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RESEARCH ARTICLE

Modulation of growth, antioxidant system in seedling of mustard under different levels of Nickel in adaptive response to metal resistant bacteria

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Abstract **Plant growth is hindered by high concentration of metals in soil by disturbing various physiological processes. However, some bacteria with plant growth promoting features have been recognized to alleviate stress in plants even under elevated levels of metal concentration. The two bacterium NWM 71 and NWM 103, identified as *Pseudomonas* sp. and *Bacillus* sp. respectively were found to be resistant to the toxic effects of nickel (Ni^{2+}) and were identified with plant growth promoting features. Both the strains showed the production of indole acetic acid (IAA) and solubilisation of phosphate. *Brassica juncea* (mustard) was used as a test plant to identify the plant growth promoting activity of the selected strains of bacteria. The growth was positively influenced by the inoculation of both the strains. The tests for the measurement of chlorophyll contents and antioxidative activity were carried out to determine the level of stress in plants. High levels of Ni decreased the growth and chlorophyll content, however, significant increase in the antioxidant activity was recorded along the treatment. Inoculation of both the selected strains of bacteria increased the shoot and root biomass of mustard grown in both unspiked and spiked soil. This positive influence on growth can be attributed to the solubilisation of phosphate and production of IAA. Furthermore the observed high levels of antioxidant enzymes led to decrease in the toxic effects of Ni. This led to enhanced growth and chlorophyll content which in turn might have enhanced the photosynthetic capacity of the plants.**

Keywords nickel, phytoremediation, PGPB, ACC, antioxidant enzymes

Introduction

The contamination of agricultural lands with heavy metals is fast becoming one of the most serious environmental concerns. The heavy metals are non-degradable. These metals are hazardous and the threat is aggravated due to their persistence nature in soil for indefinite periods. Among these heavy metals in soil, nickel contamination is fast becoming one of the major environmental issues around the globe. The level of Ni contamination in polluted areas has reached several times higher than unpolluted areas. Soils polluted with this metal can therefore, be a threat to ecosystems and the human health.

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However, plants are dependent upon some of the transition heavy metals such as Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, and also Ni^{2+} for normal growth and development. These elements are central to cellular function due to their involvement in various redox reactions which in turn are fundamental to cellular functions. Nickel is a constituent of urease, and small quantities of Ni (0.01 to 5 μg/g dry wt.) are essential for some plant species (Seregin and Kozhevnikova, 2006). Even though Ni has only been recognized as a trace element in plants but its function is very decisive for certain enzymatic activities and plays a pivotal role in various biochemical and physiological processes. However, elevated levels of Ni can generate toxicity symptoms such chlorosis, necrosis, growth inhibition (Prasad et al., 2005). Like other heavy metals Ni also restricts mineral uptake and causes Fe-deficiency either by hampering the uptake or by causing its immobilization in roots (Taylor and Crowder, 1983). Furthermore it has been reported that Ni displaces Mg^{2+} ion of chlorophyll molecule

and therefore affects the photosynthetic system (Ewais, 1997). The results from numerous experiments provide evidence that high levels of Ni is responsible for oxidative stress in plant tissues which may eventually lead to death. However, the actual mechanism behind the generation of reactive oxygen species (ROS) by excess amounts of nickel is not fully well understood.

Remediation of soils contaminated with any of the metals is therefore a challenging task. Conventional clean-up technologies are generally too costly and therefore cannot be used for the restoration of contaminated sites. The emerging phytoremediation techniques i.e. the use of green plants for the removal or detoxification of pollutants, with their lower cost and environmental friendly nature, have received increasing attention in the last two decades (Tak et al., 2013). More recent findings have suggested that application of microorganisms could not only help in increasing the bioavailability of metals in soils thereby stimulating the heavy metal accumulation in plants but even reduce heavy metal toxicity in plants (Dell'Amico et al., 2008; Wang et al., 2009). Among the biological factors governing metal extraction, the role of microorganisms, in particular plant growth promoting bacteria (PGPB) is of immense importance. There have been reports that even in soil having low concentration of metal, bacteria can boost their transfer in plants (Abou-Shanab et al., 2006; Braud et al., 2006).

