

Lithium stress tolerance of horse gram [*Macrotyloma uniflorum* (Lam.) Verdc.] plants in association with rhizobia

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ABSTRACT

The aim of the present study was to evaluate the ability of lithium (Li) tolerance in rhizobia and enhancing the symbiotic efficiency and biosorption potential in the rhizobia inoculated horse gram [*Macrotyloma uniflorum* (Lam.) Verdc.] plants. Among the thirty-two rhizobial isolates, four isolates have shown tolerance towards Li in their preliminary screening. Among these thirty-two rhizobia, four strains HGR-4, HGR-6, HGR-13, and HGR-25 were selected for further studies based on their Li tolerance levels. These Li tolerant strains grown under different concentrations of Li were inoculated individually to horse gram plants. Triplicates were maintained for each treatment. Among them, the maximum number of pods was formed upon inoculation with the strain HGR-4 and HGR-6 at 30 $\mu\text{g g}^{-1}$ of Li but followed by HGR-13 and HGR-25 at 10 $\mu\text{g g}^{-1}$ of Li. Horse gram plants inoculated with the strain HGR-6 showed the maximum nodulation at 50 $\mu\text{g g}^{-1}$ of Li. The amount of leghaemoglobin content was maximum at 30 $\mu\text{g g}^{-1}$ of Li only, later it was decreased with an increase in Li concentration. The isolate HGR-6 (GQ483458 *Rhizobium* sp., ATCC 2336) showed the maximum biosorption of Li in root nodules and as well as in soil samples. This study demonstrated that the horse gram plants inoculated with Li tolerant *Rhizobium* strains HGR-4, HGR-6, HGR-13, and HGR-25 enhanced pod formation, symbiotic efficiency, and biosorption potential, besides having the nitrogen-fixing ability, also have the ability to grow in Li contaminated soils.

Keywords: Lithium, *Rhizobium*, stress tolerance, bioremediation

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INTRODUCTION

Soil contamination by heavy metals is a widespread occurrence due to human, agricultural and industrial activities (Beladi *et al.*, 2011). This results in the accumulation of heavy metal traces in agricultural soils. It leads to a threat to food safety and overall public health (Dary *et al.*, 2010). Metals adversely influence microorganisms (Shi *et al.*, 2002) by affecting their growth, morphology, and activities (Khan *et al.*, 2007) including symbiotic N_2 fixation (McGrath and Lane, 1989). These metals exert selective pressure on the organisms, resulting in microbial populations with higher tolerance to

metals (Baath *et al.*, 1998). Application of heavy metal tolerant *Rhizobium* species with the plant 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 also.

Lithium (Li) is the 25th most abundant element and it exists naturally in the earth's crust to the extent of about 0.006%, its concentrations in soil are from 20 to 30 mg/kg (Aral and Vecchio-Sadus, 2008). It is considered as a non-essential element, but it has a benefit for organisms (Robinson *et al.*, 2018). Silicates, micas, and phosphates are the main Li

minerals, it can be found also in brine lakes, which contain about 17 million tons of Li in total, besides in water seas about 2.5×10^{14} kg (Cicek *et al.*, 2018). There are several industrial applications where Li might be used such as ceramics and special glass industries, rocket propellants, production of aluminum (Al), pharmaceutical and nuclear industries, the manufacture of greases and lubricants, synthesis of vitamin A, batteries, synthesis of organic compounds and underwater buoyancy devices (Cicek *et al.*, 2018). The wide use of Li in various fields of industry and waste management (Hull *et al.*, 2014) leads to environmental pollution. The Li at a concentration of 50–100 $\mu\text{g dm}^{-3}$ in water used for field irrigation has shown an effect on the chemical composition of plants and the uptake of elements essential for plant growth and development (Kabata-Pendias and Mukherjee, 2007).

The Li is taken up by all plant species, even though it is not essential for growth and development. But stimulation of plant growth was observed (Aral and Vecchio-Sadus, 2008) at low concentrations and is toxic to all plants at high concentrations. The Li is a mobile element that translocates easily from roots to the above-ground parts (Jurkowska and Rogo, 1991). The contamination of soil by Li is becoming a serious problem, which might be a threat to crop production in the near future. The Li enters the environment when the products containing Li disposed as waste without treatment. The common pathways that Li enters the food chain are drinking water and plants (Franzaring *et al.*, 2016).

