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ANALYSIS OF MICROBIOLOGICALLY STIMULATED BIOMASS OF *SALIX VIMINALIS* L. IN THE PRESENCE OF Cd^{2+} UNDER *IN VITRO* CONDITIONS – IMPLICATIONS FOR PHYTOREMEDIATION

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The efficiency of phytoremediation might be highly affected by plant-associated microorganisms, and understanding of the underlying mechanisms is still a great challenge. The primary aim of this study was to evaluate the efficiency parameters for Cd^{2+} accumulation in the biomass of willow (*Salix viminalis*) as well as to define the biochemical response of the host plant when it is inoculated with selected bacterial strains (*Massilia* sp. and *Pseudomonas* sp.) or saprophytic fungus (*Clitocybe* sp.) under controlled *in vitro* conditions. Inoculation of plants with bacterial strains affected the efficiency of phytoremediation process and was expressed as the quantity of accumulated Cd (Q), the bioaccumulation factor (BCF) and the translocation index (Ti); however, the effect was strain and plant organ specific. The level of hydrogen peroxide (H_2O_2), which is both an indicator of plant response to biological and/or abiotic environmental stress and a molecule involved in plant-microbial interactions, decreased under the influence of Cd^{2+} in uninoculated plants (plant growth was inhibited by Cd^{2+}) and increased in the inoculated variants of plants growing in the presence of Cd^{2+} (microbiologically stimulated biomass). The saprophytic fungus *Clitocybe* sp. generally stimulated biomass and increased the level of H_2O_2 synthesis in all the investigated plant organs and variants of the experiment. We suggest that determination of phytoremediation efficiency, and biochemical response (H_2O_2) of the host plant under *in vitro* conditions can help in predicting the final effect of plant-microbial systems in further field trials.

Key words: willow, bioremediation, cadmium, hydrogen peroxide (H_2O_2)

INTRODUCTION

Increase in heavy metal-contaminated soils is considered one of the most serious threats to both the environment and human health (Yoon et al., 2006; Moosavi and Seghatoleslami, 2013). Among all heavy metals, cadmium (Cd^{2+}) deserves special attention and was considered by the Agency for Toxic Substances and Disease Registry (ATSDR) one of the most toxic substances that is present in the environment. Moreover, this metal is in 7th place on the list of hazardous substances (The Comprehensive Environmental Response, Compensation, and Liability Act – CERCLA; ATSDR, 2013), which comprises 275 compounds that are the most harmful to human health. Cadmium is a chemical element that shows high toxicity even at relatively low concentrations because of its high solubility in water (Pinto et. al., 2003), rapid bioaccumulation in the food chain and lack of any biological function (Gallego et al., 2012). Therefore, use of suitable recultivation technologies has been extensively studied over the last two decades. The most promising method is phytoextraction (one of the phytoremediation types), which is an effective, eco-friendly and profitable method for restoration of ecosystems contaminated

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primarily with heavy metals (Ali et al., 2013; Mani and Kumar, 2014; Padmavathiamma and Li, 2007; Sun et al., 2011). The costs of applying this technology are comparable to expenses incurred during farming (Graves, 2007) (ca. 5–40 US$/ton) and are several dozen times lower compared with conventional techniques (Glass, 1999). Phytoextraction is a process that consists in uptake of contaminants present in the soil or water through the plant root system as well as their translocation to the above ground parts (shoots and leaves), where they are accumulated (Rafati et al., 2011).

Phytoextraction efficiency of heavy metals is a result of synergic interactions between plants and their surrounding environment. Microorganisms play a special role in this system, and they significantly influence heavy metal uptake by plant roots (Bell et al., 2014). Microorganisms can enhance the efficiency of heavy metal accumulation during phytoextraction process by increasing their bioavailability in the soil environment due to, i.a., changes in the pH of the soil solution, which releases biosurfactants and metal-chelating substances (i.e. siderophores and low molecular weight organic acids), or through redox reactions (Rajkumar et al., 2012). Because success of phytoextraction process depends on a plant's tolerance to metal toxicity and its overall biomass growth, many researchers have focused on the microorganisms that promote plant growth through, inter alia, the following: (i) increased availability of nutrients and water, dissolution of sparingly soluble phosphorus and ferric sources (through a decreased pH and synthesis of siderophores); (ii) synthesis of phytohormones (auxins and gibberellins) that stimulate elongation and division of plant root cells; and (iii) decreased toxicity of heavy metals (through reduction of ethylene synthesis, chelation of heavy metals ions with organic acids and siderophores, and enhancement of antioxidative enzyme activity) (El Aafi et al., 2012; Kuffner et al., 2010; Ma et al., 2011). Indirect promotion of plant growth by microorganisms can play a key role in extraction and removal of trace elements from the soil because an increase in the biomass causes an increase in the total efficiency of phytoremediation process at the same time (Sessitsch et al., 2013). The microbial stimulation of plants growing in a Cd^{2+}-polluted environment, especially by rhizosphere bacteria and/or mycorrhizal fungi, was already confirmed by many experiments, e.g., Baum et al. (2006), Azcon et al. (2010), Garg and Aggarwal (2011) and Luo et al. (2011). Much less attention has been paid to the role of saprophytic fungi in the plant growth promotion process, especially in the presence of heavy metals (Babu et al., 2014). Studies conducted on *Paecilomyces lilacinus* NH1 and *Solanum nigrum* L. (Gao et al., 2010), as well as *Trichoderma* spp. and *Zea mays* (Babu et al., 2014), indicate the high potential of saprophytic fungi (especially those that originate from areas contaminated with heavy metals) for plant protection against Cd^{2+} toxicity and stimulation of Cd^{2+} accumulation in plant biomass. A key factor in evaluating the potential of selected plant-microorganism systems in phytoremediation treatments is to determine the efficiency of this process. To determine the efficiency of phytoextraction of plant-microorganism systems used in our *in vitro* experiment, we analysed the following two parameters: the bioconcentration factor, or BCF (the ratio of the metal concentration in the plant tissues to the metal concentration in the medium), and the translocation index, or Ti (the transfer efficiency of accumulated metal from the roots to the aboveground parts of the plant) (Ali et al., 2013). In the improved phytostabilization process, the values of both factors should be $<<1$, and during the increased efficiency of phytoextraction, both values should be $>>1$ (Peuke and Rennenberg, 2005; Mendez and Maier, 2008). Analysis of these parameters can be hampered in natural conditions, in which the chemical composition and sorption properties of soil can affect metal mobility and bioavailability (Kłos et al., 2012) and consequently influence the phytoextraction efficiency (Ali et al., 2013). Meharg (2005) suggests that absorption of heavy metals by plants and associated phytotoxic reactions should be analysed under controlled conditions and recommends hydroponic cultures as a suitable way to search for and identify metal-tolerant plants before starting field studies. Mala et al. (2010) emphasize that short-term hydroponic cultures can be highly effective in general evaluation of heavy metal accumulation efficiency by tree species, especially in fast-growing willow species. Experiments conducted *in vitro* enable strict control of sterility, thus allowing for determination of the direct influence of the investigated microbial strains on the plant while excluding the effects of interactions with other microorganisms (Mhadhbi, 2012). The factors that may be key influences in phytoextraction success are compounds responsible for plant stress. The negative influence of abiotic and/or biotic factors may reportedly lead to increasing reactive oxygen species (ROS) production (Polle and Rennenberg, 1993). Exposure to Cd^{2+} may cause oxidative stress, which is indicated by, e.g., lipid peroxidation, oxidative bursts or H_2O_2 accumulation (Schützendübel and Polle, 2002). Cadmium contributes indirectly to increased ROS production through a significant diminution in the glutathione (GSH) pool or by inhibiting the activity of antioxidant enzymes, which is reflected in higher H_2O_2 accumulation in plant tissues (Schützendübel et al., 2001). An elevated concentration of H_2O_2 leads to greater ion leakage from the roots and leaves and

