Heavy Metal Toxicity to Symbiotic Nitrogen-Fixing Microorganism and Host Legumes

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Ees Ahmad, Almas Zaidi, Mohammad Saghir Khan, and Mohammad Oves

Abstract

Legume species of the flowering family *Fabaceae* are well known for their ability to fix atmospheric nitrogen and enhance nitrogen pool of soil, leading to increase in crop especially legumes both in conventional or derelict soils. The interaction between Rhizobia and legumes provides nutrients to plants, increases soil fertility, facilitates plant growth and restores deranged/damaged ecosystem. These characteristics together make legume extremely interesting crop for evaluating the effect of heavy metals. Environmental pollutants like heavy metals at lower concentrations are required for various metabolic activities of microbes including Rhizobia and legume crops. The excessive metal concentrations on the other hand cause undeniable damage to Rhizobia, legumes and their symbiosis. Currently, little is, however, known about how free-living Rhizobia or the legume–*Rhizobium* symbiosis is affected by varying metal concentration. We focus here that how the nitrogen-fixing root nodule bacteria, the "rhizobia," increase plant growth and highlight gaps in existing knowledge to understand the mechanistic basis of how different metals affect rhizobia–legume symbiosis which is likely to help to manage legume cultivation in metal contaminated locations.

2.1 Introduction

Heavy metals discharged from industrial operations and upon consequent accumulation in various ecological systems cause a massive threat to the varied agroecosystems (Ceribasi and Yetis 2001; Cheung and Gu 2007). When heavy metals accumulate into soil to an abnormal level, it causes dramatic changes in microbial composition and

E. Ahmad (✉)
Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh 202002, Uttar Pradesh, India
e-mail: eesahmad@gmail.com

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their activities (Paudyal et al. 2007; Wani et al. 2008a; Khan et al. 2009a; Krujatz et al. 2011), leading consequently to losses in soil fertility. As a result of depleted soil nutrient pools resulting from direct or indirect metal effect, the health of plants including legumes like greengram [*Vigna radiata* (L.) Wilczek] (Fig. 2.1a) (Wani et al. 2007a), pea (*Pisum sativum* L.) (Fig. 2.1b) (Wani et al. 2008a) and chickpea (*Cicer arietinum* L.) (Fig. 2.1c) (Wani et al. 2008c; Wani and Khan 2010) growing in metal-enriched soil is adversely affected either due to nutrient deficiency or due to direct effects of toxicants. For instance, the higher concentrations of metals have shown toxicity to various physiological processes like synthesis of chlorophyll pigments in various plants (Feng et al. 2010) including legumes (Bibi and Hussain 2005; Wani et al. 2007b, c; Ahmad et al. 2008a), inactivated protein synthesis (Van Assche and Clijsters 1990; Brahima et al. 2010) and consequently led to the severe reduction in crop yields (Wani et al. 2007a, 2008b). In addition, there are numerous reports where elevated amounts of heavy metals have been found to limit the rhizobial growth and their host legumes (Heckman et al. 1987; Broos et al. 2005) and concomitantly reduce the crop yields (Moftah 2000). For example, a single strain of *Rhizobium leguminosarum* could survive well in the metal contaminated plots, but this strain did not fix N with white clover (*Trifolium repens* L.), although it resulted in N formation with *Trifolium subterraneum* (Hirsch et al. 1993). In a similar manner, a profound toxic effect of metal on N_2 fixing ability of culture inoculated white clover was observed (Broos et al. 2004). In other reports, when sludge was applied for field trials in Braunschweig, it was found that the increasing sludge rates reduced the number of indigenous populations of *R. leguminosarum* bv. *trifolii* to low or undetectable levels (Chaudri et al. 1993). Similarly, adverse effect of sludge application on N_2 fixation in faba bean (*Vicia faba*) (Chaudri et al. 2000) is reported. The reduction in growth and symbiosis in white clover were due to cadmium, lead and zinc, when plants were grown in soils highly contaminated with these metals (Rother et al. 1983).

