



Potential of phytohormone producing rhizobacteria to improve growth, SPAD value and Cd uptake of mustard under Cd stress

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Abstract

Heavy metal pollution due to irrigation with wastewater is the major issue for crop production around the big cities and metropolitans. Farmers grow mustard and other fodder crops in the peri-urban areas to meet the fodder requirements of animals resulting incorporation of heavy metals into food chain. Cadmium, being the most toxic heavy metal, severely affects the plant systems. The indole-3-acetic acid (IAA) and siderophore producing bacterial strains may be helpful to improve crop growth by reducing Cd uptake through stabilizing it in the soil. Present study was conducted to evaluate Cd tolerant IAA and siderophores producing rhizobacterial strains for improving germination, growth, and physiology of mustard crop under Cd stress. Results showed that the Cd-stress significantly decreased the germination, SPAD value, seedling growth and seedling vigor index of mustard plants. Inoculation with Cd-tolerant, IAA and siderophores producing rhizobacterial strains reduced the negative effects of Cd but strain FN13 was significantly better in decreasing the inhibitory effects of Cd-stress on germination, growth and vigor index of mustard. The Cd-stress also raised the Cd uptake and accumulation in mustard seedlings while the inoculation significantly reduced the Cd concentration in root and shoot of *Brassica juncea* (L.) seedlings under Cd²⁺ stress. It can be concluded that the *Bacillus safensis* strain FN13 can be a potential phytostabilizing biofertilizer to grow mustard in heavy metal contaminated soils.

Keywords: Wastewater, fodder, metal stress, phytostabilization, growth

Introduction

Untreated use of wastewater and industrial effluents cause accumulation of heavy metals in fodders grown around the big cities particularly in developing countries (Ferronato and Torretta, 2019). Due to scarcity of irrigation water, the farmers use this untreated wastewater for fodder production (Khalid *et al.*, 2018). This activity enhances crop productivity on one hand but incorporates significant amount of potential heavy metals in soils and plants on the other hand (Chibuike and Obiora, 2014).

In Pakistan, mustard crop is important not only due to its contribution in local oil production but also used as fodder crop (Shahid *et al.*, 2010). Unfortunately, in most cases, it is cultivated on marginal lands and peri-urban areas. As fodder, the mustard crop is being grown by farmers around the vicinity of big cities where it meets the fodder requirements of main herds of dairy animals in the cities. The use of industrial effluents, untreated sewage water, agrochemicals and farm wastes is the major source of water for growing

fodders and vegetables in these soils which are major contributors of organic and inorganic pollutants (Zwolak *et al.*, 2019). As most of these lands are used for growing leafy vegetables and fodder crops the heavy metals present there thus are accumulated in edible portion/above ground parts of growing crops (Chaoua *et al.*, 2019) which ultimately enter the food chain due to consumption of vegetables and fodders by human and animals.

Heavy metals may be toxic or beneficial for soil microbes depending upon their bioavailability and nature (Jaleel *et al.*, 2009; Ayangbenro and Babalola, 2017). Plants respond to heavy metal stress by producing oxidants and free radicals, and activation of different stress responsive genes (Maruyama *et al.*, 2019). Metal stresses induce the production of several reactive oxygen species (ROS) in plants at toxic concentrations.

Among heavy metals, Cd is the most toxic element for plants and other organisms (Farid *et al.*, 2015) and its compounds have more solubility than other toxic metals

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which favors its bioavailability, absorption and accumulation in biological systems. It is promptly absorbed and accumulated in plant tissues due to its high solubility that makes it highly toxic to plants leading to disruption of the entire plant metabolic functions (DalCorso *et al.*, 2010). Plant responds to Cd stress by synthesizing stress-related proteins and signaling molecules (DalCorso *et al.*, 2010) along with activation of enzymatic and non-enzymatic defense systems.

Use of metal tolerant plant growth promoting rhizobacteria (PGPR) for remediation of metal-polluted soils has been well documented (Guo *et al.*, 2020). These PGPR use different mechanisms for the solubilization or stabilization of heavy metals in metal polluted environment (Hamidpour *et al.*, 2019). Although microbial inoculation can also be used as a tool to enhance heavy metal uptake leading to remediation of contaminated soils and water (Ning *et al.*, 2019), the phytostabilization of heavy metals using soil microbes can, however, be an effective strategy in this regard (Saran *et al.*, 2020). Reducing bioavailability of heavy metals can decrease their toxic effects on food web by lowering their uptake and accumulation in crop plants (Saengwilai *et al.*, 2019). Bacterial strains may produce exopolysaccharides, plant hormones and siderophores (Khan and Bano, 2019) which can be effective to improve plant physiology under stressful environments. These strains have been well documented to have multifarious plant growth promoting (PGP) traits in addition to phytohormone producing ability and thus can be used for inducing heavy metal stress tolerance in crop plants and phytostabilization of heavy metals in metal polluted agricultural soils (Pramanik *et al.*, 2017).

The complex biomolecules produced by bacteria contain significant quantity of negatively charged carboxyl and hydroxyl functional groups which indicate that these biomolecules have high carbohydrates to protein ratio (Biswas *et al.*, 2020). It has been documented that IAA-producing PGPR can help in heavy metal detoxification (Yang *et al.*, 2012) and mitigate plant's stress in polluted soils through various PGP traits including nutrient solubilization, siderophores, ammonia and HCN production, and nitrogen fixation (Ahmed and Khan, 2012; Shan *et al.*, 2019). The IAA producing microbes can regulate plant hormone level and help them to acclimatize stressed conditions (Treesuntorn *et al.*, 2018). Moreover, phytohormone producing microbes can reduce the uptake and translocation of heavy metals by plant roots thus help them adopt stressful environments (Das and Sarkar, 2018). Present study was conducted to evaluate the effectiveness of phytohormone producing *Bacillus safensis* strain FN13 for improving growth of *Brassica juncea* (L.) under Cd stress.