Since phytoextraction is a very slow process therefore, for economically viable decontamination, the agricultural land under phytoremediation needs to be kept productive. The use of *Brassica* spp. as metal extraction plant would give productive value to the land and at the same time reduce the cost of remediation (Salt et al., 1995). Number of plants have been identified with metal-accumulating capacity that can be used in the removal of toxic metals from soil (Rajkumar et al., 2006; Zaidi et al., 2006). Also, there are reports available where in the interactions between plants and some rhizospheric microbes have been reported to be beneficial. The microbial inoculation increase the tolerance of the plants to heavy metals and therefore help in increasing the biomass production, thus serving as an important component of phytoremediation technology (Glick, 2003). Higher levels of metals however may lead to impaired metabolic activity resulting in reduced growth and development. One of the mechanisms by which the plants resist the heavy metal stress is the strong antioxidant system. However, under severe stressful conditions the antioxidant capacity may not be sufficient to minimize the harmful effect of oxidative injury. Plant survival under stressful conditions mainly depends on the ability of plants to perceive the stimulus, and then generate and transmit signals and to adjust metabolism by inducing biochemical changes (Gao et al., 2010). This may seem to be over simplification, however, it is difficult to fully understand the physiological, biochemical and molecular mechanisms involved in metal hyper accumulation.

This study was therefore undertaken with an objective to isolate Ni resistant bacteria from local mining area, and to select Ni resistant plant growth-promoting bacterial strains and study their impact on plant growth and development. Therefore, the aim of the experiment was to: (1) to explore and elaborate the ameliorative role of PGPB, in plants subjected to nickel (Ni) stress; (2) to investigate a possible correlation between antioxidant system and the degree of resistance developed by PGPB, against the stress, in *Brassica* seedlings. We tested the hypothesis that the application of PGPB will ameliorate the toxic effect of nickel on the growth of *Brassica* plants.

Materials and methods

Isolation of Ni mobilizing bacteria

The strains of PGPB were isolated from the soil collected from the mining area in North West province of South Africa. After serial dilution of about 1 g of soil samples using 25 mM phosphate buffer, the drops were spread over on Luria-Bertani (LB) medium previously amended with 40 mg/L of Ni ($NiCl_2$). The plates were then incubated at 37°C for 48 h. From these Ni resistant colonies, different strains were picked and purified on LB medium containing 40 mg/L of Ni. To isolate the bacteria with plant growth-promoting features, all of the nickel resistant isolates were tested for their capability to grow on minimal medium with ACC as the only source of nitrogen. Finally, these nickel-resistant strains of bacteria that were also able to grow on ACC, were then tested for their ability to produce siderophores. Based on the idea that bacterial siderophores might facilitate the uptake of nickel by plants, the best siderophore producing strain designated NWM-71 and NWM-103. These were identified as *Pseudomonas* sp. and *Bacillus* sp. respectively using API 20NE and API 50CHB kits. These two strains were subsequently selected for inoculation to study their effect on plant growth promotion.

Plant growth and experimental design

The seeds were first sterilized and then incubated for 1h at room temperature in sterile distilled water as a blank control and in bacterial suspension adjusted to an absorbance of 0.025 (3.0×10^7 CFU/mL) at 600 nm. The pots were spiked with three levels of nickel (100, 300 and 500 mg $NiCl_2$) ten days before the date of sowing for allowing stabilization of metal. The inoculated and un-inoculated seeds were then sown in the earthen pots which were previously spiked with different levels of $NiCl_2$. The pots were regularly watered with equal amount of water. The pots were relocated to

change their position every alternate day. The plants were then harvested (one plant from each replicate) at 21 days after sowing (DAS) by carefully removing the whole plant along with the soil. The roots of the freshly harvested plants were first thoroughly washed under running tap water and then dipped in a bucket, filled with water. The adhering soil particles were carefully removed to ensure the integrity of the roots. The uprooted plants were then placed on blotting paper and the lengths of root and shoot were then recorded. The fresh mass of these harvested plants was then recorded and the roots and shoots were subsequently dried in an oven at 80°C for 72 h to record the dry mass. Leaf area of randomly selected leaves from each treatment was determined by tracing the outline of the leaf on the graph sheet and counting the number of squares covered by leaf.

