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ORIGINAL PAPER

Effective plant-endophyte interplay can improve the cadmium hyperaccumulation in *Brachiaria mutica*

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Abstract

Soil contamination due to cadmium (Cd) is a ubiquitous environmental problem for which inexpensive remediation alternatives are required. Phytoaccumulation, the use of plants to extract and accumulate heavy metals from the contaminated environment, is such an alternative. In this study, we aimed at establishing effective plant-bacteria interplay between *Brachiaria mutica* and Cd-resistant endophytic bacteria eventually leading to improved phytoremediation. *B. mutica* was grown in a Cd-contaminated soil and inoculated with three Cd-tolerant endophytic bacteria individually as well as in combination. Plant physiological parameters, biomass production, bacterial colonization, and Cd-accumulation were observed at four different Cd exposures, i.e., 100, 200, 400 and 1000 mg kg^{-1} of soil. The combined application of endophytic bacteria was more effective as compared to their individual applications at all concentrations. Nevertheless, highest performance of consortium was seen at 100 mg Cd kg^{-1} of soil, i.e., root length was enhanced by 46%, shoot length by 62%, chlorophyll content by 40%, and dry biomass by 64%; which was reduced with the increase in Cd concentration. The bacterial population was highest in the root interior followed by rhizosphere and shoot interior. Concomitantly, plants inoculated with bacterial consortium displayed more Cd-accumulation in the roots (95%), shoots (55%), and leaves (44%). Higher values of BCF_{root} (> 1), and lower values for BCF_{shoot} and TF (< 1) indicates capability of *B. mutica* to accumulate high amounts of Cd in the roots as compared to the aerial parts. The present study concludes that plant-endophyte interplay could be a sustainable and effective strategy for Cd removal from the contaminated soils.

Keywords *Brachiaria mutica* · Cadmium · Heavy metals · Phytoaccumulation · Phytoremediation · Plant-endophyte interplay

Introduction

Cadmium (Cd) contamination of soil can pose serious threats to agricultural land. It can lead to bioaccumulation in the food chain or can cause groundwater contamination ultimately affecting human health (Jarup 2003; Zhang et al. 2013). Cadmium is a non-essential element with unknown physiological functions in the animal body (Adriano 2001; Jarup and Alfven 2004; Sharma et al. 2010). Major routes of Cd into the natural and agricultural environment are human activities such as mining, industrial wastes, and the application of pesticides/fertilizers (Mahar et al. 2016). Once released in the environment, high solubility of Cd makes it enormously toxic for living organisms (Adriano 2001; Chaudhuri et al. 2014). Even at low concentrations, soil contamination due to Cd can be a serious risk to human health through soil-crop-human exposure pathway (Liu et al.

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2010). For instance, direct intake of Cd-contaminated food can lead to several disorders in humans such as pulmonary edema, pneumonitis and renal abnormalities (Cheng et al. 2002; Jarup and Alfven 2004; Astier et al. 2014).

The available conventional approaches to remediation are expensive and do not provide adequate solutions. Using plants in phytoremediation, however, is an environment-friendly and cost-efficient alternative for the cleanup of a variety of heavy metals (HMs) (Prapagdee et al. 2013; Mani et al. 2015). A typical phytoremediation system mainly relies on rhizodegradation, phytostabilization, phytoextraction, and phytoaccumulation. Since HMs such as Cd cannot be chemically degraded, phytostabilization and/or phytoextraction are the primary options for decontamination of soil (Salt et al. 1995). Nevertheless, the high toxicity of Cd reduces the plant performance by inhibiting growth, disrupting photosynthesis, inducing chlorosis, and eventually causing plant death (Das et al. 1997; Karthikeyan and Kulakow 2003; Pietrini et al. 2009; Adediran et al. 2015). Recently, combined application of plant and Cd-tolerant plant growth-promoting (PGP) bacteria has been proposed to overcome these issues (Sessitsch et al. 2013). In this partnership, bacteria ensure availability of HMs to the plant through releasing chemical compounds as a result of chelating, phosphate solubilizing, and acidification processes, whereas plant provides nutrients and residency to perform their services effectively. Moreover, bacteria improve plant growth by producing phytohormones such as cytokinin, siderophores, gibberellic acid, and indole acetic acid (IAA); and reduce abiotic stress by enzymatic hydrolyzation of 1-aminocyclopropane-1-carboxylate (ACC), i.e., an ethylene precursor responsible for inhibition of plant growth (Glick 2010; Sessitsch et al. 2013; Hua et al. 2010; Vejan et al. 2016). The role of endophytic bacteria, however, has always been more prominent in these services as they establish intimate relationships with their host *in planta* (Ijaz et al. 2016).

Terrestrial grasses can grow successfully in HMs contaminated soils due to their dense rooting system (Mohanty and Patra 2012). Their potential in phytoremediation depends on hyperaccumulation rates. Efforts are being made to increase the biomass of hyperaccumulators in the presence of HMs for increased phytoaccumulation within plant tissues. *Brachiaria mutica* (Forssk) Stapf—also known as a para grass—have been reported to possess a good potential in phytoextraction of Cd, nevertheless, its behavior in the presence of bacterial endophytes is rarely investigated. In this study, we established plant-endophyte interplay for increased performance of *B. mutica* in combination with three metal resistant and PGP bacterial endophytes for successful Cd-phytoaccumulation. In earlier studies, this bacterial consortium appeared to be an effective choice in phytostabilization of chromium (Cr) and displayed the highest potential in plant growth promotion (Khan et al. 2015; Ahsan et al. 2018); therefore, its potential in removing other toxic HMs including Cd is worthwhile investigating. This study presents detailed insights on the performance of *B. mutica* in the presence of Cd toxicity and the role of bacterial consortium towards improved plant growth, biomass production, Cd-hyperaccumulation, and plant-bacteria synergism at different exposure concentrations, i.e., 100, 200, 400, and 1000 mg Cd kg^{-1} soil. The chosen concentrations were higher than from what is typically found in the environment which was mainly due to the reason that we wanted to study the impact of Cd-tolerant bacteria in the mobilization of Cd followed by its effective phytoextraction from moderate to extremely high concentrations. Furthermore, we know that Cd is a non-essential element and its high concentrations can inhibit plant growth, photosynthesis, as well as other metabolic processes (Hasan et al. 2009); therefore, we it is worthwhile investigating if a plant-endophyte based system can be employed in places which are contaminated with high concentrations of Cd. Finally, phytoaccumulation ability was interpreted by estimating bioconcentration and translocation factor. We hypothesized that *B. mutica* can establish an effective partnership with the Cd-resistant endophytic bacteria eventually leading to improved remediation.

