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Interactions between accumulation of trace elements and macronutrients in *Salix caprea* after inoculation with rhizosphere microorganisms

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ABSTRACT

Although the beneficial effects on growth and trace element accumulation in *Salix* spp. inoculated with microbes are well known, little information is available on the interactions among trace elements and macronutrients. The main purpose of this study was to assess the effect of phytoaugmentation with the rhizobacteria *Agromyces* sp., *Streptomyces* sp., and the combination of each of them with the fungus *Cadophora finlandica* on biomass production and the accumulation of selected trace elements (Zn, Cd, Fe) and macronutrients (Ca, K, P and Mg) in *Salix caprea* grown on a moderately polluted soil. Dry matter production was significantly enhanced only upon inoculation with *Agromyces* sp. Regarding the phytoextraction of Cd and Zn, shoot concentrations were mostly increased after inoculation with *Streptomyces* sp. and *Agromyces* sp. + *C. finlandica*. These two treatments also showed higher translocation factors from roots to the leaves for both Cd and Zn. The accumulation of Cd and Zn in shoots was related to increased concentrations of K. This suggests that microorganisms that contribute to enhanced phytoextraction of Cd and Zn affect also the solubility and thus phytoavailability of K. This study suggests that the phytoextraction of Zn and Cd can be improved by inoculation with selected microbial strains.

1. Introduction

Contamination of soils with heavy metals is one of the most serious environmental problems and several technologies have been developed to remove contaminants from soil or render them harmless. Plants with the natural capability to accumulate heavy metals and metalloids in their aboveground biomass were selected and tested for the development of phytoextraction technologies (e.g. Baker and Brooks, 1989; Raskin et al., 1997; Wenzel and Jockwer, 1999; Prasad and Freitas, 2003; Pulford and Watson, 2003; Gamalero et al., 2009). Most trace elements do not have any known nutritional function (e.g. Cd, Hg, Pb), but others are essential micronutrients (e.g. Fe, Cu, Zn), however, all are toxic when available at high levels in the soil. Plants have developed several mechanisms to control the accumulation of nutrients. Such mechanisms ensure that the plant obtains adequate levels of essential nutrients, while prevent their excessive accumulation (Grusak et al., 1999). The uptake of toxic metals by plants is depending on several mechanisms that are both specie and metal specific. Heavy metals are taken up in root tissue via transport proteins of cells in the root cortex (Marschner, 1995). It has been reported that toxic metals, such as Cd, compete for the same transmembrane carriers with other essential nutrients (e.g. Ca, Mg, K, Fe, Zn) due to relative lack of selectivity of these transport systems (Clarkson and Lüttge 1989; Ghosh and Singh, 2005). After heavy metals have passed the membranes they are either stored in the root or translocated to the shoots. Inside the cell heavy metals are rendered less toxic through several mechanisms including chelation with phytochelatins, vacuolar compartmentalisation and sequestration, induction of mechanisms to compensate the effects of reactive oxygen species such as the biosynthesis of antioxidant molecules and stress proteins, and upregulation of peroxidase synthesis (Salt et al., 1995; Sanità di Toppi and Gabbrielli, 1999; Bricker et al., 2001). The cell wall and vacuole are the main sites of heavy metal sequestration.

The genus *Salix* is considered highly tolerant to excess heavy metal concentrations and is potentially suitable for Cd and Zn phytoextraction (van der Heijden, 2001; Pulford and Watson, 2003; Kuzovkina et al., 2004; Dos Santos Utmazian and Wenzel, 2007; Unterbrunner et al., 2007). Phytoextraction efficiency depends on metal tolerance and accumulation capacity, and can be improved by beneficial interaction between plant roots, soil and microorganisms

