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RESEARCH ARTICLE

Challenging synergistic activity of poplar–bacteria association for the Cd phytostabilization

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Abstract The synergistic activity between plants and microorganisms may contribute to the implementation of proactive management strategies in the stabilization of contaminated sites, although heavy metals, such as cadmium (Cd), are potentially toxic to them. The aim of this study was to evaluate the degree of tolerance to Cd contamination (supplying twice 40 mg kg^{-1} of Cd) in poplar cuttings [clone I-214, *P.* × *euramericana* (Dode) Guinier] inoculated or not with two concentrations of *Serratia marcescens* strain (1×10^7 CFU/g and 2×10^7 CFU/g of potting mix). The response of the plant–bacteria system to excess Cd was investigated with special reference to the structural traits of plants and the functional efficiency of bacteria. Bacterial colonization and substrate components were previously assessed in order to define the best solution for formulating the experimental plant growth media. The tested plant–bacteria association, especially when bacteria were provided in double concentration, stimulated specific tolerance mechanisms to Cd through the promotion of the poplar growth. Inoculated plants produced larger leaves and increased stem diameter, while roots grew longer and wider in Cd-treated plants. The effect of bacterial inoculum on plant growth traits and metal partitioning in plant organs was assessed in order to define the potential of this poplar clone to be a suitable candidate for phytostabilization of Cd-contaminated soil. The final effect of the inoculation with bacteria, which alleviated the metal load and Cd phytotoxicity due to their bioaccumulation ability, suggests promising phytostabilization potential of these plant–bacteria associations.

Keywords Bioremediation · Heavy metal · Inoculum · Phytostabilization · *Populus* · *Serratia marcescens*

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Introduction

Heavy metals have been excessively released into the environment due to rapid industrialization and have created great global concern (Ali et al. 2013). Heavy metals are not biodegradable; they accumulate in living tissues through the food web, causing global environmental problems and major threats to humans after chronic exposure. Anthropogenic activities have resulted in severe and widespread contamination of land and underground water by toxic heavy metals, such as cadmium (Cd), which may become extremely persistent in the environment (Gallego et al. 2012). Cadmium can be absorbed by roots and transported to shoots, causing a range of physiological and growth disorders in plants (Liu et al. 2011; Zacchini et al. 2011). Once structural barriers have been overcome, Cd can affect cell biochemical mechanisms by suppressing the control of cell redox state and by altering the interaction of reactive oxygen species or antioxidants with hormones (Castagna et al. 2013, 2015; He et al. 2015). Cadmium has been found to trigger a series of changes in gas exchange, stomatal behavior, water relations, nutrient uptake, and redox status that can lead to phytotoxicity and,

eventually, bring to growth inhibition and plant death (Lux et al. 2011; Azevedo et al. 2011).

In some cases, plants have evolved strategies allowing them to grow in metal-contaminated soils, where they extract high concentrations of metals and store them in various tissues without signs of toxicity (Gallego et al. 2012). However, the mechanisms that allow enhanced pollutant uptake exclusion and root-to-shoot metal translocation are not fully understood (Mench et al. 2009; Dal Corso et al. 2013). Nevertheless, mechanisms regulating the formation of apoplastic barriers, xylem loading and efflux-influx of contaminants, concentration and chemical speciation in xylem/phloem, and metal storage, detoxification and tolerance processes related to stress resistance and use of plants for remediation purposes were reported in herbaceous and woody species (He et al. 2011, 2013a, b). This knowledge can be conveniently implemented in the use of plants and associated organisms as a tangible alternative with great potential for affordable remediation of polluted sites. Nowadays, "phytotechnologies" offer efficient solutions for the cleanup of contaminated soil and water (Pilon-Smits 2005; Evangelou et al. 2012). These green technologies provide options involving plants ("phytoremediation") and associated microbes ("bioremediation") (Afzal et al. 2013; Sessitsch et al. 2013).

Microbial inoculation of plant roots plays a practical role in improving the Cd mobility and availability to the plant through the release of chelating agents, acidification, phosphate solubilization, and redox changes, and therefore, it has the potential to enhance phytoremediation processes (e.g., Elobeid et al. 2012; Cocozza et al. 2014). Plant growth promoting rhizo-bacteria (PGPR) have been shown of being able to detoxify and transform metals into bioavailable and soluble forms through the action of siderophores, organic acids, biosurfactants, biomethylation, and redox process (Ullah et al. 2015). More, PGPR harbor plasmids, which encode for resistance genes to many toxic/heavy metals and metalloids, e.g., Ag^{+}, Cd^{2+}, Hg^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Sb^{3+} (Ullah et al. 2015). Interaction of bacteria with plants revealed a reduction in the harmful effects of Cd, indicating important biochemical and molecular mechanisms involved in Cd stress tolerance (Farinati et al. 2011; Luo et al. 2014; Ma et al. 2014). Indeed, microbes may increase the efficiency of biological systems in accumulating and absorbing heavy metals due to their large specific surface area and capability to activate and remove heavy metals (Wu et al. 2010). After the activation of heavy metal particles, the ion may be accumulated by roots and successively transported to shoots through the xylem for phytoextraction (Moore et al. 2006) or sequestered and stored in the rhizosphere for soil stabilization (Todeschini et al. 2007). Another advantage of this approach for the cleanup of polluted environments is represented by the capacity of several microbes to produce antibiotics enhancing plant immunity (Wu et al. 2010) or release essential nutrients and even plant growth hormones (De Maria et al. 2011), with a synergistic action, leading to improved plant growth. Thus, the symbiotic system (plant–microbe), as efficient plant–soil–microbe combination, represents a promising solution in removing hazardous metals (Wu et al. 2010; Afzal et al. 2013; Ali et al. 2013).

In poplars (*Populus* spp.), the effects of heavy metals and the ability of the plant to tolerate their toxicity have been widely studied (e.g., Borghi et al. 2008; Zacchini et al. 2009; Polle et al. 2013; Di Baccio et al. 2011, 2014). Poplars, in particular, have perennial growth cycles, developmental phase changes, secondary growth and metabolism, wide and deep root system, easy vegetative propagation, and exhibit articulated resistance/tolerance features to counteract different environmental conditions (Durand et al. 2011; Trupiano et al. 2014). Indeed, the fast growth rate and marked environmental adaptability of poplars make them good candidates for combining biomass production, carbon sequestration, and metal absorption (Tognetti et al. 1999, 2004; Laureysens et al. 2004; Calfapietra et al. 2010; Fernàndez et al. 2012; Romeo et al. 2014). In this manner, the evaluation of poplar ability to overcome growth limitations in polluted soils is an important step to be considered in order to assess the real potential in terms of phytoremediation in open-field applications (Tognetti et al. 2013). Insights on the association between roots and microorganisms can contribute to a more integrated understanding of the complex Cd tolerance and removal potential of plants for modern phytotechnologies.

The main objective of this study was to elucidate the effect of excess Cd in the growth substrate on the growth of poplars inoculated with and without rhizosphere-associated bacteria strains. We hypothesized that the bacteria used in our tests, *Serratia marcescens* (isolated from a common natural wood peat in a local deciduous oak forest stand and able to growth in Cd-polluted substrate), cope with Cd stress by inducing/enhancing plant resistance to metal. The poplar clone I-214 (*P.* × *euramericana*) was chosen because of its high adaptability to Cd stress, which makes this clone promising for a potential use in phytoremediation programs and intensive use as model system to investigate the response to heavy metal treatments (e.g., Cocozza et al. 2014; Di Baccio et al. 2014; Fernàndez-Martínez et al. 2014). In this context, the establishment of the association between roots and microorganisms could benefit poplars through the adaptation and alleviation of Cd stress or, alternatively, rhizosphere microorganisms could alter soil environment (pH, competition between populations of microorganisms, etc.), making Cd more available to the plant.

Materials and methods

Bacteria strain selection

Twenty-five bacteria strains (Fig. 1) were isolated from natural wood peat in Casacalenda (Campobasso, Italy) and were used for in vitro screening according to the methodology described by Boulter et al. (2000). Bacterial strains were routinely cultivated in minimum salt liquid medium [K_2HPO_4 2.5 g L^{-1}, KH_2PO_4 2.5 g L^{-1}, $(NH_4)2HPO_4$ 2.5 g L^{-1}, $MgSO_4 \times 7H_2O$ 0.2 g L^{-1}, $FeSO_4 \times 7H_2O$ 0.01 g L^{-1}, $MnSO_4 \times 7H_2O$ 0.007 g L^{-1}, 10 g L^{-1} of sucrose for gram-positive strains or dextrose for gram negative strains; pH 7.5] and nutrient broth (NB, Carlo Erba, Italy) at 28 °C and 120 g. Bacterial cells were re-suspended in sterile PBS (NaCl 170 mM, KCl 3 mM, Na2HPO$_4$ 12 mM, KH_2PO_4 4 mM; pH 7.4), and cell concentration was adjusted spectrophotometrically (600 nm). Bacterial strains were suspended in 30 % glycerol and stored at −80 °C.

Bacterial isolates were chosen on the basis of isolation source, morphological aspect, and tolerance to different Cd concentrations (Cocozza et al. 2014). Successively, they were characterized by the routine bacteriological tests (Borrero et al. 2006) and by sequencing the small 16S ribosomal subunit (16S rDNA).

