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RESEARCH ARTICLE

Effect of cadmium stress and inoculation with a heavy-metal-resistant bacterium on the growth and enzyme activity of *Sorghum bicolor*

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Abstract In this study, the influence of the heavy-metal-resistant rhizobacterial inoculant *Rhodococcus ruber* N7 on the growth and enzyme activity of *Sorghum bicolor* (L.) Moench. under cadmium stress was investigated in quartz sand pot experiments. The effect of cadmium and bacterium on the plant biomass accumulation, photosynthetic pigments, protein content, and the activities of plant-tissue enzymes such as peroxidase, laccase, and tyrosinase were estimated. It was shown that the presence of cadmium in the sand influenced the roots to a greater extent than it influenced the aerial parts of sorghum. This is manifested as increased protein content, reduced activity of peroxidase, and increased activity of laccase. Compared with cadmium stress, inoculation of plants with rhizobacterium *R. ruber* N7 has a stronger (and often opposite) effect on the biochemical parameters of sorghum, including a decrease in the concentration of protein in the plant, but increased the activity of peroxidase, laccase, and tyrosinase. Under cadmium contamination of sand, *R. ruber* N7 successfully colonizes the roots of *Sorghum bicolor*, survives in its root zone, and contributes to the accumulation of the metal in the plant roots, thereby reducing the concentration of the pollutant in the environment.

Keywords Cadmium · *Sorghum bicolor* · *Rhodococcus ruber* · Peroxidase · Laccase

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Introduction

Environmental pollution by heavy metals has become a global problem, with the ever-increasing intake of organic and inorganic compounds from anthropogenic flows worldwide. Accumulation of heavy metals in living organisms and their transfer through the food chains pose a threat to human health because of the heavy metal toxicity associated with violation of the biochemical processes in the plant and animal organisms.

Among heavy metals, cadmium is the most toxic because of its high mobility and ability to bioaccumulate. The World Health Organization has classified cadmium as one of the most dangerous to human health, and its content in the environment is governed by the lowest maximum permissible concentration (Hygienic Standards GN 2.1.7.020-94). The widespread use of cadmium in industrial production (nuclear power, electroplating, battery manufacture, etc.) and its distribution in fuels, ore dumps, and fertilizers are responsible for the increasing concentration of this metal in the environment. In this regard, control over the cadmium content in natural and economic facilities and soil and water purification from its compounds are topical environmental issues.

Currently, phytoremediation, i.e., the use of plants for phytosequestration, phytoextraction, and phytoaccumulation of inorganic pollutants, is considered the most promising technology for cleaning up the soil from heavy metals, cadmium in particular. There are a number of reports on metal-accumulating plants used to remove toxic metals from soil (Glick 2003; McGrath and Zhao 2003; Nunes da Silva et al. 2014).

The ability of plants to resist and grow under pollution stress is a key issue in their successful application for phytoremediation. The mechanisms of plant tolerance to heavy metals can be divided into avoidance strategies, leading

to limitation of cadmium uptake, and tolerance strategies, including accumulation and storage of cadmium by binding it to amino acids, proteins, and peptides (Tran and Popova 2013). Other mechanisms that plants have developed to cope with cadmium damage are related to stress compounds such as salicylic acid, jasmonic acid, nitric oxide, ethylene, and hydrogen peroxide, which are induced by cadmium and are involved in cell response to cadmium toxicity. These compounds act as signaling molecules in the activation of enzymes such as peroxidase, laccase, and polyphenol oxidase (tyrosinase), involved in antioxidation and lignification as stress responses (Yang et al. 2007). Thus, the mechanism of cadmium tolerance in plants can include both antioxidant defense and/or hyperaccumulation defense (Tran and Popova 2013).

The success of phytoremediation may depend not only on the plant itself but also on the interaction of the plant roots with bacteria and the concentrations of heavy metals in the soil. Certain heavy-metal-resistant bacteria have an exceptional ability to promote the growth of host plants by various mechanisms including nitrogen fixation; solubilization of minerals; transformation of nutrient elements; and production of phytohormones, siderophores, and specific enzymes (Glick 2003). Therefore, improvement of the interactions between plants and beneficial rhizosphere microbes can enhance biomass production and tolerance of plants to heavy metals (Glick 2003). In studying rhizosphere bacteria, Wang et al. (2000) found that in the plant root zone, microorganisms are able to accept and express genes from soil bacteria from contaminated sites that code for resistance to heavy metals. This makes topical the use of microorganisms markedly resistant to metal pollutants for the inoculation of plants to remediate heavy-metal-contaminated soils.

Although many metal-resistant bacteria, belonging to genera such as *Pseudomonas*, *Mycobacterium*, *Alcaligenes*, *Bacillus*, and *Rhizobium*, potentially can promote plant growth and reduce stress symptoms in plants (Dell'Amico et al. 2008; Mohamed and Gomaa 2012; Wani and Khan 2013), only a few attempts have been made to study the influence of nonphytopathogenic *Rhodococcus* plant tolerance to and uptake of heavy metals (Belimov et al. 2005; Trivedi et al. 2007).

In this study, we investigate sorghum [*Sorghum bicolor* L. (Moench.)], a heat-loving, drought-resistant, salt-tolerant crop that has universal use in agriculture and industry. This plant is also widely used in experiments on the phytoremediation of heavy metal contamination of soil (Marchiol et al. 2007; Soudek et al. 2014; Zhuang et al. 2009). The objective of our research was to elucidate the influence of the cadmium-resistant bacterial inoculant *Rhodococcus ruber* on the growth and activity of some antioxidative and lignification enzymes of sorghum under cadmium stress.

Materials and methods

Plant and cultivation

Seeds of sorghum [*Sorghum bicolor* (L.) Moench] were obtained from the Scientific Research Institute of Agriculture in the South-East (Saratov, Russia).