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(Kidd et al., 2009; Wenzel, 2009). Several studies reported an increase of heavy metal concentrations in the tissues of terrestrial plants by application of rhizosphere microorganisms that can influence the bioavailability of contaminants and nutrients, the composition of root exudates and plant root development through the release of chelating substances, the acidification of the microenvironment, and the induction of changes in redox potential (Lebeau et al., 2008; Wenzel, 2009; Glick, 2010). Moreover, microbial inoculation of the soil, i.e. phytoaugmentation, can improve nutrient supply and enhance plant tolerance to excess amounts of toxic trace metals when grown on contaminated soil (Leyval et al., 2002; Krupa and Kozdrój, 2007; Gamalero et al., 2009). It is well known that some bacteria can synthesise siderophores which solubilise and sequester Fe from the soil solid phase, thus increasing its phytoavailability (Crowley et al., 1991; Wang et al., 1993). It has been demonstrated that some bacteria are able to synthesise several plant growth regulators including indole-3-acetic acid, auxins and cytokinins (Glick et al., 1998), which can increase the root surface absorption area resulting in a better uptake of water and nutrients (Lasat, 2002; Wu et al., 2005). Chen and Zao (2009) reported improved nutrient uptake in *Astragalus sinicus* grown on contaminated soil, when inoculated with arbuscular mycorrhiza (AM). It has been also reported that soil bacteria may affect gene expression in plants by providing additional nutrients from the soil (Gamalero et al., 2009); positive effect on nutrient uptake was reported also in *Trifolium pratense* when inoculated with both mycorrhizal and bacterial strain (Vivas et. al., 2006).

Although some recent studies have focused on the effects of microbial inoculation on growth and heavy metal accumulation in *Salix* sp. (Baum et al., 2006; Dos Santos Utmazian et al., 2007; Kuffner et al., 2008; Kuffner et al., 2010), the interaction of microorganisms on biomass production, nutrient and heavy metal accumulation are still poorly understood and results are often contrasting.

The main objective of this study was to assess the efficiency of three rhizosphere microorganisms (two bacterial and one fungal strain) on the Cd and Zn phytoextraction capacity of *Salix caprea*. All inocula were already tested before on a different heavily polluted soil (Dos Santos Utmazian et al., 2007; Kuffner et al., 2008). However, since phytoextraction will be mainly feasible on moderately contaminated soil, we have chosen such a soil to assess the effects of phytoaugmentation on Zn and Cd phytoextraction on a less polluted substrate. A second objective addressed the question whether the combined inoculation of bacteria and fungi results in higher phytoextraction efficiency (i.e. Cd and Zn concentration and biomass production) compared to the phytoaugmentation with one microbial species only. In addition, the effect of rhizosphere microorganisms on the accumulation of selected nutrients (Fe, Ca, K, P and Mg) was assessed and compared with the Zn and Cd concentrations in order to clarify if enhance nutrient uptake can explain differences in phytoextraction efficiency.

2. Material and methods

2.1. Bacterial and fungal strains

The bacterial strains tested were isolated from rhizosphere soil and were affiliated by sequencing of the 16S rRNA gene and analysis of 16S rDNA to the genera *Streptomyces* (AR17) and *Agromyces* (AR33) in a previous study conducted by Kuffner et al. (2008). The AM fungal strain *Cadophora finlandica* PRF15 was isolated and characterised by PCR-amplification and sequencing of the ITS-region in a previous research of Dos Santos Utmazian et al. (2007).

2.2. Inoculation of plants

Cuttings of *S. caprea*, clone Boku 04 CZ-024 (derived from Kutna Hora, Czech Republic, as described in Puschenreiter et al., 2010 and Kuffner et al., 2010) were pre-grown for 1 year in a sand–soil mixture under controlled environmental conditions (14/20 °C day/night temperature; 80% air moisture, 16 h light d^{-1}). The heavy metal contaminated soil used in this experiment was collected in Celje (Slovenia), air dried, sieved (2 mm) and gamma-ray irradiated with 25 kGy for 24 h by Mediscan GmbH (Seibersdorf, Austria). The main properties of the experimental soil are given in Table 1. Each pot was filled with 800 g of soil. The bacterial inocula were previously described in Kuffner et al., 2010 and were prepared as written in Kuffner et al. (2010). The fungal inoculums (*C. finlandica* PRF15) was previously described by Dos Santos Utmazian et al. (2007) and prepared as written there.