The Genomic DNA (gDNA) of each bacterial strain was purified by using a Puregene Yeast/Bacteria Kit (Qiagen, USA) and processed by PCR, using the 16S universal primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Amplification was carried out in 25 μl of reactions under standard conditions using 12.5 μl PCR Master Mix (Promega, USA), 0.4 μM of each primer, 50 ng of gDNA, and sterile water to volume. PCRs were performed in a ThermalCycler (Techne, mod. 512) using the following cycling protocol: an initial step of denaturation (3 min at 95 °C) followed by 35 cycles of 15 s at 95 °C, 15 s at 49 °C, and 40 s at 72 °C. PCR products were observed on 1.5 % (*w*/*v*) agarose gel. The amplicon was sequenced by the Parco Scientifico e Tecnologico Padano (Lodi, Italy). Species-

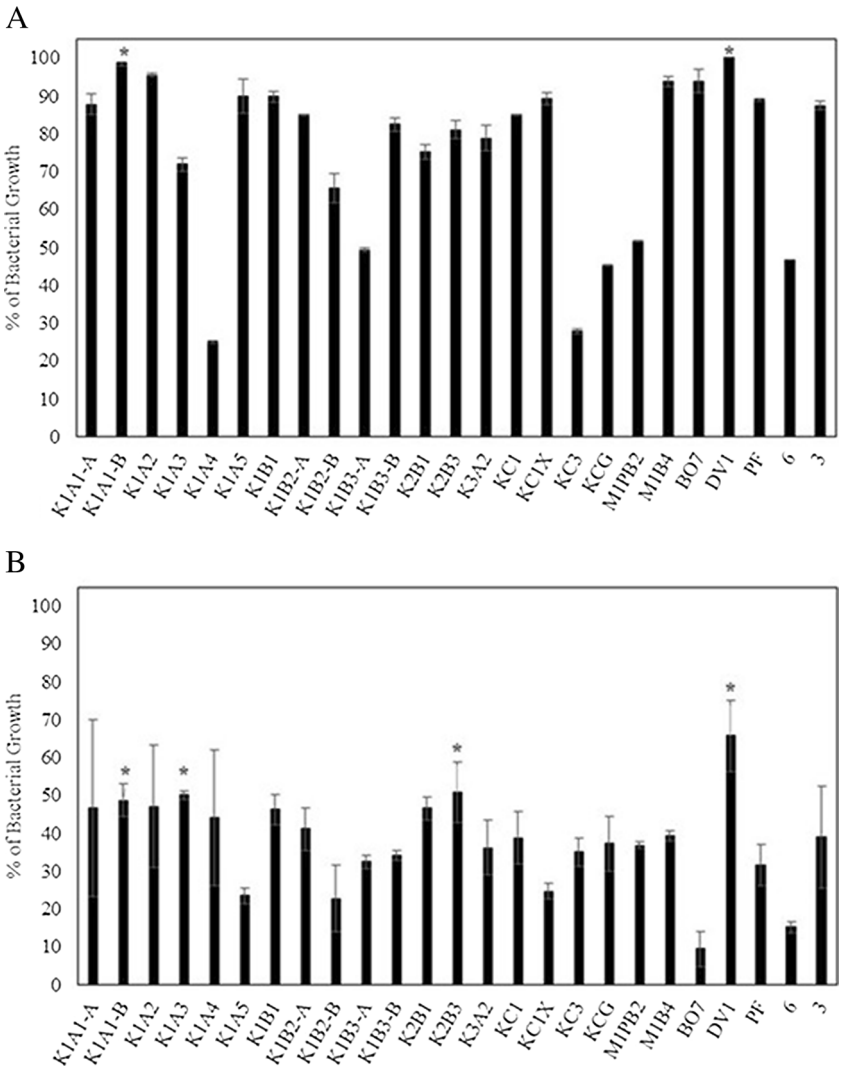

Fig. 1 In vitro screening of bacterial grown at concentrations of 40 (**a**) and 160 mg (**b**) of Cd kg^{-1} substrate. Bacterial growth was determined spectrophotometrically at 595 nm, after 72 h of incubations at 28 °C. Values were reported as % average of bacterial growth. Data represent the mean (n=4)± standard error. The strains K1A3, K1A4, K1B2-A, K1B3-A, KC1X, KCG, M1PB2, DV1, PF, 6 and 3 are Gram negative, while the remaining strains are Gram positive. Mean values marked with an asterisk are statistically different (LSD test, $P \leq 0.05$)

level identification was further refined through BLASTn searches of sequence data from the partial 16S rDNA gene in GenBank.

Activity of bacterial strains in vitro

The bacterial growth of 25 strains was initially assessed in vitro by two different final Cd concentrations (40 and 160 mg kg^{-1}: the first treatment is widely used in similar studies, while the second treatment, 4-fold higher than the first, was used to observe and compare Cd effects at very high concentrations) in liquid medium assays by using 96-well microliter plates in accordance with Vitullo et al. (2012). In the multiwell plate, each sample (total volume 200 μl) contained 190-μl standard liquid medium (NB) alone or added at two different Cd concentrations and 10-μl of bacterial suspension in phosphate buffer solution (PBS, NaCl 0.14 M, KCl 2.7 mM, $Na_2HPO_4 \times 2H_2O$ 8.1 mM, KH_2PO_4 1.8 mM, pH 7.4) at 0.1 O.D. (600 nm), which corresponds to about 1×10^8 CFU ml^{-1}. Plates were incubated at 28 °C on a rotary shaker (200*g*) for 72 h, and the increase in bacterial biomass was determined spectrophotometrically (595 nm) through the use of Microplate Reader (mod. 550, Bio-Rad). Non-inoculated NB was used as a blank. The experiment was repeated three times.

Substrate components and bacterial inoculum

Different substrate components were used for the experimental trial: (i) expanded clay prepared by blending light sphagnum peat with coarse horticultural grade expanded clay aggregates (Laterlite SpA, Lentella, CH, Italy) at a ratio of 3:2, (ii) light peat amended with 2 kg m^{-3} Osmocote (NPK 14:13:13+7SO_3, Scott, Italy), and (iii) a mixture of 40 and 60 % of clay and peat respectively.

A preliminary greenhouse screening of 5 days was performed with three different substrate components in pots without plants and without bacteria in order to assess the efficacy of the substrate component to hold or release the metal, measuring the availability of Cd, and to choose the best formulation for the plant experiment. The experimental setup included a control treatment (0 mg kg^{-1} Cd) and Cd treatment (40 mg kg^{-1} Cd, using $CdSO_4$-Sigma, St. Louis, USA); this Cd addition to the substrate was considered unharmful to bacterial populations studied in vitro. Six treatment combinations (three pots each) were defined by different substrate components and Cd treatments: (i) control expanded clay, (ii) Cd-treated expanded clay, (iii) control peat, (iv) Cd-treated peat, (v) control clay+peat, and (vi) Cd- treated clay+peat. The Cd content in each treatment was analyzed (Table 1).

A subsequent greenhouse screening was performed in pots filled with the clay+peat substrate without plants in order to assess the availability of Cd and to test the bacterial inoculum.

Table 1 Exchangeable or environmentally available Cd content in substrate components without plants and without bacterial inoculum (mean values±standard error). Cd content in control samples was below the detection limit (<0.1 mg kg^{-1}). Two-way ANOVA was applied to weigh the effects of Cd treatments, substrate components and their interactions (*P* level values are reported)

	Substrate	Contamination	Inoculum	Cd mg kg^{-1}
First treatment				
5 days	Expanded clay	Control	No ino	<0.1
	Expanded clay	Cd-treated	No ino	5.30±1.54
	Peat	Control	No ino	<0.1±
	Peat	Cd-treated	No ino	7.60±1.70
	Clay+peat	Control	No ino	<0.1±
	Clay+peat	Cd-treated	No ino	6.97±0.79
ANOVA *p-level*				
Cd treatment *0.01*				
substrate component *0.32*				
Cd treatment x substrate component *0.04*				

S. marcescens was selected from the in vitro screening for the high percentage of growth. The clay+peat substrate was inoculated with two bacterial concentrations of *S. marcescens* strain: 1×10^7 CFU/g (DV1) and 2×10^7 CFU/g of substrate (dDV1). The two bacterial concentrations were carried out in order to assess differences in the plant response to excess Cd, defining different bacteria levels and consequently different root colonization. Four treatment combinations (six pots each) were defined by different Cd treatments and bacterial inoculums: (i) control clay+peat, (ii) Cd-treated clay+peat, (iii) Cd-treated and DV1-inoculated clay+peat, and (iv) Cd-treated and dDV1-inoculated clay+peat. The Cd content in each treatment was analyzed (Table 2), and the bacterial viability and activity in inoculated clay+peat was determined (Fig. 2).

Then, the inoculated clay+peat substrate was incubated for 7 days at 20–25 °C before transplanting of poplar rooted cuttings. The non-inoculated clay+peat substrate was used for the uncontaminated treatments (control) (Table 3; Fig. 3). Substrate samples were harvested to assess the initial microbial population in the potting mixture.

Plant material and experimental setup

Two-year-old woody cuttings of the hybrid poplar clone I-214, *Populus deltoides*×*Populus nigra* (*P.* × *euramericana* (Dode) Guinier) were rooted in February 2013 in 1.5 L pots containing vermiculite and kept in a growth chamber under controlled environmental conditions (25 °C air temperature, 50–70 % relative humidity, 15-h photoperiod) for 45 days. Forty-eight homogeneously rooted cuttings, with similar morphological traits, were selected for the experiment. This poplar clone was selected because several experiments demonstrated

Table 2 Cadmium content in clay+peat substrate without plants, in non-inoculated and DV1- and dDV1-inoculated conditions during the first and second Cd application. Cd content in control samples was below the detection limit (<0.1 mg kg^{-1}). Mean values (± standard error) marked with the same letter are not statistically different (lowercase letters for the comparison of day of treatment; capital letters for the comparison of bacterial inoculum per each day of treatment) (LSD test, $P \leq 0.05$). Two-way ANOVA was applied to weigh the effects of days of treatment, bacterial inoculums, and their interactions (p level values are reported)

		Inoculum	Cd	
	LSD ($P \leq 0.05$) first and second day of treatment			LSD ($P \leq 0.05$) each day of treatment
First treatment				
5 days	c	No ino	6.97±0.97	A
		DV1	11.88±3.29	A
		dDV1	15.14±0.41	A
30 days	ac	No ino	21.89±0.25	A
		DV1	11.86±0.32	B
		dDV1	14.40±0.31	C
Second treatment				
5 days	b	No ino	28.67±0.47	B
		DV1	51.60±1.91	A
		dDV1	32.60±0.59	B
30 days	a	No ino	8.11±0.20	C
		DV1	16.55±0.45	B
		dDV1	40.85±3.28	A
ANOVA *p level*				
Day of treatment *0.00*				
Bacterial inoculum *0.12*				
Day of treatment×bacterial inoculum *0.00*				

that it could be a good candidate for phytoremediation purposes (e.g., Cocozza et al. 2014; Di Baccio et al. 2014; Fernàndez-Martínez et al. 2014).

