Acta fytotechn zootechn, 25, 2022(1): 54–59
http://www.acta.fapz.uniag.sk

Original Paper

Iron effect on symbiotic efficiency of horse gram

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Article Details: Received: 2021-07-16 | Accepted: 2022-02-25 | Available online: 2022-03-31

https://doi.org/10.15414/afz.2022.25.01.54-59

Four horse gram *Rhizobium* (HGR) strains HGR-4, 6, 13 and 25 grown under different concentrations of Iron (Fe) were inoculated individually to horse gram plants. These plant sets were screened for their net photosynthetic rate (P_N), pod formation and symbiotic efficiency. Horse gram plants inoculated with the strain HGR-4 have shown high P_N values at 100 µg g^{-1} of Fe. The number of pods formed were maximum upon inoculation with HGR-4 at 300 µg g^{-1} of Fe. Nodulation was maximum with the prior inoculation of the strains HGR-6, 13 and 25 at 500 µg g^{-1} of Fe. Leghaemoglobin content was maximum on inoculation with HGR-6, 13 and 25 at 300 µg g^{-1} of Fe. This study demonstrated that the horse gram plants associated with rhizobia, besides having nitrogen fixing capacity also have shown Fe stress tolerance and the ability to remove Fe from soils. Hence, the study confirms the ability of HGR-4, HGR-6, HGR-13 and HGR-25 isolates of *Rhizobium* to have tolerance of the metal Fe at the plant nodule during pot experiments. Therefore these isolates could be suggested for cultivation of horse gram plants in Fe contaminated soils. These findings imply that horse gram-rhizobia symbiosis is an essential element of plant adaptation to metal stress.

Keywords: Iron, rhizobia, metal tolerance, symbiotic efficiency

1 Introduction

Contamination of soils by metals is widespread 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 soil fertility loss since the composition of microbial flora and microbial activities are affected

severely (Krujatz et al., 2011). The *Rhizobium*-legume association has the advantage that the organisms may influence metal solubility, bioavailability, mobility and renders plants more tolerance to excess metal concentrations (Sanchez-Pardo et al., 2012). Among metals, iron (Fe) represent the most important micronutrient that is critical for plant growth and crop yields (Osmolovskaya et al., 2019). All plants require this micronutrient for their optimum growth. Legumes, which develop symbiotic relationships with N2 fixing bacteria have an increased demand for Fe (Tang et al., 1990). Iron plays major role in nitrogen fixation, and is a component of several key proteins such as nitrogenase, leghaemoglobin and ferredoxin. It is the key factor of many metabolic reactions involved in symbiotic nitrogen fixation (SNF). In the early stages of nodulation, heminic iron is critical for catalase-mediated free radical detoxification (Jamet et al., 2003). Upon nodule maturation, Fe is required for nitrogenase and leghaemoglobin activity (Manuel et al., 2014). The application of metal tolerant *Rhizobium* species with the plant symbiosis provides high efficiency for phytoremediation. It also has the additional advantage of providing N-compounds to the soil by biological nitrogen fixation in root nodules under metal pollution (Hao et al., 2014). This enhances soil fertility too.

Horse gram [*Macrotyloma uniflorum* (Lam.) Verdc. = *Dolichos biflorus* (Linn.)] is an important pulse crop and it is extensively cultivated on light red and gravel soils of peninsular India in 1.1 million hectares during Kharif and Rabi seasons. The significance of the crop is its adaptability to 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. The current studies target to analyze the effect of

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Fe tolerant rhizobial-plant symbiosis on photosynthetic performance, pod formation and symbiotic efficiency of horse gram plants upon inoculation with the chosen rhizobial strains.

2 Materials and methods

2.1 Isolation and analysis of rhizobial strains

Soil samples were collected from 32 different regions of Andhra Pradesh, India for the study. Locally available horse gram seeds were sown in these soil samples. Root nodules were collected from the horse gram plants were surface sterilized and used for isolation of rhizobia. Rhizobial strains were isolated on yeast extract mannitol (YEM) agar medium with 0.0025% Congo red dye (Vincent, 1970). All these isolates were confirmed as rhizobia by using biochemical parameters and 16S rRNA sequence analysis.

