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## PHYTOREMEDIATION OF HEAVY AND TRANSITION METALS AIDED BY LEGUME-RHIZOBIA SYMBIOSIS

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*Legumes are important for nitrogen cycling in the environment and agriculture due to the ability of nitrogen fixation by rhizobia. In this review, we introduce an important and potential role of legume-rhizobia symbiosis in aiding phytoremediation of some metal contaminated soils as various legumes have been found to be the dominant plant species in metal contaminated areas. Resistant rhizobia used for phytoremediation could act on metals directly by chelation, precipitation, transformation, biosorption and accumulation. Moreover, the plant growth promoting (PGP) traits of rhizobia including nitrogen fixation, phosphorus solubilization, phytohormone synthesis, siderophore release, and production of ACC deaminase and the volatile compounds of acetoin and 2,3-butanediol may facilitate legume growth while lessening metal toxicity. The benefits of using legumes inoculated with naturally resistant rhizobia or recombinant rhizobia with enhanced resistance, as well as co-inoculation with other plant growth promoting bacteria (PGPB) are discussed. However, the legume-rhizobia symbiosis appears to be sensitive to metals, and the effect of metal toxicity on the interaction between legumes and rhizobia is not clear. Therefore, to obtain the maximum benefits from legumes assisted by rhizobia for phytoremediation of metals, it is critical to have a good understanding of interactions between PGP traits, the symbiotic plant-rhizobia relationship and metals.*

**KEY WORDS:** Legume-Rhizobia Symbiosis, Phytoremediation, Metals Toxicity

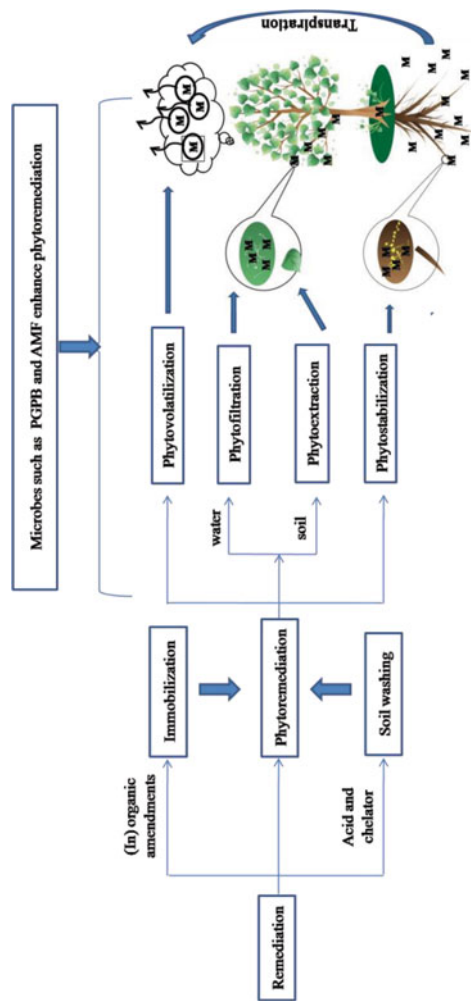
### INTRODUCTION

Metals and metalloids including Pb, Cu, Zn, Cd, Ni, Cr, As, Se etc. are toxic to organisms at high concentration. They are persistent pollutants in soil because they are

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non-degradable (Pajuelo *et al.* 2011). Heavy metal pollution affects soil structure (clay ionic structure) and fertility (McGrath *et al.* 1995), population and diversity of vegetation and microbes (Martensson and Witter 1990; McGrath *et al.* 1995), and is a significant threat to livestock as well as human beings via the food chain (Volesky and Holan 1995). Phytoremediation has received a great deal of attention in recent years. Phytoremediation includes phytoextraction, phytostabilization, rhizofiltration, phytovolatilization (Ghosh and Singh 2005; Dary *et al.* 2010) (Fig. 1), and is considered a promising, energy and cost effective way to rehabilitate metal-contaminated environments (Glick 2004; Kuiper *et al.* 2004; Denton 2007). Plants that are able to hyperaccumulate metals are an optimum option for phytoextraction because they can accumulate large amounts of metal(loid)s or trace metals in shoots (at least 0.01% dry weight for As, Cd and some trace metals; 0.1% dry weight for Cu, Pb, Co, and Cr; 1% dry weight for Ni, Zn, and Mn) without any visible symptoms such as wilting or necrosis of leaves, root discoloration, and plant growth (Piechalak *et al.* 2002; Vara Prasad and de Oliveira Freitas 2003). Over 400 plant members throughout the various plant families including *Asteraceae*, *Brassicaceae*, *Fabaceae*, *Poaceae*, and *Violaceae* have been reported as hyperaccumulators with a potential in metal remediation (Vara Prasad and de Oliveira Freitas 2003). Reeves & Baker (2000) summarized reported hyperaccumulators of Ni, Zn, Pb, Cd, Cu, Co, Cr, and Se all over the world. Some of them could accumulate higher concentrations (more than the metal concentration of the soil) of various elements as an innate property but the variation of uptake rates was wide and varied according to the plant species. However, high plant biomass with better ground cover is also important to successful phytoremediation. Factors such as slow growth rate and low biomass limit the application of most hyperaccumulators, especially in contaminated soils where nitrogen and phosphorus are seriously deficient (Dary *et al.* 2010). In this context, studies have been conducted to assess the impact of various plant growth promoting bacteria (PGPB) on effectiveness of phytoremediation. These include stimulation of plant growth, enhancement of plant resistance to metals and protection of plants from pathogens in distinct ways such as fixing nitrogen (Pajuelo *et al.* 2008a), improving nitrogen and phosphorus availability, phytohormone production (indole-3-acetic acid (IAA), cytokinins and auxins) (Vivas *et al.* 2006; Dodd *et al.* 2010), release of volatile components (acetoin and 2, 3-butanediol) (Ryu *et al.* 2003), secretion of siderophores and organic acids (Ma *et al.* 2009), synthesis of 1-aminocyclopropane-1-carboxylate deaminase (ACC) (Glick *et al.* 2007), as well as biosorption and accumulation of metals (Chen *et al.* 2008). Depending on the study and system, there were observed increases or decreases in metal accumulation in plant tissues.