Calibrated seeds were sown in 1-L pots filled with 1 kg of quartz sand (particle size of 1–2 mm) uncontaminated and contaminated with cadmium. Cadmium-contaminated sand was prepared by treatment with a water solution of $CdC1_2 \times 2.5H_2O$ (concentration of 15.2 mg kg^{-1}, corresponding to a level 15-fold higher than the maximum permissible level for sand (Hygienic Standards GN 2.1.7.020-94). The control substrate was treated with water of equal volume. Plants were cultivated in a growth chamber with a 14/10-h day/night regime (light intensity 8000 lx, temperature of 24/20 °C, relative humidity of 70 %) for 7 weeks. The water content of the quartz sand was maintained at 80 % of the maximum water-holding capacity by adding deionized water or Ruakura nutrient solution according to Smith et al. (1983) with modifications. The salts used to make up the Ruakura solutions were as follows: macronutrient stock solution A (g L^{-1}): Mg $(NO_3)_2 \cdot 6\ H_2O$, 4.94; Ca $(NO_3)_2 \cdot 4\ H_2O$, 16.78; NH_4NO_3, 8.48; KNO_3, 2.28; macronutrient stock solution B (g L^{-1}): KH_2PO_4, 2.67; K_2HPO_4, 1.64; K_2SO_4, 6.62; Na_2SO_4, 0.60; NaCl, 0.33; and micronutrient supplement (mg L^{-1}): H_3BO_4, 128.80; $CuCl_2 \cdot 2\ H_2O$, 4.48; $MnCl_2 \cdot 4\ H_2O$, 81.10; $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, 0.83; $ZnCl_2$, 23.45. The original constituent, iron citrate pentahydrate (809.84 mg L^{-1}), was replaced by an equimolar amount of $FeCl_3 \cdot 6\ H_2O$ (2.18 g L^{-1}). The dilute solution applied to the plants was prepared by mixing 200 mL of each macronutrient stock solution with 100 mL of the micronutrient supplement and by diluting the mixture to 4.5 L with deionized water (pH 6.0).

Microorganism and inoculation

R. ruber strain N7 (IBPPM 496) from the Collection of Rhizosphere Microorganisms (IBPPM RAS, Saratov, Russia) was used in this study. This microorganism was isolated as a nickel-resistant strain on Tris medium containing 1 mM $NiCl_2$ from the rhizosphere of *Phragmites australis* L. grown in hydrocarbon-contaminated soil (Bondarenkova 2009). A study of heavy metal resistance of this microorganisms had revealed that *R. ruber* N7 can grow with 0.57 mg mL^{-1} of Ni^{2+}, 0.33 mg mL^{-1} of Pb^{2+}, 0.38 mg mL^{-1} of As^{3+}, and 0.13 mg mL^{-1} of Cd^{2+} (data not shown).

In this study, the minimal inhibitory concentration (MIC; the lowest concentration of metal ions at which no growth of the microorganism was observed) of cadmium was determined for *R. ruber* N7. The microorganism was cultivated in

test tubes with 5 mL of Tris medium (Mergeay et al. 1985) containing 0.2 to 2.0 mM ion concentration for cadmium (as $CdCl_2$). The growth medium was inoculated with a fresh microbial culture grown in LB agar medium to an initial absorbance of the culture medium about of 0.2 at 600 nm. Incubation was performed at 29 °C for 5 days. Growth was monitored turbidimetrically at an absorbance of 600 nm.

The plant-growth-promoting properties of *R. ruber* N7 were revealed by determining phosphate-solubilizing activity, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, and production of siderophores and the phytohormone indole-3-acetic acid (IAA). Phosphate-solubilizing activity was determined with Muromtsev's agar medium (Muromtsev 1957) amended with tricalcium phosphate. The appearance of clear zones caused by dilution of phosphate around the strain's colonies was indicative of phosphate-solubilizing activity of *R. ruber* N7. Siderophore production was detected by the universal method of Schwyn and Neilands (1987). The ACC deaminase activity was determined by colorimetric monitoring of the amount of α-ketobutyrate generated by enzymatic hydrolysis of ACC, as described by Belimov et al. (2005). IAA production was measured according to Golubev et al. (2009). Briefly, the culture was grown in an L-tryptophan-containing liquid medium at 29–30 °C for 72 h, after which IAA content in the cultural liquid was measured by HPLC.

Inoculation of 4-day-old sorghum seedlings with *R. ruber* N7 was performed by watering them with a cell suspension in Ruakura's solution to a final concentration in the sand of 10^{-6} CFU g^{-1}. For preparation of microbial suspensions, a 2-day culture grown on LB agar was washed with phosphate buffer (pH 7.2) and resuspended in the medium used for plant watering. In the control treatment, the seedlings were treated with the same volume of Ruakura's solution without microbial cells.

The number of *R. ruber* N7 cells in the sand and in the zone of the plant root was determined by plating serial dilutions on Tris medium containing 1 mM nickel. Plates were incubated at 30 °C for 7 days, after which, red colonies morphologically similar to colonies of *R. ruber* were counted. With account taken of the selective conditions for isolation of cadmium-resistant microorganisms and the appearance of characteristic colonies from the inoculated samples, only the isolated microorganisms were considered reisolates of *R. ruber* N7.

Analyses of plants

At the end of cultivation, the plants were lifted out of the pots and the soil was shaken off the roots. The roots were separated from the shoots, transferred into a beaker, and washed carefully with tap water. After that, both tissues were dried at 70 °C until dry weight constancy.

For determining the content of chlorophylls *a* and *b* (Chl *a* and Chl *b*), a weighed leaf sample (20–30 mg) was crushed in 96 % ethanol with quartz sand and $CaCO_3$. The homogenate was centrifuged at 4000 rpm for 10 min, the supernatant liquid was collected, and the volume of the extract was adjusted to 5 mL. Absorbance spectra were measured over the range 400–700 nm by using Evolution 60 spectrometer (Thermo Scientific, USA). The concentrations of Chl *a*, Chl *b*, and carotenoids were quantified by using the absorbance at 648, 665, and 440.5 nm and calculation from the equations according to von Wettstein (1957).

Measurement of enzymatic activities

Shoot and root samples (0.2 g) were ground in a mortar with quartz sand and were suspended in 2 mL of 0.1 M Na/K-phosphate buffer (pH 6.0). The homogenate was centrifuged (5000 g for 10 min), and the supernatant liquid was filtered (0.2 μm) and dialyzed for 24 h against 2 l of distilled water. Protein content in the root and shoot extract samples was determined according to Bradford (1976).

