

Enhancing phytoextraction of Cd by combining poplar (clone “I-214”) with *Pseudomonas fluorescens* and microbial consortia

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Received: 8 July 2013 / Accepted: 12 August 2013 / Published online: 25 August 2013
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Abstract The plant–microorganism combinations may contribute to the success of phytoextraction of heavy metal-polluted soil. The purpose of this study was to investigate the effects of cadmium (Cd) soil concentration on selected physiological parameters of the poplar clone “I-214” inoculated at root level with a strain (BT4) of *Pseudomonas fluorescens* and a commercial product based on microbial consortia (Micosat F Fito®). Plants were subjected to Cd treatment of 40 mg kg⁻¹ in greenhouse. The effects of plant–microbe interactions, plant growth, leaf physiology, and microbial activity were periodically monitored. Metal concentration and translocation factors in plant tissues proved enhanced Cd uptake in roots of plants inoculated with *P. fluorescens* and transfer to shoots in plants inoculated with Micosat F Fito®, suggesting a promising strategy for using microbes in support of Cd uptake. Plant–microbe integration increased total removal of Cd, without interfering with plant growth, while improving the photosynthetic capacity. Two major mechanisms of metal phytoextraction induced by microbial inoculation may be suggested: improved Cd accumulation in roots inoculated with *P. fluorescens*, implying phytostabilization prospective and high Cd transfer to shoots of inoculated plants, outlining enhanced metal translocation.

Keywords *Populus* · Heavy metal · Gas exchange · Plant–microbe interaction

Introduction

Contamination of soils and waters by toxic heavy metals poses a great potential threat to the environment and human health (McGrath and Zhao 2003). Among the technologies available for the decontamination of waters and soils, an increasing interest has been devoted to phytoremediation (Mench et al. 2009; Iori et al. 2012; Ali et al. 2013). Heavy metals can be extracted from polluted soils through roots and transported through tissues where they can be sequestered (Memon and Schröder 2009; Wu et al. 2010). In particular, Salicaceae are known for tolerating heavy metals to a certain extent and for a remarkable metal absorption capacity, as they produce high amounts of easily harvestable leaf biomass and develop a massive root system in the topsoil (Pulford and Watson 2003). Salicaceae shows high variability between and within species and clones (e.g., Zacchini et al. 2009; Pietrini et al. 2010a; Marmioli et al. 2011; Castagna et al. 2013). The effectiveness of phytoremediation can be greatly enhanced by adding biological agents, already used against plant pathogens, in combination with suitable plants (Cicatelli et al. 2012; Ho et al. 2012). The use of plants and their associated microbes for environmental cleanup also yields other benefits including carbon sequestration, soil stabilization (Todeschini et al. 2007), and the possibility of biofuel or fiber production (Pilon-Smits 2005). Rhizosphere plays a significant role in the promotion of plant growth by providing access of compounds or enhancing water uptake (Pilon-Smits 2005). Bacteria may increase metal mobility and availability to the plant or else decrease metal solubility due to precipitation (Gadd 2010; De Maria et al. 2011; Regier et al. 2012). Rhizosphere bacteria and bacteria colonizing the interior of plants (e.g., endophytes) may contribute to metal uptake and

Responsible editor: Elena Maestri

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tolerance (Cicatelli et al. 2010; De Paolis et al. 2011). Moore et al. (2006) found a variety of endophytic bacteria in association with poplar, which may be efficiently used to enhance in situ phytoextraction of heavy metals.

Cadmium (Cd) is a heavy metal of anthropogenic origin that accumulates in soil and water, making it extremely persistent in the environment. Cadmium has no biological function and it is toxic to living organisms even at low concentrations. Although Cd is not an essential element, poplars can accumulate it to a certain extent in different plant parts (Castagna et al. 2013; Wu et al. 2010). The root is the first organ in contact with the various components of rhizosphere, and Cd can be translocated from root tips, where it may cause browning to shoots (Uraguchi et al. 2009), causing chlorosis, inhibition of water and nutrient uptake, photosynthesis, respiration, and growth (Sanità di Toppi and Gabbrielli 2009). Nevertheless, little is known about the specific requirements of poplars for optimal Cd uptake and about the environmental factors supporting the accumulation process (Di Lonardo et al. 2011).

We designed a combined phytoremediation process with poplar plants (clone I-214, *P. × euramericana* (Dode) Guinier) inoculated with different microorganisms. We hypothesized an increase in the potential for the removal of Cd on a contaminated substrate, by improving the extraction capacity, accumulation potential, and metal tolerance of the system (bacteria–plant). We report the short-term effect of Cd-contaminated (40 mg kg⁻¹) substrates on growth parameters and photosynthetic activity in poplars inoculated with promoting microorganisms. To this end, several bacteria strains were preliminarily isolated and screened through in vitro and in vivo experiments. To reveal the enhancement of Cd-phytoextraction capacity, we addressed the following objectives: (1) evaluate the influence of root inoculation with bacteria strains (including commercial product) on Cd uptake in I-214; (2) elucidate plant responses to Cd-contaminated substrates in relation to plant–microbe interaction.

Materials and methods

Isolation and identification of microorganisms

Eight bacteria strains were used for in vitro and in vivo experiments as isolated from compost and organic amendment, according to the methodology described in Berg et al. (2006) and Vitullo et al. (2012). The most representative bacterial isolates were characterized by the routine bacteriological tests Biolog® and API (Borrero et al. 2006) and by sequencing the small 16S ribosomal subunit (Centraalbureau voor Schimmelcultures, CBS, The Netherlands). These eight strains were identified as *Bacillus amyloliquefaciens*, strains BO7 and BO5A; *Bacillus subtilis*, strains BTd43 and BS1;

Pseudomonas fluorescens, strains FP and BT4, *Burkholderia cepacia*, strain BTd1; and *Pseudomonas* sp., strain BH.

Bacterial strains were routinely cultivated in minimum salt liquid medium (K₂HPO₄ 2.5 g L⁻¹; KH₂PO₄ 2.5 g L⁻¹; (NH₄)₂HPO₄ 2.5 g L⁻¹; MgSO₄×7H₂O 0.2 g L⁻¹; FeSO₄×7H₂O 0.01 g L⁻¹; MnSO₄×7H₂O 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, Na₂HPO₄ 12 mM, KH₂PO₄ 4 mM; pH 7.4) (Sambrook et al. 1989) and cell concentration was adjusted spectrophotometrically (600 nm). Bacterial strains were suspended in 30 % glycerol and stored at -80 °C. The commercial product Micosat F Fito®, based on consortia of different soilborne microorganisms, provided by CCS Aosta (Aosta, Italy), was also used.