Materials and methods

Bacterial strains

Three endophytic bacteria namely *Pantoea stewartii* ASI11, *Microbacterium arborescens* HU33, and *Enterobacter* sp. HU38, previously isolated from the plant interior of *Prosopis juliflora,* were used in the present study (Khan et al. 2015). These bacteria were selected based on their Cd (100 mg l^{-1}) resistance and PGP activities namely ACC deaminase, IAA and siderophores production, inorganic phosphate solubilization, as tested previously (Khan et al. 2015; Ahsan et al. 2018). The inoculum was prepared by growing bacteria in LB broth for 48 h and cells were harvested by centrifugation. Subsequently, the cell pellet was washed and suspended in 0.9% (w/v) NaCl solution. The numbers of bacterial cells were adjusted to obtain 10^{10} cells ml^{-1} for each bacterial culture (Sutton 2011), which were then mixed in an equal ratio (1:1:1) to prepare the bacterial consortium having 10^{10} cells ml^{-1}. Fifty ml of each bacterial culture and consortium was applied in each pot having 1.5 kg soil. In our earlier experiments, we found that inoculum density affects bacterial survival, colonization and phytoremediation efficacy, and maximum phytoremediation achieved at high inoculum density (Shabir et al. 2016). Thus, in this study, we employed high-density inoculum (10^{10} CFU/ml) for consortium as well as individual strains.

Experimental setup

Agricultural clay loam soil (sand: 39%, silt 32%, and clay: 29%) was collected from the fields of NIBGE, Faisalabad, Pakistan. At natural levels, soil of Faisalabad contains organic matter (> 1%) due to high temperature and low rainfall; however, earlier studies have reported a positive effect of soil humates on Cd accumulation in the plant roots as well as on alleviation of plant stress due to Cd toxicity (Baraud et al. 2005). Therefore, we added animal manure (10%, w/w), obtained from a local cattle farm, in the soil to improve the soil fertility, microbial population, and phytoremediation potential. This mixture was air-dried and sieved (2 mm mesh) to homogenize the soil. The physico-chemical properties of prepared soil are listed in Table 1. Spiking was performed by using cadmium nitrate tetrahydrate [$Cd(NO_3)_2.4H_2O$] salt. Briefly, the stock solution (10,000 mg l^{-1}) of Cd was prepared by dissolving 20.74 g of the salt in 1-l of Milli-Q water. Four concentration levels, i.e., 100, 200, 400 and 1000 mg Cd kg^{-1} soil, were achieved by sprinkling 15, 30, 60, and 150 ml of the stock solution to 1.5 kg soil. The spiked soil was mixed thoroughly and left to re-equilibrate for 6 weeks undergoing three cycles of saturation with water and air drying (Blaylock et al. 1997) with an open-air exchange, before filling the pots (1.5 kg of the soil). Surface sterilized cuttings of *B. mutica* (20 pot^{-1}) were sown in each pot whereas each cutting had an average length of 15 cm. Bacterial strains were applied separately and in combination to check their individual and combined effects on plant growth and metal uptake. A 50 and 16.66 ml of suspension (10^{10} cells ml) of each bacterial strain was added to the soil treated with individual strain and consortium, respectively. The soil treated with a single strain contained 3.3×10^9 cells g^{-1} soil, whereas soil treated with consortium contained 1.1×10^3 cells g^{-1} soil of each bacterial strain. The seedlings were allowed to grow for a week and then poorly grown seedlings were removed and a number of 15 plants were maintained per pot. In the control soil, an equal amount of 0.9% NaCl solution was added without bacterial strains. Different treatments were,

1. Control (*B. mutica* vegetated in un-contaminated soil without bacterial augmentation).
2. *B. mutica* vegetated in spiked soil, without bacterial augmentation.
3. *B. mutica* vegetated in spiked soil, augmented with ASI11, HU33, and HU38 bacterial strains, separately.
4. *B. mutica* vegetated in spiked soil, augmented with the bacterial consortium (a mixture of ASI11, HU33, and HU38).

The treatments were triplicated and pots were arranged randomly in the natural conditions of NIBGE, Faisalabad, Pakistan. Pots were water fed thrice a week. The experiment was conducted in summer between August to October 2016 in which temperature variations were recorded between 26.1 and 37.4 °C. There were two rainfall events during the experimental period and therefore, on rainy days, no water was fed manually.

Table 1 Physical and chemical properties of the agricultural soil used in the study

Parameter	Value	Reference method
pH (soil: water = 1: 2.5)	7.62	Bäckströmet al. (2004)
Bulk density (Mg m^{-1})	1.28 ± 0.05	Tejada et al. (2008)
Conductivity (µS cm^{-1})	560 ± 7	Bäckströmet al. (2004)
Organic matter (%)	3.87 ± 0.36	Peltola and Åström (2003)
Cr (mg kg^{-1})	13 ± 0.5	Brunetti et al. (2012)
Cd (mg kg^{-1})	0.4 ± 0.03	Brunetti et al. (2012)
Co (mg kg^{-1})	15 ± 0.8	Brunetti et al. (2012)
Cu (mg kg^{-1})	20 ± 2	Brunetti et al. (2012)
Mg (mg kg^{-1})	525 ± 43	Brunetti et al. (2012)
Mn (mg kg^{-1})	475 ± 41	Brunetti et al. (2012)
Ni (mg kg^{-1})	25 ± 3	Brunetti et al. (2012)
Pb (mg kg^{-1})	10 ± 0.4	Brunetti et al. (2012)
Zn (mg kg^{-1})	65 ± 7	Brunetti et al. (2012)

Values represent means ± standard deviation (n = 3)

Plant and soil analysis

After three months of sowing, plants were harvested to determine different agronomic parameters including root and shoot lengths, chlorophyll content, and dry biomass. The plant tissues particularly roots were washed carefully with the tap water following a gentle rinse in the distilled water until all the adhering soil particles were removed (Arslan et al. 2014). Dry biomass was assessed by putting the root and shoot samples in an oven at 80 °C for 48 h (Ahsan et al. 2017). Hitachi 57 double beam UV/Visible spectrophotometer (Tokyo, Japan) was used to measure the chlorophyll content (a + b) as described previously (Arnon 1949). Subsequently, pH and electrical conductivity (EC) of the soil was monitored by using 781-pH/Ion meter (Metrohm Herisau, Switzerland) and XL 30 conductivity meter (Fisher Scientific Pte Ltd. UE Tech Park, Singapore) respectively (Lotfy and Mostafa 2014). The total organic matter was determined by following the guidelines of Peltola nd Åström (2003).

