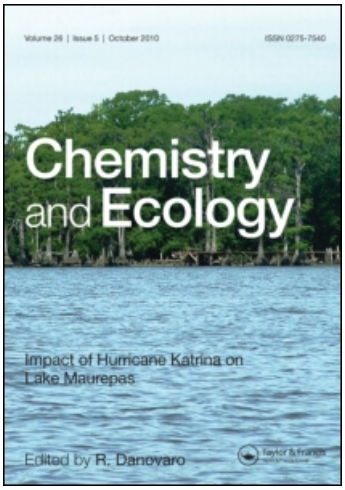

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Effect of *Pseudomonas fluorescens* on metal phytoextraction from contaminated soil by *Brassica juncea*

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Effect of *Pseudomonas fluorescens* on metal phytoextraction from contaminated soil by *Brassica juncea*

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The present study deals with metal uptake by *Brassica juncea* in the presence of *Pseudomonas fluorescens* Pf 27 for Zn, Cu and Cd removal from brass and electroplating-industry effluent-contaminated soil. Inoculation of *P. fluorescens* significantly ($p < 0.05$) increased water soluble (Ws) and exchangeable (Ex) metal content in contaminated soil in laboratory conditions and also enhanced plant biomass by 99% and chlorophyll content by 91% as compared to uninoculated plants in the greenhouse. The metal uptake by *B. juncea* followed the pattern Zn > Cu > Cd and increased with increasing plant growth duration. *P. fluorescens* inoculation increased root and shoot uptake of Zn by 3.05 and 2.69, Cu by 3.19 and 2.82 and Cd by 3.11- and 2.75-fold, respectively. BCF value for each metal was >1 and increased by 44%, 42% and 38% for Zn, Cu and Cd, respectively, in inoculated conditions, whereas TF remained unaffected and followed the order Zn > Cd > Cu. *P. fluorescens* inoculation also enhanced Ws fraction of Zn, Cu and Cd by 99%, 77% and 90% and Ex by 107%, 70% and 93%, respectively. Results depicted that association of *B. juncea* with *P. fluorescens* could be a promising strategy for enhancing soil metal bioavailability and plant growth for successful phytoremediation of heavy metal contaminated soils.

Keywords: heavy metal; phytoremediation; *Brassica juncea*; *Pseudomonas fluorescens*; bioconcentration factor; translocation factor

1. Introduction

The global industrial revolution has given rise to various environmental problems, including heavy metal pollution. Some metals/metalloids, especially cadmium, lead, mercury and arsenic are toxic even in trace amounts as they disrupt enzyme functions, replace essential metals in pigments or produce reactive oxygen species [1]. The problem of ground water and soil contamination by heavy metals is in need of an effective and affordable technological solution [2]. Conventional remediation technologies are generally too costly and often harmful to the soil characteristics [3,4]. The use of plants, i.e. phytoremediation, is a promising technology for the cleansing of both aquatic and terrestrial habitats owing to their low cost and ecofriendly nature [5], which involves various specified mechanisms, such as: (i) phytoextraction – absorption of metals from the soil by plant roots and translocation to other tissues; (ii) phytovolatilisation – volatilisation of metals

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by plants to the atmosphere; (iii) phytostabilisation – stabilisation of metals into a less soluble form; and (iv) rhizofiltration – absorption, concentration or precipitation of metals by plant roots. Plants capable of accumulating more than 1,000 mg kg^{-1} dry weight of metals like Ni, Co, Cu and Pb and 100 and 10,000 mg kg^{-1} for Cd and Zn, respectively, in their shoots are termed hyperaccumulators [6], whilst those that grow fast, produce high biomass and tend to accumulate moderate concentrations of metals are called accumulators [7].

Since remediation by plants is time consuming, it requires acceleration by exploiting rhizospheric microflora and their modifications [8]. In this context, the role of rhizobacteria enhancing plant biomass, metal uptake and nutrient availability [9] through the release of chelating agents, acidification, phosphate solubilisation, and redox changes [10] and synthesis of phytohormones precursors [11,12], vitamins, enzymes, siderophores, antibiotics [13, 14] has been elucidated. Therefore, the application of rhizobacteria in combination with plants is expected to provide high efficiency for phytoremediation [10,15]. Reports are also available on *Pseudomonas* spp. solubilising Zn, Ni and Cu, promoting growth of *Ricinus communis* [16], and increasing plant biomass and Cd accumulation in *Brassica napus* [17] in metal-stressed soil.