Plants were inoculated with DV1 and dDV1 bacterial cell suspensions. Then, plants were transplanted separately into 10 L plastic pots filled with clay+peat substrate. Plants were grown in a screened greenhouse with temperatures ranging between 15 and 30 °C. The pots were automatically irrigated each day with tap water to keep the moisture at about 70 % of the water-holding capacity. No fertilizers were applied.

Pots were prepared in advance (about 4 weeks) to allow time for the added Cd to come to equilibrium with the clay+peat substrate. Cadmium was supplied in two doses of 40 mg kg^{-1} each, the first at the end of April and the second at the end of May. The Cd treatment at the end of April was aimed at eliciting early responses of the plant–microbe association to the pollutant, whereas the Cd treatment in May was provided to assess the system responses to chronic pollutant exposure.

Six treatment combinations (six pots each) were defined by different conditions of plant growth: (i) control, (ii) Cd treatment, (iii) DV1 inoculation, (iv) Cd treatment and DV1-inoculation, (v) dDV1-inoculation, (vi) Cd treatment and dDV1 inoculation. Cadmium was supplied as 40 mg kg^{-1} of $CdSO_4$ solution. The inoculation was applied as concentrations of 1×10^7 CFU/g (named DV1) and 2×10^7 CFU/g (named dDV1) of *S. marcescens* strain to the clay+peat substrate.

The length of the experimental period and the degree of substrate contamination were aimed at eliciting short-term responses in the plant–microbe system, simulating early plant establishment on a contaminated clay+peat substrate. Since the experiment was a simulation of a field trial, substrates were not sterilized, allowing for the proliferation of spontaneous microorganisms.

The growth of plants (stem and leaf) and bacteria was monitored 30 days after the first and second addition of Cd to the growth medium, and the metal was determined in the clay+peat substrate. Additionally, at the end of experiment, the plants were washed free of substrate, and biomass allocation, root morphology, and Cd concentrations in leaves, stem, and roots analyzed.

Dynamics and viability of bacterial populations

Dynamics of the total bacterial population and total ATP content were evaluated during the experiment: 0, 15, and 30 days after Cd treatment.

For population dynamics, 1 g of clay+peat substrate was taken from each pot, was suspended in 9 ml PBS (pH 7.4), and kept at 28 °C on a rotary shaker (150*g*) for 30 min. Suspensions were filtered through four layers of sterile cheesecloth, mixture water extracts were serially diluted (from 10^{-3} to 10^{-7}), and 100 μl of each diluted suspension was plated in Petri dishes containing Nutrient Agar medium (beef extract 3 g l^{-1}, peptone 2 g l^{-1}, dextrose 2.5 g l^{-1}, agar 15 g l^{-1}). Plates were incubated at 28 °C for 2 days, and bacterial colonies were enumerated and expressed as colony forming unit per gram of soil.

Total ATP content was tested with total ATP assay (Ranalli et al. 2005). A portable bioluminometer Biocounter model P 1.500 (Lumac B.V., Landgraaf, The Netherlands), equipped with a photomultiplier tube (PTM) and set at 7200 RLU (Relative Luminose Unit) with 200 pg ATP in 100 μl, was adopted. For the evaluation of ATP content, the Microbial Biomass Test Kit and Standard ATP assay (Celsis-Lumac B.V., Landgraaf, The Netherlands) were used. Triplicate bioluminescent assays were performed in a Tris-HCl buffer solution (0.025 M; pH 7.75) by adding an adequate dilution of standard ATP to the sample as internal standard (Ranalli et al. 2003).

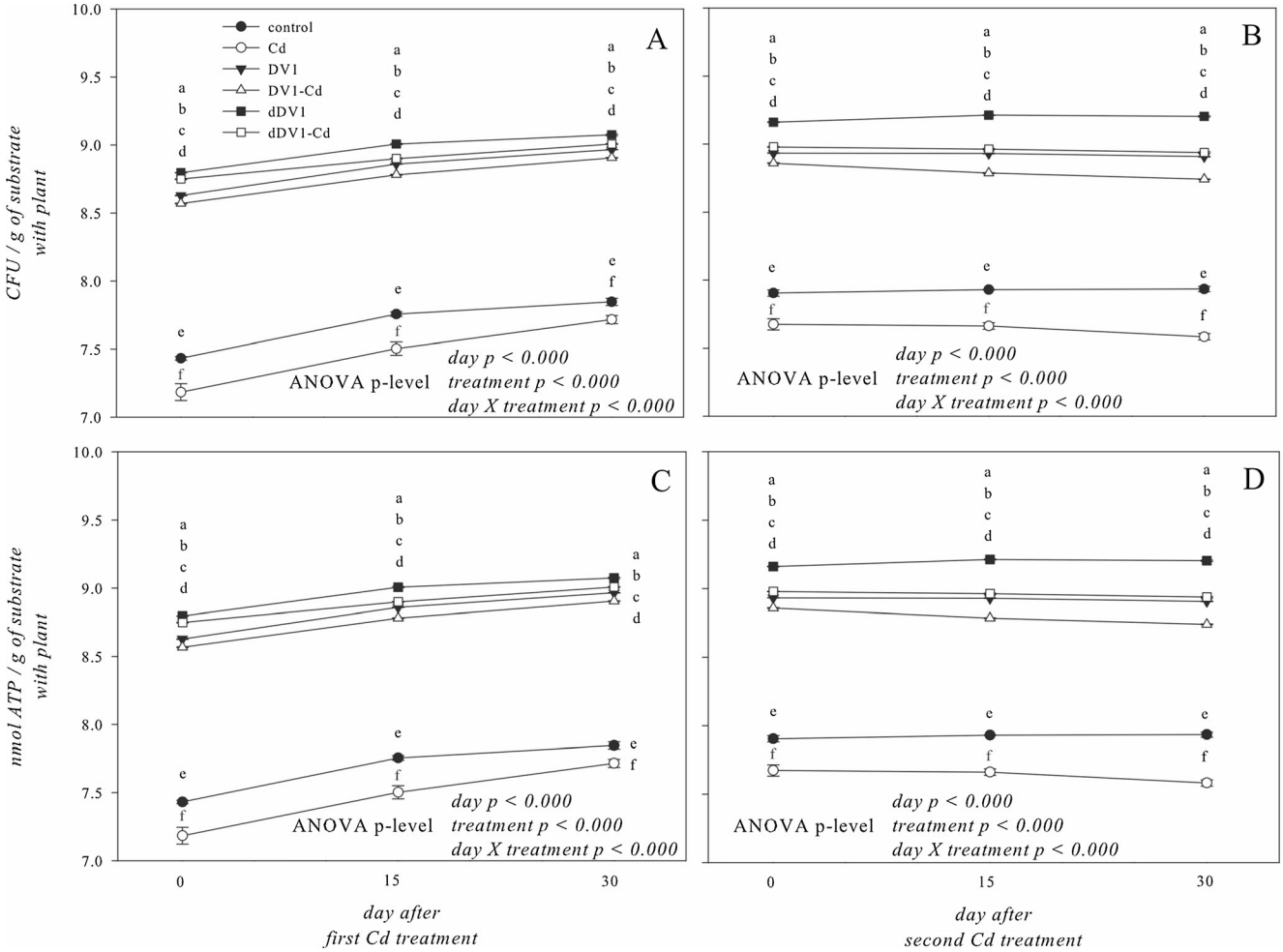

Fig. 2 Dynamics of the total bacterial population (**A**–**B**) and total ATP content (**C**–**D**) in control, Cdtreated, DV1- and dDV1-inoculated and non-inoculated substrates with plants were evaluated 0, 15, and 30 days after the first and second Cd addition. Data represent the mean (n=3)± standard error. A logarithmic scale is used for the y-axis. Mean values marked with the same letter are not statistically different among treatments in each day (LSD test, $P \leq 0.05$)

Plant analysis

The main morphometric parameters were measured 30 days after the first and second Cd treatment: LA=leaf area, D1=leaf length, D2=leaf width, LN=leaves number, LP=leaf perimeter, RL=root length, RD=root diameter, RN=roots number, S/R=shoot to root ratio, SB=stem branching, SD=stem diameter, SH=stem height. Digital images were captured with a Nikon Coolpix camera and the Image J 1.41 (http://rsb.info.nih.gov/ij/) and WinRhizo 2003.b version (Regent Instruments Ltd., Canada) software were used for analysis. Biomass allocation in the different organs (roots, leaves, and stems) of inoculated and non-inoculated, Cd-treated, and untreated plants were determined before (fresh weight, FW) and after (dry weight, DW) 24 h of drying in the oven at 80 °C. The shoot-to-root ratio (S/R) was calculated by dividing the sum of leaf and stem DW by the root DW.

Chlorophyll analysis

Chlorophylls were measured 30 days after the first and second Cd treatment. Chlorophylls were extracted from three randomly sampled leaf discs (10 mm) with *N*,*N* dimethylformamide (DMF). Extraction was carried out for 48 h at 4 °C in the dark at a ratio of 1:20 (plant material/solvent, *w*/*v*) (Moran and Porath 1980). The extinction coefficients proposed by Inskeep and Bloom (1985) were used for the quantification of chlorophylls a (Chla) and b (Chlb) and total Chl by spectrophotometric analysis. The following equations were used: $\mathrm{Chla} = 12.70_{A664.5} - 2.79_{A647}$; $\mathrm{Chlb} = 20.70_{A647} - 4.62_{A664.5}$; total $\mathrm{Chl} = 17.90_{\mathrm{A647}} + 8.08_{A664.5}$, where A=absorbance in 1.0 cm cuvettes and Chl=mg per liter. Successively, the leaf area Chl content was calculated (mg cm^{-2}). Furthermore, the Chla/Chlb ratio was also calculated.

