S deficiency was observed initially in the crops that were raised in dry land and in rice in wet land. The increased application of low S fertilizers and reduced use of organic manure resulted in S deficiency in Indian soil. Under the condition of S deficiency, protein synthesis is impaired and the level of chlorophyll in the photosynthetic organism declines. During the early phases of sulfur deficiency in leguminous plants, the rate of nitrogen fixations decline more than the rate of photosynthesis. Sulfur deficiency in plants usually appears on the leaves in the form of light green coloration, intervein chlorosis, stripes on upper leaves, etc. These symptoms can be overcome by foliar application of elemental S in the form of dust. This normally alleviates S deficiency in the crop besides protecting it from rust disease. To overcome the issue of deficiency, S fertilizers are added mainly in reduced form such as elemental S. This form must be oxidized to sulfate by the involvement of microorganisms. Plants can easily take up S in the form of sulfates. Sulfur occurs in soil in different forms mainly as elemental S, mineral sulfide,

sulfates, hydrogen sulfide, organic S compounds, etc. Inorganic form of S in soil occurs as sulfate and in anaerobic conditions, it is present in a reduced form.

During the uptake process, sulfate is activated by forming adenosine-5′ phosphosulfate and thereafter, 3-phosphoadenosine-5′ phosphosulfate that initially gives rise to the amino acid cysteine, which acts as a precursor for methionine and later as the building block of proteins. Kertesz and Mirleau (2004) demonstrated that the sulfate ester and sulfonate pools of soil sulfur are also plant bioavailable, probably due to interconversion of carbon-bonded sulfur and sulfate ester sulfur to inorganic sulfate by soil microorganisms. An important role of S in leguminous plants was observed. Its deficiency in soil resulted in poor quality of crop production. Application of elemental S adequately is required in enhancing pod yield in peanut, oil contents in sesame, mustard, soya bean, sunflower, etc. An early seedling emergence and growth promotion in rice, maize, field bean, cotton, and other crops due to S application was recorded.

Sulfur interacts with phosphorus (P) available in soil and such process allows a greater absorption of $PO_4^{3-}$ in comparison to that of $SO_4^{2-}$ by growing crop plants. During this process, desorption of $SO_4^{2-}$ also occurs due to substitution of $PO_4^{3-}$ on the absorption site of $SO_4^{2-}$. This may cause leaching of S in soil, resulting in S deficiency following application of phosphate. Lime (CaO) releases $SO_4^{2-}$ in soil and makes the soil neutral, which is a desirable characteristic to neutralize acid soil. Conversely, application of elemental S to alkaline soil increases the availability of other nutrients such as Fe, Zn, and Mn. The effect of S oxidation on the availability of these nutrients is to lower redox potential of the soil and reduce the insoluble form of $Fe^{3+}$ to the soluble form, i.e., $Fe^{2+}$, similar to the iron-chelating action of siderophores. Sulfur has synergism with nitrogen, phosphorus, potassium, magnesium, and zinc.

Various microbes utilize sulfur compounds as electron donors and oxidize them in the soluble form of sulfate during the biogeochemical cycle. A diverse range of bacteria of different physiological natures, viz. chemolithotrophs, photolithotrophs, photohetrotrophs, etc., play different roles in S oxidation. In fact, S oxidation occurs due to involvement of the S 4 intermediate pathway (S 41) which includes formation and oxidation of tetrathionate, thionate, polythionate, and/or S from thiosulfate and another in the *Paracoccus* Sulfur oxidation (PSO) pathway that oxidize thiosulfate directly into sulfate. But studies are required to be performed in different soils in a diverse range of crops growing in agroclimatic zones.