2.2 What Are Nitrogen-Fixing Microbes?

All organisms capable of transforming atmospheric dinitrogen to biologically available form of N, for example, ammonia through a process called biological nitrogen fixation (BNF), are in general collectively referred to as nitrogen-fixing organisms (NFO). Among the two most widely studied nitrogen-fixing groups, asymbiotic represented for example by *Azotobacter* spp. (Plate 2.1a) and symbiotic bacteria (Plate 2.1b) capable of forming nitrogen-fixing organ nodules (Fig. 2.2) on leguminous plants have classically been named "Rhizobia." In the beginning, all bacteria able to nodulate legumes were included in a single genus, *Rhizobium* (Frank 1889), within the family Rhizobiaceae (Conn 1938). This genus had four fast-growing species: *R. leguminosarum* (Frank 1889), *R. phaseoli*, *R. trifolii* and *R. meliloti* (Dangeard 1926) and two slow-growing species: *R. japonicum* (Buchanan 1926) and *R. lupini* (Eckhardt et al. 1931). Later, on the basis of infection data, *R. leguminosarum* was found as microsymbiont for *Vicia*, *Pisum* and *Lens*; *R. phaseoli* for *Phaseolus*; *R. trifolii* for *Trifolium*; and *R. meliloti* for

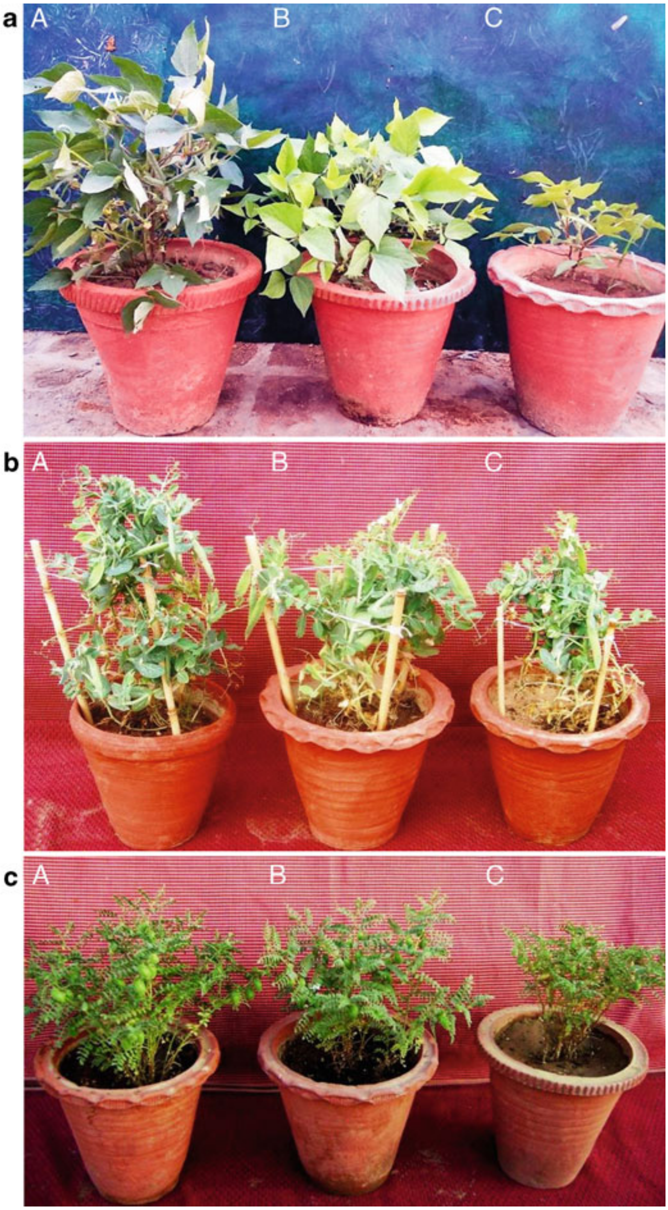

Fig. 2.1 (**a**) Greengram plants grown in sandy clay loam soil treated with *Bradyrhizobium* sp. (Vigna) alone (A), *Bradyrhizobium* sp. (Vigna) with cadmium (B) and cadmium alone (C) in a pot trial experiment. (**b**) Pea plants grown in sandy clay loam soil treated with *Rhizobium* alone (A), *Rhizobium* with copper (B) and copper alone (C) in a pot trial experiment. (**c**) Chickpea plants grown in sandy clay loam soil treated with *Mesorhizobium ciceri* alone (A), *Mesorhizobium ciceri* with chromium (B) and chromium alone (C) in a pot trial experiment