Enzyme assays

For the assay of antioxidative enzymes, phosphate buffer, i.e. 50 mM (pH 7) containing 1% (w/v) soluble polyvinyl pyrrolidone, was used for homogenization of the leaf tissue. This was followed by centrifugation of homogenate at 15000 r/min for 10 min at 4°C. The supernatant was used as source of enzymes catalase, peroxidase and superoxide dismutase.

The activity of peroxidase and catalase was measured by following the method as described by (Chance and Maehly, 1955). The estimation of catalase was carried out by titration of reaction mixture, consisting of phosphate buffer (pH 6.8), 0.1 M H_2O_2, enzyme extract and 2% H_2SO_4, against 0.1N potassium permanganate solution. The reaction mixture for peroxidase consisted of pyrogallol phosphate buffer (pH 6.8), 1% H_2O_2 and enzyme extract. The change in absorbance due to catalytic conversion of pyrogallol to perpurogalline, was noted at an interval of 20 s for 2 min, at 420 nm on a spectrophotometer (Spectronic 20D, Milton Roy, USA).A control set was prepared by using double distilled water instead of enzyme extract. The activity of superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium using the method of Beauchamp and Fridovich (1971). The reaction mixture contained 50mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium (NBT), 2 mM riboflavin, 0.1 mM EDTA and 0–50 µL enzyme extract and was placed under 15W fluorescent lamp. The reaction was started by switching on the light and was allowed to run for 10 min. The reaction was stopped by switching off the light. Fifty per cent inhibition by light was considered as one enzyme unit.

Statistical analysis

Four samples representing each treatment were collected from four different pots to make the observations. The data was analyzed statistically using SPSS, 17.0 for windows (SPSS, Chicago, IL-USA). Standard error was also calculated.The analysis of variance (ANOVA) was performed on data to determine the least significance difference (LSD) between treatment means at $p \leqslant 0.05$ level of significance.

Results and discussion

Plant growth

The growth of plants in pots (soil) amended with Ni was affected and significant decline in growth parameters was observed as compared to control. Therefore, the toxicity generated by Ni clearly affected the growth and development. It was observed in the form of stunted root length, shoot length and reduced shoot fresh and dry mass. As is evident from Figs. 1 and 2 the growth of *Brassica juncea* as observed at 21 days after sowing (DAS), significant differences among different Ni treatments was observed. Further clear reduction in shoot fresh and dry mass was observed at high Ni level (Ni + 500). The inhibition of growth by excess Ni concentration has also been reported by (Ewais, 1997). However, the application of inoculants of both the bacteria was found to be effective in overcoming stress generated by excess level of Ni and significantly promoted plant growth compared to un-inoculated under uniform Ni treatments. Also at low levels of