Tolerance of bacterial isolates to different Cd concentrations in vitro

The activity of bacterial strains was preliminarily assessed in vitro. The ability of the bacterial strains to survive at five different concentrations (10, 20, 40, 80, and 160 mg kg⁻¹) of Cd was tested in liquid medium assays by using 96-well microliter plates according to Romano et al. (2011) and 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 different Cd concentration, and 10-µl of bacterial suspension in PBS at 0.1 O.D. (600 nm), which corresponds to about 1×10⁸ cfu ml⁻¹. Plates were incubated at 28 °C on a rotary shaker (200 g) for different periods (24, 48, and 72 h), and the increase in bacterial biomass was determined spectrophotometrically (595 nm) by using a Microplate Reader (mod. 550, Bio-Rad). Uninoculated NB was used as a blank. The experiment was repeated three times. Therefore, only one bacteria strain (*P. fluorescens*, strains BT4) was chosen for the investigation in planta, considering two selective parameters involved in bacterial growth and activity: (1) the ability to tolerate or resist to different cadmium concentration and (2) the capacity of fast growth in a synthetic medium.

Plant material and experimental setup

Woody cuttings (90 units), 2 years old, of the hybrid poplar clone I-214 *Populus deltoides* × *Populus nigra* (*P. × euramericana* (Dode) Guinier) were rooted in early spring (March, 2011), 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 2 months. After this establishment period, 72 homogenously rooted cuttings, which were uniform in size, were selected for the experiment. Plants were inoculated,

when required, with bacterial cell suspensions. An unsterilized substrate was used in close-to-nursery conditions to evaluate the ability of bacterial cells to compete with the natural microflora and colonize roots and the substrate. Rooted (inoculated) cuttings were transplanted separately into 10-L plastic pots filled with a commercial mixture of peat, Osmocote (NPK 14:13:13+7SO₃, Scott, Italy), and expanded clay.

Plants were then grown in a screened greenhouse with temperatures ranging between 15 and 30 °C, from the beginning of May to the end of August. The pots were irrigated every other day with tap water to keep the moisture at about 70 % of the water-holding capacity. No fertilizers were applied.

The time of exposure to metal contamination (4 weeks) was defined by control condition (0 mg kg⁻¹ Cd) and Cd addition to the substrate (40 mg kg⁻¹ Cd, using CdSO₄—Sigma, St. Louis, USA), defined as the metal concentration not damaging bacterial population. Pots were prepared in advance (about 4 weeks) to allow time for the added Cd to come to equilibrium with the substrate. The length of experimental period and degree of substrate contamination were aimed at eliciting short-term responses in the plant–microbe system, simulating early plant establishment on a contaminated substrate. Treatment groups were defined by common plant growth conditions, considering reference settings (T1, T3, T5) and Cd treatments (T2, T4, T6), as the following: T1 and T2, plants without inoculum; T3 and T4, plants inoculated at the root level with *P. fluorescens*, strain BT4; T5 and T6, plants inoculated at the root level with Micosat F Fito®, following the protocol described in Vitullo et al. (2008). Plant containers were moved frequently in the greenhouse to avoid positional effects.

Plant growth analysis

Stem length and leaf number were measured once a week from the initial pot arrangement to the end of Cd treatment. At the end of the experiment, all plants were harvested and separated into roots, shoots, and leaves.

All plant tissues were dried to a constant weight at 80 °C and the dry weight recorded before they were ground in a metal-free mill (Retsch GmbH, Haan, Germany). Aliquots (0.1 g) of ground root, leaves, and substrate material were digested using a mixture of HNO₃/HClO₄ at 225 °C using an automated heating block (Digester DK 42/26, Velp Scientifica, Milano, Italy). Cadmium concentrations in digests were determined using the Flame Atomic Absorption Spectroscopy (GF-AAS PerkinElmer 2100). Chemical analyses were validated by blanks and reference materials.

The translocation factor (leaf/root ratio) was calculated for each treatment according to the ratio between metal concentration (in milligrams per kilogram) in leaves and metal

concentration (in milligrams per kilogram) in roots. The total metal content was calculated for each treatment by multiplying the dry weight of leaves (in kilograms) by the metal concentration in leaves (in milligrams per kilogram). Bioconcentration factors (BCF) were calculated as the ratio between metal concentration (in milligrams per kilogram) in leaves and total metal concentration (in milligrams per kilogram) in soil, see Dos Santos Utmazian et al. (2007).

Photosynthesis and fluorescence measurements

One plant out of the four cuttings in each replicate was randomly selected for the physiological measurements. The first fully expanded leaf, Leaf Plastochron Index, LPI 5, i.e., fifth leaf from the apex (Dickmann 1971) of each plant was sampled for chlorophyll fluorescence measurements on different dates during the experiment. Chlorophyll fluorescence kinetics parameter (F_v/F_m =ratio between variable and maximum fluorescence) was measured with a PAM chlorophyll fluorometer (PAM 2100, Walz, Effeltrich, Germany). Leaf samples were placed in the dark for 30 min using a clasp. The minimal and the maximal fluorescence, F_o and F_m of a dark-adapted leaf, respectively, were measured. The maximum quantum yield of PSII photochemistry (F_v/F_m), was determined as $(F_m - F_o)/F_m$.

Photosynthetic response curves (A/C_i curves), derived from the response of net photosynthesis (A) to intercellular CO₂ concentration (C_i), were weekly measured on the same cuttings and leaves that were used for chlorophyll fluorescence analysis with a LI-COR photosynthesis system (LI-6400; LI-COR Inc., Lincoln, NE, USA) (cf., Tognetti et al. 2004). Leaf chamber temperature and humidity were adjusted to maintain a leaf-to-air vapor pressure deficit of 1.1 kPa and irradiance maintained at a saturating value of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Maximum carboxylation rate (V_{cmax}), electron transport rate (J_{max}), triose phosphate use (TPU), mesophyll conductance (g_m), CO₂ compensation point (T_c), and day respiration (R_{day}) were computed from the A/C_i curves using the A/C_i curve fitting model developed by Sharkey et al. (2008). TPU was determined as the highest A , regardless of whether symptoms of TPU were present, which makes TPU similar to the parameter A_{max} reported by other investigators.

Instantaneous photosynthesis rate (A_{sat}) and corresponding stomatal conductance (g_s) and ratio of intercellular (C_i) to ambient (C_a) CO₂ concentration (C_i/C_a) were measured at saturating light and ambient CO₂ concentration, at the end of experiment on the same plants sampled for the A/C_i curves and with the same cuvette conditions.

