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Symbiotic efficiency, biosorption and the growth of rhizobia on Horse gram plants under aluminium stress

Učinkovitost simbioze, biosorpcije in rasti rizobijev pri vrsti *Macrotyloma uniflorum* zaradi aluminijevega stresa

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Abstract: The aim of the present study was to evaluate the tolerance potential of Horse gram rhizobia to aluminium (Al) toxicity, the enhancement in pod formation, symbiotic efficiency and biosorption potential in the rhizobia inoculated Horse gram (*Macrotyloma uniflorum* (Lam.) Verdc.) plants. Initially, 32 isolates of Horse gram rhizobia were screened for their tolerance of Al in growth media. Among the 32 strains, HGR 4, 6, 13 and 25 that were more tolerant were inoculated individually to Horse gram plants and the plants were then screened for the ability of pod formation, symbiotic efficiency and biosorption potential. Among them, maximum pod formation was observed in Horse gram upon inoculation with HGR-6 and grown at 400 μg g^{-1} of Al. Maximum nodulation was observed in Horse gram upon inoculation with HGR-6 and HGR-13 grown at 200 μg g^{-1} Al. Leghaemoglobin content was maximum on inoculation with HGR-13 at 400 μg g^{-1} of Al. The strain HGR-13 has shown biosorption potential in soil and as well as in root nodules even at 300 μg g^{-1} of Al though it was maximum at 100 μg g^{-1}. This study demonstrated that the Horse gram plants inoculated with *Rhizobium* strains HGR - 4, 6, 13 and 25, besides having nitrogen fixing ability also have the ability to grow in Al contaminated soils. Hence, Horse gram plants associated with these strains of rhizobia could be used in phytoremediation of metal (Al) contaminated soils.

Key words: Aluminium, biosorption, metal tolerance, phytoremediation, *Rhizobium*, symbiotic efficiency.

Izvleček:
Namen študije je bil ovrednostiti tolerančni potencial rizobijev za aluminij pri vrsti *Macrotyloma uniflorum* (Lam.) Verdc. Preučevali smo tvorbo strokov, učinkovitost simbioze in biosorpcijski potencial 32 izolatov rizobijev. Med njimi so bili najbolj tolerantni sevi HGR 4, 6, 13 in 25, ki smo jih v nadaljevanju inokulirali individualno na rastline in spremljali 3 parametre. Največjo tvorbo strokov smo izmerili pri sevu HGR-6 in rasti pri 400 μg g^{-1} Al. Največ nodulov je bilo pri inokulaciji s HGR-6 in HGR-13 pri 200 μg g^{-1} Al. Vsebnost leghemoglobina je bila najvišja pri inokulaciji s HGR-13 pri 400 μg g^{-1} Al. Sev HGR-13 je imel biosorpcijski potencial tako v tleh

kot v koreninskih nodulih celo pri 300 µg g^{-1} Al, največji pa je bil pri 100 µg g^{-1} Al. Raziskava kaže, da imajo rastline vrste *Macrotyloma uniflorum* , ki so inokulirane s sevi *Rhizobium* HGR - 4, 6, 13 in 25 poleg sposobnosti vezave dušika tudi sposobnost rasti v tleh, onesnaženih z Al. Zato so lahko take rastline primerne za fitoremediacijske postopke s kovinami (Al) onesnaženih tal.

Ključne besede: aluminij, biosorpcija, toleranca za kovine, fitoremediacija, *Rhizobium*, učinkovitost simbioze.

Introduction

Contamination of soils by metals is wide spread due to human, agricultural and industrial activities (Beladi et al. 2011). These activities result in the accumulation of traces of metals in agricultural soils which pose a threat for food safety and public health (Dary et al. 2010). This accumulation of metals leads to loss of soil fertility, since the composition of microbial flora and microbial activities are affected severely (Krujatz et al. 2011). Some metals, though essential in micro quantities for organisms, are harmful in excess. Aluminium (Al), the third most abundant element in the earth's crust after oxygen and silicon, comprises approximately 7% of its mass (Foy et al. 1978). Al toxicity limits world's agricultural productivity, as Al becomes more soluble in acidic conditions and is the major toxic element in acidic soils and water (Sledge et al. 2005). Excess of Al resists the crop is as a result of direct inhibition of nutrient uptake or impairment in root cell function (Kochaian 1995, Matsumoto 2000) and productivity of plants (Kochian et al. 2004).