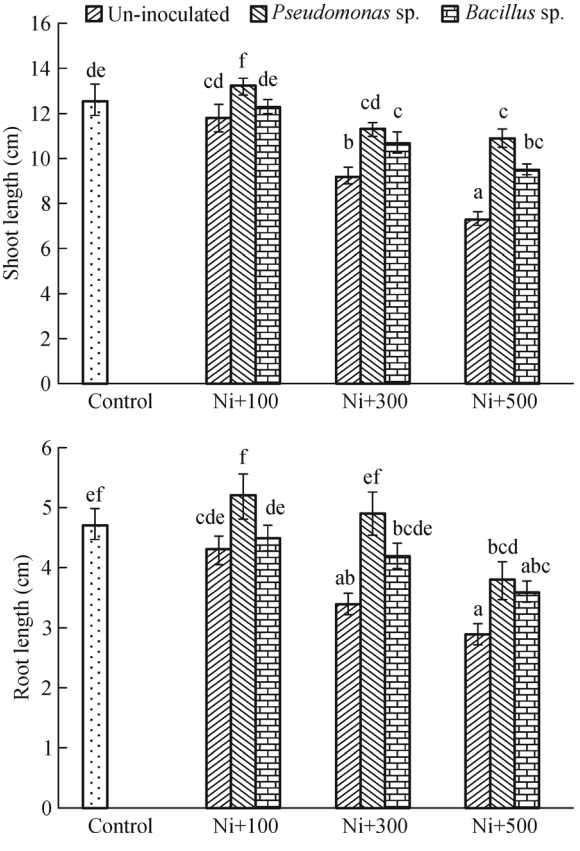

Figure 1 Effect of *Pseudomonas* spp. and *Bacilus* spp. on shoot length (cm), root length (cm) of *Brassica juncea* exposed to different levels of Ni (100, 300 and 500 mg).

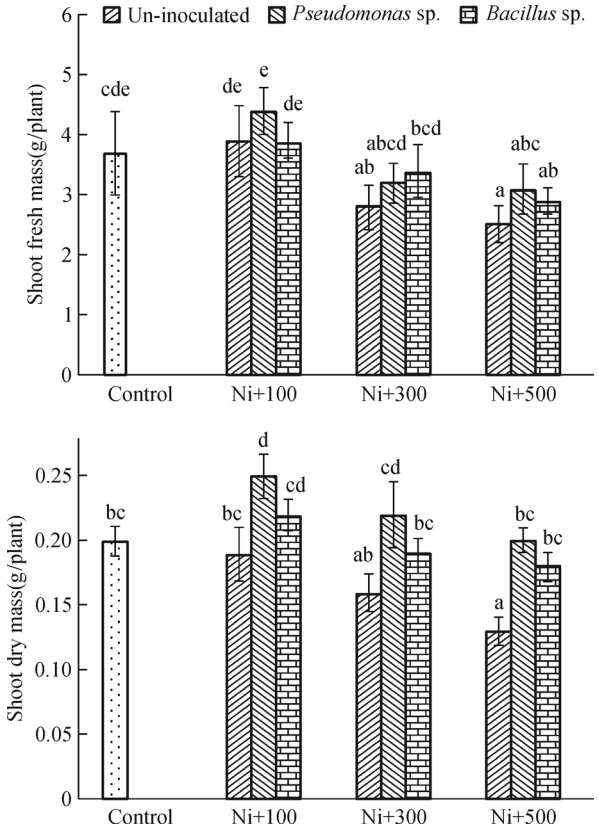

Figure 2 Effect of *Pseudomonas* spp. and *Bacillus* spp. onshoot fresh mass (g/plant) and shoot dry mass (g/plant) of *Brassica juncea* exposed to different levels of Ni (100, 300 and 500 mg).

Ni, there was no significant deleterious effect on the growth of the plants (Figs. 1 and 2). Furthermore the shoot fresh mass of the plants under the application of both the bacterial strains in the pots spiked with 100 mg Ni even exceeded the biomass under un-spiked soil.

The ability of plant growth promoting rhizobacteria (PGPR) in exhibiting various plant growth promoting traits such as siderophore production, phosphate solubilisation, ACC deaminase (ACCD) activity has been well documented by numerous researchers (Forchetti et al., 2007; Jha and Kumar, 2007). Both the strains used for the inoculation in the present set of experiment were found to be positive for the production of siderophore, solubilization of phosphate and exhibited ACCD activity. Thus the better growth even under stressful condition is possibly because of the growth promoting activities of bacteria. Though the levels of ethylene was not tested however, under stressful conditions it is known that the level of ACC in the roots is considerably increased. Plant stress generated by metal-contaminated soils can be countered by strengthening the plant defense response (Burd et al., 1998). One way of countering the stress is by alleviating its impact on plants by enzymatic hydrolysis of ACC which is an intermediate in the biosynthetic pathway of ethylene (Karthikeyan et al., 2012). The ACC molecules then diffuse from plants and are imported into PGPR cells where they are subjected to the action of ACC deaminase (Tak et al., 2013). However, the exact mechanism of the transfer of ACC to bacterial cells is unknown (Fig. 3). Because of this, microbes and plants are more tolerant to stress-induced growth inhibition that is mediated by ethylene. Ma et al. (2009) reported significant increase in the root length, shoot length fresh mass and dry mass of *Brassica juncea* plants after inoculation with *A. xylosoxidans* Ax10 as compared to the control both under normal and Cu amended conditions. These authors attributed the effect to the utilization of ACC, production of IAA and solubilization of phosphate.