Brass and electroplating industries, located at Moradabad in north-west Uttar Pradesh, India, release large amounts of effluent having a pH of 6.1 and total Zn 7.6, Cu 6.10, Cd 4.1, Cr 5.2 and Ni 4.4 mg/l [18]. The effluent, while passing through the drain before joining a tributary of the River Ganges, i.e. Ramganga, overflows and spreads over the surrounding arable land. Also, the effluent is used as unlevied irrigation water by local farmers, which has resulted in the accumulation of heavy metals, viz. Cu, Cr, Cd, Ni and Zn, up to toxic levels in soil [18], which need to be reclaimed. The present investigation was undertaken to assess the phytoextraction potential of Indian mustard (*Brassica juncea*) in combination with *Pseudomonas fluorescens* strain Pf 27 for Zn, Cu and Cd removal from the effluent-contaminated soil.

2. Materials and methods

2.1. *Soil sampling and analysis*

The soil samples S1 (effluent-contaminated) and S2 (not contaminated by effluent or fertilisers) were collected from the site about 10 m and 1000 m longitudinal distance from the effluent drain, respectively. The samples were air dried, sieved through 2 mm screens and mixed thoroughly. Soil chemical properties such as pH (measured by a digital pH meter), EC (measured by an EC meter), organic carbon (measured by the Walkley-Black method) [19] were determined. The total metal (Zn, Cu and Cd) content was assessed using an atomic absorption spectrophotometer (AAS, GBC Avanta Ver. 1.33, Australia) after digesting 0.5 g of dried soil samples with 15 mL of HNO_3, H_2SO_4 and $HClO_4$ in a 5:1:1 ratio at 80 °C [20] and filtered through Whatman No. 42 filter paper, followed by dilution up to 50 mL with triply distilled water. The water soluble (Ws) metal content was assessed after adding distilled water in a ratio of 1:1 (w/v) and the exchangeable (Ex) fraction by extraction with 0.01 mol/l $CaCl_2$ (1:10 w/v, soil: $CaCl_2$), followed by acidification with HNO_3 and using AAS [21].

2.2. *Metal extraction from soil using* **P. fluorescens** *supernatant*

P. fluorescens strain Pf 27, obtained from the Department of Plant Pathology, College of Agriculture, G.B. Pant University of Agriculture and Technology, Pantnagar, possesses the properties of rhizospheric colonisation, production of siderophores, phytohormones like indole acetic acid (IAA), cytokinins and ethylene, antifungal compounds viz. β-1-3 glucanase, chitinase and

antibiotics, besides nutrient-solubilising ability [22]. Therefore, the ability of *P. fluorescens* to enhance water soluble (Ws) and exchangeable (Ex) fractions of Zn, Cu and Cd in S1 soil was tested in *in vitro* conditions, as these fractions are most readily available for plant uptake [23]. For this, the strain was grown overnight in 500 mL Erlenmeyer flasks containing 300 mL of sterilised nutrient broth [24] in a shaker at 150 rpm at 30 °C. Another flask containing sterile nutrient broth was uninoculated as a sterile (axenic) control. From each flask, 50 mL of medium was centrifuged at 8000 *g* for 15 min; the supernatant was decanted and vacuum filtered through a sterile filter (0.22 μm pore size). The ability of the filtrate to extract metal from the soil was determined after 0, 12, 24, 36, 48 and 60 h of bacterial growth by shaking a 2 g soil sample with 10 mL of each of the bacterial or axenic filtrates for 2 h in triplicate. The soil suspensions were centrifuged at 4000 *g* for 15 min, filtered and acidified with HNO_3 to determine Ws and Ex metal fractions using AAS.