Materials and Methods

Laboratory and growth room experiments were conducted to evaluate the phytohormone producing rhizobacterial strains for improving germination and growth of *Brassica juncea* (L.) under Cd stress.

Collection of bacterial strains

The *Bacillus safensis* strain FN13 along with unidentified strains FN14 and FN16 were collected from Soil Microbiology and Biotechnology Laboratory, Department of Soil Science, IUB. The strains were previously characterized for their ability to tolerate Cd along with production of IAA and exopolysaccharides (Nazli, 2020). The strain FN13 was identified as *Bacillus safensis* and submitted to National Center for Biotechnology Information (NCBI) database under accession number MT229105 (Nazli *et al.*, 2020). Fresh culture of the strain was prepared and used for the experiments.

Collection of *Brassica juncea* (L.) seeds and determination of its EC₅₀

The seeds of latest commercial mustard variety, "Pioneer 45S42", were collected and the EC₅₀ of seeds was determined using different concentrations of Cd²⁺ (0-300 µg mL⁻¹). The mustard seeds were surface sterilized using 2% sodium hypochlorite. Solutions with different concentrations of Cd²⁺ were poured in separate Petri plates having Whatman filter paper. Ten surface-sterilized mustard seeds were placed in the Petri plates with three replications. Petri plates were incubated in dark at 30 ± 1°C and germination percentage was calculated after 48 to 72 hours of incubation using the following formula.

$$\text{Germination percentage} = \frac{\text{No. of seeds germinated}}{\text{Total number of seeds}} \times 100$$

Jar trial to evaluate the Cd-tolerant, IAA-producing rhizobacterial strains for improving growth of *Brassica juncea* (L.) under Cd stress

The phytohormone producing rhizobacterial strains were evaluated in jar trial to improve germination and growth of *Brassica juncea* (L.) seedlings under Cd stressed axenic conditions.

Mustard seeds were kept in 2% solution of sodium hypochlorite for 10 minutes to surface sterilize them. The surface sterilized mustard seeds were rinsed with sterile distilled water for four to five times and were soaked in sterile filter paper. Seeds were inoculated with 48-hour old culture of respective bacterial strains (OD = 0.45) by



dipping in broth culture for 30 minutes. For un-inoculated control, mustard seeds were dipped in sterile broth for 30 minutes. Eighty seeds of each inoculated and un-inoculated treatment were planted in seed beds made by using sterile absorbent cotton with approximately 2 cm height and sterile Whatman filter paper placed on cotton in 200 mL glass beakers.

All treatments were replicated thrice. All jars were labelled with respective treatments and about 25 mL Hoagland solution (Ahmad *et al.*, 2016) was used in each glass jar. In Cd-stressed treatments, 100 and 150 $\mu\text{g mL}^{-1}$ Cd^{2+} was used in the Hoagland solution. The beakers were placed at $32 \pm 1^\circ\text{C}$ under dark conditions in growth room for 3 days. After germination, the sets were arranged on shelf in the growth room adjusted to 10 hours light and 14 hours dark period, for 9 days.

3.1.1.1. Recording of morphological parameters

After 12 days of sowing, morphological parameters of plant related to growth were recorded according to experimental standards. Seedling Vigor Index (SVI) was calculated through the formula as described by Bal *et al.* (2013).

$$SVI = (\text{mean shoot length} + \text{mean root length}) \times \text{germination percentage}$$

3.1.1.2. Analysis of SPAD value

The chlorophyll contents of mustard leaves were measured in terms of SPAD value. The SPAD meter was used for these measurements as described by Hussain *et al.* (2000).

3.1.1.3. Cadmium accumulation in mustard seedlings

Cadmium concentration was analyzed using atomic absorption spectrophotometer (AAS) model AAS-240FS, Agilent, USA, according to the method of Yang *et al.* (2009). Bioaccumulation and translocation factor(s) of Cd for mustard seedlings were estimated by using the method modified from Ahmad *et al.* (2014).

$$\text{Bioaccumulation factor} = \frac{\text{Cd concentration in mustard shoot}}{\text{Cd concentration given in seed bed}}$$

$$\text{Translocation factor} = \frac{\text{Cd concentration in mustard shoot}}{\text{Cd concentration given in mustard roots}}$$

Statistical analysis

Data of the bioassay and growth trial were statistically analyzed. For this purpose, the CRD design was used in factorial arrangement. The Tukey's test was used to compare means (Steel *et al.*, 1997) and the Excel (MS office

365) was used to calculate standard error, means and to draw graphs.

Results

Experiments were conducted to determine the EC_{50} level of mustard seedlings for Cd, followed by evaluating the efficacy of rhizobacterial strains for improving growth of *Brassica juncea* (L.) under Cd stress.

Collection of *Brassica juncea* (L.) seeds and determination of its EC_{50}

The germination of seeds was 100% without stress (Cd^{2+}) that decreased significantly with increasing level of Cd^{2+} (Figure 1). The germination was reduced to 78.33, and 53.33% at 100 and 150 $\mu\text{g mL}^{-1}$ of cadmium, respectively, that was further decreased to 33.33 and 0.0% at 200 and 300 $\mu\text{g mL}^{-1}$ of cadmium thus the EC_{50} recorded for "Pioneer 45S42" was 150 $\mu\text{g mL}^{-1}$. The Cd stress levels of 100 and 150 $\mu\text{g mL}^{-1}$ of Cd^{2+} (EC_{50}) were further studied to evaluate the effect of stress on growth of *Brassica juncea* (L.) in comparison to un-stressed control.