may act as an indicator of the oxidative stress level as well as changes in the antioxidant defence of plants (Anjum et al., 2011; Garg and Bhandri, 2014). However, H_2O_2 is also involved in the regulation of plant cell expansion and cell wall plasticity. This process directly influences the rate of biomass increase (Schopfer, 2002; Liszkay et al., 2004).

The primary goal of our study was to determine the efficiency of microbiologically assisted (by the rhizosphere bacteria *Massilia* sp. and *Pseudomonas* sp. and the saprophytic fungus *Clitocybe* sp.) phytoextraction of Cd^{2+} ions by willow (*Salix viminalis*) under *in vitro* conditions. The willow species used in the experiment is considered one of the most economically important tree species cultivated on a wide scale for biomass production and used in the processes of phytoremediation due to its high heavy metals extraction capacity (Wojciechowicz and Kikowska 2009; Hrynkiewicz et al., 2012). During the experimental analysis, both the metal accumulation (which is expressed as the total amount of accumulated metal (Q), the bioconcentration factor (BCF), and the translocation index (Ti)) and the biomass were correlated with the intensity of oxidative stress (based on the H_2O_2 level) in the leaves, shoots and roots of the investigated plants. To provide controlled conditions and eliminate possible interference, the experiment was performed in hydroponic culture.

MATERIALS AND METHODS

THE EXPERIMENTAL DESIGN

In the conducted experiment plants were cultured in two variants of medium: (i) control – without addition of Cd^{2+} (-Cd) and (ii) medium enriched with 1 mM Cd^{2+} (+Cd). For both mediums four variants of microbial inoculation were conducted: control – uninoculated plants (i), plants inoculated with bacterial strains *Massilia* sp. III-116-18 (ii) or *Pseudomonas* sp. IV-111-14 (iii), and fungus strain *Clitocybe* sp. (iv). In each variant of our experiment (8 variants in total) we analysed 5 plants for biomass production (40 plants in total) – leaves, shoots an roots were analysed separately. For each analysed plant organ we measured: concentration of cadmium and H_2O_2 synthesis in triplicate (360 measurements for each parameter in total).

PLANT MATERIAL

In the *in vitro* experiment, we used a willow (*Salix viminalis*) clone obtained from the Institute of Plant Genetics at the Polish Academy of Sciences in Poznan. *S. viminalis* explants were cultured in 250 mL glass jars for *in vitro* cultures that contained 40 mL of MS medium (Murashie and Skoog, 1962: 4.26 g MS, 30 g sucrose, 8 g agar, and 1000 mL H_2O_{dist}, pH 5.8) for 6 weeks (with continuous lighting at 45 μmol $m^{-2}s^{-1}$, T 26±1°C). After this time, 2 fragments of plant shoots (each with one internode of 2–3 cm length) from the middle part of the shoot were cut and transferred into glass jars containing 40 mL of MS liquid medium (without agar). To stabilize the plants in the liquid medium, 40 cm^3 of sterile glass beads (∅ 10 mm, Carl Roth GmbH + Co. KG) covered with sterile gauze was used in each glass jar in the *in vitro* culture.

MICROBIAL MATERIAL AND CULTURE CONDITIONS

The saprophytic fungus *Clitocybe* sp. was isolated from the fruiting bodies collected in the post-mining area in the vicinity of Bolesław, which is strongly influenced by pollutants emitted from a smelter in Bukowno and the Upper Silesian Industrial Region. This area is characterized by its high concentrations of heavy metals in the soil, such as Pb, 428.8 mg; Zn, 1559 mg; Cu, 10.5 mg; and Cd, 14.9 mg per kilogram dry weight of soil. A detailed analysis of the soil collected from this area was described in our earlier work, Złoch et al. (2014). According to Mleczko (2004) and our own observations from 2012, *Clitocybe* sp. was the most frequently occurring fungal species in this area. This species was earlier classified on the basis of its phylogenetic analysis as a saprobiont (Matheny et al., 2006), although there are reports indicating that it belongs to the ectomycorrhizal fungi (Högberg et al., 1999).The collected fungus strain was identified on the basis of ITS sequence in accordance with the procedure given by Hrynkiewicz et al. (2010). A phylogenetic analysis of these sequences is presented in Fig. 1. The mycelia were cultured and stored on slants with PDA (Difco™). The suspension used for inoculating the plants growing in the *in vitro* cultures was prepared from 2-week-old mycelia sampled from PDA medium (Difco™) and rubbed in a flask filled with 5 mL of sterile physiological solution (0.9% NaCl).

