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ORIGINAL PAPER

Effect of Al and heavy metals on enzymes of nitrogen metabolism of fast and slow growing rhizobia under explanta conditions

N. K. Arora · Ekta Khare · S. Singh · D. K. Maheshwari

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Abstract Most of the legume crops are affected by metal stress present in the soil mainly due to contaminated agrochemicals and sewage sludge. The effect of aluminium, and heavy metals copper, iron and molybdenum on growth and activity of enzymes of fast and slow growing rhizobial sps. was studied. *Sinorhizobium meliloti* RMP_5 was found to be more tolerant to metal stress than *Bradyrhizobium* BMP_1. Both the strains were extremely sensitive to Al than other metals. Al was much more deleterious for the enzymatic activities (nitrate reduction, nitrite reduction, nitrogenase and uptake hydrogenase) of strain RMP_5 and BMP_1. Cu showed inhibitory effect on growth and enzyme activities of *Bradyrhizobium* strain at all concentrations. However, in *S. meliloti* RMP_5 all the tested enzymatic activities increased up to the concentration of 0.1 mM Cu. Fe enhanced the growth and enzyme activities of *S. meliloti* RMP_5 and *Bradyrhizobium* BMP_1 up to 100 mM concentration. Mo enhanced 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 got enhanced only up to 0.5 mM Mo. Both Fe and Mo are the key components of the enzyme nitrogenase and nitrate reductase and enhanced the growth and enzyme activities of both the sps. The study of physiology of nitrogen fixing ability of both fast and slow growing rhizobial strains reported that the supplementation of Mo and Fe in soils along with the biological formulations will enhance the process of symbiotic nitrogen fixation.

Keywords Hydrogenase · Nitrate reduction · Nitrite reduction · Nitrogenase · Nitrogen fixation

N. K. Arora (✉) · E. Khare · S. Singh
Department of Microbiology, Institute of Biosciences and Biotechnology, C.S.J.M. University, Kanpur 208024, India
e-mail: nkarora_net@rediffmail.com

D. K. Maheshwari
Department of Botany & Microbiology, Gurukul Kangri University, Hardwar 249404, India

Introduction

In agricultural lands although biological nitrogen fixation is the sole alternative for chemicals, several environmental conditions are limiting factors for growth and activity of both the nitrogen fixing plants and root nodulating bacteria (Chen et al. 2003; Antoun and Prévost 2005). In rhizobial cells there are two pathways for the assimilation of nitrogen to ammonia. One pathway involves enzyme nitrogenase, which directly converts atmospheric N_2 to NH_4^+ with reduction of protons and liberation of H_2 (Gupta and Maheshwari 1991). A H_2-recycling mechanism utilizing uptake hydrogenase should increase the overall efficiency of N_2-fixing process under conditions in which photosynthate is limiting (Schubert et al. 1977). The second pathway involves reduction of nitrate by the enzyme nitrate reductase (NR). Subsequently nitrite is converted to NH_4^+ by the enzyme nitrite reductase (NiR) (Hervas et al. 1991). If nitrates are present in plentiful amount, legumes and their symbionts prefer the second pathway for the synthesis of amino acids (Sawhney et al. 1991).

Optimum level of physiological function can be achieved in suitable environment and adequate nutrient supply (Wang et al. 2009). Some elements, such as heavy metals, though essential for organisms, are harmful if present in excess. Most of the cultivated legumes are exposed to agrochemicals, which not only contain essential nutrients but also

comprise of contaminants such as Aluminium (Al) and heavy metals causing a threat to symbiotic nitrogen fixation (Mårtensson 1992; Lebeau et al. 2008). These metals are not quickly degraded and therefore, there concentration is slowly building up especially in soils treated with agrochemicals and sewage sludge and this may results in decline of rhizobial population or its efficiency of nitrogen fixation (Giller et al. 1989). Aluminium is an abundant element in the soil occurring in a wide variety of mineral forms (Flis et al. 1992). Sewage sludges and compost are reported to be heavily contaminated with copper, commonly applied in fields as nitrogen fertilizers (Chaudri et al. 2000). There are some elements (iron, molybdenum, cobalt, copper etc.) essential for growth and functioning of various important enzymes (Roy and Chakrabarty 2000). Exposure of the organisms to Al and heavy metals may result into either stimulation or no change or inhibition of the enzyme activity, which ultimately depends upon the extent of exposure, dose and type of the metals used (Giller et al. 1998).