For elemental analysis, soil samples and grounded plant material were digested in a microwave oven (Multiwave 3000, Anton Paar GmbH Graz, Austria) using method 3051 (USEPA 1994) and 3052 (USEPA 1996). Cadmium concentrations were determined by using inductively coupled plasma-optical emission spectrometry

(ICP-OES) (ICAP6500, Thermo Scientific, Cambridge, UK). The chemicals used in samples and standards preparation were of analytical grade. For quality control purposes, blanks and duplicates were run in parallel. The results were verified by using NIST-certified standards and spikes (Jiang et al. 2008; Brunetti et al. 2012). The detection limit for Cd in the soil and plant samples was 0.01 mg l^{-1} at 98% confidence level.

The phytoaccumulation ability of *B. mutica* was estimated by measuring bioaccumulation factor (BCF) and translocation factor (TF) using these expressions:

$$\mathrm{BCF}_{\mathrm{roots}} = \frac{Cd\ concentration\ in\ roots}{Cd\ concentration\ in\ soil}, \quad \mathrm{BCF}_{\mathrm{shoots}} = \frac{Cd\ concentration\ in\ shoots}{Cd\ concentration\ in\ soil}$$

$$\mathrm{TF} = \frac{Cd\ concentration\ in\ shoots}{Cd\ concentration\ in\ roots}$$

Survival/persistence of inoculant bacteria

Bacterial survival/persistence was estimated from plant rhizo- and endosphere to see how effective was the partnership of inoculated strains with their host. Briefly, plant roots and shoots were surface-sterilized by washing them for 10 min in 70% ethanol; this was followed by 1-min rinse in 2% NaClO solution amended with 0.01% Tween 20 solution. The surface-sterilized shoots were cut into small pieces (size was about 1 cm). Accordingly, rhizospheric soil was obtained by gently shaking the roots. One gram of both rhizospheric soil and sterilized plant tissues were crushed with a pestle and mortar and mixed in 0.9% NaCl solution to make a slurry suspension. After settling of the soil and plant particles, aqueous phase (100 µl) of 10^{-5} dilutions were plated onto LB plates containing 50 mg l^{-1} of Cd (Afzal et al. 2012). Incubation was performed for 48 h at 37 °C. Bacterial colonies were quantified in terms of colony-forming units (CFU). The identity of isolates was confirmed through restriction fragment length polymorphism (RFLP) on previously amplified 16-23S rDNA intergenic spacer region (IGS). The PCR reaction was set up with the following primers: reverse (5′-GGCTGCTTCTAAGCCAAC-3′) and forward (5′-TGC GGCTGGATCACCTCCT-3′). The amplified IGS product was used to set up RFLP experiment. The RFLP reaction constituted 7 µl PCR product, 1 µl HindIII enzyme, 1.5 µl R-buffer, and 5.5 µl deionized water, to make a total of 15 µl reaction. Each RFLP reaction was placed at 37 °C for 3 h. The RFLP product was confirmed by comparing with controls on gel electrophoresis run in 2% agarose.

Data processing and statistical analysis

OriginPro v2016 software package was used to analyze the experimental data. After conducting *the Shapiro–Wilk* test, two-way ANOVA was performed and statistical differences were assessed by multiple comparisons between treatments while using the Bonferroni posthoc test ($p < 0.05$). The results of RFLP were plotted by using correspondence analysis in order to reveal the relationship between Cd concentration and distribution of inoculated bacteria in the plant compartments, i.e., rhizosphere, root interior, and shoot interior.

Results

Plant response in the presence of Cd contamination and bacterial inoculation

Effect of Cd contamination and endophytes inoculation was determined in terms of plant growth parameters such as root and shoot lengths, chlorophyll content (a + b), and dry biomass (Table 2; Figs. 1, 2). A decreasing trend was observed for all parameters with an increasing level of Cd concentration in soil. However, bacterial inoculation supported plant growth by enhancing plant root and shoot length, chlorophyll content, and dry biomass as compared to the uninoculated treatments. This increase was statistically relevant up to the concentration of 400 mg Cd kg^{-1} soil. Nevertheless, highest plant growth was observed when consortium was applied as compared to their individual applications (Table 2). Briefly, bacterial consortium increased shoot length by 62%, 48%, 37%, 24%; root length by 46%, 39%, 30%, 19%; chlorophyll content by 40%, 33%, 27%, 13%; and dry biomass by 64%, 57%, 45%, and 36% of *B. mutica* grown in 100, 200, 400 and 1000 mg Cd kg^{-1} soil, respectively. The statistical significance of the results was confirmed at the 5% level using a two-way ANOVA model, separately exploring treatment (bacterial inocula) and Cd doses. These results confirm our hypothesis that endophytic bacteria, when equipped with the appropriate metal-tolerant and plant growth-promoting activities, can help plants survive under conditions of elevated levels of Cd.

Cadmium accumulation in *B. mutica* and effect of bacterial endophytes

Cadmium concentration within plant tissues was detected to evaluate the effect of inoculated endophytic bacteria on phytoaccumulation (Table 3). Although *B. mutica* displayed a good potential of Cd accumulation in the roots, shoots, and

Table 2 Effect of bacterial inoculation on shoot length, root length and chlorophyll (mg g^{-1} fresh weight) of *Brachiaria mutica* grown in cadmium contaminated soil