The bacterial strains *Massilia* sp. III-116-18 and *Pseudomonas* sp. IV-111-14 were isolated from the rhizosphere of willows (*Salix viminalis*) growing in anthropogenically degraded soils. The strains were selected on the basis of their high enzymatic activity, their capacity to synthesize siderophores (Hrynkiewicz et al., 2010) and their high efficiency in accumulation of Cd^{2+} ions in the biomass (Hrynkiewicz et al., 2015), which indicated their promising application in the bioremediation processes. The bacterial inoculum was prepared from 3-day-old cultures incubated on R2A medium (Difco™), suspended in physiological solution

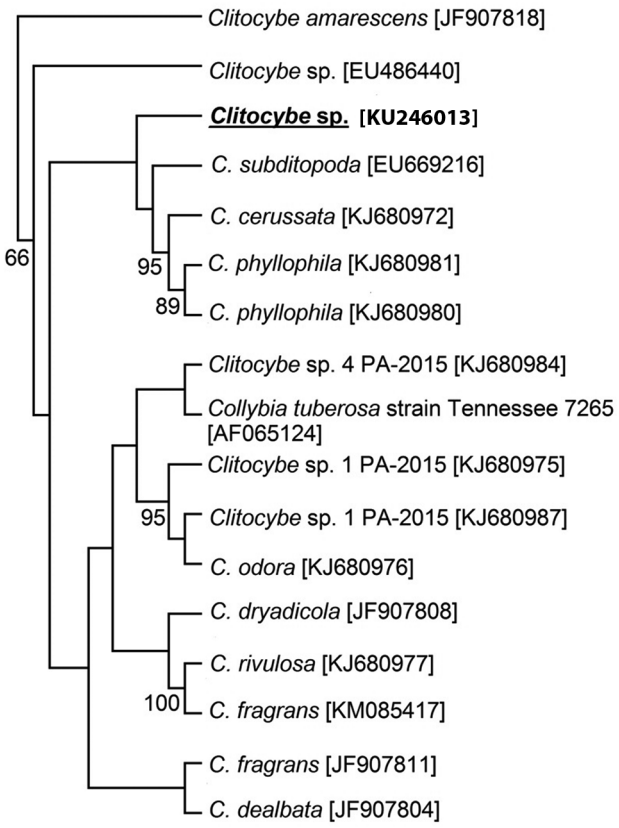

Fig. 1. Phylogenetic relationships of *Clitocybe* sp. Neighbour-joining analysis of internal transcribed spacer (ITS), using Kimura two-parameter genetic distances, combined with bootstrap analysis from 1,000 replicates (bootstrap values <50% not shown).

(0.9% NaCl) and diluted to OD = 0.5 (OD – optical density, measured at 600 nm).

PLANT-MICROBIAL CO-CULTURE AND TREATMENTS WITH CADMIUM (CD^{2+})

The plant inoculation was conducted 4 weeks after transferring the willow transplants to the *in vitro* cultures. The plants were inoculated with 150 µL of bacterial suspension or 0.5 mL of mycelial suspension (per 40 mL of liquid MS medium). Two days after microbial inoculation, Cd^{2+} in the form of a sterile cadmium sulphate ($3CdSO_4 \times 8H_2O$) solution was added to the culture medium to a final concentration of 1 mM Cd^{2+}. Parallel to the plant inoculation with microbial strains receiving Cd^{2+} treatment, the inoculated plants, which were grown on the medium without Cd ions, were subjected to analysis. Furthermore, the control, which was not inoculated or treated with Cd^{2+}, was included in the study.

ANALYSIS OF HYDROGEN PEROXIDE (H_2O_2) SYNTHESIS IN PLANT ORGANS

An analysis of H_2O_2 level was performed after 31 days of *in vitro* culture and 24 hours after adding Cd^{2+} ions (1 mM Cd^{2+}) to contaminated variants of the experiment. The analysis was performed according to Veljovic-Jovanovic et al. (2002) with our own modifications by the spectrophotometric DMAB-MBTH-POX method. The reaction was based on H_2O_2 reduction by peroxidase with the simultaneous formation of indamine dye during the process of 3-methyl-2-benzothiazoline hydrazone (MBTH) and 3-(dimethylamino) benzoic acid (DMAB) coupling. One hundred milligram portions of plant tissues (leaves, shoots and roots) were homogenized in liquid nitrogen and extracted with 5 mL of 0.1% TCA at 4°C. The resulting homogenate was centrifuged at 10 000 × g (10 min., 4°C). The supernatant was then neutralized with 0.4 N KOH to pH 7.5 and centrifuged at 1000 x g for 1 minute. To 1 mL of neutralized supernatant, 250 µL of 19.8 mM DMAB (in 0.5 M phosphate buffer, pH 6.5), 230 µL of 0.456 mM MBTH and 0.250 µL of peroxidase (1 KU/ml) were added. The samples were incubated for 20 minutes at 25°C, and the absorbance of the resulting coloured product was measured at 590 nm. The results were compared with a standard curve made for known H_2O_2 concentrations. To account for non-specific absorbance, the experiments were accompanied by control reactions without peroxidase and/or controls in which the plant extract was replaced with neutralized 0.1% TCA solution.