The activities of oxidase, peroxidase, and tyrosinase in the root and shoot extracts were measured spectrophotometrically with Evolution 60 spectrometer (Thermo Scientific, USA). The final volume of 2.0 mL of the reaction mixture contained 100 μL of enzyme extract diluted in an appropriate buffer and added with the substrate of the enzyme studied.

Oxidase activity in the plant root and shoot extracts was measured with 1 mmol L^{-1} 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) (Niku-Paavola et al. 1988), 30 μmol L^{-1} 4-hydroxy-3,5-dimethoxybenzaldehyde azine (syringaldazine (SGZ)) (Leonowicz and Grzywnowicz 1981), and with 23 μmol L^{-1} 2,7-diaminofluorene (2,7-DAF) (Criquet et al. 2000), by using 50 mmol L^{-1} sodium tartrate buffer (pH 3.5) and 50 mmol L^{-1} Na/K-phosphate buffer (pH 6.0). The oxidation rates of the substrates were monitored at λ=436, 525, and 600 nm, respectively, for the three substrates.

The activity of peroxidase was measured by monitoring the oxidation rate of the same substrates at the same pH values and with 0.3 mmol L^{-1} *o*-dianisidine (DAZ) in 50 mmol L^{-1} Na/K-phosphate buffer (pH 6.0) at 460 nm in the presence of 0.1 mmol L^{-1} H_2O_2.

The activity of tyrosinase was measured with 4 mmol L^{-1} 3,4-dihydroxy-DL-phenylalanine (DL-DOPA) and 50 mmol L^{-1} Tris HCl buffer at pH 7.5 (Criquet et al. 2000). The oxidation rate of the substrate was monitored at 475 nm.

All the activities are expressed in enzyme units (U) defined as micromole oxidized substrate per minute and per milligram protein.

Cadmium

Cadmium content in sand and in different plant tissues was determined by atomic absorption spectrophotometry. Samples were digested in a MARS Xpress high-pressure microwave system (USA) in Teflon vessels by using concentrated HNO_3 (and 30 % H_2O_2 solution, only for plant tissues) with constant control of temperature and analyzed with an atomic absorption spectrometer iCE3500 (Thermo Scientific, USA).

The transfer of cadmium from spiked sand to plant was measured by calculating the bioconcentration factor (BCF). According to McGrath and Zhao (2003), the BCF is defined as the ratio of metal concentration in plant shoots to metal concentration in soil. To assess the ability of *Sorghum bicolor* to translocate cadmium from roots to shoots, we calculated the translocation factor (TF). According to Ruiz et al. (2009), the TF is the ratio of metal concentration in plant shoots to metal concentration in roots.

Data analysis

Three replicates of each treatment were included in all pot experiments. In vitro experiments were conducted in three replicates. All samples were analyzed in three replicates. Analysis of variance (ANOVA) was used to estimate statistically significant differences between groups of samples. The means were compared by using standard deviations, confidence intervals, and LSD Fisher's test (p=0.05). Statistica software version 7 (StatSoft Russia) and Microsoft Excel 2003 were used for statistical analysis.

Results

Ecological characteristics of *R. ruber* N7

A study of metal resistance of *R. ruber* N7 showed that the lowest concentration of cadmium inhibiting bacterial growth was 1.2 mM.

A study of the plant growth-promoting potential of *R. ruber* N7 allowed us to reveal ACC deaminase activity that corresponded to 0.50±0.01 μmol α-KB mg^{-1} h^{-1}. It was established that cadmium at 0.2 mM did not inhibit the production of ACC deaminase but rather increased it up to 0.56± 0.02 μmol α-KB mg^{-1} h^{-1}. This strain was also siderophore and weak IAA producer but could not solubilize a calcium phosphate.

Effect of cadmium and *R. ruber* N7 on plant growth and biomass

Neither rhizobacterial inoculum nor cadmium had any marked effect on the germination and germination rate of sorghum either in untreated or in cadmium-contaminated soil (data not shown). This is because at the seed stage, many plants are resistant to toxicants in the environment owing low permeability rind seeds for many pollutants, including heavy metals.

The accumulation of biomass is an integral characteristic of plant growth and development. In exploring the effect of the bacterial inoculum and cadmium on the growth of sorghum, we found that *R. ruber* N7 significantly increased sorghum shoot dry weight (by 37 %) in the absence but not in the presence of cadmium (Fig. 1).

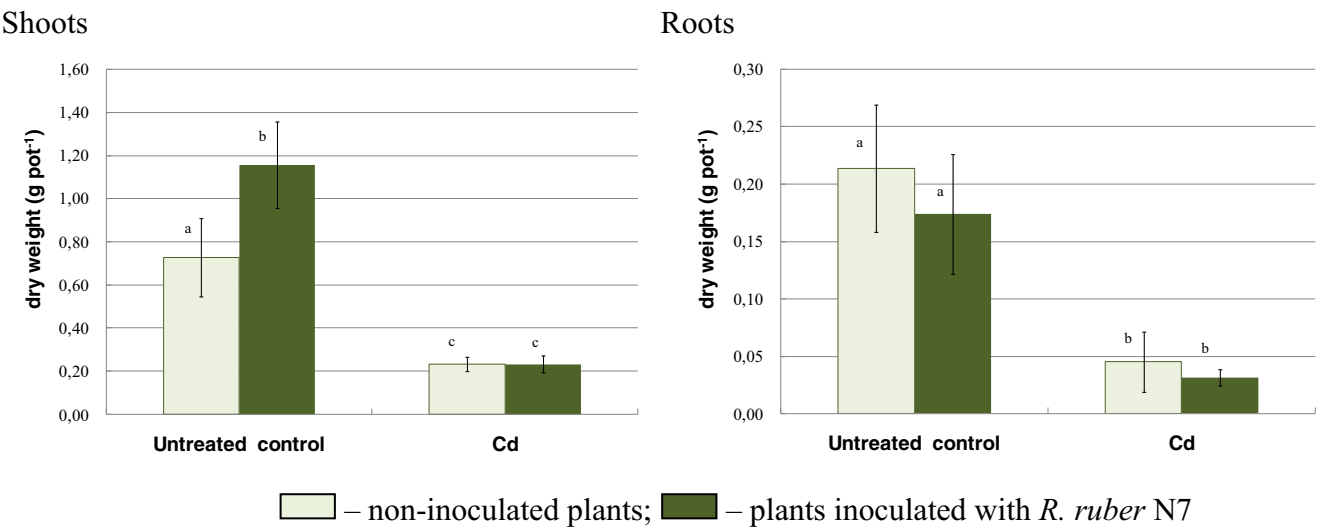

Fig. 1 Effect of cadmium and *R. ruber* N7 on the shoot and root dry weights of *Sorghum bicolor* grown in Cd-treated and Cd-untreated sand. The *error bars* indicate the standard deviation of the means (n=3), and the *same letters* mean no significant difference ($p \leq 0.05$)