Horse gram plants were inoculated with the selected four horse gram rhizobial (HGR) strains HGR-4 (GQ483457), HGR-6 (GQ483458), HGR-13 (GQ483459) and HGR-25 (GQ483460). The rhizobial suspension of these four isolates, grown in YEM broth in flasks at 28 ±2 °C, 120 rpm for three days (cell density of 6×10^9 cells mL^{-1}) were inoculated to horse gram plants. The inoculation was performed on sterilized seeds coated with the rhizobial strain (by soaking the seeds in liquid culture medium for 2 h using 10% (wt/vol) gum Arabic adhesive to deliver approximately 10^9 cells $seed^{-1}$). The inoculated seeds (20 seeds pot^{-1}) were sown in clay pots using 2 kg sterilized soil (autoclaved at 121 °C for 3 h for three successive days). Controls were maintained with seeds treated in sterilized distilled water. Iron tolerance of horse gram plants was also analyzed with Fe supplements i.e. 100, 300, 500 and 700 µg g^{-1} of kg^{-1} in unsterilized soil separately. A total number of three pots were used, for each of the triplicate three pots were used.

2.2 Analysis of plants for Photosynthetic performance and symbiotic efficiency

After 40 d of treatment the Net photosynthetic rate (P_N) was determined with a Portable Photosynthetic System ADC Bioscientific, U.K. The measurements were made at ambient CO_2 concentrations between 09.00 and 11.00 h on a clear sky day. The pots were watered when required and were maintained in open field conditions and allowed to grow. The plants were observed for nodulation regularly after the seedlings came out by plucking and checking. Five plants from three replicate/triplicates in each treatment were picked up randomly and nodulation characteristics viz., number, size, shape, color, and distribution of the nodules were taken 40 days after sowing, as previously observed that highest nodulation of horse gram occurred at 40 days (Edulamudi et al., 2021) The number of pods formed was also counted after 40 days of sowing. For biochemical analysis, nodules were collected from the plants raised in different concentrations of Fe. Nodule samples were frozen before leghaemoglobin extraction. Leghaemoglobin content was estimated (Tu et al., 1970) in triplicates. The nodule samples (500 mg to 1 g) were homogenized in 5 mL of 0.1 N potassium hydroxide (KOH) and centrifuged for 10 min at 12,000 rpm and 1.5 mL of supernatant was taken, to this 1mL of water, 0.5 mL of 5 N KOH, and 0.1 g of sodium dithionite was added for reduction. Optical density (OD) of leghaemoglobin was determined at 537, 557, and 577 nm wavelengths after mixing by using a spectrophotometer (ELICO, SL171, MINISPEC). The leghaemoglobin content was calculated using the formula mg of leghaemoglobin = OD_{557} - 1/2 (OD_{537} + OD_{577}). Nitrogen content of the samples as dry weight was estimated using micro- Kjeldahl 'N' method (AOAC, 1978). Oven dried samples (0.1 g) were digested with 3 mL of concentrated sulphuric acid (H_2SO_4) in the presence of potassium and copper sulphate mixture till the solution became colorless or clear. The digest was then neutralized with 40% sodium hydroxide, distilled and the liberated ammonia was collected in 4% boric acid containing indicator. The distillate was titrated against the standard acid. It was taken that N/50 H_2SO_4 is equal to 0.00028 g of total nitrogen.

$$\% \text{ N sample} = \frac{\text{¼ sample titre - blank titre} \times \text{N of } H_2SO_4 \times \text{molecular weight of } N_2 \times 100}{\text{sample weight (g)}}$$

Soil pH, organic matter, total nitrogen (Jackson 1973), and total phosphorus (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 washed in tap water. They were again washed with 0.1 N hydrogen chloride (HCl). Finally the nodules were washed twice with distilled water. The nodules were dried at 80 °C for 48 h in hot air oven and they were ground to a fine powder.

From this, 0. 5 g of powdered tissue was added to 5 mL of concentrated nitric acid (HNO_3) for cold digestion at room temperature. Then 5 mL of concentrated HNO_3 and hydrogen peroxide was 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, Billerica, MA) and used for elemental analysis. Soil samples were also subjected to acid digestion with slight modifications and were

used in elemental analysis. Iron concentration present in the sample was determined 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 lg L^{-1} gas flow, 0.2 nm band width, and 72 s of furnace programme total time.