Scanning electron microscopy observations

Root portions (1–2 mm in length) were fixed with 3 % glutaraldehyde (ν/ν) in 0.1-M phosphate buffer (pH 7.2) for 6–

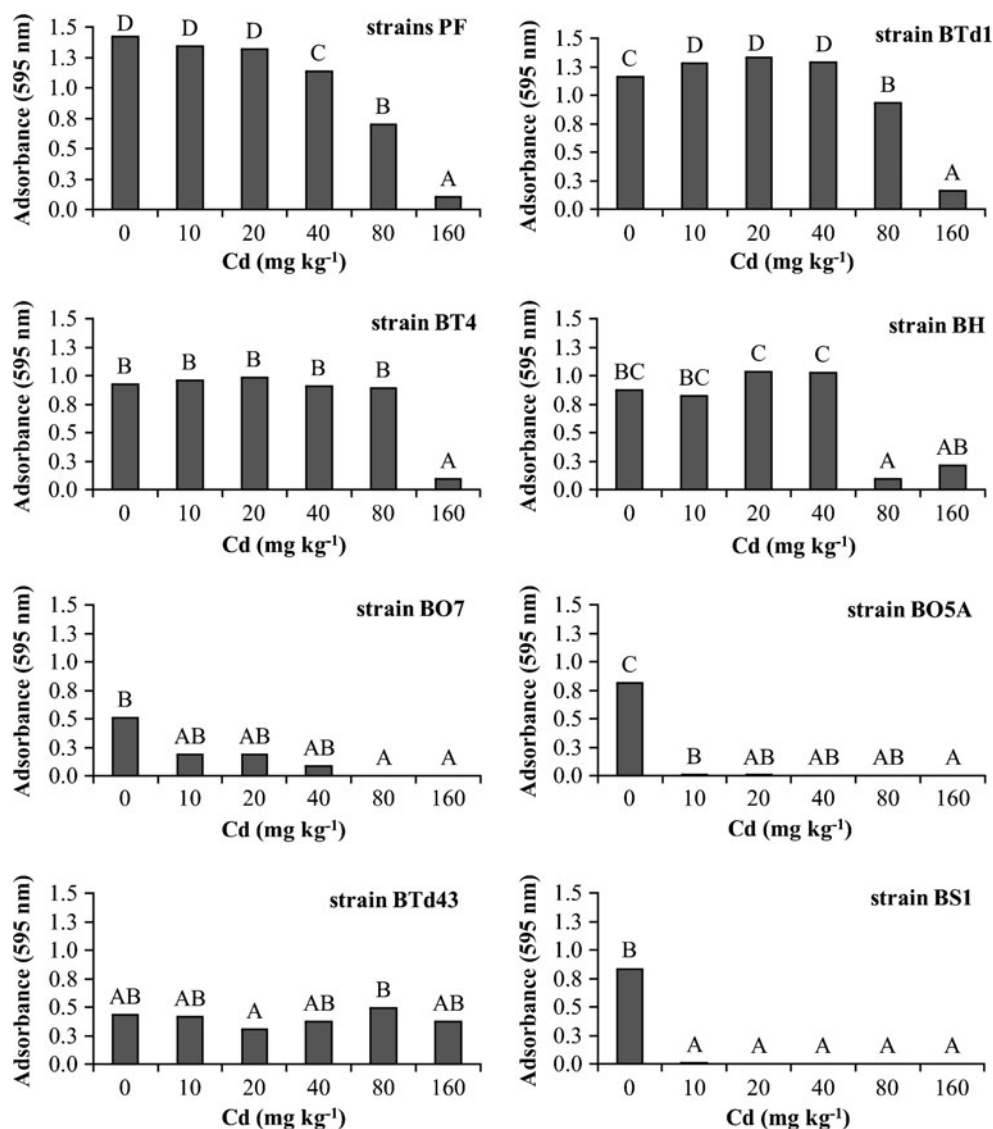
8 h under 4 °C, post-fixed in 1 % osmium tetroxide for 1 h, and immersed in 0.1-M phosphate buffer (pH 7.2) for 1–2 h. The root samples were then dehydrated in a graded ethanol series (50, 60, 70, 80, 90, 95, and 100 %) with a last wash in acetone for a better CO₂ substitution during the dehydration procedure at a pressure of 1,200 bars. Afterward, root samples were dried to critical point (critical point drier K850—Emitech, Ashford, UK) to produce an electrically conductive surface and thus prevent charging under the electron beam, and then covered with gold through sputtering (sputter coater K550—Emitech, Ashford, UK).

Dynamics of bacterial population in the substrate

The total bacterial population in the mixtures subjected to different treatments was periodically evaluated after 7, 14, 21, and 28 days. Hence, 1 g of bulk soil mixture taken from

each pot was suspended in 9 ml of phosphate buffer (NaCl 0.14 M, KCl 2.7 mM, Na₂HPO₄×2H₂O 8.1 mM, KH₂PO₄ 1.8 mM, pH 7.4) and kept at 28 °C on a rotary shaker (150 g) for 30 min (De Curtis et al. 2010). 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 were plated in Petri dishes containing King's B medium (20 g L⁻¹ proteose peptone, 10 g L⁻¹ glycerol, 1.5 g L⁻¹ K₂HPO₄, 1.5 g L⁻¹ MgSO₄×7H₂O, pH 7.2). Plates were incubated at 28 °C for 2 days and bacterial colonies were enumerated and expressed as colony forming unit per gram of soil, while in order to enumerate the total fluorescent bacterial colonies, plates were observed by a transilluminator (Gel Doc 2000, Bio-Rad) under UV light, which allows the observation of fluorescent pigments released in the agarized medium by bacterial colonies of fluorescent *Pseudomonas* spp.