Withering of sorghum leaves was recorded for all plants grown in cadmium-contaminated sand. Cadmium decreased the accumulation of aboveground biomass both in inoculated and in non-inoculated sorghum by 82 and 68 %, respectively. The accumulation of belowground biomass was inhibited by the metal to a greater extent: by 88 and 84 % for inoculated and non-inoculated plants, respectively.

Effect of cadmium and *R. ruber* N7 on photosynthetic pigments of sorghum

Cadmium at the concentration used had no significant effects on the content of Chl *a* and Chl *b* and carotenoids in the leaves of non-inoculated sorghum plants (Fig. 2). In untreated sand, the inoculation had no effects on chlorophyll and carotenoid content either, but in cadmium-contaminated sand, *R. ruber*

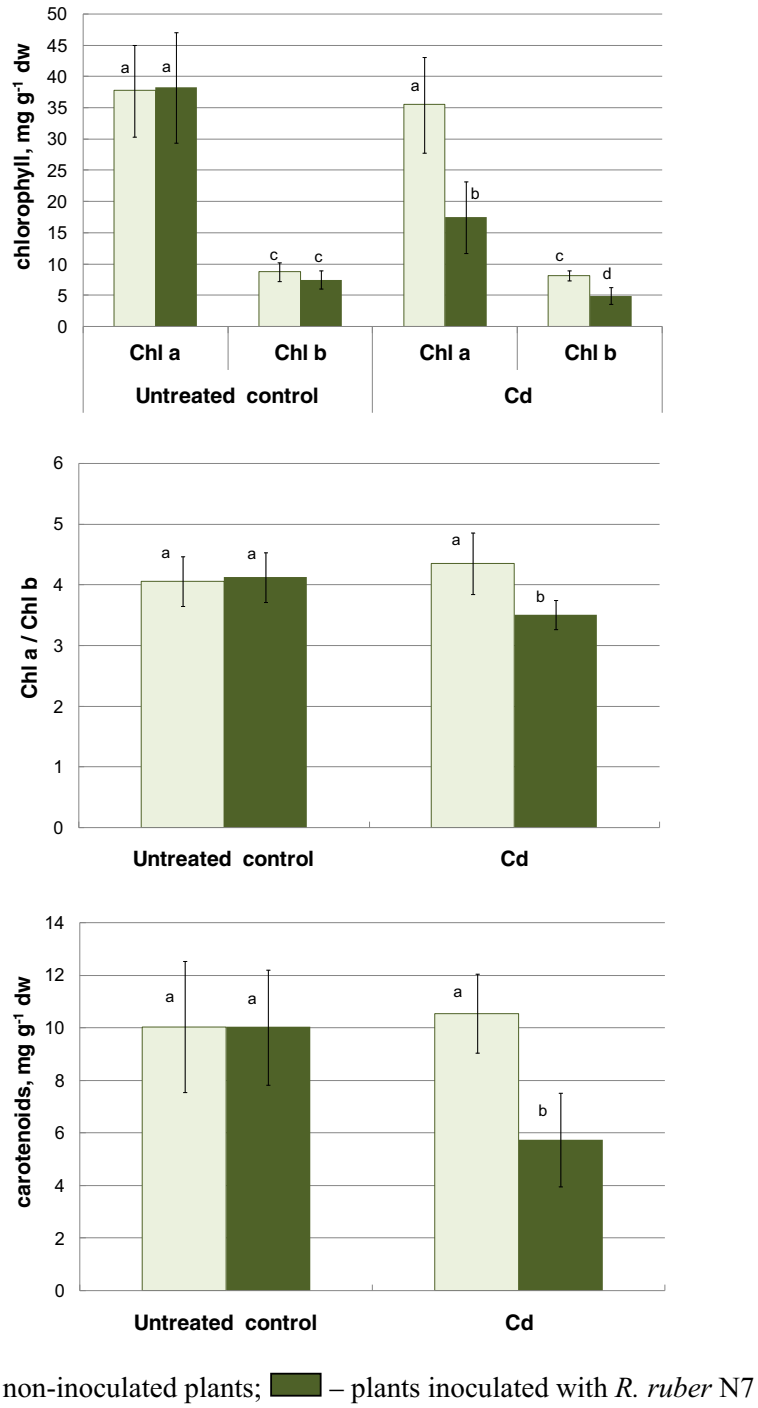

Fig. 2 Effect of cadmium and *R. ruber* N7 on chlorophyll and carotenoid content in leaves of *Sorghum bicolor* grown in Cd-treated and Cd-untreated sand. The *error bars* indicate the standard deviation of the means (n=6), and the *same letters* mean no significant difference (p<0.05)

N7 reduced Chl *a* and Chl *b* by 54 and 34 %, respectively, and carotenoids by 43 %. This effect of the microbial inoculant in cadmium-contaminated sand was reflected in the Chl *a*/Chl *b* ratio, which decreased by 15 % in comparison with non-inoculated plants grown both in untreated and in cadmium-contaminated sand and with inoculated plants grown in untreated control pots.

Protein content in sorghum biomass

Under the influence of cadmium, protein content was not altered in shoots, but significantly increased (by 61 %) in roots of non-inoculated plants (Fig. 3).