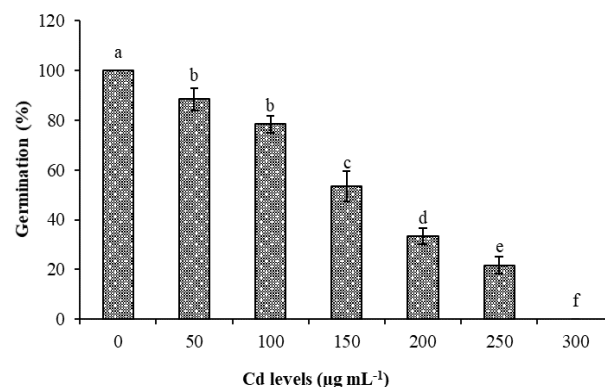


Figure 1: Effect of Cd stress on germination of *Brassica juncea* (L.) under axenic conditions

(n = 3); Means with same alphabets do not differ statistically from one another ($p \leq 0.05$)

Effect of Cd-tolerant, IAA-producing rhizobacterial strains on germination of *Brassica juncea* (L.) under Cd stress

Germination is the most crucial factor in the performance of crop varieties under various climatic conditions. An improvement in seed germination under stress can have significant influence on overall performance of crop variety. The Cd-tolerant, IAA-producing rhizobacterial strains were assessed for their potential to reduce the impact of Cd-stress on germination of *Brassica juncea* (L.) seeds. The Cd-levels used were 100 and 150 $\mu\text{g mL}^{-1}$ of Cd^{2+} along with un-stressed control. The Cd-stress significantly reduced the germination of *Brassica* and the



minimum germination 51.67% was observed at 150 $\mu\text{g mL}^{-1}$ of Cd^{2+} . Inoculation of *Brassica* seeds with Cd-tolerant, IAA-producing rhizobacterial strains significantly reduced the impact of stress on germination. Maximum improvement in germination was observed by the inoculation with strain FN13 at 150 $\mu\text{g mL}^{-1}$ of Cd^{2+} followed by FN16 at the same level (Figure 2).

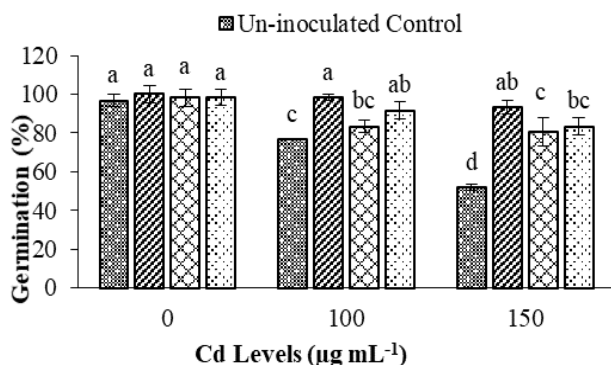


Figure 2: Effect of Cd-tolerant, IAA-producing rhizobacterial strains on germination of *Brassica juncea* (L.) under Cd stress

(n =3); Means with same alphabets do not differ statistically from one another ($p \leq 0.05$)

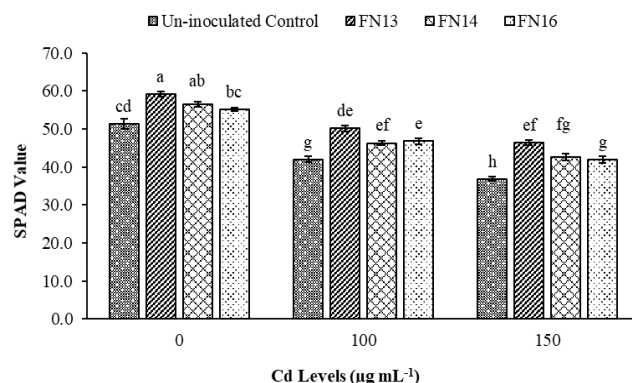


Figure 3: Effect of Cd-tolerant, IAA-producing rhizobacterial strains on SPAD value of *Brassica juncea* (L.) seedlings under Cd stress in jar trial

(n =3); Means with same alphabets do not differ statistically from one another ($p \leq 0.05$)

Effect of Cd-tolerant, IAA-producing rhizobacterial strains on growth and physiology of *Brassica juncea* (L.) under Cd stress

Results (Figure 3) revealed that Cd-stress caused a significant reduction in SPAD value of mustard seedlings

that was improved due to inoculation. The decrease in SPAD value due to Cd-stress was 18.2 and 28.2% at 100 and 150 $\mu\text{g mL}^{-1}$ of Cd^{2+} stress, respectively. At highest level of Cd-stress i.e. 150 $\mu\text{g mL}^{-1}$ of Cd^{2+} , the FN13 was the most efficient strain to improve SPAD value where the improvement was 25.9% higher than control. At lower level of Cd-stress, same trend was observed where the maximum improvement in SPAD value (19.5%) was observed due to inoculation with FN13. Overall, the inoculation was more effective at higher level of Cd-stress than the lower level and un-stressed control.

Results (Table 1) showed that Cd-stress significantly reduced the shoot length of mustard seedlings. The severity of stress increased with increasing level of stress.