Rhizobia are a subset of PGPB that may play an important role in phytoremediation. The legume-rhizobia symbiosis is one of the beneficial plant-microbe interactions which have traditionally been used in agricultural practice to provide nitrogen to plant and thus enhance plant growth (De Hoff and Hirsch 2003). Recently, this symbiotic relationship has been proposed to be applied in metal contaminated soil to improve soil fertility and extract or stabilize metals simultaneously (Carrasco *et al.* 2005; Zheng *et al.* 2005; Ike *et al.* 2007; Pajuelo *et al.* 2008a; Dary *et al.* 2010). The rhizobia can directly enhance phytoremediation through nitrogen fixation and production of the plant growth promoting factors as mentioned above. This would result in increased metal uptake and translocation from soil to plant due to the change in bioavailability. On the other hand, microbial metabolism such as extracellular polymeric substance (EPS) production and enzyme activities can immobilize and/or change the redox state of metals to lessen their toxicity to plants. Some *Rhizobium* strains can also adsorb and accumulate metal, which indirectly helps phytostabilization. Although the legume-rhizobia symbiosis is particularly sensitive to some metals, especially to Cd



**Figure 1** Prevalent remediation approaches for cleaning up metal contaminants in soil. The selection of the appropriate remediation approach depends on the type of metal contamination in soil (Raymond and Felix 2011). All approaches are not mutually exclusive and could be applied together according to the condition of the polluted areas. "M" in circle indicate metal(oid)s in gaseous forms (Color figure available online).

**Table 1** Metal(loid)s tolerance range of *Rhizobium* strains isolated from metal-contaminated soil

Species	Cu (mM)	Zn (mM)	Cd (mM)	Ni (mM)	Pb (mM)	Cr (mM)	As (mM)	Reference
<i>Rhizobium</i> sp.	0.5–2	0.5–2	0.1–1	0.25–1	3–6	—	1–10	(Pajuelo <i>et al.</i> 2008a)
<i>Sinorhizobium</i> strains	1.4	—	—	—	—	—	10	(Pajuelo <i>et al.</i> 2008b; Fan <i>et al.</i> 2011)
<i>Mesorhizobium</i> strains	2.2	16–32	0.3–0.5	—	—	9.6	—	(Wani <i>et al.</i> 2008a; Vidal <i>et al.</i> 2009; Hao <i>et al.</i> 2012a)
<i>Bradyrhizobium</i> strains	1.5	0–21.5	<0.5	5.1	2	—	2	(Wani <i>et al.</i> 2007b; Dary <i>et al.</i> 2010)
<i>Azorhizobium caulinodans</i>	—	—	4–5	—	—	—	—	(Zheng <i>et al.</i> 2005)

— data was not available.

(Zheng *et al.* 2005), there are strains of *Rhizobium* that have been isolated from metal-contaminated soil that are resistant to some metals (Table 1). These resistant rhizobia are able to nodulate and effectively fix nitrogen with their host legumes in polluted environments. This review focuses on the reports over the past 30 years of using the legume-rhizobia symbiosis as a tool for heavy metal bioremediation, the interaction between metal(loid)s and the legume-rhizobia symbiosis, as well as the effects and problems of employing this particular symbiotic interaction in metal remediation.

## THE LEGUME-RHIZOBIA SYMBIOSIS AS A TOOL FOR PHYTOREMEDIATION

### Legumes as Pioneer, Survivor and Introduced Plants for Metal Phytoremediation

Soil contaminated with high concentrations of heavy metals often support limited plant growth, resulting in limited water retention, periodic erosion, and loss of organic matter and nutrients (Wong 2003). These kinds of harsh conditions are adverse for soil-forming processes and limit the survival of plants and microbes. However, over time metals in soil were shown to be much less phytotoxic than laboratory spiked soil at equivalent total metal concentration, especially when soil pH is neutral or calcareous. Therefore, microbes were either sufficiently tolerant or selected for survival in this environment with less bioavailable metals (Smolders *et al.* 2003; Smolders *et al.* 2004). This is the main reason why most soil microbial processes were still found even in highly contaminated field soils (Renella *et al.* 2002; Smolders *et al.* 2003). Moreover, it has become increasingly clear that soils must be made calcareous to prevent current and future metal phytotoxicity to both plants and microbes (Giller *et al.* 1998; Brown *et al.* 2009; O'Day and Vlassopoulos 2010).

### Necessity of Soil Amendments for Successful Phytoremediation by Rhizobia-Legumes Symbiosis

Heavy metal toxicity in addition to phosphorus and nitrogen deficiencies are the three major factors limiting revegetation in contaminated soil. Soil pH and other soil characteristics have a much larger effect on plant species than metal contaminants due to

their effects on metal bioavailability (Giller *et al.* 1998). Although N-fixation is the greatest advantage legumes have when competing with other plants in metal toxic sites, it should be noted that most rhizobia and legumes are quite sensitive to excessive Zn in soil and none of the known metal hyperaccumulators or highly Zn tolerant plant species reported are legumes. Therefore, it is not enough to produce a persistent plant cover simply by seeding with metal tolerant rhizobia, PGPB or using chemical fertilizers. Soil amendments, including excess limestone to make calcareous soil, high phosphate and organic matter to improve soil fertility, are necessary to reduce phytotoxicity in soil before planting and inoculation (Chaney *et al.* 2000b). It was reported that the yields and diversity of plant species in calcareous sites were much better than in acidic soil (Smith and Bradshaw 1979). Because there are no truly metal tolerant legumes, if these calcareous and fertilizing steps are taken to protect legumes from phytotoxicity in soil, then it is very much a possibility that legumes and rhizobia with enough inherent or selected metal resistance will thrive on phytostabilized metal contaminated sites. This model for phytoremediation was described well by Chaney *et al.* (2010).

Overall, compared to soil amendments, PGPB have little power to support legumes growth unless phytotoxicity is greatly reduced by soil amendments (Ibekwe *et al.* 1996; 1998). Similarly with other plants used for revegetation, Zn phytotoxic soils were made calcareous and metal phytoavailability was greatly reduced by soil amendments before planting and inoculation, enabling the legumes and rhizobia to survive in the presence of high Zn soils (Chaney *et al.* 2000a; Li *et al.* 2000). Therefore, reduction of soil phytotoxicity is the precondition for growth of legumes on high metal contaminated sites. Making soil metals such as Zn and Cd less phytoavailable for the rhizobia-legumes symbiosis is crucial for the successful use of this model.