Table 3 Cadmium content in substrate with non-inoculated (no ino) and inoculated (DV1 and dDV1) plants 5 and 30 days after the first and the second Cd application

	LSD ($P \leq 0.05$)	Inoculum	Cd first and second day of treatment	LSD ($P \leq 0.05$) each day of treatment
First treatment			mg kg^{-1}	
5 days	c	No ino	14.94±0.24	A
		DV1	8.90±0.80	B
		dDV1	8.50±0.05	B
30 days	b	No ino	21.97±0.59	B
		DV1	9.90±0.10	C
		dDV1	24.10±0.63	A
Second treatment				
5 days	a	No ino	33.63±0.86	A
		DV1	19.40±2.63	B
		dDV1	38.13±0.55	A
30 days	b	No ino	11.21±0.23	C
		DV1	20.79±0.26	A
		dDV1	13.80±0.19	B
ANOVA *p level*				
day of treatment *0.00*				
bacterial inoculum *0.00* day of treatment x bacterial inoculum *0.00*				

Cd content in control plants was below detection limit (<0.1 mg kg^{-1}). Mean values (± standard error) marked with the same letter are not statistically different (lowercase letters for the comparison of day of treatment; capital letters for the comparison of bacterial inoculum per each day of treatment) (LSD test, $P \leq 0.05$). Two-way ANOVA was applied to weigh the effects of days of treatment, bacterial inoculums and their interactions (p level values are reported)

Cd determination

Roots, leaves, and stems of all plant samples were harvested at the end of experiment and dried at 80 °C until constant weight and ground in a metal-free mill (Retsch GmbH, Haan, Germany). Substrates were sampled 0 and 30 days after the first and second Cd addition. After the removal of visible plant residues, the substrates were oven-dried at 30 °C to a constant weight and homogenized; then, a portion of each sample was powdered with a pestle in a ceramic mortar. For total Cd concentrations in the substrates and in the plant compartments, 1.0 g of samples was digested using the US EPA 3050B method (US EPA, 1996) and an automated heating block (Digester DK 20, Velp Scientifica, Milano, Italy). Extraction with HNO_3 should release maximum potentially mobile fraction of metal (exchangeable or environmentally available), without releasing metal associated with solid phases, within the structure of insoluble minerals (see Kovacs et al. 2000). After digestion, the samples were filtered and transferred quantitatively into 100 ml volumetric flasks and diluted to volume with double deionized water. The final solutions were stored in polyethylene bottles at 4 °C until analysis. Determination of Cd was performed using a Varian SpectrAA 220FS atomic absorption spectrophotometer with a graphite furnace GTA 100. Samples were analyzed at λ= 228.8 nm using pyro-coated graphite tubes and a $Pd(NO_3)2$-citric acid matrix modifier. The signals were measured with background correction (deuterium lamp). The presence of possible contaminants during the digestion process was controlled by reagent blank in each analytical batch. Each sample was run in triplicate to guarantee that the measured absorbance values were constant. Metal concentrations calculated from each replicate absorbance value were then used to calculate an average metal sample concentration. Successively, to assess the Cd uptake and tolerance in plants, the total and organ specific (leaves, stems and roots) bio-concentration factor (BCF), tolerance index (TI), and translocation factor (TF) were calculated. The BCF was calculated as the ratio of Cd concentrations in organ/plant and in the clay+peat substrate. The TI was calculated as the ratio of DW of Cd-treated plants and DW of control plants. The TF was calculated as the ratio of Cd concentration in aboveground (leaves and stems) and belowground (roots) plant compartments.

Statistical analysis

A completely randomized design with six replicates for each treatment was set up for the experiment. The

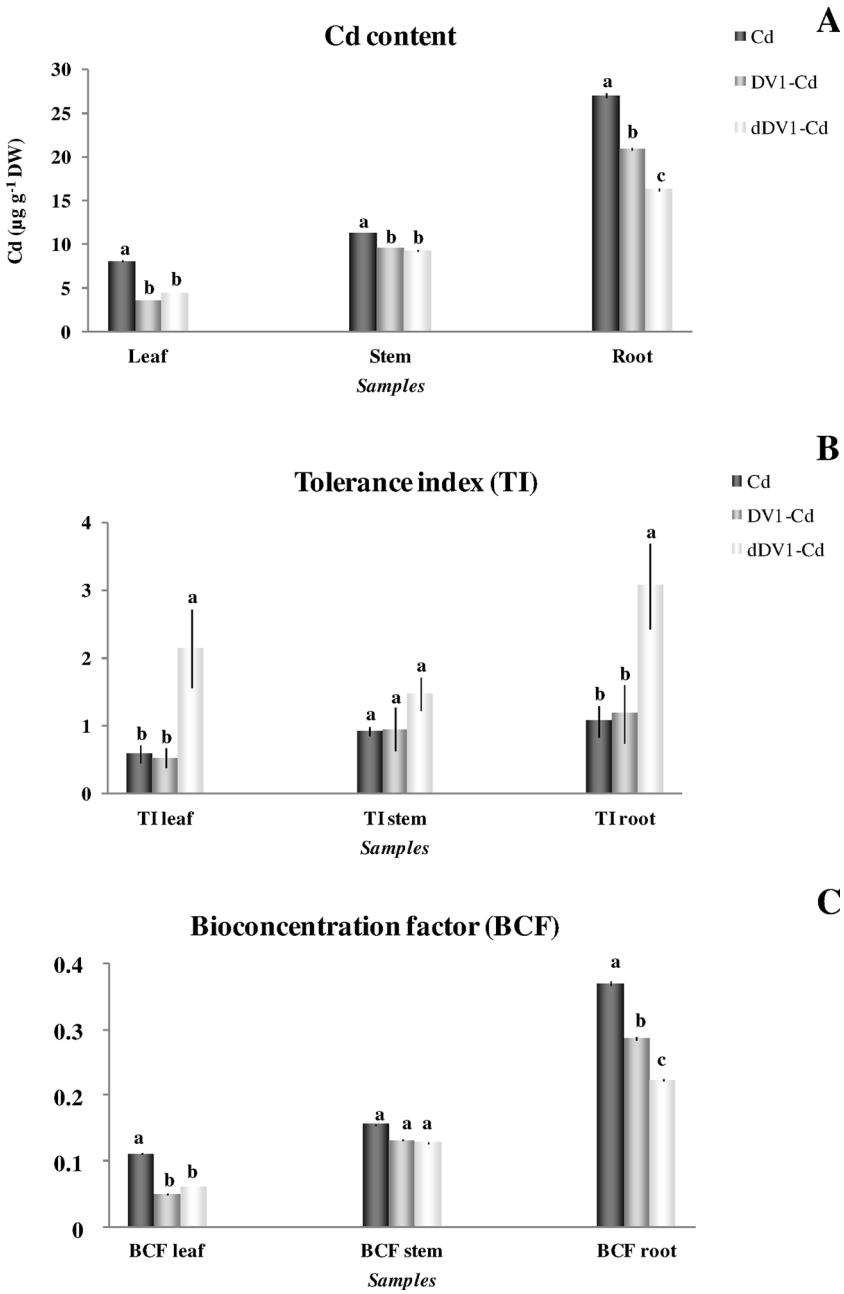

Fig. 3 Exchangeable Cd content (**A**), tolerance index (TI) (**B**), and bioconcentration factor (BCF) (**C**) in leaves, stems, and roots of Cd-treated (Cd), DV1- and dDV1-inoculated poplar plants. Cd content was expressed as µg/g of dry mass (DW). Data represent the mean (n=3)±standard error. Mean values marked with the *same letter* are not statistically different (LSD test, $P \leq 0.05$)

analysis of variance (ANOVA) was applied in order to evaluate the effects of the different Cd and inoculum treatments (two-way ANOVA), and the day of treatment, Cd treatment, and bacterial inoculum (three-way ANOVA), considering the effect of single factors and interaction effects among factors. To assess the differences between treatments for the measured parameters, a post hoc comparison of means was performed using the Fisher least significant difference (LSD) test at the 0.05 significance level. Statistical analysis was conducted with OriginPro version 8.5.1 (OriginLab, Northampton, MA).

Results

Selection of bacteria strains and Cd concentration

The in vitro bacteria screening showed that the gram-negative strains were relatively tolerant to the highest Cd concentration (160 mg kg^{-1} of Cd), whereas gram-positive strains were more susceptible to the tested Cd concentrations (Fig. 1a, b). On this basis, 40 mg kg^{-1} of Cd was defined as the metal concentration undamaging bacterial populations growing in vitro, and the DV1 strain was considered the best candidate for in vivo application

(Fig. 1a, b). Based on morphological characterization and BLASTn search comparisons of the 16S rDNA amplicon sequence data, the DV1 bacterial strain was identified as *S. marcescens* (99 % of identity; data not shown). Two different *S. marcescens* strain concentrations were selected for the in vivo experiment (1×10^7 CFU/g, named DV1, and 2×10^7 CFU/g of substrate, named dDV1).

Cadmium was differently available in the three different potting mixtures (expanded clay, peat, and clay+peat) 5 days after the addition of 40 mg kg^{-1} Cd (Table 1). The Cd adsorption capacity was comparable among the three potting mixtures (about 80 % of Cd was bound). However, higher Cd contents were in clay+peat substrate 7.0±0.8 mg kg^{-1} and in peat 7.6±1.7 mg kg^{-1} than in expanded clay 5.3±1.5 (Table 1). Although Cd content was slightly lower in comparison with the peat substrate, the clay+peat was further used as it was considered the best formulation for growing plants, as a mixture of organic (useful for the plant growth) and inorganic (suitable for the bacteria colonization) matter.

Characterization of substrate components

In substrates without plants, the Cd content showed a different availability per day of experiment in relation to the bacterial inoculum, increasing after the second Cd addition (Table 2). In particular, 30 days after the first Cd addition, the metal content increased in the non-inoculated substrate, whereas the Cd content increased in dDV1 inoculated substrates 30 days after the second Cd addition (Table 2).

In substrates with plants, available Cd varied between days of treatment ($P<0.001$) and bacterial inoculum ($P<0.001$) (Table 3). In fact, 5 days after the first Cd addition, a higher proportion of Cd (37.38 % of added Cd) was found in the non-inoculated clay+peat substrate than in the inoculated one, whereas after 30 days, a higher Cd content was observed in dDV1 inoculated than in DV1 non-inoculated. In the second Cd treatment, after 5 days, the lowest Cd content was found in DV1 inoculated, whereas after 30 days, DV1 inoculated showed the highest values (Table 3).