### *15.5.5 Zinc Stress*

Soil nutrient enhancement is of considerable significance because in various regions of globe the soil is deficient in one or the other form of minerals. Regular application of micronutrient fertilizers in the form of chemicals may pose several environmental problems. Zn stands at the 12 position (IIB) in the periodic table, and several of its forms such as Zinc sulfide (ZnS), Zinc oxide (ZnO), Zinc silicate

($ZnSiO_3$), Smithsonite ($ZnCO_3$), Sphalerite (ZnFe)S, Willemite ($ZnSiO_4$), etc., are present in reduced state in soil. Although, Indian soil contains adequate amount of Zn, it is inadequate to meet the requirements of the crop plants (Singh 2001). Therefore, the role of bacteria lies in solubilizing insoluble source of zinc compounds into more accessible forms similar to the phosphate-solubilization process involving using certain phosphate-solubilizing microorganisms. By such a process, Zn compounds may benefit the crop in the fields. Zinc plays an important role in protein and starch synthesis, and therefore a low zinc concentration induces accumulation of amino acids and reducing sugars in plant tissue (Marschner 1995; Römheld and Marschner 1991). As an activator of Cu/Zn-SOD, Zn is involved in membrane protection against oxidative damage through the detoxification of superoxide radicals (Cakmak 2000). Application of Zn to the soil reduced infections by *Fusarium graminearum* (Schwabe) and root rot diseases, caused by *Gaeumanomyces graminis* (Sacc.) in wheat (Graham and Webb 1991; Grewal et al. 1996).

Under extreme $Zn^{2+}$ deficiency, carbon fixation activity is completely abolished and decline in photosynthetic activity occurs owing to less availability of $HCO_3^-$, a substrate for $CO_2$ fixation. Zinc-deficiency symptoms such as chlorosis of leaves occur due to its nonavailability and alterations. The most common feature of zinc deficiency is stunted growth and smaller leaves on account of disturbance in the auxin and indole-3-acetic acid metabolism, an indicator of Zn deficiency in the plant tissues. Baruah and Barthakur (1999) observed that Zn solubility by certain groups of microorganisms increased corresponding to decrease in pH. This kind of reaction mechanism involved the action of micromediated organic acid production responsible for Zn solubilization. White et al. (1997) reported the involvement of phytoextraction, nutrient enhancement, and breakdown of compounds during the process of Zn solubilization by *Thiobacillus ferrooxidans*. A few other bacteria, namely, *Microbacterium saperidae*, *Pseudomonas monteilli*, *Enterobacter cancerogens* (Whiting et al. 2001), *P. fluorescens* (Di Simine et al. 1998), and *P. aeruginosa* also solubilize Zn. The efficient ZnO-solubilizing strains of *M. saperidae*, *P. monteilli*, and *E. cancerogenes* have dominant potential of Zn solubilization which can be concluded by the significance of their habitat linked with Zn hyperaccumulated sites. Saravanan et al. (2007) reported the role of gluconic acid and its derivatives secreted by *Gluconacetobacter diazotrophicus* in the Zn-solubilization process. Certain fungi namely, *Abisidia cylindrospora*, *A. glauca*, *A. spinosa*, *Penicillium aurantiogriseum*, *P. brevicompactum*, and *P. simplicissiumare* also exhibit Zn solubilization. for the benefit of plants. When *Pseudomonas putida* was coinoculated with AM fungi, it increased the mineral concentration and uptake of Zn in some gymnosperms. The Zn-solubilizing bacteria confer certain mechanism-transforming insoluble forms of Zn into solubilized forms that could easily be taken up by plants. In such cases, the PGPR group of bacteria, in particular *Bacillus* spp. and fluorescent pseudomonads, showed Zn solubilization of a wide range of Zn compounds. On the other hand, *Azotobacter chroococcum*, *Bacillus megaterium*,

and *B. edaphicus* increased the availability of Zn in the soil, but molecular mechanisms of Zn-solubilization process are still a subject of research interest for microbiologists.

It may be concluded that Zn solubilization by bacteria plays two important roles. One is as a suitable source of rhizoremediation of Zn compounds accumulating as soil contaminant and the other during its transformation as a nutrient. Certain plant root systems also secrete chemical root exudates that pose extra benefit to the Zn-solubilization process.