**Advantages of using legumes as pioneer plants.** The effect of heavy metals on the diversity of plants and microbes in soil exposed to heavy metals were evaluated both in short-term or long-term experiments (McGrath *et al.* 1995; Giller *et al.* 1998; Ranjard *et al.* 2006). Surveys of plant species surviving in long-term metal-contaminated environments showed legumes as the dominant portion of these populations. When up to 99 different plant species that survived after the toxic spill of the Aznalcóllar mine were identified, 15 of the species were shown to be in the legume family (Del Rio *et al.* 2002). Vara Prasad *et al.* (2003) listed 26 species of *Fabaceae* plants growing in mine refuse. In the Aznalcóllar pyrite mine polluted area, Carrasco *et al.* (2005) isolated metal resistant rhizobia from native legume plants including *Medicago*, *Trifolium*, *Viciae*, *Lotus*, and *Lupinus*. These *Rhizobium* species formed normal-sized pink nodules on their host plants. Herbaceous and woody legumes such as *Medicago* sp., *Sesbania rostrata*, and *Leucaena leucocephala* were also reported as pioneer plants colonizing Pb/Zn mine tailings in China (Yang *et al.* 1997; Zhang *et al.* 2001). Using legumes as pioneer plants has the following advantages (de-Bashan *et al.* 2011): First, pioneer legumes improve soil characteristics (immobilize contaminants, increase organic content, modify rhizosphere population), which makes the growth of other plant species in harsh environments possible; Second, the increased diversity of microbes including rhizobacteria and arbuscular mycorrhiza fungi (AMF) will help immobilize metal and enhance the growth of plants and finally, improve and stabilize the ecology of the contaminated environment. A key feature of the utility of legumes as a resource for phytoremediation is their role in providing additional N-compounds to the soil, thus improving its fertility and ability to support biological growth.

Almost all reported legumes used in metal remediation could be sorted into two types of application: phytostabilization and phytoextraction. The main contribution of rhizobia

on the two types of phytoremediation is plant growth enhancement (Glick 1995). For phytostabilization, due to the contribution of rhizobia, metal uptake by both roots and shoots increased as biomass increased. However, since the increased biomass of shoots is much greater than the increased biomass of roots, metal concentration in roots is still larger than in shoots (Fritioff *et al.* 2005). This point is evident from previous studies that most of the legumes showed higher concentrations of metals in roots than in shoots (Table 2). However, there are also some legumes that show a different pattern with high metal accumulation in shoots such as some *Lupinus* and *Astragalus* species, which could be used for phytoextraction (Pastor *et al.* 2003; Vara Prasad and de Oliveira Freitas 2003). For these legumes, metal accumulation in shoots keeps increasing with the assistance of rhizobia. Moreover, metal concentrations are not diluted by the increased biomass for unknown reasons (Rate *et al.* 2004). It should be noted that the metal concentration in shoots of most of the legumes used for phytostabilization were reported to be below the threshold allowed for animal grazing (Pajuelo *et al.* 2011), indicating it is safe to apply legumes. However some legumes, such as *Lupinus albus*, have been reported to hyperaccumulate metal (Pastor *et al.* 2003; Vázquez *et al.* 2008) and thus would be problematic in areas where grazing was allowed. Pastor *et al.* (2003) reported that in acidic soil, the accumulation of Zn in both roots (4.65 g/kg) and shoots (3.61 g/kg) of *Lupinus albus* L. were well over the limit allowed for animal consumption (500 mg/kg) when Zn was applied to the soil at 300 mg/kg. Dary *et al.* (2010) also showed translocation of Zn to the shoot of *L. albus* was high (748.3 mg/kg Zn) when the soil pollution index (PI) was above 3, a moderate level of contamination. The soil pollution index is a formula to determine the level of contamination in soil. When the PI is  $>1$ , the average levels of metals in the soil are above the permissible standard level (Dary *et al.* 2010). Although the Cu uptake of the root (150.7 mg/kg) was much higher than uptake in the shoot (52.1 mg/kg) when the PI was 2, the concentration in the aerial parts was still over the safe threshold for consumption (40 mg/kg). Moreover, some *Astragalus* species were reported to be Se hyperaccumulators with up to 6000 mg/kg Se in leaves and 10000 mg/kg Se in fruits and seeds (Vara Prasad and de Oliveira Freitas 2003; Freeman *et al.* 2006). These reports revealed that these legumes while unsafe for grazing in highly contaminated areas, could be used for phytoextraction.

### **RHIZOBIA THAT BOTH POSSESS PGP PROPERTIES AND SHOW INCREASED METAL RESISTANCE, ASSISTED LEGUME GROWN UNDER METAL STRESS CONDITIONS**

Rhizobia, particularly those with both increased heavy metal resistance and plant growth promoting capacity are of great interest for their ability to aid in phytoremediation of metals. In addition to their inherent ability to fix nitrogen, these *Rhizobium* species could also assist plant growth via adsorption and tolerance to metals. Those that nodulate their hosts may increase metal accumulation in root nodules, while those that remain in the rhizosphere would reduce metal toxicity in the rhizosphere by precipitation, chelation, immobilization and biosorption (Fig. 2). Moreover, once the symbiosis is established, nodules could serve as metal buffer areas which provide plants an extra place to stock metals and reduce the risk of direct exposure of the plant to metals. Nodules containing high concentrations of rhizobia could also serve as biosorbents or storage for metals. Chen *et al.* (2008) examined the metal uptake capacities of Pb, Cu and Cd in the symbiosis between *Mimosa pudica* with *Cupriavidus taiwanensis*. They found that the uptake by nodulated *M. pudica* for Pb, Cu, and Cd was 86%, 12%, and 70% higher than that of nodule-free plants,

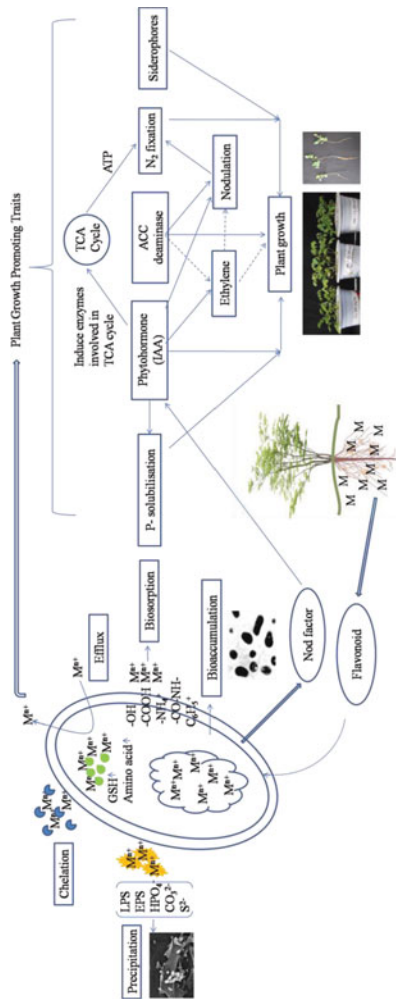
**Table 2** Metal accumulation in plant tissue when using different legume-rhizobium symbiosis for phytoremediation