2.3. *Pot experiment*

The soil of S1 and S2 were separately mixed with fertilisers (0.44 g of urea and 0.88 g KH_2PO_4 kg^{-1} of soil) and filled in pots. Seeds of *B. juncea* var. Kranti, obtained from the Crop Research Centre of G.B. Pant University of Agriculture and Technology, Pantnagar, were first sterilised with a mixture of ethanol and 30% H_2O_2 (1:1) for 10 min and then washed with sterile water to remove surface contaminations. Ten seeds per pot (having 4 kg of soil) were allowed to grow for 15 days and after germination, the seedlings were thinned to three per pot. The experiment consists of three harvests (i.e. 30, 60 and 90 days) with three replicates of each of the two treatments (inoculated and uninoculated), performed in a greenhouse at 25 ± 4 °C temperature. Pot soil moisture was maintained throughout the experiment at 70% water holding capacity. A corresponding control (plant free) was also maintained.

2.4. P. fluorescens *inoculation in soil*

P. fluorescens was grown in sterilised nutrient broth. Cells in exponential phase were collected after 16 h, followed by centrifugation at 16,099 *g* for 10 min, and the pellets were then washed twice with sterile distilled water. Bacterial inoculum was prepared by resuspending pelleted cells in sterile distilled water to get an inoculum density of ca. 10^8 cfu/mL using a dilution plate technique. The soil was inoculated with bacterial suspension (10 mL/pot) after 10 days of seedling emergence, whereas in uninoculated pots an equal amount of sterile distilled water was sprayed.

2.5. *Post-harvest plant and soil analysis*

Three plants per treatment were harvested after 30, 60 and 90 days of seedling emergence. After removing the plants, the roots and shoots were separated, washed with deionised water and were dried at 105 °C for biomass determination. Total leaf chlorophyll was estimated by the Arnon method [25]. The milled plant material (0.5 g per treatment) was digested with a mixture of concentrated HCl:HNO_3 (4:1 v/v) [26] and analysed for Zn, Cu and Cd using AAS. After each harvest, pot soil was also analysed for Ws and Ex fraction of metals.

2.6. *Statistical analysis*

Experimental data were presented as mean values ± standard deviation (SD) using MS Excel (2003). To verify the statistical significance of difference among treatments, the data were analysed using Student's *t*-test as available in the SPSS statistical package (Statgraphics Plus V. 11), and were expressed at a 0.05 probability level.

3. Results and discussion

3.1. *Soil characteristics*

The metal-contaminated soil S1 was found to be acidic (pH 6.34) with lower Ec values and higher moisture content than the uncontaminated soil S2 (Table 1), which can be attributed to the exposure of the soil to acidic effluent. Relatively low organic matter in S1 may be accounted for by the high concentration of metals, which might have suppressed microbial mineralisation of organically bound carbon [27,28]. Further, the water soluble Zn, Cu and Cd in S1 soil were ca. 6.0, 2.0 and 4.0-fold higher, respectively, than in S2, whilst the corresponding increase in Ex fraction was ca. 8.5, 5.5 and 15.0-fold. Higher metal content in S1 was due to the high frequency of intentional and/or unintentional irrigation with metal rich effluent, which declined in S2. The relatively higher bioavailable metal content in S1 is well correlated with its lower pH, which invariably resulted into increased competition between H^+ ions and positively charged metal ions for negatively charged binding sites in soil [1].

3.2. *Bacterial efficiency for metal solubilisation*

P. fluoresens inoculation for 24 h resulted in a significant ($p < 0.05$) increase in soil Ws and Ex metal fractions (Figure 1). Maximum Ws Zn (5.01 mg kg^{-1}) was recorded after 60 h of bacterial growth, whereas maximum Cu (2.59 mg kg^{-1}) and Cd (0.08 mg kg^{-1}) were obtained after 48 h. The values increased by 66%, 44% and 58% for Zn, Cu and Cd, respectively, as compared to the control. However, maximum Ex Zn, Cu and Cd (8.22, 4.54 and 0.75 mg kg^{-1}) increased by 59%, 45% and 54%, respectively, over the uninoculated control. In general, Ex metal content was higher than the Ws fraction in both *P. fluorescens* in inoculated as well as uninoculated soil, and this depends on the texture and other physicochemical properties, viz. organic meter, pH, CEC of soils [29]. Apart from this, various microbial metabolites, such as biosurfactants [30,31], siderophores [32,33] and/or organic acids [34,35] have been shown to increase soil metal bioavailability [36]. Huang et al. [37] also reported the marked increase in metal solubility in soil inoculated with *P. putida*.