2.3 Statistical analysis

Statistical analysis was done in triplicates for each treatment. The mean and standard error (SE) were calculated using Microsoft Office Excel 2007 (Microsoft, Redmond, WA, USA). To know the statistical significance all the values were analyzed by analysis of variance, using IBM SPSS Statistics, Version 20.

3 Results and discussion

All the 32 isolates were subjected to cultural, biochemical, and plant growth promoting rhizobial (PGPR) activities. After that we have subjected all the data to cluster analysis. Basing on these clusters we have selected four isolates HGR-4, HGR-6, HGR-13, and HGR-25 from each cluster having most representative characters were used for further studies like 16S rRNA and all the strains were used for metal (Fe) tolerance studies. All the 32 isolates were able to grow at all the concentrations tested. The amount of N (%) and phosphorus (%) present in the soil is 0.85 and 1.24, respectively. The total organic matter content in soil (1.20), sand (18), silt (16), and clay were 1.20, 18, 16, and 42, respectively with 6.44 as the pH of soil.

Horse gram plants showed significant changes in their net photosynthetic rate (P_N) upon inoculation with *Rhizobium* strains grown under different concentrations of Fe (Figure 1). The plants inoculated with the strain HGR-4 showed their maximum P_N performance (11.49 µ mol CO_2 m^{-2} s^{-1}) at 100 µg g^{-1} of Fe (mean 4.882; SD 4.443 and SE 3.848) and HGR-13 inoculated plants showed (mean 4.98, SD 0.676 and SE 0.585) at 300 µg g^{-1} (5.75 µ mol CO_2 m^{-2} s^{-1}). Two strains HGR-6 (mean 4.99, SD 0.947 and SE 0.820) and 25 showed (mean 5.277, SD 1.440 and SE 1.247) enhanced P_N values (6.41 µ mol CO_2 m^{-2} s^{-1} and 7.28 µ mol CO_2 m^{-2} s^{-1} with increase in metal concentration up to 700 µg g^{-1}. In control plants (mean 3.925, SD 2.077 and SE 1.799) even though P_N increased with increase in metal concentration, the P_N of horse gram plants was low when compared to the inoculated plants. They were statistically significant at 5% level of significance (*P* value 0.03). The critical Fe concentrations were about 65 µg g^{-1} for *Lupinus angustifolitus* and *Lupinus luteus* and 52 µg g^{-1} for *Lupinus pilosus* (Tang et al., 1990). The optimum concentration of Fe for growing peas in nutrient solution culture depends on experimental condition. At 2.0 mg L^{-1} Fe is optimum, below this it is deficient and above 3.0 mg L^{-1} is toxic in peas (Nenova 2009). Two applications of 0.8% Fe increased photosynthetic rate by 42% in alfalfa (Chun-xia et al., 2017). But, deficiency of this micronutrient leads to inhibition of photosynthesis in *Phaseolus vulgaris* (Urwat et al., 2021). The number of pods formed were maximum at 300 µg g^{-1} of Fe, when they were inoculated with the strain HGR-4 (mean 39.5, SD 1.914 and SE 1.658). But, the plants inoculated with the strains HGR-6 (mean 35.5, SD 2.986 and SE 2.586), 13 (mean 35.5, SD 2.516 and SE 2.179) and 25 (mean 42.5, SD 3 and SE

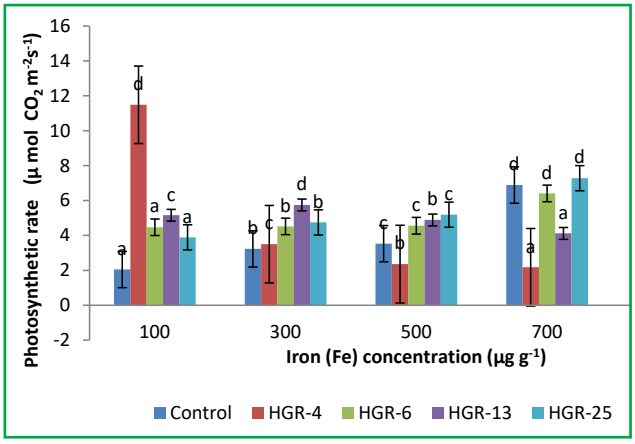

Figure 1 Fe effect on photosynthetic performance of horse gram plants inoculated with four *Rhizobium* strains. Bars indicate mean ±SE. Bars with similar letters indicate that they are statistically significant