Inoculation with Cd-tolerant, IAA-producing rhizobacterial strains was significantly effective in lessening the negative effect of Cd-stress on mustard growth. Under un-stressed control, maximum improvement (11.6%) in shoot length over control was given by FN13 that was non-significant with FN14. Under Cd-stress, all the Cd-tolerant, IAA-producing rhizobacterial strains significantly enhanced the shoot length of mustard over the control. Maximum improvement (45.8%) in shoot length was observed due to inoculation with FN13 at 150 $\mu\text{g mL}^{-1}$ of Cd^{2+} . Inoculation with Cd-tolerant, IAA-producing rhizobacterial strains also improved the root length of *Brassica juncea* (L.) seedlings under un-stressed conditions and Cd-stress (Table 1). Under un-stressed conditions, the results of all strains were non-significant with each other in improving the root length of mustard, but this improvement was significantly better than control. Under Cd-stress, the impact of inoculation was more pronounced as compared to normal conditions. At 150 $\mu\text{g mL}^{-1}$ Cd^{2+} level, the maximum improvement (48.4%) in root length over control was observed by the strain FN13.

The data (Table 1) revealed that Cd-stress significantly decreased the shoot dry weight and root dry weight of mustard seedlings at both levels. Inoculation with Cd-tolerant, IAA-producing rhizobacterial strains lessened the negative effect of Cd-stress resulting in an improvement of shoot dry weight. The inoculation with these strains was, however, more effective at higher level of Cd-stress i.e. 150 $\mu\text{g mL}^{-1}$ of Cd^{2+} . The decrease in shoot dry weight was up to 33.9% at 150 $\mu\text{g mL}^{-1}$ of Cd^{2+} . Maximum response was observed by the strain FN13 at both levels of Cd-stress as well as under un-stressed conditions. Inoculation improved the root dry weight of mustard seedlings by decreasing the inhibitory effect of stress (Table 1). Strains showed variable response in improving the root dry weight under un-stressed conditions and Cd-stress. Maximum improvement (49.5%) in root dry weight was noted by strain FN13 under un-stressed



conditions. The same strain was also better to improve the root dry weight under Cd-stress where the increase was 35.6 and 31.4% at 100 and 150 $\mu\text{g mL}^{-1}$ of Cd^{2+} . The effect of other two strains on root dry weight was not significantly different than control at both levels under Cd-stress.

As observed in case of shoot dry weight, the Cd-stress significantly decreased the total dry matter of mustard seedlings at both levels (Figure 4). Inoculation lessens the negative effect of Cd-stress thus resulted in improvement of total dry matter. Inoculation with these strains was, however, more effective in enhancing the total dry matter under unstressed conditions than Cd-stressed conditions. Under Cd-stress, the maximum effect was noted due to inoculation with strain FN13 at both levels. Overall, inoculation with Cd tolerant strains was effective under stress. Data regarding seedling vigor index (SVI) showed that the inoculation was effective in enhancing the SVI of mustard seedlings under un-stressed conditions and Cd-stress (Figure 5). It was observed that Cd-stress significantly reduced the seedling vigor index and the minimum SVI was observed at 150 $\mu\text{g mL}^{-1}$ of Cd^{2+} . Under Cd-stress, all the strains significantly improved the SVI of

mustard seedlings at both levels of Cd^{2+} . At 100 and 150 $\mu\text{g mL}^{-1}$ of Cd^{2+} , the maximum improvement in SVI was given by the strain FN13 that showed significantly better results than other strains and the un-inoculated control.

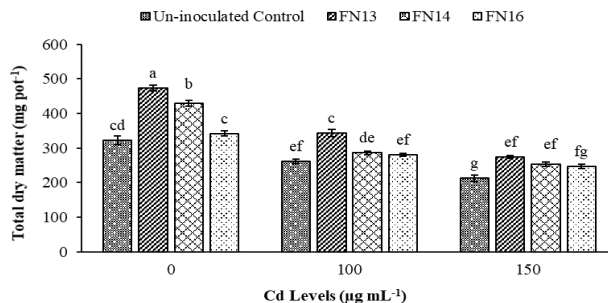


Figure 4: Effect of Cd-tolerant, IAA-producing rhizobacterial strains on total dry matter of *Brassica juncea* (L.) seedlings under Cd stress in jar trial

(n =3); Means with same alphabets do not differ statistically from one another ($p \leq 0.05$)

Table 1: Effect of Cd-tolerant, IAA-producing rhizobacterial strains on shoot growth of *Brassica juncea* (L.) seedlings under Cd stress in jar trial; (n =3)

Isolate	Cadmium level ($\mu\text{g mL}^{-1}$)		
	0	100	150
Shoot length (cm)			
Un-inoculated Control	5.167 \pm 0.03 cd	4.03 \pm 0.07 f	3.27 \pm 0.07 g
FN13	5.767 \pm 0.03 a	5.47 \pm 0.09 a-c	4.77 \pm 0.09 e
FN14	5.533 \pm 0.07 ab	4.87 \pm 0.09 de	4.20 \pm 0.06 f
FN16	5.367 \pm 0.07 bc	4.77 \pm 0.03 e	4.23 \pm 0.07 f
LSD ($p \leq 0.05$)	0.3363		
Root length (cm)			
Un-inoculated Control	4.67 \pm 0.07 c	4.10 \pm 0.10 d	3.10 \pm 0.06 e
FN13	5.43 \pm 0.09 a	5.20 \pm 0.06 ab	4.60 \pm 0.06 c
FN14	5.20 \pm 0.12 ab	4.87 \pm 0.03 bc	4.07 \pm 0.09 d
FN16	5.27 \pm 0.03 a	4.67 \pm 0.07 c	4.07 \pm 0.03 d
LSD ($p \leq 0.05$)	0.3638		
Shoot dry weight (mg pot⁻¹)			
Un-inoculated Control	261 \pm 9.54 cd	212 \pm 4.73 e	172 \pm 7.87 f
FN13	381 \pm 6.39 a	276 \pm 7.52 c	221 \pm 3.85 e
FN14	345 \pm 6.81 b	231 \pm 3.79 de	205 \pm 4.64 e
FN16	275 \pm 5.87 c	226 \pm 2.91 c	201 \pm 4.92 ef
LSD ($p \leq 0.05$)	30.726		
Root dry weight (mg pot⁻¹)			
Un-inoculated Control	62 \pm 2.65 bc	50 \pm 1.20 de	40 \pm 2.34 f
FN13	93 \pm 1.45 a	67 \pm 2.34 b	53 \pm 1.16 de
FN14	84 \pm 1.67 a	55 \pm 1.77 cd	49 \pm 1.16 d-f
FN16	67 \pm 1.45 b	54 \pm 1.45 c-e	46 \pm 1.77 ef
LSD ($p \leq 0.05$)	8.9921		