Table 1. Chemical characteristics of contaminated (S1) and uncontaminated (S2) soil as affected by brass and electroplating industry effluent (mean ± SD, $n = 3$).

Parameters	S1 soil	S2 soil
pH	6.34 ± 0.37	7.04 ± 0.45
EC (ds/m)	0.50 ± 0.05	0.61 ± 0.03
Organic carbon (%)	1.78 ± 0.45	2.30 ± 0.41
Soil moisture (%)	21.08 ± 1.22	16.5 ± 1.10
Total Zn (mg/kg)	77.08 ± 5.24	19.12 ± 1.83
Ws Zn (mg/kg)	2.03 ± 0.26	0.33 ± 0.02
Ex Zn (mg/kg)	3.67 ± 0.38	0.43 ± 0.05
Total Cu (mg/kg)	67.32 ± 5.2	23.39 ± 1.62
Ws Cu (mg/kg)	1.11 ± 0.08	0.53 ± 0.02
Ex Cu (mg/kg)	2.33 ± 0.26	0.42 ± 0.03
Total Cd (mg/kg)	6.46 ± 0.40	2.10 ± 0.17
Ws Cd (mg/kg)	0.04 ± 0.01	0.01 ± 0.008
Ex Cd (mg/kg)	0.66 ± 0.14	0.04 ± 0.01

Note: Ws and Ex denote water soluble and exchangeable fraction of metal, respectively.