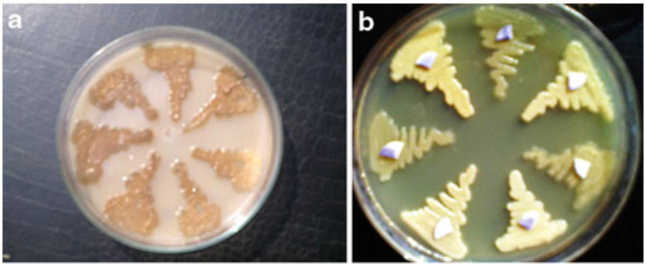

Plate 2.1 (**a**) *Azotobacter* and (**b**) Rhizobia

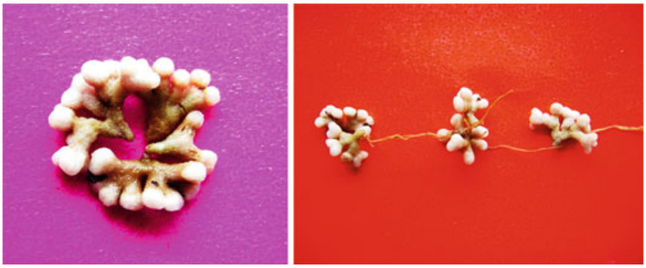

Fig. 2.2 Nodules morphology

Table 2.1 Current information on available rhizobial species

Genus	No. of species	Major host plants
Rhizobium	33	*Pisum*, *Phaseolus*, etc.
Sinorhizobium	12	*Acacia*, *Medicago*, etc.
Mesorhizobium	19	*Cicer*, *Prosopis*, etc.
Bradyrhizobium	08	*Glycine*, *Pachyrhizus*, etc.
Azorhizobium	02	*Sesbania*

(Compiled from: Rivas et al. (2009))

Medicago (Jordan and Allen 1974). The slow-growing species, *R. lupini* (Eckhardt et al. 1931), was found to nodulate *Lupinus* and *R. japonicum* (Buchanan 1926) mainly *Glycine max* (Jordan and Allen 1974). However, even after the role of Rhizobia was well established, this genus was less explored in terms of its diversity and functionality. Recently, with the advent of some newer molecular techniques and interest of rhizobiologist in exploring them as microbial inoculants for raising the productivity of crops especially legumes, the identification of rhizobial species from various hosts and locations has received a renewed attention. As a result, currently, Rhizobia have been reported to include more than 50 species (Table 2.1) distributed in genera *Rhizobium*, *Ensifer* (*Sinorhizobium*), *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium* (Velázquez et al. 2010). These rhizobial species carry symbiotic genes (located on plasmids or symbiotic islands) which codes for nodulation and N_2 fixation. Interestingly, the host range of these genes can be extended to individual/groups of Rhizobia which do not have such genes. And therefore, upon acceptance by the recipient Rhizobia, such genes confer them the ability to nodulate legumes and fix atmospheric N.

2.3 *Rhizobium*–Legume Pairing: An Overview

Nitrogen is one of the prime elements required essentially for the synthesis of enzymes, proteins, chlorophyll, DNA and RNA. And hence, N plays a critical role in determining the health of living organisms including microbes and plants. For nodulating legumes, the N demand is fulfilled through symbiotic N_2 fixation (SNF) wherein atmospheric N_2 is converted to usable N (NH_3) by nitrogenase of Rhizobia (Shiferaw et al. 2004). The BNF accounts for about 65% of the total N currently utilized in agricultural practices which of course is believed to be continuously required in future sustainable crop production systems (Matiru and Dakora 2004). Rhizobial species of the genera *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium* and *Sinorhizobium* intimately interact with legumes using flavonoid molecules as signal compounds, released by the legume host. These plant-generated compounds systematically induce the expression of nodulation (nod) genes in Rhizobia, which in turn produce lipo-chitooligosaccharide (LCO) signals called Nod factors (Perret et al. 2000; Shaw et al. 2006; Cooper 2007; Maj et al. 2010). These signal compounds trigger the mitotic cell division in roots, leading finally to nodule formation (Dakora 1995; Matiru and Dakora 2004; Jones et al. 2007; Batut et al. 2011). Inside the central nodule cells, Rhizobia are housed as symbiosomes that are horizontally acquired organelles and are involved in the enzymatic reduction of atmospheric N to NH_3 and make this N accessible to their hosts. In return, the Rhizobia get carbohydrates from their host. The host plants, however, regulate the number of nodules formed, the maturation of nodules and the N_2 fixation process.

