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MITIGATION OF CADMIUM TOXICITY INDUCED STRESS IN WHEAT BY ACC-DEAMINASE CONTAINING PGPR ISOLATED FROM CADMIUM POLLUTED WHEAT RHIZOSPHERE

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Abstract

Heavy metals induced stress is a serious threat to crop productivity all over the world. Heavy metals enter the food chain through their uptake by plants and thereby affect human health. Among all the heavy metals, cadmium (Cd) is ubiquitously toxic due to its high water solubility. Cadmium can cause cancer, cardiovascular diseases and neural abnormalities in humans when taken up in the form of food. Keeping in mind the importance of wheat as widely used staple food, a hydroponic glass jar experiment was performed under axenic condition for the screening of most efficient Cd tolerant ACC deaminase containing PGPR from Cd polluted (4.3 mg kg^{-1} Cd) wheat rhizosphere. Out of 20 strains, only nine strains were capable of growing at five mg L^{-1} Cd level. The most efficient Cd tolerant ACC deaminase containing PGPR that significantly enhanced morphological growth attributes in wheat plants under various levels of Cd (2.5 and 5.0 mg L^{-1}) were identified as *Agrobacterium fabrum* ($CdtS_5$) and *Stenotrophomonas maltophilia* ($CdtS_7$). The photosynthetic pigments were also enhanced significantly in wheat seedlings where *Agrobacterium fabrum* and *Stenotrophomonas maltophilia* were applied as an inocula. It is concluded that *Agrobacterium fabrum* and *Stenotrophomonas maltophilia* are ACC-deaminase containing PGPR strains which are Cd tolerant, have ability to reduce the Cd uptake by the plant and enhance the wheat growth under Cd toxicity.

Key words: ACC-deaminase PGPR, Cadmium, Morphological attributes, Photosynthetic pigments, Wheat.

Introduction

Industrial pollution is continuously deteriorating the environment (Ahmad *et al.,* 2014). According to a survey, about 20 million hectare agricultural land of the world is irrigated with industrial waste water (Iram *et al.,* 2012). Mostly industrial waste and sewage waters have a large concentration of heavy metals (e.g., As, Cr, Cd, Ni and Pb etc.) which become hazardous for human health on reaching their accumulation beyond threshold limit (Ahmad *et al.,* 2014). Frequent intake of heavy metal contaminated food can cause serious disorders like cancer, cardiovascular, neuronal, renal as well as mutagenesis issues in human body (Steenland & Boffetta, 2000; Jarup, 2003; Radwan & Salama, 2006).

The higher rate of anthropogenic activities is also responsible for the buildup of heavy metals toxicity in soil (Hassan & David, 2014). It is documented that the mobility of these trace elements in soil is very high due to which they become part of human food chain (Malik, 2004). In recent past, it has been estimated that due to anthropogenic activities 25,000 Mg Cd $year^{-1}$ becomes the part of our environment (Azevedo *et al.,* 2012).

Cadmium (Cd) is one of ubiquitous heavy metals found in soil with its high solubility in water (Hassan *et al.,* 2013). It reduces the rate of photosynthesis and transpiration resulting in retarded plant growth (Lamoreaux & Chaney, 1978). Moreover, in plants, Cd can cause the accumulation of high level of ethylene (Hassan *et al.,* 2016), instability of lipid membrane, chlorosis (Khan & Lee, 2013), imbalance of water and alteration in the permeability of membrane (Azevedo *et al.,* 2012; Hassan *et al.,* 2016). Uptake of Cd beyond the threshold level can also cause nutritional imbalance (Greger *et al.,* 1991; Larbi *et al.,* 2002; Dong *et al.,* 2006; Khanmirzaei *et al.,* 2013). Most of the plants become iron (Fe) deficient due to antagonistic relationship of Cd with Fe regarding bioavailability to plants. It also decreases the bioavailability of potassium (K) and magnesium (Mg) in plants (Greger *et al.,* 1991; Larbi *et al.,* 2002; Dong *et al.,* 2006).

Higher level of ethylene biosynthesis in plants under abiotic stress like presence of heavy metals (Shahzadi, 2013) in soils is one of the causes of decrease in plant growth and yield (Grichko *et al.,* 2000; Glick *et al.,* 2007; Naz *et al.,* 2013; Zheng, 2014; Akhtar *et al.,* 2015). Plant growth promoting rhizobacteria (PGPR) are capable to mobilize or immobilize the heavy metals in soil (Gadd, 1990). The role of heavy metals tolerant PGPR that can mitigate the heavy metals effects on plant growth is being studied around the globe (Belimov *et al.,* 2005). Some of the PGPR having ability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase can be used as an amendment in reducing the stress generated by ethylene accumulation in plants (Hall *et al.,* 1996). The ACC deaminase breaks the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) into α-ketobutyrate and NH_3 (Burd *et al.,* 1998; Grichko & Glick, 2001; Glick *et al.,* 2007; Naz *et al.,* 2013).