The total bacterial population was larger in the inoculated (DV1 and dDV1) substrates than in the non-inoculated substrates (Cd-treated and control) throughout the entire monitoring period (0, 15, and 30 days after the first and second Cd addition) (Fig. 2A, B). The ATP content showed the same pattern of the population dynamics, with higher values in inoculated than in non-inoculated (Fig. 2C, D).

Inoculum effect and effect of Cd treatment on biomass and morphology

Significant differences in morphological traits between inoculated and non-inoculated plants were found (Table 4). After the first treatment, LA, LP, D1, D2, SH, and SD were higher in DV1 and dDV1 than in control plants (Table 4), while LN and SB were higher in control than in inoculated plants (Table 4).

At the end of the experiment, total DW was about 2- and 5-fold higher in DV1 and dDV1, respectively, than in control plants due to a significant increment of DWL and DWS; however, the highest DW value was reached in dDV1 (Table 5). Furthermore, S/R increased significantly in DV1 and dDV1, achieving, also in this case, the highest value in dDV1 (Table 5). Root morphometric parameters changed between inoculated treatments (Table 5). In detail, RN was higher in control than in DV1 and dDV1 plants, while RL, RD, DW, and S/R showed higher values in dDV1 than in DV1 and control plants (Table 5).

Significant differences in growth parameters were recorded between treatments (Table 4). In detail, after the first Cd treatment, significantly higher values were observed for LN and SB in control plants; D1 in dDV1; LA, LP, and D2 in DV1 Cd; and SD in dDV1 plants (Table 4). After the second Cd treatment, significantly higher values were found for LN in dDV1-inoculated plants, and for SB in control plants; LA, LP, D1, D2, SH, and SD in dDV1-inoculated plants (Table 4).

In Cd-treated plants (Cd, DV1-Cd, and dDV1-Cd), RL and RD were higher than in control plants, while RN was higher in control plants (Table 5). Total DW and S/R were significantly higher in dDV1 not Cd-treated plants due to high values of DW L, DW S, and DW R (Table 5). However, S/R did not show differences between control and dDV1-Cd plants (Table 5).

Cd uptake/intake in leaves, stems, and roots

In Cd-treated plants, the Cd content increased by 14-fold, 24-fold, and 107-fold in leaves, stem, and root, respectively. In DV1-Cd treated plants, the Cd content increased by 6-fold, 20-fold, and 83-fold in leaves, stem, and root, respectively. In dDV1-Cd treated plants, the Cd content increased by 8-fold, 20-fold, and 65-fold in leaves, stem, and root, respectively (Fig. 3A). However, the Cd content was lower in plant tissues of DV1-Cd and dDV1-Cd than in Cd-treated non-inoculated plants (Fig. 3A). Additionally, in all samples, the highest Cd content was observed in roots (Fig. 3A), showing values of the translocation factor index (Cd_{shoot}/Cd_{root}) 0.72 in Cd-treated, 0.63 in DV1-Cd, and 0.84 in dDV1-Cd plants (data not shown). The tolerance index was significantly higher in dDV1-Cd than control plants, in particular in leaves (about 3.5-fold) and roots (about 2-fold) (Fig. 4B). No significant variation was observed in DV1-Cd treated plants. The total BCF of DV1 and dDV1Cd-treated plants was lower than in non-inoculated Cd-treated plants. In DV1-Cd and dDV1-Cd plants, the BCF_{leaves} decreased about 2-fold, while BCF_{roots} decreased in comparison with Cd-treated plants (Fig. 3C). No significant variation was observed in TI and BCF of stem in all treated plants.

Table 4 Main plant morphometric parameters measured 30 days after the first and second Cd application: LN=leaves number; LA=leaf area; LP=leaf perimeter; D1=leaf length; D2=leaf width; SH=stem height; SD=stem diameter; SB=stem branching. Data represent the mean (n=3)±standard error. Mean values marked with the same letter are not statistically different (lowercase letters for the comparison of first day of treatment; capital letters for the comparison of second day of treatment) (LSD test, $P \leq 0.05$). Three-way ANOVA was applied to weigh the effects of Cd treatments, days of treatment, bacterial inoculums, and their interactions (P level values are reported)

Samples	Treatment	Inoculum	LN	LA (cm^2)	LP (cm)	D1 (cm)	D2 (cm)	SH (cm)	SD (cm)	SB
First treatment										
Control	no Cd	No ino	56.33±2.52 a	20.42±4.19 d	17.47±1.97 d	6.56±0.64 d	4.58±0.69 d	48.75±0.25 d	0.85±0.10 b	3.67±0.58 a
Cd	Cd	No ino	23.00±0.58 d	24.22±2.02 d	18.63±0.62 d	6.29±0.34 d	5.67±0.26 d	68.00±3.00 b	0.97±0.19 b	1.00±0.00 d
DV1	no Cd	DV1	34.00±1.00 c	29.11±1.21 c	20.26±0.80 c	7.16±0.50 c	6.11±0.18 c	72.00±2.00 b	1.03±0.14 b	2.00±0.00 c
DV1-Cd	Cd	DV1	19.00±0.58 e	69.58±7.85 a	31.27±1.62 a	10.54±0.97 a	9.92±0.76 a	71.50±0.50 b	1.08±0.28 b	1.00±0.00 d
dDV1	no Cd	dDV1	49.00±2.00 b	61.08±2.96 a	30.14±0.76 a	10.96±0.64 a	8.70±0.19 b	57.50±4.50 c	1.14±0.20 a	1.67±1.15 d
dDV1-Cd	Cd	dDV1	54.00±1.00 a	46.56±3.59 b	26.20±1.33 b	9.67±0.37 b	7.99±0.49 b	81.50±1.50 a	1.12±0.05 a	2.33±1.15 b
Second treatment										
Control	no Cd	No ino	59.30±1.00 B	32.26±7.70 D	21.48±2.36 D	7.28±0.71 D	6.68±0.96 D	51.25±0.25 E	1.26±0.21 C	4.00±0.00 A
Cd	Cd	No ino	27.33±6.51 C	57.57±5.01 C	29.68±1.31 C	9.28±0.29 C	9.37±0.62 C	81.00±1.00 C	1.37±0.21 C	1.00±0.00 C
DV1	no Cd	DV1	33.00±1.00 C	96.51±23.62 B	37.59±5.69 B	12.35±1.70 B	11.19±0.93 B	86.50±0.50 B	1.31±0.05 C	2.00±0.00 B
DV1-Cd	Cd	DV1	17.00±2.00 D	98.12±39.88 B	37.28±7.50 B	11.83±2.44 B	11.78±1.99 B	77.50±0.50 D	1.46±0.00 B	1.00±0.00 C
dDV1	no Cd	dDV1	79.00±1.00 A	184.93±42.49 A	53.73±6.61 A	17.14±1.86 A	16.62±2.05 A	110.50±1.50 A	1.82±0.16 A	1.77±1.18 B
dDV1-Cd	Cd	dDV1	20.33±3.51 D	99.65±13.76 B	38.69±2.59 B	12.52±0.55 B	12.25±0.90 B	86.25±0.25 B	1.34±0.10 C	2.43±1.65 B
ANOVA *p level*										
Day of treatment			*0.000*	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Cd treatment			0.000	0.451	0.847	0.544	0.372	0.000	0.228	0.000
Bacterial inoculum			0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
Day of treatment×Cd treatment×bacterial inoculum			0.000	0.000	0.000	0.000	0.000	0.000	0.138	0.012

Table 5 Main root morphometric parameters measured at the end of experiment: RL=root length; RD=root diameter; RN=roots number; DW L=leaf dry weight; DW S=stem dry weight; DW R=root dry weight; Total DW=total dry weight; S/R=shoot to root ratio. Data represent the mean (n=3)±standard error. Mean values marked with the same letter are not statistically different (lowercase letters for the comparison of first day of treatment) (LSD test, $P \leq 0.05$). Two-way ANOVA was applied to weigh the effects of days of treatment, bacterial inoculums and their interactions (P level values are reported)

Sample	Treatment	Inoculum	RL		RD		RN		DW L		DW S		DW R		Total DW		S/R	
Second treatment																		
Control	No Cd	No ino	12.46±0.26	c	0.09±0.01	c	12.67±0.58	a	13.60±4.53	c	9.28±0.60	c	10.40±0.31	b	33.28±3.72	c	18.09±1.44	c
Cd	Cd	No ino	24.12±5.12	b	0.17±0.02	b	8.67±1.53	b	7.70±0.57	d	8.60±0.67	d	8.21±2.68	b	27.52±1.21	d	9.76±2.04	d
DV1	No Cd	DV1	18.18±2.58	b	0.14±0.02	b	7.33±2.52	b	34.60±5.61	b	12.74±1.92	b	13.16±4.99	b	60.49±6.82	b	38.14±1.28	b
DV1-Cd	Cd	DV1	22.34±2.49	b	0.17±0.02	b	4.33±0.58	c	7.63±4.42	d	8.81±2.60	d	12.33±4.71	b	28.77±8.94	d	7.79±0.83	d
dDV1	No Cd	dDV1	29.32±3.03	a	0.29±0.07	a	8.33±3.51	b	72.71±26.09	a	61.47±22.16	a	42.60±5.14	a	176.79±43.25	a	70.51±6.86	a
dDV1-Cd	Cd	dDV1	25.37±4.85	b	0.21±0.02	a	8.33±2.52	b	27.57±2.99	b	13.78±2.85	b	12.02±4.75	b	53.37±5.45	b	21.60±4.53	c
ANOVA p level																		
Cd treatment			0.032		0.595		0.041		0.000		0.002		0.000		0.000		0.000	
Bacterial inoculum			0.002		0.000		0.008		0.000		0.000		0.000		0.000		0.000	
Cd treatment×bacterial inoculum			0.007		0.003		0.286		0.034		0.001		0.000		0.000		0.037	

Chlorophyll determination

The total Chl and Chlb contents increased in inoculated plants after the first Cd application (Table 6). Lower values of Chla/Chlb ratio were observed in DV1 plants. Nevertheless, after the second treatment, total Chl contents were lower in dDV1 because of the reduction in Chlb. The Chla/Chlb ratio was the highest in dDV1 plants, owing to the lowest Chlb amount (Table 6). The decline in total Chl was probably related to the appearance of slight leaf chlorosis (not shown).