2.4 How Rhizobia Promote Legume Growth?

Primarily, Rhizobia is known for its ability to provide N exclusively to legumes through BNF. However, the biologically available form of N produced by Rhizobia can also facilitate the overall growth of associated non-legumes directly by transferring symbiotically formed N to crops like cereals, growing in intercrops (Snapp et al. 1998) or to subsequent crops rotated with legumes (Hayat 2005; Hayat et al. 2008a, b). In addition to N_2 fixation, Rhizobia promote the growth of plants by other mechanism also (Table 2.2). For example, species of Rhizobia isolated from various sources such as conventional (Zaidi et al. 2003; Ahmad et al. 2008b; Ahemad and Khan 2011a) or stressed environment (Carrascoa et al. 2005; Wani et al. 2007c, 2008a) have shown the production of plant growth-promoting substances like phytohormones; auxins, cytokinins and abscisic acids; lumichrome, riboflavin, LCOs and vitamins (Keating et al. 1998; Wani et al. 2008c, 2009;

Table 2.2 Examples of plant growth-promoting substances synthesized by symbiotic nitrogen fixers

Symbiotic N_2 fixer	Crop enhancer	References
Bradyrhizobium MRM6	IAA, HCN, siderophore, ammonia, EPS	Ahemad and Khan (2011c)
Rhizobium MRL3	IAA, HCN, siderophore, ammonia	Ahemad and Khan (2011d)
Sinorhizobium strain	Chitinase	Qing-xia et al. (2011)
Rhizobium leguminosarum var. *phaseoli*	IAA	Stajkovic et al. (2011)
Rhizobium spp.	IAA, siderophore	Mehboob et al. (2011)
Sinorhizobium meliloti	IAA, P-solubilization	Bianco and Defez (2010)
Bradyrhizobium	IAA, gibberellic acid	Afzal et al. (2010)
Mesorhizobium	IAA	Ahemad and Khan (2010a)
Rhizobium spp.	IAA	Chakrabarti et al. (2010)
Rhizobium leguminosarum	IAA, siderophore	Ahemad and Khan (2010c)
Mesorhizobium	IAA, HCN, siderophore, ammonia, P-solubilization	Ahmad et al. (2008b)
Rhizobium strain TAL 1145	ACC-deaminase	Tittabutr et al. (2008)
Rhizobium spp.	IAA, gibberellic acid, zeatin	Boiero et al. (2007)
Mesorhizobium loti MP6	IAA, HCN, siderophore, P-solubilization	Chandra et al. (2007)
Rhizobium etli USDA9032	Phenazine, antibiotic	Krishnan et al. (2007)

Ahemad and Khan 2009, 2010b). Other plant growth-enhancing traits for which Rhizobia have been exploited includes synthesis of siderophore (Wani et al. 2008c; Ahemad and Khan 2011b), solubilization of inorganic P (Abd-Alla 1994; Chabot et al. 1996: Khan et al. 2007, 2009a, b, 2010) and as biocontrol agents (Khan et al. 2002; Deshwal et al. 2003a, b). Rhizobia isolated from nodules of some tropical legumes have also been shown to infect roots of crops other than legumes such as rice (*Oryza sativa*), wheat (*Triticum aestivum*) and maize (*Zea mays*) via crack entry mechanism (Webster et al. 1997).