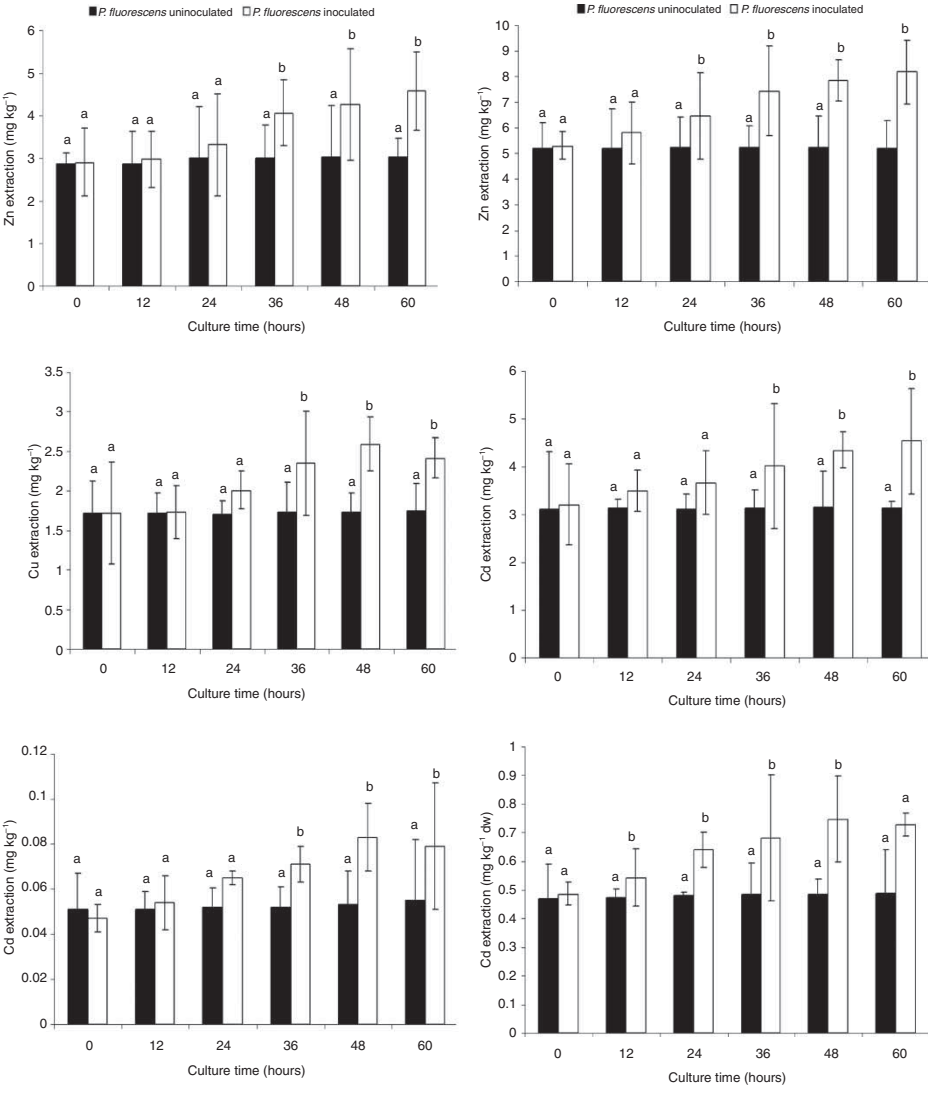

Figure 1. Extraction of water soluble (a) and exchangeable (b) Zn, Cu and Cd (mean ± SD) from metal contaminated soil inoculated by *P. fluoresens* in *in vitro* conditions. Different letters indicate significant difference at $p < 0.05$ ($n = 3$).

3.3. *Effect of* P. fluorescens *inoculation on plant growth*

The growth inhibition and pigment contents in the leaves are good parameters to be included as a phytotoxicity index. The plant grown in contaminated soil (S1) for 90 days showed toxicity symptoms in terms of a reduction of biomass and chlorophyll content by 45% and 70%, respectively, compared to the plant grown in S2 soil (Figures 2 and 3). Similar phytotoxic effects of heavy metals on other accumulators and hyperaccumulators were reported [38–41], particularly in dicots such as *B. juncea* [42]. The relatively significant ($p < 0.05$) decrease in chlorophyll content in S1-grown plants in present study exhibited similarity with the observations of Fargasova [43] for Cd, Zn, Pb, Cu, and Fe in mustard, and of Samantary [44] for Cr in mung bean, and may be attributed to metal-induced ultrastructural disorders in leaves [45] and inactivation of photosystem II [46], leading to the impaired chlorophyll biosynthesis and its destruction. However, inoculation of

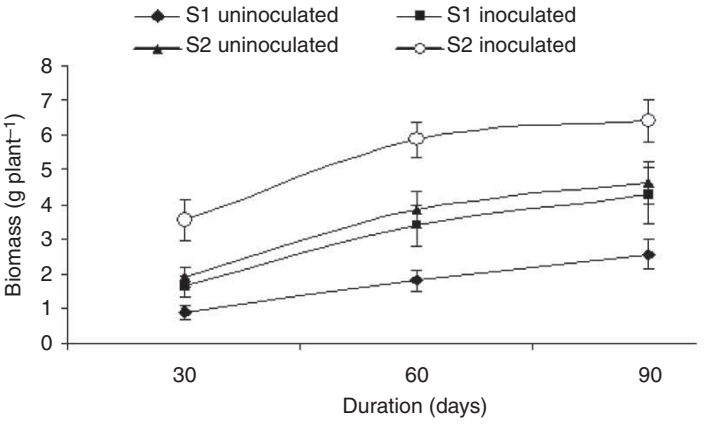

Figure 2. Biomass of *B. juncea* grown in S1 and S2 soils under *P. fluoresens* inoculated and uninoculated conditions. Error bars indicate ± SD ($n = 3$).

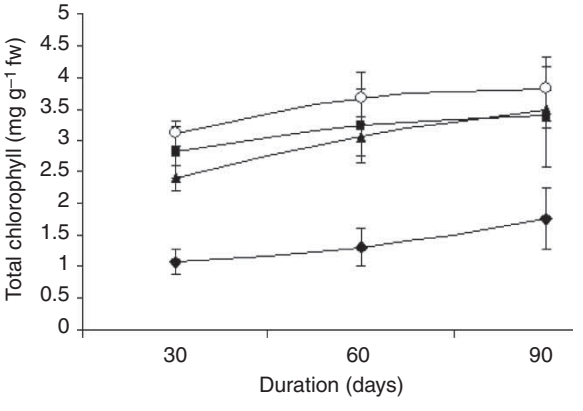

Figure 3. Total chlorophyll content of *B. juncea* grown in S1 and S2 soils under *P. fluoresens* inoculated and uninoculated conditions. Error bars indicate ± SD ($n = 3$). Symbols are the same as in Figure 2.