Discussion

Bacteria screening

Bacteria strains showed different responses to the Cd treatment, suggesting that microorganisms dwelling in the contaminated substrates were able to develop different strategies to tolerate metal stress (Oyetibo et al. 2010). The dynamics of the total bacterial population and total ATP content showed similar trends, as observed by Falappi et al. (1994). The population size and the ATP content increased after the inoculum of the clay+peat substrate with bacteria and with the growth of plants. Bacteria have been found to adjust their level of ATP, thus benefiting the population in contaminated substrates, by enabling individual cells to adopt different tolerance strategies under severe growth conditions (Yaginuma et al. 2014). The ability of bacteria to grow in polluted substrates might have resulted in an increase of the population size, providing more binding sites and wider surface areas available for the metal (e.g., Hayat et al. 2010). In the short term of this experiment, this might have favored bacteria in outcompeting plants in the Cd uptake. The level of variation within bacteria strains in this experiment was notable, suggesting that the colonization by members of different consortia can differ within each host plant. Future research to characterize specific inoculum or strain consortia is needed to explain the variation observed under excess Cd, as well as the specific interaction between the host plant, the bacteria community, and the growth substrate.

The effect of inoculum on plant growth

S. marcescens inoculum, in single and double concentrations (DV1 and dDV1), had positive effects on the growth of poplar plants. Indeed, plants grew taller with greater stem diameter in inoculated rather than in non-inoculated plants, whereas stem branching was higher in control plants. Branch formation within poplar species is known to be under strong genetic control (Wu and Hinckley 2001), where the variance observed within the same genotype is the result of environmental variation (Wu and Stettler 1997, 1998; Ma et al. 2008). Indeed, the

Table 6 Chlorophyll content 30 days after the first and second Cd application. The following parameters were measured: chlorophyll a – Chla, chlorophyll b – Chlb, total chlorophyll – Chl, and Chla-to-Chlb ratio – Chla/Chlb. Data represent the mean (n=3)±standard error. Mean values marked with the same letter are not statistically different (lowercase letters for the comparison of first day of treatment; capital letters for the comparison of second day of treatment) (LSD test, $P \leq 0.05$). Three-way ANOVA was applied to weigh the effects of Cd treatments, days of treatment, bacterial inoculums, and their interactions (P level values are reported).

Samples	Treatment	Inoculum	Chla (mg cm^{-2})		Chlb (mg cm^{-2})		Chl (mg cm^{-2})		Chla/Chlb	
First treatment										
Control	No Cd	No ino	0.26±0.00	b	0.18±0.02	b	0.44±0.02	c	1.49±0.07	b
Cd	Cd	No ino	0.30±0.01	a	0.18±0.02	b	0.48±0.02	b	1.71±0.09	a
DV1	No Cd	DV1	0.27±0.02	b	0.23±0.02	a	0.50±0.02	a	1.20±0.06	e
DV1-Cd	Cd	DV1	0.29±0.00	a	0.21±0.01	a	0.50±0.00	a	1.30±0.07	d
dDV1	No Cd	dDV1	0.30±0.02	a	0.20±0.01	a	0.50±0.00	a	1.51±0.08	b
dDV1-Cd	Cd	dDV1	0.29±0.01	a	0.21±0.01	a	0.50±0.00	a	1.41±0.07	c
Second treatment										
Control	No Cd	No ino	0.36±0.02	A	0.23±0.02	A	0.59±0.01	A	1.61±0.08	C
Cd	Cd	No ino	0.32±0.02	B	0.21±0.01	A	0.53±0.01	B	1.19±0.07	D
DV1	No Cd	DV1	0.35±0.01	A	0.22±0.01	A	0.58±0.00	A	1.58±0.08	C
DV1-Cd	Cd	DV1	0.31±0.01	B	0.16±0.01	B	0.47±0.01	C	1.91±0.10	B
dDV1	No Cd	dDV1	0.33±0.01	B	0.14±0.01	C	0.47±0.01	C	2.31±0.12	A
dDV1-Cd	Cd	dDV1	0.33±0.02	B	0.18±0.00	B	0.51±0.02	B	1.87±0.09	B
ANOVA p level										
Day of treatment			0.000		0.003		0.002		0.001	
Cd treatment			0.000		0.013		0.000		0.270	
Bacterial inoculum			0.003		0.000		0.000		0.004	
Day of treatment×Cd treatment×bacterial inoculum			0.000		0.012		0.000		0.026	

resource availability is critical for branching, as the vigor of the main stem is positively correlated with the branching pattern (Wu and Hinckley 2001).

Inoculated and Cd-treated plants had fewer primary roots, though showing greater root diameter and length in comparison with control plants. As for the branch development, *S. marcescens* inoculum can drive root morphogenesis by regulating root elongation and/or formation (Alonso et al. 2003; Swarup et al. 2002), commonly associated with an enhanced performance of plants in their response to a challenging environment (Lynch and Ho 2005). Synergistic interactions between the soil microbial community and plant roots benefited poplar growth, probably optimizing nutrient allocation. These interactions can alter the root development and the plant growth by stimulating the production of growth regulation substances, such as hormones (auxin and/or ethylene) and volatile compounds, disease suppression, enhancement of nutrient availability, and the increase of other beneficial microorganisms to plants (Gerhardson and Wright 2002; Ping and Boland 2004; Ryu et al. 2003).

The shoot-to-root ratio was also greater in plants inoculated with bacteria and not treated with Cd, which is affected by the balance between shoot-specific (carbon supply) and root-specific (mineral and water uptake) activities. Besides the experimentally proven ability of bacteria to enhance water and mineral uptake by host plants, they also participate, with unknown mechanisms, in the partitioning of carbon compounds within the plant (Murty and Ladha 1988; Sarig et al. 1988). The response of poplar plants to inoculation treatments was probably the result of combined interactions between the host genotype, the growth substrate, and the specific inoculation. Given the correlation between growth characteristics and wood traits, further investigation is needed to determine the potential for inoculation with bacteria to tailor the productivity of multipurpose poplar plantations.

The chlorophyll content, a parameter directly related to the photosynthesis efficiency and to plant growth and health, was higher in inoculated plants after the first treatment period, though slightly decreasing thereafter. Indeed, a specific bacterial strain inoculation can promote neutral or phytopathogenic effects on plant growth, depending on the inoculation dose and environmental conditions (Belimov et al. 2007). Again, inoculations contributed to increase total leaf area in Cd-treated plants. Although we did not measure

photosynthesis, total CO_2 assimilation and subsequent growth are likely to have increased in plants with greater leaf area, even with the same net CO_2 assimilation rate per leaf area. Nevertheless, the influence of specific host plant–microbial community interactions on growth promotion warrants further studies (Knoth et al. 2013).

The effect of inoculum on plant tolerance to Cd

Differences in Cd content in roots between inoculated and non-inoculated plants would suggest a controlled metal absorption in inoculated plants (non-inoculated plants showing higher Cd content). However, the root length was comparable between inoculated and non-inoculated Cd-treated plants, highlighting similar root elongation in Cd-treated and inoculated and non-inoculated plants, differing from control plants. The combined effect of Cd and inoculum on the root structure might affect xylem water transport to leaves. The presence of *Serratia* spp. has been reported to explain high levels of metal tolerance in plants (Visioli et al. 2015). Indeed, Anjum et al. (2011) observed that the root hydraulic conductivity decreased from two to four times, depending on the applied Cd stress and species feature. We hypothesize that, after the Cd addition, the absorption of water and nutrients and metals were not impaired, especially in DV1 and dDV1-inoculated plants, as instead observed in hydroponic Cd-polluted conditions (Cocozza et al. 2011). The plant growth is function of the complex interplays between limitations in sources and sinks at the root and shoot level, which establish a dynamic equilibrium. Microorganisms in the rhizosphere can variably alter bioavailability of contaminants and nutrients through different processes (release of chelating substances, acidification of the microenvironment, changing the redox potential), thus influencing metal uptake in plants (Whiting et al. 2001; Gadd 2004; Jiang et al. 2004; Kidd et al. 2009; De Maria et al. 2011). In the present case, similar mechanisms may have promoted the stabilization of Cd at root level of poplar plants. Indeed, the Cd content was higher in root of non-inoculated than inoculated plants, indicating that the bacteria association probably defined specific processes to diverge Cd uptake, by roots, thus promoting the growth and survival of plants under excess Cd in the substrate.

In inoculated plants, Cd contents were higher in roots than leaves and stems. Indeed, *Salicaceae* spp. tolerate heavy metal stress by enhancing metal compartmentalization in roots and preventing its translocation to other organs of the plant, reducing its toxic effects (Vaculík et al. 2012). Overall, these results suggest a complex role of bacterial inoculum in maintaining the plant growth and health under excess Cd by exploiting a wide range of mechanisms, in particular enhancing root absorption area and physiological tolerance (Glick 2012). Many investigations have reported the effect of Cd on inhibiting several biometric parameters of poplar plants (e.g., biomass accumulation, plant height, root length, leaf area) (e.g., Di Baccio et al. 2014). In the present study, bacteria were able to assist poplar plants in counteracting excessive disorders in biomass distribution between shoots and roots induced by excess Cd in the clay+peat substrate, thus maintaining water and nutrient balance within the plant. However, follow-up greenhouse studies and long-term field trials are required to determine whether or not the positive contribution of inoculations with bacteria to the plant growth traits is maintained in multipurpose poplar plantations with environmental restoration purposes.

The inoculum enhanced the accrual of biomass; inoculated Cd-treated plants produced wider leaves and longer stems than non-inoculated plants, which resulted in an increase of total biomass in dDV1 plants. Bacteria have the potential to support the mechanisms coordinating nutrient uptake and photosynthetic activity, thus ensuring metal tolerance in plants (Hossain et al. 2012). Additionally, the inoculum had positive effects on chlorophyll contents (Chlb and total Chl), especially evident after the first Cd application. Chlorophyll contents decreased with the proceeding of experiment. Chlb is the key pigment of PSII light harvesting complexes (Hikosaka and Terashima 1995). We hypothesize that the amount of Cd added to the clay+peat substrate was not high enough to induce permanent damages to the photosynthetic apparatus. In contrast, the second Cd treatment might have supplied a sufficient amount of Cd to replace Mg in the chlorophyll molecule (Xue et al. 2013). The decrease in chlorophyll contents with increasing metal concentration in the growth media has been previously reported by Di Baccio et al. (2011) for the same clone I-214 exposed to excess Zn. In our study, the chlorophyll contents were consistent with metal stress tolerance and potential oxidative damages in poplar plants, as a consequence of the increasing Cd concentration in the growth solution (Pietrini et al. 2010).