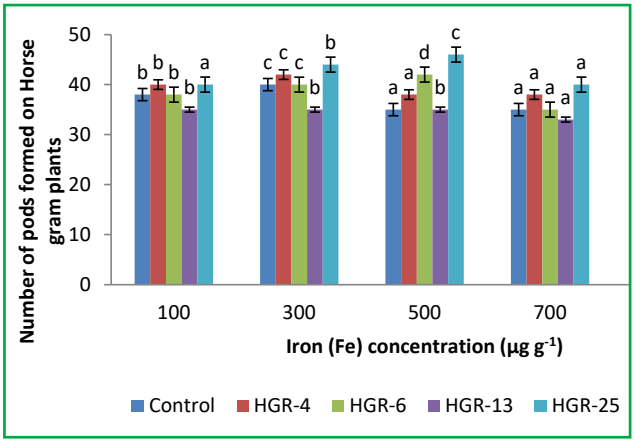

Figure 2 Number of pods formed in response to iron in horse gram plants inoculated with four *Rhizobium* strains. Bars indicate mean ±SE. Bars with similar letters indicate that they are statistically significant

2.598) showed at 500 µg g^{-1}, later they were decreased (Figure 2).

Legumes required high amount of Fe during nitrogen fixing symbiosis (Tang et al., 1990). Application of 20 kg Fe along with *Rhizobium* inoculation increased the seed yield in cowpea (Mundra and Bhati 1991). The number of fruits per plant, number of seeds per fruit and their yield were high at 25 ppm concentration of Fe but it was decreased at 100 ppm concentration (Malik and Kumar, 2013).The number of pods formed were maximum when the plants were treated at 20 kg ha^{-1} and 25.5 to 24.8 kg ha^{-1} Fe.

The yield of chickpea significantly increased at different concentrations of Fe (Khan et al., 2014). In chickpea the grain yield of 465 kg ha^{-1} was obtained with Fe applied at 2 kg ha^{-1}, while minimum grain yield of 307 kg ha^{-1} in control. More pods were found in two genotypes of chickpea when Fe applied at 2 kg ha^{-1} (Khan et al., 2014). Fe application at 0–20 kg ha^{-1} increased yield in cowpea (Singh and Varun 1989) and maximum yield in urd bean was observed at 2–20 kg hg^{-1} over control (Salam et al., 2004).The application of Fe up to 10 kg ha^{-1} increased pods in chickpea over control (Kumar et al., 2009).

These four horse gram *Rhizobium* strains showed their ability to form effective nodules at all the metal (Fe) concentrations tested. In the present study, nodules appeared after 13 days of sowing and were observed both on tap root and as well as on lateral roots. The total number of nodules formed per plant ranged from 11 to 16 (Figure 3).

The strain HGR-4 inoculated plants showed maximum nodulation (mean 13.20, SD 4.112 and SE 3.561) at 100 µg g^{-1} of Fe concentration, whereas the strains HGR-6 (mean 13, SD 4.690 and SE 4.062), 13 (mean 6.771, SD 1 and SE 0.868) and 25 (mean 13.25, SD 0.957 and SE 0.829) showed at 500 µg g^{-1}. Two strains HGR-13 and HGR-25 inoculated plants showed more nodules up to 500 µg g^{-1} than at control (mean 12.75, SD 3.201 and SE 2.77). These are statistically significant (*P* value 0.008). Legumes involved in a nitrogen fixing symbiosis has been shown to be greater requirement for Fe. Iron requirement for nodule formation was greater than that for the growth of host plants (Tang et al., 1990). The soil application of Fe at 25 kg ha^{-1} increased nodulation in soybean (Bhanavasa et al., 1994). Iron enhanced nodulation up to 25 mM, after that it showed negative effect (Paudyal et al., 2007). Fe (25 ppm) increased the number of nodules in *Vigna radiata* over the control. Further increase in Fe level (50 ppm) enhanced the number of nodules when compared to control, but they were lesser than at 25 ppm Fe. The number of nodules decreased with increase in Fe concentration at 100 ppm concentration (Malik and Kumar, 2013). In chickpea, the number of nodules formed were maximum (54) at 25 kg ha^{-1} Fe concentration, and were minimum (25) at 20 kg ha^{-1} (Khan et al., 2014). At 2 kg ha^{-1}, Fe supported maximum nodulation and the number of nodules ranged from 8.5 to 9.9 $plant^{-1}$ (Togay et al., 2015). Iron enhanced nodule number and it was maximum at about 5 µM. At 5 or 20 µM Fe concentration, nodules were observed after 15 days of sowing. But, nodulation was not observed up to 17 days and even later when the plants were treated with 1 µM (Tang et al., 1990). The number of nodules $plant^{-1}$ were significantly affected in chickpea when received Fe at 2 kg ha^{-1} (Khan et al., 2014).

