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Research Article

Exploitation of IAA Producing PGPR on mustard (*Brassica nigra* L.) seedling growth under cadmium stress condition in comparison with exogenous IAA application

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Abstract

Soil pollution by cadmium (Cd) is a global threat for plants and animals. Exogenous auxin application reduces the Cd stress on plants. Moreover, IAA production by rhizospheric bacteria can play a key role in plant growth and development by promoting cell division, cell elongation, cell differentiation, flowering and lateral root formation. The present study was to evaluate the efficiency of IAA producing, Cd tolerant plant growth promoting rhizobacteria for plant (*Brassica nigra* L.) growth under Cd stressed condition comparing with the external synthetic auxin application. *Lysinibacillus varians* and *Pseudomonas putida* were isolated previously as IAA producing PGPR and selected in this study for their exploitation in plant growth development under Cd stressed condition. The impact of external synthetic IAA application significantly increased the plant growth under Cd amended soil. Whereas, PGPR inoculation also showed significant (p<0.05) elevation in germination percentage, root and shoot length, chlorophyll content and other growth parameters of Cd stressed *Brassica* plants which were comparative to synthetic IAA application. So, these selected PGPRs (*L. varians* and *P. putida*) can be used as biofertilizer which ameliorate the adverse effect of cadmium.

Keywords: Cadmium stress; IAA; plant growth promoting rhizobacteria; plant growth.

Citation
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Introduction

The world population is increasing day by day. Agricultural production has increased by the use of high yielding varieties along with chemical fertilizers and pesticides application. The pursuit for producing more crop yield by excessive use of chemicals cause deterioration effects in the chemical, physical and biological health of cultivable land (1). So, the futuristic agricultural development is absolutely important for the sustainable advances (2). Moreover, soil pollution by different heavy metals is one of the major threats for agricultural development. The primary source of soil pollution includes the accumulation of heavy metals through emissions from the rapidly expanding industrial areas, disposal of high metal wastes, metal forging, spillage of petrochemicals, application of fertilizers, sewage sludge application,

pesticides, wastewater irrigation in agronomic practices (3). Though these sewage effluents are a good source of organic matter with other nutrients but also they elevate different heavy metals like Fe, Mn, Cu, Zn, Pb, Cr, Ni, Cd and Co (4,5). Heavy metals can found on the surface and in the tissue of fresh vegetables. Prolonged consumption of unsafe concentrations of heavy metals in foodstuffs may cause the disruption of numerous biological and biochemical processes in the human body.

Cadmium slows down the plant growth by inhibiting many enzymes, involved in respiration and photosynthesis (6,7). Cadmium stresses also inhibits the germination percentage, root or shoot development and other plant growth parameters (8,9). Yoan and Huang showed that Cd increased the nitric oxide (NO) accumulation in the root, which hold back the transport of auxin by inhibiting PIN1 protein activity. Inhibited PIN1 protein reduces the root apex auxin concentration resulting reduced root meristem size (10). PINFORMED1 (PIN1) protein is an auxin efflux carrier, involved in the maintenance of the root meristem growth (11). Moreover, PIN1 was also reported as a key protein involved in the auxin circulation under heavy metal stress condition (12).

Cadmium stress decrease the endogenous level of auxin by disturbing the IAA homeostasis in the root tips (13) that suppress the primary root elongation (14). Exogenous application of auxin or its precursor L-TRP can increase the endogenous levels of auxins which ultimately enhanced the plant growth and yield under Cd stress (15). So, external application of auxin reduces the Cd stress on plants. Furthermore, IAA producing plant growth promoting rhizobacteria (PGPR) can be an attractive alternative for replacing the synthetic auxin application to reduce the Cd stress and to decrease the use of chemical fertilizers for reclamation of environmental pollution (9). Cd tolerant PGPR can maintain the soil fertility in Cd contaminated soil by different direct or indirect mechanism such as to solubilise insoluble phosphate or potassium, nitrogen fixation, siderophore production, ammonium production, phytohormone such as IAA production etc (8,9,16).

The objective of the study was to determine the effectiveness of IAA producing PGPR strains in comparison with the external synthetic auxin application for the plant growth and development under Cd stress condition.

