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Microbial assisted phytoextraction of Cd²⁺ by *Salix viminalis* under *in vitro* culture conditions

Abstract: Microbially assisted phytoremediation is considered as the most promising eco-friendly solution for recultivation of heavy metal polluted soils. *In vitro* experiments can be favorable systems that allow assessing compatibility and efficiency of both partners (e.g. plant-microorganism) which reduces time and space in the initial stages of this technology.

The main objective of this study was: (1) to calculate the Cd²⁺ accumulation factors (e.g. BCF, Ti, AF) using willow (*Salix viminalis* L.) inoculated with three *Streptomyces* sp. strains under *in vitro* conditions and (2) to compare obtained results with that derived from pot experiments by Złoch et al. (2017).

Our results reveal significantly increase in Cd²⁺ accumulation capacity of *Streptomyces* spp. inoculated willow plants, indicating microbial stimulation of phytoextraction. Additionally, inoculated plants showed higher biomass production and lower lipids peroxidation level. The results revealed significant increase of MEA, BCF, Ti, MER by Strep-1 and Strep-2 in the above-ground parts of inoculated plants. Moreover, of the three strains tested, Strep-1 (*Streptomyces* sp. SIIB-Zn-R8) demonstrated the highest impact on the Cd²⁺ phytoextraction efficiency.

In conclusion, the proposed *in vitro* model system allowed predicting Cd²⁺ phytoextraction capacity performed using inoculated willow plants with the significant reduction of both time and space.

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Introduction

Increasing soil contamination with heavy metals (HMs) has become one of the most serious environmental problems worldwide, especially in areas of rapid industrialization and intense agricultural activities (Bhargava et al., 2012; Usman et al., 2013). Many engineering-based techniques such as vitrification and landfilling can be used to restore HMs polluted areas (Keller, 2006). However, they irreversibly affect soil properties, fertility and biodiversity which may make the soil unsuitable for future colonization

by plants (Padmavathiamma & Li, 2007). Furthermore, application of conventional methods is often associated with very high costs amounting from 100 000 to 1 000 000 USD per hectare (Russel et al., 1991). Currently, many researchers are focused on developing cost-effective and sustainable biological solutions for soil remediation of contaminated environments (Bhargava et al., 2012). Thus, employing plants for their capacity to accumulate HMs in the harvestable parts (phytoextraction) has been proposed as an economical and ecologically friendly technique (Zacchini et al., 2009; Yang et al., 2015).

Plant's suitability for use in the phytoextraction processes depends on several properties. An ideal plant should possess multiple traits e.g.: (i) fast growth, (ii) deep and extended root system, (iii) ability to accumulate large quantities of contaminants and translocate them efficiently from the roots to the aerial parts, (iv) tolerance to accumulated toxic elements, (v) be easy to manage, dispose of and/or recycle, (vi) fast adaptation to different habitat conditions (Keller, 2006; Ali et al., 2013; Krzciuk, 2015). However, the choice of plant species for a given situation is always a compromise (Keller, 2006). Initially, much of the research focused mainly on the hyperaccumulators that accumulate exceptionally high concentrations of metals in their aboveground biomass (Yang et al., 2015). However, hyperaccumulators exhibit many disadvantages such as a low biomass, slow growth rate, shallow root system as well as large annual variations of yield and metal concentrations which may hinder the phytoextraction efficiency (Keller, 2006; Deram et al., 2007; Doumett et al., 2008). Recent phytoremediation efforts have focused on the use of high-yielding, fast-growing tree species (e.g. *Salix* sp., *Populus* sp.) for phytoextraction of metals (Doty, 2008; Capuana, 2011). Under this approach, lower accumulation of HMs in the plant tissues is compensated by the high biomass production (Hernández-Allica et al., 2008; Zacchini et al., 2009). In this context, an alleviation of toxic effect of HMs on plant growth rate is crucially important since most plants demonstrate relatively low biomass when grown at metalliferous sites (Glick, 2003).

It is well known that soil microorganisms can protect plants against adverse effect of HMs. Among them, siderophore producing bacteria (SPB) deserve special attention. These microorganisms indirectly promote plant biomass production (e.g. facilitating uptake of Fe and other elements) and have the potential to facilitate metal accumulation within plant organs (Hrynkiewicz et al., 2018).