2.5 Heavy Metal Toxicity: A General Perspective

Heavy metals when present in lower concentration play an important role in the activities of many enzymes like proteinases, dehydrogenases and peptidases. Among metals, zinc, for example, is required in the synthesis of carbohydrates, proteins, phosphate, auxins, RNA and ribosome. Likewise, copper plays critical roles in various physiological processes such as respiration, photosynthesis, N and cell wall metabolism, carbohydrate distribution and seed production (Kabata-Pendias and Pendias 2001). However, in addition to some toxic metals, when the concentrations of even the biologically significant metals become higher, they cause toxicity. For example, cadmium even-though is not involved in any biological processes but may become quite toxic after it is accumulated inside the organisms. Some of the nuisance of cadmium includes (1) disturbed enzyme activities, (2) inhibition of DNA-mediated transformation in microorganisms, (3) reduced symbiosis between microbes and plants and (4) increased plant predisposition to fungal invasion (Kabata-Pendias and Pendias 2001; Mohanpuria et al. 2007). In addition, stressors like heavy metal have also been reported to convert the viable bacterial cells to non-culturable form (Paton et al. 1997; Paudyal et al. 2007). Therefore, once the soil is destructed by heavy metals, metals found naturally within soil or accumulated as a result of anthropogenic activities (Giller et al. 1989; McGrath et al. 1995; Robinson et al. 2001; Lei et al. 2011), it becomes uninhabitable for microbial communities or unsuitable for crop production. For example, numerous metals (e.g. Cu, Ni. Zn, Cd, As) have been reported to inhibit the growth, morphology and activities of various groups of microorganisms (Khan and Scullion 2002; Shi et al. 2002; Lakzian et al. 2002; Bondarenko et al. 2010) including symbiotic N_2 fixers (McGrath et al. 1988; Santamaría 2003; Stan et al. 2011) like *R. leguminosarum*, *Mesorhizobium ciceri*, *Rhizobium* sp. and *Bradyrhizobium* sp. (Vigna) and *Sinorhizobium* (Wani 2008; Arora et al. 2010; Bianucci et al. 2011). On the other hand, Rhizobia among soil bacteria have been the organism of great interest for agronomist in general and legume growers in particular primarily due to their ability to provide N to plants. Considering the benefits of Rhizobia in N economy and the role of legumes in animal and human health, attention in recent times has been paid onto understanding how metals could affect the very survival of Rhizobia either present as free-living organism or when they are in intimate relationship (symbiosis) with legumes (Ibekwe et al. 1995; Khan et al. 2009b). Heavy metals are inhibitory to rhizosphere microorganisms, and processes mediated by them, like nitrogen-fixing ability of Rhizobia, are lost when they are in symbiotic association with the legume host, growing in metal-enriched locations (Vasseur et al. 1998; Barajas-Aceves and Dendooven 2001; Hernandez et al. 2003). For example, Arora et al. (2010) in a study assessed the impact of aluminium and copper, iron and molybdenum on growth and enzyme activity of fast- and slow-growing rhizobial species. Of the tested rhizobial strains, *Sinorhizobium meliloti* RMP_5 showed greatest tolerance to metal stress compared to *Bradyrhizobium* BMP_1. Both the strains were, however, extremely sensitive to Al than other metals. In addition, Al was found extremely toxic and reduced the various enzymatic activities like nitrate reduction, nitrite reduction and nitrogenase and hydrogenase uptake, by strains RMP_5 and BMP_1. Among the metals, copper had strong inhibitory effect on growth and enzyme activities of *Bradyrhizobium* strain at all concentrations. In comparison, all the tested enzymatic activities of *S. meliloti* RMP_5 increased up to the concentration of 0.1 mM Cu, while Fe enhanced the growth and enzyme activities of *S. meliloti* RMP_5 and *Bradyrhizobium* BMP_1 up to 100 mM concentration. Molybdenum increased all the tested enzymatic activities of *S. meliloti* RMP_5 up to 1 mM. Nitrate and nitrite reduction activities of *Bradyrhizobium* BMP_1 increased up to 1 mM concentration. However, nitrogenase and hydrogenase activities of *Bradyrhizobium* BMP_1 were enhanced only up to 0.5 mM Mo. In a similar study, Paudyal et al. (2007) determined the effect of three heavy metals (Al, Fe and Mo) on two strains of Rhizobia isolated from root nodules of two tropical legume species, *Mucuna pruriens* and *Trigonella foenum-graecum*. All tested concentrations of aluminium had detrimental effect on rhizobial strains when grown in vitro and in vivo conditions. Iron, in contrast, supported bacterial growth and enhanced the symbiotic parameters such as biomass production and nodulation up to 25 μM which however had negative effect thereafter. Molybdenum at 75 μM improved bacterial growth, while up to 20 μM, molybdenum increased plant production and nodulation of test legumes. Hirsch et al. (1993), for example, demonstrated that the population of *R. leguminosarum* bv. *trifolii* was radically altered by long-term exposure to heavy metals, and this *Rhizobium* lost the ability to form functional symbiosis with white and red clover. In other study, Chaudri et al. (2000) in a long-term field trial reported a decrease in two agriculturally important species of Rhizobia, *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii*, in soils, which were irrigated with sewage sludge containing Zn or Cu or mixture of Zn and Cu. Besides the potential toxicity of heavy metals on the growth and survival of Rhizobia, nodulation in legumes is also considerably affected (Khan et al. 2008). In sludge-treated soils, even though the nodulation on the root systems of clover was observed, the nodules were ineffective (McGrath et al. 1988; Giller et al. 1989). Similarly, Singh et al. (2003) noted that Pb reduced number and size of root hairs of greengram and also the darkness and total area of the leaves. Significant decrease in acetylene reduction by nodules or free-living heterotrophic nitrogen fixers in the presence of heavy metals has also been reported by others (Obbard et al. 1993; Shvaleva et al. 2010). McGrath et al. (1988) has shown a decrease in yield of white clover in monoculture on sludge-treated plots compared to plots receiving farm yard manure. If soils' heavy metal contents were at acceptable level after the amendment of the soil with sewage sludge, there was no negative effect on yield and N contents of alfalfa plants (Rebah et al. 2002).