Therefore, the present study was designed with hypothesis that Cd tolerant ACC deaminase containing PGPR strains, isolated from Cd contaminated soil would improve wheat (*Triticum aestivum* L.) growth under Cd polluted condition.

Material and Methods

Rhizosphere soil collection: The soil samples were collected from the rhizosphere of wheat, cultivated in naturally Cd-contaminated soil (with 4.3 mg kg^{-1} Cd level) at Ada Biliwala (30°04'42.3"N 71°30'35.0"E). Wheat plants were uprooted quite carefully to prevent the loss of soil attached to the roots. All the samples were

preserved in sterilized plastic bags and brought in the Laboratory of Soil Microbiology and Biochemistry (30°25-87' N, 71°51-45' E), Department of Soil Science, Bahauddin Zakariya University, Multan. The roots were tapped gently to remove soil particles and then rubbed with a spatula to remove the stuck particle of soil from roots. The samples were collected and homogenized by mixing manually with a spatula.

Isolation and incubation of PGPR isolates: One g rhizosphere soil was weighed on digital weight balance and serial dilutions (10^{-1} to 10^{-7}) were made in sterilized deionized water. Using ACC as the sole source of nitrogen, DF minimal salt medium (Dworkin & Foster, 1958) was prepared for the isolation of ACC deaminase containing PGPR. All the petri dishes were labelled and incubated at 25°C for 72h. The isolates were grown in petri dishes again and again to obtain pure strains.

Screening of Cd-tolerant ACC deaminase containing PGPR strains: After getting ACC deaminase containing PGPR strains, DF nutrient medium (Dworkin & Foster, 1958) was supplemented with $CdCl_2$ to screen out the Cd-tolerant PGPR. The 5.0 mg L^{-1} toxicity of Cd was used to assess the survival of 20 strains to find out Cd-tolerant ACC deaminase containing PGPR. The Cd toxicity was introduced in the DF nutrient media using the salt of $CdCl_2$ as described by Ahmad *et al.,* (2014). Finally, 9 out of 20 strains which were survived at 5.0 mg L^{-1} Cd toxicity were used for further experimentation.

Experimental design and treatments: For screening of most efficient Cd-tolerant ACC deaminase PGPR, three sterilized seeds of wheat (Sahar 2006 variety) in each sterilized glass jar were inoculated with 9 strains individually. There were 2 levels of Cd; 2.5 mg L^{-1} as control and 5.0 mg L^{-1} to introduce toxicity (using $CdCl_2$). Hydroponic cultivation of wheat was carried out in sterilized glass jars with 10 treatments and 3 replications following factorial CRD design.

Nutrients and water: The nutrients were applied using Hoagland solution (Hoagland & Arnon, 1950). After each 9 days, 5 ml of Hoagland solution was provided to seedlings. However, 30 ml water was maintained on regular basis by using sterilized deionized water.

Seeds sterilization and inoculation: The wheat seeds were surface sterilized by dipping seeds in sodium hypochlorite (5%) solution for 5 min. After that, the seeds were washed thrice with ethanol (95%) and finally with sterilized deionized water (Ahmad *et al.,* 2014). Three seeds were placed on sterilized filter paper and inoculum was poured on them. At the end, another piece of sterilized filter paper was used to sandwich the seeds which were rolled and placed in sterilized glass jar.

Harvesting and plant growth attributes analysis: The harvesting was done after 21 days of sowing. The germination percentage was calculated at 7th day, vigor index (VI) (Shehzad *et al.,* 2012) and coefficient of velocity (Maguire, 1962) were calculated using the formulae:

$$\mathbf{G}\ (\%) = \frac{\text{Total seeds germinated at 7th day x 10}}{\text{Total seed sown}}$$

$$\text{VI} = \text{Seedling length (cm) x Emergence Energy}$$

$$\text{CV} = \frac{(\text{G1} + \text{G2} + \text{G3} \ldots\ldots\ldots\ldots\ldots \text{Gn})}{(1 \text{ x G1} + 2 \text{ x G2} \ldots\ldots\ldots \text{n x Gn})}$$

where n G = germinated seeds and n is last day of germination. Emergence energy (EE) was determined (Farooq *et al.,* 2006) using the equation:

$$\text{EE}\ (\%) = \frac{\text{No. of seedings emerged at 4th day of sowing x 100}}{\text{Total number of seeds sown}}$$

Morphological attribute: Fresh weight of root and shoot were taken soon after the harvesting, on analytical grade electrical weight balance in the laboratory. The shoot and root length were measured using a scale. For determination of dry weight of root and shoot, samples were oven dried for 72 h at 60°C in an oven. Dry weight was noted on analytical grade electrical weigh balance.