There are several reports of high tolerance ability of *Sinorhizobium meliloti* and *Bradyrhizobium* strains to various heavy metals (Kinkle et al. 1994; Wani et al. 2007). Although there are various reports on the detrimental effects of Al and different heavy metals on a range of aspects including growth and nodulation capacity, their impact on enzymes involved in nitrogen fixation has not been given much attention (Chaudri et al. 2000). Because of the relationship between the soil productivity and symbiotic nitrogen fixation, accurate test methods for investigating effects of various metals on the legume symbiosis are needed. In the present study the effect of four metals, Al, molybdenum (Mo), iron (Fe) and copper (Cu) on rhizobial growth and enzymes activities involved in nitrogen assimilation was determined.

Materials and methods

Microorganisms

The fast growing *Sinorhizobium meliloti* RMP_5 and slow growing *Bradyrhizobium* sp. BMP_1 were selected from corresponding author's collection (Arora et al. 2000). The strains were grown in yeast extract mannitol broth (YEMB, HiMedia Mumbai) at 30°C and 150 rpm and maintained at 4°C in yeast extract mannitol agar (YEMA, HiMedia Mumbai).

Effect of heavy metals on growth kinetics

A loopful of log phase culture of each strain was inoculated separately in YEMB amended with aluminium chloride (0–100 μM), ferric chloride (0–300 mM), sodium molybdate (0–300 mM) and cupric chloride (0–100 mM) (Paudyal et al. 2007). To screen the tolerance of heavy metals by rhizobial strains the growth was measured up to stationary phase at 610 nm by Shimadzu UV–VIS sphectrophotometer model, UV-1601.

Nitrate reductase (NR) and nitrite reductase (NiR) assay

To determine the effect of heavy metals on NR and NiR activity the rhizobial strains were inoculated in YEMB amended with different doses of aluminium chloride (0–100 mM), cupric chloride (0–100 mM), ferric chloride (0–300 mM) and sodium molybdate (0–300 mM). The rhizobial cultures (48 h) were centrifuged at 19319 × *g* (Remi, Model No. CM-12, Mumbai) for 15 min at 4°C. The bacterial pellet obtained was treated with 1 mM EDTA and 1% lysozyme. The treated pellet was again centrifuged and then washed repeatedly with distilled water. The pellet was dissolved in tris HCl buffer so as to obtain 1 mg bacterial protein, which was used for the determination of NR and NiR activity.

NR activity was assayed in dark at 30°C using test mixture, 1 mg bacterial protein in 50 mM phosphate buffer (pH 7.5), 1 ml of 1% sulphanilamide in 1 N HCl, 0.02% *N*-napthyl ethylenediamine dihydrochloride and 100 mM KNO_3. Nitrite formed was determined after 30 min incubation by measuring the optical density at 540 nm and NR activity was reported in terms of μM of nitrite formed h^{-1} mg^{-1} $protein^{-1}$ (Snell and Snell 1949; Stephens and Neyra 1983). To determine NiR activity nitrate salt was replaced by nitrite salt in test mixture and reported in terms of μM of nitrite reduced h^{-1} mg^{-1} $protein^{-1}$ (Snell and Snell 1949). NR and NiR activities were assayed in five replicates.

Nitrogenase assay

The ex-planta nitrogenase activity was determined in CS7 agar medium (Pagan et al. 1975) by acetylene reduction assay (Gibson et al. 1976). The culture of rhizobial strains (14 days) from YEMA amended with different concentrations of Al and heavy metals were suspended in 9 ml sterile distilled water and 0.06 ml spread over the surface of the CS7 agar tubes, plugged with 'Suba-seal' rubber stoppers and incubated at 30°C for 24 h. The culture tubes were then flushed with a gas mixture of O_2: C_2H_2: Ar (2: 1: 7) to 1 atmosphere. After incubation for 24 h the gas sample in the tubes were analysed for ethylene produced using a Nucon 5500 Gas chromatograph with a flame ionization detector (FID) and Porapak T column (32 mm × 1.8 m). The temperature of oven was kept at 93°C and that of injector and detector at 110°C. The flow rate of gas mixture was maintained at 30 ml/min. The specific nitrogenase activity was determined by evaluating

the peak height of ethylene according to Somasegaran and Hoben (1994) and reported as nmole C_2H_4 mg^{-1} bacterial dry weight. The experiment was conducted in five replicates.