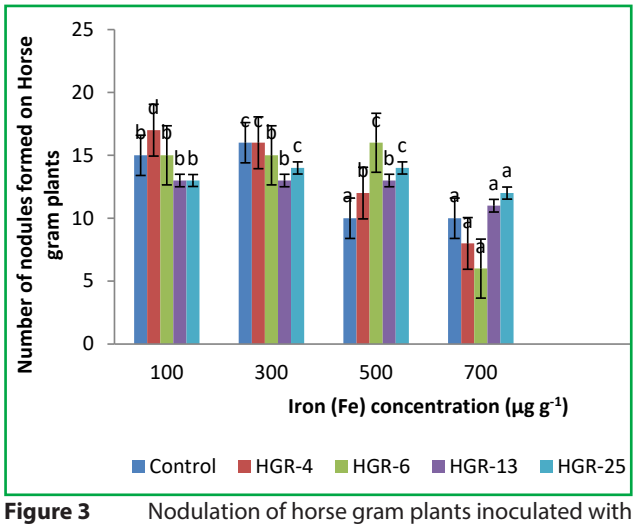

Figure 3 Nodulation of horse gram plants inoculated with *Rhizobium* strains in response to iron. Bars indicate mean ±SE. Bars with similar letters indicate that they are statistically significant

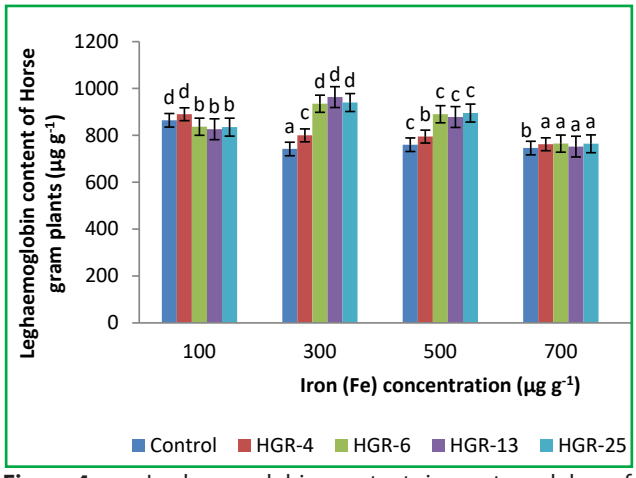

Figure 4 Leghaemoglobin content in root nodules of horse gram plants inoculated with *Rhizobium* strains in response to iron (Fe). Bars indicate mean ±SE. Bars with similar letters indicate that they are statistically significant

The amount of leghaemoglobin was maximum i. e. 935 (mean 856.75, SD 73.113 and SE 63.318), 963 (mean 854.75, SD 88.774 and SE 76.881) and 940 µg g^{-1} (mean 858.5, SD 76.282 and SE 66.062) when the plants were inoculated with the strains HGR-6, 13 and 25 at 300 µg g^{-1}. But, the strain HGR-4 (mean 811.75, SD 54.823 and SE 47.478) inoculated plants showed at 100 µg g^{-1} along with control (mean 778, SD 57.850 and SE 50.099) plants and were significant at 5% level of significance (*P* value 0.03) (Figure 4). Iron is an important nutrient for nitrogen fixation in legume root nodules. The amount of leghaemoglobin was high at low concentrations of Fe, i.e. at 25 ppm and increased at 50 ppm. At 100 ppm also leghaemoglobin content was increased when compared to control but it was less than in 50 ppm concentration (Malik and Kumar, 2013). *Lupines* require a greater supply of Fe when relying on SNF for the supply of nitrogen when compared to plants growth with nitrogen fertilizer (Tang et al., 1990).