Symbiosis	Rhizobium	Contaminated degree in soil	Metal accumulation in roots (mg/kg)						Metal accumulation in shoots (mg/kg)						Ref.
			Cu	Zn	Cd	Pb	Cu	Zn	Cd	Pb	Cu	Zn	Cd	Pb	
<i>Lupinus luteus</i>	<i>Bradyrhizobium</i> sp.	multi-metal contaminated soil with PI = 1–2	27.5 ± 4.3	165.1 ± 17.9	1.2 ± 0.2	11.0 ± 2.2	12.6 ± 1.9	135.1 ± 10.0	0.6 ± 0.1	<1.5					(Dary <i>et al.</i> 2010)
<i>Lupinus luteus</i>	<i>Bradyrhizobium</i> sp.	multi-metal contaminated soil with PI = 2–3	64.7 ± 9.8*	642.0 ± 144.3*	4.1 ± 0.9	26.6 ± 7.7	21.5 ± 3.6	472.0 ± 156.8	1.6 ± 0.4	3.5 ± 1.5				(Dary <i>et al.</i> 2010)	
<i>Lupinus luteus</i>	<i>Bradyrhizobium</i> sp.	multi-metal contaminated soil with PI > 3	150.7 ± 17.9*	806.3 ± 24.4*	4.8 ± 1.7	80.7 ± 23.0	52.1 ± 14.7*	748.3 ± 167.8*	2.0 ± 0.6	35.3 ± 14.6				(Dary <i>et al.</i> 2010)	
<i>Lupinus</i>	<i>Bradyrhizobium</i> sp.	Guadamar riverbed near the Aznalcollar mine spills	65.0*	755*	4.0	27.0	21.5	472	1.6	—				(Pajuelo <i>et al.</i> 2008a)	
<i>Lablab purpureus</i>	<i>Rhizobium</i> sp.	100 mg/kg metal treated soil	~24	~47	~120*	—	~12	~30	~85	—				(Younis 2007)	
<i>Lablab purpureus</i>	<i>Rhizobium</i> sp.	200 mg/kg metal treated soil	~53*	~92	~134*	—	~33	~43	~67*	—				(Younis 2007)	
Pea plant	<i>Rhizobium</i> sp.	24 mg/kg Cd; 136 mg/kg Cr; 1388 mg/kg Cu	14.4	—	1.5	—	8.5	—	0.62	—				(Wani <i>et al.</i> 2008c)	
Pea plant	<i>Rhizobium</i> sp.	9780 mg/ kg Zn	—	~400	—	—	—	~300	—	—				(Wani <i>et al.</i> 2008b)	
<i>Sesbania rostrata</i>	—	Tailing	30 ± 9.8	605 ± 235*	—	277 ± 122*	11 ± 4.3	216 ± 72	—	33 ± 14				(Yang <i>et al.</i> 2003)	
<i>Sesbania sesban</i>	—	Tailing	57 ± 17*	383 ± 119	—	100 ± 25*	12.4 ± 1.1	209 ± 30	—	1.3 ± 2.3				(Yang <i>et al.</i> 2003)	

\* represents metal concentration accumulated in plant tissue (roots or shoots) was over the safe threshold of consumption (Cu 40 mg/kg, Zn 500 mg/kg, Cd 10 mg/kg, Pb 100 mg/kg)

— data was not available.





**Figure 2** Pathways by which rhizobia can act as plant growth promoting bacteria to give a positive effect on the growth of legumes. Phytoremediation using a legume-rhizobia symbiotic system is mainly based on two characteristic traits, metal resistance determinants and PGP bacterial properties to assist legumes to grow under metal stress conditions. Interaction of different properties of PGP bacteria is represented by arrows. LPS and EPS in the figure correspond to lipo-polysaccharide and extracellular polymeric substance, respectively. “M” and “M<sup>n+</sup>” indicate metal(loid)s and metal(loid) ions, respectively. The arrows show a positive effect, while the dashed arrows represent a negative effect (Color figure available online).

respectively. As plants with a similar biomass (with or without nodules) were used in the metal biosorption during the same cultivation period, this greater uptake of nodulated *M. pudica* was only due to the increased metal uptake, demonstrating the effectiveness of using nodulated plants for removal of metals. The different available legume-rhizobia symbiotic systems in relation to the types of metal contaminated soil are shown in Table 2.

### **Plant Growth Promoting Bacteria Properties are Necessary for Legume Growth, Nodulation and Nitrogen Fixation, Especially Under Metals Stress**

Multiple mechanisms in rhizobia assisting growth of legumes played important roles in metal phytoremediation. IAA (indole-3-acetic acid), an important phytohormone influencing cell division and differentiation, stimulated plant growth and nodule performance in legume-rhizobia symbioses. It has been reported that almost 80% of rhizobacteria are capable of producing IAA to varying degrees (Khalid *et al.* 2005). Our study on the IAA producing capacity of *A. tumefaciens* CCNWGS0286 (IAA overproduction, isolated from nodules of *Robinia pseudoacacia* growing in zinc-lead mine tailings) in the presence of copper and zinc showed that although the level of IAA synthesis decreased with increasing concentrations of copper/zinc, there were still large amounts of IAA that could be detected in presence of 2.0 mM Zn<sup>2+</sup>. Moreover, compared to the *A. tumefaciens* mutant strain with lower IAA production, legume growth was enhanced greatly by inoculating with the IAA overproducing strain in spite of the presence of Zn<sup>2+</sup> (Hao *et al.* 2012b). Three synthetic pathways for production of IAA have been identified in rhizobia: the IAM (indole-3-acetamide), the Tra (tryptamine) and the IPyA (indole-3-pyruvic acid) pathway (Patten and Glick 1996; Theunis *et al.* 2004). Bianco and Defez demonstrated that recombinant *Rhizobium* strains overproducing IAA showed improved nitrogen fixation ability relative to the wild-type strain (Bianco and Defez 2010). IAA induces the activity of some key enzymes such as citrate synthase and  $\alpha$ -ketoglutarate dehydrogenase in the TCA cycle (tricarboxylic acid cycle) which provides the energy required by bacteroids to fix nitrogen. Mutants lacking *gltA* (citrate synthase) in *S. meliloti* (Mortimer *et al.* 1999), *icd* (isocitrate dehydrogenase) in *S. meliloti* (McDermott and Kahn 1992), or *dct* (dicarboxylic acid transport) in *S. meliloti* (Bolton *et al.* 1986), *R. leguminosarum* bv. *viciae* (Finan *et al.* 1983), and *R. trifolii* (Ronson *et al.* 1981) were unable to fix nitrogen. However, the relationship between the concentration of IAA released by PGPB and root growth could not be predicted because of interference by various environmental factors and other bacterial metabolites. Environmental factors such as oxidation and dilution may lessen the influence of IAA to the threshold of the level needed for growth promotion (Barazani and Friedman 1999). An increased rooting development stimulated by low concentration of IAA was beneficial to plant mineral and nutrient uptake and bacterial colonization as shown by Dazzo *et al.* (2000). They found the uptake of N, P, K, Fe was enhanced in rice inoculated with IAA producing rhizobia and increased nutrient uptake was independent of biological N-fixation. In addition, roots with nodules contained higher concentrations of IAA than un-nodulated ones (Hirsch and Fang 1994; Ghosh and Basu 2006) partly due to the high concentration of IAA produced by *Rhizobium* in the nodules (Spaepen *et al.* 2007). However, similar to the effect of IAA on rooting development, only low concentrations of IAA (around 10<sup>-8</sup> M) could enhance nodulation, while high concentrations inhibited nodulation (Spaepen *et al.* 2007). IAA could be involved in controlling nodule numbers (Penmetsa *et al.* 2003) and nodulation efficiency (Kaneshiro and Kwolek 1985; Camerini *et al.* 2008). Rhizobial Nod factors induced by plant flavonoids are signaling molecules mediating host recognition