Focus on use of legume plants associated with microorganisms for bioremediation of metals is growing every day (Carrasco et al. 2005). The *Rhizobium*-legume association has an advantage in which both the microorganisms and plants may influence metal solubility, bioavailability. Added to this is an advantage of enhanced nitrogen content of soils when used for bioremediation or phytoremediation (Pajuelo et al. 2008, Dary et al. 2010). Therefore, isolation of rhizobia, capable to tolerate these metal stress is essential for efficient metal remediation and also the nitrogen fixation under metal pollution (Woldeyohannes et al. 2007). The significance in choice of Horse gram for our study is its adaptability to poor adverse climatic conditions, which are unsuitable for other pulse crops. Horse gram is cultivated as a grain legume and fodder crop in the states of Tamil Nadu, Karnataka, Andhra Pradesh and Orissa of South India during Kharif and Rabi (the two agricultural monsoon seasons). Our study targets to analyze the effect of Al tolerant *Rhizobium* strains on pod formation, symbiotic efficiency content and Al biosorption potential of Horse gram plants upon inoculation with the chosen rhizobial strains.

Materials and methods

Isolation and analysis of rhizobial strains

Soil samples were collected from various regions in united Andhra Pradesh, India for sowing the plants. Root nodules were isolated from these plants. Root nodules were surface sterilized and the rhizobial strains were further isolated on Yeast Extract Mannitol (YEM) agar medium (Vincent 1970) with 0.0025% Congo red dye. All these isolates were subjected to biochemical and 16S rRNA sequence analysis. YEM agar medium was prepared with varying concentrations of $Al_2(SO_4)_2$ i.e. 50, 100, 200, 300, 500, 750 and 1000 µg g^{-1}. After solidification, all the isolates were inoculated and incubated at room temperature for 72 hrs. After incubation, the colony diameter was monitored. Replicates were maintained for each metal concentration. Initially 32 isolates of Horse gram rhizobia (HGR's) were screened for the tolerance of aluminium.

Inoculation with rhizobial strains grown under different concentrations of $Al_2(SO_4)_2$

Seeds of Horse gram used during the study were obtained from local fields of Andhra Pradesh, India. The pots of the study were filled with soil sterilized in an autoclave at 121°C for 3 hrs each on three alternative days. Horse gram seeds were surface sterilized with 70% ethanol for 3 min, followed by sodium hypochlorite treatment for 3 min and then rinsed six times with sterilized water, dried. The rhizobial suspension of isolates used in the study were grown in YEM broth in flasks shaken at 120 rpm at 28±2°C for 3 days to obtain a cell density of 6×10^9 cells ml^{-1}. Horse gram plants were inoculated with the selected strains HGR-4 (GQ483457), HGR-6 (GQ483458), HGR-13 (GQ483459) and HGR-25 (GQ483460) which performed well during the initial screening on Al tolerance assay. To perform the inoculations, sterilized seeds were coated with the rhizobial strain by soaking the seeds in liquid culture medium for 2 hrs using 10% (wt/vol) gum Arabic as adhesive to deliver approximately 10^9 cells $seed^{-1}$. Respective controls were maintained with seeds treated in sterilized distilled water. The inoculated seeds (20 seeds pot^{-1}) were sown in clay pots using 2 kg sterilized soil. In order to evaluate the effect of Al metal on the Horse gram plants inoculated with 4 HGR strains, plants were maintained with $Al_2(SO_4)_2$ supplements of 50, 100, 200, 300 and 400 µg g^{-1} kg^{-1}. Negative controls were also maintained using the Horse gram plants natively available with $Al_2(SO_4)_2$ supplements of 50, 100, 200, 300 and 400 µg g^{-1} kg^{-1}. For the purpose of comparison controls were also maintained without adding Al. Three replicates were maintained for each treatment. The pots were watered regularly and were maintained in an open field conditions and allowed to grow.

Analysis of plants for nodulation, symbiotic efficiency and biosorption potential

The number of pods formed were counted post 40 days of sowing. The plants were observed for nodulation regularly after the seedlings emerged. Five plants in each treatment were picked up randomly and nodulation characteristics were evaluated 40 days after sowing, as it was previously observed during the study that highest nodulation of Horse gram occurred on 40^{th} day. For biochemical analysis, plants raised in different concentrations of Al were collected, the amount of leghaemoglobin was estimated (Tu et al. 1970) post 40 days of sowing. Soil pH, organic matter and total nitrogen (N) (Jackson 1973) and total phosphorus (P) (Olsen et al. 1954) were also estimated. The amount of sand, silt and clay present in the soil were also analyzed (Black 1965).