Fig. 1 Preliminary in vitro screening of bacterial growth. Experiment was carried out on a 96-multiwell plate by using a standard liquid medium added to CdSO₄ at five different concentrations (Cd treatment from 10 to 160 mg kg⁻¹). Bacterial growth was determined spectrophotometrically at 595 nm, after 72 h of incubations at 28 °C. Gram-negative bacteria: *PF* and *BT4* *Pseudomonas fluorescens*, *BTd1* *Burkholderia cepacia* and *BH* *Pseudomonas* sp. Gram-positive bacteria: *BO7* and *BO5A* *Bacillus amyloliquefaciens*, *BTd43* and *BS1* *Bacillus subtilis*. Mean values of histograms marked with the same letter are not statistically different (Tukey's test, $P \leq 0.01$)



Statistical analysis

The experimental layout was a completely randomized block design with two factors (Cd treatment and microbial inoculation) and three replicates for each Cd level $\times$ microbe–inoculum combination. Plants were allotted into six homogeneous treatment groups of 12 individuals each that were split randomly into four plants for each of the three replicates. Data were averaged on a plant basis and the individual means were used for the analysis. Data sets were tested for homogeneity of variance and for the assumption of compound symmetry of the variance–covariance matrix using the Bartlett–Box F test and the Mauchly criterion, respectively. The effects of treatments on the development traits and photosynthetic parameters were tested using repeated measures analyses of variance (ANOVA). Data referring to one sampling only were subjected to one-way ANOVA, without considering the effects of time of exposure.

To assess the differences between treatments for the measured parameters, a post hoc comparison of means was performed using the least significant difference (LSD) test or Tukey's test at the 0.05 significance level; percentages were converted into Bliss angular values ($\arcsin \sqrt{\%}$) before analysis. Statistical analysis was performed with the statistical package Statistica (StatSoft Inc., Tulsa, OK, USA) and SPSS software (version 16.0 for Windows; SPSS Inc., Chicago, IL, USA).

Results

In vitro screening performed on bacterial strains grown under experimental conditions showed a different microbial growth under excess Cd (Fig. 1). Gram-negative strains (BT4, BTd1, BH, and PF) were relatively tolerant to the different Cd concentrations; consistent growth inhibition occurred only at 80 and 160 mg kg⁻¹ of Cd. By contrast, gram-positive strains (BO7, BO5A, BTd43 and BS1) were more susceptible to all tested Cd concentrations, except for strain BTd43 of *Bacillus subtilis* that proved to be somewhat tolerant to Cd. Overall, strain BT4 of *P. fluorescens* was considered as the best candidate for the application in vivo biological metal extraction assays.

Stem length increased constantly throughout the experiment in all treatments, without being influenced markedly by excess Cd. In parallel, leaf number also augmented with plant development, showing higher values in non-inoculated than inoculated plants. However, fewer leaves did not correspond to shorter stem, and symptoms of leaf chlorosis were not observed. Leaf dry mass and the root/shoot dry mass ratio did not vary among treatments, averaging 3.10 $\pm$ 0.11 and 1.15 $\pm$ 0.11 g, respectively, across treatments; whereas the dry mass of stem and roots (and total dry mass) was higher in T4 and

T6, averaging 3.97 $\pm$ 0.09 and 8.94 $\pm$ 0.38 g (and 15.96 $\pm$ 0.01 g), respectively, in comparison with T1 and T2, averaging 3.31 $\pm$ 0.06 and 6.43 $\pm$ 0.39 g (and 12.89 $\pm$ 0.49 g), respectively.

The amount of Cd in metal-treated plants was higher than background values observed in non-contaminated plants. Cadmium concentrations were significantly higher in tissues of inoculated plants; indeed, a remarkable accumulation of metal in roots of T4, and in stems and leaves of T4 and T6 was observed (Table 1). The highest BCF was found in plants inoculated with Micosat F Fito®; whereas, *P. fluorescens* was less effective in augmenting the capability of I-214 to extract and accumulate Cd. The efficacy of Micosat F Fito® to complement plants for the translocation of the metal absorbed by roots to the shoots was further substantiated by the values of Tf. The highest Tf were observed in T6, even if differences between the two microbial inoculations were not significant. Excess Cd did not affect significantly F_v/F_m , which remained stable during days of treatment (DOT), regardless of the treatment (Tables 2). Nevertheless, F_m was not affected significantly by Cd treatment, while metal addition and microbial inoculation increased F_o . In general, gas exchange parameters were marginally influenced by Cd contamination and microorganism inoculation (Tables 3, 4). Values of V_{cmax} did not vary significantly among treatments or DOT; this was not the case for J_{max} and TPU, which varied among treatments and DOT. Within DOT, V_{cmax} showed higher values in T4 in DOT 21, in comparison with other treatments; J_{max} and TPU were lower in T3 and T5, in DOT 28. Moreover, R_{day} was apparently reduced by excess Cd with

Table 1 Cadmium content (milligram per kilogram dry mass) in tissues of poplar clone I-214 treated with 40 mg kg⁻¹ Cd for 4 weeks

Microbe inoculum	T2 None	T4 <i>Pseudomonas</i>	T6 Micosat
Cd content			
Roots	42.82ab (0.3)	88.85b (0.9)	27.88a (3.2)
Stem	13.58a (0.3)	31.03ab (1.8)	49.72b (4.5)
Leaves	20.68a (1.6)	29.54a (2.8)	31.85a (4.3)
BCF	0.10a (0.02)	0.19a (0.07)	0.32b (0.08)
Tf	1.13a (0.59)	1.17a (0.54)	6.08a (3.97)

Cd content was not detectable in control plants. Bioconcentration factor (BCF) and translocation factor (Tf) are reported

Mean values ($\pm$ standard errors) followed by the same letter within rows are not significantly different (LSD, $P \leq 0.05$)

Table 2 Treatment effects on plant development traits (stem length (cm) and leaf number (n)) and on chlorophyll fluorescence parameters (minimal fluorescence (F_o), and photochemical efficiency (F_v/F_m)), measured during the experiment (DOT 7, 14, 21, and 28)

Cd level	DOT 7									
	T1					T2				
	Control	T3	T5	T6		Control	T3	T5	T6	
Microbe inoculum	None					Micosat				
	Pseudomonas					Micosat				
	None					Micosat				
Stem length	67.6a (3.8)	60.7a (3.0)	59.1a (3.4)	62.8a (3.4)		65.6a (4.1)	64.5a (3.2)	61.8a (3.6)	68.7ab (3.2)	67.5ab (3.7)
Leaf number	33.9c (2.8)	28.8abc (3.3)	22.5a (3.9)	25.1ab (2.8)		31.8b (3.0)	24.8ab (2.8)	40.2abc (5.7)	54.9abc (6.0)	49.7b (4.2)
F_o	38.7a (10.4)	83.5ab (50.5)	61.0ab (25.5)	47.33a (29.62)		154.0b (50.5)	48.0ab (15.0)	111.5b (11.5)	98.0b (34.0)	18.5a (4.5)
F_v/F_m	0.7b (0.1)	0.9b (0.1)	0.4ac (0.2)	0.8bc (0.1)		1.0b (0.2)	0.7bc (0.1)	0.6a (0.2)	0.6a (0.1)	0.7a (0.2)
DOT 21										
Cd level	T1					T2				
	Control					Cd				
	None					Micosat				
Microbe inoculum	Pseudomonas					Micosat				
Stem length	81.3b (4.6)	73.2ab (3.6)	65.4a (4.0)	73.6ab (4.3)		74.6ab (5.2)	75.1ab (3.4)	69.4ab (4.5)	79.3a (4.3)	81.0ab (4.0)
Leaf number	104.3c (7.5)	85.2ab (5.9)	63.4a (8.2)	70.2a (6.0)		102.8bc (6.3)	81.9a (9.0)	82.5a (8.9)	91.4ac (8.8)	89.8a (8.2)
F_o	50.50a (3.5)	121.5a (63.5)	45.7a (14.5)	108.0a (58.0)		102.0a (8.0)	129.5a (63.5)	63.5ab (15.5)	123.5bc (2.5)	135.3c (15.0)
F_v/F_m	0.8a (0.1)	0.7a (0.1)	0.7a (0.1)	0.9a (0.1)		0.6a (0.1)	0.7a (0.4)	0.5a (0.2)	0.7a (0.3)	0.5a (0.1)