Materials and Methods

Collection of Bacteria

Two potent cadmium tolerant plant growth promoting rhizobacteria were isolated previously by standard microbiological techniques. These bacteria were already characterized and identified as *Lysinibacillus varians* (NCBI GenBank accession number MG976681) and *Pseudomonas putida* (NCBI GenBank accession number MG976684) for further research work.

Characterization of Bacteria

The collected Cd resistant PGPR isolates were previously characterized by morphological and biochemical properties such as amylase, Catalase, methyl red, citrate utilization, gelatine hydrolysis, indole production, urease with standard protocol (9).

Plant growth promoting ability of the bacterial isolates was determined such as IAA production (17), Phosphate solubilization (18), ammonia production (19), HCN production (20), Nitrogen-fixing ability (21) and siderophore production (22).

Exploitation of PGPR on growth and development of mustard *(Brassica nigra* L.*)* seedlings in heavy metal (Cd) stress condition

Collection of seed

Mustard (*Brassica nigra* L.) was used as test plant in this experiment. Seeds were collected from Bidhan Chandra Krishi Viswavidyalaya, W.B.

Dose determination

Initially mustard seeds were treated with different concentration of Cd (5, 10, 20, 40, 80, 160, 320, 640 ppm) for dose determination of the seeds. The concentration of Cd, at which the growth of seedling was affected to some extent but not too much leading to death (*i.e.* 10 ppm of Cd), was taken for consideration in this study. In the second set of experiments different IAA concentrations (0, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) were applied from the germination state to seedling state of *Brassica*. On the basis of effective seedling growth, 10^{-6} M of IAA was determined for further experiments.

Seed germination test

The Mustard seeds were surface sterilized/ disinfected to avoid the presence of any pathogenic microorganisms on the seed surface by dipping the seeds for 3 minutes in 0.1% $HgCl_2$ and washing 4 times in sterilized water. Initially bacterial cultures were prepared in NA slant. 48 hrs old fresh cultures was scrapped with sterile inoculating needle and suspended in sterile distilled water. Seeds were then inoculated with water suspension of bacterial culture (~ 10^6 cells/ml). After 10 hrs of inoculation the seeds were placed on the sterile blotting paper in different experimental setups. Number of seed germination was recorded after 24 hrs intervals up to 6 days. The different experimental setups are as follows:

- Control
- Cadmium (10ppm)
- IAA (10-6M)
- Cd+IAA
- Cd+A
- Cd+T

Table 1. Colony morphology, Gram nature, Biochemical characterization and PGP ability of the Bacterial isolates

Bacterial isolates	Minimal Inhibitory Concentration of Cadmium	Colony Morphology & Gram nature	Biochemical characterization	Plant growth promoting ability
A (***L. varians***)	150 ppm	Creamy yellow, circular with serrated margins, opaque with a glossy surface, Gram-positive, rod	Catalase, methyl red, citrate utilization tests positive and amylase, gelatin hydrolysis, indole production, urease tests negative	ammonia production, IAA production (20.43 ± 0.318 µg/ml), Nitrogen-fixing ability, low phosphate solubilization and low siderophore production positive
T (***P. putida)***	150 ppm	Creamy white, circular entire margin with a glossy surface, Gram-negative rod	Catalase, methyl red, citrate utilization positive and amylase, gelatine hydrolysis indole production, urease test negative	phosphate solubilisation, ammonia production, IAA production (20.05 ± 0.266 µg/ml), low siderophore production

NB: Cd=10 ppm, IAA=10^{-6}M, A=*Lysinibacillus varians* and T=*Pseudomonas putida*

Germination percentage were calculated by the following formula:

Germination percentage = (Total no of germinated seeds / Total no of seeds) x 100

Pot Experiments

For exploitation of PGPR, pots were prepared with sterile sleeved soil and sand as one third depth of pot were filled with sterile sand and upper remaining portion were filled with fine sterile soil. Imbibed seeds were shown in the different experimental set up as described earlier, but in case of bacterial inoculation *Brassica* seeds were imbibed in the water suspension of selected bacteria separately. All the experimental setups were triplicated and continuously altered their position for elimination of position effect.