Reliable evaluation of the proposed plant-microorganism system efficiency is based on the calculation of many metal accumulation parameters, e.g. bioconcentration factor (BCF), translocation index (Ti) or accumulation factor (AF). For such purposes,

the use of *in vitro* model systems are proposed as it enables reduction of both time and space which is particularly important in the case of tree species considering their long life cycle (Marmiroli et al., 2011).

In a previous study by Złoch et al. (2017), SPB strains of *Streptomyces* spp. were used to investigate their influence and role on the HMs phytoextraction efficiency of *Salix dasyclados* L. through pot experiments (4 different naturally contaminated metalliferous soils were used). With this background, the present aim of our study was formulated: (i) to assess the effect of inoculation with SPB strains on Cd²⁺ phytoremediation efficiency by willow plants growing under *in vitro* conditions and (ii) to compare their effects with the results derived from the pot experiment described in the work of Złoch et al. (2017). We hypothesise that the proposed *in vitro* model system will enable to predict and analyse Cd²⁺ phytoextraction efficiency of willow plants in a short time duration in comparison to the pot experiments and the results from both these approaches will be comparable.

Materials and methods

Plant material

For the *in vitro* experiment we used a willow (*Salix viminalis*) clone obtained from the Institute of Plant Genetics in Poznan (Poland). Plants were cultured and maintained in sterile conditions using 250 mL glass jars with 40 mL of MS medium (Murashige & 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⁻²s⁻¹, T 26±1 °C).

Bacterial strains and culture conditions

We have used three bacterial strains *Streptomyces* sp.: SIIB-Zn-R8 (Strep-1), SIIB-Cd-R4 (Strep-2) and SIA-Zn-R4 (Strep-3) to prepare the microbial inoculum. These strains were previously selected based on their ability to synthesise siderophore under heavy metal stress conditions and were very effective in heavy metals phytoextraction from three different soils (Złoch et al., 2016). Bacterial strains were isolated from the rhizosphere of birch (*Betula pendula* – Strep-1 and Strep-2) and alder (*Alnus glutinosa* – Strep-3) trees growing at heavy metal contaminated sites in the vicinity of the mining-metallurgic plant ZGH "Bolesław" in Bukowno in southern Poland (Złoch et al., 2017). Bacterial inoculum was prepared from 3-day-old cultures incubated on R2A medium (Difco™), suspended in physiological solution (0.9% NaCl) and diluted to OD=0.25 (OD – optical density, measured at 600 nm).

Experimental design

All experimental setup included 12 different variants: growth medium supplemented with and without Cd^{2+}, non-inoculated (control) and inoculated plants (with 3 different bacterial strains).

Explants used in the experiment were obtained by cutting top parts of the shoots and transferring them into glass tubes containing 10 mL of MS liquid medium (without agar). To stabilize plant's growth, the explants were put on sterile paper previously inserted into the culture tubes (Supplementary material, Figure A). Plant inoculation was conducted 7 days after transferring the willow explants to the *in vitro* cultures. The plants were inoculated with 25 μL of bacterial suspension (per 10 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 reach final concentration of 25 μM and 100 μM (sterile distilled water was added to the control variant). Plants (12 replicates per treatment) were cultivated 8 weeks. After this time the plant material was collected for analysis.

Analysis of lipid peroxidation level in plant organs

The level of lipid peroxidation was determined in the roots, shoots and leaves of *S. viminalis* for all experimental variants. The analysis was performed after 8 weeks of *in vitro* explants culture. The samples consisted in two hundred milligram portions of each plant organ (leaves, shoots and roots), homogenized in liquid nitrogen. Lipid peroxidation was assessed as described by Hodges et al. (1999). The method was based on the measurement of malondialdehyde (MDA) level which reacts with thiobarbituric acid (TBA) under acidic conditions and high temperature, leading to formation a colored (reddish) product (Taulavuori et al., 2001). The analyses were carried out in three biological and three technical replicates (nine in total).