Means with same alphabets do not differ statistically from one another ($p \leq 0.05$)



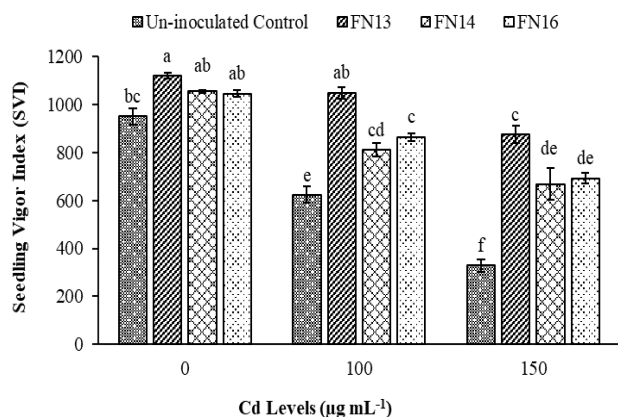


Figure 5: Effect of Cd-tolerant, IAA-producing rhizobacterial strains on seedling vigor index (SVI) of *Brassica juncea* (L.) seedlings under Cd stress in jar trial

(n =3); Means with same alphabets do not differ statistically from one another ($p \leq 0.05$)

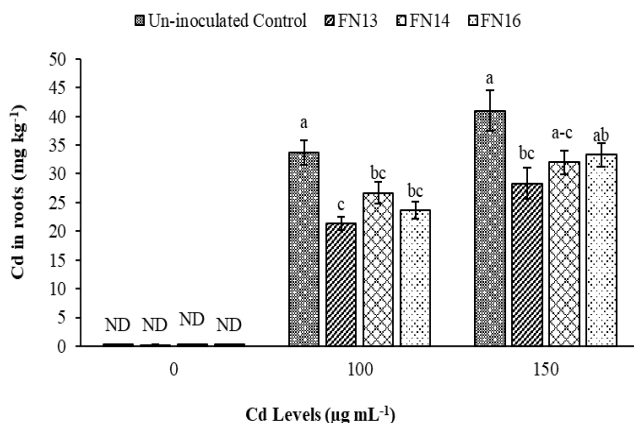


Figure 6: Effect of Cd-tolerant, IAA-producing rhizobacterial strains on Cd concentration in root of *Brassica juncea* (L.) seedlings under Cd stress in jar trial

(n =3); Means with same alphabets do not differ statistically from one another ($p \leq 0.05$)

Effect of Cd-tolerant, IAA-producing rhizobacterial strains on Cd uptake and translocation in *Brassica juncea* (L.) under Cd stress

It was observed that Cd-stress elevated the Cd concentration on root of mustard seedlings but at both levels of Cd-stress the difference in Cd concentration in roots was non-significant (Figure 6). Inoculation significantly reduced the Cd concentration in root of *Brassica juncea* (L.) seedlings at both levels of Cd²⁺ stress. At 150 $\mu\text{g mL}^{-1}$ of

Cd²⁺, maximum Cd concentration (41.0 mg kg⁻¹) was observed in the roots of plants under un-inoculated control. At 100 $\mu\text{g mL}^{-1}$ of Cd²⁺, maximum Cd concentration (33.67 mg kg⁻¹) was also observed in the roots of plants under un-inoculated control. The strains FN14 and FN16 also decreased the Cd concentration in roots of mustard seedlings up to 20.8 and 29.8%, respectively, but this decrease was not significantly different than control.

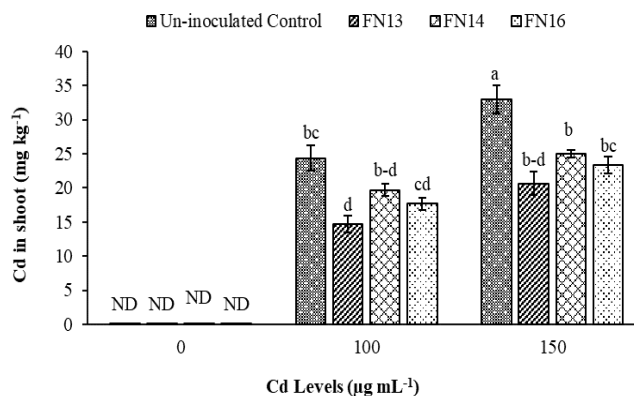


Figure 7: Effect of Cd-tolerant, IAA-producing rhizobacterial strains on Cd concentration in shoot of *Brassica juncea* (L.) seedlings under Cd stress in jar trial

(n =3); Means with same alphabets do not differ statistically from one another ($p \leq 0.05$)

As observed in case of Cd concentration in roots, Cd-stress conditions also increased the Cd concentration in shoot of mustard seedlings but in contrast to Cd concentration in roots, the shoot Cd was significantly different at both levels of Cd-stress (Figure 7). The Cd concentration in shoot of *Brassica juncea* (L.) seedlings was also significantly decreased due to inoculation with Cd-tolerant, IAA-producing rhizobacterial strains at both levels of Cd²⁺ stress. It was observed that Cd-concentration in shoot was less as compared to Cd in roots of mustard plants at both levels. At 150 $\mu\text{g mL}^{-1}$ of Cd²⁺, maximum Cd concentration (33.0 mg kg⁻¹) was observed in the shoot of plants under un-inoculated control treatment. The strain FN13 was the most effective treatment in reducing the Cd concentration in shoot of mustard plants that reduced the Cd concentration up to 37% less than control. As observed in case of higher-level Cd-stress, at 100 $\mu\text{g mL}^{-1}$ of Cd²⁺, maximum Cd concentration (24.33 mg kg⁻¹) was also observed in the shoot of plants under un-inoculated control. Minimum Cd concentration was observed in the shoot of plants inoculated with rhizobacterial strain FN13 where 39.7%

less Cd was observed, and it was significantly less than control.