The contaminated soil was inoculated with microorganisms to test six treatments: autoclaved inoculum of *Streptomyces* sp. and *Agromyces* sp. as control (C); *Streptomyces* sp. (AR17); *Agromyces* sp. (AR33); *C. finlandica* plus autoclaved inoculum of AR17+AR33 (F); *C. finlandica*+AR17 (F_AR17); *C. finlandica*+AR33 (F_AR33).

For all treatments with *C. finlandica*, the fungal inoculum was mixed into the soil before planting the willows. For all treatments inoculated with bacterial strains, 10 mL of bacterial suspension were applied to the soil surface surrounding the plantlet. The pots were positioned in a greenhouse in a randomised design, and each treatment was replicated four times. After a growth period of 12 wk, plants were harvested, separated into roots and shoots and washed using tap water. For the analysis of Zn and Cd concentrations, a fraction of leaf material was treated separately. To remove metals from the apparent free space of the root tissues, roots were sonicated for 10 min in 0.05 M $CaCl_2$ and rinsed with deionised water. All samples were dried at 80 °C for 24 h before determining the dry matter (DM). Plant samples were finely ground and a subsample of 0.2 g was digested using a mixture of $HNO_3/HClO_4$ (4:1) in an open digestion system. P, Cd and Zn concentrations were determined by using an ICP-MS (Elan 9000 DRCe, Perkin Elmer), whereas concentrations of Ca, Mg, K and Fe were determined with an ICP-OES (Zeiss, Plasmaquant 100). The translocation factor (TF) was calculated as element concentration in the leaves divided by the concentration in roots.

2.3. Statistical analysis

Statistical analysis was performed by R software (version 2.10.1). All variables were tested with one way Analysis of Variance

Table 1
Main characteristics of the experimental soil.

Soil property	Value
Sand (g kg^{-1})	450
Silt (g kg^{-1})	340
Clay (g kg^{-1})	210
pH (H_2O)	7.54
Carbonate content (g kg^{-1})	7.30
Cation exchange capacity (mmol kg^{-1})	273
Organic carbon (g kg^{-1})	38.5
Total element concentration (*aqua regia*)	
Zn (mg kg^{-1})	608
Cd (mg kg^{-1})	4.9
Fe (mg kg^{-1})	2.3
Ca (g kg^{-1})	24.6
K (g kg^{-1})	3.8
Mg (g kg^{-1})	15.8
Extractable element concentration (1 M NH_4NO_3)	
Zn (mg kg^{-1})	0.3
Cd (μg kg^{-1})	12.0

(ANOVA) followed by Duncan's test. A Student's test was performed to determine statistical differences between Cd and Zn TFs within the same treatment. Normalised element concentration values were calculated through a z score standardisation. The z score value is given by $z = (x - \mu)/\sigma$, where x is the element concentration of each sample; μ and σ are respectively the mean and the standard deviation of each element.

3. Results

3.1. Biomass production

In contrast to root biomass, the microbial treatments had no significant effect on the shoot biomass of *S. caprea*; however, some non-significant tendency for increased shoot biomass was found after inoculation with *Agromyces* AR33 (Fig. 1A). With the same bacterial inoculum, root biomass was significantly higher than the control, whereas no significant differences were observed for the other microbial treatments (Fig. 1B), including *Agromyces* AR33 that was ineffective when co-inoculated with the AM fungus *C. finlandica* (Fig. 1A).

3.2. Cadmium and zinc concentrations in leaves and roots

The concentrations of Zn and Cd in roots and leaves of *S. caprea* were significantly enhanced by phytoaugmentation (Fig. 2). For both elements, foliar concentrations always exceeded those in the roots. Foliar Zn concentrations were significantly increased for both F_AR33 and AR17 compared with the other treatments which were not significantly different from each other. In all treatments root Zn concentrations were statistically different compared to the control. As for Zn, the highest Cd foliar concentrations were