Inoculation had a more distinct effect on protein content in sorghum plants. Under the influence of *R. ruber* N7, the protein concentration in the leaves and roots of inoculated plants grown in untreated sand decreased by more than two times and, in cadmium-contaminated sand, by three and nine times, respectively.

Peroxidase activity in sorghum tissues

According to the data obtained, cadmium inhibited the peroxidase activity in the green parts of the sorghum plants (Table 1). Under the influence of cadmium, the ABTS and DAZ oxidation rates decreased by three and six times, respectively. Inoculation of plants with *R. ruber* N7 did not affect peroxidase activity in the aboveground biomass of sorghum grown in untreated control sand but increased it by three times for all test substrates when plants were grown in the presence of cadmium.

The root peroxidase activity was, on average, five times higher than that in shoots and more clearly reflected the effects of cadmium and the bacterial inoculant on the plant. Cadmium inhibited DAZ, DAF, and ABTS peroxidase activities by 9, 24, and 61 %, respectively. The inoculant increased root peroxidase activity by an average of 3 times (226, 334, and 293 % for DAZ, DAF, and ABTS, respectively) in sorghum grown in untreated sand and by an average of 12 times (1149, 1235, and 1202 % for DAZ, DAF, and ABTS, respectively) in sorghum grown in the presence of cadmium.

Oxidase activity in sorghum tissues

Oxidase activity in sorghum shoots and roots was determined with ABTS, DAF, SGZ, and L-DOPA as test substrates (Table 2). In sorghum shoots, ABTS oxidase activity was very low, but it was possible to observe an inhibiting effect of cadmium: the ABTS oxidation rate decreased by more than by 60 % in non-inoculated and rhodococcus-inoculated plants, respectively. The effect of the microorganism was revealed only with DAF in plants grown in the presence of cadmium: inoculation increased DAF oxidase activity in leaves by more than two times (156 %).

Sorghum root oxidase activity was much higher and was determined by using both ABTS and SGZ. As with the leaves, ABTS oxidase activity in non-inoculated plants was inhibited by cadmium by 2.5 times, but in *R. ruber*-inoculated plants, it was not changed significantly. The opposite trend was observed for SGZ oxidase. Under the influence of cadmium, the oxidation of SGZ increased by 56 and 147 % in non-inoculated and inoculated plants, respectively. Inoculation of plants increased

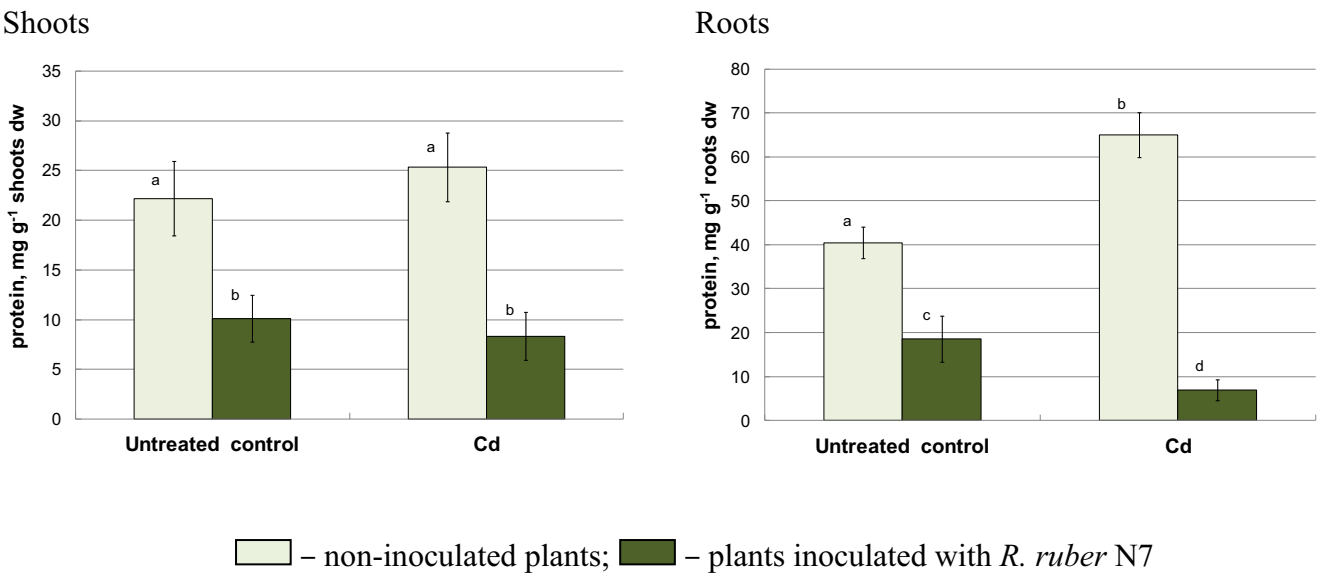

Fig. 3 Effect of cadmium and *R. ruber* N7 on protein content in sorghum shoots and roots. The *error bars* indicate the standard deviation of the means (n=6), and the *same letters* mean no significant difference (p<0.05)

Table 1 Effect of cadmium and *R. ruber* N7 on peroxidase activity in sorghum shoots and roots (mM min^{-1} mg^{-1} protein)

Test substrates	Untreated control		Cd	
	Noninoculated	+ *R. ruber* N7	Noninoculated	+ *R. ruber* N7
Shoots				
DAZ	2.467 a±1.243	2.397 a±1.070	0.389 b±0.239	1.354 a±0.359
DAF	0.970 a±0.535	1.042 a±0.486	0.635 a±0.392	1.605 b±0.091
ABTS	0.732 a±0.375	0.786 a±0.447	0.264 b±0.173	0.847 a±0.265
Roots				
DAZ	14.689 c±2.371	33.138 b±2.926	11.939 d±0.992	137.293 a±43.622
DAF	5.263 c±3.173	17.567 b±2.099	4.027 d±2.075	49.747 a±16.273
ABTS	3.334 c±1.677	9.770 b±0.066	1.311 d±0.603	15.768 a±3.835

The values are means±standard deviations (p=0.05) of at least three samples. Mean values followed by the same letters in the line are not significantly different at p=0.05

ABTS oxidase activity by 2.6 and 12 times (162 and 1133 %) and SGZ oxidase activity by 2 and 3 times (111 and 234 %) in untreated and cadmium-contaminated sand, respectively. We supposed that the oxidation of SGZ allowed us to reveal laccase activity in sorghum tissues.