For elemental analysis, root nodules were collected and washed under tap water to remove sediments and soil. Then they were washed in 0.02% detergent (tween-20) and once again in tap water. They were again washed with 0.1 N HCl. Finaly, the nodules were washed twice with distilled water. The nodules were dried at 80ºC for 48 hrs in hot air oven and they were ground to a very fine powder. From this, 0.5 grams of powdered tissue was added to 5 ml of conc. HNO_3 for cold digestion at room temperature. Then 5 ml of conc. HNO_3 and H_2O_2 were added to the digested sample in 10:4 ratio, the samples were heated to a volume of 2 ml. The clear solution obtained was made up to 25 ml with deionized water (millipore) and used for elemental analysis. Soil samples were also subjected to acid digestion with slight modifications and were used in elemental analysis. Al concentration present in the sample was determined (APHA 22nd Edition, 3111 B) by Atomic Absorption Spectroscopy (AAS) (THERMO AAS Model No: ICE 3000). The system was operated using the Thermo scientific SOLAAR data station V 11.02 software. Argon was used as inert gas during operation. The instrument's operating conditions included Furnace instrumental mode, Lamp current at 15 mA, Wavelength of 232 nm, 0.2 µg/l Gas flow, 0.2 nm band width and 72 sec of Furnace programme total time.

Statistical analysis

Statistical analysis was done in three replicates for each treatment. The mean and standard error (SE) were calculated using Microsoft Office Excel 2007. To test the statistical significance, all the values were analyzed by ANOVA, using SPSS Statistics, Version 20 (Armonk, 2011). Bars indicate means ± SE and were significant at 5% level of significance (P value < 0.05).

Results

Most of the 32 rhizobial isolates were able to grow on YEM agar plates containing 1000 µg g^{-1} concentration of Al, but the diameter of the colonies varied with the isolate i.e. from 2 mm (HGR-16) to 14 mm (HGR-13). At this concentration the colonies are round, white, translucent, raised and convex with entire margins. Similar results were obtained in YEM broth also. Hence four isolates of the study HGR - 4, 6, 13 and 25 which performed better were further used for evaluation of other parameters.

Al supported good growth of Horse gram plants up to 300 µg g^{-1}, when the plants were inoculated with the four rhizobial strains. Later, the growth of the plants decreased with increase of metal concentration. The plants inoculated with the strains HGR-4, 6, 13 and 25 have shown their maximum pod formation at 100 to 400 µg g^{-1} of Al. Horse gram rhizobial inoculation increased pod formation when compared to control. Among the four selected strains the strain HGR-4 inoculated plants have shown maximum pod formation at 200 µg g^{1}, HGR-6 at 400 µg g^{-1}, HGR-13 at 300 µg g^{-1} and HGR-25 has shown at 100 µg g^{-1} of Al only (Fig. 1). They were significant at 5% level (P value < 0.05).

In the present study, nodules appeared post 13 days of sowing on tap root and as well as on lateral roots at all the Al concentrations experimented. The total number of nodules formed per plant ranged from 8 to 22. The plants inoculated with the strain HGR-6 and 13 have shown highest nodulation at 200 µg g^{-1} of Al. The number of nodules formed were more when rhizobia inoculated to Horse gram plants when compared to control. They were significant at 5% level (P value < 0.05). However, the increased concentration of Al reduced the number of nodules. HGR-4 and 25 inoculated plants had maximum number of nodules at 100 µg g^{-1} of Al only (Fig. 2). The leghaemoglobin content was maximum in the Horse gram plants inoculated with the strains HGR-6 and HGR-13 at 400 µg g^{-1} of Al (Fig. 3). The plants inoculated with HGR-4 and HGR-25 have shown their maximum at 50 µg g^{-1} only. But, these values were more than in control. They were significant at 5% level (P value < 0.05).