Hydrogenase activity

The effect of Al and heavy metals on hydrogenase activity of rhizobial strains was determined in Hup^+ medium (Maier et al. 1978). The rhizobial cultures were transferred from YEMA as described earlier, to the surface of Hup^+ medium. The medium was filled with standard 10% hydrogen and incubated for 24 h at 30°C. The gas phase in the test tubes was analysed by a Nucon 5500 gas chromatograph having a thermal conductivity detector (TCD) and a 5Å molecular sieve column. The temperature of the column was kept at 60°C, while injector and detector were kept at 90°C. Nitrogen was used as carrier gas. The hydrogenase activity was reported in nmole hydrogen consumed h^{-1} mg^{-1} cell dry weight (Bertelsen 1985). Hydrogenase activity was assayed in five replicates.

Results

Effect of Al and heavy metals on growth

Of the four metals selected, Al was found to be the most lethal to the survival of both *S. meliloti* RMP_5 and *Bradyrhizobium* BMP_1. *S. meliloti* RMP_5 was found to be more tolerant to all the four metals in comparison to *Bradyrhizobium* BMP_1. The growth of *S. meliloti* strain was completely inhibited at 100 µM and that of *Bradyrhizobium* at 75 µM Al (Fig. 1a). The growth of *S. meliloti* RMP_5 was not affected by Cu up to 0.1 mM concentration. The *S. meliloti* RMP_5 and *Bradyrhizobium* BMP_1 were able to tolerate Cu up to 50 and 1 mM, respectively (Fig. 1b). The growth of both the strains was stimulated up to 50 mM of Fe and Mo, but further increase in concentration inhibited the growth (Fig. 1c, d).

Effect of Al and heavy metals on NR and NiR activity

Aluminium was found to be much more deleterious for both the enzymatic activities of strains RMP_5 and BMP_1