Cd (mg kg^{-1} soil)	Treatment	Shoot length (cm)	Root length (cm)	Chlorophyll (mg g^{-1})
0	Uninoculated	103 ± 7.4	42.2 ± 3.1	2.11 ± 0.17
100	Uninoculated	$66^{cde} \pm 5.4$	$29.8^{bcde} \pm 2.2$	$0.88^{bcd} \pm 0.05$
	Pantoea stewartii ASI11	$73^{cd} \pm 6.2$	$33.9^{bcd} \pm 2.5$	$0.96^{ab} \pm 0.05$
	Microbacterium arborescens HU33	$77^{bcd} \pm 5.3$	$35.5^{bc} \pm 1.8$	$0.92^{bc} \pm 0.07$
	Enterobacter sp. HU38	$76^{bcd} \pm 4.8$	$34.7^{bc} \pm 2.3$	$0.97^{ab} \pm 0.06$
	Consortium*	$107^{a} \pm 7.9$	$43.4^{a} \pm 3.5$	$1.23^{a} \pm 0.12$
200	Uninoculated	$56^{efgh} \pm 3.2$	$26.3^{e} \pm 1.6$	$0.67^{efg} \pm 0.03$
	P. stewartii ASI11	$61^{efg} \pm 4.1$	$28.7^{cde} \pm 1.9$	$0.72^{cde} \pm 0.06$
	M. arborescens HU33	$66^{cde} \pm 5.3$	$29.4^{cde} \pm 2.3$	$0.75^{cde} \pm 0.04$
	Enterobacter sp. HU38	$62^{def} \pm 3.7$	$28.5^{cde} \pm 2.1$	$0.77^{cde} \pm 0.05$
	Consortium*	$83^{b} \pm 6.8$	$36.6^{ab} \pm 2.9$	$0.89^{bcd} \pm 0.04$
400	Uninoculated	$43^{h} \pm 2.4$	$22.4^{e} \pm 2.6$	$0.49^{gh} \pm 0.05$
	P. stewartii ASI11	$47^{fgh} \pm 3.7$	$26.4^{e} \pm 1.5$	$0.51^{gh} \pm 0.03$
	M. arborescens HU33	$48^{fgh} \pm 2.9$	$27.3^{de} \pm 1.1$	$0.53^{gh} \pm 0.02$
	Enterobacter sp. HU38	$46^{gh} \pm 3.1$	$25.8^{e} \pm 1.8$	$0.52^{gh} \pm 0.04$
	Consortium*	$59^{efg} \pm 4.3$	$29.2^{cde} \pm 2.1$	$0.62^{fgh} \pm 0.04$
1000	Uninoculated	$29^{ij} \pm 1.3$	$17.2^{f} \pm 0.8$	$0.31^{i} \pm 0.02$
	P. stewartii ASI11	$32^{ij} \pm 2.1$	$18.2ef \pm 0.6$	$0.32^{i} \pm 0.2$
	M. arborescens HU33	$31^{ij} \pm 1.7$	$18.5^{ef} \pm 1.1$	$0.33^{i} \pm 0.03$
	Enterobacter sp. HU38	$33^{ij} \pm 1.1$	$18.8^{ef} \pm 0.7$	$0.32^{i} \pm 0.02$
	Consortium*	$36^{i} \pm 2.3$	$20.4^{ef} \pm 1.3$	$0.35^{i} \pm 0.02$

The values presented are the means ± standard deviation (n = 3). Significant differences ($p < 0.05$) were determined using a two-way ANOVA followed by a Bonferroni posthoc test

Treatment groups with at least one common letter are not significantly different from each other

*Plants were inoculated with a mixture of *P. stewartii* ASI11, *M. arborescens* HU33, and *Enterobacter* sp. HU38

leaves; endophytes augmentation enhanced the accumulation rates significantly. The augmentation of all individual strains enhanced Cd accumulation in the plant, however, their effect was almost non-significant as compared to control at all five dosages of Cd. Moreover, they also performed almost equal at all concentrations of Cd in soil. In comparison to the individual bacterium, consortium application appeared to be more promising at all concentrations. More precisely, at a contamination level of 100, 200, 400 and 1000 mg Cd kg^{-1} soil, bacterial consortium helped increase in Cd accumulation within roots by 95%, 41%, 29%, 21%; in shoots by 55%, 44%, 38%, 24%; and in leaves by 54%, 44%, 38%, 24%, respectively. Nevertheless, maximum Cd accumulation was recorded at the highest Cd concentration (1000 mg kg), i.e., 889 mg kg^{-1} in the roots, 83 mg kg^{-1} (in the shoots, and 71 mg kg^{-1} in the leaves. The increasing trend of Cd accumulation in *B. mutica* can be written in the following order: roots > shoots > leaves.

The phytoextraction/phytoaccumulation potential of *B. mutica* was evaluated in terms of BCF and TF (Table 4). A pronounced effect of bacterial augmentation was recorded for both BCF and TF, nevertheless, bacterial consortium displayed significant accumulation ($p < 0.01$). Briefly, at contamination levels of 100, 200, 400, and 1000 mg Cd kg^{-1} soil, BCF_{roots} increased from 1.57 to 3.06, 1.59 to 2.24, 0.97 to 1.25, and 0.73 to 0.89, respectively. However, BCF_{shoots} and TF were < 1 mg Cd kg^{-1} soil at all levels even in the presence of endophytic bacteria.

Bacterial survival/persistence in the rhizo- and endosphere

The survival/persistence of inoculated endophytes was enumerated in the rhizosphere, root interior, and shoot interior in order to verify their potential role in the phytoremediation (Table 5). The inoculated bacteria displayed persistence in the rhizospheric soil, and root and shoot the interior of *B. mutica*. Nevertheless, high survival was observed in the root interior followed by the rhizosphere and shoot interior. On the other hand, poor survival was observed in the presence of higher Cd levels. Except for *Enterobacter* at 200 mg Cd kg^{-1} for rhizosphere or *Enterobacter* and *M. arborescens* for the root interior at 1000 mg Cd kg^{-1}, only bacterium consortium was effective as protectors on bacterial