Cd in shoot and root: The oven dried shoot and root samples were ground and passed through 0.2 mm sieve. After that 0.5g sieved sample was transferred into a 250-mL digestion conical flask. Five mL H_2SO_4 (98%) in combination with 3g digestion mixture (K_2SO_4 (100): $CuSO_4.5H_2O$ (10): $FeSO_4$ (1)), were added in the samples. All the flasks were placed on the hot plate at 380°C until the color changed from black to green. When the samples were digested, dilutions of 50 ml were made using distilled water. After that, all the digested samples were passed through Whatman No. 42 filter paper. The Cd concentration was determined by pre-calibrated Atomic Absorption Spectrophotometer.

$$\text{Cd}\ (\mu\text{g g} - 1)\ = \text{Cd}\ (\mu\text{g g} - 1) \text{ from calibration vurve x } \frac{\text{Dilution Factor}}{\text{Weight of sample}}$$

Cholorophyll analysis: For examining the cholorophyll a, cholorophyll b and total cholorophyll in the leaves, 0.1g fresh leaf was cut from shoot. Crushing was made in a mortar by adding 5ml of 80% acetone solution. When the leaf samples became like a thick paste in acetone, the final volume of 10 mL was made. At the end, filtration was done by using Whatman No.42 filter paper. For determination of absorbance at 645 and 663 nm wavelength spectrophotometer was used. The final chlorophyll 'a', 'b' and 'total' contents in wheat leaves were evaluated by using the formula of Arnon (1949):

$$\text{Chlorophyll a (mgg}-1\text{ f. wt)} = \frac{12.7\ (\text{OD } 663) - 2.69\ (\text{OD } 645)\text{V}}{1000\ (\text{W})}$$

$$\text{Chlorophyll b (mgg}-1\text{ f. wt)} = \frac{22.9\ (\text{OD } 645) - 4.68\ (\text{OD } 663)\text{V}}{1000\ (\text{W})}$$

where, V = final volume made, W = gram of fresh leaf sample and f. wt. = fresh weight.
However, total chlorophyll was calculated by the addition of chlorophyll a and chlorophyll b.

Identification of Cd-tolerant ACC deaminase PGPR: The Cd-tolerant ACC deaminase rhizobacterial strains showing maximum growth promoting activity were identified as *Agrobacterium fabrum* ($CdtS_5$) and *Stenotrophomonas maltophilia* ($CdtS_7$) by Macrogen Online Sequencing using PCR primer 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' (Lonergan *et al.*, 1996). The BLAST analysis was used to align the 16S rRNA gene sequences (Altschul *et al.*, 1997).

Biochemical characterization of PGPR: The indole acetic acid (IAA) production by PGPR was assessed by using Salkowski reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$ solution) with and without L-tryptophan (L-TRP; Sigma). The PGPR strains were grown in DF broth, incubated at 28 ±2°C for 72h. Supernatant was collected after centrifugation and 1 ml of supernatant was mixed with 2 ml Salkowski reagent. When pink color was developed after 30 minutes, reading of sample was taken at 530 nm at spectrophotometer (Glickmann & Dessaux, 1995). The production of IAA by *Agrobacterium fabrum* ($CdtS_5$) and *Stenotrophomonas maltophilia* ($CdtS_7$) was 7.46 and 6.46 (µg/ml) in the presence of tryptophan while 4.34 and 3.04 (µg/ml) without tryptophan respectively. The presence of ACC deaminase activity was determined in cell-free extracts on spectrophotometer at 540 nm wavelength by examining the α-ketobutyrate synthesis in inoculum (El-Tarabily, 2008). All the isolates were grown on DF broth which prepared at 28 ±2°C shaking for 5 days on rotatory shaker (250 rpm). The cells were collected and suspended in 0.1 M Tris–HCl. For the rupturing of cells, the cells were kept in water bath 3 times at 25 °C for 5 min followed by dipping in liquid nitrogen for 1 min (Shah *et al.*, 1998). Finally, centrifugation was done to remove lysate at 60,000 × g for 60 min to get supernatant for determination of ACC deaminase activity by quantifying α-ketobutyrate through 2,4-dinitrophenylhydrazine (derivative of α-ketobutyrate) as described by Honma & Shimomura, (1978). The production of ACC deaminase was 432.6 and 71.78 (µmol α-ketobutyrate nmol g^{-1} protein h^{-1}) by *Agrobacterium fabrum* ($CdtS_5$) and *Stenotrophomonas maltophilia* ($CdtS_7$), respectively.

Statistical analysis

Statistical analysis was done using statistical software (SPSS version 18.0). All the treatments were compared using Tukey's-HSD test at $p \leq 0.05$ (Steel *et al.*, 1997).

Results

Germination, emergence energy, vigor index and germination index: Main effects of PGPR and Cd levels (2.5 and 5.0 mg L^{-1}) were significant for germination, emergence energy, vigor index, and germination index of wheat (Table 1). Moreover, the germination, emergence energy, vigor index and germination index were found to be significantly better at 2.5 mg L^{-1} than at 5.0 mg L^{-1} Cd level, germination, however, the interactive effect of PGPR and Cd levels did not differ in these parameters. All the PGPR strains were statistically alike to each other for germination. However, the isolates $CdtS_5$, $CdtS_6$, $CdtS_7$ and $CdtS_8$ remained significant as compared to control (No PGPR) for germination. In case of vigor index, inoculation of $CdtS_7$ and $CdtS_8$ remained significantly better as compared to control. However, $CdtS_5$ performed significantly best among all the treatments for vigor index. The isolates $CdtS_1$, $CdtS_3$, $CdtS_5$, $CdtS_6$, $CdtS_7$ and $CdtS_8$ differ significantly as compared to control for germination index. In case of emergence energy $CdtS_5$ performed the best and differ significantly as compared to control. Maximum increase of 2.00, 4.64, 2.45 and 1.50-fold in germination, vigor index, germination index and emergence energy were noted as compared to control respectively where $CdtS_5$ was inoculated in wheat.