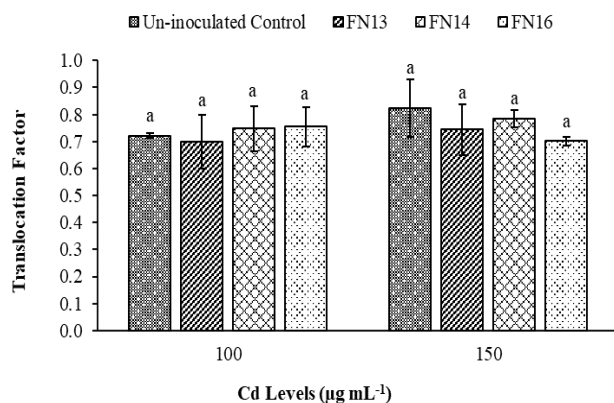


Figure 8: Effect of Cd-tolerant, IAA-producing rhizobacterial strains on translocation of Cd from root to shoot of *Brassica juncea* (L.) seedlings under Cd stress in jar trial

(n =3); Means with same alphabets do not differ statistically from one another ($p \leq 0.05$)

Results showed that increased concentration of Cd in the root zone enhanced the translocation the translocation factor of Cd for mustard seedlings. The increase was, however, non-significant (Figure 8). Inoculation with Cd-tolerant, IAA-producing rhizobacterial strains showed variable response on the translocation of Cd from root to shoot of *Brassica juncea* (L.) seedlings at both levels of Cd stress. At low level of Cd-stress i.e. $100 \mu\text{g mL}^{-1}$ of Cd^{2+} , the strain FN13 decreased the translocation factor while the strains FN14 and FN16 enhanced Cd-translocation from root to shoot but this impact was non-significant for all strains. At higher level of Cd-stress i.e. $150 \mu\text{g mL}^{-1}$ of Cd^{2+} , all the strains decreased the translocation factor thus helping in reduction of Cd translocation from root to shoot. The impact of these strains was non-significant when compared with respective un-inoculated control. The minimum translocation factor was observed for the strain FN16 followed by FN13 while the un-inoculated control plants showed the maximum translocation factor for Cd.

Results of the present studies showed that with increase in concentration of Cd in the sowing bed the bioaccumulation factor of Cd was decreased for mustard seedlings. However, this decrease was non-significant in case of un-inoculated plants (Figure 9). Inoculation with Cd-tolerant, IAA-producing rhizobacterial strains showed a significant decrease in Cd bioaccumulation factor in shoot of *Brassica juncea* (L.) seedlings at both levels of Cd-stress. At both levels of Cd^{2+} , the strain FN13 was the

most promising one in decreasing the bioaccumulation of Cd in mustard seedlings followed by FN16. Both these strains showed significantly less bioaccumulation of Cd in mustard shoot when compared with respective un-inoculated control at both levels of Cd-stress. The results of FN14 were however, non-significant with respective un-inoculated control at both levels of Cd-stress.

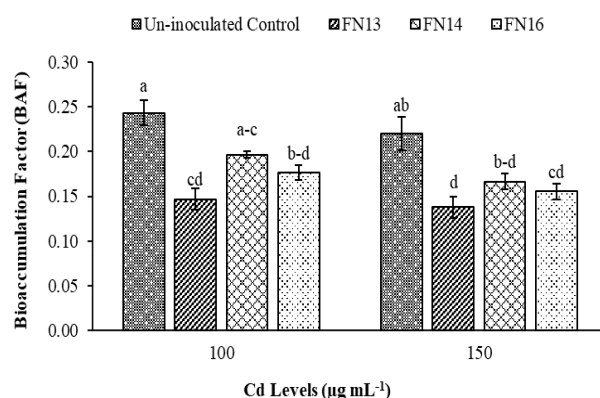


Figure 9: Effect of Cd-tolerant, IAA-producing rhizobacterial strains on Bioaccumulation of Cd by *Brassica juncea* (L.) seedlings under Cd stress in jar trial

(n =3); Means with same alphabets do not differ statistically from one another ($p \leq 0.05$)

Discussion

Heavy metal pollution due to use of untreated wastewater and industrial effluents for crop production (Khalid *et al.*, 2018) is the major problem for vegetables and fodder crops especially around metropolitans and industrial cities all over the globe (Ghorani-Azam *et al.*, 2016). These heavy metals are accumulated in edible portion/above ground parts of growing crops (Chaoua *et al.*, 2019) which ultimately enter the food chain due to consumption of vegetables and fodders by human and animals. Heavy metals are toxic for soil, plants, animals and human beings and cadmium among the most toxic ones (Farid *et al.*, 2015).