DETERMINATION OF BIOMASS, CD^{2+} CONTENT AND PHYTOEXTRACTION PARAMETERS

A dry biomass analysis was performed on 6-week-old plants growing in the *in vitro* cultures for 14 days after microbial inoculation and 12 days after adding Cd^{2+} ions. The resulting plant material was dried for 24 hours at 60°C to obtain dry biomass. Twenty milligrams of dry root, shoot and leaf samples was mineralized in a mixture of nitric and hydrochloride acids (1:3 v/v, 180°C, 3 h) and then dissolved in 0.1% HNO_3, filtered (Ø 0.45 µm) and measured by atomic absorption spectroscopy (AAS) with a Perkin Elmer 4100 apparatus (PerkinElmer, USA) to analyse the Cd^{2+} accumulation level in the plant biomass. The results were presented as indicators of the phytoextraction efficiency for (i) Cd^{2+} concentration (Cd^{2+} concentration was given in mg/g of dry weight); (ii) total concentration of Cd^{2+} accumulated in the biomass (Q = Cd^{2+} ion concentration × dry biomass); (iii) the bioconcentration factor (BCF = Cd^{2+} ion concentration in biomass/Cd^{2+} concentration in culture medium); and (iv) the index of translocation (Ti = [concentration of Cd^{2+} in the

TABLE 1. Microbiologically stimulated biomass of *S. viminalis* (leaves, shoots, roots) in the medium without (-Cd) or with addition of Cd^{2+} (+Cd). (n=5; mean in mg (standard deviation), *p≤0.05)

Variant	Leaves		Shoots		Roots	
	-Cd	+Cd	-Cd	+Cd	-Cd	+Cd
Control-uninoculated plants	59.2 (5.9)	56.2 (6.9)	15.8 (3.2)	12.2 (4.3)	92.8 (8.6)	31.8* (13.1)
Massilia sp. III-116-18	88.4 ↑ (17.7)	94.4 ↑ (9.6)	20.8 ↑ (2.7)	24 ↑ (2.9)	73.6 (12.6)	47.0* (5.0)
Pseudomonas sp. IV- 111-14	134.8 ↑ (15.1)	80.6 ↑* (13.5)	26.6 ↑ (2.6)	21.8 ↑* (2.8)	98.4 (20.7)	34.4 * (12.7)
Clitocybe sp.	91.6 ↑ (15.1)	84.2 ↑ (12.1)	25.0 ↑ (3.7)	22.0 ↑ (1.6)	62.2 ↓ (3.2)	66.2 ↑ (8.9)

↑ or ↓ – significant increase or decrease in biomass compared to control – uninoculated plants (p≤0.05) (U Mann Whitney test); * – significant difference compared to the control variant (inoculated with the respective microbe but unsupplemented with Cd^{2+}) (U Mann Whitney test).

aboveground plant tissues / concentration of Cd^{2+} in roots] × 100).

STATISTICAL ANALYSIS

Significant differences in plant biomasses, hydrogen peroxide levels and accumulated Cd^{2+} amounts between the inoculation variants, as well as differences between the variants in culture medium (with or without Cd^{2+} amendments), were determined using a nonparametric U Mann-Whitney test due to lack of normal distribution of analyzed data and significant differences in variance within the compared variants. The relations among the investigated parameters were analysed by Pearson's correlation separately for leaves, shoots and roots of *S. viminalis* under 1 mM Cd^{2+} addition on the basis of mean values calculated for each variants (n=4). All the analyses were conducted with Statistica software (Statistica v. 7, Statsoft).

RESULTS

The microbial inoculation of willow performed during the *in vitro* experiment significantly stimulated the growth of the above-ground parts of the plants (leaves and shoots) (Tab. 1). This effect was observed in both the control and the medium supplemented with Cd^{2+}, as well as for both inoculation bacterial strains, that is *Massilia* sp. III-116-18 and *Pseudomonas* sp. IV-111-14, and the saprophytic fungus *Clitocybe* sp. In the case of the control medium without cadmium (-Cd), the highest biomass of the above-ground parts (leaves and shoots) was reported in the variant inoculated with *Pseudomonas* sp. IV-111-14. In the medium enriched with Cd^{2+} (+Cd), the most significant impact on the growth of the above-ground plant parts was observed for the variant inoculated with *Massilia* sp. strain III-116-18. The biomasses of leaves and shoots derived from the variants inoculated with *Clitocybe* sp. fungus always had intermediate values compared with the variants inoculated with both bacterial strains. The biomasses of plants derived from variants inoculated with *Pseudomonas* sp. IV-111-14 were characterized by significantly lower growth in the medium supplemented with Cd^{2+} (+Cd) compared with the control medium. We did not observe a negative impact of Cd^{2+} in the remaining experimental variants (uninoculated control plants and plants inoculated with *Massilia* sp. III-116-18 bacteria and *Clitocybe* sp.). The bacterial strains used in our experiment did not affect the growth of the root biomass, unlike the saprophytic fungus, which either stimulated or inhibited root growth depending on the medium variant (Tab. 1). Moreover, we observed a significant decrease in the root biomass in all the variants (except the variants inoculated with *Clitocybe* sp.) in the medium supplemented with Cd^{2+}. The dry biomasses of the plants (expressed as percentage values in relation to the uninoculated variants) showed that all the microorganisms used in the experiment for inoculation: the bacteria *Pseudomonas* sp. IV-111-14 and *Massilia* sp. III-116-18, as well as the fungus *Clitocybe* sp., stimulated the biomasses of the leaves and shoots in the medium without Cd^{2+} by 30–130% for the bacteria and 55–60% for the fungus. In the medium supplemented with Cd^{2+}, the bacterial strains stimulated the biomass by 43–80% and the fungus stimulated the biomass by 50–62% (Fig. 2). The root biomasses revealed the inhibitory effect of the control medium when it was inoculated with *Massilia* sp. III-116-18 and *Clitocybe* sp. (20% and 30%, respectively). The same strains had a stimulatory effect on the root biomass in the medium supplemented with Cd^{2+} (15% and 35%, respectively). *Pseudomonas*