and nodule formation in the legume-rhizobium symbiosis (Denarie and Cullimore 1993). These lipo-oligosaccharide compounds were demonstrated to have regulatory activity and were proposed to be involved in the control of plant development. IAA transport could also be affected by Nod factors (Theunis *et al.* 2004) and in some *Rhizobium* species such as *S. meliloti* and *R. leguminosarum*, the presence of flavonoids was also able to promote IAA synthesis (Prinsen *et al.* 1991), indicating that rhizobial IAA synthesis and transport were regulated by specific host plants. Overall, a complex relationship exists between IAA and the members of the legume/rhizobia symbiosis, thus more attention in future research should be focused on the effects that IAA has on the success of the symbiosis, especially in metal contaminated environments.

A large amount of IAA in plants could trigger the synthesis of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylate) and thus increase the level of ethylene, which inhibits the growth of the root system (Glick 2005). Moreover, nodulation which was effectively inhibited by ethylene/ACC ( $ED_{50} \leq 5 \mu\text{M}$ ) has been reported in some legumes such as *Medicago truncatula* (Penmetsa and Cook 1997), *Lotus japonicas* (Nukui *et al.* 2000), *Pisum sativum* L. (Goodlass and Smith 1979; Lee and Larue 1992), *Trifolium repens* L. (Goodlass and Smith 1979) and *Glycine max* (Hunter 1993) by inducing cross-linking of matrix glycoproteins causing failure of the infection thread (Penmetsa and Cook 1997; Ma *et al.* 2003a). *Bradyrhizobium elkanii* has been reported to have evolved a mechanism to overcome ethylene stress by producing rhizobitoxine (an ethylene synthesis inhibitor) thus enhancing nodulation (Fuhrmann 1996; Yuhashi *et al.* 2000). Another method used by PGPB to overcome ethylene inhibition of plant growth is via production of ACC deaminase, an enzyme that cleaves ACC thus preventing ethylene biosynthesis. ACC deaminase-containing rhizobacterial species can assist nodulation by *Rhizobium* (Belimov *et al.* 2009). Several rhizobial species including *M. loti* (Sullivan *et al.* 2002), *Sinorhizobium* sp. (Di Gregorio *et al.* 2006) and *Rhizobium* spp. (Ma *et al.* 2003b) were reported to possess ACC deaminase, which in some cases was shown to assist plant survival and metal uptake. Plants inoculated with rhizobacteria possessing ACC deaminase activity exhibited an increase in biomass and longer roots in spite of growth inhibition caused by heavy metals (Di Gregorio *et al.* 2006; Safronova *et al.* 2006). Ma *et al.* (Ma *et al.* 2003a; Ma *et al.* 2003b) isolated one strain of *R. leguminosarum* bv. *viciae* with ACC deaminase activity and created mutant strains, via insertional mutagenesis, in the ACC deaminase structural gene *acdS* and the regulatory gene *lrpL*. They demonstrated that in comparison to the wild-type strain, deficiency of ACC deaminase resulted in a decrease in nodulation efficiency on *P. sativum* L. In another study it was shown that when *acdS* along with *lrpL* were introduced into *S. meliloti* strain Rm1021, which lacks an ACC deaminase gene and ACC deaminase activity, there was a 40% increase in nodulation (Ma *et al.* 2004). Moreover, *S. meliloti* with *acdS* and *lrpL* induced became more competitive when nodulating alfalfa compared to the wild-type strain. All these results suggested that rhizobia with ACC deaminase activity may be valuable for use in phytoremediation due to their enhanced nodulation abilities and positive effects on plant growth.

Besides nitrogen, phosphorus (P) is another major nutrient necessary for plant growth. P deficiency is a problem in many soils, especially in mining or metal-polluted areas because most P present in soil is immobilized and thus unavailable for plants (Rodríguez and Fraga 1999; Shilev *et al.* 2001). In addition to fertilization and enzymatic activity in soil, P-mobilization by PGPB is a major natural way to increase free P in soil. The genera *Rhizobium*, *Pseudomonas* and *Bacillus* were thought to be the most effective P-solubilizers among the P-solubilizing bacteria reported (Rodríguez and Fraga 1999). The solubilization