Mean values ($\pm$ standard errors) followed by the same letter within a parameter, in each DOT, are not significantly different (LSD, $P \leq 0.05$)

Table 3 Treatment effects on photosynthetic response curves

Cd level	DOT 7						DOT 21					
	T1 Control		T3		T5		T2 Cd		T4		T6	
	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>
Microbe inoculum	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>
V_{cmax}	82.7a (7.5)	70.2a (11.0)	72.4a (4.9)	89.5a (15.4)	75.4a (19.7)	93.3a (17.3)	78.1b (5.65)	67.6ac (1.83)	56.2ac (7.51)	82.2a (20.7)	79.2a (4.23)	89.8ab (7.13)
J_{max}	98.1a (3.6)	74.8a (1.0)	91.7a (7.3)	72.6a (38.4)	83.1a (13.5)	107.5a (13.6)	108.0b (2.6)	103.7b (1.1)	87.6a (11.3)	122.3c (5.1)	140.5b (8.9)	132.0bc (5.4)
TPU	6.6a (0.3)	5.0a (0.1)	5.8a (0.5)	4.4a (2.6)	4.6a (0.1)	6.0a (1.6)	7.3b (0.0)	6.5a (0.2)	6.0a (0.0)	8.6c (0.2)	8.5c (0.0)	8.4c (0.0)
R_{day}	0.02a (0.0)	0.02a (0.0)	3.9c (0.3)	2.7b (0.6)	2.3b (0.4)	2.5b (0.4)	3.6b (1.83)	1.5a (0.28)	1.3a (0.26)	2.1b (0.31)	2.4bc (0.42)	2.9b (0.81)
Γ_c	3.1a (0.2)	3.3a (0.5)	7.5c (0.9)	5.2bd (0.4)	6.6bc (0.8)	4.5ad (0.6)	5.7a (1.9)	4.7a (0.2)	5.0a (0.2)	3.3a (0.7)	4.0a (1.1)	4.5a (0.7)
g_m	30.0b (0.2)	29.1b (0.8)	25.9ab (3.0)	26.8ab (1.1)	23.9a (1.6)	27.6ab (1.9)	29.6a (0.7)	30.5acd (0.4)	31.0bde (0.6)	22.8a (4.09)	26.6a (0.45)	26.4a (1.13)

Cd level	DOT 28						DOT 21					
	T1 Control		T3		T5		T2 Cd		T4		T6	
	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>
Microbe inoculum	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>	None	<i>Pseudomonas</i>
V_{cmax}	68.6ab (11.05)	104.5d (8.87)	74.6c (11.13)	101.8b (8.76)	82.6a (3.08)	84.6ab (8.94)	79.2a (4.23)	89.8ab (7.13)	82.2a (20.7)	79.2a (4.23)	89.8ab (7.13)	82.2a (20.7)
J_{max}	107.7b (8.9)	98.5ab (3.5)	98.0ab (1.7)	129.1bc (0.0)	75.5a (3.4)	83.0a (3.4)	140.5b (8.9)	132.0bc (5.4)	122.3c (5.1)	140.5b (8.9)	132.0bc (5.4)	122.3c (5.1)
TPU	7.0bc (0.5)	6.2a (0.1)	6.3a (0.1)	8.5c (0.0)	4.8a (0.2)	5.3b (0.2)	8.5c (0.0)	8.4c (0.0)	8.6c (0.2)	8.5c (0.0)	8.4c (0.0)	8.6c (0.2)
R_{day}	2.3ab (0.23)	1.9ab (0.31)	0.9a (0.46)	1.7bc (0.61)	0.1a (0.15)	0.4a (0.43)	2.4bc (0.42)	2.9b (0.81)	2.1b (0.31)	2.4bc (0.42)	2.9b (0.81)	2.1b (0.31)
Γ_c	4.5a (1.0)	4.3a (0.2)	4.3a (0.2)	3.1a (1.5)	2.8a (0.6)	2.4a (1.1)	4.0a (1.1)	4.5a (0.7)	3.3a (0.7)	4.0a (1.1)	4.5a (0.7)	3.3a (0.7)
g_m	31.5bc (0.3)	30.0ac (0.5)	29.7a (0.2)	24.9a (2.27)	18.5a (9.67)	25.8a (1.77)	26.6a (0.45)	26.4a (1.13)	22.8a (4.09)	26.6a (0.45)	26.4a (1.13)	22.8a (4.09)

V_{cmax} (in micromoles per square meter per second); J_{max} (in micromoles per square meter per second); TPU (in micromoles per square meter per second); R_{day} (in micromoles per square meter per second); Γ_c (in percentage); g_m (in millimoles per square meter per second per Pascal)

Gas exchange parameters from A/C_i response curves were measured on fully expanded leaves (LPI 5). Effects measured on plants during the experiment (DOT 7, 21, and 28)

Mean values ($\pm$ standard errors) followed by the same letter within a parameter, in each DOT, are not significantly different (LSD, $P \leq 0.05$)

Table 4 Significance of Cd treatment (Cd), microbe inoculum (I) and day of experiment (DOT)