Tyrosinase activity in sorghum tissues

Tyrosinase activity in sorghum roots and leaves was revealed by oxidation of L-DOPA as a test substrate. In non-inoculated plants, the activity of this enzyme was not changed under the influence of cadmium, whereas inoculation of sorghum with *R. ruber* had a significant effect on tyrosinase (Table 2). In the leaves, the effect of bacterization was evident only in cadmium-contaminated sand: tyrosinase activity increased 10-fold (by 944 %) in comparison with that in the uncontaminated plants. In the roots, under the influence of the bacterium, tyrosinase activity increased by 3 and 12 times (198 and 1126 %) in untreated and cadmium-contaminated sand, respectively.

Cadmium accumulation in sorghum tissues

The concentrations of Cd in sorghum plants are given in Fig. 4. As can be seen, cadmium taken up by sorghum was mostly concentrated in the roots (1003 and 1075 mg kg^{-1} for non-inoculated and inoculated plants, respectively). Inoculation of sorghum with *R. ruber* N7 significantly (p<0.1) increased cadmium content in roots but had no marked effects on metal accumulation in shoots.

In this study, the concentrations of Cd in shoots of inoculated and non-inoculated plants were 32.0 and 34.7 mg kg^{-1}, and the corresponding BCF values were 4.26 and 4.62, respectively. No significant effect of inoculation on cadmium accumulation by sorghum shoots was observed at p>0.05.

The TF values for inoculated and non-inoculated plants were 0.030 and 0.035, respectively. Inoculation of sorghum with *R. ruber* N7 had no significant effect on this parameter.

Survival of *R. ruber* N7

After 7 weeks of plant cultivation, a reisolation of *R. ruber* from the rhizosphere of sorghum was successful with a high

Table 2 Effect of cadmium and *R. ruber* N7 on oxidase activity in sorghum shoots and roots (mM min^{-1} mg^{-1} protein)

Test substrates	Untreated control		Cd	
	Noninoculated	+ *R. ruber* N7	Noninoculated	+ *R. ruber* N7
Shoots				
ABTS	0.008 a±0.004	0.009 a±0.005	0.003 b±0.002	0.003 b±0.002
DAF	0.033 ab±0.016	0.040 ab±0.014	0.023 a±0.010	0.059 b±0.023
L-DOPA	0.238 b±0.172	0.207 b±0.119	0.135 b±0.086	1.410 a±1.815
Roots				
ABTS	0.008 b±0.004	0.021 a±0.005	0.003 c±0.001	0.037 a±0.026
SGZ	0.471 c±0.050	0.994 b±0.603	0.736 b±0.246	2.459 a±1.287
L-DOPA	0.384 c±0.135	1.144 b±0.537	0.345 c±0.127	4.291 a±1.553

The values are means±standard deviations (p=0.05) of at least three samples. Mean values followed by the same letters in the line are not significantly different at p=0.05

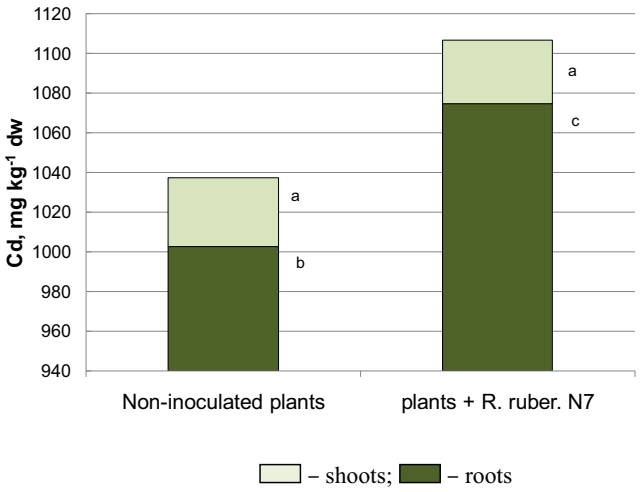

Fig. 4 Effect of inoculation of sorghum with *R. ruber* N7 on the cadmium concentration in the shoots and roots of plants grown in metal-contaminated sand. The *same letters* mean no significant difference ($p<0.05$; $n=9$)

degree of probability. On the basis of the ability of strain N7 to grow in the presence of 1 mM nickel and to form distinctive red colonies, the colonies that appeared on Tris medium with Ni after 5–7 days of incubation of rhizosphere samples were considered *R. ruber* N7. In the non-inoculated samples, no colonies similar to those of the original inoculant strain in their cultural–morphological characters were found.

According to the data obtained, the strain numbers were 4.1×10^4 cells per gram of sand in the sorghum rhizosphere without the pollutant and 2.6×10^5 cells per gram of sand in the cadmium-contaminated rhizosphere. This indicated the competitive advantage of *R. ruber* N7 in the metal-contaminated environment.

Discussion

Previous studies on the resistance and accumulation of cadmium by *Sorghum bicolor* plants demonstrated that sorghum is tolerant to cadmium at a concentration of 0.5 mM (Epelde et al. 2009; Kuriakose and Prasad 2008; Marchiol et al. 2007; Soudek et al. 2014). In our experiments on the cultivation of sorghum in quartz sand contaminated with 70 μM cadmium, we observed clearly the toxic effects of the heavy metal. The main symptoms of cadmium phytotoxicity are visually manifested through inhibition of plant growth, leaf chlorosis, and necrosis of aboveground and underground organs (Hernandez and Cooke 1997). In this study, accumulation of shoot and root biomass was reduced by more than 80 %, indicating a toxic effect of cadmium. Unfortunately, inoculation of sorghum with *R. ruber* N7 had no positive effect on plant biomass accumulation in cadmium-treated sand, although microbial stimulation of shoot biomass accumulation in uncontaminated sand was clearly observed.