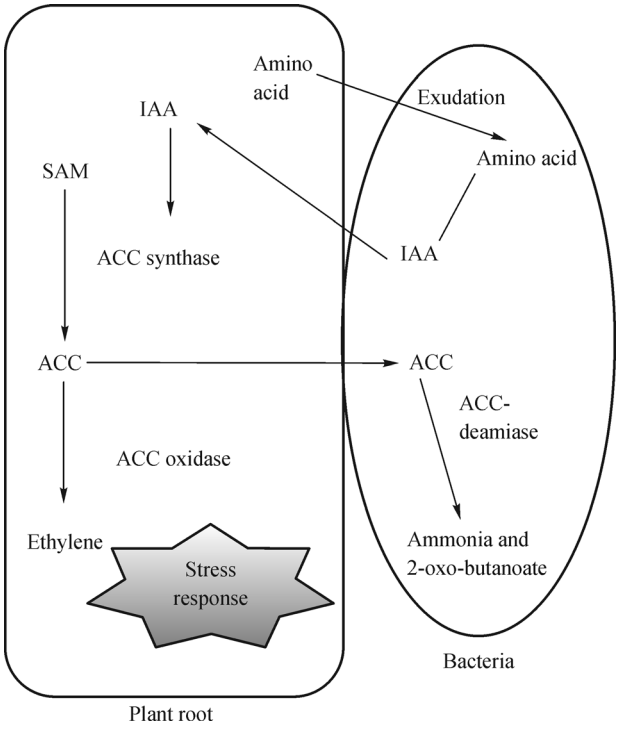

Figure 3 Diagrammatic model showing reduction of ethylene levels in roots by bacterial 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. ACC synthesized in plant tissues is believed to be exuded from plant roots and is taken up by rhizobacteria where ACC ishydrolyzed to ammonia and 2-oxobutanoate (Adapted from Tak et al., 2013).

No visual toxicity symptoms were reported in plants treated with Ni + 100 and thus clearly showing Ni tolerance at this level. However with the increase in Ni concentration the biomass was significantly reduced compared to control. The level of decrease was however, significantly lowered by the use of either of the inoculant. Thus the contribution of both inoculants to alleviate Ni stress on plant growth was observed and the strain *Pseudomonas* sp. proved more efficient in alleviating stress and maximum biomass was recorded for plants treated with this bacteria at all the three Ni levels. The bacterial inoculant of *Pseudomonas* sp. under Ni stress enhanced the plant biomass when compared to the un-inoculated treatments.

Antioxidant response

Chlorosis is one of the most common symptoms of toxicity due to heavy metals, including Ni (Pandey and Sharma, 2002). The effect on the chlorophyll usually shows positive correlation with the lipid peroxidation which corresponds for cellular oxidative damage (Sinha and Saxena, 2006). Ni toxicity generally cause a decrease in chlorophyll content through inhibition of chlorophyll biosynthesis or by accelerating its degradation (Sheoran et al., 1990) as was also reported in this experiment (Fig. 4). The decrease in content of chlorophyll pigment due to Cd, Pb and Ni has also been reported by (Ewais, 1997). The possible reason for the decrease of the photosynthetic pigment may be ascribed to the fact that excess of Ni hampers the uptake of Mg and Fe (Piccini and Malavolta, 1992). However, inoculation of both the species of bacteria, improved the chlorophyll content significantly (Fig. 4). The chlorophyll content in the plants treated Ni + 300 and inoculated with *Pseudomonas* spp. and *Bacillus* spp. was 1.74 and 1.45 respectively as compared to 1.32 in un-inoculated plants. The finding that bacteria in stressed and non-stressed plants increased the chlorophyll content is in tune with the results obtained by Grichko and (Glick, 2001).