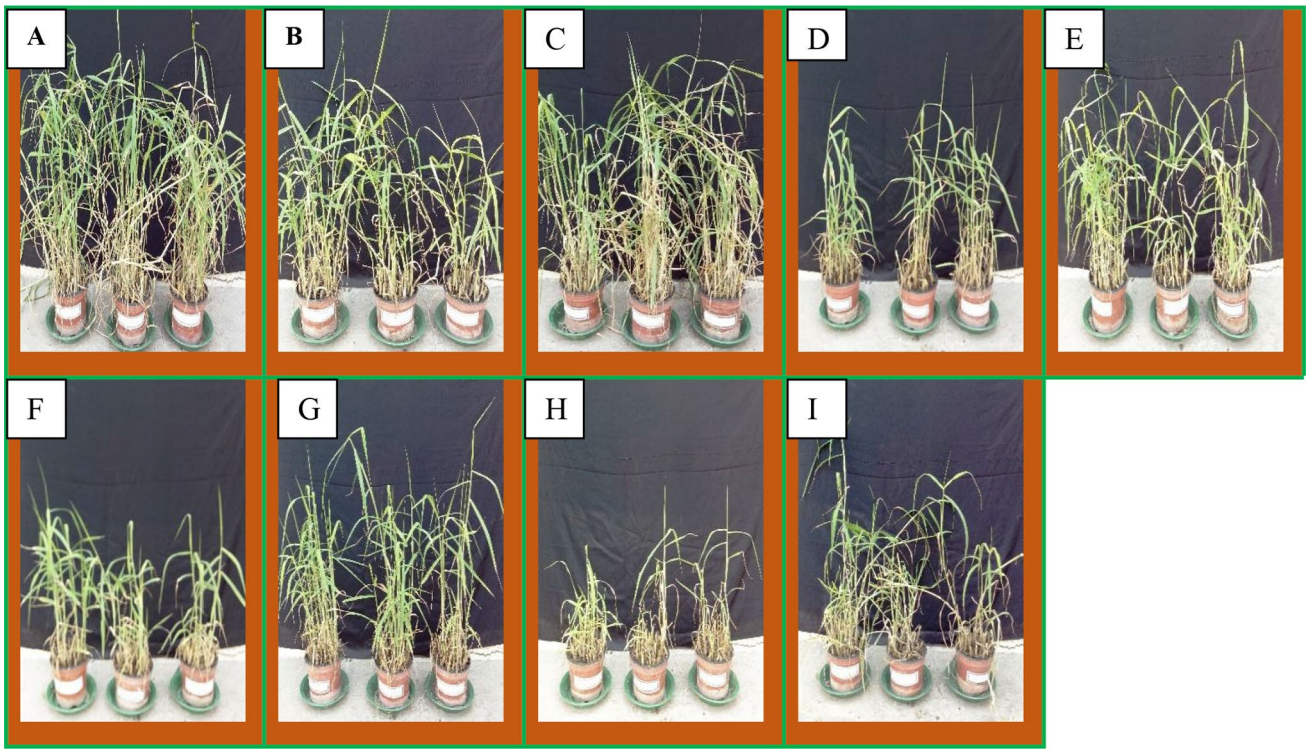

Fig. 1 Effect of Cd contamination level and endophytes inoculation on growth of *Brachiaria mutica* vegetated in Cd contaminated soil. **a** Uncontaminated soil; **b** soil contaminated with Cd 100 mg kg^{-1} soil; **c** soil contaminated with Cd 100 mg kg^{-1} soil and inoculated with bacterial consortium; **d** soil contaminated with Cd 200 mg kg^{-1} soil; **e** soil contaminated with Cd 200 mg kg^{-1} soil and inoculated with bacterial consortium; **f** soil contaminated with Cd 400 mg kg^{-1} soil and G, soil contaminated with Cd 400 mg kg^{-1} soil and inoculated with bacterial consortium; **h** soil contaminated with Cd 1000 mg kg^{-1} soil and **i** soil contaminated with Cd 1000 mg kg^{-1} soil and inoculated with bacterial consortium

survival/persistence. The bacterial population was highest (i.e., $6.8 \pm 0.29 \times 10^6$ CFU g^{-1}) in the root interior at 100 mg Cd kg^{-1} soil. RFLP analyses elucidated that 53–76% were our inoculated strains in the root interior, 47–59% in the shoot interior, and 67–74% in the rhizosphere. Correspondence analysis conducted on RFLP dataset revealed that the concentration of Cd had a positive effect on survival and colonization of inoculated bacteria (Fig. 3). Briefly, concentration up to 400 mg Cd kg^{-1} had less significant effect on the distribution of inoculated bacteria whereas their pattern in the root and shoot interior were quite similar. Likewise, concentration of 1000 mg Cd kg^{-1} soil displayed a pronounced effect on the bacterial profile both at individual and consortium level.

Discussion

The ability of plants to hyperaccumulate HMs in their tissues is extensively linked to their root and shoot biomass. Plants with less aerial biomass are unsuitable for phytoextraction whereas, plants with less root biomass are unfavorable for phytostabilization (Gołda and Korzeniowska 2016). Recently, fast-growing plants with high-biomass are actively tested for their potential to accumulate moderate levels of HMs in their roots and shoots. Among these plants, grasses displayed a significant tolerance/accumulation against HM pollution. Therefore, efforts are being made to improve the phytoremediation potential by helping plants gain more biomass as well by minimizing the phytotoxic effects caused by HMs (Liu et al. 2010; Prapagdee et al. 2013; Hechmi et al. 2015). This study suggests the potential of using plant-endophyte interplay in the line of these efforts for increased removal of Cd from the contaminated environment.

We found that root length, shoot length, chlorophyll content, plant biomass were decreased with an increase in Cd contamination. This observation is consistent with the earlier reports where higher Cd contaminations decreased plant development (Chen et al. 2003; Lin et al. 2014; Gołda and Korzeniowska 2016). However, the application of Cd-tolerant PGP endophytic bacteria expedites phytoremediation capacity of plants which can be attributed to the production of plant growth hormones as well as stress alleviation properties of the inoculated bacteria (Ahemad 2015; Ozyigit and Dogan 2015). These bacteria previously displayed positive results for ACC-deaminase, phosphorus solubilization,