2.5.1 Are Legumes Safe to Grow in Metal Contaminated Soils?

Legume–*Rhizobium* interactions occurring in either conventional or stressed environment have been one of the most widely studied and practical aspect in biological sciences. Rhizobia in general are used as inoculants for legume production in different agroecological environment and have shown a significantly higher pulse yields (Zaidi et al. 2003; Wani et al. 2007c; Ahemad and Khan 2011a). However, when grown in soils treated intentionally with heavy metals for experimental purpose or in soils already contaminated with heavy metals mainly due to contaminated agrochemicals and sewage sludge, most legume crops are not safe and affected negatively. The deleterious effects of heavy metals on nodulation and N_2 fixation of *Rhizobium*–legume symbiosis are probably due to their inhibitory effects on the growth and activity of both symbionts. For example, when 50–200 mg kg^{-1} soil of Co, Cu, Cd and Zn was added deliberately to soils used for *Lablab purpureus* cultivation, these metals invariably affected adversely the growth, nodulation and nitrogenase activity of plants in both pot and field trials. Apart from the effects of those tested metals on measured parameters, these metals also reduced substantially the level of nutrient elements like Na, K and Ca within shoots of this plant which of course increased with increasing rates of metals applied (Younis 2007). Sepehri et al. (2006) in a greenhouse experiment showed that 2 mg Cd/kg soil had a variable effect on symbiotic properties of *S. meliloti* strains and consequently on *S. meliloti*–alfalfa symbiosis. A decreasing effect of cadmium concentration on root nodules and N concentration in plants inoculated with sensitive rhizobial strains in comparison with plants bacterized with tolerant strains was 68% and 41%, respectively.

Heavy metals when present in excess have also been found to delay the nodulation process in some legume crops. For example, with increasing concentration of arsenic (As) in the nutrient solution, there was greater time required for *Bradyrhizobium japonicum* strain CB1809 inoculated soybean (*Glycine max*) cv. Curringa plants to produce nodules, and the number of nodules per plant decreased at harvest. In addition, the inoculated plants had poor root hairs and dry matter contents in roots and shoots as the concentration in the solution increased (Reichman 2007). The abnormally higher concentrations of metal also limit the uptake of water and nutrients by plants (Terry 1981; Karpiscak et al. 2001) and concomitantly the health of plants. However, when a single or mixture of metals get a chance to enter within plant tissues and are translocated subsequently to various plant organs, they can interact directly with cellular components and disrupt the metabolic activities, causing cellular injuries and in some cases even may lead to the death of the plants (Fig. 2.3). As an example, cadmium even at considerably lower concentration was found toxic for the microsymbiont (Pereira et al. 2006; Younis 2007) and (1) inhibited the nitrogenase activity; (2) affected the plant biomass production; (3) disrupted nodule ultrastructure number of nodules and induced nodule senescence; (4) reduced dry matter accumulation in roots, shoot and leaf; and (5) adversely affected metabolic activities like photosynthesis of legumes (Balestrasse et al. 2004; Mumtaz et al. 2006; Wani et al. 2006; Noriega et al. 2007). Furthermore, cadmium-induced oxidative stress has led to the reduction in carbohydrate and protein (leghaemoglobin) synthesis within nodule and inhibited antioxidant enzyme activity. The increase in lipid peroxidation and thiols has also been found to result from cadmium toxicity for other crops (Balestrasse et al. 2003; Benavides et al. 2005; Garg and Aggarwal 2011). The increasing concentrations of heavy metals like cadmium, zinc and lead significantly decreased nodule index: the number of nodules per gramme of the total fresh biomass, at about 2.64 mg Cd kg^{-1}, 300 mg Zn kg^{-1} and 130 mg Pb kg^{-1}. From this study, it was proposed that the nodulation index of white clover could serve as a suitable bioindicator of increased heavy metal toxicity in soil (Manier et al. 2009). The effects of metals on rhizobial composition within soil or nodule environment and different legume genotypes, however, have been contradictory (Wani et al. 2007a, b, 2008a, b; Wani 2008). To validate this concept of conflicting effects of metals on Rhizobia, Paudyal et al. (2007) conducted an experiment which revealed that Rhizobia grew poorly in culture medium supplemented with even lower concentration of aluminium, while rhizobial growth was completely inhibited at 50 mM Al concentration (Wood and Cooper 1988; Chaudri et al. 1993; Broos et al. 2004). On the contrary, no reasonable changes in dynamics of *B. japonicum* and