Determination of growth parameters, Cd^{2+} content, and phytoextraction factors

Analysis of the root and shoot length, as well as the weight of the fresh and dry biomass of the leaves, shoots and roots was performed on 8-week-old explants. To obtain dry biomass, the plant material was dried for 24 hours at 60 °C. To analyse the Cd^{2+} content in the plant biomass 100 mg of dry roots, shoots and leaves samples was mineralized in 12 mL of mixture of nitric and hydrochloric acids (1:3 v/v) in quartz tubes according to USEPA 3052-HCl method using microwave digestion system Novawave SA (SCP Science, US). Cd concentration was measured by atomic absorption spectroscopy (AAS) using AAnalysit 800 (PerkinElmer, US) (Złoch et al., 2015). For each variant of the experiment, 3 plants were chosen (biological replicates) and measured in three technical replicates. The results were presented as indicators of the phytoextraction efficiency: BCF – Bioconcentration factor, Ti – Translocation index, AF – Accumulation factor, MEA – metal extraction, MER – metal extraction ratio. The descriptions of these factors including formulas are demonstrated in the Table 1.

Statistical analysis

Significant differences in plant growth parameters (shoots and roots length, fresh and dry biomass), lipid peroxidation levels and accumulated Cd^{2+} amounts between inoculation variants were determined using ANOVA with Newman Keuls post-hoc test. All analyses were made with Statistica software (Statistica v. 7, Statsoft).

Results

Effect of *Streptomyces* sp. inoculation on *S. viminalis* growth under increasing Cd^{2+} concentrations

Inoculation of *S. viminalis* L. with *Streptomyces sp.* strains SIIB-Zn-R8 (Strep-1), SIIB-Cd-R4 (Strep-2)

Table 1. The factors used for describing the capacity and efficiency of Cd accumulation in the investigated plants (modified after Li et al. 2011 and Krzciuk, 2015)

Parameter	Acronym	Formula
Translocation index	Ti	Ti = [concentration of Cd in the aboveground plant tissues / concentration of Cd in the roots] $\times$ 100
Bioconcentration factor	BCF	BCF = Cd concentration in biomass/Cd concentration in culture medium
Metal extraction amount	MEA	MEA = Cd concentration in plant tissue $\times$ dry biomass
Metal extraction ratio	MER	MER = [(Cd concentration in the leaves/shoots $\times$ biomass of the leaves/shoots)/(Cd concentration in the culture medium $\times$ volume of the medium)] $\times$ 100
Accumulation factor	AF	AF = Cd content in the plant/Cd content in the culture medium

and SIA-Zn-R4 (Strep-3) significantly increased plant growth and biomass without and with Cd^{2+} (mainly at $25 \mu M$ Cd^{2+}) (Table 2). Strep-2 and Strep-3 significantly stimulated shoot at $25 \mu M$ Cd^{2+} while Strep-1 and Strep-2 significantly increased root length at $100 \mu M$ Cd^{2+} (Table 2). Dry biomass of leaves increased in all the inoculated variants. Moreover, all strains stimulated root biomass at $100 \mu M$ Cd^{2+} and Strep-1 and Strep-2 were consistent in increasing this parameter at $25 \mu M$ (Fig. 1).

Lipid peroxidation in *S. viminalis* organs

Decrease in MDA levels in leaves, shoots and roots after bacterial inoculation was evident in all the experimental variants, grown in Cd^{2+} supplemented medium (both at $25 \mu M$ and $100 \mu M$ Cd^{2+}) (Fig. 2). This effect was significant in comparison to the non-inoculated control. However, increase in MDA level in leaves and roots was observed in plants grown in Cd^{2+} – free medium ($0 \mu M$ Cd^{2+}), but subjected to the bacterial treatments (Strep-2 and Strep-3). Positive effect (reduced MDA levels) was observed mainly with Strep-2 inoculation at $25 \mu M$ Cd^{2+} for all plant organs.