Fresh and dry weight of root and shoot: Main effects of PGPR and Cd were significant on root and shoot fresh weight of wheat seedlings (Table 2). However, the interaction was non-significant. The isolates $CdtS_1$, $CdtS_2$, $CdtS_5$, $CdtS_7$, and $CdtS_8$ remained statistically similar to each other but differ significantly as compared to control for shoot fresh weight. Inoculation of $CdtS_3$, $CdtS_4$, $CdtS_6$ and $CdtS_9$ remained statistically alike with control for shoot fresh weight. In the case of shoot dry weight, $CdtS_1$, $CdtS_2$, $CdtS_5$, $CdtS_7$ and $CdtS_8$ were statistically alike to each other but remained significant as compared to control. The isolates $CdtS_1$, $CdtS_2$, $CdtS_3$, $CdtS_4$, $CdtS_5$, $CdtS_7$ and $CdtS_8$ performed significantly better as compared to control for root fresh weight. In case of root dry weight, $CdtS_1$, $CdtS_2$, $CdtS_5$, $CdtS_7$ and $CdtS_8$ remained significant as compared to control. Shoot fresh weight, shoot dry weight, root fresh weight and root dry weight were significantly higher at 2.5 mg Cd L^{-1} as compared to 5.0 mg Cd L^{-1}. Maximum increase of 1.14, 1.15, 1.05 and 1.17-fold was noted in the shoot fresh weight, shoot dry weight, root fresh weight and root dry weight respectively as compared to control where $CdtS_7$ was inoculated.

Shoot and root length: Main effects of PGPR and Cd were significant, but their interaction remained non-significant for shoot and root length under various levels of Cd (2.5 and 5.0 mg L^{-1}) (Table 3). The strains $CdtS_5$ and $CdtS_7$ though statistically similar with each other but improved the shoot length significantly as compared to control. The isolate $CdtS_5$ remained significantly best for improvement in root length as compared to control. Both shoot and root length were significantly higher at 2.5 as compared to 5.0 mg Cd L^{-1}. Maximum increase in the shoot (1.01-fold) and root length (1.69-fold) was noted as compared to control where $CdtS_5$ was inoculated.

Table 1. Effect of ACC deaminase PGPR on germination (%), vigor index, germination index and emergence energy (%) of wheat seedlings grown under various levels of Cd hydroponically.

PGPR	Cadmium levels (mg L^{-1})											
	2.5	5.0	Mean	2.5	5.0	Mean	2.5	5.0	Mean	2.5	5.0	Mean
	Germination (%)			Vigor index			Germination index			Emergence energy (%)		
Control	41.7±14.4	16.7±14.4	29.2 ^{B}	682±239.5	267±232.6	475 ^{D}	0.30±0.08	0.10±0.08	0.20 ^{C}	33.3±14.4	16.7±14.4	25.0 ^{B}
$CdtS_1$	66.7±14.4	58.3±14.4	62.5 ^{AB}	1484±497.4	926±218.9	1205 ^{BCD}	0.50±0.08	0.37±0.10	0.43 ^{B}	41.7±14.4	33.3±14.4	37.5 ^{AB}
$CdtS_2$	66.7±14.4	41.7±14.4	54.2 ^{AB}	1339±341.7	749±292.3	1044 ^{BCD}	0.50±0.08	0.30±0.08	0.40 ^{BC}	41.7±14.4	8.30±14.4	25.0 ^{B}
$CdtS_3$	66.7±14.4	58.3±14.4	62.5 ^{AB}	1522±446.3	806±207.9	1164 ^{BCD}	0.48±0.12	0.39±0.08	0.43 ^{B}	33.3±14.4	25.0±25.0	29.2 ^{AB}
$CdtS_4$	66.7±14.4	41.7±14.4	54.2 ^{AB}	1504±116.5	691±256.8	1097 ^{BCD}	0.48±0.12	0.30±0.08	0.39 ^{BC}	41.7±28.9	16.7±28.9	29.2 ^{AB}
$CdtS_5$	91.7±14.4	83.3±14.4	87.5 ^{A}	3285±753.1	2075±335.7	2680 ^{A}	0.71±0.15	0.66±0.10	0.69 ^{A}	66.7±14.4	58.3±14.4	62.5 ^{A}
$CdtS_6$	75.0±25.0	58.3±14.4	66.7 ^{A}	1155±396.6	679±162.8	917 ^{CD}	0.54±0.14	0.39±0.14	0.47 ^{AB}	41.7±14.4	33.3±14.4	37.5 ^{AB}
$CdtS_7$	83.3±14.4	58.3±14.4	70.8 ^{A}	2073±414.0	1281±301.4	1677 ^{BC}	0.61±0.12	0.37±0.10	0.49 ^{AB}	58.3±14.4	33.3±14.4	45.8 ^{AB}
$CdtS_8$	83.3±14.4	75.0±25.0	79.2 ^{A}	1967±246.6	1649±862.2	1808 ^{B}	0.57±0.05	0.52±0.17	0.55 ^{AB}	58.3±14.4	25.0±25.0	41.7 ^{AB}
$CdtS_9$	75.0±25.0	33.3±28.9	54.2 ^{AB}	1425±592.1	451±400.5	938 ^{CD}	0.49±0.14	0.21±0.18	0.35 ^{BC}	33.3±14.4	8.30±14.4	20.8 ^{B}
Mean	71.7 ^{A}	52.5 ^{B}		1643 ^{A}	957 ^{B}		0.52 ^{A}	0.36 ^{B}		45.0 ^{A}	25.8 ^{B}	