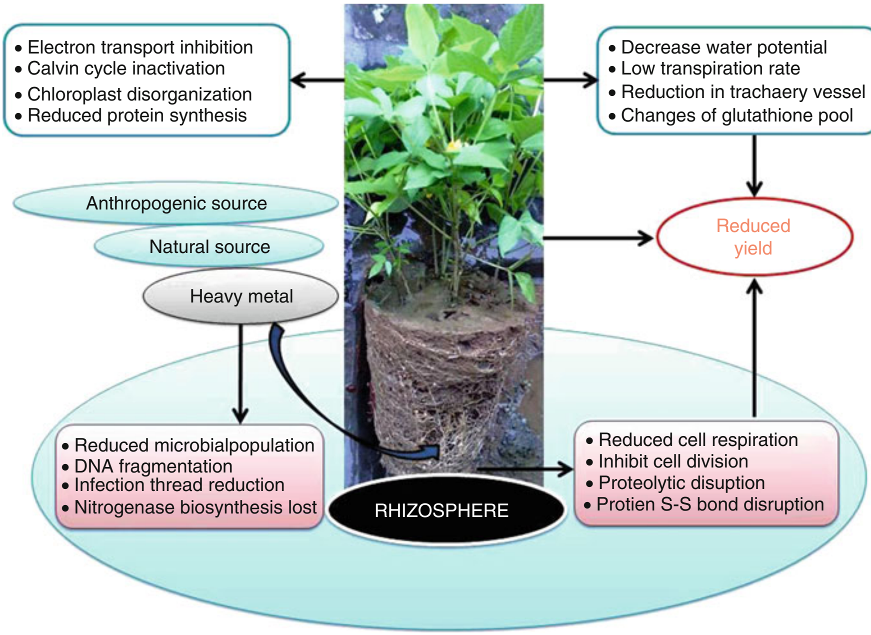

Fig. 2.3 Toxicity of heavy metal to various metabolic stages of plants including *Rhizobium*–legume symbiosis

growth and N_2 fixation by host plant, when grown in metal contaminated soils, were observed by others (Kinkle et al. 1987; El-Aziz et al. 1991; Smith and Giller 1992). Furthermore, it is suggested that there exist a relationship between *Rhizobium's* tolerance, heavy metal soil contamination and alterations in protein pool. Due to this, the assessment of variation in protein contents is considered a good indicator to estimate the level of stress imposed on *Rhizobium* populations exposed to heavy metal contamination (Pereira et al. 2006).

Conclusion

Heavy metal toxicity to some plants and microorganism is well documented, but its effect on legumes, *Rhizobium* and legume–*Rhizobium* symbiosis is poorly understood. These metals can arrest the growth and multiplication of Rhizobia in rhizosphere and may also have depressive effect on the steps involved in legume–*Rhizobium* symbiosis, resulting in low nitrogen fixation. In addition, metals can also cause severe toxicity to various metabolic activities of legumes including photosynthesis, synthesis of proteins, enzymes and carbohydrates. Therefore, understanding the metal–rhizobia–legume interaction in metal-enriched environment is urgently required for growing legumes in soils contaminated with heavy metals.

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