properties of organic and mineral P by various PGPB isolated from different environments, as well as their effects on plants have been described (Halder *et al.* 1990; Nahas 1996; Shilev *et al.* 2001; Rivas *et al.* 2006). Peix *et al.* (2001) showed that available P in soils treated with insoluble phosphates, where barley and chickpea were grown, increased up to 100% and 125% respectively, when inoculated with *Mesorhizobium mediterraneum*. Both the plants' biomass and their ability to take up other nutrients such as N, K, Ca, and Mg increased significantly. The mechanism of P-solubilization was thought to be via release of various organic acids such as gluconate, succinate, fumarate to complex the cations binding to phosphate and make P available to plants (Kpombekou-a and Tabatabai 1994). The *phoCDET* operon or *pstSCAB* homologs encoding a high-affinity transport system and the *pit* operon encoding a low-affinity system were considered to be the major P transport systems in rhizobia (Botero *et al.* 2000; Bianco and Defez 2010). The correlation of IAA synthesis and P-solubilization activity was reported previously (Vassilev *et al.* 2006). Recombined *S. meliloti* 1021 strain RD64 with IAA overproduction capacity could not only promote nitrogen fixation (Imperlini *et al.* 2009), but also mobilized P effectively under P deficient conditions compared to wild-type strain 1021 (Bianco and Defez, 2010). Bianco and Defez (2010) demonstrated that the expression of *phoBCDET* was upregulated under P-limited conditions compared to wild-type strain 1021 in the IAA overproducing strain RD64. Under P-sufficient conditions, three genes in the operon, *phoB*, *phoC*, and *phoT* were downregulated in strain RD64 compared to their expression in wild-type strain 1021. These results indicate that IAA synthesis promotes P solubilization. Treating wild-type strain 1021 with IAA also resulted in up-regulation of the *phoBCDET* operon under P-limited conditions and down-regulation of *phoB*, *phoC*, and *phoT* under P-sufficient conditions.

Therefore, synthesis of IAA, ACC deaminase, and organic acids were not only effective plant growth promoting traits, but also essential to the legume-rhizobia symbiosis for effective nodulation and nitrogen fixation as described above. However, other bacterial traits such as the production of siderophores to increase Fe uptake and protection of plants against pathogens (Ma *et al.* 2011), release of the volatile compounds of acetoin and 2, 3-butanediol (Chen *et al.* 2007) were also regarded as important properties for plant growth promotion. The different magnitude of the effect that PGPB can have on nodulation, N-fixation, as well as on phytoremediation performance is further explained in Table 3. Interestingly, PGPB including rhizobia were reported to regularly possess two or more plant growth promoting properties. Antoun (1998) tested the potential plant growth promoting properties of rhizobia isolated from nodules. Among 266 tested rhizobia (mainly *Bradyrhizobium* and *Rhizobium*), 83% of them produced siderophores, 58% synthesized IAA and 54% solubilized phosphorus. A balanced interplay of these factors (Fig. 2), such as IAA synthesis and ACC deaminase, IAA synthesis and P solubilization or IAA synthesis and siderophore production appears to be important. The exact combination of factors necessary to provide optimal plant growth promotion needs to be further explored. In addition, some rhizobia with these traits also greatly improved plant growth in a metal polluted environment, which became the basis of using this symbiosis for phytoremediation.

**Metal resistance determinants protect rhizobia from metal toxicity and enable them to enhance plant growth.** In addition to the plant growth promoting traits of rhizobia, and their role in indirect assistance for phytoremediation, the mechanisms responsible for conferring metal resistance by the rhizobia have also been examined. The existence of high concentrations of metals will select for more metal resistant rhizobia, and numerous plant growth promoting rhizobia have been isolated from various plants in

**Table 3** Direct and indirect effects of PGPB traits and metal tolerance on symbiotic properties, metal resistance and phytoremediation performance

PGP traits	Effect factors	Nodulation	N fixation	phytoremediation performance	Metal resistance	PGP traits
	IAA	+++ (low concentration); — (high concentration)	++ <sup>a</sup>	+++ (low concentration); — (high concentration)	N	N
	ACC deaminase	++ <sup>b</sup>	++ <sup>b</sup>	+++	N	N
	P solubilization	+ <sup>c</sup>	+ <sup>c</sup>	+++	+ <sup>d</sup>	N
	Siderophores	+ <sup>e</sup>	+ <sup>e</sup>	+++	N	N
	Volatile compounds of acetoin and 2, 3-butanediol	N	N	+++	N	N
	Produce EPS, LPS	+++	N	+	++	N
	Operon related to resistance	N	N	+	+++	N

+++ : significant enhancement/improvement, directly; + + : significant enhancement/improvement, indirectly; + : enhancement/improvement, indirectly.

N : no reported effect.

<sup>a</sup>: 1) affect some key enzymes in TCA cycle and thereby affect N-fixation; 2) affect nodulation. <sup>b</sup>: prevent ethylene biosynthesis. <sup>c</sup>: affect IAA level. <sup>d</sup>: release of various organic acids could bind metal forming organic acid-metal complex, reducing metal toxicity and enhance metal resistance indirectly. <sup>e</sup>: competitive advantage.

heavy metal contaminated environments (Wani *et al.* 2008b; Glick 2010). Rhizobia contain different metal resistance systems to maintain metal homeostasis in cells using putative mechanisms that are similar to those in other microbes. For example, increased levels of glutathione (GSH), the amino acid-like thiol, proline, urease, exopolysaccharides (EPS) and cell inclusions were reported in various metal tolerant species of *Rhizobium* exposed to Cd, Cu, Ni, and Zn, respectively (Purchase *et al.* 1997; Singh *et al.* 2001; Lima *et al.* 2006). Lakzian *et al.* (2007) demonstrated that increased levels of metals induced the transfer of naturally occurring plasmids between rhizobia, which played an important role in conferring enhanced tolerance. In addition, with more *Rhizobium* genome sequences available, many genes related to metals resistance/homeostasis could be identified in whole genome sequences. Although most of these genes have not been characterized in these rhizobial strains, they still provide abundant information to better understand mechanisms of metal resistance in rhizobia.

The process of nitrogen fixation is considered to be one of the most important and unique traits of rhizobia compared to other microbes used in metal phytoremediation. Therefore, it is crucial whether this process can still work well under increased metal stress. Metal resistance determinants provide protection for rhizobia to survive and maintain a certain threshold for effective nodulation of legumes, allowing them to play a role in promoting plant growth (Fig. 1). Although big differences in metal tolerance/resistance exist among different species of rhizobia (Giller *et al.* 1993), rhizobia which could effectively nodulate with host plants and increased metal levels were also reported in some studies (Martensson and Witter 1990; Obbard and Jones 1993). Pajuelo *et al.* (2008a) showed that a *S. meliloti* strain with high As resistance could form an effective symbiosis with *Medicago sativa*, although a reduced number of nodules were formed. Ibekwe (1995) reported an increase in nodulation of different inoculated legumes grown in soil with Ni and Zn. The symbiotic effectiveness including nodulation and N-fixation abilities of metal tolerant strains of *R. leguminosarum* and *S. meliloti* was also reported (El-Aziz *et al.* 1991; Smith and Giller 1992). However, the negative effect of metals on formation of an effective symbiosis cannot be ignored.