A check on the bioavailability of heavy metals can reduce their toxic effects on food web by decreasing their uptake and accumulation (Saengwilai *et al.*, 2019). The reduced uptake and translocation of heavy metals to upper parts of plants is employed as adaptive strategy under heavy metal stress (Ghani *et al.*, 2010). Although microbial inoculation can also be used as a tool to enhance heavy metal uptake leading to remediation of contaminated soils and water (Ning *et al.*, 2019), the phytostabilization of heavy metals using soil microbes can be an effective strategy in this regard (Saran *et al.*, 2020).



Effect of Cd-tolerant, IAA-producing rhizobacterial strains on germination of *Brassica juncea* (L.) under Cd stress

Seed germination is important for better crop stand and ultimately the yield of the crops. Seeds of the crops sense the environmental stresses and reduced germination is the first defense mechanism used by seeds to cope with environmental stresses (Li *et al.*, 2005). Heavy metals have severe effect on seed germination of crop plants. Cadmium is more toxic to seed germination than other heavy metals (Bautista *et al.*, 2013). In the present study, Cd-stress significantly decreased the seed germination and the effect was more pronounced at higher levels of Cd-stress. The germination is completely ceased at 300 $\mu\text{g mL}^{-1}$ of Cd-stress. This might be due to the damage caused by Cd-stress to the living endosperm (Sung *et al.*, 2008) that acts as protective barrier that encloses the embryo (Kranmer and Colville, 2011).

Soil microorganisms can influence germination of crop plants through production of different kind of plant growth regulators (Egamberdiyeva, 2007). Seed germination is directly related to the endogenous hormone production especially auxins and gibberellins (GAs) by crop plants as they play a major role in tissue differentiation, cell division and elongation and root initiation (Nadeem *et al.*, 2014). The phytohormone-producing bacteria can be beneficial to improve crop growth and germination under stressed environments (Bokhari *et al.*, 2020).

In our studies, inoculation with Cd-tolerant, IAA-producing rhizobacterial strains improved the germination of *Brassica juncea* (L.) under Cd stress. This improvement of seed germination under Cd-stress might be due to production of IAA and exopolysaccharides by inoculated strains which decreased the inhibitory effects of Cd-induced osmotic stress thus positively influencing the seed germination. Previous studies report the variable response on seed germination of crop species. For example, Egamberdiyeva (2007), during bioassay, confirmed the improvement of seed germination due to inoculation with *Bacillus* strains and they attributed this improvement with production of PGP substances by the inoculated strains.

Effect of Cd-tolerant, IAA-producing rhizobacterial strains on growth and physiological parameters of *Brassica juncea* (L.) under Cd stress

Environmental stresses affect the growth of crop plants at different growth stages. Plants respond to these stresses by changing their morphology, root and shoot architecture,

and physiology. Cadmium reduces the uptake of water and nutrients, and poses injuries to growing roots (Jibril *et al.*, 2017). It reduces the root length, root surface area and overall volume of roots (Wang *et al.*, 2015). In our study, Cd-stress decreased the root and shoot growth by severely affecting the parameters under study. This might be due to reduction in water and nutrient uptake of mustard plants exposed to Cd-stress that decreased the root growth leading to decrease in shoot growth by maintaining the root/shoot ratio. Our studies are supported the work of Dutta *et al.* (2018) who reported that Cd-stress negatively affected the growth of *B. juncea* thus decreased root and shoot growth of mustard plants under Cd-stress. Cadmium stress also decreases the root volume thus changes the size of the root system (Wang *et al.*, 2015) that affects the exposure of roots to soil and nutrients that decreases the crop performance.

Stress tolerance can be induced in crop plants by inoculation with PGPR having ability to produce different plant growth regulators or exopolysaccharides (Zhou *et al.*, 2017). It has also been observed that bacterial strains involved in improving plant growth under stress are not effective under un-stressed conditions. For example, Bokhari *et al.* (2020) reported that *Bacillus circulans* strains PK3-15 and PK3-109 enhanced plant growth as compared to control, but these strains lost their effectiveness under un-stressed conditions. However, *Bacillus* strains have been well documented to be involved in inducing physiological alterations in plants as an adaptive strategy under stress (Radhakrishnan *et al.*, 2017). The role of Cd immobilizing bacterial strains in combination with different immobilizing amendments have also shown their worth under stress (Saeed *et al.*, 2019)

In the present study, inoculation with Cd-tolerant, IAA-producing PGPR improved the growth of *Brassica juncea* L. under Cd-stress. These strains enhanced the root and shoot growth thus improving the total dry matter of mustard plants. This increase in plant biomass is attributed to improvement in root growth due to production of IAA by these strains that led to enhanced shoot growth. The IAA production by inoculated strains thus might have helped in enhanced root proliferation leading to improvement in root-shoot growth and biomass accumulation by crop plants. It is well documented that soil bacteria synthesize auxins using L-tryptophan as precursor thus help in improvement of plant growth (Ahmad *et al.*, 2013b; Hussain *et al.*, 2014). It has been reported that *Bacillus aryabhattai* SRB02 can tolerate oxidative stress thus promote soybean growth by changing the phytohormones level of plants (Park *et al.*, 2017)

This increase in plant growth might also be due to production of exopolysaccharides and siderophores by



inoculated strains as observed in laboratory studies. The EPS produced by bacterial strains help in binding the soil particles, improve soil structure, increase moisture retention and help in biofilm formation by the bacterial strains (Marchal *et al.*, 2011). Inoculation with ESP-producing bacteria thus can help to enhance nutrients bioavailability leading to increase crop stand and biomass under stressed conditions (Zhou *et al.*, 2017). Previous studies report that PGPR increase crop tolerance to Cd-stress due to production of exopolysaccharides, siderophores, and plant hormones along with other PGP traits (Lin *et al.*, 2016).