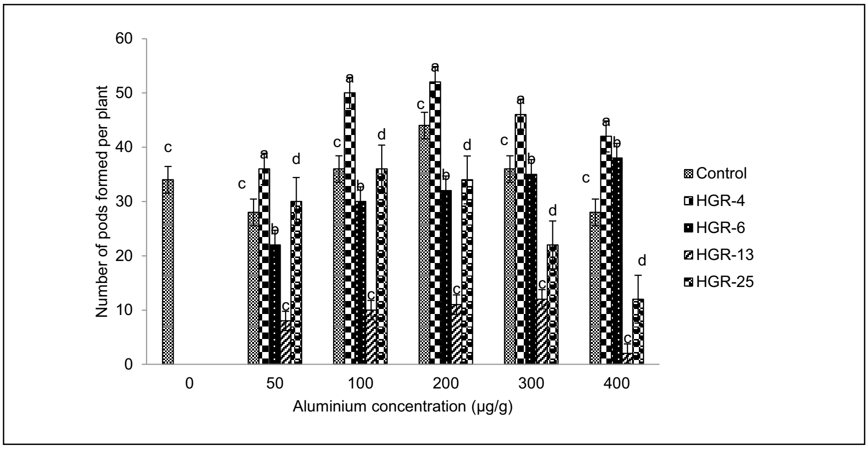

Figure 1: Formation of pods on Horse gram plants inoculated with the four *Rhizobium* strains in response to varying concentrations of aluminium. Data show mean value ± SE.

Slika 1: Tvorba strokov pri rastlinah *Macrotyloma uniflorum,* inokuliranih s štirimi sevi rizobija pri različnih koncentracijah aluminija. Prikazane so povprečne vrednosti ± SN.

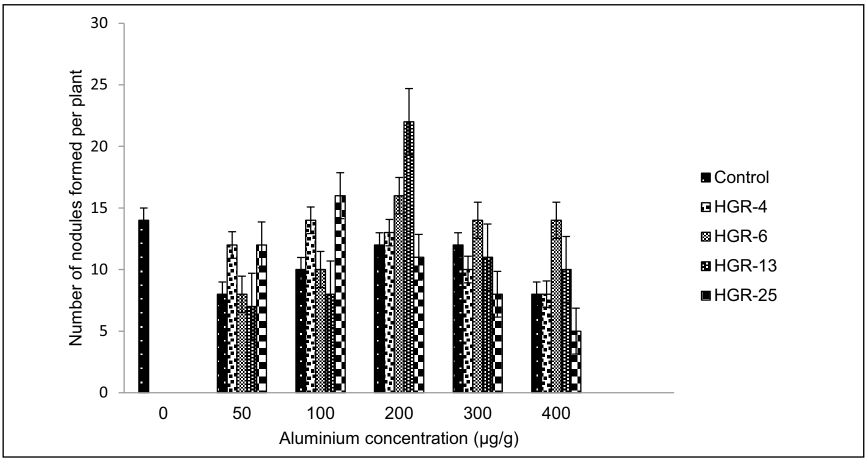

Figure 2: Formation of root nodules on Horse gram plants inoculated with the four *Rhizobium* strains in response to varying concentrations of aluminium. Data show mean value ± SE.

Slika 2: Tvorba koreninskih nodulov pri rastlinah *Macrotyloma uniflorum,* inokuliranih s štirimi sevi rizobija pri različnih koncentracijah aluminija. Prikazane so povprečne vrednosti ± SN.

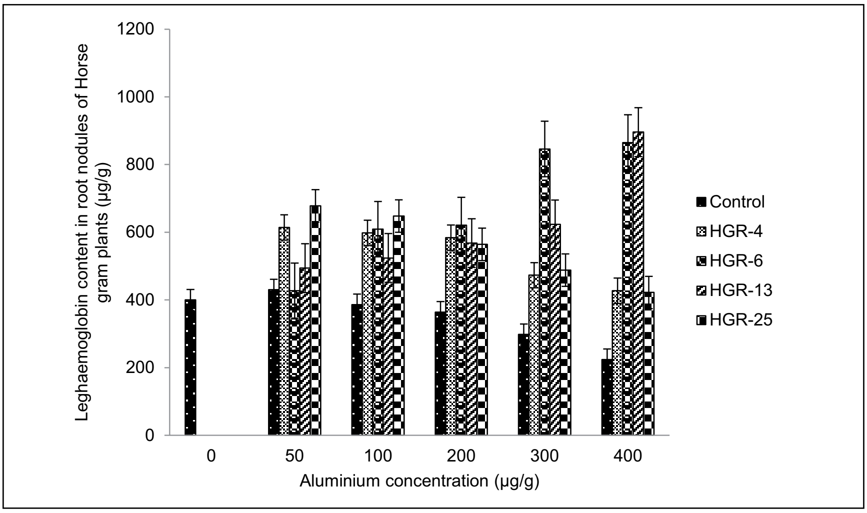

Figure 3: Leghaemoglobin content in root nodules of Horse gram plants inoculated with the four *Rhizobium* strains in response to varying concentrations of aluminium. Data show mean value ± SE.