P. fluorescens in S1 increased plant biomass by 99% and chlorophyll content by 91% as compared to the uninoculated plant. Such an increase in plant growth is due to detoxification of heavy metals by redox changes [47] and concomitant production of growth promoters, viz. IAA, siderophore, ammonia, HCN and P-solubilisation [48–50] by *P. fluorescens*, as has been observed by others [51,52]. Bacterial strains of *Pseudomonas*, *Xanthomonas*, *Azomonas* and *Bacillus* have also been reported to enhance plant growth and metal accumulation in *Brassica napus* [53].

3.4. *Metal uptake in* B. juncea *influenced by* P. fluorescens

The metal uptake in *B. juncea* increased with increasing plant growth duration. Soil inoculation with *P. fluorescens* increased root and shoot uptake of Zn by 3.05 and 2.69 times, Cu by 3.19 and 2.82 times and Cd by 3.11 and 2.75 times, respectively (Figure 4). From both inoculated and uninoculated soils, Zn uptake was maximum followed by Cu and Cd, which may be attributed to the higher mobility of the former [54]. The higher uptake of Zn and Cu was also due to their essentiality for plant growth [55] in contrast to Cd, which has no known biological and/or physiological functions [56]. This also explains why Cd accumulation was least in *B. juncea* in the present study. Further, higher metal concentration observed in roots than shoots is possibly