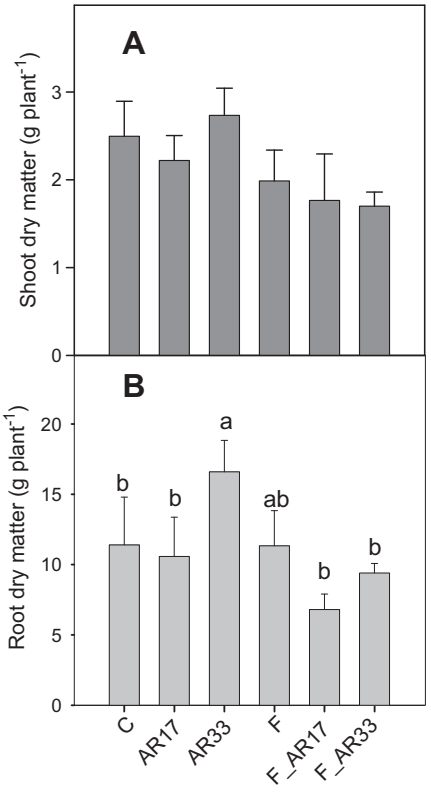

Fig. 1. Effect of microbial inoculation on: (A) shoots and (B) roots DM. Error bars indicate the standard error of the mean ($n = 4$); bars with same letter are not significantly different for $p < 0.05$ based on Duncan's test.

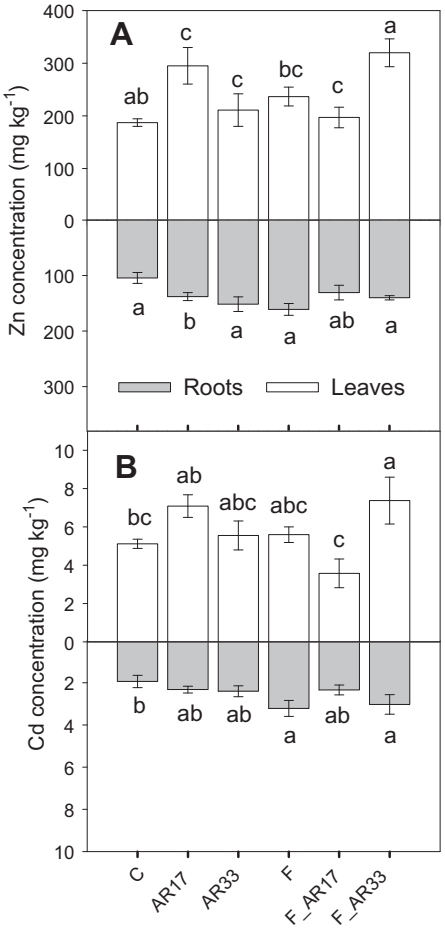

Fig. 2. Zn (A) and Cd (B) concentration in leaves and roots of *S. caprea* for the different treatments. Error bars indicate the standard error of the mean ($n = 4$). Bars with the same letter are not significantly different ($p < 0.01$) based on Duncan's test.

observed in the treatments F_AR 33 and AR 17, whereas the lowest was found for F_AR17 and C. In the roots, the highest Cd concentrations were found in F and in F_AR33 and the lowest in C, whereas no differences were found among the other treatments. Considering all treatments, a close relationship was found between Zn and Cd concentrations in plant tissues ($r^2 = 0.80$) (Fig. 3).

Table 2 shows the TF from roots to leaves for Cd and Zn. A one-way ANOVA showed that the investigated parameters were significantly affected by treatments ($p < 0.05$). Zinc TF was significantly higher in the treatment F_AR33, and significantly lower in the treatments AR33 and F; however no significant differences were observed between individual microbial treatments and the control. The TF for Cd showed that the co-inoculation of fungus *C. finlandica* generally decreased translocation from roots to leaves with respect to the corresponding treatments without the fungus, except for F_AR33 which was not different from the bacterial strain AR33. Independent from the treatments, TF for Cd were generally higher than for Zn; comparing Cd and Zn TF in each microbial treatment, Student's test showed significant differences ($p < 0.05$) only for the control and for both bacterial treatments AR17 and AR33 (Table 2).