Horse gram [*Macrotyloma uniflorum* (Lam.) Verdc. or *Dolichos biflorus* (Linn.)] is an important pulse crop that is extensively cultivated on light red and gravel soils of peninsular India. The significance of this crop is its adaptability to poor and adverse climatic conditions, which are unsuitable for other pulse crops. It is widely cultivated as a grain legume and fodder crop in the states of Tamil Nadu, Karnataka, Andhra Pradesh, and Orissa of South India. To the best of our knowledge, there are no previous reports on the Li stress effect on horse gram plants and the rhizobia associated with it. Hence, the current studies target to analyze the effect of Li tolerant rhizobia on plant growth,

pod formation, nodulation, nitrogen fixation, and 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 thirty-two different regions based on their geographical locations in the United Andhra Pradesh State of India for the study. Seeds were sown in these soil samples. Root nodules collected from the horse gram plants were surface sterilized and used for isolation of rhizobia. Soil pH, organic matter, total nitrogen (Jackson, 1973), and total phosphorus (Olsen *et al.*, 1954) were also estimated. The amounts of sand, silt, and clay present in the soil were also analyzed (Black, 1965).

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. The agar dilution method on YEM was used to determine the metal (Li) tolerance among the horse gram rhizobia. Minimum inhibitory concentration (MIC) of horse gram rhizobia was performed by using various concentrations of lithium sulphate (Li_2SO_4), i.e., 10–1,000 mg g^{-1} . Then, 10 μL of the bacterial suspensions (2×10^8 c.f.u./mL) was inoculated on the surface of each plate and incubated at room temperature for 72 hours. The isolates were considered resistant when visible growth occurred. YEM agar medium was supplemented with various Li concentrations (10, 30, 50, and 100 $\mu\text{g g}^{-1}$) and was sterilized in an autoclave and poured into Petri plates.

Inoculation with Rhizobial Strains Grown under Different Concentrations of Lithium Sulphate (Li_2SO_4)

Seeds of horse gram obtained from local fields of Andhra Pradesh in India were used in the experimentation. The pots in the study were filled with soil sterilized in an autoclave at 121°C for 3 hours each on three successive days. Horse gram

seeds were surface sterilized with 70% ethanol for 3 minutes followed by sodium hypochlorite for 3 minutes and were then rinsed six times with sterilized water and dried. The rhizobial suspensions of isolates used in the study were grown in YEM broth in flasks shaken at 120 rpm at $28 \pm 20^\circ\text{C}$ for 3 days to a cell density of 6×10^9 cells mL^{-1} .

Horse gram plants were inoculated with the selected four Li tolerant *Rhizobium* strains which were selected based on their tolerance levels, i.e., HGR-4 (GQ483457), HGR-6 (GQ483458), HGR-13 (GQ483459) and HGR-25 (GQ483460). To perform the inoculations, sterilized seeds were coated with the rhizobial strains by soaking the seeds in a liquid culture medium for 2 hours using 10% (wt/vol) gum arabic as adhesive to deliver approximately 10^9 cells seed^{-1} . The 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. To evaluate the effect of Li on horse gram plants, separate sets of plants were maintained with Li supplements (10, 30, 50, 100, and $200 \mu\text{g g}^{-1}$) of kg^{-1} in unsterilized soil. Triplicates were maintained for each treatment.

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. Five plants in each treatment were picked up randomly and nodulation characteristics viz., number, size, shape, colour, and distribution of the nodules were taken 40 days after sowing, as it was previously observed the highest nodulation of horse gram occurred at 40 days.

For biochemical analysis, nodules were collected from the plants raised in different concentrations of Li. Nodule samples were frozen before leghaemoglobin extraction. Leghaemoglobin content was estimated (Tu *et al.*, 1970) in triplicates. The 500 mg to 1 g of nodules were homogenized in 5 mL of 0.1 N KOH and centrifuged for 10 minutes at 12,000 rpm and 1.5 mL of supernatant were taken to

this. Then, 1 mL of water, 0.5 mL of 5 N KOH, and 0.1 g of $\text{Na}_2\text{S}_2\text{O}_4$ were added for reduction. The optical density of leghaemoglobin was determined at 537, 557, and 577 nm wavelengths after mixing by using a spectrophotometer (ELICO, SL171, MINISPEC, Hyderabad, India). The leghaemoglobin content was calculated using the formula: μg of leghaemoglobin = $\text{OD}_{557} - \frac{1}{2}(\text{OD}_{537} + \text{OD}_{577})$. Li concentration present in control as well as in inoculated soil samples at $100 \mu\text{g g}^{-1}$ was determined by atomic absorption spectroscopy (AAS, US) before and after horse gram plantation.