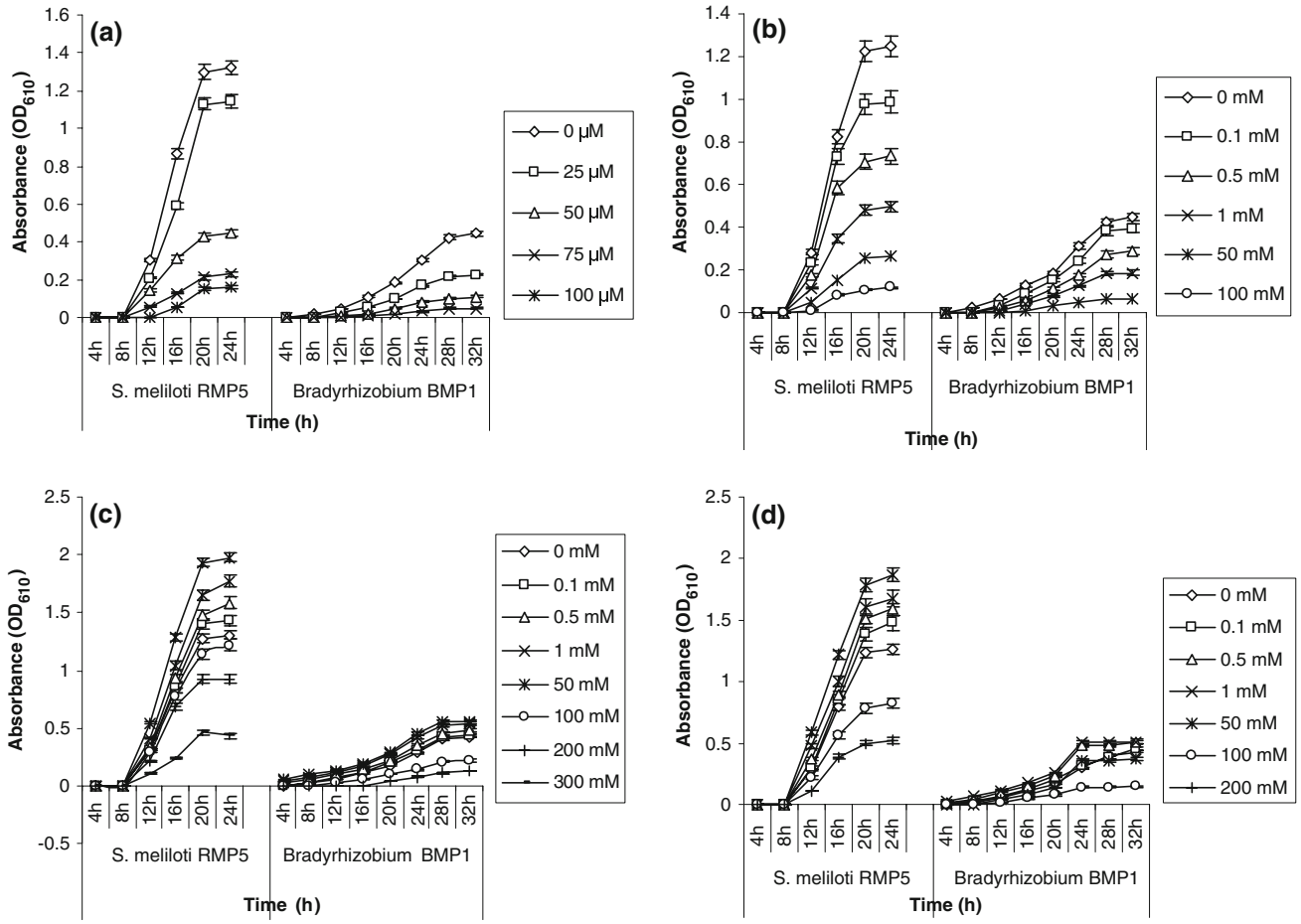

Fig. 1 Effect of Al and heavy metals on growth of *S. meliloti* RMP_5 and *Bradyrhizobium* BMP_1. **a** Aluminium, **b** Copper, **c** Iron, **d** Molybdenum

(Fig. 2a, b). NR activity was reduced to 50% of control (without metal) at 60 and 30 μM Al concentration in *S. meliloti* and *Bradyrhizobium* strains, respectively and NiR activity was reduced to 50% of control at 55 and 35 μM Al. There was complete inhibition of NR activity at 100 and 1 mM Cu concentration in RMP_5 and BMP_1, respectively. The inhibition of NiR activity by Cu followed similar trend as NR activity in case of *Bradyrhizobium* BMP_1. However, there was enhancement in NiR activity of *S. meliloti* RMP_5 at 0.1 mM Cu concentration followed by reduction.

Iron enhanced both NR and NiR activities by 45.2 and 33.9% over control at 100 mM concentration in *S. meliloti* RMP_5. There was 28.1 and 41.7% enhancement in NR and NiR activities of *Bradyrhizobium* BMP_1 at 100 mM Fe. Above 100 mM concentration of Fe there was a sharp decline in NR and NiR activities (Fig. 2c). NR and NiR activities were also enhanced up to 1 mM Mo concentration, in both the strains. However, further increase in the concentration of Mo was inhibitory for both the enzymes (Fig. 2d).

Nitrogenase and hydrogenase activity

The nitrogenase and uptake hydrogenase activities of *S. meliloti* RMP_5 was reduced to 90 and 82% from the control at 75 μM concentration of Al. At this concentration there was complete inhibition of these enzymes activity in *Bradyrhizobium* BMP_1. There was almost a linear decline in both nitrogenase and hydrogenase activities of BMP_1 with increase in copper concentration. Up to 0.1 mM Cu nitrogenase and hydrogenase activities were slightly enhanced to 5.88 and 5.13%, respectively in RMP_5 followed by reduction with further increase in concentration to 100 mM (Fig. 2a, b).