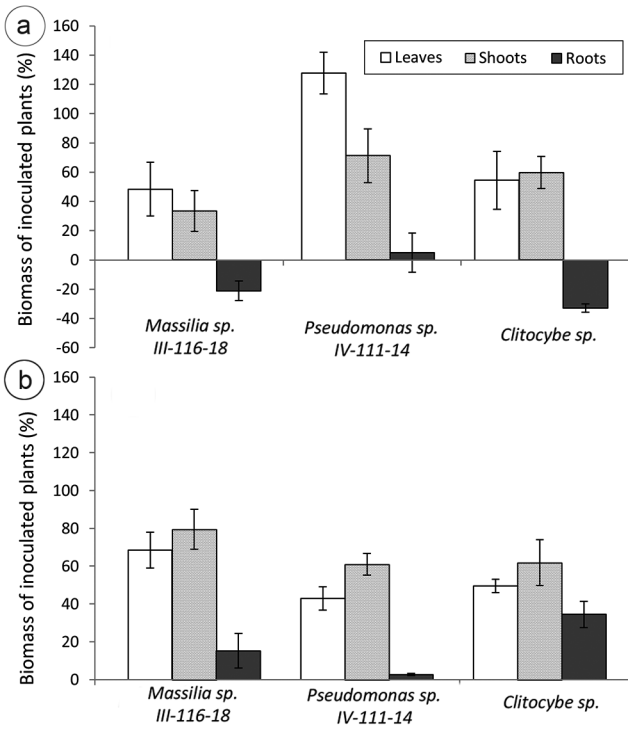

Fig. 2. Microbiologically stimulated biomass of *S. viminalis* (leaves, shoots, roots) in the medium without Cd^{2+} (-Cd) (**a**) or with addition of Cd^{2+} (+Cd) (**b**) presented as mean percentage values (%) compared to the uninoculated control (n=5; mean ± standard deviation, *p≤0.05)

sp. IV-111-14 bacteria did not affect the biomass of the roots growing in both -Cd and +Cd media (growth stimulation at 2% compared with the uninoculated variant).

The analysis of Cd^{2+} that accumulated in the biomass (leaves, shoots and roots) revealed the significant stimulatory effect of inoculation (with the exception of shoots in the variant of plants inoculated with *Clitocybe* sp.) (Tab. 2). In the case of leaves and roots, microorganisms significantly increased the Cd^{2+} concentration in the biomass of the inoculated plants and in shoots, the Cd^{2+} level depended on the microbial strain: the concentration decreased with *Massilia* sp. III-116-18, it increased for *Pseudomonas* sp. IV-111-14 and *Clitocybe* sp. did not influence the Cd^{2+} concentration.

The bioconcentration factor (BCF) was determined as the Cd^{2+} concentration in the biomass (leaves, shoots and roots)/Cd^{2+} concentration in the culture medium, depending on the microorganism used for inoculation as well as the analysed plant organ. *Pseudomonas* sp. IV-111-14 decreased the BCF in the leaves and increased it in the shoots and roots. *Massilia* sp. III-116-18 only decreased the BCF in the shoots, and the *Clitocybe* sp. fungus did not affect this parameter in the shoots, leaves or roots (Tab. 2).

An analysis of the Cd^{2+} translocation index from the roots to the aboveground parts revealed a significant inhibitory effect of inoculation only in the leaves of plants inoculated with *Pseudomonas* sp. IV-111-14 (Tab. 2).

An analysis of the H_2O_2 level under the microbiological stimulation of the phytoextraction process revealed significantly higher levels in all the plant tissues inoculated with the *Clitocybe* sp. fungus when exposed to a 1 mM Cd^{2+} concentration (Tab. 3). The increase in the percentage of H_2O_2 in the leaves, shoots and roots of the inoculated plants in relation to the control plants ranged from 35% to 112% (Fig. 3b). When the medium was not supplemented with Cd^{2+} (-Cd) and the plants were inoculated with saprophytic fungus, there was a significantly higher level of H_2O_2 only in the leaves and shoots (1729.5 and 3983.8 ng/g of fresh weight, respectively) (Tab. 3). For the bacterial strains, we recorded a sig-

TABLE 2. Factors of phytoremediation efficiency: quantity of accumulated Cd (Q), bioconcentration factor (BCF), and translocation index (Ti) in the microbiologically stimulated biomass of *S. viminalis* (leaves, shoots, roots) in the medium with addition of Cd (+Cd) (n=5; mean (standard deviation), *p≤0.05).

Variant	Leaves			Shoots			Roots	
	Q	BCF	Ti	Q	BCF	Ti	Q	BCF
Control uninoculated plants	38.33 (1.39)	5.72 (0.49)	9.55 (1.79)	3.86 (2.33)	1.78 (0.57)	2.99 (1.01)	253.86 (55.09)	61.16 (8.29)
Massilia sp. III-116-18	62.86 ↑ (6.82)	5.64 (0.80)	9.89 (1.26)	0.89 (0.17) ↓	0.31 ↓ (0.07)	0.54 ↓ (0.12)	317.62 ↑ (15.30)	57.04 (4.31)
Pseudomonas sp. IV-111-14	48.68 ↑ (3.07)	5.02 ↓ (0.56)	7.23 ↓ (1.14)	8.14 ↑ (2.28)	3.11 ↑ (1.06)	4.43 (1.46)	309.14 ↑ (47.57)	70.64 ↑ (10.61)
Clitocybe sp.	58.45 ↑ (2.97)	5.74 (0.38)	9.88 (0.88)	2.62 (1.81)	1.04 (0.75)	1.72 ↓ (1.11)	468.56 ↑ (13.80)	58.44 (4.90)

Q – quantity of accumulated Cd in μg; BCF – bioconcentration factor: Cd concentration in the biomass (leaves, shoots, roots)/ Cd concentration in the medium; Ti – translocation index: Cd concentration in the biomass of leaves and shoots/ Cd concentration in the roots; ↑ or ↓ – see Table 1.

TABLE 3. The influence of inoculation with bacteria (*Massilia* sp. III-116-18 and B2 – *Pseudomonas* sp. IV-111-14) and the saprophytic fungus (*Clitocybe* sp.) on the level of hydrogen peroxide synthesis in the leaves, shoots and roots of *S. viminalis* clones growing in the medium without (-Cd) or with addition of Cd^{2+} (+Cd) in the *in vitro* culture expressed as ng H_2O_2 per gram of fresh weight of biomass (mean (standard deviation), n=5, *p≤0.05).