Slika 3: Vsebnost leghemoglobina v koreninskih nodulih pri rastlinah *Macrotyloma uniflorum,* inokuliranih s štirimi sevi rizobija pri različnih koncentracijah aluminija. Prikazane so povprečne vrednosti ± SN.

The amount of total nitrogen (%) and phosphorus (%) present in the soil is 0.85 and 1.24 respectively. The total content of organic matter in soil is 1.20, sand 18, silt 16, clay 42 and the pH of the soil is 6.44. Among these four strains HGR-13 has shown more number of nodules and maximum leghaemoglobin content at high concentrations of Al. So, we have selected HGR-13 to estimate biosorption potential of the Horse gram rhizobia. Biosorption potential of the strain HGR-13 was

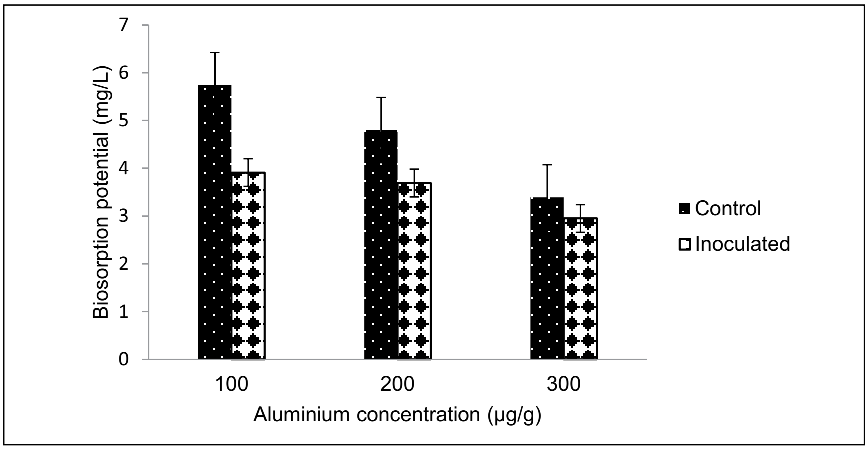

Figure 4: Biosorption potential of the strain HGR-13 in soil after deplantation of Horse gram plants at different concentrations of aluminium. Data show mean value ± SE.
Slika 4: Biosorpcijski potencial seva HGR-13 po presaditvi rastlin *Macrotyloma uniflorum,* v tla z različno koncentracijo aluminija. Prikazane so povprečne vrednosti ± SN.

determined by Atomic Absorption Spectroscopy (AAS), as the amount of metal present in the nodule and soil samples after the treatment with the isolate HGR-13. After analyzing the treated samples in AAS, the inoculated strain HGR-13 has shown maximum biosorption of Al. The results have shown that the Al present in the soil was decreased after deplantation of Horse gram plants when they were inoculated with the Al tolerant rhizobia (HGR-13) i. e. from 5.74 mg/L (control without inoculation) to 3.91 mg/L at 100 µg g^{-1}, 4.8 mg/L to 3.69 mg/L at 200 µg g^{-1} and 3.39 mg/L to 2.95 mg/L at 300 µg g^{-1} of Al concentration. In nodules, the biosorption potential was maximum at 200 µg g^{-1} (1.69 mg/L to 0.99 mg/L), at 100 µg g^{-1} it was 1.92 mg/L to 1.89 mg/L and at 200 µg g^{-1} it was 1.28 mg/L to 1.27 mg/L. The results have shown that the isolate was able to adsorb Al at a concentration of 300 µg g^{-1} in root nodules and also in soil samples inoculated with the strain HGR-13 (Fig. 4, 5).

Discussion

In our study all the tested Horse gram rhizobia are able to show growth at all the Al concentrations tested. But, they have shown variations in their growth. Al stress reduced the growth of rhizobial species under laboratory conditions and also in natural environment (Paudyal et al. 2007; Avelar Ferreira et al. 2012). Some cowpea rhizobia tolerate up to 20 m mol_c dm^{-3} of Al^{+3} (Bruno et al. 2013). *Rhizobium* species like *Sinorhizobium meliloti* and *Bradyrhizobium* growing explanta were extremely sensitive to Al (Arora et al. 2001). The rhizobia from Bambara groundnut were able to grow in a medium pH as low and Al concentration of 50 µM (Laurette et al. 2015). The growth of the *Rhizobium* strain CB756 was also not affected by 50 µM Al at pH 4.5 (Keyser and Munns, 1979). But 50 µM Al at pH 4.6 decreased the growth of *Bradyrhizobium* spp. (Shamsuddin 1987) and the growth of rhizobia was completely inhibited at 50 mM Al (Broos et al. 2004). At high concentrations Al is a potent inhibitor of rhizobial growth. It was observed that root nodule bacteria grow after a lag period in culture medium containing 75 µM Al (Whelan and Alexander 1986). Wild rhizobia from Soybean have shown growth at 200 µ $mol.L^{-1}$ of Al^{3+} (Ping et al. 2014). It has shown negative effect on the survival and growth of *Rhizobium trifolii*. *Bradyrhizobium* strains were able to show growth in the presence of 100 µM $AlCl_3$ (Lesueur et al. 1993) and some *Bradyrhizobium japonicum* strains were tolerant to Al (Tayler et al. 1991).