Values are mean ± standard deviation of 3 replicates. Different letters are representing statistical differences at $p \leq 0.05$

Table 2. Effect of ACC deaminase PGPR on root and shoot fresh weight (g) and dry weight (g) of wheat seedlings grown under various levels of Cd hydroponically.

PGPR	Cadmium levels (mg L^{-1})											
	2.5	5.0	Mean	2.5	5.0	Mean	2.5	5.0	Mean	2.5	5.0	Mean
	Shoot fresh weight (g)			Shoot dry weight (g)			Root fresh weight (g)			Root dry weight (g)		
Control	0.9±0.09	0.5±0.46	0.7 ^{CD}	0.017±0.002	0.010±0.009	0.013 ^{DE}	0.141±0.013	0.075±0.065	0.108 ^{D}	0.008±0.004	0.004±0.002	0.006 ^{CD}
$CdtS_1$	1.6±0.20	1.1±0.11	1.3 ^{AB}	0.029±0.004	0.021±0.002	0.025 ^{ABC}	0.237±0.030	0.166±0.017	0.201 ^{AB}	0.014±0.001	0.010±0.001	0.012 ^{AB}
$CdtS_2$	1.4±0.14	1.2±0.18	1.3 ^{AB}	0.027±0.003	0.022±0.003	0.024 ^{ABC}	0.212±0.020	0.174±0.028	0.193 ^{AB}	0.012±0.002	0.010±0.001	0.011 ^{AB}
$CdtS_3$	1.3±0.16	0.8±0.02	1.1 ^{ABC}	0.025±0.003	0.015±0.001	0.020 ^{BCD}	0.196±0.024	0.122±0.004	0.159 ^{BC}	0.011±0.000	0.007±0.003	0.009 ^{BC}
$CdtS_4$	1.2±0.32	0.8±0.01	1.0 ^{BCD}	0.022±0.006	0.015±0.000	0.018 ^{CDE}	0.173±0.048	0.124±0.001	0.148 ^{BC}	0.010±0.000	0.007±0.002	0.009 ^{BCD}
$CdtS_5$	1.7±0.27	1.1±0.17	1.4 ^{AB}	0.032±0.005	0.021±0.003	0.026 ^{AB}	0.249±0.040	0.165±0.025	0.207 ^{AB}	0.014±0.001	0.010±0.000	0.012 ^{AB}
$CdtS_6$	0.7±0.04	0.4±0.22	0.6 ^{D}	0.014±0.001	0.008±0.004	0.011 ^{E}	0.109±0.006	0.063±0.033	0.086 ^{D}	0.006±0.002	0.004±0.002	0.005 ^{D}
$CdtS_7$	1.6±0.20	1.3±0.17	1.5 ^{A}	0.031±0.004	0.025±0.003	0.028 ^{A}	0.244±0.030	0.199±0.026	0.221 ^{A}	0.014±0.001	0.011±0.001	0.013 ^{A}
$CdtS_8$	1.4±0.16	1.1±0.13	1.2 ^{AB}	0.026±0.003	0.020±0.003	0.023 ^{ABC}	0.204±0.023	0.163±0.020	0.184 ^{AB}	0.012±0.001	0.009±0.002	0.011 ^{AB}
$CdtS_9$	1.1±0.24	0.5±0.39	0.8 ^{CD}	0.020±0.005	0.008±0.007	0.014 ^{DE}	0.161±0.036	0.067±0.059	0.114 ^{CD}	0.009±0.003	0.004±0.000	0.007 ^{CD}
Mean	1.3 ^{A}	0.9 ^{B}		0.024 ^{A}	0.016 ^{B}		0.193 ^{A}	0.132 ^{B}		0.011 ^{A}	0.008 ^{B}	

Values are mean ± standard deviation of 3 replicates. Different letters are representing statistical differences at $p \leq 0.05$

Table 3. Effect of ACC deaminase PGPR on shoot length, root length, chlorophyll a, b and total chlorophyll of wheat seedlings grown under various levels of Cd hydroponically.