	Cd	I	DOT	Cd × I	Cd × DOT	I × DOT	Cd × I × DOT
Stem length	0.3NS	6.1**	9.4***	5.9**	0.0NS	0.3NS	0.9NS
Leaf number	0.3NS	39.0***	128.1***	6.5**	0.1NS	2.3*	0.6NS
F_o	1.6NS	5.9**	0.8NS	1.9NS	5.2**	3.5**	1.7NS
F_m	1.7NS	2.1NS	19.6***	0.3NS	0.3NS	0.4NS	0.2NS
F_v/F_m	0.0NS	0.6NS	0.9NS	1.4NS	0.6NS	1.4NS	0.4NS
V_{cmax}	2.9NS	0.8NS	2.9NS	3.8*	2.6NS	2.1NS	1.2NS
J_{max}	7.2*	3.4NS	10.2***	3.4*	6.2**	3.4*	1.1NS
TPU	2.2NS	4.8*	13.2***	4.5*	8.9***	1.4NS	1.1NS
R_{day}	12.4**	2.6NS	0.8NS	5.4**	7.8**	8.6***	4.4**
Γ_c	1.6NS	0.1NS	10.1***	5.1*	3.4*	2.5NS	3.8*
g_m	0.0NS	0.9NS	10.2***	0.2NS	1.3NS	0.2NS	1.9NS

Effects on stem length, leaf number, minimal fluorescence (F_o), maximal fluorescence (F_m), photochemical efficiency (F_v/F_m), maximum carboxylation rate (V_{cmax}), electron transport rate (J_{max}), triose phosphate utilization (TPU), day respiration (R_{day}), CO_2 compensation point (Γ_c), and mesophyll conductance (g_m)

NS not significant

F value and P level: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

little variation during DOT, whereas, Γ_c and g_m changed with DOT. A significant variability among treatments was observed in A_{sat} ($P = 0.04$) and g_s ($P = 0.04$) (Fig. 2). However, Cd treatment did not reduce A_{sat} , when considering inoculated and non-inoculated treatments separately (T1 vs. T2; T3 vs. T4; T5 vs. T6). High values of A_{sat} and g_s were observed in inoculated plants with Micosat F Fito®. Stomatal conductance was generally higher ($P = 0.04$) in inoculated plants at the end of the experiment, whereas no significant differences in C_i/C_a were observed among treatments.

Unstained preparations of the test microorganisms before and after metal treatment were subjected to SEM observation (Fig. 3), to further investigate and confirm the microbial localization on root tissues. Inoculated root tissues revealed a rather diffuse and homogenous microbial colonization. Internal and external characteristics of primary and secondary roots were not strongly affected by the Cd treatment, despite the consistent accumulation of the metal. Indeed, no tissue necrosis was observed in roots of I-214 after Cd treatment.

Dynamics of total and fluorescent bacterial populations were weekly monitored in the growth substrate for 28 days, starting from the addition of bacterial strains into the substrate. During the monitoring period and for each tested treatment, the bacterial populations remained stable, with a ratio around 100:1 between total bacterial and fluorescent populations. Specifically, the level of fluorescent populations was approximately 5.0 log colony forming unit per gram, whereas those of total bacterial populations were around 7.0 log colony forming unit per gram in both the treated and untreated substrate.

Discussion

Clone I-214 responded to concomitant Cd contamination (40 mg kg^{-1}) and microbial inoculation through controlling impairments of plant growth, gas exchange, and photosynthetic capacity, at least in the short term of the study. The biomass of roots, stems, and leaves in I-214 subjected to Cd treatment was similar in inoculated and control plants. The biomass of shoot tissues was somewhat balanced by root biomass, which is important in defining the potential uptake of heavy metals (Wang and Jia 2010). The root/shoot biomass ratio provided an indication of change in the overall fitness of I-214, comparing control plants (T1) with those inoculated with Micosat F Fito® (T6), in metal phytoextraction terms. The relatively higher root/shoot biomass ratio in T6 would suggest a positive role of microbial inoculation in exploring a larger fraction of soils, for the increasing proportion of root size. Considering that leaf biomass did not change significantly among Cd-treated and untreated plants, and that the highest Cd accumulation was in leaves of inoculated plants, it may be concluded that the extraction and translocation capacity of I-214 was increased by microbial inoculation.

Metal accumulation was quite low in relation to the high Cd concentration in the substrate, considering the short-term nature of the experiment, which lasted only 28 days, while poplars trees are expected to continue phytoremediation processes in successive years. However, the variability of Cd uptake and distribution in the various plant organs was affected by microbial inoculation. Indeed, Cd content was higher in roots inoculated with *P. fluorescens* than in those inoculated with Micosat F Fito®. Although the presence of the rhizosphere strain BT4 (*P. fluorescens*) favored Cd import into the