Growth parameters

Up to 75 days of seedling development, different growth parameters such as shoot length (cm), root length (cm), number of leaves, leaf area (cm^2), inflorescence length (cm), fresh weight (mg), dry weight (mg), seed weight (mg), chlorophyll content (mg/gm of tissue), vigour index (cm) and relative water content were recorded.

Estimation of chlorophyll

0.5g of each plant material was taken in the test tubes containing 10ml of methanol and kept in dark (23). After 24 hrs, the supernatant solution from each test tube was taken for measurement of absorbance at 470, 652 and 665 nm [A470, A652 and A665] by the help of Spectrophotometer (Microprocessor visible, model no-LI-722, Lasany, Made in India).

Chl a (mg/gm tissue) = 16.29×A665-8.54×A652

Chl b (mg/gm tissue) = 30.66×A652-13.58×A665

Chl a+b (mg/gm tissue) = 22.12×A652+2.71×A665

Vigour index

Vigour index was calculated using the following formula suggested by Abdul-Baki and Anderson (24) and expressed as whole number.

Vigour index = Germination (%) x Total seedling length (cm)

Relative Water Content

RWC was calculated using the following formula

RWC (%) = (FW−DW / TW−DW) × 100.

Where, FW = Fresh weight

DW = Dry weight

TW = Turgid weight.

Statistical analysis

Standard error (SE) of all experiments was intended from triplicates (n=3) and represented in the Table (value ± SE**)** and figures (error bar). Variations within the experimental groups were considered unpaired t-test. Difference in between control and cadmium treated groups are denoted by lower case alphabet 'a'. Whereas, lower case alphabet 'b' denotes the differences between Cd treated groups and respective other groups. Asterisk (*) above the bars indicate significant level.

Results and Discussion

IAA is one of the major phytohormone which can be produced by many rhizobacteria. In this study two potent PGPR were selected as potential IAA producers. *L. varians* and *P. putida* both produced approximately 20 ppm of IAA along with other plant growth promoting abilities (Table 1).

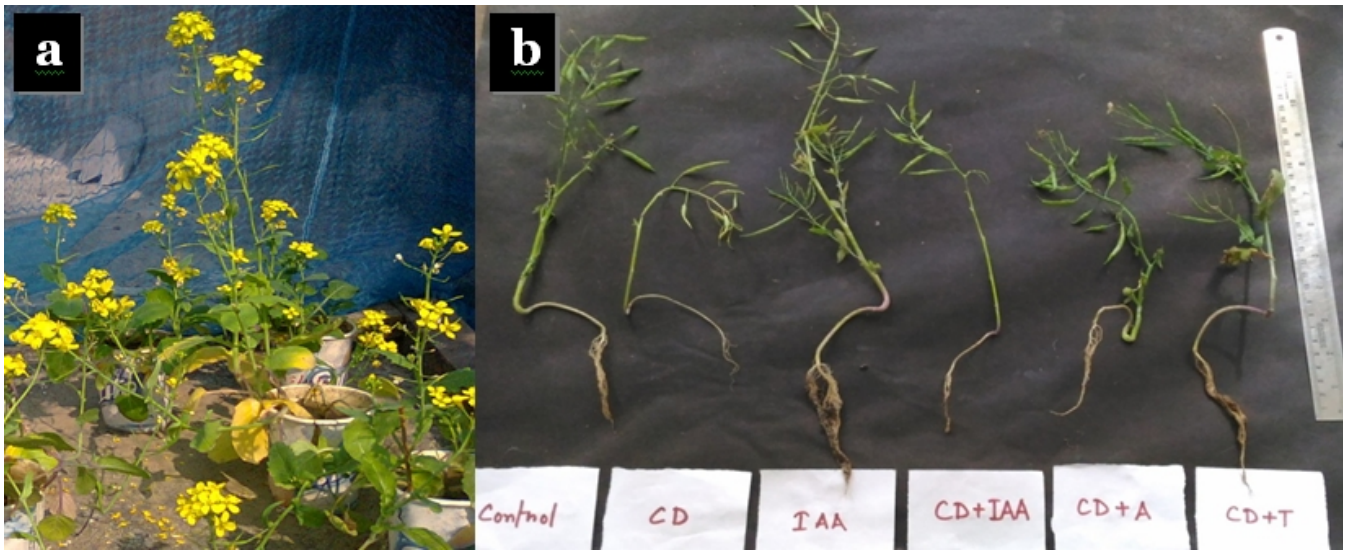
Effect on plant growth

Cadmium application adversely affected the seed germination (Fig. 1). But it was rather improved with the exogenous IAA application by 1.09 fold.