Variant	Leaves		Shoots		Roots	
	-Cd	+Cd	-Cd	+Cd	-Cd	+Cd
Control, uninoculated plant	784.69 (219.20)	339.19 * (125.59)	2394.09 (890.58)	1255.76 * (752.63)	1394.01 (536.00)	776.56 * (352.69)
Massilia sp. III-116-18	370.10 ↓ (230.47)	812.33 ↑* (450.62)	1646.04 (1373.34)	726.72 (368.03)	724.63 ↓ (86.07)	962.16 * (287.85)
Pseudomonas sp. IV-111-14	469.85 ↓ (243.66)	625.27 ↑ (240.36)	2369.68 (1067.96)	1163.99 * (466.10)	851.14 ↓ 186.46)	778.56 (285.38)
Clitocybe sp.	1729.50 (282.98)	693.94 ↑* (289.32)	3983.82 ↑ (1072.58)	3032.04 ↑* (871.48)	1103.96 (358.27)	984.13 ↑ (117.91)

Abbreviations: see Table 1

nificant inhibitory effect of the inoculation on the H_2O_2 quantity in the leaves and roots of plants growing in the medium not supplemented with Cd^{2+} (-Cd) (Tab. 3). The H_2O_2 levels were 60% (*Massilia* sp. III-116-18) and 38% (*Pseudomonas* sp. IV-111-14) higher in the leaves and 48% (*Massilia* sp. III-116-18) and 38% (*Pseudomonas* sp. IV-111-14) higher in the roots (Fig. 3). The inoculation of plants with bacterial strains in the media supplemented with Cd^{2+} resulted in significant increase in H_2O_2 synthesis only in the leaves (812.3 ng/g fresh weight for *Massilia* sp. III-116-18 and 625.3 ng/g fresh weight for *Pseudomonas* sp. IV-111-14) (Tab. 3).

An analysis of the metal's influence on the H_2O_2 synthesis level revealed a significant decrease in all the investigated plant tissues in uninoculated willow and in the aboveground parts (leaves and shoots) of the plants inoculated with *Clitocybe* sp. compared with the -Cd variants. In the variants inoculated with bacterial strains, a similar decrease was noted only for the shoots of plants growing in the presence of *Pseudomonas* sp. IV-111-14. In plants inoculated with *Massilia* sp. III-116-18, the presence of Cd^{2+} in the medium caused a significant increase in the synthesis of H_2O_2 in both leaves and roots (Tab. 3).

As a result of a linear correlation analysis of the H_2O_2 level with other measured parameters, a significant correlation was noted only for the leaves. A higher quantity of hydrogen peroxide was accompanied by higher biomass growth (in terms of the dry biomass and the percentage change in biomass compared with the uninoculated variant) as well as by a greater total quantity of accumulated Cd^{2+} (Tab. 4). Moreover, in all the analysed plant organs (leaves, shoots and roots), we observed a significant correlation of the accumulated Cd^{2+} with the bioconcentration factor (BCF) as well as a correlation of the total quantity of metal accumulated in the biomass of leaves and roots with the concentration of Cd^{2+} and BCF for shoots (Tab. 4).

An ITS phylogenetic tree was constructed on the basis of a BLAST search of the ITS sequences within NCBI database to identify the closest evolutionary relative of the investigated fungus strain. An NJ tree

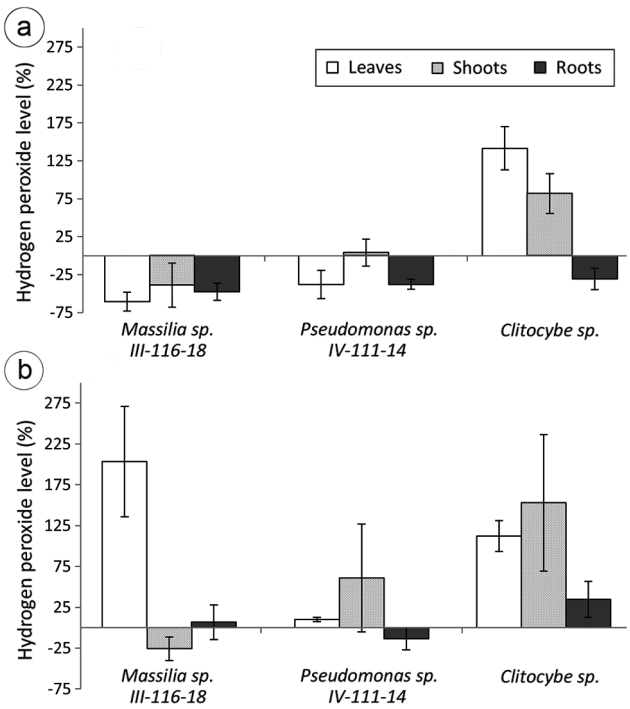

Fig. 3. Hydrogen peroxide (H_2O_2) synthesis in the microbiologically stimulated biomass (leaves, shoots and roots) of *S. viminalis* clones growing without (**a**) or in presence (**b**) of Cd^{2+} in the culture medium. The level of hydrogen peroxide was presented as percentage stimulation or inhibition compared to the control (100%) representing uninoculated plants growing in the medium without (a) or with addition of Cd^{2+} (b) (mean standard deviation).

TABLE 4. Analysis of the correlation between investigated parameters: level of H_2O_2 synthesis, biomass (DW), percentage increase/decrease of biomass (DWΔ%), quantity of accumulated Cd^{2+}(Q), and bioconcentration factor (BCF) determined in the leaves, shoots and roots of willow *S. viminalis* in the presence of 1mM Cd^{2+} in the medium. Bold type – significant correlation (linear correlation coefficient r/ p level; n=4).