Effect of Cd²⁺ accumulation in aboveground organs of *S. viminalis*

The calculated accumulation factor (AF) revealed that microbial *Streptomyces sp.* inoculation for both variants of medium (25 and $100 \mu M$ Cd^{2+}) significantly increased Cd^{2+} accumulation in whole aboveground part of the *S. viminalis* in comparison to the control plants (Fig. 3). At $100 \mu M$ Cd^{2+}, the 3 *Streptomyces* sp. strains recorded a twofold increase in Cd^{2+} accumulation. However, Strep-1 and Strep-3
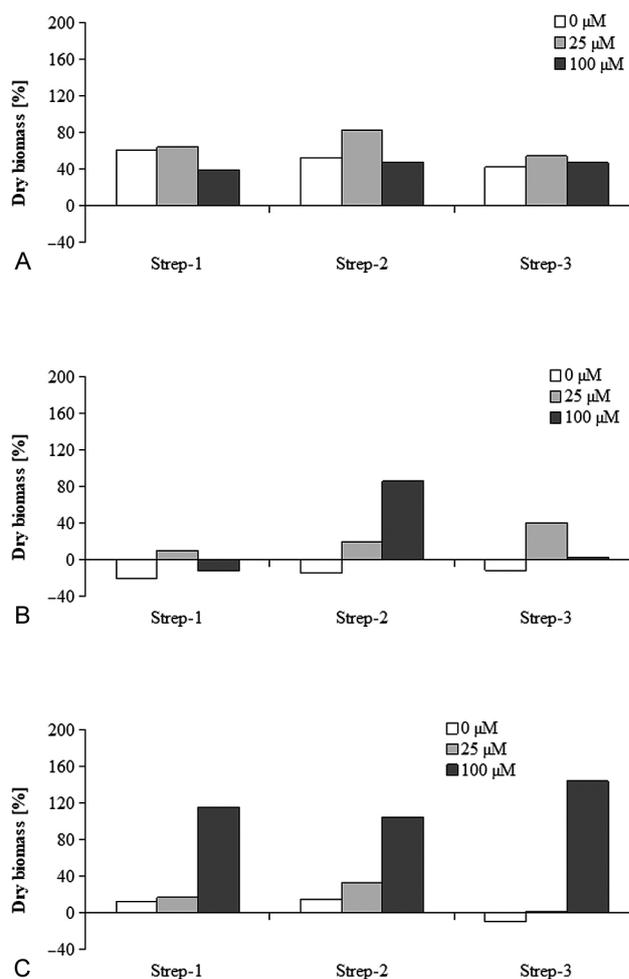
Fig. 1. Dry biomass of leaves (A), shoots (B) and roots (C) of *S. viminalis* clones inoculated with *Streptomyces* sp. strains growing at $0 \mu M$, $25 \mu M$ and $100 \mu M$ Cd^{2+} in the culture medium. The results are presented as percentage stimulation or inhibition with respect to their control (100%) representing uninoculated plants (mean ± standard deviation)

* – significantly different compared to control (p≤0.05).

Table 2. Growth parameters of *Salix viminalis* growing in MS medium without ($0 \mu M$) or with addition of Cd^{2+} (25 and 100 μM). (n=5; mean ± standard deviation, significant differences marked with different letters, p≤0.05)

	Shoot length [mm]	Roots length [mm]	Leaves DW [g]	Shoots DW [g]	Roots DW [g]
			$0 \mu M$		
Control	89.0±8.0b	152.0±30.0a	0.188±0.042a	0.087±0.018a	0.196±0.031ab
Strep-1	97.0±5.0b	187.0±12.0b	0.295±0.023b	0.069±0.013a	0.217±0.006b
Strep-2	95.0±3.0b	189.0±11.0b	0.280±0.017b	0.074±0.015a	0.223±0.015b
Strep-3	78.0±2.0a	131.0±10.0a	0.261±0.021b	0.075±0.004a	0.177±0.024a
			$25 \mu M$		
Control	80.0±5.0a	199.0±14.0b	0.116±0.004a	0.062±0.003a	0.199±0.011a
Strep-1	76.0±3.0a	192.0±8.0b	0.190±0.003b	0.069±0.009a	0.233±0.013b
Strep-2	88.0±3.0b	195.0±17.0b	0.212±0.008c	0.075±0.012a	0.265±0.012c
Strep-3	93.0±8.0b	146.0±7.0a	0.179±0.017b	0.088±0.010b	0.203±0.017a
			$100 \mu M$		
Control	50.0±3.0a	98.0±18.0a	0.115±0.017a	0.056±0.006a	0.087±0.016a
Strep-1	57.0±7.0a	113.0±16.0b	0.158±0.003b	0.050±0.009a	0.186±0.022b
Strep-2	60.0±10.0a	119.0±13.0b	0.168±0.013b	0.105±0.019b	0.176±0.023b
Strep-3	55.0±2.0a	96.0±29.0a	0.168±0.012b	0.058±0.004a	0.208±0.004b