Table 2. Plant growth parameters in different experimental setups

TREATMENT	SHOOT LENGTH (cm)					Root length (cm) (75 days)	NUMBER OF LEAVES					INFLORESCENCE LENGTH (45 DAY)	NUMBER OF FRUITS		FRESH WEIGHT (g) (75 day)	DRY WEIGHT (mg) (75 day)	SEED WEIGHT (mg) (75 day)	Leaf area (cm^2) (45 day)
	15 DAY	30 DAY	45 DAY	60 DAY	75 DAY		15 DAY	30 DAY	45 DAY	60 DAY	75 DAY		60 DAY	75 DAY				
CONTROL	5.5 ±0.35	5.623 ±0.27	19.38 ±0.26	24.88 ±0.44	25.80 ±0.37	13.98 ±1.09	6	8	8	8	0	6.49 ±0.37	9	12	1.54 ±0.148	25.45 ±9.43	6.8 ±0.1	87.54 ±1.68
Cd	3.49 ±0.26 a***	4.43 ±0.41 a**	8.41 ±0.13 a***	11.62 ±0.169 a***	14.66 ±0.43 a***	8.25 ±0.85 a***	3	5	5	4	0	4.2 ±0.44 a**	5 a**	5 a**	0.84 ±0.07 a*	15.71 ±0.68 a***	2.75 ±0.21 a***	46.06 ±1.58 a***
IAA	4.98 ±0.237 b***	5.756 ± 0.08 b**	17.616 ±0.336 b***	21.85 ±0.189 b***	24.75 ±0.478 b***	16.93 ±0.99 b***	4	8	9	6	0	6.57 ±0.26 b***	8 b*	13 b***	1.54 ±0.126 b*	36.13 ±1.39 b***	7.52 ±0.25 b***	104.46 ±1.12 b***
Cd + IAA	4.83 ± 0.21 b**	5.756 ±0.329 b**	17.76 ±0.37 b***	21.11 ±0.24 b***	25.44 ±0.59 b***	11.19 ± 0.63 b***	3	5	7	5	0	4.86 ±0.42 NS	5 NS	9 b**	1.1 ±0.09 b*	24.91 ±0.25 b***	4.45 ±0.12 b***	84.77 ±2.89 b***
Cd + A	6.37 ±0.26 b***	6.43 ±0.181 b***	20.72 ±0.33 b***	24.623 ±0.298 b***	25.30 ±0.48 b***	17.16 ± 1.12 b***	4	4	7	5	1	5.93 ±0.50 b**	5 NS	8 b*	1.03 ±0.12 NS	22.95 ±1.13 b***	3.69 ±0.14 b**	106.92 ±0.80 b***
Cd+ T	6.103 ±0.24 b***	6.123 ± 0.44 b***	17.62 ±0.19 b***	20.08 ±0.46 b***	23.8 ±0.39 b***	17.52 ±0.87 b***	3	5	7	6	1	5.906 ±0.14 b**	6 NS	10 b**	1.36 ±0.092 b*	26.133 ±1.48 b***	4.8 ±0.254 b***	101.86 ±4.18 b***

Difference in between control and cadmium treated groups are denoted by lower case alphabet ‘a’. Whereas, lower case alphabet ‘b’ denotes the differences between Cd treated groups and respective other groups. Asterisk (*) indicates significant level (A- *L. varians*, B- *P. putida*).