In our study, it was found that neither cadmium nor bacterial inoculant separately had any effect on the photosynthetic apparatus of sorghum, whereas their combined effect led to a significant decrease in the content of photosynthetic pigments (chlorophylls *a* and *b* and carotenoids) in sorghum leaves. The effect of cadmium on photosynthesis is quite widely reported in the scientific literature (Prasad 1995; Tran and Popova 2013). It is known that cadmium destroys the structure and function of chloroplasts, as well as reduces the content and ratio of photosynthetic pigments as a consequence of inhibition of the biosynthesis and degradation of chlorophyll (Tran and Popova 2013; Zengin and Munzuroglu 2005). The inhibiting effect of heavy metals on carotenoids is less pronounced compared to that on chlorophylls (Prasad 1995). Decreases in the content of these pigments under the influence of cadmium were reported for *Artemisia annua* (Li et al. 2012) and *Helianthus annuus* (Saidi et al. 2014). At the same time, by using *Juncus maritimus* and *Phragmites australis* as examples, it was reported that the carotenoid content increased under cadmium stress (Nunes da Silva et al. 2014). The authors suggested that carotenoids might be involved in protective mechanisms to counteract the absorption and translocation of cadmium in the plant. Analysis of the reported data shows that the inhibiting effect of cadmium on photosynthetic pigments depends on the concentration of metal ions in the medium (Soudek et al. 2014; Wani and Khan 2013).

In contrast to heavy metal stress, less is known about the impact of microorganisms on plant photosynthetic pigments. It has been reported that the effect of microbial inoculants on the photosynthetic pigments of plants may be different for phytopathogenic and plant-growth-promoting microorganisms. Inoculation of plants with pathogenic microorganisms (e.g., fungus *Colletotrichum lindemuthianum* and the bacteria *Uromyces appendiculatus* and *Xanthomonas campestris*) was reported to decrease chlorophyll and carotenoid content in legumes (Berova et al. 2007; Lobato et al. 2009; Lopes and Berger 2001). In contrast, inoculation of stressed plants with plant-growth-promoting microorganisms, e.g., *Rhizobium* sp., *Bacillus subtilis*, and *Pseudomonas fluorescens*, resulted in increases in chlorophyll and carotenoid content (Dell'Amico et al. 2008; Mohamed and Gomaa 2012; Wani and Khan 2013).

In addition to the total chlorophyll content, the effect of cadmium on plants manifests itself in a change in the pigment ratio in leaves. It is believed that the change in the chlorophyll *a*/*b* ratio is an adaptive response of the assimilation apparatus of plants to stress: when the level of the main photosynthetic pigment chlorophyll *a* decreases, the auxiliary chlorophyll *b* converts to chlorophyll *a*, decreasing the concentration of chlorophyll *b* greater than the concentration of chlorophyll *a*, which ultimately increases the chlorophyll *a*/*b* ratio

(Breckle 1991; Prasad and Prasad 1987). Wan et al. (2012) reported that the photosynthetic pigment content in the leaves of *Solanum nigrum* grown under cadmium-contaminated conditions significantly increased from inoculation with *Serratia nematodiphila*. Soudek et al. (2014) showed an increase in Chl *a*/*b* in the leaves of *Sorghum bicolor* under cadmium stress. However, the increase in chlorophyll *a*/*b* ratio was statistically significant only for the cadmium concentration of 5000 μM, whereas at lower concentrations of the metal (10–2000 μM), no significant changes in this ratio compared to the control were detected, which was also shown in this study. In contrast to cadmium stress, in response to inoculation with plant-growth-promoting bacteria, the Chl *a*/*b* ratio decreased in the leaves of *Lolium multiflorum* and *Glycine max* inoculated with *Bradyrhizobium* sp., as reported by Guo and Chi (2014).

In our study, a combined effect of cadmium and a bacterial inoculant on sorghum plants, which manifested itself in a decrease in the photosynthetic pigment content in leaves, was observed. It is possible that the bacterial inoculant increased cadmium uptake by the plant, which was followed by an increase in the toxic effect of the metal on the photosynthetic apparatus of sorghum. This explanation may be supported by the data demonstrating an enhancement of cadmium accumulation by sorghum (Fig. 4). However, in this case, an increase in the Chl *a*/*b* ratio should have been expected but was not observed. That is why the induction of bacterial phytotoxicity by cadmium may also be considered as a possible explanation for this phenomenon. This may also explain the absence of stimulation of sorghum growth by *R. ruber* N7 in cadmium-treated sand. From this point of view, the physiological response of bacteria to cadmium stress should be deeply understood before any metal-resistant and/or plant-growth-promoting microbial strains can be recommended for use in inoculation of plants to remediate metal-contaminated soil.

Changes in protein abundance under stressful conditions can be molecular markers for the manifestation of plant response to stress (Tran and Popova 2013). It is known that in plants growing under cadmium stress, the content of some proteins, e.g., the heat shock protein (HSP70), and the metal-binding proteins phytochelatins is increased (Cobbett and Goldsbrough 2002). A protein designated as cadmium-stress-associated protein (CSAP) from wheat seedlings was purified and characterized by Mittra et al. (2008). In our study, protein content in sorghum tissues also increased markedly in sorghum roots and ended to increase in shoots under the influence of cadmium. The opposite effect was obtained for bacterization. Inoculation of sorghum with rhodococcus extremely reduced protein content in sorghum both in presence and in absence of cadmium in the sand. According to the data reported by other researchers, the effect of microbial inoculants on protein content in plants may be various and differed between bacterial species (strains) and between plant species. For example, Burd et al. (2000), in inoculating Indian mustard and canola plants with *Kluyvera ascorbata* SUD165, observed a decrease in protein content in the leaves of plants grown in artificial soil untreated and treated with lead. In contrast to this, Dell'Amico et al. (2008) showed that the concentration of protein increased in leaves of *Brassica napus* under the influence of inoculation with *Pseudomonas fluorescens* and *Pseudomonas tolasii* in the presence of cadmium in the growth medium.

From these results, it is interesting to note that the extreme decrease in plant protein content under the influence of bacterial inoculation was accompanied with an increase in the activity of antioxidative enzymes, especially in plant roots. These findings confirm the data by Siciliano et al. (1998) to show that microbial inoculation of plants stimulates enzymatic activity associated with pollutant degradation but has little effect on protein content.