Statistical Analysis

Statistical analysis was done in three replicates for each treatment. To know the statistical significance, all the values were analyzed by the one-way analysis of variance using IBM SPSS Statistics, Version 20.

RESULTS AND DISCUSSION

Horse gram plants showed significant change in their growth under different concentrations of Li. They were able to show growth up to $200 \mu\text{g g}^{-1}$ concentration. At above $100 \mu\text{g g}^{-1}$ concentration of Li, these plants had stunted growth with pale leaves and were unable to survive after 20–25 days. Horse gram plants showed nodulation, pod formation, and leghaemoglobin at a concentration up to $100 \mu\text{g g}^{-1}$ of Li.

Horse gram plants inoculated with the strain HGR-4 (42 pods) and HGR-6 (50 pods) showed the maximum number of pods at $30 \mu\text{g g}^{-1}$ of Li. Whereas, HGR-13 (47 pods) and HGR-25 (48 pods) inoculated plants performed the highest number of pods at $10 \mu\text{g g}^{-1}$ of Li (Table 1). Control plants (horse gram plants without rhizobial inoculation) showed the maximum pod formation (40 pods) at $30 \mu\text{g g}^{-1}$ of Li, but the number was low when compared to rhizobia inoculated to horse gram plants.

Table 1 Number of pods formed in response to lithium (Li) in horse gram plants inoculated with four *Rhizobium* strains

Metal concentration (ppm)	Control	HGR-4	HGR-6	HGR-13	HGR-25
10	38	40	45	47	48
30	40	42	50	44	45
50	36	30	44	15	40
100	28	26	30	10	34

Note: F-ratio value = 6.5156, P-value = 0.004

In the present study, nodules appeared after 13 days and were observed both on tap roots and as well as on lateral roots. The total number of nodules formed per plant ranged from 6 to 23 (Table 2). The number of nodules formed in control plants was low when compared to rhizobial strains inoculated to horse gram plants. Nodulation was maximum with the strain HGR-6 at 50 $\mu\text{g g}^{-1}$ of Li. While the strains HGR-13 and HGR-25 inoculated plants had the highest number of nodules at 10 $\mu\text{g g}^{-1}$ of Li. But the plants inoculated with the strain

HGR-4 showed an increase in nodule number with increasing Li concentration up to 100 $\mu\text{g g}^{-1}$.

The amount of leghaemoglobin was maximum at 30 $\mu\text{g g}^{-1}$ of Li with the prior inoculation of the strains HGR-6 (930 μg) and HGR-13 (935 μg). But the strains HGR-4 (940 μg) and HGR-25 (880 μg) inoculated plants showed the highest leghaemoglobin content at 10 $\mu\text{g g}^{-1}$ of Li (Table 3). The leghaemoglobin content decreased in control plants with increasing Li concentrations and it was maximum (860 μg) at 10 $\mu\text{g g}^{-1}$ of Li.

Table 2 Number of nodules formed of horse gram plants inoculated with *Rhizobium* strains in response to lithium (Li)

Metal concentration (ppm)	Control	HGR-4	HGR-6	HGR-13	HGR-25
10	10	8	11	12	13
30	12	8	13	12	12
50	8	8	23	8	10
100	6	14	6	6	8

Note: F-ratio value = 0.8475, P-value = 0.4880

Table 3 Leghaemoglobin content (μg) of in root nodules of horse gram plants under lithium (Li) stress

Metal concentration (ppm)	Control	HGR-4	HGR-6	HGR-13	HGR-25
10	860	940	855	860	880
30	744	920	930	935	865
50	546	875	860	910	850
100	482	850	820	865	800

Note: F-ratio value = 1.9452, P-value = 0.1630

The amounts of total nitrogen and phosphorus in the soil were 0.85% and 1.24%, respectively. The organic matter in the soil was 1.20%. The soil contained 18% sand, 16% silt, and 42% clay with a pH of 6.44. In the present study, biosorption potential of the strain HGR-6 was determined by AAS, as the amount of metal present in the medium after the treatment with the isolate. After analyzing the treated samples in AAS, the isolate HGR-6 showed the maximum biosorption of Li. The results indicated that the isolate was able to absorb Li at a concentration of 100 $\mu\text{g g}^{-1}$ in root nodules (0.46 to < 0.32 mg/L) and also in soil samples (0.63 to < 0.48 mg/L) inoculated with the strain HGR-6.