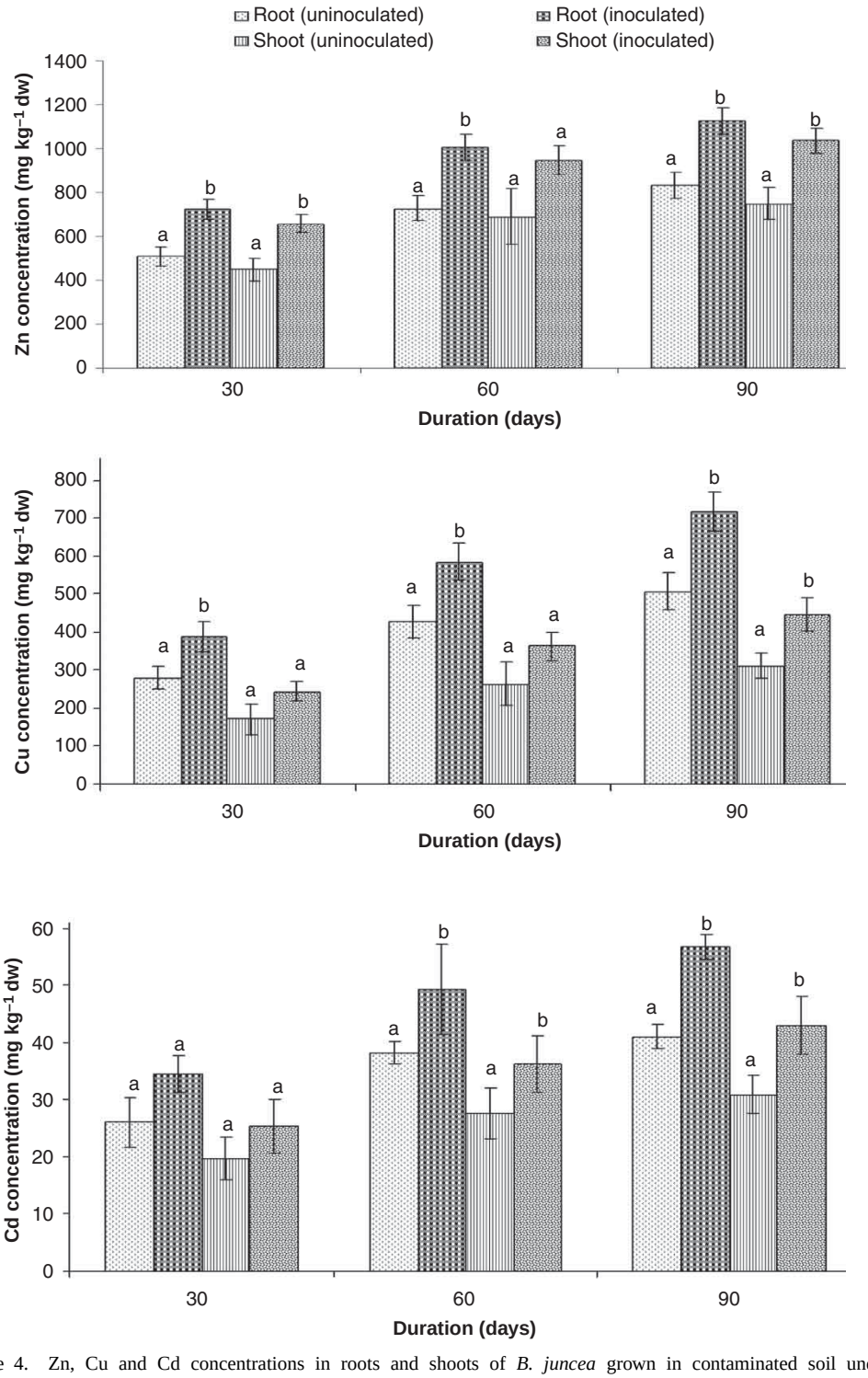

Figure 4. Zn, Cu and Cd concentrations in roots and shoots of *B. juncea* grown in contaminated soil under *P. fluoresens* inoculated and uninoculated conditions. Different letters indicate significant difference at $p < 0.05$ ($n = 3$).

due to an active defence system, leading the synthesis of antioxidant enzyme, viz. glutathione reductase, which protected the plant roots from the oxidative stress induced by heavy metals [57]. Substantial increase in the bioavailability of metals in soil [36] and plant biomass led to increased metal accumulation in the plant under *P. fluorescens* inoculated conditions.

In the present study, the concentrations of Zn, Cu and Cd in plant tissues were found to be less than the threshold values prescribed for hyperaccumulators (>10,000 mg/kg, >1000 mg/kg and >100 mg/kg for Zn, Cu and Cd, respectively). But as phytoextraction efficiency is related to both plant metal concentration and dry matter yield [58], *B. juncea* can be regarded as a good accumulator of Zn, Cu and Cd owing to its high biomass production capacity, as observed in the present study as well as elsewhere [59].

3.5. *Bioconcentration factor (BCF) and translocation factor (TF)*

BCF and TF (ratio of metal concentration in roots to soil and shoots to roots, respectively) are an index of phytoremediation capacity of plants. BCF values of >1 for metals indicate a better phytoextraction potential of a plant, whereas TF values of <1 indicate its lesser translocation to above ground parts [60]. In the present study, the BCF values for Zn, Cu and Cd were >1 (Table 2), which indicated the good phytoextraction potential of *B. juncea* for these metals. In uninoculated conditions, a maximum BCF was found for Zn (10.77) followed by Cu (7.57) and Cd (6.34), which increased due to bacterial inoculation by 44%, 42% and 38%, respectively, through increasing metal bioavailability to plants [36]. Further, TF values of <1 for Zn, Cu and Cd (Table 2) indicated low metal translocation to shoots, and this may be accounted for by their strong binding to root cell walls and/or compartmentalisation into vacuoles [61], which might have restricted the movement of metals from roots to shoots. The order of TF was Zn > Cd > Cu, which may be due to the fact that Zn and Cd are more mobile and were hence retained less strongly by the soil matrix as compared to Cu [62]. The results showed conformity with the observations of others (Fischerova et al. [63] and Bennet et al. [64]), who reported metal translocation in grass and Indian mustard in the order of Mn > Zn > Cd > Cr > Cu. Although the TF value for each metal was <1, the higher shoot biomass of *B. juncea* compensated for the lower metal concentration in shoots compared to roots and this it could be regarded as a good phytoextracter.

3.6. *Soil Ws and Ex metal fractions after phytoremediation*

The bioavailable metal fraction in contaminated soil S1 was found to be concentration dependent of total metal content, depicting maximum availability of Zn followed by Cu and Cd in phytoremediated soil (Table 3). Both Ws and Ex metal fractions increased with increasing phytoremediation duration, while in unplanted soil, negligible change was observed in metal bioavailability (data not shown). Water soluble fractions of Zn, Cu and Cd were 37%, 30% and 35% higher in *B. juncea* planted soil as compared to unplanted soil after 90 days, while the corresponding

Table 2. Bioconcentration (BCF) and translocation factors (TF) of *B. juncea* for Zn, Cu and Cd in *P. fluorescens* inoculated and uninoculated soil (mean ± SD, $n = 3$).