Similar to growth both nitrogenase and uptake hydrogenase activities were enhanced to 69 and 53% with increase in concentration of Fe up to 100 mM in *S. meliloti* RMP_5. There was 69.1 and 40.2% enhancement in activities of both the enzymes in *Bradyrhizobium* BMP_1 followed by progressive inhibition (Fig. 2c). Molybdenum enhanced nitrogenase and uptake hydrogenase activities of both

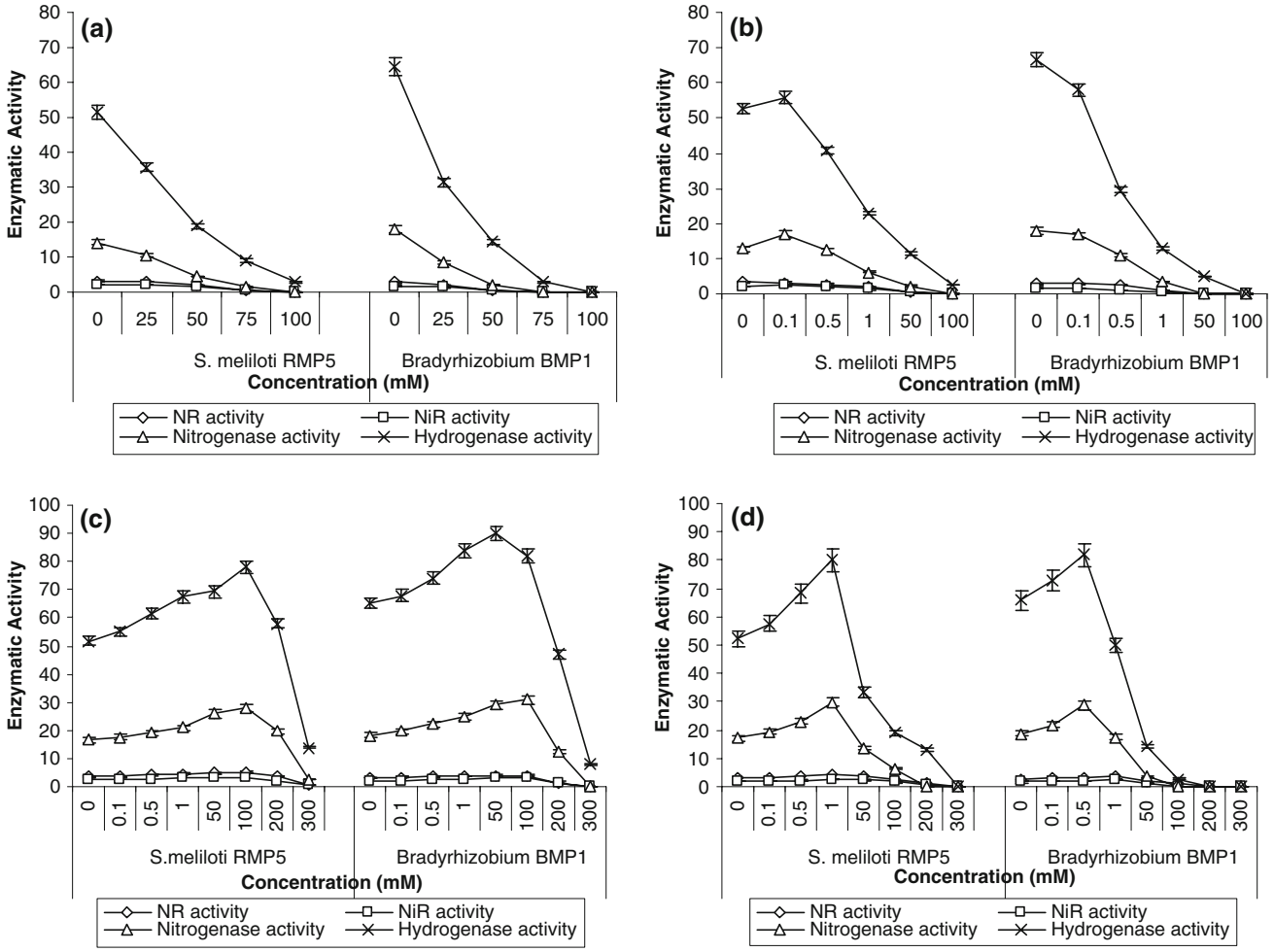

Fig. 2 Effect of increasing concentration of Al and heavy metals on enzymes activity of fast and slow growing rhizobial sps. **a** Aluminium, **b** Copper, **c** Iron, **d** Molybdenum