PGPR	Cadmium levels (mg L^{-1})														
	2.5	5.0	Mean	2.5	5.0	Mean	2.5	5.0	Mean	2.5	5.0	Mean	2.5	5.0	Mean
	Shoot length (cm)			Root length (cm)			Chlorophyll a (mg g^{-1})			Chlorophyll b (mg g^{-1})			Total chlorophyll (mg g^{-1})		
Control	10.2±1.9	6.90±6.0	8.60 ^{CD}	6.10±1.9	3.80±3.3	4.9 ^{B}	0.96±0.16	0.50±0.44	0.73 ^{C}	1.19±0.18	0.63±0.55	0.91 ^{DE}	2.16±0.34	1.13±0.99	1.65^{CD}
$CdtS_1$	11.9±1.8	8.90±0.6	10.4 ^{BCD}	10.0±1.6	7.10±1.5	8.5 ^{AB}	1.41±0.17	1.19±0.17	1.30 ^{A}	1.78±0.23	1.51±0.22	1.64 ^{AB}	3.19±0.40	2.69±0.39	2.94 ^{A}
$CdtS_2$	11.5±1.1	10.1±0.8	10.8 ^{BCD}	8.50±2.8	7.70±2.0	8.1 ^{B}	1.64±0.17	1.28±0.17	1.46 ^{A}	2.09±0.23	1.60±0.22	1.84 ^{AB}	3.73±0.39	2.88±0.39	3.30 ^{A}
$CdtS_3$	12.4±2.5	6.60±2.1	9.50 ^{BCD}	10.2±0.4	7.20±0.7	8.7 ^{AB}	1.46±0.17	0.92±0.05	1.19^{AB}	1.86±0.20	1.14±0.10	1.50 $^{A-C}$	3.32±0.37	2.06±0.15	2.69 ^{AB}
$CdtS_4$	13.3±2.7	10.2±2.2	11.7 ^{BC}	9.80±1.2	6.80±1.7	8.3 ^{AB}	1.46±0.23	0.92±0.02	1.19^{AB}	1.70±0.20	1.06±0.03	1.38 ^{BCD}	3.16±0.42	1.98±0.05	2.57^{ABC}
$CdtS_5$	20.9±2.3	13.6±5.0	17.3 ^{A}	14.7±2.9	11.7±0.9	13.2 ^{A}	1.90±0.31	1.27±0.19	1.59 ^{A}	2.23±0.24	1.56±0.23	1.90 ^{AB}	4.13±0.55	2.83±0.42	3.48 ^{A}
$CdtS_6$	7.00±0.6	4.70±0.9	5.80 ^{D}	8.40±1.5	7.00±0.2	7.7 ^{B}	0.83±0.05	0.50±0.20	0.66 ^{C}	1.00±0.06	0.62±0.29	0.81 ^{E}	1.83±0.11	1.12±0.50	1.47 ^{D}
$CdtS_7$	15.0±1.2	13.1±1.6	14.0 ^{AB}	9.80±0.1	8.90±0.7	9.4 ^{AB}	1.62±0.15	1.44±0.17	1.53 ^{A}	2.04±0.21	1.77±0.21	1.91 ^{A}	3.66±0.35	3.21±0.38	3.43 ^{A}
$CdtS_8$	13.9±1.8	12.8±0.6	13.3 ^{ABC}	9.80±2.8	8.20±5.0	9.0 ^{AB}	1.53±0.20	1.24±0.16	1.39 ^{A}	1.94±0.27	1.55±0.22	1.74 ^{AB}	3.47±0.47	2.79±0.38	3.13 ^{A}
$CdtS_9$	11.0±0.5	6.30±5.4	8.60 ^{CD}	8.10±6.7	2.70±3.2	5.4 ^{B}	1.21±0.28	0.48±0.42	0.85^{BC}	1.52±0.34	0.61±0.53	1.06 ^{CDE}	2.73±0.62	1.09±0.95	1.91^{BCD}
Mean	12.7 ^{A}	9.3 ^{B}		5.40 ^{A}	4.10 ^{B}		1.40 ^{A}	0.97 ^{B}		1.74 ^{A}	1.20 ^{B}		3.14 ^{A}	2.18 ^{B}	

Values are mean ± standard deviation of 3 replicates. Different letters are representing statistical differences at $p \leq 0.05$

Photosynthetic pigments: Main effects of PGPR and Cd levels were significant for cholorophyll contents in wheat (Table 3). However, the interaction of various Cd levels and PGPR remained non-significant for cholorophyll contents. For cholorophyll a and total chlorophyll $CdtS_1$, $CdtS_2$, $CdtS_3$, $CdtS_5$, $CdtS_7$ and $CdtS_8$ performed best and remained statistically alike to each other but differ significantly as compared to control. For cholorophyll a, inoculation of $CdtS_4$ differ significantly as compared to control. However, for total cholorophyll $CdtS_4$ remained statistically alike with control. In case of chlorophyll b, the strains $CdtS_1$, $CdtS_2$, $CdtS_3$, $CdtS_5$, $CdtS_7$ and $CdtS_8$ were statistically alike and remained significant as compared control. Maximum increase in chlorophyll a (1.18-fold) and total chlorophyll (1.11-fold) was noted in $CdtS_5$ while chlorophyll b (1.10-fold) in $CdtS_7$ as compared to control under various levels of Cd.