Increase in anitoxidative enzyme activity in all treatments under increasing Ni concentration was observed irrespective of the bacterial inoculation (Fig. 4). However under similar Ni treatment the highest antioxidant activity was observed in plants inoculated with *Pseudomans* spp. As a natural course plants when exposed to any stress produce large quantities of reactive oxygen species (ROS) such as O_2^- and H_2O_2 as a by-product of several metabolic processes. These ROS can oxidize proteins, lipids and nucleic acids resulting in abnormalities at the level of cell (Sanita di Toppi and Gabbrielli, 1999). To counteract these reactive oxygen species, nature has equipped plants with the ability to induce the synthesis of antioxidant metabolites (proline, ascorbate, glutathione etc.) and enzymes (superoxide dismutase, catalase etc.) that neutralize the toxic effects of ROS generated through stress. An increase in antioxidant system in all treatments as influenced by Ni concentration was noticed in the present work (Fig. 4). The synergistic role played by enzymes and metabolites helps to carry out ROS detoxification. SOD catalyzes dismutation of O_2^- to H_2O_2 and O_2^- It is considered to be the first line of defense against ROS. CAT is then involved in the scavenging of H_2O_2 thereby converting it into H_2O and O_2. The POD is also involved in the elimination of H_2O_2 and is considered to be the key enzyme in the