Although the enhanced allocation of biomass towards shoots, inoculating plants with bacteria, did not increase the partitioning of Cd to aboveground organs, as shown by BCF values. This occurred regardless of the active translocation of Cd from the roots to the leaves through the stem. Nevertheless, the plant tolerance to excess Cd increased with the inoculum, particularly in leaves and roots and in dDV1. These indices provide a good evaluation of the distribution of metals between the aboveground and belowground biomass and their tolerance in poplar plants (e.g., Di Baccio et al. 2014). Di Baccio et al. (2014) found that leaves of I-214 exposed to excess Cd retained preferentially the metal in the roots and were able to counteract oxidative stress through

anatomical and morpho-physiological mechanisms. Considering the complexity of the rhizosphere, more insights are necessary in order to unravel the role of microbial inoculation on biomass production and metal accumulation in different poplar clones, and the benefit in field applications, such as phytoremediation and ecological restoration of contaminated sites.

Conclusions

The inoculum of *S. marcescens* had positive effects on the plant growth, also with excess Cd, especially in double concentration. The investigated plant–bacteria–substrate system provided a promising framework for testing phytotechnologies (poplar plantations), underlining a more appropriate remedial approach for the use of this poplar clone in association with *S. marcescens* in the phytostabilization of metal-contaminated sites. The plant–bacteria association highlighted a major partition of the metal towards the root system, alleviating metal load due to bioaccumulation properties of *S. marcescens*. The investigation started with the initial isolation of bacteria from natural wood peat in a region characterized by riparian ecosystems. Considering the vulnerability of riparian ecosystems to pollution, the application of bacterial inoculum methodologies appears cost-effective and simple to use for multipurpose canal or riverbank plantations (*Salicaceae* spp.) and other agroforestry practices. The study confirms the suitability of the poplar clone I-214, also in phytoprotective microbial association against Cd, as a biological tool for the reclamation of Cd-contaminated sites. Inoculation procedures, which ensure efficient bacterial survival and plant colonization, especially in land reclamation, warrant further studies and represent major environmental challenges in modern plantation forestry. The restoration of polluted sites with poplars under limiting environmental conditions (e.g., the Mediterranean semi-arid systems) requires the selection of rhizosphere bacteria that are able to resist the high toxicity of heavy metals, reducing the toxicity of Cd and promoting the growth of plants.

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Compliance with Ethical Standards The manuscript is an original study that has not been submitted to other journal for simultaneous consideration. The authors declare their consent. The authors declare that there is no conflict of interest and principles of ethical and professional conduct have been followed.

References

Afzal M, Khan S, Iqbal S, Mirza MS, Khan QM (2013) Inoculation method affects colonization and activity of *Burkholderia phytofirmans* PsJN during phytoremediation of diesel-contaminated soil. Int Biodeter Biodegr 85:331–336

Ali H, Khan E, Sajad MA (2013) Phytoremediation of heavy metals—concepts and applications. Chemosphere 91:869–881

Alonso JM, Stepanova AN, Solano R, Wisman E, Ferrari S, Ausubel FM, Ecker JR (2003) Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis. Proc Natl Acad Sci U S A 100:2992–2997

Anjum SA, Xie X, Wang L, Saleem MF (2011) Morphological, physiological and biochemical responses of plants to drought stress. Afr J Agric Res 6:2026–2032

Azevedo RA, Carvalho RF, Cia MC, Gratão PL (2011) Sugarcane under pressure:an overview of biochemical and physiological studies of abiotic stress. Trop Plant Biol 4:42–51

Belimov AA, Dodd IC, Safronova VI, Hontzeas N, Davies WJ (2007) *Pseudomonas brassicacearum* strain Am3 containing 1-aminocyclopropane-1-carboxylate deaminase can show both pathogenic and growth-promoting properties in its interaction with tomato. J Exp Bot 58:1485–1495

Borghi M, Tognetti R, Monteforti G, Sebastiani L (2008) Responses of two poplar species (*Populus alba* and *Populus* x *canadensis*) to high copper concentrations. Environ Exp Bot 62:290–299

Borrero C, Ordovása J, Trillasb MI, Avilésa M (2006) Tomato *Fusarium* wilt suppressiveness. The relationship between the organic plant growth media and their microbial communities as characterised by Biolog®. Soil Biol Biochem 38:1631–1637

Boulter JI, Boland GJ, Trevors JT (2000) Compost: a study of the development process and end-product potential for suppression of turfgrass disease. World J Microbiol Biotechnol 16:115–134

Calfapietra C, Gielen B, Karnosky D, Ceulemans R, Scarascia-Mugnozza G (2010) Response and potential of agroforestry crops under global change. Environ Pollut 158:1095–1104

Castagna A, Di Baccio D, Tognetti R, Ranieri A, Sebastiani L (2013) Differential ozone sensitivity interferes with cadmium stress in poplar clones. Biol Plant 57:313–324

Castagna A, Di Baccio D, Ranieri AM, Sebastiani L, Tognetti R (2015) Effects of combined ozone and cadmium stresses on leaf traits in two poplar clones. Environ Sci Pollut Res 22:2064–2075

Cocozza C, Maiuro L, Tognetti R (2011) Mapping Cadmium distribution in roots of *Salicaceae* through scanning electron microscopy with x-ray microanalysis. iForest 4:113–120

Cocozza C, Vitullo D, Lima G, Maiuro L, Marchetti M, Tognetti R (2014) Enhancing phytoextraction of Cd by combining poplar (clone "I-214") with *Pseudomonas fluorescens* and microbial consortia. Environ Sci Pollut Res 21:1796–1808

Dal Corso G, Fasani E, Furini A (2013) Recent avances in the analysis of metal hyperaccumulation and hypertolerance in plants using proteomics. Front Plant Sci 4:280

De Maria S, Rivelli R, Kuffner M, Sessitsch A, Wenzel WW, Gorfer M, Strauss J, Puschenreiter M (2011) Interactions between accumulation of trace elements and macronutrients in *Salix caprea* after inoculation with rhizosphere microorganisms. Chemosphere 84:1256–1261

Di Baccio D, Galla G, Bracci T, Andreucci A, Barcaccia G, Tognetti R, Sebastiani L (2011) Transcriptome analyses of *Populus*× *euramericana* clone I-214 leaves exposed to excess zinc. Tree Physiol 31:1293–1308

Di Baccio D, Castagna A, Tognetti R, Ranieri A, Sebastiani L (2014) Early responses to cadmium of two poplar clones that differ in stress tolerance. J Plant Physiol 171:1693–1705

Durand TC, Sergeant K, Renaut J et al (2011) Poplar under drought: comparison of leaf and cambial proteomic responses. J Proteomics 74:1396–1410

Elobeid M, Gobel C, Feussner I, Polle A (2012) Cadmium interferes with auxin physiology and lignification in poplar. J Exp Bot 63:1413–1421

Evangelou MWH, Deram A, Gogos A, Studer B, Schulin R (2012) Assessment of suitability of tree species for the production of biomass on trace element contaminated soils. J Hazard Mater 209–210: 233–239

Falappi D, Farini A, Ranalli G, Sorlini C (1994) Effects of simulated acid rain on some microbiological parameters of subacid soil. Chemospere 6:1087–1095.

Farinati S, Dal Corso G, Panigati M, Furini A (2011) Interaction between selected bacterial strains and *Arabidopsis halleri* modulates shoot proteome and cadmium and zinc accumulation. J Exp Bot 62:3433–3447

Fernàndez J, Zacchini M, Fleck I (2012) Photosynthetic and growth responses of *Populus* clones Eridano and I-214 submitted to elevated Zn concentrations. J Geochem Explor 123:77–86

Fernàndez-Martínez J, Zacchini M, Fernández-Marín B, García-Plazaola JI, Fleck I (2014) Gas-exchange, photo- and antioxidant protection, and metal accumulation in I-214 and Eridano *Populus* sp. clones subjected toelevated zinc concentrations. Environ Exp Bot 107: 144–153

Gadd GM (2004) Microbial influence on metal mobility and application for bioremediation. Geoderma 122:109–119

Gallego SM, Pena LB, Barcia RA, Azpilicueta CE, Iannone MF, Rosales EP, Zawoznik MS, Groppa MD, Benavides MP (2012) Unravelling cadmium toxicity and tolerance in plants: insight into regulatory mechanisms. Environ Exp Bot 83:33–46

Gerhardson B, Wright S (2002) Bacterial associations with plants: beneficial, non N-fixing interactions. In: Sivasithamparam K, Dixon KW, Narrett RL (eds) Microorganism in plant conservation and biodiversity. Kluwer Academic Press, London, pp 79–103

Glick BR (2012) Plant growth-promoting bacteria: mechanisms and applications. Scientifica 12:1–15

Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60:579–598

He JL, Qin JJ, Long LY, Ma YL, Li H, Li K, Jiang XN, Liu TX, Polle A, Liang ZS, Luo ZB (2011) Net cadmium flux and accumulation reveal tissue-specific oxidative stress and detoxification in Populus x canescens. Physiol Plant 143:50–63

He JL, Li H, Luo J, Ma CF, Li SJ, Qu L, Gai Y, Jiang XN, Janz D, Polle A, Tyree M, Luo ZB (2013a) A transcriptomic network underlies microstructural and physiological responses to cadmium in Populus x canescens. Plant Physiol 162:424–439

He JL, Ma CF, Ma YL, Li H, Kang JQ, Liu TX, Polle A, Peng CH, Luo ZB (2013b) Cadmium tolerance in six poplar species. Environ Sci Pollut Res 20:163–174

He J, Li H, Ma C, Zhang Y, Polle A, Rennenberg H, Cheng X, Luo Z-B (2015) Overexpression of bacterial γ-glutamylcysteine synthetase mediates changes in cadmium influx, allocation, and detoxification in poplar. New Phytol 205:240–254