### *15.5.6 Iron Stress*

Iron (Fe) is one of the most important micronutrients for animals and humans and the interaction between Fe nutrition and human or animal health has been well studied, as it is involved in the induction of anemia. Several plant pathogens, e.g., *Fusarium*, have higher requirements for Fe or higher utilization efficiency compared with higher plants. Therefore, Fe differs from the other micronutrients such as Mn, Cu, and B, for which microbes have lower requirements. The addition of Cu, Mn, and B to deficient soils generally benefits the host, whereas the effect of Fe application is not as straightforward as it can have a positive or negative effect on the host. Fe can control or reduce the severity of several diseases such as rust in wheat leaves, smut in wheat, and *Colletotrichum musae* in banana (Graham and Webb 1991; Graham 1983). Foliar application of Fe can increase the resistance of apple and pear to *Sphaeropsis malorum* and cabbage to *Olpidium brassicae* (Graham 1983). Rhizosphere microorganisms can synthesize siderophores which can lower Fe level in the soils. These siderophores can suppress germination of chlamydospores of *F. oxysporum f. sp. cucumerinum* in vitro. Biswas et al. (2000) reported a significant increase in Fe uptake in lowland rice through inoculation of *Rhizobium leguminosarum* bv. *trifolii* E11. They suggested that the increased uptake of Fe, P, and K was associated with higher N rates but higher N was a result of mechanisms other than biological N fixation. Patel et al. (2011) reported the production of siderophores by *Pseudomonas putida* and *Pseudomonas pseudoalcaligenes* under salinity stress. Pandey et al. (2005) reported the presence of a new type of hydroxamate type siderophore in rhizosphere competent *Pseudomonas aeruginosa* that solubilizes iron and enhances growth of *Brassica campestris*. Tank and Saraf (2010) reported the production of siderophore by PGPR adapted at 6% NaCl concentration. Pandya and Saraf (2010) showed a significant increase in iron uptake by producing siderophore in the presence of PGPF. Jha and Saraf (2011) showed siderophore production by PGPR isolated from Jatropha rhizosphere. The PGPR genera produce a different type of siderophore that mediates support for better plant growth and development (Maheshwari 2010).

### 15.5.7 Calcium Stress

Ca is important for the stability and function of plant membranes and in case of Ca deficiency, there is membrane leakage of low-molecular-weight compounds, e.g., sugars and amino acids, from the cytoplasm to the apoplast, which stimulate infection by the pathogens (Marschner 1995). Further, Ca is an important component of the cell wall structure, as calcium polygalacturonates are required in the middle lamella for cell wall stability. When Ca concentration drops, there is an increased susceptibility to fungi which preferentially invade the xylem and dissolve the cell walls of the conducting vessels, which leads to wilting symptoms. In addition, plant tissues low in Ca are also much more susceptible than tissues with normal Ca levels to parasitic diseases during storage. Ca confers resistance against *Pythium*, *Sclerotinia*, *Botrytis*, and *Fusarium* (Graham 1983). Ca can be mobilized in alfalfa lesions caused by *Colletotrichum trifolli* and supports the growth of the pathogen by stimulating the macerating action of the pectolytic enzyme polygalacturonic acid transeliminase (Kiraly 1976).

### 15.5.8 Manganese Stress

Manganese (Mn) is probably the most studied micronutrient as regards its effects on disease and is important in the development of resistance in plants to both root and foliar diseases (Graham and Webb 1991; Huber and Graham 1999; Heckman et al. 2003). Mn availability in the soil varies and depends on many environmental and soil biotic factors. Mn is required in much higher concentration by higher plants than by fungi and bacteria and there is opportunity for the pathogen to exploit this difference in requirement (Marschner 1995). Manganese fertilization can control a number of pathogenic diseases such as powdery mildew, downy mildew, take-all, tan spot, and several others (Brennan 1992; Huber and Graham 1999; Heckman et al. 2003; Simoglou and Dordas 2006). Mn has an important role in lignin biosynthesis, phenol biosynthesis, photosynthesis, and several other functions (Marschner 1995; Graham and Webb 1991). Mn inhibits the induction of aminopeptidase, an enzyme which supplies essential amino acids for fungal growth and pectin methylesterase, a fungal enzyme that degrades host cell walls.