### **INHIBITION OF PRODUCTIVE LEGUME-RHIZOBIA INTERACTIONS BY THE TOXICITY OF METALS**

Generally, the toxicity of heavy and transition metals to the legume-rhizobia symbiosis is considered to be caused by a direct inhibitory effect on legumes and nodules, or indirectly via inhibition of the symbiotic rhizobia (Oves *et al.* 2010). Leguminous plants exposed to excessive heavy metals will suffer chlorosis, tissue damage, root browning and growth inhibition, thereby affecting photosynthesis and symbiosis (Wani *et al.* 2007a; 2008c). In addition, high levels of metals also exert adverse effects on the survival, population, diversity, metabolic activity and nitrogen-fixing efficiency of rhizobia (Pereira *et al.* 2006). However, estimation on the effect of heavy metal toxicity on rhizobia, the legume-rhizobia interaction and effective symbiotic nitrogen fixation varies between bacterial species and different contaminated soils.

The negative effects of heavy and transition metals on *Rhizobium* populations, diversity and symbiotic nitrogen fixation have been analyzed. Earlier studies reported the indigenous population of *R. leguminosarum* bv. *trifolii* was decreased to undetectable levels, and nitrogen fixation was inhibited significantly with increased soil metal concentrations (McGrath *et al.* 1988; Giller *et al.* 1989; Chaudri *et al.* 1993). Studies on *R. leguminosarum*

bv. *viciae* also displayed a reduced population when Zn increased from 50 to 400 mg/kg (Lakzian *et al.* 2002). Castro *et al.* (1997) studied the effects of heavy metals on the *R. leguminosarum* bv. *trifolii* symbiosis in an area polluted by industrial effluents for nearly 40 years and found metal did not affect the size of the population but affected the effectiveness of N-fixation and the genetic diversity of the population. Low genetic diversity and ineffective rhizobia were also found in other studies (Giller *et al.* 1989; Martensson and Witter 1990; Giller *et al.* 1993; Hirsch *et al.* 1993). Lakzian *et al.* (2007) proposed that although zinc-tolerant rhizobia could survive in polluted soil, nitrogen fixation would be impaired due to the reduction in the number of tolerant rhizobia below the threshold needed for nodulation (50 cells/g soil). Giller *et al.* (1989) reported that nodulation-competent *Rhizobium* did not survive in soil contaminated with metal containing sludge, but strains unable to perform N-fixation became more tolerant to metals. Chaudri *et al.* (1992) reported that no viable *Rhizobium* was found when the concentrations of Zn and Cd exceeded 385 mg/kg and 7.1 mg/kg, respectively. In contrast, 225 mg/kg Cu also resulted in a decrease in *Rhizobium* numbers, but with no effect on nodulation efficiency. Ni had little effect on the population. Therefore the order for metal toxicity on rhizobia and nitrogen fixation was Cd>Zn>Cu>Ni. It seems that some rhizobia species living under long-term metal stress could adapt to maintain their population in a harsh environment but at the cost of losing their symbiotic function (Casella *et al.* 1988; Castro *et al.* 1997).

However, other studies suggested that the survival of symbiotic rhizobia was most influenced by presence of the host in contaminated soils (Smith and Giller 1992; Obbard and Jones 1993; Smith, 1997; Giller *et al.* 1998; Broos *et al.* 2005). For example, *R. leguminosarum* bv. *trifolii* could be isolated from clover-growing sites even at high metal concentrations. In addition, N content in clover grown in polluted soil was not affected by the smaller number of rhizobia present, due to metal stress (Chaudri *et al.* 1993). Broos *et al.* (2004) also confirmed that log MPN (most probable number) and nitrogen fixation were not affected in soils where the host plants existed even with up to 1926 mg/kg Zn, suggesting the presence of host plants was the most important factor on nitrogen fixation. Moreover, indigenous host legumes are thought to be very important for effective symbiosis and nitrogen fixation in metal contaminated soils because of frequent failed nodulation occurring in non-indigenous plants (Obbard and Jones 1993). It appears that the existence of a symbiont provides protection for the survival of rhizobia facing metal toxicity and free-living rhizobia which are more sensitive to metal toxicity are most affected in nitrogen fixation in metal contaminated soil. Chaudri *et al.* (2000) reported that indigenous populations of free-living rhizobia in soil were decreased due to Zn toxicity. Nodulation and nitrogen fixation were also found to be affected when using this soil for planting. It should be noticed that although the presence of host plants could protect symbiotic rhizobia in nodules and release a number of rhizobia into soil, populations of free-living rhizobia in soil would still decrease with increasing metal concentration. Carrasco *et al.* (2005) demonstrated that although metal toxicity led to a reduction of nodule numbers, the nodule size and nitrogenase activity were not affected. Once the nodule formed and the symbiotic relationship was established successfully, nitrogen fixation appeared similar to that in non-contaminated areas. This report also described high genetic diversity in the highly metal polluted soil. Similar results on the diversity of rhizobia not being influenced by Cu in a medium-term field were also obtained in another study (Laguerre *et al.* 2006). In addition, a survey on the *R. meliloti* and alfalfa collected from soils contaminated by a Zn smelter operating for over 90 years demonstrated that there was no correlation between *R. meliloti* population, metal tolerance and metal concentration in soil. No adverse

effect was found on the rhizobial population and symbiotic relationship (Angle and Chaney 1991).

Furthermore, other studies considered that the adverse effects to the symbiosis were due to metal toxicity on the host legumes, not necessarily the bacteria. It was reported that compared to shoot and root growth, nodulation showed a significant sensitivity to various heavy metals (Werner *et al.* 2002; Gupta *et al.* 2007). Rhizobia infections on *M. sativa* were reduced 90% when in the presence of 25–35  $\mu\text{M}$  As as observed by inoculating *Sinorhizobium* sp. strain MA11 with a *lacZ* reporter gene. The reduced infection rate was considered to be due to root hair (especially the tip areas) damage, an increased necrotic zone and a shortened infective root zone (Pajuelo *et al.* 2008b). Similar damage and inhibition on roots by other metals such as Cu, Cd and Hg were also reported (Ortega-Villasante *et al.* 2005; Kopittke *et al.* 2007). Root damage would affect the legume responses to Nod factors and thereby affect nodule formation directly. Therefore, the reduced infective zone was believed to be the major reason for reduced nodule numbers not the reduced rhizobial population (Fig. 3).