Cd concentration in shoot and root: Both main and interactive effects of Cd and PGPR differ significantly for root and shoot Cd concentration (Fig. 1). At both Cd levels, $CdtS_5$ and $CdtS_7$ showed minimum Cd concentration in root and shoot. The isolates $CdtS_5$ and $CdtS_7$ significantly reduced the Cd concentration in the root and shoot at 5 mg L^{-1} Cd, as compared to control. The strain $CdtS_5$ showed the maximum reduction in concentration of Cd in root (0.25-fold) and shoot (0.90-fold) of wheat seedlings as compared to the control at 2.5 mg L^{-1} Cd. At 5 mg L^{-1} Cd toxicity, the $CdtS_7$ performed significantly better for maximum decrease in wheat shoot (0.40-fold) Cd concentration. However, at 5 mg L^{-1} Cd toxicity the $CdtS_5$ performed significantly better for maximum reduction in wheat root (0.31-fold) Cd concentration.

Biochemical analysis of PGPR: The most efficient Cd-tolerant PGPR strains, $CdtS_5$ (*Agrobacterium fabrum*) and $CdtS_7$ (*Stenotrophomonas maltophilia*) were also capable of producing indole acetic acid (IAA). In the presence of L-tryptophan, *Agrobacterium fabrum* produce 0.16-fold more IAA as compared to *Stenotrophomonas maltophilia.* However, without L-tryptophan, the production of IAA was 0.43-fold higher in *Agrobacterium fabrum* as compared to *Stenotrophomonas maltophilia*. Both the strains also showed ACC deaminase activity. However, ACC deaminase activity was 5.03-fold higher in *Agrobacterium fabrum* as compared to *Stenotrophomonas maltophilia*.

Discussion

It is well documented that the toxicity of Cd restricts the shoot and root growth adversely, affects the homeostasis and nutrients uptake in plants (Sanita di Toppi & Gabbrielli, 1999; Fiaz *et al.,* 2014). Such abiotic stress condition usually enhances the level of ethylene synthesis in higher plants that exert negative effect on the growth attributes (Glick, 2014). An improvement in the morphological growth attributes (shoot and root length, shoot and root fresh and dry

weight) of wheat plants in our experiment under various levels of Cd toxicity might be due to reduction in synthesis of ethylene in plants (Salehuzzaman *et al.,* 1998; Ganesan, 2008; Zafar-ul-Hye *et al.,* 2014). According to Penrose & Glick, (2001) the enzyme ACC-deaminase ultimately cleaved the ACC (ethylene precursor) into NH_3 and α-ketobutyrate. The reduction in rhizospheric ethylene due to continuous cleaving of rhizospheric ethylene resulted in movement of root ethylene, out in rhizosphere due to concentration gradient (Glick *et al.,* 1998). The NH_3 produced as a result of ethylene breakdown, is utilized by PGPR as a source of N that also restricts the reformation of ACC (Penrose & Glick, 2001). Results of current study showed that shoot length and root length was significantly increased as compared to control where PGPR $CdtS_7$ (*Stenotrophomonas maltophilia*) and $CdtS_5$ (*Agrobacterium fabrum*) were inoculated on seeds under Cd toxicity. Such enhancement in the growth traits is in complete agreement with Nadeem *et al.,* (2006) findings under abiotic stress. Results indicated that wheat seedlings inoculated with PGPR $CdtS_7$ (*Stenotrophomonas maltophilia*) and $CdtS_5$ (*Agrobacterium fabrum*) also showed less concentration of Cd in root and shoot. Our finding was in favor of results documented by Gadd, (2004) regarding immobilization of metals by PGPR. The most prominent Cd-tolerant PGPR $CdtS_7$ (*Stenotrophomonas maltophilia*) and $CdtS_5$ (*Agrobacterium fabrum*) in our experiment were also capable of producing IAA which might be one of the causes of improvement in root elongation of wheat seedlings under Cd stress. The results documented by Burd *et al.,* (1998) and Tripathi *et al.,* (2005) also favor our argument of significant root elongation by IAA. The improvement in germination, vigor index, emergence energy, and germination index in our study was might be due to IAA production by $CdtS_7$ (*Stenotrophomonas maltophilia*) and $CdtS_5$ (*Agrobacterium fabrum*). However, the less accumulation of ethylene due to ACC deaminase containing PGPR in the wheat seedlings under even Cd stress might also be a reason of better root elongation and improvement of morphological growth attributes (Fuhrer, 1982). The increase in the fresh and dry weights of shoot and root in wheat plants were due to better uptake of nutrients through better developed roots (Biari *et al.,* 2008). Results documented by Safronova *et al.,* (2006) justified that the inoculation of PGPR significantly enhanced the uptake of nutrients under abiotic stress via better root elongation. Similar findings were also reported by Belimov *et al.,* (2001) in spring rape when they inoculated the seeds with ACC deaminase containing PGPR. Higher accumulation of ethylene starts degradation of lipids in the cell wall. Due to lipid degradation ethylene activates chlorophyllase (chlase) gene by contacting with chloroplast. This chlorophyllase (chlase) gene degrades chlorophyll resulted in chlorosis and poor photosynthesis (Matile *et al.,* 1997). However, a significant improvement in synthesis of cholorophyll a, cholorophyll b and total cholorophyll in current study signified the efficacious functioning of Cd tolerant ACC deaminase containing PGPR (*Stenotrophomonas maltophilia* and *Agrobacterium fabrum*) as compared to control regarding mitigation of Cd induced stress in wheat seedlings.