displayed similar accumulation rates and were significantly different from Strep-2. Moreover, the Cd^{2+} accumulation factor for both 25 μM and 100 μM Cd^{2+} was the highest in the variants inoculated with Strep-1, where AF values amounted to 0.53 and 0.86, for 25 μM and 100 μM Cd^{2+}, respectively.
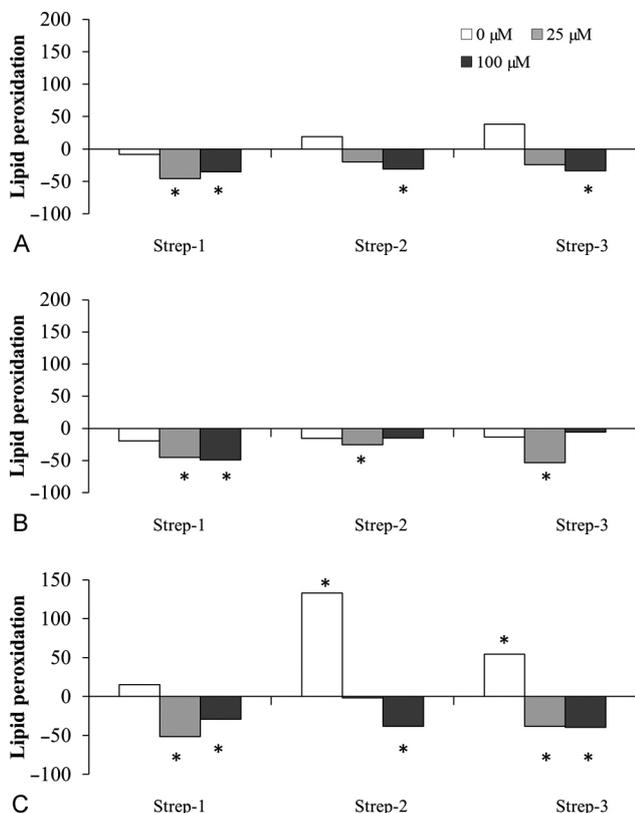
Fig. 2. The level of lipids peroxidation of leaves (A), shoots (B) and roots (C) of *S. viminalis* clones after *Streptomyces* sp. strains inoculation growing at 0 μM, 25 μM and 100 μM Cd^{2+} in the culture medium. The level of lipids peroxidation is represented as the percentage stimulation or inhibition compared to the control (100%) representing uninoculated plants (mean ± standard deviation) * – significantly different compared to control (p≤0.05).
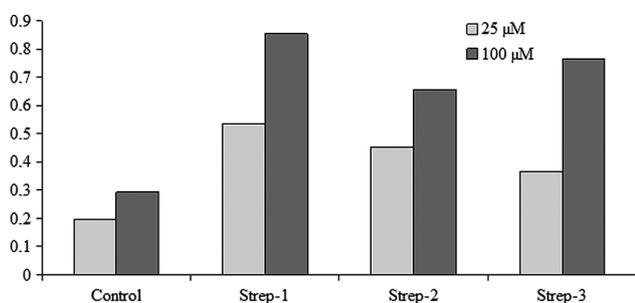
Fig. 3. The accumulation factor (AF) in the *S. viminalis* above-ground parts (shoots and leaves) of the non-inoculated control and inoculated with (Strep-1–Strep-3) growing in medium supplemented with 25 μM and 100 μM Cd^{2+}. Different letters indicate significant differences (p≤0.05) at 25 μM (lower cases) or 100 μM (upper cases) Cd^{2+} concentration