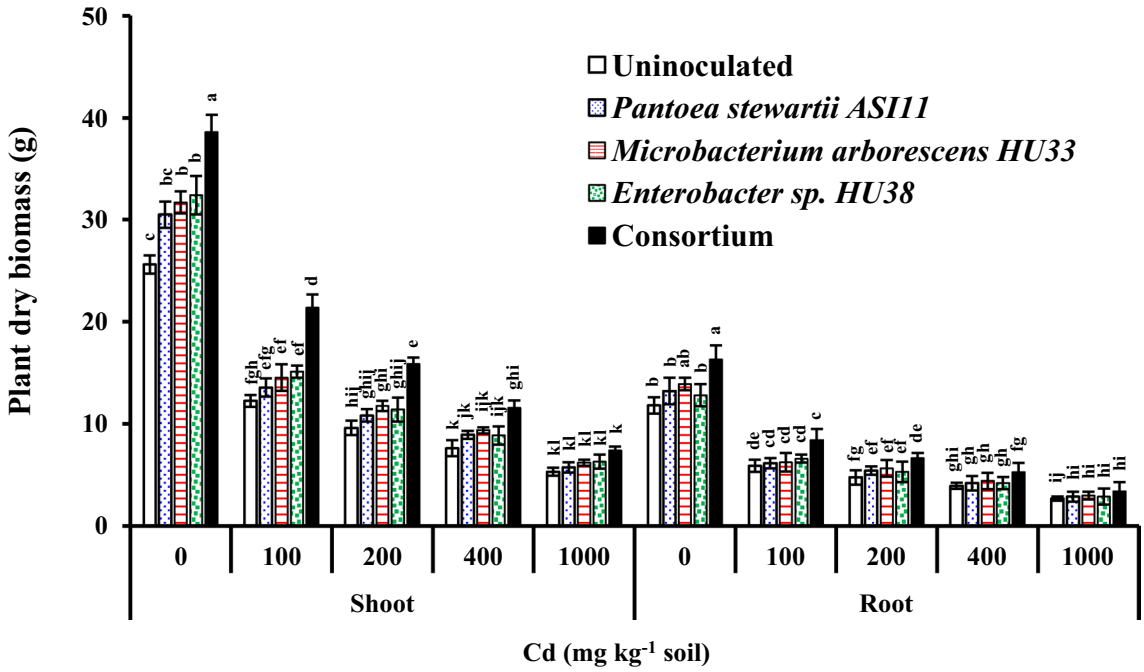

Fig. 2 Effect of bacterial inoculation on dry biomass of shoot and root of *Brachiaria mutica* grown in Cd amended soils at different contamination levels. Significant differences ($p < 0.05$) were determined using a two-way ANOVA followed by a Bonferroni posthoc test. Treatment groups with at least one common letter are not significantly different from each other. Each error bar represents the standard deviation (SD) of the measurements

Table 3 Effect of bacterial inoculation on the accumulation of cadmium in the root, shoot and leaves of *Brachiaria mutica* grown in Cd contaminated soils

Cd (mg kg^{-1})	Treatment	Root (mg kg^{-1})	Shoot (mg kg^{-1})	Leaves (mg kg^{-1})
100	Uninoculated	$157^{g} \pm 6.8$	$11^{f} \pm 0.8$	$9^{g} \pm 0.4$
	Pantoea stewartii ASI11	$168^{g} \pm 4.6$	$13^{ef} \pm 0.6$	$10^{fg} \pm 0.6$
	Microbacterium arborescens HU33	$172^{g} \pm 9.3$	$15^{ef} \pm 1.1$	$10^{fg} \pm 0.3$
	Enterobacter sp. HU38	$171^{g} \pm 7.2$	$13^{ef} \pm 0.5$	$11^{fg} \pm 0.8$
	Consortium*	$306^{f} \pm 13.4$	$17^{e} \pm 0.7$	$13^{f} \pm 0.7$
200	Uninoculated	$318^{f} \pm 13$	$16^{e} \pm 1.4$	$13^{f} \pm 1.2$
	P. stewartii ASI11	$328^{ef} \pm 11$	$19^{de} \pm 1.2$	$15^{ef} \pm 1.1$
	M. arborescens HU33	$332^{ef} \pm 15$	$18^{de} \pm 1.4$	$15^{ef} \pm 0.8$
	Enterobacter sp. HU38	$323^{ef} \pm 17$	$22^{de} \pm 1.1$	$16^{ef} \pm 1.3$
	Consortium*	$447^{cd} \pm 24$	$23^{cde} \pm 1.2$	$18^{def} \pm 1.5$
400	Uninoculated	$386^{def} \pm 28$	$21^{de} \pm 1.3$	$19^{def} \pm 1.3$
	P. stewartii ASI11	$396^{def} \pm 23$	$25^{cde} \pm 1.3$	$22^{cd} \pm 1.7$
	M. arborescens HU33	$399^{de} \pm 31$	$24^{cde} \pm 1.7$	$20^{cde} \pm 1.5$
	Enterobacter sp. HU38	$401^{de} \pm 21$	$27^{cd} \pm 1.6$	$21^{cde} \pm 1.8$
	Consortium*	$498^{c} \pm 18$	$29^{c} \pm 2.1$	$26^{cd} \pm 1.7$
1000	Uninoculated	$734^{b} \pm 33$	$67^{b} \pm 5.3$	$59^{b} \pm 3.4$
	P. stewartii ASI11	$741^{b} \pm 22$	$73^{b} \pm 3.7$	$61^{b} \pm 2.9$
	M. arborescens HU33	$758^{b} \pm 27$	$75^{ab} \pm 4.2$	$66^{ab} \pm 3.4$
	Enterobacter sp. HU38	$748^{b} \pm 31$	$72^{b} \pm 5.7$	$63^{b} \pm 3.8$
	Consortium*	$889^{a} \pm 39$	$83^{a} \pm 7.6$	$71^{a} \pm 6.1$

The values presented are the means ± standard deviation (n = 3). Significant differences ($p < 0.05$) were determined using a two-way ANOVA followed by a Bonferroni posthoc test

Treatment groups with at least one common letter are not significantly different from each other

*Plants were inoculated with a mixture of *P. stewartii* ASI11, *M. arborescens* HU33, and *Enterobacter* sp. HU38

Table 4 Bioconcentration and translocation factors of *Brachiaria mutica* grown in cadmium contaminated soils

Cd (mg kg^{-1})	Treatment	BCF		TF
		Roots	Shoots	
100	Uninoculated	1.57	0.11	0.07
	Pantoea stewartii ASI11	1.68	0.13	0.08
	Microbacterium arborescens HU33	1.72	0.15	0.09
	Enterobacter sp. HU38	1.71	0.13	0.08
	Consortium*	3.06	0.17	0.06
200	Uninoculated	1.59	0.08	0.05
	P. stewartii ASI11	1.64	0.10	0.06
	M. arborescens HU33	1.65	0.09	0.05
	Enterobacter sp. HU38	1.62	0.09	0.06
	Consortium*	2.24	0.12	0.05
400	Uninoculated	0.97	0.05	0.05
	P. stewartii ASI11	0.99	0.06	0.06
	M. arborescens HU33	1.00	0.06	0.06
	Enterobacter sp. HU38	1.00	0.06	0.06
	Consortium*	1.25	0.07	0.06
1000	Uninoculated	0.73	0.07	0.07
	P. stewartii ASI11	0.74	0.07	0.07
	M. arborescens HU33	0.75	0.08	0.08
	Enterobacter sp. HU38	0.74	0.07	0.07
	Consortium*	0.89	0.08	0.08