3.3. Shoot concentrations

The concentration of Ca, Cd, Fe, K, Mg, P, and Zn in shoots is shown in Table 3. Except for Fe, all elements were significantly affected by the microbial treatments. Ca and Mg concentrations were generally higher in the co-inoculated treatments compared to those without *C. finlandica* (Table 3). In particular, the co-inoculated treatment

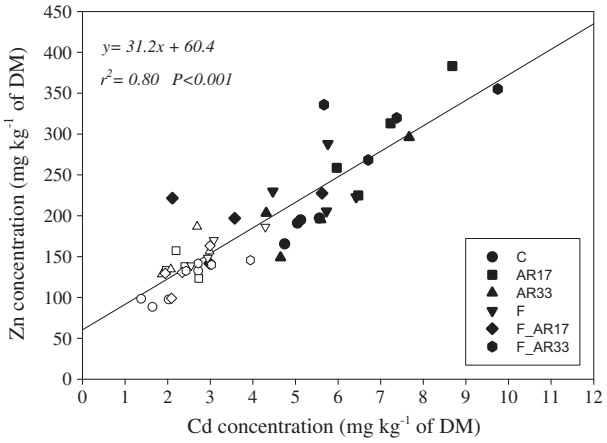

Fig. 3. Relationship between Zn and Cd concentrations in roots (white symbols) and leaves (black symbols). Each data point represents measurements from an individual plant.

Table 2
Root to leaves translocation factors (TF) for Zn and Cd. Values are means ± s.e. ($n = 4$). Means with the same letter within a column are not significantly different according to Duncan's test. The statistical analysis was a one-way ANOVA. Significant differences between means within a line ($p \leqslant 0.05$ of *t*-student test) are marked with asterisks (*).

Treatment	Translocation factor		*p*-value
	Zn	Cd	
C	1.83 ± 0.17 abc	2.80 ± 0.38 a	0.05*
AR17	2.17 ± 0.30 ab	3.05 ± 0.20 a	0.05*
AR33	1.42 ± 0.26 c	2.42 ± 0.47 ab	0.02*
F	1.48 ± 0.10 c	1.78 ± 0.17 bc	0.25
F_AR17	1.57 ± 0.30 bc	1.48 ± 0.22 c	0.86
F_AR33	2.28 ± 0.13 a	2.44 ± 0.19 ab	0.51
F probability	0.04*	0.008*	

F_AR33 showed a significant increase of 33% of Ca and 27% of Mg compared to AR33. The shoot concentrations of *P* were significantly increased compared to the control for all treatments except AR33. For K, no differences were observed for all treatments except for AR17 which was significantly higher compared to the corresponding treatment with *C. finlandica* (F_AR17). As for leaves, the highest Cd and Zn concentrations in shoots were found in F_AR33 and the lowest in F_AR17; in this latter, Zn and Cd concentrations were reduced by 30% and 46% respectively, with respect to the corresponding treatments without *C. finlandica* (AR17) (Table 3).

The *z* scores (Fig. 4) of Ca, Cd, Fe, K, Mg, P, and Zn indicate that the elements are grouped differently for different treatments. Comparing the bacterial strain AR17 and the co-inoculated F_AR17 treatment, standardised values are arranged in two clearly distinct groups, i.e. Ca–Mg–P and Cd–Zn–K. In particular, high levels of Cd–Zn–K scores in AR17 treatment match high Ca–Mg–P values in F_AR17 and vice versa. To the contrary, in both AR33 and the corresponding combined treatment F_AR33, *z* scores values are grouped all together with the highest values in the combined treatment. Instead, in C and in F treatments *z* scores values are similar and generally coupled together.

4. Discussion

Interaction between plants and soil microorganisms can affect plant growth, accumulation and tolerance to elevated trace element concentrations, as well as nutrient acquisition (Chen and Zao, 2009; Wenzel, 2009; Weyens et al., 2009). In the phytoaugmentation experiment reported here, no significant differences were observed on shoot DM after inoculation with microbes, except for AR33 where a significant increase of root DM and a slight trend of enhanced shoot dry matter was found (Fig. 1). Although several studies showed that root growth is associated with enhanced nutrient accumulation (Marschner, 1995; Vessey, 2003), our results showed that the increase of root biomass observed for AR33 inoculation did not cause an increased concentration the investigated elements, except for Zn in roots. On the other hand, AR17 and F_AR33 showed an increase of all investigated elements in shoots, independently of changes in biomass production. These results suggest that a mechanism other than root growth promotion was responsible for the increase of shoot concentrations observed after inoculation with AR17 and with F_AR33.