Manganese controls lignin and suberin biosynthesis (Römheld and Marschner 1991; Vidhyasekaran 1997) through activation of several enzymes of the shikimic acid and phenylpropanoid pathways (Marschner 1995). Both lignin and suberin are important biochemical barriers to fungal pathogen invasion (Kolattukudy et al. 1994; Rioux and Biggs 1994; Hammerschmidt and Nicholson 2000; Vidhyasekaran 1997, 2004), since they are phenolic polymers resistant to enzymatic degradation (Agrios 2005). Lignin and suberin are believed to contribute to wheat resistance against powdery mildew and to all diseases caused by *G. graminis* (Sacc.) (Rovira et al. 1983; Graham and Webb 1991; Huber 1996b; Krauss 1999). It has also been

shown that Mn soil applications reduce common scab of potato (Keinath and Loria 1996), *Fusarium* spp. infections in cotton, and *Sclerotinia sclerotiorum* (Lib. de Bary) in squash (Graham and Webb 1991; Agrios 2005). The yield of cereals on calcareous soils is frequently limited by Mn deficiency caused by low Mn availability, rather than low Mn content in the soil (Rengel 2000). Mn-efficient genotypes take up more Mn from soils with limited Mn availability, but the physiological mechanisms underlying Mn efficiency are poorly understood (Rengel 2000, 2001). As a result of root uptake and poor mobility, depletion of Mn in the rhizosphere is greater than replenishment from bulk soil, resulting in a lower concentration of Mn in the rhizosphere compared to the bulk soil. Such Mn depletion is more prominent in the rhizospheres of Mn-efficient than Mn-inefficient *Triticum aestivum* genotypes (Marschner et al. 2003). A list of microbial genera affecting manganese availability has been shown in Table 15.4.

*M. sativa* plants exude a variety of carboxylates under Mn deficiency. The amounts of exuded citrate and malonate (and to a lesser extent fumarate, malate, oxalate, and lactate) under Mn deficiency were positively correlated with the Mn efficiency of *M. sativa* genotypes (Gherardi and Rengel 2003).

### *15.5.9 Chemical Fertilizers and Other Synthetic Chemicals' Stress*

Chemical fertilizer are considered to have enhanced the yield of agricultural crops, but with advancements of technologies and increase in environmental awareness it is evident that continuous use of synthetic chemicals affects soil health and mismanages its ecology, provides microbial resistance, kills beneficial microorganisms, and imparts residual effect in edible parts resulting in overall disturbance in the ecosystem (Paul and Savitri 2003). Along with these constraints, their cost and adverse effect on crop plants' quality frequently deters farmers (Finck 1994). In addition, the presence of poisonous heavy metals such as cadmium and lead in synthetic chemical fertilizers may also build up toxicity among plants, animals, and human health if used for prolonged periods. The overuse of chemical fertilizers has been demonstrated to cause economic loss and environmental hazard (Ayala and Rao 2002). Although, chemical fertilizers have played a significant role in the green revolution, their unbalanced use has led to reduction in soil fertility and environmental degradation (Gyaneshwar et al. 2002) and hence a new approach of blending the low dose of chemical fertilizers with bacterial fertilizers for a long-term sustainable approach of an evergreen revolution has been adopted (Kumar et al. 2009).

Agrochemicals including chemical fertilizers reduced the population of beneficial microbes due to their inhibitory effect on bacterial growth (Smiley 1981). In an old report, Strzeleowa (1970) found abnormal morphological changes in rhizobia by growing them on Throton's agar medium containing 2.5 mg/ml urea. Increased

generation time and disrupted protein synthesis in *Acetobacter diazotrophicus* (renamed as *Glucoacetobacter diazotrophicus*) by excessive nitrogenous fertilization was reported by Becking (1995). Further, Muthukumarasamy et al. (2002) noticed higher population of *G. diazotrophicus* in the presence of low nitrogenous fertilization in comparison to excessive N-fertilized soil. Maheshwari and Nishimura (1994) and Maheshwari and Saraf (1994) examined the effect of 2,4-D and carbaryl on morphological features and cell physiology of *Rhizobium leguminosarum* and *B. japonicum*. Further, they also described the effect of two organocarbamate nematicides (aldicarb and carbofuran) on N assimilation (Maheshwari and Gupta 1991a) and growth on oxygen uptake (Maheshwari and Gupta 1991b) of *B. japonicum*. Similar effects of fertilizers and other agrochemicals have been reported by a number of workers around the world (Dragun 1988; Kantochote et al. 2001; Joshi et al. 2006).