S. meliloti and *Bradyrhizobium* strains up to 1 and 0.5 mM concentration, respectively (Fig. 2d).

Discussion

Effect of increasing concentrations of Al and selected heavy metals was studied on growth and enzymatic activities of fast and slow growing rhizobial sps. Although *Bradyrhizobium* BMP_1 was also tolerant, *S. meliloti* RMP_5 was slightly more tolerant to all the four metals. Various workers have reported high tolerance abilities of *S. meliloti* strains to various metals (Kinkle et al. 1994). Of the four metals Al proved to be highly toxic while Mo and Fe (up to certain concentrations) enhanced the growth as well as enzyme activities of both fast and slow growing strains. Aluminium adversely affected the growth, nitrogenase, hydrogenase, NR and NiR ability of both the strains. Aluminium is known to be toxic to growth and various other aspects of root nodulating bacteria including the nitrogen fixation ability (Flis et al. 1992).

Wood and Cooper (1988) reported inhibition of multiplication of rhizobial strains at 50 μM Al concentration. Paudyal et al. (2007) reported detrimental effect of aluminiun in all its concentration on rhizobial growth. Our study reported a *S. meliloti* strain that can tolerate 100 μM concentration of aluminium. Copper was found to be inhibitory to the growth and enzyme activities of *Bradyrhizobium* strain at all concentrations. However, in *S. meliloti* RMP_5 enzymatic activities were increased up to a concentration. Chaudri et al. (2000) reported that soil concentrations of Cu at 7250 mg/Kg considerably reduced soil rhizobial population. The variation in Cu sensitivity levels in different rhizobial lineage groups was reported by Laguerre et al. (2006). Casella et al. (1988) observed that the exposure of bacteroids with metal chelator, diethyldithiocarbamate, prevented reduction of nitrite, indicating the presence of a Cu-containing nitrite reductase. Abdel Wahab and Abd-Alla (1995) reported that bacteroid nitrogenase is sensitive to nitrite, therefore the increase in nitrogenase and uptake hydrogenase activities in RMP_5 was due to the ability to remove nitrite because of enhanced NiR activity.

Iron and molybdenum enhanced the growth and enzyme activities of *S. meliloti* RMP_5 and *Bradyrhizobium* BMP_1 up to certain concentrations. Both Fe and Mo are the key components of the enzyme nitrogenase and nitrate reductase. Enhancement of nitrogenase and nitrate reductase stimulated uptake hydrogenase and nitrate reductase activities, respectively (Palacios et al. 2005). According to Palacios et al. (2005) one of the most relevant biogenic hydrogen sources is the nitrogen fixation process. Apart from being the important cofactors of various enzymes Fe is an important component of various other cellular components and products like leghaemoglobin, etc. (Dudeja et al. 1997). Fu and Tabatabai (1989) reported the inhibitive effects of Cu, Fe and Mo on nitrate reductase activities of soil microbes after a certain limit.

Bradyrhizobium BMP_1 showed better nitrogenase and uptake hydrogenase activities in comparison to *S. meliloti* RMP_5. However, NR and NiR activities were less than that of RMP_5. This study clearly reported the better nitrogen fixing ability of slow growing *Bradyrhizobium* strain over *S. meliloti* but less tolerance to Al and heavy metals. The work unfold the hidden effect of Al and heavy metals on the physiology of nitrogen fixing ability of both fast and slow growing rhizobial strains, the neglected part of most of the stress studies. This study recommends the supplementation of Mo and Fe (up to certain concentrations) in soils along with the biological formulations so as to enhance the process of symbiotic nitrogen fixation.

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