Photograph 1. Shoot & root length measurement of brassica plant (b). (a- experimental set) (A- *L. varians,* B- *P. putida*)

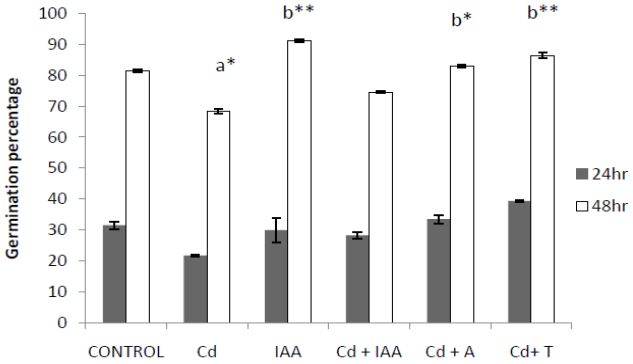


Fig. 1. Seed germination under Cd stress condition

Difference in between control and cadmium treated groups are denoted by lower case alphabet 'a'. Whereas, lower case alphabet 'b' denotes the differences between Cd treated groups and respective other groups. Asterisk (*) above the bars indicate significant level. (A- *L. varians, B- P. putida*).

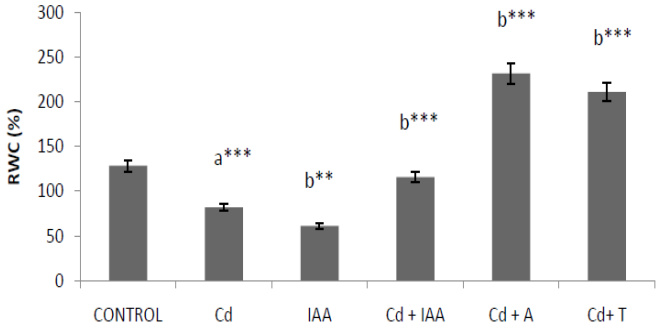


Fig. 2. RWC (%) under Cd stress condition

Difference in between control and cadmium treated groups are denoted by lower case alphabet 'a'. Whereas, lower case alphabet 'b' denotes the differences between Cd treated groups and respective other groups. Asterisk (*) above the bars indicate significant level. (A- *L. varians, B- P. putida*).

Whereas, PGPR mediated seed germination was also increased under Cd stressed condition by 1.21 and 1.26 fold respectively for *L. varians* and *P. putida* inoculation. It was previously reported that Cd stress decreased seed germination of different crops (8,9,25,26). It was important to note that the PGPR isolates have some positive effects on seed germination. So, this study indicates that PGPR can safely use for enhancement of seed germination in heavy metal contaminated soils.

Application of Cd in the soil system reduced the plant growth than the control plant (Table 2; Photograph 1). Application of synthetic auxin significantly (p<0.05) increased the shoot length and root length under cadmium stressed soil than the heavy metal stressed condition alone. Both the selected IAA producing PGPRs showed somewhat similar kind of growth enhancement like that of exogenous auxin application under Cd stress. Inoculation of *L. varians* and *P. putida* certainly reduced the adverse effects of Cd stress. It was previously reported that the bad effects of Cd become more severe with the increased heavy metal concentration (26). Aydinalp and Marinova also showed that 20 ppm of Cd reduced the alfalfa shoot length by 62% (26). In our study shoot length and root length of mustard was decreased by 43.18% and 40.99% with Cd application respectively. Whereas, *L. varians and P. putida* increased shoot length by 1.73 and 1.62 fold or root length by 2.08 and 2.12 fold correspondingly than the un-inoculated Cd stressed plants (Photograph 1). Similar type of PGPR mediated plant growth enhancement under Cd stress was noticed by different researchers (8,9,27). Literature revealed that Cd inhibited PIN1 protein activity which decreased the root meristem by reducing auxin concentration (10). Exogenous synthetic auxin or PGPR mediated IAA application can help the plant growth under Cd stress even though, the internal auxin concentration of plant was low.