BCF bioconcentration factor, *TF* translocation factor

*Plants were inoculated with a mixture of *P. stewartii* ASI11, *M. arborescens* HU33, and *Enterobacter* sp. HU38

siderophore production, and indole acetic acid formation; as well as showed high resistance up to 300–400 mg l^{-1} for As, Pb, U, and Zn (Khan et al. 2015; Ahsan et al. 2017). It is well established that the phytohormone IAA released by the bacteria enhance plant growth, promote nutrients absorption, and increase metals uptake by the plant roots (Glick 2003; Prapagdee et al. 2013; Ahemad 2015). Similarly, siderophores help chlorophyll biosynthesis and improve plant development by facilitating iron availability to the stressed plants (Rajkumar et al. 2009, 2010). The ACC deaminase activity, however, reduces HMs stress by hydrolyzing ACC and reducing the ethylene level in the roots, which is a growth-inhibiting signal in plants (Glick et al. 2007; Ma et al. 2011; Hao et al. 2012; Arslan et al., 2014). Previously, potential of *P. stewartii* J1-13-7 was also tested for PGP activities in the presence of Cu contamination (Zhang et al. 2011), *M. arborescens* NE1E3 for Cu, Zn, Pb, and As (Román-Ponce et al. 2016), and several other species of *Enterobacter* for many HMs including Cd (Nie et al. 2002; Egidi et al. 2016; Ma et al. 2016; Tirry et al. 2018). Likewise, their potential in developing effective plant-bacteria partnership with *Leptochloa fusca* has also been reported for treating tannery effluent containing high HM content (Ashraf et al. 2018), and of *P. stewartii* J1-13-7 strain for *Brassica napus* (Zhang et al. 2011). Since plant recruits endophytic bacteria from external environment, it can be argued that these bacteria may have possessed necessary genes that help them adopt plant environment adequately.

Although single strain augmentation improved plant growth, statistical analysis showed that the relative increase in shoot and root length, chlorophyll content and dry biomass were less significant ($p < 0.05$) than the treatments with the bacterial consortium, when compared with the uninoculated treatments. The augmentation of bacterial consortium resulted in maximum root and shoot length, chlorophyll content and dry biomass (Table 2; Fig. 2). Enhanced growth of *B. mutica* in the presence of bacterial consortia may be attributed to synergistic PGP activities of bacterial endophytes which were less prominent alone (Lee et al. 2004; Dary et al. 2010; Srivastava et al. 2013). This has been seen several times where single inoculant had a poor effect on plant growth whereas consortium improved the growth greatly (Kumar et al. 2016; Khan et al. 2015; Ahsan et al. 2018). For instance, Kumar et al. (2016) reported that the combined application of PGP rhizobacteria strengthens the synergistic effect under drought stress and result in improved plant growth and development. Likewise, Dary et al. (2010) reported similar results where co-inoculation of metal resistant PGP rhizobacteria (*Bradyrhizobium* sp., *Pseudomonas* sp. and *Ochrobactrum cytisi*) produced an additional improvement of plant biomass in lupines. The inoculated bacteria can also enhance the mobility/bioavailability of Cd by solubilizing the mineral salt thus enhancing the metal uptake by plant roots (Ahemad 2015). Based on these observations, it can be argued that application of metal resistant PGP bacteria in the form of consortium is a more promising strategy as compared to the individual applications.

All plants are capable of HMs uptake to a certain extent. *B. mutica* also showed a good tendency to accumulate Cd *in planta*. Nevertheless, the application of bacterial consortia significantly improved the overall phytoaccumulation potential. This can be attributed to the effective plant-bacteria interplay which resulted in increased plant performance for pollutant removal (Khan et al. 2015). However, high accumulation within plant roots could be due to the less mobility of Cd while transporting from root to the aerial tissues (Gjengedal and Steinnes 1994; Marques et al. 2013; Prapagdee and Khonsue 2015; Gołda and Korzeniowska 2016; Mohammadzadeh et al. 2016). This was further confirmed by high BCF_{root}, low BCF_{shoot} and low TF values. This shows that *B. mutica* has a natural potential for phytoaccumulation of Cd-contaminated soils however the phytaccumulation capacity can be increased significantly by application of Cd-tolerant PGP bacteria. A similar relationship for different

Table 5 Effect of metal contamination on bacterial survival and persistence (CFU g^{-1} dry weight) in the rhizosphere (RH), root interior (RI) and shoot interior (SI) of *Brachiaria mutica* grown in cadmium contaminated soil

Cd (mg kg^{-1})	Treatment	RH (CFU g^{-1} soil $\times 10^4$)	RI (CFU g^{-1} root $\times 10^4$)	SI (CFU g^{-1} shoot $\times 10^4$)
100	*Pantoea stewartii* ASI11	$65.8^{b} \pm 2.2$	$493^{bc} \pm 19$	$2.6^{bc} \pm 0.14$
	Microbacterium arborescens HU33	$64.3^{b} \pm 2.4$	$514^{bc} \pm 16$	$3.2^{b} \pm 0.11$
	Enterobacter sp. HU38	$68.4^{b} \pm 2.0$	$478^{cd} \pm 24$	$2.4^{bc} \pm 0.08$
	Consortium*	$86.3^{a} \pm 3.4$	$687^{a} \pm 29$	$5.2^{a} \pm 1.1$
200	*P. stewartii* ASI11	$46.7^{c} \pm 1.8$	$384^{f} \pm 17$	$1.9^{cd} \pm 0.09$
	M. arborescens HU33	$47.6^{c} \pm 1.4$	$398^{f} \pm 22$	$2.2^{bc} \pm 0.11$
	Enterobacter sp. HU38	$51.3^{c} \pm 3.1$	$423^{de} \pm 18$	$2.1^{cd} \pm 0.13$
	Consortium*	$63.8^{b} \pm 2.9$	$543^{b} \pm 31$	$2.8^{c} \pm 0.12$
400	*P. stewartii* ASI11	$35.1^{d} \pm 1.1$	$231^{f} \pm 10$	$1.1^{e} \pm 0.06$
	M. arborescens HU33	$33.4^{d} \pm 1.7$	$264^{f} \pm 12$	$1.3^{e} \pm 0.08$
	Enterobacter sp. HU38	$31.5^{d} \pm 2.7$	$243^{f} \pm 11$	$1.2^{e} \pm 0.05$
	Consortium*	$48.4^{c} \pm 3.1$	$386^{e} \pm 14$	$1.8^{d} \pm 0.09$
1000	*P. stewartii* ASI11	$7.3^{fg} \pm 0.3$	$93^{i} \pm 2.6$	$0.8^{f} \pm 0.02$
	M. arborescens HU33	$10^{f} \pm 0.4$	$102^{h} \pm 6.4$	$0.6^{f} \pm 0.04$
	Enterobacter sp. HU38	$11^{f} \pm 0.6$	$113^{h} \pm 8.3$	$0.7^{f} \pm 0.03$
	Consortium*	$23^{e} \pm 0.5$	$142^{g} \pm 7.6$	$1.1^{e} \pm 0.06$