Anoxybacillus sp. SK 3-4 was found to be the most resistant to Al and significant growth was observed at 300 mg L^{-1} to 800 mg L^{-1} of Al (Lim et al. 2015). To the best of our knowledge this is the first report that rhizobia nodulating Horse gram were able to grow in culture medium containing 1000 µg g^{-1} Al concentration.

Al effects the growth and nodulation of many legumes (Kim et al. 1985). Kushwaha et al. 2017 observed in a study with cowpea, the number of pods formed were more in soil upon treatment with 40 ppm concentration of Al. In the present study the number of pods formed was more at 100 to 400 µg g^{-1} of Al. The inoculation of acid tolerant, Al tolerant *Bradyrhizobium japonicum* strains could increase number of pods in soybean (Situmorang et al. 2009). Previous studies reported that at high Al concentrations, nodulation was partially or totally inhibited in several species such as common bean (*Phaseolus vulgaris*), clover (*Trifolium repens*), *Stylosanthes* species and also in other tropical legumes (de Carvalho et al. 1981, Paudyal et al. 2007). de Carvalho et al. 1981 reported that Al has shown its effect on reduction or inhibition of nodulation at 25 iM concentration. Kushwaha et al. 2017 during their study reported that the more number of nodules formed at lower concentrations of Al (0 and 20 ppm) and number of nodules were reduced at higher concentration of Al. Brady et al. 1990 reported that Al at < 5 µM has shown reduction in nodulation of soybean. Inoculation of *Sinorhizobium mexicanum* ITTG 27^T to *Acaciella angusstissime* plants enhanced nitrogen content (Rosales et al. 2011). Al toxicity has shown negative effect on symbiotic nitrogen fixation in common bean plants that were grown hydroponically in acidic nutrient solution containing 70 µM $AlCl_3$ (Mendoza-Soto et al. 2015). Blamey and Chapman, 1982 reported that in groundnut, poor nodulation and nitrogen fixation was observed under Al toxicity. Horse gram rhizobia i.e. HGR-6 and HGR-13 inoculated plants have shown increased leghaemoglobin content even under 400 µg g^{-1} of Al stress. de Carvalho et al. 1982 suggest that the effects of Al on N_2 fixation may be indirect. The rhizospheric microorganisms have intrinsic ability to reduce/detoxify the metal stress by several mechanisms. These mechanisms include the efflux of metal ions outside the cell, biostimulation, bioaugmentation, metal reduction and biosorption (Outten et al. 2000). Mammaril et al. 1997 proved that the metal concentration decreased after rhizobial inoculation, which shows the ability of their rhizobial strain RP5 in the removal of metals through adsorption-desorption mechanism. Bacterial biosorption/bioaccumulation mechanisms together with other plant growth promoting features accounted for improved plant growth in metal contaminated soils (Zaidi et al. 2006). Horse gram rhizobia (HGR-13) have the ability to remove Al even at 300 µg g^{-1} Al, even though it was maximum at 100 µg g^{-1} in the inoculated plants when compared to control plants. Hence, rhizobia nodulating their hosts may increase metal accumulation in root nodules and may lead to chelation, immobilization and biosorption (Hao et al. 2014).

Conclusions

Results clearly show that the accumulation of Al in soils reduced upon inoculation of Horse gram plants with Horse gram rhizobia of the current study. The present study demonstrated that the Horse gram plants inoculated with Al tolerant *Rhizobium* strains HGR-4, 6, 13 and 25 besides having nitrogen fixing capacity also have the ability to grow in Al contaminated soils. Hence, these Horse gram plants upon inoculation of the rhizobia associated with them during the study i. e HGR 4, 6, 13 and 25 could be used in phytoremediation of metal from Al contaminated soils.

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