Average number of leaves was increased slightly with the inoculation of PGPR it was not significantly altered in all the cases, but leaf area significantly (p<0.05) increased with the PGPR

application. *L. varians* and *P. putida* enhanced the leaf area by 2.29 and 2.18 fold respectively under Cd amended condition (Table 2). This study revealed that Cd-stress on mustard plant was somehow reduced by *L. varians* and *P. putida* for the leaf development. Synthetic auxin application abridged the Cd stress and improved the inflorescent length by 1.16 fold than the only Cd treated plants. On the other hand, *L. varians* and *P. putida,* under Cd contamination, improved the length of inflorescence by 1.41 and 1.4 fold as compared to Cd stressed condition. Simultaneously, fruit number was also increased with the application of synthetic IAA (1.8 fold) or by any of the PGPR under Cd stress (Table 2). *L. varians* and *P. putida* increased the number of fruits by 1.8 and 2 fold accordingly under heavy metal amended condition. Great increment was noticed in case of IAA application for the seed weight production. IAA application, *L. varians* and *P. putida* inoculation lessen the heavy metal stress and helped the plant to increase the seed weight by 1.61, 1.34 and 1.74 fold correspondingly. Fruit development and number of seed in mustard plant is proportionately dependent on the inflorescence length and flower number. So, it was evident from this study that PGPR application helped to increase productivity of mustard. Cd contamination drastically (p<0.05) reduced fresh weight as well as dry weight by 45.45 and 38.11% than the control plants (Table 2). In case of fresh weight or dry weight, *L. varians* and *P. putida* produced better performance than the un-inoculated Cd stress. IAA producing *L. varians* and *P. putida* enhanced these growth parameters significantly (p<0.05) which were comparable to synthetic IAA application.

It was previously reported by Pal *et al*. that *L. varians and P. putida* enhanced different plant growth parameters of chilli plant such as germination percentage, root length, shoot length, number of leaves, leaf area, fresh weight, dry weight and chlorophyll content under Cd or Pb stressed condition (9). Rajkumar and Freitas observed that *Pseudomonas* and *Arthrobacter* produce IAA that helped for better growth and development (27). PGPR increases dry weight under heavy metal stress condition (28). The increased plant biomass is due to production of different phytohormones like Gibberellins, IAA etc (27). According to Aydinalp and Marinova (26), under increasing Cd stress, root length and shoot length were gradually decreased. It was previously reported that Cd damages many plants by Cd-ROS-MAP kinase signal which was diminished by IAA producing PGPR to enhance the rice seedling growth under Cd stress (29). PGPR-produced IAA enhanced plant growth by increasing nutrient uptake with accelerated root growth under Cd stressed condition (30,31). Pishchik *et al.* reported Cd tolerant *Klebsiella mobilis* decreased Cd content and increased grain yield in barley (*32*). Abbass and Okon suggested that IAA and other plant hormones could be responsible for improved growth of canola, tomato, and wheat inoculated with *Azotobacter paspali* (33). Similar type of observation was made recently by Kamran *et al* in case of *Eruca sativa,* where inoculation of bacteria facilitated to overcome the adverse effect of heavy metal (28). Cd inhibits root elongation by accumulation nitric oxide (NO) in root meristem suppressing PIN 1 protein (10). Cd hampers root endogenous auxin concentration by inhibiting PINFORMED1 (PIN1), which act as an auxin efflux protein (13,11). According to Farooq *et al.*, exogenous auxin application enhanced plant growth under Cd stress (15).

So, this study revealed that the PGPR influenced the plant growth improvement under Cd stressed condition by different PGP traits. The observation in this study therefore supports the previous views as mentioned above.

Relative water content (Fig. 2) was drastically affected by Cd application comparing to control set. IAA treatment increased the RWC by 1.41 fold whereas; *L. varians* and *P. putida* enhanced 2.83 and 2.57 fold respectively than the only Cd treated plants. Vigour index was significantly (p<0.05) (Fig. 3) reduced with the Cd treatment by 48.63 % than control setup. Whereas, increased VI was noticed in case of IAA application (1.81 fold) or PGPR inoculation under Cd stress. *L. varians* and *P. putida* appreciably boosted the VI by 1.28 and 2 fold accordingly than Cd amended condition alone.