It has been reported that rhizosphere microorganisms can differently alter bioavailability of contaminants and nutrients through the release of chelating substances, acidification of the microenvironment and by changing the redox potential, modifying soil conditions that influence metal accumulation in plants (Whiting et al., 2001; Lasat, 2002; Gadd, 2004; Jiang et al., 2004; Kidd et al., 2009). Our results (Fig. 2 and Table 3) showed that in each microbial treatment Cd and Zn concentrations in tissues of *Salix* were affected similarly and Cd and Zn concentrations in shoots and roots were well correlated ($r^2 = 0.80$). Similar results were reported by Kuffner et al. (2008) who, by testing several bacterial strains, suggested that released bacterial metabolites may affect Zn and Cd concentration similarly. It is interesting to note that for AR17 enhanced both Cd and Zn concentration in shoots were found while the concentration decreased compared to the control in the corresponding treatment F_AR17. Interestingly, the opposite result was found for AR33 and F_AR33. These results confirm the findings of Gamalero et al. (2009), who reported that metal uptake by plants treated with AM fungi can vary significantly as a function of the interaction with a specific bacterial strain. In addition it was also reported that mycorrhizal associations may reduce trace elements translocation from roots to shoots by binding large amounts on cell wall components such as chitin, extrahyphal slime or trough intracellular immobilisation with metallothioneins and polyphosphates or compartmentalisation within vacuoles (Leyval et al., 1997), suggesting a beneficial symbiosis of fungi and bacteria association.

In our study, the phytoaugmentation affected also the concentration of the investigated macro nutrients. Interestingly, Fe was the only nutrient for which no significant differences were found, probably because the two investigated bacterial strains did not produce

Table 3
Concentrations of selected elements in shoots of *S. caprea*. Values are means ± s.e. ($n = 4$). Means with the same letter within a column are not significantly different according to Duncan's test following a one-way ANOVA.

Treatments	Ca (g kg^{-1} DM)	Mg (g kg^{-1} DM)	K (g kg^{-1} DM)	P (g kg^{-1} DM)	Fe (mg kg^{-1} DM)	Cd (mg kg^{-1} DM)	Zn (mg kg^{-1} DM)
C	20.6 ± 2.1 b	5.4 ± 0.2 bc	18.8 ± 1.6 b	1.62 ± 0.04 c	57.7 ± 4.5	5.6 ± 0.1 abc	156 ± 6.70 c
AR 17	23.3 ± 1.2 ab	5.5 ± 0.2 bc	23.6 ± 0.9 a	1.78 ± 0.03 b	62.6 ± 2.9	6.6 ± 0.4 ab	238 ± 24.1 ab
AR 33	20.0 ± 0.5 b	4.9 ± 0.1c	20.1 ± 0.7 ab	1.67 ± 0.05 c	58.8 ± 2.8	4.8 ± 0.5 cd	169 ± 20.8 c
F	24.5 ± 1.7 ab	5.5 ± 0.3 bc	20.1 ± 0.7 ab	1.81 ± 0.03 b	71.1 ± 4.9	5.2 ± 0.3 bc	202 ± 14.7 bc
F_AR 17	26.7 ± 2.7 a	6.0 ± 0.2 ab	18.0 ± 1.9 b	1.71 ± 0.03 bc	6 0.1 ± 3.3	3.5 ± 0.5 d	167 ± 13.9 c
F_AR 33	26.6 ± 1.1 a	6.2 ± 0.1a	21.2 ± 0.9 ab	1.98 ± 0.04 a	5 9.7 ± 3.3	6.6 ± 0.8 a	259 ± 18.5 a
F probability	$p < 0.05$	$p < 0.01$	$p = 0.052$	$p < 0.001$	$p = 0.15$	$p < 0.001$	$p < 0.01$