Citrus plants are relatively sensitive to Li (Aral and Vecchio-Sadus, 2008). In maize, the growth stimulating effect of Li was observed at a concentration of 5 mg Li dm^{-3} . Li influence on plants is dose-dependent and its ions can exert toxicity at high concentrations (50 mg Li dm^{-3}) or stimulate growth at low concentrations (5 mg Li dm^{-3}) (Hawrylak-Nowak *et al.*, 2012). Li is taken up by all plant species, even though it is not to be essential for proper growth and development. But it has shown stimulation on plant growth (Aral and Vecchio-Sadus, 2008) at ppb range (Schweigart, 1962). But the presence of Li at high concentrations in the soil is toxic to all plants and causes chlorosis-like symptoms. In our study, horse gram plants showed better growth, pod formation, and nodulation up to 100 $\mu\text{g g}^{-1}$ of Li. After that, the growth of the plants rapidly declined. Nightshade plant species are tolerant to Li up to 1,000 $\mu\text{g g}^{-1}$. At low concentrations, Li may enhance faster maturation and increase resistance to plant diseases (Anderson *et al.*, 1988). They also reported that plants grown under low concentrations of Li may display improved productivity. Li concentrations ranging from 1 to 32 Li dm^{-3} showed stimulation on total maize yield. But it showed a negative effect on maize yield at 64, 128, and 256 mg Li dm^{-3} concentration (Antonkiewicz, 2017). Li concentration in a solution of 50 mg dm^{-3} was unfavorable and reduced maize yield. According to Jurkowska *et al.* (1998), 25 mg Li kg^{-1} dm and 40 Li kg^{-1} dm soil decreased the yield of oats and maize. It indicates

that maize is more resistant to high concentrations of Li (Hawrylak-Nowak *et al.*, 2012).

Heavy metals adversely affected nodulation and N_2 fixation of legumes (McGrath and Lane, 1989). Metal toxicity led to a reduction in nodule number and size but nitrogenase activity was not affected (Carrasco *et al.*, 2005). The toxicity of heavy metals to the legume-rhizobia symbiosis and symbiotic nitrogen fixation varied with the species of legumes, rhizobia, types of soil, metal, the degree of pollution, as well as the nodulation and plant growth promotion activities of rhizobia under metal stress (Oves *et al.*, 2010). Inoculation of white clover plants grown in metal-contaminated soil with an effective strain of *R. leguminosarum* bv. *trifolii* promoted N_2 fixation (Giller *et al.*, 1989). A profound toxic effect of metal on the N_2 -fixing ability of culture inoculated white clover was observed (Broos *et al.*, 2005). Heavy metals are inhibitory to rhizosphere microorganisms and processes like the N_2 -fixing ability of rhizobia are lost when they are in symbiotic association with the legume host growing in metal-enriched locations (Hernandez *et al.*, 2003).

The rhizosphere microorganisms, with intrinsic ability to reduce/detoxify the heavy metal stress by several mechanisms. These include the efflux of metal ions outside the cell, biostimulation, bioaugmentation, metal reduction, and biosorption (Outten *et al.*, 2000). Bacterial biosorption/bioaccumulation mechanism together with plant growth promoting features accounted for improved plant growth in metal-contaminated soils (Zaidi *et al.*, 2006). The isolate HGR-6 showed the maximum biosorption potential of Li at a concentration of 100 $\mu\text{g g}^{-1}$. Rhizobia 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 (Hao *et al.*, 2014). Symbiotic relationship applied in metal-contaminated soil improves soil fertility and extracts or stabilizes metals simultaneously (Carrasco *et al.*, 2005; Dary *et al.*, 2010). The application of rhizobacteria along with seeds increased growth and yield of *Cicer arietinum*, *Vigna radiata*, and *Pisum sativum* and

decreased metal toxicity (Gupta *et al.*, 2004). This study demonstrated that the Li tolerant *Rhizobium* strains HGR-4, HGR-6, HGR-13, and HGR-25 besides having nitrogen-fixing capacity, also have the ability to remove Li from soils. Hence, horse gram plants and the rhizobia associated with them could be used in phytoremediation of metal (Li) contaminated soils.

plants inoculated with Li tolerant *Rhizobium* strains HGR-4, HGR-6, HGR-13, and HGR-25 besides having nitrogen-fixing capacity also have the ability to grow in Li contaminated soils. Hence, these horse gram plants upon inoculation of the rhizobia associated with them during the study i.e., HGR 4, HGR-6, HGR-13, and HGR-25 could be used in the removal of metal (Li) from contaminated soils.

CONCLUSIONS

Results clearly showed that the accumulation of Li in soils reduced upon inoculation of horse gram plants with horse gram rhizobia in the current study. The present study demonstrated that the horse gram

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