Several forms of stress are overcome by ACC deaminase-containing PGPR in bacteria, which eliminate the effect of deleterious phytopathogens and tolerance to stress from polyaromatic hydrocarbons from heavy metals such as $Ni^{2+}$ and $Co^{2+}$ in salinity and drought as observed by Glick et al. (2007).

## 15.6 Nutrient Management and Disease Control

It is important to provide a balanced nutrition, and at the time when the nutrient can be most effective for disease control, and also for higher yield. Not only can the application of the fertilizer affect disease development, but also any factor that affects the soil environment such as pH modification through lime application, tillage, seedbed firmness, moisture control (irrigation or drainage), crop rotation, cover crops, green manures, manures, and intercropping for sustainable agriculture (Kumar et al. 2011a, b).

Addition of microorganisms such as bacteria, fungi which form mycorrhizae, and any PGP organisms can increase nutrient uptake (P, Zn, and Mn) by influencing minor element availability through their oxidation–reduction reactions or siderophore release (Huber and McCay-Buis 1993). In some cases, the application of fertilizers to the soil is not always effective, such as in the case of Mn, Zn, and Fe in high pH soils with high concentrations of free $CaCO_3$, or where rapid oxidation by microorganisms makes Mn unavailable in the soil. Many times, it is recommended to conduct foliar applications which relieve aboveground deficiency symptoms. Increasing the nutrient content in the grains was actively pursued as a means of improving human nutrition and may concurrently increase plant resistance to a variety of diseases (Graham 1983; Graham and Webb 1991). Take-all is one of the most important diseases of wheat and occurs in many countries of the world. It was found that 12 of the 14 principal nutrients required for plant growth affect take-all. Application of N fertilizer and especially $NH_4^+$ can reduce the losses from take-all: $NH_4^+$ also increases the availability of Mn, Zn, and Fe. Crop rotation can decrease the incidence of the disease. Also, it was found that long-term

monocropping of wheat provides a natural biological control of this disease called take-all decline.

## 15.7 Integrated Nutrient Management

Most Indian soil contains sufficient amount of agrochemicals due to their continuous applications since the time of the green revolution in 1972. Hence, an integrated approach proved significant wherein microbial inoculants were applied along with reduced level of fertilizers so as to obtain better growth and yield. Application of chemical fertilizers and agrochemicals lowers the microbial diversity. Soil bacteria are more sensitive to chemical N fertilizer application during the plant growth cycle. Temporal changes in bacterial functionality have been observed to disappear in conventional cultivation with chemical inputs. The effects of different chemicals on microbes were well documented by Dragun (1988) and Kantochote et al. (2001).

Declining soil fertility and mismanagement of plant nutrients have made the task of providing food for the world's population in 2020 and beyond more difficult. However, some responses can ameliorate these difficulties. The responses highlighted here comprise the approach commonly known as INM. Sustainable agricultural production incorporates the idea that natural resources should be used to generate increased output and incomes, especially for low-income groups, without depleting the natural resource base. In this context, INM maintains soils as storehouses of plant nutrients that are essential for vegetative growth. INM's goal is to integrate the use of all natural and man-made sources of plant nutrients, so that crop productivity is increased in an efficient and environmentally benign manner, without sacrificing soil productivity of future generations. INM relies on a number of factors, including appropriate nutrient application and conservation and the transfer of knowledge about INM practices to farmers and researchers. Balanced application of appropriate fertilizers is a major component of INM. Fertilizers need to be applied at the level required for optimal crop growth, based on crop requirements and agroclimatic considerations.