The improvement in root and shoot growth leading to total plant biomass due to inoculation with EPS-producing rhizobacteria of wheat under drought-induced osmotic stress has also been reported in previous studies (Hussain *et al.*, 2014). The EPS produced by rhizobacteria initiate biofilm formation and help to protect plants from environmental anomalies (Vanderlinde *et al.*, 2010). The EPS-producing bacteria also help plants to extract more water and nutrients from the soil due to better bioavailability. These exopolymers bind the cations in soil (Gadd, 2004) thus can help in phytostabilization of heavy metals including Cd in soil.

In our study, inoculation with Cd-tolerant, IAA-producing rhizobacterial strains reduced the negative effects of Cd-stress on SPAD value thus causing a significant improvement. The improvement in SPAD value under Cd-stress might be due to EPS and IAA- production ability of these strains. Similarly, improvement in SPAD value and relative water contents of maize and wheat crops under Cd-stress has been reported by Ahmad *et al.* (2014). In another study, Nadeem *et al.* (2009) reported that inoculation with rhizobacterial strain *P. fluorescens* increased the chlorophyll contents and relative water contents of maize under salinity-induced osmotic stress. Ahmad *et al.* (2011) conducted a series of experiments on mungbean under salinity stress and reported an improvement in SPAD value and relative water contents as compared to control plants. They attributed these positive results of inoculation to production of IAA, and ACC-deaminase activity of inoculated strains (Ahmad *et al.*, 2013b) along with other growth promoting attributes (Tang *et al.*, 2019).

Effect of Cd-tolerant, IAA-producing rhizobacterial strains on Cd uptake and translocation in *Brassica juncea* (L.) under Cd stress

Cadmium is toxic to plants at all stages of growth. It is transported in plants through several processes, metal transporters and channels before its accumulation in edible parts (Ismael *et al.*, 2019). Moreover, its bioavailability,

uptake, translocation in plant tissues and toxicity is affected by many factors (Bali *et al.*, 2020). Higher Cd uptake in crop plants negatively affects plant physiology due to interruption in mineral uptake and water uptake in plants (Jibril *et al.*, 2017). It alters the membrane permeability, lowers leaf water potential, and affects the efficiency of proton pumps in crop plants (Karcz and Kurtyka, 2007). Moreover, Cd reduces root hydraulic conductivity due to lower activity of aquaporins leading to reduced turgor pressure and leaf growth (Ehlert *et al.*, 2009). The increased Cd uptake thus can seriously damage the root-shoot growth and plant physiology. In the present study, Cd-stress significantly enhanced the Cd concentration in root and shoot of mustard plants as compared to un-stressed control plants. The higher Cd uptake also induces higher production of ROS which change the plant physiology thus shifts the plant metabolic activities (Tripathi *et al.*, 2017). The enhanced bioaccumulation of heavy metals also alters the plant metabolic functions and cause ROS-induced damages to plants (Gill *et al.*, 2011).

In our studies, inoculation with Cd-tolerant PGPR reduced the uptake and translocation of Cd to above ground parts of mustard crop. This might be due to EPS and IAA-producing ability of these PGPR strains. It has been well documented that cadmium tolerant PGPR strains help to reduce Cd uptake by crop plants by stabilizing it in the soil through production of exopolysaccharides (Zhang *et al.*, 2019). For example, Lin *et al.* (2016) reported the reduced uptake and accumulation of Cd in rice grains.

The PGPR can use different mechanisms for the solubilization or stabilization of heavy metals in metal polluted environment (Hamidpour *et al.*, 2019). Phytostabilization of heavy metals by using EPS, and IAA producing bacterial strains has also been reported by Ojuederie and Babalola (2017). Rajkumar *et al.* (2013) reported that *Bacillus megaterium* effectively improved plant growth by decreasing the uptake and translocation of heavy metals in mustard and other crops. Recently, Zeng *et al.* (2020) reported that Cd-tolerant PGPR in combination with hydroxyapatite helped in the immobilization of Cd in soil thus reducing their uptake. They argued that enhanced sequestration of Cd in soil was due to production of phytohormones, siderophores and polymeric substances. Although, inoculation with PGPR may enhance the uptake and translocation of heavy metals in plants as reported by Liu *et al.* (2020), the inoculation with Cd-tolerant PGPR can, however, be used as an effective strategy to reduce the uptake and translocation of Cd to above ground parts (Okolie *et al.*, 2020).

In a recent study conducted by Naveed *et al.* (2020b), *Enterobacter* sp. MN17 inoculation in combination with



gravel sand and biochar improved the nutritional quality and crop growth of *Pisum sativum* by reducing the Cd uptake. They attributed the improvement in nutritional quality with reduced Cd uptake that resulted due to enhanced Cd immobilization in the inoculated treatments. The enhanced immobilization was linked with the Cd removal by bacterial strain and production of metal-chelating proteins thus resulting in reduction in available fraction of Cd in soil (Naveed *et al.*, 2020a). They suggested the combined use of *Enterobacter* sp. MN17, gravel sand, and biochar as an effective approach to grow pea and other vegetables under Cd contaminated soils.

Conclusion

Due to high efficiency of Cd removal, and siderophores, exopolysaccharides and IAA production ability, the strain FN13 can be a potential phytostabilizing agent for heavy metal contaminated soils that should be effective to grow vegetables, fodders and oilseed crops in heavy metal polluted soils irrigated with wastewater around industrial areas and big cities. The bioremediation potential of *Bacillus safensis* has also been reported along with multiple PGP traits (Priyalaxmi *et al.*, 2014) but little is known about the phytostabilization potential and Cd accumulation in body by *Bacillus safensis*. It is concluded that the strain FN13 can be effective to stabilize heavy metals in polluted soils thus can help to grow crop plants with less concentration of heavy metals in edible portions of plants.

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