Recently, in order to promote metal removal, several studies using recombinant rhizobia in symbiosis with legumes and recombinant rhizobia alone have been reported. In one, expression of *MTL4* (tetrameric human metallothionein) under the control of *nifH* and *nolB* in *Mesorhizobium huakuii* subsp. *rengei* B3 increased the Cd accumulation in free-living cells and in the nodules of *Astragalus sinicus* inoculated with the recombinant strain, but no significant increase was found in Cu accumulation (Sriprang *et al.* 2002). Similar increased Cd accumulation was also reported in another study using recombinant *M. huakuii* B3 carrying *PCS<sub>At</sub>* (phytochelatin synthase from *A. thaliana*) (Sriprang *et al.* 2003) or carrying both *MTL4* and *AtPCS* (*PCS<sub>At</sub>*) (Ike *et al.* 2007). In a further study (Ike *et al.* 2008), *AtIRT1* (iron-regulated transporter 1 gene from *A. thaliana*) was introduced into recombinant rhizobium B3 (pMPnolBMTL4nifHPCS). This new recombinant strain was more sensitive and accumulated higher concentrations of Cd in free-living cells than B3, suggesting that the presence of *AtIRT1* led to more Cd uptake. In a symbiotic relationship between this strain and *A. sinicus* a greater accumulation of Cu and As in nodules was observed, but not of Cd and Zn. In these studies, the introduced foreign genes were under the control of *nif* or *nol* promoters so that they would be specifically expressed in nodules. The recombinant rhizobia could respond to the compounds secreted by legumes and produce Nod factors as well as the foreign proteins, either as free-living rhizobia in the rhizosphere or attached on roots, or in nodules following differentiation into bacteroids. Wherever the recombinant rhizobia found a habitat, they all contributed to an increase of metal accumulation in cells, plant roots, and nodules. However, as described above, the introduction of foreign genes might lead to a preference for specific metals and a higher sensitivity to some specific metals. Therefore, the characteristics of the encoded foreign gene products, their effect on rhizobial resistance, symbiosis and accumulation in legumes, as well as on the rhizobial population and diversity when applied to the environment should be considered.

Overall, the effects of metal on symbiosis are complex. The toxicity of heavy metals to the legume-rhizobia symbiosis and symbiotic nitrogen fixation varied with the species of legumes and rhizobia, types of soil and metal, the degree of pollution, as well as the nodulation and plant growth promotion activities of rhizobia under metal stress (Oves *et al.* 2010). Therefore, all the parameters described above should be considered before using a symbiotic system as a tool for phytoremediation. In addition, more studies at the molecular level should be carried out on how to build a successful symbiotic relationship





**Figure 3** Morphological changes of roots and nodules of *Medicago lupulina* on different  $\text{Cu}^{2+}$  concentration in the nutrient solution. A–G are root hairs of *M. lupulina* inoculated with *Sinorhizobium meliloti* after 3 days in the presence of 0, 5, 10, 25, 50, 100 and 200  $\mu\text{M}$   $\text{Cu}^{2+}$ , respectively. The obvious damage of root hair could be observed when the Cu concentration was over 50  $\mu\text{M}$ . The number of root hair was reduced significantly with an increase of Cu concentration. H is root necrotic area (3 days) in the presence of 200  $\mu\text{M}$   $\text{Cu}^{2+}$  in the nutrient solution. I–K are nodule primordia (8 days inoculation) in the presence of 0, 10 and 100  $\mu\text{M}$   $\text{Cu}^{2+}$ , respectively. L and M are young nodules (14 days) with 0 and 25  $\mu\text{M}$   $\text{Cu}^{2+}$  in the nutrient solution, respectively. N–P are mature nodules (28 days) in the presence of 0, 50 and 100  $\mu\text{M}$   $\text{Cu}^{2+}$ , respectively (Color figure available online).

with high nitrogen-fixing efficiency under metals stress, and elaborating how metals affect the legume-rhizobia symbiosis in detail.

## FUTURE TRENDS

The legume-rhizobia symbiosis, with its unique benefits in reclamation of metal polluted environments, has been successfully established in many studies. As discussed previously, the effects of metals on the legume-rhizobia symbiosis were complex and several factors could affect the performance of the symbiosis for phytoremediation. However, most studies using the legume-rhizobia symbiosis for phytoremediation were carried out in pots or under green house conditions; few field results are currently available. There are several things in the symbiotic interaction that should be taken into consideration for field tests. First, due to the complexity and unpredictability of *in situ* phytoremediation in metal polluted soil, factors such as the different chemical nature of pollutants, type of polluted soil and optional symbiotic combinations should be taken into account before designing field tests. Second, most data on symbioses were obtained with one or two pollutants treated with a single legume-rhizobia pair. It should also be taken into consideration that the combined use of rhizobia with other PGPBs and AMF (Arbuscular mycorrhizal fungi) was suggested to improve plant yields and metal accumulation in some studies (Pajuelo *et al.* 2008a; Teng *et al.* 2008; Dary *et al.* 2010). However, competition among inoculants or competition between inoculants and native populations might lessen the growth promoting effect, which was the major reason why native bacteria were recommended first. Therefore, the challenge of how to combine the advantages of all the different parts in symbiosis in order to achieve the maximum benefits for phytoremediation without leading to other ecological problems should be studied further. Finally, although most of the reported legume-rhizobia symbioses were used for phytostabilization, which was safer for consumption, translocation from roots to shoots was still occurring with some metals like Zn (Dary *et al.* 2010). Considering the possibility of a multi-contaminated environment, monitoring work should first be done in field tests.

## CONCLUSION

The legume-rhizobia symbiosis has been promoted as a novel and effective method to employ plant-microbe interactions for phytoremediation, especially phytostabilization. Since both rhizobia and legumes are quite sensitive to metals, reduction of metal phytoavailability by soil amendments is the precondition of successful use of rhizobia-legumes symbiosis for phytoremediation (Ibekwe *et al.* 1996; Chaney *et al.* 2010). Rhizobia, possessing both metal resistance mechanisms and plant growth promoting traits, in addition to their intrinsic nitrogen fixing capability promote legume growth in polluted soil by increasing legume yields, metal accumulation and N content in both plants and soil. The interaction among different kinds of plant growth promoting traits was shown to be a delicate and complicated network. Most of these traits such as phytohormone synthesis, P solubilization, and ACC deaminase appear to be associated with maintenance and performance of nodulation and nitrogen fixation. However, the exact target of metal toxicity and the effect of transition and heavy metals on these traits and the symbiotic relationship are still unknown. Overall, as a novel remediation technology, phytostabilization by legume-rhizobia symbioses offers a promising strategy for restoration of metal contaminated areas.

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