Cd drastically reduced the chlorophyll content of mustard plant than the control plants (Fig. 4). Synthetic IAA application or PGPR inoculation enhanced the chlorophyll content significantly (p<0.05) under Cd amended situation. *L. varians* and *P. putida* under Cd amended condition increased the total chlorophyll content respectively by 4.34 and 4.48 fold than Cd stressed alone. There was similar sterile soil condition, minimal position effects, same environmental condition, similar agronomic practices and randomised data sets collected from three triplicates of each treatment for this study. So, it can be apparently confirm that the growth enhancement under Cd stressed condition was due to the PGPRs. This PGPR mediated increment in the chlorophyll content was even better than the synthetic auxin application. These data coincided with the study of few others (8,9,34). They examined that Cd reduces the chlorophyll content in wheat and rice respectively. Cd hampers the chlorophyll biosynthesis and reduces the amount of chlorophyll in green plants (35,36). In this study rhizobacterial isolates positively increased the chlorophyll content under Cd stress condition which coincide the previous observations on different plants (3,8,9,31). Pramanik et al. (2017) found that PGPR strain enhanced Chl-a, Chl-b and total chlorophyll content markedly (> 2.5 fold) in Cd amended condition than the control plants (31) which corroborate this work. The results in this

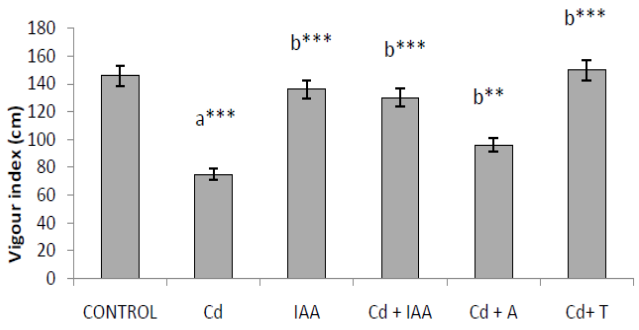


Fig. 3. Vigour index (cm) under Cd stress condition

Difference in between control and cadmium treated groups are denoted by lower case alphabet 'a'. Whereas, lower case alphabet 'b' denotes the differences between Cd treated groups and respective other groups. Asterisk (*) above the bars indicate significant level. (A- *L. varians*, B- *P. putida*).

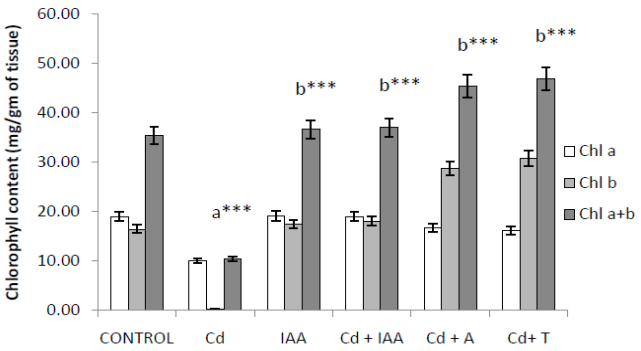


Fig. 4. Chlorophyll content (mg/gm tissue) under Cd stress condition (45 day)

Difference in between control and cadmium treated groups are denoted by lower case alphabet 'a'. Whereas, lower case alphabet 'b' denotes the differences between Cd treated groups and respective other groups. Asterisk (*) above the bars indicate significant level. (A- *L. varians*, B- *P. putida*).

study showed that the growth improvement under Cd stressed condition was better than the exogenous synthetic IAA mediated growth.

Conclusion

Both the selected bacterial isolates, *L. varians* and *P. putida* having very promising PGP activities including IAA producing ability, showed growth promotion of mustard plants under cadmium stressed condition. This comparative analysis revealed that exogenous synthetic IAA application improved different growth parameters of *Brassica* plant to some extent in Cd amended condition. Moreover, very interesting results were obtained in case of PGPR application where *L. varians* and *P. putida,* ameliorated the Cd stress and showed various morphological improvement of mustard plants. In this observation it can be indicated that the application of IAA producing PGPR improved the vigor of mustard by reducing the deleterious effects of metal toxicity. Moreover PGPR mediated growth enhancement was better than the growth improvement by exogenous IAA application and even better than the unstressed control set. Hence, IAA producing *L. varians* and *P. putida* could be exploited for the sustainable agricultural development under Cd-contaminated soil.

Author's contribution

AKP conducted the whole experiment, collected the data, performed statistical analysis and written up the whole manuscript. SM supported the experimental works for different plant growth parameters. CS hypothesized the paper concept, designed the experiment, supervised throughout the process.

Competing interests

The authors declared that they have no competing interest.

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