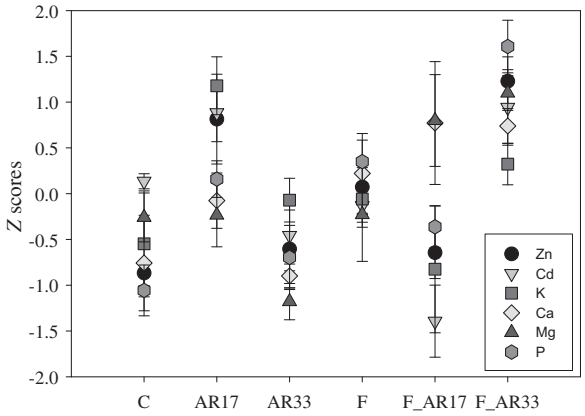

Fig. 4. Mean values of *z*-scores performed on concentrations of Cd, Zn, Ca, Mg, K and P in shoots of *S. caprea* inoculated with different microorganisms. Error bars indicate the standard error.

siderophores (Kuffner et al., 2008). It is well known that some bacteria can synthesise siderophores which solubilise and sequester Fe from the soil solid phase, thus increasing its phytoavailability (Crowley et al., 1991; Wang et al., 1993). Except for Fe, among microbial treatments the uptake of macronutrients generally increased in the co-inoculated *C. finlandica* plus *Agromyces* (F_AR33) treatment. Leyval and Berthelin (1991) suggested a positive interaction between ectomycorrhizal fungi and soil bacterial strains, hypothesising that bacteria modify availability of nutrients in soil, whereas fungi may increase the absorptive area around roots. This was not the case for the co-inoculation of *C. finlandica* with *Streptomyces* (F_AR17) where a lower Cd, Zn and K concentration in shoots and a higher P, Ca and Mg concentrations was found compared to the *Streptomyces* (AR17) treatment. Although it is known that bacteria affect bioavailability of elements in the rhizosphere modifying their accumulation, our results showed that the co-inoculation of a fungus and bacterial strains modifies the rhizosphere in different ways with respect to treatments with only one species.

Regarding the effect of phytoaugmentation on the accumulation of macro nutrients, different results were found for K compared to Ca, Mg and P. Standardised values of K in all microbial treatments showed always values near to those of Cd and Zn (Fig. 4), indicating that increased Cd concentration in shoots were associated with a concomitant increase of K concentrations. This trend can indicate a possible synergistic effect between Cd and K as previously found in tomato (Moral et al., 1994) and in Indian mustard (Jiang et al., 2004). Wenzel and Jockwer (1999) hypothesised that K may be related to compartmentation of toxic metals in several hyperaccumulator plants. Gussarssonn (1994) suggested that trace elements including Cd may interfere with nutrient uptake, including K, by altering the plasma membrane permeability and by affecting element transport processes across the cell membrane. As for Zn, Cd and K, standardised values of P, Ca and Mg concentrations in shoots were similar (Fig. 4), suggesting a similar mechanism of solubilisation in soil on the one hand and accumulation in the plant tissues on the other.

5. Conclusions

Among the tested treatments, the bacterial strain *Streptomyces* AR17 was most efficient to increase the accumulation of Zn and Cd in leaves and shoots of *S. caprea*, thus this particular strain is a good candidate to increase the phytoextraction efficiency of *S. caprea* on both highly and moderately contaminated soil. However, also the combination of *C. finlandica* plus *Agromyces* AR33 resulted in an enhanced accumulation of Cd in shoots. We could show that the accumulation of Cd (and Zn) in shoots was related to increased concentrations of K. This suggests that microorganisms that contribute to enhanced phytoextraction of Cd affect also the solubility and thus phytoavailability of K. Thus, the enhanced plant uptake of K seems to play a significant role in the phytoextraction efficiency of *S. caprea*. Considering the complex role of rhizosphere in phytoremediation technologies, further studies are necessary to further investigate the role of bacteria, mycorrhiza and their combinations to exploit their synergistic mechanisms for phytoextraction of Zn- and Cd-contaminated soils after phytoaugmentation.

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