Over-application of fertilizers, while inexpensive for some farmers in developed countries, induces neither substantially greater crop nutrient uptake nor significantly higher yields (Smaling and Braun 1996). Rather, excessive nutrient applications are economically wasteful and can damage the environment. Under application, on the other hand, can retard crop growth and lower yields in the short term, and in the long term jeopardize sustainability through soil mining and erosion.

Nutrient conservation in the soil is another critical component of INM. Soil conservation technologies prevent the physical loss of soil and nutrients through leaching and erosion and fall into three general categories. First, practices such as terracing, alley cropping, and low-till farming alter the local physical environment of the field and thereby prevent soil and nutrients from being carried away. Second, mulch application, cover crops, intercropping, and biological nitrogen fixation act as physical barriers to wind and water erosion and help to improve soil

characteristics and structure. Lastly, organic manures such as animal and green manures also aid soil conservation by improving soil structure and replenishing secondary nutrients and micronutrients (Kumwenda et al. 1996).

Integrated exploitation of biofertilizers with growth-regulating chemicals to maintain the higher leaf-area index (LAI) throughout the growth period and enhanced dry matter production have been observed by many workers, and combined application of biofertilizers, and reduced dose of chemical fertilizers resulted in increased wheat yield as compared to the single application and recommended dose of fertilizers. INM supply seems to be essential not only for increasing the crop productivity but also for the maintenance and possible improvement of soil fertility for sustainable crop productivity. Results from various cropping systems showed the positive interaction of the integrated use of mineral fertilizers, organic manures, and biofertilizers for maintaining growth throughout the crop duration. INM is an endeavor to blend ecology and economy in a cost–benefit framework. It takes systemic and simultaneous account of the environment aspects, the quality of the produce, and the profitability of the agriculture. Recently, Cakmakci et al. (2006) investigated seed inoculation with five $N_2$-fixing and two phosphate-solubilizing bacteria and mineral fertilizers (N and P) application. Conversely, N-fertilizer significantly reduced sugar content when compared with control and PGPR. Improvement in agricultural sustainability requires optimal use and management of soil fertility and soil physical properties, which rely on soil biological processes and soil biodiversity.

Integrated uses of organic and inorganic fertilizers improve crop productivity and sustain soil health and fertility. Concentrating on nutrients' management alone, we can increase the agricultural productivity up to 15–20%; but there is a gap of 10 million tons of plant nutrient between removal by crop and replenishment through fertilizers. Of late, biofertilizers are being promoted as an important component of an integrated plant nutrient system (IPNS). It is a good beginning but the country has a long way to go. Application of the organic–inorganic combination has proved effective in realization of high yield and high response to added nutrients, while the imbalanced use of nutrient has a detrimental effect.

The interactive effect of biofertilizers and INM has been studied on oilseed crops such as mustard, soybean, groundnut, and sunflower (Dubey and Maheshwari 2011). The combined application of biofertilizers and reduced (low) dosages of chemical fertilizers increased wheat yield. Reasonable results have been observed from fertilized fields using fertilizer-tolerant strains of an indigenous rhizobial population.

## 15.8 Conclusion and Future Perspective

Understanding of the role that root exudation of organic compounds plays in increasing nutrient availability in the rhizosphere is sketchy at present. The regulation of the complete exudation process and the underlying genetics need to be elucidated. Future prospects for genetically manipulating plants to enhance their capacity to alter the biology and chemistry of the rhizosphere and increase nutrient

availability under given environmental conditions will be underpinned by (1) understanding the regulation of the exudation process, (2) elucidating the regulation of root morphology, (3) characterizing the synthesis and activity of membrane-embedded nutrient transporters, and (4) understanding the interactions among root exudation, indigenous rhizosphere microorganisms, and nutrient availability. Bioengineering the rhizosphere by adding beneficial microorganisms will require understanding of microbe–microbe and microbe–plant interactions, enabling introduced microorganisms to show full activity in the targeted rhizosphere. It is also necessary to find the best integrated pest-management approaches with disease-resistant varieties, which can be combined with specific cultural management techniques and can efficiently control plant disease. In addition, more research is required to find how the nutrients increase or decrease disease tolerance or resistance, what the changes are in plant metabolism, and how this can be used to control plant disease.

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