Phytoremediation efficiency of *S. viminalis* in the presence and absence of *Streptomyces* sp. strains

Overall the phytoremediation efficiency of *S. viminalis* leaves and shoot were significantly higher in all the inoculated variants as compared to the non-inoculated controls (Table 3). At 25 μM Cd^{2+} concentration, significant increase of MEA, BCF, Ti, MER by Strep-1 and Strep-2 were seen (Table 3A). A similar effect on inoculation of *Streptomyces sp.* was also observed in variants supplemented with 100 μM Cd^{2+} (Table 3B). Analysing the important factor i.e. MER which represents the percentage of Cd^{2+} removed from the culture medium, revealed a several fold increase after *Streptomyces sp.* inoculation both in the leaves and shoots in all variants (Table 3). Overall, the highest MER values were observed in variants inoculated with Strep-1 with the exception of shoots at 25 μM Cd^{2+} respectively.

Correlation analyses among variants and the studied attributes

Cd^{2+} concentration and dry weight attributes in shoots for all *Streptomyces sp.* inoculated variants were significantly and positively correlated with phytoextraction factors: BCF and Ti, except the association of MDA levels which was negative but significant (Table 1– Supplementary material). In leaves with Strep-1, the MDA levels were negatively correlated to all the studied attributes, while in Strep-2 and Strep-3 where strength of MDA level's association with some attributes were positive but not-significant. Furthermore, a positive correlation of leaves dry weight with BCF and Ti variables was significant for Strep-1 and Strep-2 variants, respectively.

According to PCA analysis performed on the basis of the phytoremediation efficiency factors, lipid peroxidation level and the biomass revealed differences both between variants of inoculation as well as investigated plant organs (Fig. 4). The oxidative stress parameters (lipid peroxidation) were negatively correlated to the non-inoculated variants of plants growing in the 25 and 100 μM Cd^{2+} medium. Based on the results of the phytoremediation efficiency factors, Strep-1 and Strep-3 in the medium supplemented with 100 μM Cd^{2+} show the positive correlation in leaves and shoots, and Strep-2 and Strep-3 at 100 μM Cd^{2+} were positively correlated with roots.

Table 3. The factors of phytoremediation efficiency: metal extraction amount (MEA), the bioconcentration factor (BCF), translocation index (Ti) and metal extraction ratio (MER) in the microbiologically stimulated biomass of *S. viminalis* (leaves, shoots, roots) in the medium with 25 μM (A) and 100 μM (B) Cd²⁺ concentration. (n=5; mean $\pm$ standard deviation). $\uparrow$ or $\downarrow$ – the significant increase or decrease (bold font) in the level of analyzed parameter compared to uninoculated plants (p$\leq$0.05) (Newman Keuls test)

A

Variant	Leaves				Shoot				Roots	
	MEA	BCF	Ti	MER	MEA	BCF	Ti	MER	MEA	BCF
Control	12.87 (1.98)	17.07 (2.15)	87.11 (3.79)	20.07 (3.09)	1.36 (0.19)	3.42 (0.32)	15.65 (1.33)	2.11 (0.29)	24.24 (3.03)	19.61 (2.51)
Strep-1	**88.07** $\uparrow$ **(18.15)**	**72.47** $\uparrow$ **(15.91)**	**295.60** $\uparrow$ **(48.59)**	**62.91** $\uparrow$ **(12.96)**	**3.10** $\uparrow$ **(0.67)**	**7.12** $\uparrow$ **(1.47)**	**30.65** $\uparrow$ **(5.73)**	**4.84** $\uparrow$ **(1.05)**	**35.9** $\uparrow$ **(2.24)**	24.35 (1.73)
Strep-2	**54.65** $\uparrow$ **(15.53)**	**38.75** $\uparrow$ **(9.23)**	**250.56** $\uparrow$ **(75.38)**	**49.68** $\uparrow$ **(14.11)**	**3.41** $\uparrow$ **(0.67)**	**7.17** $\uparrow$ **(1.36)**	**48.89** $\uparrow$ **(8.69)**	**5.31** $\uparrow$ **(1.05)**	26.41 (2.21)	15.72 (1.30)
Strep-3	**21.2** $\uparrow$ **(4.60)**	17.98 (2.91)	**56.36** $\downarrow$ **(18.66)**	**33.05** $\uparrow$ **(7.17)**	5.36 $\uparrow$ (1.28)	9.24 $\uparrow$ (1.41)	29.60 $\uparrow$ (10.51)	8.36 $\uparrow$ (1.99)	43.66 $\uparrow$ (13.16)	33.53 $\uparrow$ (8.01)