	BCF		TF	
Heavy metal	*B. juncea*	*B. juncea* + *P. fluorescens*	*B. juncea*	*B. juncea* + *P. fluorescens*
Zn	10.77 ± 1.61a	15.44 ± 1.31b	0.90 ± 0.12a	0.92 ± 0.02a
Cu	7.50 ± 1.17a	10.62 ± 1.21b	0.61 ± 0.09a	0.62 ± 0.01a
Cd	6.34 ± 1.10a	8.78 ± 0.30b	0.75 ± 0.08a	0.75 ± 0.02a

Note: Different letters in the same row for a parameter indicate significant difference ($p < 0.05$).

Table 3. Water soluble (Ws) and exchangeable (Ex) metal content in *B. juncea* planted soil under *P. fluorescens* inoculated and uninoculated conditions after 30, 60 and 90 days (mean ± SD, $n = 3$).

Treatment		30 days		60 days		90 days	
		Ws	Ex	Ws	Ex	Ws	Ex
Zn	Uninoculated	2.30 ± 0.21a	4.31 ± 1.4a	2.42 ± 0.12a	4.64 ± 0.88a	2.79 ± 0.77a	5.63 ± 1.2a
	Inoculated	4.34 ± 0.98b	8.62 ± 1.23b	4.52 ± 0.66b	9.50 ± 2.08b	5.54 ± 0.56b	11.65 ± 1.34b
Cu	Uninoculated	1.24 ± 0.08a	2.53 ± 0.6a	1.31 ± 0.46a	2.69 ± 0.64a	1.45 ± 0.25a	2.87 ± 0.48a
	Inoculated	2.16 ± 0.23b	4.28 ± 0.95b	2.20 ± 0.65b	4.47 ± 0.77b	2.57 ± 0.57b	4.90 ± 0.44b
Cd	Uninoculated	0.05 ± 0.04a	0.76 ± 0.17a	0.05 ± 0.01a	0.81 ± 0.05a	0.06 ± 0.02a	0.92 ± 0.08a
	Inoculated	0.09 ± 0.06a	1.48 ± 0.76a	0.98 ± 0.09b	1.55 ± 0.32b	0.12 ± 0.03b	1.78 ± 0.14b

Note: Different letters in the same column for a metal indicate significant difference ($p < 0.05$).

values for Ex Zn, Cu and Cd content were 53%, 23% and 40% higher, respectively. The increase in metal bioavailability after phytoremediation might be due to the prevalence of acidification, complexation/chelation and reduction/oxidation [65] by plants having high metal uptake potential [66]. Apart from this, release of root exudates by *B. juncea* plants might have also contributed to increased metal bioavailability [67,68]. A relatively smaller increase in Cu biovailabilty was probably due to its high affinity to organic matter [69], leading to the formation of strong metal–organic complexes. Further, *P. fluorescens* inoculated soil showed a significant ($p < 0.05$) increase in both Ws and Ex metal content (Table 3). The increase in water soluble Zn, Cu and Cd content was 99%, 77% and 90%, respectively, and in exchangeable content by 107%, 70% and 93%, respectively, as compared to the uninoculated one. This clearly depicted the ability of *P. fluorescens* to enhance metal bioavailability by acidifying the rhizosphere [1] with plant growth-promoting substances viz. IAA [70], organic acids and siderophores [71] to facilitate the phytoextraction of Zn, Cu and Cd by *B. juncea*.

4. Conclusions

The present study clearly demonstrated that *B. juncea*, unlike hyperaccumulators, accumulated a moderate amount of metals but possessed an attribute of high biomass production, which led us to acclaim it a good phytoextractor of Zn, Cu and Cd. The study also revealed that the metal uptake efficiency of *B. juncea* was enhanced substantially by the presence of *P. fluorescens* Pf 27, which also promoted plant growth and ensured better bioavailability of the metals for phytoextraction. As such, microbe-assisted phytoremediation appears a valued technology for reclamation of metal contaminated soils, but warrants further investigation, especially on the suitability of plant-rhizobacteria combinations for its wider application.

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