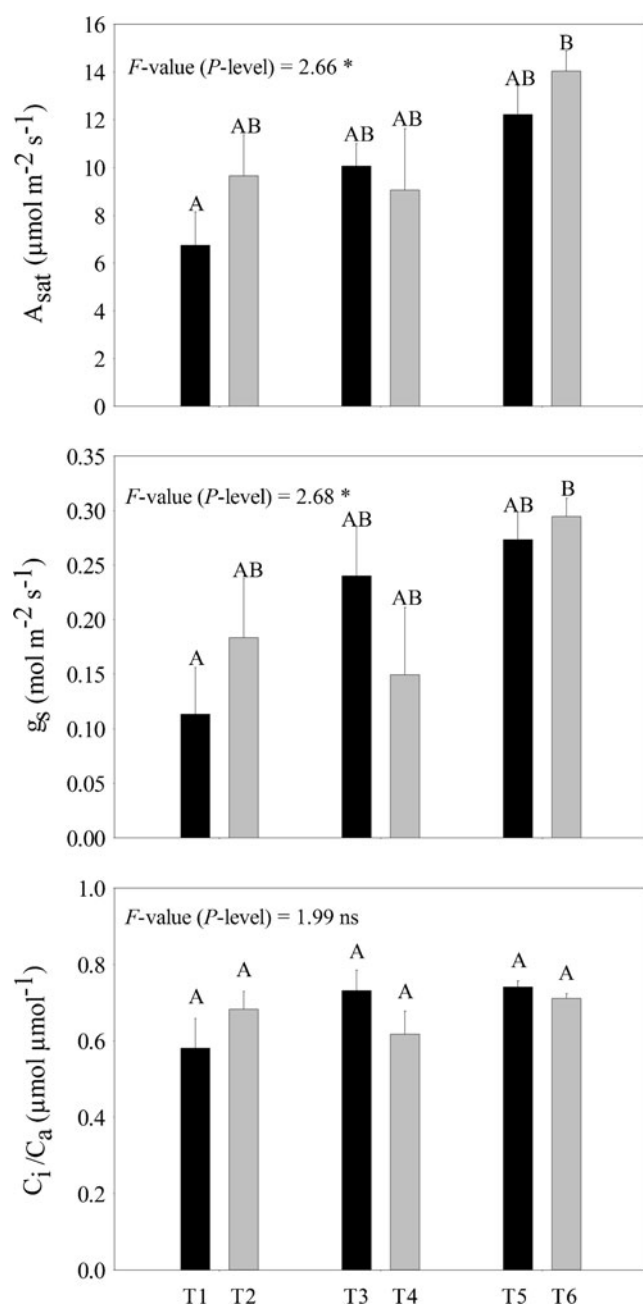


Fig. 2 Instantaneous gas exchange under saturating light conditions in poplar clone I-214, with or without root inoculation with *P. fluorescens* and Micosat F Fito®, and subjected to Cd treatment of 0 or 40 mg kg⁻¹, at the end of experiment (DOT 28). Mean values of histograms ($\pm$ standard errors) followed by the same letter within a parameter within DOT are not significantly different (LSD, $P \leq 0.01$). Significance of treatment effect (ANOVA) for each variable is indicated by *F* value and *P* level: * $P < 0.05$; NS not significant

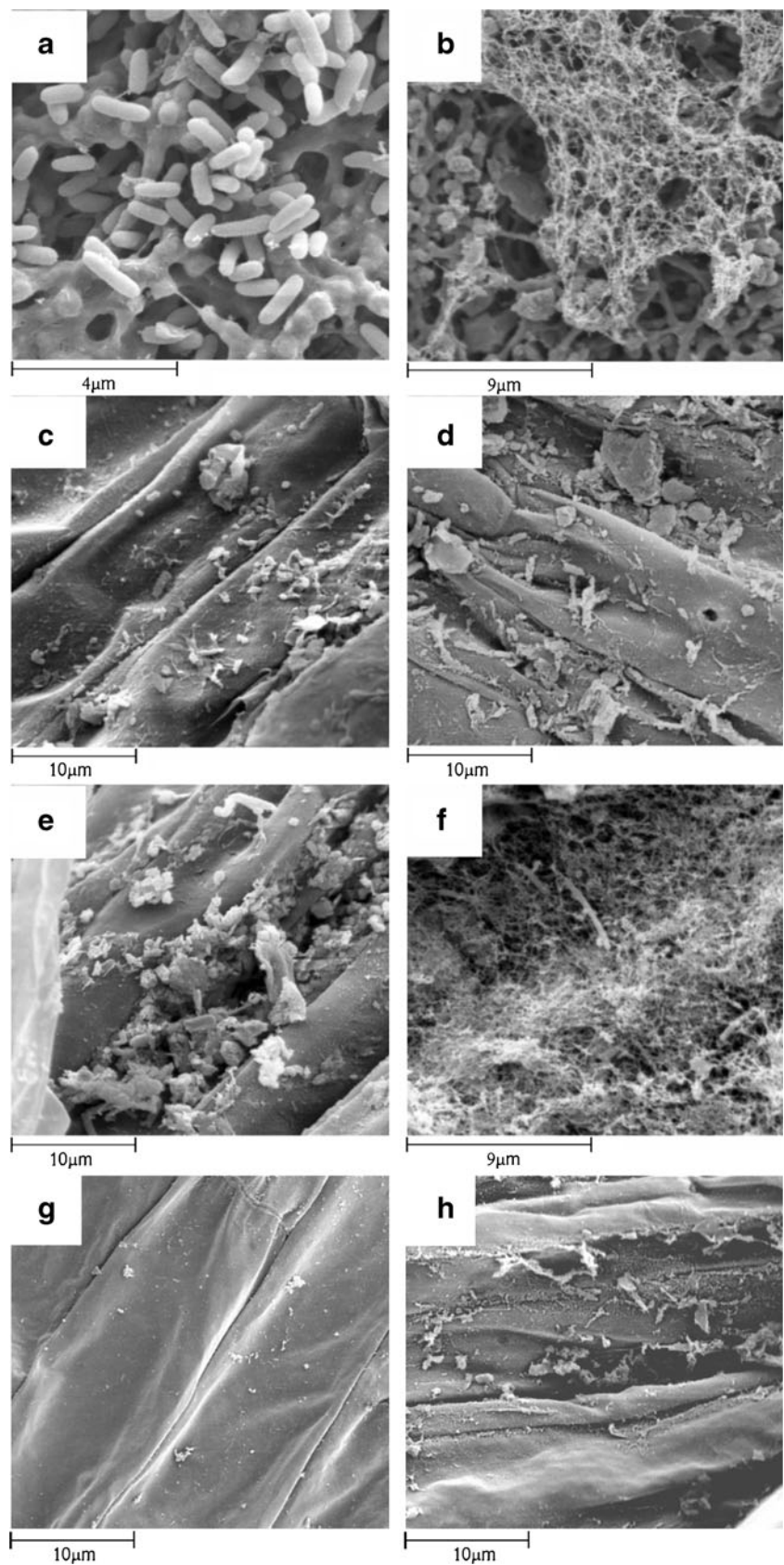
root of I-214, where it accumulated preferentially, it did not affect the amount of heavy metal transferred to shoots. Micosat F Fito® was particularly effective in mobilizing the metal toward the shoot. In particular, BCF values confirmed that Micosat F Fito® was the most efficacious in bio-accumulating Cd in plant tissues. The preferential

accumulation of Cd in shoots of I-214 as enhanced by Micosat F Fito® was also illustrated, at least partly, by *Tf* values. Thus, microbial inoculation improved metal accumulation in I-214, and overall Cd tolerance, supporting the potential practice of this clone in early establishment of multipurpose tree plantations on contaminated sites. Evaluation of BCF and *Tf* confirms that Salicaceae has a considerable potentiality to remove Cd from contaminated sites that could be increased by improving the translocation of the metal from roots to shoots (Zacchini et al. 2009; Wu et al. 2010). The role that bacteria, eventually entered the plant interior, may have in inducing the expression of heavy metal transporters needs further investigations.

These results confirm previous observations of the role of microbial communities on Cd accumulation in plant tissues (Cicatelli et al. 2010), supporting growth of the accumulating biomass. Positive interactions occurring in the plant–microbe system improved plant tolerance or resistance, and uptake potential of Cd (Cicatelli et al. 2012; Lingua et al. 2008), which may be conveniently exploited to increase the phytoremediation of environmental contamination (De Maria et al. 2011). Babu et al. (2013) showed that inoculating *Alnus firma* Siebold & Zucc. with *Bacillus thuringiensis* GDB-1 alleviated the metabolic perturbations and stress induced by high concentrations of heavy metals and enhanced biomass and metal accumulation by the plant. Compiling the present observations and the results of other studies, microbial-assisted phytoremediation of heavy metals by fast growing tree species is a promising tool at contaminated sites.