Hikosaka K, Terashima I (1995) A model of the acclimation of photosynthesis in the leaves of C3 plants to sun and shade with respect to nitrogen use. Plant Cell Environ 18:605–618

Hossain MA, Piyatida P, Teixeira da Silva JA, Fujita M (2012) Molecular mechanism of heavy metal toxicity and tolerance in plants: central role of glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. J Bot Vol. 2012, ID 872875

Inskeep WP, Bloom PR (1985) Extinction coefficients of chlorophyll a and B in n,n-dimethylformamide and 80% acetone. Plant Physiol 77:483–5

Jiang W, Luo YM, Liu Q, Liu SL, Zhao QG (2004) Effects of cadmium on nutrient uptake and translocation by Indian Mustard. Environ Geochem Health 26:319–324

Kidd P, Barceló J, Pilar Bernal M, Navari-Izzo F, Poschenrieder C, Shilev S, Lemente R, Monterroso C (2009) Trace element behaviour at the root-soil interface: implications in phytoremediation. Environ Exp Bot 67:243–259

Knoth JL, Kim S-H, Ettl GJ, Doty SL (2013) Effects of cross host species inoculation of nitrogen-fixing endophytes on growth and leaf physiology of maize. GCB Bioenergy 5:408–418

Kovacs B, Prokisch J, Gyori Z, Kovacs AB, Palencsar A (2000) Studies on soil sample preparation for inductively coupled plasma atomic emission spectrometry analysis. Commun Soil Sci Plant Anal 31: 1949–1963

Laureysens I, Blust R, De Temmerman L, Lemmens C, Ceulemans R (2004) Clonal variation in heavy metal accumulation and biomass production in a poplar coppice culture: I. Seasonal variation in leaf, wood and bark concentrations. Environ Pollut 131:485–494

Liu Z, Chen W, He X (2011) Cadmium-induced changes in growth and antioxidative mechanisms of a medicine plants (*Lonicera japonica* Thunb.). J Med Plants Res 5:1411–1417

Luo ZB, Wu C, Zhang C, Li H, Lipka U, Polle A (2014) The role of ectomycorrhizas in heavy metal stress tolerance of host plants. Environ Exp Bot 108:47–62

Lux A, Martinka M, Vaculík M, White PJ (2011) Root responses to cadmium in the rhizosphere: a review. J Exp Bot 62:21–37

Lynch JP, Ho MD (2005) Rhizoeconomics: carbon costs of phosphorus acquisition. Plant Soil 269:45–56

Ma CX, Yu QB, Berg A, Drost D, Novaes E, Fu GF, Yap JS, Tan AX, Kirst M, Cui YH et al (2008) A statistical model for testing the pleiotropic control of phenotypic plasticity for a count trait. Genetics 179:627–636

Ma YL, He JL, Ma CF, Luo J, Li H, Liu TX, Polle A, Peng CH, Luo ZB (2014) Ectomycorrhizas with *Paxillus involutus* enhance cadmium uptake and tolerance in *Populus* x *canescens*. Plant Cell Environ 37: 627–642

Mench M, Schwitzguébel J-P, Schroeder P, Bert V, Gawronski S, Gupta S (2009) Assessment of successful experiments and limitations of phytotechnologies: contaminant uptake, detoxification and sequestration, and consequences for food safety. Environ Sci Pollut Res 16: 876–900

Moore FP, Barac T, Borremans B, Oeyen L, Vangronsveld J, van der Lelie D, Campbell CD, Moore ERB (2006) Endophytic bacterial diversity in poplar trees growing on a BTEX-contaminated site: the characterisation of isolates with potential to enhance phytoremediation. Syst Appl Microbiol 29:539–556

Moran R, Porath D (1980) Chlorophyll determination in intact tissues using n, n-dimethylformamide. Plant Physiol 65:478–479

Murty MG, Ladha JK (1988) Influence of *Azospirillum* inoculation on the mineral uptake and growth of rice under hydroponic conditions. Plant Soil 108:281–285

Oyetibo GO, Ilori MO, Adebusoye SA, Obayori OS, Amund OO (2010) Bacteria with dual resistance to elevated concentrations of heavy metals and antibiotics in Nigerian contaminated systems. Environ Monit Assess 168:305–314

Pietrini F, Zacchini M, Iori V, Pietrosanti L, Ferretti M, Massacci A (2010) Spatial distribution of cadmium in leaves and its impact on photosynthesis: examples of different strategies in willow and poplar clones. Plant Biol 12:355–363

Pilon-Smits E (2005) Phytoremediation. Annu Rev Plant Biol 56:15–39

Ping L, Boland W (2004) Signals from the underground: bacterial volatiles promote growth in *Arabidopsis*. Trends Plant Sci 9:263–266

Polle A, Klein T, Kettner C (2013) Impact of cadmium on young plants of *Populus euphratica* and *P. x canescens*, two poplar species that differ in stress tolerance. New For 44:13–22

Ranalli G, Zanardini E, Pasini P, Roda A (2003) Rapid biodeteriogen and biocide diagnosis on artwork: a bioluminescent low-light imaging technique. Ann Microbiol 53:1–13

Ranalli G, Alfano G, Belli C, Lustrato G, Colombini MP, Bonaduce I, Zanardini E, Abbruscato P, Cappitelli F, Sorlini C (2005) Biotechnology applied to cultural heritage: biorestoration of frescoes using viable bacterial cells and enzymes. J Appl Microbiol 98:73–83

Romeo S, Trupiano D, Ariani A, Renzone G, Scippa GS, Scaloni A, Sebastiani L (2014) Proteomic analysis of *Populus* x *euramericana* (clone I-214) roots to identify key factors involved in zinc stress response. J Plant Physiol 171:1054–1063

Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Pare PW, Kloepper JW (2003) Bacterial volatiles promote growth in *Arabidopsis*. Proc Natl Acad Sci U S A 100:4927–4932

Sarig S, Blum A, Okon Y (1988) Improvement of the water status and yield of field-grown grain sorghum (*Sorghum bicolor*) by inoculation with *Azospirillum brasilense*. J Agric Sci 110:271–277

Sessitsch A, Kuffner M, Kidd P, Vangronsveld J, Wenzel WW, Fallmann K, Puschenreiter M (2013) The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. Soil Biol Biochem 60:182–194

Swarup R, Parry G, Graham N, Allen T, Bennett M (2002) Auxin cross-talk: integration of signaling pathways to control plant development. Plant Mol Biol 49:411–426

Todeschini V, Franchin C, Castiglione S, Burlando B, Biondi S, Torrigiani P, Berta G, Lingua G (2007) Responses to copper of two registered poplar clones inoculated or not with arbuscular mycorrhizal fungi. Caryologia 60:146–155

Tognetti R, Longobucco A, Raschi A, Miglietta F, Fumagalli I (1999) Responses of two *Populus* clones to elevated atmospheric CO_2 concentration in the field. Ann For Sci 56:493–500

Tognetti R, Sebastiani L, Minnocci A (2004) Gas exchange and foliage characteristics of two poplar clones grown in soil amended with industrial waste. Tree Physiol 24:75–82

Tognetti R, Cocozza C, Marchetti M (2013) Shaping the multifunctional tree: the use of *Salicaceae* in environmental restoration. iForest 6: 37–47

Trupiano D, Rocco M, Scaloni A, Renzoni G, Rossi M, Viscosi V, Chiatante D, Scippa GS (2014) Temporal analysis of poplar woody root response to bending stress. Physiol Plant 150:174–193

Ullah A, Sun H, Munis MFH, Fahad S, Yang X (2015) Phytoremediation of heavy metals assisted by plant growth promoting (PGP) bacteria: a review. Environ Exp Bot 117:28–40

Vaculík M, Konlechner C, Langer I, Adlassnig W, Puschenreiter M, Lux A, Hauser M-T (2012) Root anatomy and element distribution vary between two *Salix caprea* isolates with different Cd accumulation capacities. Environ Poll 163:117–126

Visioli G, D'Egidio S, Sanangelantoni AM (2015) The bacterial rhizobiome of hyperaccumulators: future perspectives based onomics analysis and advanced microscopy. Front Plant Sci 5:752

Vitullo D, Romano A, Di Pietro A, Lanzotti V, Lima G (2012) Role of new bacterial surfactins in the antifungal interaction between *Bacillus amyloliquefaciens* and *Fusarium oxysporum*. Plant Pathol 61:689–699

Whiting SN, de Souza MP, Terry N (2001) Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. Environ Sci Technol 35:3144–3150

Wu RL, Hinckley TM (2001) Phenotypic plasticity of sylleptic branching: genetic design of tree architecture. Crit Rev Plant Sci 20:467–485

Wu R, Stettler RF (1997) Quantitative genetics of growth and development in *Populus*. 2. The partitioning of genotype x environment interaction in stem growth. Heredity 78:124–134

Wu R, Stettler RF (1998) Quantitative genetics of growth and development in *Populus* III Phenotypic plasticity of crown structure and function. Heredity 81:299–310

Wu G, Kang H, Zhang X, Shao H, Chu L, Ruan C (2010) A critical review on the bio-removal of hazardous heavy metals from contaminated soils: issues, progress, eco-environmental concerns and opportunities. J Hazard Mater 174:1–8

Xue XC, Gao HY, Zhang LT (2013) Effects of cadmium on growth, photosynthetic rate and chlorophyll content in leaves of soybean seedlings. Biol Plant 57:587–590

Yaginuma H, Kawai S, Tabata KV, Tomiyama K, Kakizuka A, Komatsuzaki T, Noji H, Imamura H (2014) Diversity in ATP concentrations in a single bacterial cell population revealed by quantitative single-cell imaging. Sci Rep 4:6522. doi:10.1038/srep06522

Zacchini M, Pietrini F, Mugnozza GS, Iori V, Pietrosanti L, Massacci A (2009) Metal tolerance, accumulation and translocation in poplar and willow clones treated with cadmium in hydroponics. Water Air Soil Pollut 197:23–34

Zacchini M, Iori V, Scarascia Mugnozza G, Pietrini F, Massacci A (2011) Cadmium accumulation and tolerance in *Populus nigra* and *Salix alba*. Biol Plant 55:383–386