		H_2O_2 [ng/gsm]	DW [mg]	DWΔ%	Cd [mg/gsm]	Q [µg]	BCF
H_2O_2 [ng/gsm]	Leaves		**0.999/0.001**	**0.999/0.001**	-0.091/0.909	**0.970/0.030**	-0.091/0.909
	Shoots		0.079/0.921	0.037/0.963	-0.111/0.889	-0.110/0.890	-0.111/0.889
	Roots		0.901/0.099	0.901/0.099	-0.868/0.132	0.753/0.247	-0.755/0.245
DW [mg]	Leaves	**0.999/0.001**		**0.999/0.000**	-0.140/0.860	0.957/0.043	-0.140/0.860
	Shoots	0.079/0.921		**0.998/0.002**	-0.260/0.740	-0.136/0.864	-0.260/0.740
	Roots	0.901/0.099		**1.000/**---	-0.684/0.316	**0.956/0.044**	-0.575/0.425
DWΔ%	Leaves	**0.999/0.001**			-0.130/0.870	**0.959/0.041**	-0.130/0.870
	Shoots	0.037/0.963	**0.998/0.002**		-0.295/0.705	-0.172/0.828	-0.295/0.705
	Roots	0.901/0.099	**1.000/**---		0.684/0.316	**0.956/0.044**	0.575/0.425
Cd [mg/gsm]	Leaves	-0.091/0.909	-0.140/0.860	-0.130/0.870		0.149/0.851	**1.000/**---
	Shoots	-0.111/0.889	-0.260/0.740	-0.295/0.705		**0.992/0.008**	**1.000/**---
	Roots	-0.868/0.132	-0.684/0.316	0.684/0.316		-0.442/0.558	**0.979/0.021**
Q [µg]	Leaves	**0.970/0.030**	**0.957/0.043**	**0.959/0.041**	0.149/0.851		0.149/0.851
	Shoots	-0.110/0.890	-0.136/0.864	-0.172/0.828	**0.992/0.008**		**0.992/0.008**
	Roots	0.753/0.247	**0.956/0.044**	**0.956/0.044**	-0.442/0.558		-0.322/0.679
BCF	Leaves	-0.091/0.909	-0.140/0.860	-0.130/0.870	**1.000/**---	0.149/0.851	
	Shoots	-0.111/0.889	-0.260/0.740	-0.295/0.705	**1.000/**---	**0.992/0.008**	
	Roots	-0.755/0.245	-0.575/0.425	0.575/0.425	**0.979/0.021**	-0.322/0.679	

(Fig. 1) clearly showed a membership within the genus *Clitocybe* with the closest relative to *C. subditopoda* [EU669216], *C. cerrusata* [KJ680972] as well as *C. phyllophila* [KJ680981] and *C. phyllophila* [KJ680980].

DISCUSSION

In the experiments performed in this work both investigated bacterial strains (*Massilia* sp. III-116-18 and *Pseudomonas* sp. IV-111-14) and the saprophytic fungus (*Clitocybe* sp.) were revealed to increase the total amount of Cd^{2+} that significantly accumulated in the biomass of the roots and leaves. The observations by Dos Santos Utmazian et al. (2007), who studied the effects of inoculating with *Cadophora finlandica* mycorrhizal fungus and indigenous soil microorganisms on the accumulation of Cd and Zn by *S. smithiana* and *S. caprea*, were opposite. These researchers revealed that heavy metal contents of the biomass were generally lower in inoculated plants compared with uninoculated control variants. Simultaneously, they noted a significant increase in the value of Cd bioconcentration for *S. smithiana* when it was inoculated with fungus and microorganisms as well as for *S. caprea* inoculated with microorganisms only. On this basis, the researchers formed the hypothesis that a higher bioconcentration factor may indicate the contribution of bacterial secretions (i.e. siderophores) in increasing Cd availability to willows (Whiting et al., 2001; Abou-Shanab et al., 2003). Dimpka et al. (2009) revealed that the siderophores synthesized by *Streptomyces tendae* strain F4 significantly enhanced Cd^{2+} uptake by sunflowers. A similar effect was noted by Sheng et al. (2008) for tomato, maize and rape plants inoculated with *Bacillus* sp. J119, demonstrating the ability to synthesize biosurfactants (lipopeptide). In our studies, higher Cd^{2+} contents in the plant biomass (compared with uninoculated plants) were not accompanied by an increase in the BCF factor. This finding suggests that under conditions of high availability of the metal, which appeared in the culture medium, the effect of microbial secretions in increasing the heavy metal bioavailability is less important than in the case of soil. Weyens et al. (2013) revealed that inoculating the roots with selected rhizobacteria and endophytes (for high Cd^{2+} resistance and activity from the synthesis of siderophores, organic acids and IAA) isolated from a *Salix schwerinii* × *S. viminalis* cv. Tora clone growing in metalliferous areas caused decreased

phytoextraction efficiency in soil contaminated with Cd^{2+} and toluene. Moreover, a single inoculation with the selected strains decreased the Cd^{2+} contents in both the leaves and roots of the investigated willows. The researchers noted that the characteristic physiological abilities of the strains are not sufficient for predicting their impact on the host plant. This observation may also indicate the appropriateness of using *in vitro* experiments involving both plants and microorganisms, which (as a selection step) should precede more time-consuming pot and field experiments.