Cadmium contamination did not affect the photosynthetic apparatus significantly, and plants maintained the assimilation potential throughout the experimental period. By contrast, Pietrini et al. (2010b) observed a significant decrease in photosynthetic capacity in Salicaceae with acute Cd stress. Again, detrimental effects of excess Cd on instantaneous gas exchange of Salicaceae have been often found (Pietrini et al. 2010a, 2010b). Our results on instantaneous gas exchange measured at the end of experiment diverge from these findings. Microbial inoculation had positive effects on photosynthetic (A_{sat}) and transpiration (g_s) parameters, suggesting that sequestration mechanism was also strongly supported by effective protection. It may be hypothesized that the metal amount absorbed by I-214 was probably compartmented in structures, such as the vacuole or trichomes and veins (Vollenweider et al. 2006), and turned not harmful, thus providing a powerful protection of the photosynthetic machine. Maintenance of C_i/C_a ratio across treatments would indicate stable instantaneous water use efficiency and, therefore, an efficient regulation capacity of water balance and stomatal water-vapor diffusion (g_s). An explanation of the high capacity of I-214 to tolerate Cd in leaves could be that the main sinks in this plant compartment were not in the mesophyll but

Fig. 3 Scanning electron micrographs of poplar clone I-214 roots: micro-colonies of *P. fluorescens* strain BT4 (a) and microbial consortia Micosat F Fito® (b) on paper filter in water-nutrient medium suspension; micro-colonies of BT4 (c) and Micosat F Fito® (d) on roots after 2 h of inoculation in water medium suspension; micro-colonies of BT4 (e) and Micosat F Fito® (f) on poplar roots after 4 weeks of Cd treatment; uninoculated roots before (g) and after (h) 4 weeks of Cd treatment



in the veins (cf., Gilmore et al. 1996), as long as the storage capacity for this metal remained under control. Indeed, the photosynthetic capacity (V_{cmax} , J_{max} , TPU) and internal CO_2 diffusion (g_m) were only marginally affected. In addition, photosynthetic potentials were not limited by high R_{day} (the release of CO_2 in the light by processes other than photorespiration) or Γ_c (photorespiration) in Cd-treated plants. In a recent study, Castagna et al. (2013) found that the combination of Cd and ozone stress, which share some common toxicity pathways, induced responses in I-214 that are not always predictable on the basis of the known effects induced by the single factors. More specifically, Cd was effective in reducing g_s and in promoting H_2O_2 and NO accumulation and in decreasing carotenoid content; ozone exposure, on the other hand, induced a significant increase in the xanthophyll de-epoxidation state.

In the present study, the photochemical efficiency was not affected by Cd addition. The F_v/F_m ratio depends on the variations of F_o and F_m . In particular, F_o increases when the photochemical apparatus is damaged or, more specifically, when the number of functional chlorophylls are not connected to the reaction centers of PSII increases (Gilmore et al. 1996). In I-214, Cd addition significantly increased F_o . The decrease of F_o is, on the contrary, an indication of high-energy dissipation in the minor antenna. However, adjustments in F_m proportional to F_o would explain the maintenance of optimal F_v/F_m ratio, indicating balanced damage, or close regulation of, energy harvesting and energy conversion capacities (Pietrini et al. 2010b). Pietrini et al. (2010b) observed that Cd addition did not cause a reduction in F_v/F_m in poplar and willow clones, while a reduction in photosynthetic electron transport was induced.

Visual symptoms of Cd phytotoxicity were not observed in roots, where the metal was consistently accumulated without affecting tissue integrity. Coccozza et al. (2011) found high metal contents in root epidermis, suggesting slow or reduced metal mobility towards the central cylinder in I-214 under excess Cd. The central cylinder was relatively preserved from Cd accumulation, identifying an anatomical barrier to radial mobility of the metal. On the other hand, Cd localization in root epidermis would indicate preferential sites for metal–microbe interaction. Other than exclusion strategy, detoxification by bacteria would represent an important factor in metal tolerance, particularly considering the toxicity effects of Cd on plant mineral metabolism (Coccozza et al. 2008). The scanning electron microscope imaging showed the ability of the chosen microbial community to colonize poplar roots, which poses the basis for exploiting complementary traits of different plant–microbe combinations for metal tolerance. Considering that plants subjected to long-term Cd treatment may show alterations of the root structure, which would influence redistribution and

accumulation of the metal in different root tissues and/or cell compartments (Stoláriková et al. 2012), investigations of root morphological variation (e.g., vessel lumina, wall strength, and effects of Cd on plant transport system) in response to metal-induced stress in poplar clones warrants further investigations.

Conclusion

Results showed that I-214 associated with *P. fluorescens* (strain BT4) and the microbial consortia Micosat F Fito®, in a Cd-polluted substrate (i.e., 40 mg kg^{-1}), might be an efficient system to preliminary test phytoextraction strategies involved in early plant establishment on artificially contaminated soil. Significant depression in plant biomass, photosynthetic capacity, and gas exchange were not observed, highlighting early tolerance to Cd stress. Most likely, microbial inoculation of plant roots played a practical role in improving absorption and translocation mechanisms under excess Cd. The relatively high Cd contents measured in stem and leaves of inoculated plants would suggest improved efficiency in metal uptake and translocation through plant compartments (from roots to shoots), especially with Micosat F Fito®. The role of plant–microbe interaction in phytoremediation of metal-contaminated sites warrants further investigations under varying environmental conditions and heavy metals to elucidate the combined mechanisms underlying metal tolerance. If these results will be confirmed in long-term field experiments and with other poplar clones, inoculation with *P. fluorescens* (strain BT4) could become a promising strategy for phytostabilization of metal-contaminated sites, while Micosat F Fito® could enhance the capacity of Cd phytoextraction.

Acknowledgments We acknowledge: the Project “Molecular, physiological, and agronomic analyzes for selecting and managing *Salicaceae* in phytoremediation” (MIUR, PRIN 2008) for financial support; Prof. Arturo Alvino (University of Molise) for providing the photosynthesis system; Annachiara Di Niro and Carla Maglieri for help with measurements; *Osservatorio regionale sulla qualità degli alimenti di origine vegetale* of ARPA Molise (Environmental Protection Regional Agency) for Cd determination; CCS Aosta srl for supplying the Micosat F Fito®.

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