The rate of nitrogen fixation in nodules of *Phaseolus vulgaris* L. is positively correlated with increase in nodule Fe concentrations (Slanti et al., 2008). At the time of nodule maturity, highest Fe concentration was observed in soybean nodules. Nearly 44% of the Fe is present in soybean nodule, when compared to leaves (31%) seed (7%) and in roots (5%). At the time of maturity, the seeds have shown highest Fe concentration (35%) followed by nodules (27%), leaves (23%), roots (9%) and stem (3%) (Burton et al., 1998). It reveals that Fe at lower concentrations promotes growth and yield but at higher concentrations they have shown inhibitory effect. Iron present in the soil was decreased after deplantation of horse gram plants, i.e. from 0.48 (control plants without inoculation) to 0.32 mg L^{-1} (inoculated) with HGR-4 (SD 0.113 and SE 0.08), 0.56 to 0.42 mg L^{-1} with HGR-6 (SD 0.098 and SE 0.07), 0.62 to 0.49 with HGR-13 (SD 0.091 and SE 0.065) and 0.74 to 0.44 mg L^{-1} with HGR-25 (SD 0.212 and SE 0.15) at a concentration of 500 µg g^{-1} Fe (Figure 5). It clearly shows that the accumulation of Fe in soils was decreased when the horse gram plants were inoculated with the horse gram rhizobia.

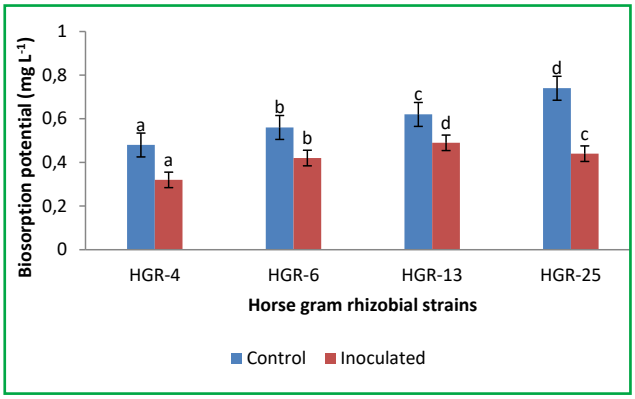

Figure 5 Biosorption potential of horse gram plants inoculated with *Rhizobium* strains in response to iron (Fe). Bars indicate mean ±SE. Bars with similar letters indicate that they are statistically significant

4 Conclusions

Iron is one among the micronutrients that could affect the survival of plant on a higher dosage. The current study demonstrates an attempt to identify Fe tolerant horse gram associated rhizobia named as HGR-4, HGR-6, HGR-13 and HGR-25. These four isolates were able to tolerate 700 µg g^{-1} of Fe amended in soil with enhanced total nitrogen and leghaemoglobin content compared to the respective controls. AAS analysis of the Fe content in the nodules and soil also revealed that at 500 µg g^{-1} concentration Fe was accumulated which reveals that these isolates can accumulate Fe thereby helping the phytoremediation of Fe contaminated soils.

This study demonstrated that the horse gram plants associated with rhizobia, besides having nitrogen fixing capacity also have shown Fe stress tolerance and the ability to remove Fe from soils. Hence, the study confirms the ability of HGR-4, HGR-6, HGR-13 and HGR-25 isolates of *Rhizobium* to have tolerance of the heavy metal Fe at the plant nodule during pot experiments. Therefore these isolates could be suggested for cultivation of orse gram plants in Fe contaminated soils. These findings imply that horse gram-rhizobia symbiosis is an essential element of plant adaptation to metal stress.

Acknowledgements

EP is grateful to University Grants Commission (UGC), New Delhi for financial assistance in the form of Post-Doctoral Fellowship (PDF).

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