Not the least important factor in describing heavy metal phytoextraction efficiency and determining the potential of plant-microorganism systems is the biomass value. When inoculating with the selected microorganisms in both control media and Cd^{2+} exposure treatments in our experiment, the biomass of the aboveground parts (leaves and shoots) of *S. viminalis* significantly increased compared with the uninoculated plants. Interestingly, while *Pseudomonas* sp. IV-111-14 demonstrated the highest stimulation of leaf and shoot growth under control conditions, *Massilia* sp. III-116-18 was the most effective at promoting plant growth in the medium supplemented with Cd^{2+}. This result corresponds with our earlier observations (Hrynkiewicz et al., 2015), in which a higher biomass of bacterial cells and higher effectiveness in terms of Cd^{2+} accumulation were noted for *Massilia* sp. III-116-18. In literature, there are numerous examples of stimulating effects of bacteria on plant growth in the presence of Cd^{2+} (e.g., Belimov et al., 2005; Tripathi et al., 2005; Zimmer et al., 2009). This knowledge is far scarcer in the case of saprophytic fungi, and detailed studies addressing their influence on plant growth (including different willow species) under heavy metal exposure is still lacking. In one of few studies, Adams et al. (2007) investigated the influence of inoculation with *Trichoderma harzianum* Rifai 1295-22 on the growth of *S. fragilis* in soil polluted with heavy metals including Cd (30 mg/kg of dry weight of soil). The researchers noted significant stimulation of *S. fragilis* growth after inoculating the plants with the fungal strain (39% higher dry biomass compared to the control). Because the experiment was conducted in soil, the influence of *T. harzianum* may be a combination of the direct impact of the fungus on the plant, interactions with microorganisms present in the soil and the stimulation of nutrient uptake by the fungal strain. Moreover, the authors noted lower stimulation of *S. fragilis* growth in heavy-metal-contaminated soil compared with the control soil. The increased metal content in the leaves and roots of plants inoculated with *T. harzianum* may suggest the essential role of the fungus in enhancing heavy metal ion uptake by plants. These observations are consistent with the results of our research, in which inoculating with the saprophytic fungus *Clitocybe* sp. caused a simultaneous increase in the biomass of leaves, shoots and roots of *S. viminalis*, as well as an increase in the Cd^{2+} content of the leaves and roots, compared with the uninoculated plants. This effect was particularly visible for root biomass. In the control medium, inoculating with *Clitocybe* sp. inhibited root growth, and when the plants were exposed to metal ions, this effect was changed to significant stimulation. Our work showed stimulation of biomass production and Cd^{2+} accumulation resulting from the direct influence of *Clitocybe* sp., which suggests that the mechanisms responsible for this effect should be investigated in the future.

The analysis of H_2O_2 synthesis in the plant tissues of willows growing in *in vitro* cultures revealed a significant decrease in the H_2O_2 level in the uninoculated plants (control) under Cd^{2+} exposure in both the aboveground organs and roots. This finding contrasts with the results of previous studies, which address the ability of Cd^{2+} to elicit oxidative stress in plants, and the general view is that the H_2O_2 level increased under Cd^{2+} stress (Schützendübel and Polle, 2002). In pine roots at 5–50 μM $CdSO_4$ (Schützendübel and Polle, 2002), in the roots, wood, bark and leaves of *Populus canescens* at 0–50 μM $CdSO_4$ (He et al., 2011), or in the roots and leaves of *Sedum alfredii* at 1.5–400 μM $CdCl_2$ (Jin et al., 2008) the hydrogen peroxide levels increased under the influence of Cd^{2+}. In contrast to the aforementioned studies, we used a higher (1 mM) Cd^{2+} concentration to challenge the plants in this study. However, it should be noted that hydrogen peroxide, in addition to contributing to oxidative stress, also plays an important role in many physiological processes, such as forming vascular bundles in the xylem, lignifying cell walls, cell elongation (i.e., root hairs) or the proper functioning of stomata (Teichmann, 2001; Cheng and Song, 2006; Nanda et al., 2010). Moreover, considering the relatively high Cd^{2+} concentration used in our experiment, the decrease in H_2O_2 synthesis in the tissues of uninoculated plants may reflect the arrest of growth and metabolic activity as an early response to the supplementation of the growth medium with the high metal concentration. This explanation was reflected in the nearly three times lower growth of the root biomass in the uninoculated plants in the medium with added Cd^{2+}. However, inoculating with the saprophytic fungus *Clitocybe* sp. caused a significant stimulation of H_2O_2 synthesis compared with uninoculated plants both in the control medium (leaves and shoots) and in the presence of 1 mM Cd^{2+} (all organs). This effect is particularly visible in the roots, in which significant increases in the H_2O_2 level were accompanied by increases in the total quantity of accumulated Cd^{2+} and plant bio-

mass. Nanda et al. (2010) observed that at the early stage of both symbiotic and pathogenic interactions between plants and microorganisms, transient increases in ROS production can occur in the plant organs. The elevated H_2O_2 accumulation in the cells was noted, e.g., in *Medicago truncatula* during the formation of mycorrhizal associations with the fungus *Glomus intraradices* (Salzer et al., 1999) or in *Castanea sativa* with *Pisolithus tinctorius* (Baptista et al., 2007). In light of these findings, the increased H_2O_2 levels in willows inoculated with *Clitocybe* sp. fungus is likely to be an early response of the plant to root colonization by the fungus rather than a stress response to the presence of Cd ions, which is indicated by the simultaneous stimulation of growth and the efficiency of metal accumulation in the plant biomass. Similar findings were reported for the leaves of plants inoculated with the studied bacterial strains in the medium supplemented with this metal.

CONCLUSIONS

In our studies, the selected microbial strains generally stimulated both biomass and the parameters of phytoextraction efficiency (when measured as Q, BCF and Ti) in the *in vitro* cultures of *S. viminalis* supplemented with Cd^{2+}; however, they were strain and plant organ specific. We suggest that increased H_2O_2 levels in the uninoculated plants in the presence of Cd^{2+} can be caused by the general negative impact of this metal on plant growth. Increasing H_2O_2 synthesis in the biomass of inoculated plants growing under the influence of Cd^{2+} indicates the beneficial effects of microorganisms on the plant growth. Because of the lack of similar studies in literature, we plan to continue similar experiments but at lower Cd^{2+} concentrations in the medium. The significant stimulation of biomass growth and the amount of accumulated metal after inoculating the plants with the saprophytic fungus *Clitocybe* sp. indicates its potential for recultivation of Cd^{2+}-contaminated areas and suggests the appropriateness of research on saprophytic fungal species in the context of their application to phytoremediation processes.

AUTHORS' CONTRIBUTIONS

The following declarations about authors' contributions to the research have been made: performed experiment (biomass measurement, heavy metal analysis), statistical analysis preparation of manuscript: MZ; responsible for plant stress analysis: JT; supervised the research design and manuscript preparation: KH.

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