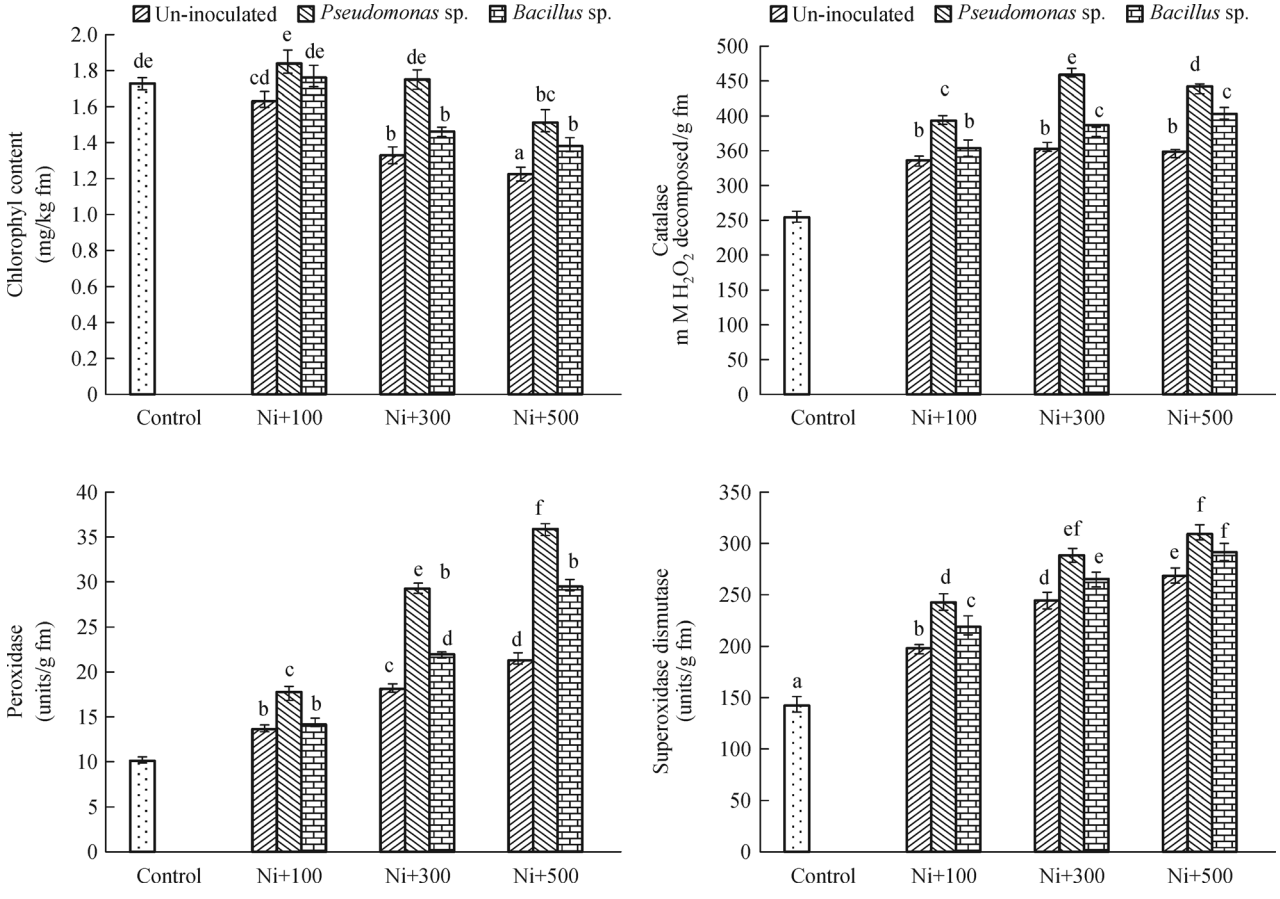

Figure 4 Effect of *Pseudomonas* spp. and *Bacillus* spp. on chlorophyll content (mg/kg fm), catalase (mM H_2O_2 decomposed/g fm), peroxidase (units/g fm), superoxidase dismutase (units/g fm) of *Brassica juncea* exposed to different levels of Ni (100, 300 and 500 mg).

synthesis of lignin (Asada, 1992). These enzymes play a key role in defending the cells against oxidative damage (Michiels et al., 1994). Also the balance between SOD and CAT activities in cells is crucial for determining the steady-state level of O_2 and H_2O_2. It is interesting here to note that bacterial inoculation increased the levels of SOD and CAT under different Ni levels conferring more resistance to Ni mediated stress in inoculated plants. Karthikeyan et al. (2007) reported an increase in the activities of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) due to the treatment with diazotrophic bacteria such as *Azospirillum* and *Azotobacter*. The above results corroborate well with the present findings that under high Ni concentration the strain of *Pseudomonas* spp. increased the levels of antioxidative enzymes thereby conferring better resistance for plants under stress generated by excess heavy metal. The importance of soil bacteria in mobilization of heavy metal and their ability to promote the host plant growth in a metal-contaminated environment have attracted attention, making them the preferred choice for the phytoremediation studies. Regardless of the precise mechanism used by the bacterium to protect plants, the experiments with plant seedlings reported here suggest that certain bacteria may eventually find a use in the development of phytoremediation strategies. In this regard, increasing the amount of plant biomass will be an important step forward. In heavily contaminated soil where the metal content exceeds the limit of plant tolerance, it may be possible to treat plants with plant growth promoting bacteria, increasing plant biomass and thereby stabilizing and remediating metal-polluted soils.

Conclusions

Our study demonstrated that the growth promoting effect of PGPR having ACCD activity was beneficial for the alleviation of stress generated by high Ni concentration. The growth of *Brassica* in the presence of toxic Ni concentrations was dependent on the bacterial strain. Pot experiments demonstrated that the application of Ni-resistant PGPR protects plants against the oxidative effects of $NiCl_2$ by strengthening the antioxidant system comprising of SOD, POX and CAT and therefore effectively help the plant to overcome stress. While ACCD activity may be responsible for better root, siderophore and production of IAA may facilitate the mobilization of nutrients, hormonal balance and, thus, plant growth. Extensive research in the areas of colonization capability, the role of rhizobacteria and plant roots in the uptake of metals and their mode of metal translocation, is required to elucidate the mechanisms of PGPR protection against toxic elements in soil to achieve the stabilization and remediation of metal-polluted soils. In conclusion the presented study has shown that strain of *Pseudomonas* sp. could be used as a suitable bioinoculant for *Brassica* plants grown under Ni affected areas. This work opens up the possibility for better exploring plant-bacterial interactions under heavy metal affected soil conditions.

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Compliance with ethics guidelines

Hamid Iqbal Tak declare that he have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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