The values presented are the means ± standard deviation (n = 3). Significant differences ($p < 0.05$) were determined using a two-way ANOVA followed by a Bonferroni posthoc test

Treatment groups with at least one common letter are not significantly different from each other

*Plants were inoculated with a mixture of *P. stewartii* ASI11, *M. arborescens* HU33, and *Enterobacter* sp. HU38

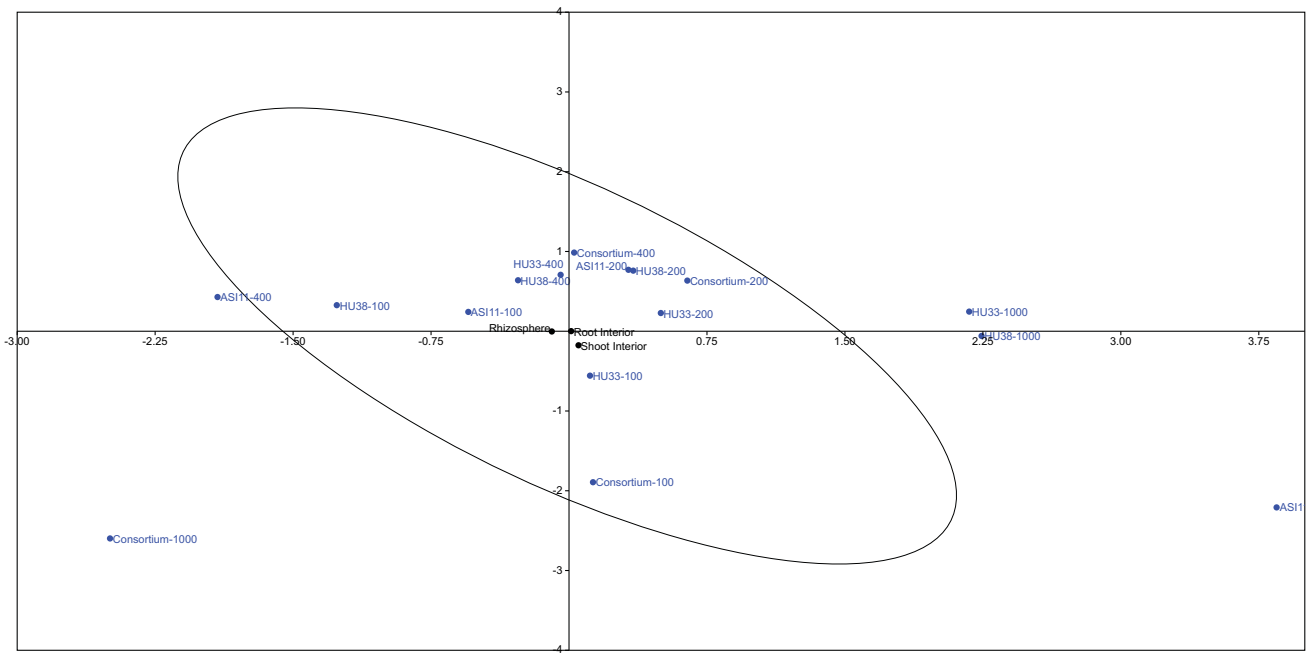

Fig. 3 Correspondence analysis conducted between the profile of inoculated bacteria and Cd concentration

plants has been observed by Sharma and Archana (2016) as well.

In a successful plant-endophyte interplay, inoculant bacteria must display the ability to survive in the plant rhizo- and/or endosphere. In our investigation, inoculated bacteria showed successful survival in the roots followed by shoots of *B. mutica*; while maximum bacteria were observed in the root interior. This observation is in-line with

the earlier studies which described that PGP bacteria can colonize the host environment ubiquitously (He et al. 2013; Yuan et al. 2014). The higher persistence of endophytic bacteria in the root endosphere may also be credited to the fact that (1) these bacteria were previously isolated from plant interior, and (2) endosphere offers more protection to the bacteria against the deleterious external environment. Moreover, it has been reported that *B. mutica* is able to recruit contaminant resistant bacteria from the external environment when exposed to the contaminants (Ashraf et al. 2018; Fatima et al. 2016). Similar behavior of bacterial colonization within plant endosphere rather than rhizosphere has also been described in different studies (Andria et al. 2009; Afzal et al. 2012). Interestingly, correspondence analysis further showed that the high concentration of Cd affected the bacterial distribution in the plant rhizo- and endosphere; this may have led to the weakened performance of the plant-bacteria partnership due to high toxicity.

Conclusions

The results mentioned above show that inoculated bacteria were able to survive/persist the plant interior mainly in the roots. Higher values of BCF_{root} (> 1), and lower values for BCF_{shoot} (< 1) and TF (< 1) indicates the capability of *B. mutica* to tolerate and accumulate a significant proportion of Cd in the roots with less translocation to aboveground parts, hence reducing the possibilities of Cd uptake in the food chain. This shows that an effective plant-bacteria interplay can help increase the phytoaccumulation potential of the plants especially for those who possess a natural tendency of bioaccumulation. Nevertheless, application of PGP endophytic bacterial consortium instead of single bacterial strain is a better option to encounter soil hostile conditions. This preliminary study set the basis for deeper studies in future regarding the genes involved in the underlying mechanisms of Cd uptake and translocation effects.

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