Plant enzymes such as peroxidase, laccase, and tyrosinase, which are involved in antioxidation and lignification, change their activity in response to stress. Peroxidase is a plant-specific oxidoreductase with a wide range of catalytic activity, which participates in several physiological and biochemical processes in plants, including photosynthesis, respiration, protein metabolism, regulation of growth processes, detoxification of some free radicals, pathogen defense, and lignin and suberin formation. This enzyme is sensitive enough to external influence, which suggests the possibility that its activity could be used as a test to determine the status of plants (Chernavskaya 1988). In this study, peroxidase activity decreased in sorghum shoot and root tissues under the influence of cadmium stress. Similar results were obtained by Soudek et al. (2014) for 4-week-old sorghum cultivated hydroponically at 0–5000 μM cadmium. However, Yang et al. (2007) observed an increase in peroxidase activity when 5-day-old soybean seedlings were treated with $CdCl_2$ at 0.2–1 μM. In the last case, the authors observed a stress reaction of the plant to cadmium, whereas in our experiments with sorghum, we monitored peroxidase activity in the plant constitutively resistant to cadmium. At the same time, we observed a very strong reaction of sorghum to the inoculant: the increase in peroxidase activity in the plant roots was so large that it was possible to speak about biotic stress. This effect of *R. ruber* N7 was more impressive in cadmium-contaminated sand, which again attests to an increasing toxicity of the inoculant in the presence of the heavy metal. An approximately 30 % induction of peroxidase in chilli plants (*Capsicum annuum* L.) following seed bacterization with *Pseudomonas aeruginosa* was reported by Siddiqui and Meon (2009).

Under different stress conditions, an increase in enzyme activity can be caused by activation of latent form and (or) the synthesis of new enzyme molecules (Seregin and Ivanov 2001). In response to cadmium stress, production of phenols and thiols in plants is induced as a defense mechanism (Lavid

et al. 2001). In addition, the activation of lignin biosynthesis is a typical plant response both to biotic and to abiotic environmental stress (Yang et al. 2007). Cadmium intensifies lignification in plant tissues, which correlates with an increase in the activity of ligninolytic enzymes, including peroxidase and laccase (Yang et al. 2007). In our study, the oxidation of SGZ by root enzymes can presumably be characterized as a manifestation of the activity of laccase in the roots of sorghum. Studies of this enzyme in sorghum are extremely limited. There are a few reports of the presence of laccase in *Sorghum bicolor* (Dubrovskaya et al. 2014; Paterson et al. 2009; Gramene. *Sorghum bicolor* enzyme: laccase); however, we have not come across any experimental studies on the characterization and purification of sorghum laccase. Yang et al. (2007) showed a significant increase in the synthesis of laccase in *Glycine max* roots in response to the presence of cadmium in the environment. The authors suggested that the increased activity of this enzyme was associated with an early stage of polymerization of lignin in the soybean root tissues under the influence of cadmium. Laccase activity detected by us in sorghum roots (but not in shoots) was also significantly stimulated by cadmium, demonstrating a positive correlation with accumulation of the metal in the roots (R^2=0.50 and p<0.05 for non-inoculated plants; R^2=0.62 and p<0.05 for inoculated plants). In general, these observations are consistent with studies of laccases from white rot fungi. Stimulation of laccase activity by cadmium was reported by Baldrian and Gabriel (2002) by using the example of *Pleurotus ostreatus*. The authors showed that cadmium (2 mM) together with copper (1 mM) added to the culture medium stimulated the production and activity of laccase by five and eight times, respectively. It was concluded that the presence of heavy metals in the environment plays an important role in the regulation of extracellular enzymes. The involvement of polyphenols, polyphenol oxidase, and peroxidase activities in cadmium accumulation by plants was reported by Lavid et al. (2001). These authors demonstrated in vivo cadmium binding by polymerized phenols in the water plants *Nymphoides peltata* and *Nymphaea* and suggested that in these water plants, the main mechanism of cadmium accumulation is based on the trapping of cadmium crystals by polymerized phenols in specialized epidermal structures and that this is due to peroxidase and polyphenol oxidase activities.

In assessing the uptake of cadmium by sorghum plants, we found that the roots accumulated almost 30 times more cadmium than the aboveground biomass. Similar results were reported by Marchiol et al. (2007) and Soudek et al. (2014). Inoculation of sorghum with the cadmium-resistant bacterium *R. ruber* significantly increased the metal uptake by the roots but had no effect on metal accumulation in plant shoots. BCF and TF are key values needed to estimate the potential of a plant for phytoextraction and phytostabilization. Plants exhibiting a shoot BCF of >1 are suitable for phytoextraction, and plants with a root BCF of >1 and a TF of <1 have the potential for phytostabilization (McGrath and Zhao 2003; Ruiz et al. 2009). This study showed that both non-inoculated and inoculated sorghum plants had TFs of <1 and BCFs of >1 for cadmium. Therefore, we conclude that the practical use of *Sorghum bicolor* for the phytoremediation of cadmium-contaminated soil is mostly in the field of phytostabilization rather then phytoextraction.

Conclusions

In summary, it can be seen that the presence of cadmium in the sand influences the roots to a greater extent than it influences the aerial parts of *Sorghum bicolor*. This is manifested as increased protein content, reduced activity of peroxidases, and increased activity of laccases. Compared with cadmium stress, inoculation of plants with rhizobacterium *R. ruber* N7 has a stronger (and often opposite) effect on the biochemical parameters of sorghum, including a decrease in the concentration of protein in the plant, but increases the activity of peroxidase, laccase, and tyrosinase. The induction of bacterial phytotoxicity by cadmium is assumed. It can also be concluded that under cadmium contamination of sand, *R. ruber* N7 successfully colonizes the roots of sorghum, survives in its root zone, and contributes to the accumulation of the metal in the plant roots, thereby reducing the concentration of the pollutant in the environment. However, the physiological response of bacterial inoculant to cadmium stress should be deeply understood before any metal-resistant and/or plant-growth-promoting microbial strains can be recommended for use in inoculation of plants to remediate metal-contaminated soil.

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