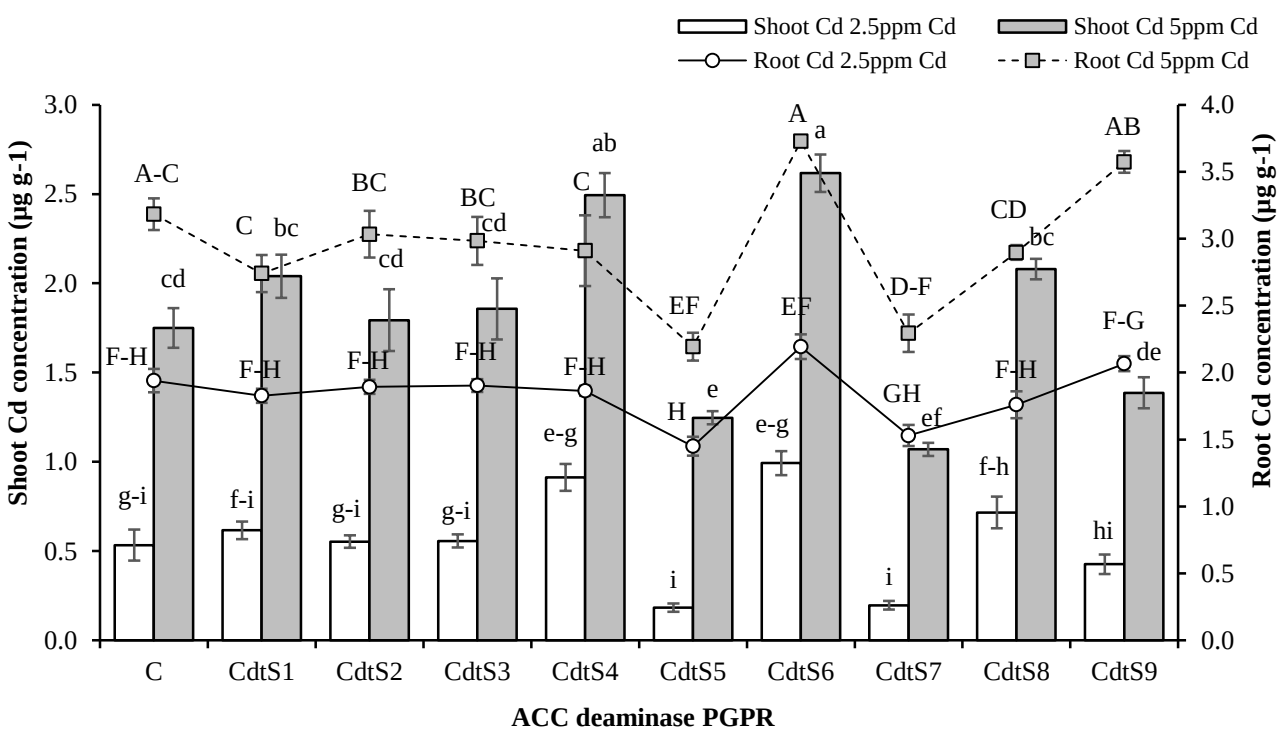

Fig. 1. Effect of Cd tolerant ACC deaminase containing PGPR on shoot and root Cd concentration (µg g^{-1}) of wheat seedlings cultivated under various levels of Cd hydroponically. Mean values of 3 replicates of shoot and root Cd concentration are represented by bars and lines respectively. Different letters (capital for root; small for shoot) on bars and lines represent the significant difference at $p \leq 0.05$ respectively.

Conclusion

It is concluded that the inoculation of ACC deaminase containing PGPR *Stenotrophomonas maltophilia* and *Agrobacterium fabrum* on seeds could promote the growth of wheat under Cd toxicity. Both strains are effective to reduce the uptake of Cd in the wheat plants. However, further investigations are recommended for the use of *Stenotrophomonas maltophilia* and *Agrobacterium fabrum* as an efficient PGPR in future for the promotion of plants growth under normal as well as Cd toxic soil condition.

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