B

Variant	Leaves				Shoot				Roots	
	MEA	BCF	Ti	MER	MEA	BCF	Ti	MER	MEA	BCF
Control	20.15 (3.00)	7.05 (0.43)	19.81 (3.43)	7.85 (1.17)	54.84 (6.09)	19.74 (0.55)	55.32 (8.29)	21.38 (2.38)	20.82 (5.22)	36.35 (5.43)
Strep-1	80.90 $\uparrow$ (10.11)	20.01 $\uparrow$ (2.66)	66.32 $\uparrow$ (5.99)	31.54 $\uparrow$ (3.94)	191.59 $\uparrow$ (18.07)	41.10 $\uparrow$ (2.51)	136.82 $\uparrow$ (9.01)	53.35 $\uparrow$ (5.03)	34.98 $\uparrow$ (4.59)	30.08 $\downarrow$ (1.48)
Strep-2	**60.62** $\uparrow$ **(12.49)**	**13.79** $\uparrow$ **(2.09)**	**38.67** $\uparrow$ **(6.16)**	**23.63** $\uparrow$ **(4.87)**	107.57 $\uparrow$ (14.73)	39.86 $\uparrow$ (2.24)	116.12 $\uparrow$ (7.70)	41.94 $\uparrow$ (5.74)	39.24 $\uparrow$ (5.40)	35.69 (0.89)
Strep-3	**54.93** $\uparrow$ **(6.35)**	**12.56** $\uparrow$ **(1.25)**	**37.08** $\uparrow$ **(3.42)**	**21.41** $\uparrow$ **(2.47)**	152.08 $\uparrow$ (31.21)	51.34 $\uparrow$ (11.10)	157.37 $\uparrow$ (32.94)	45.28 $\uparrow$ (6.04)	44.86 $\uparrow$ (0.95)	33.85 (0.24)
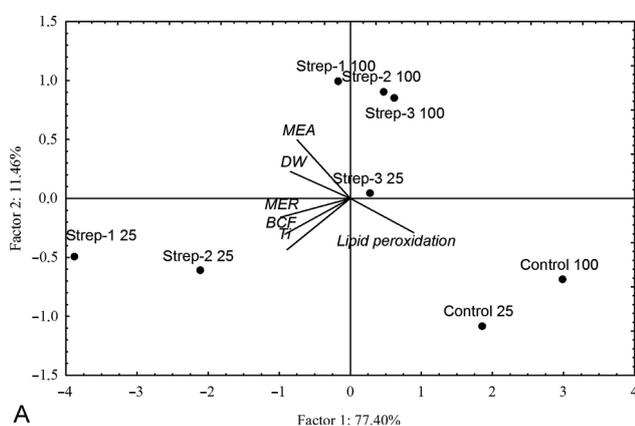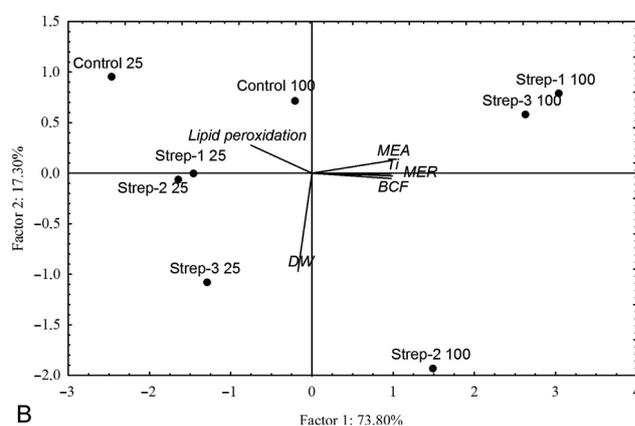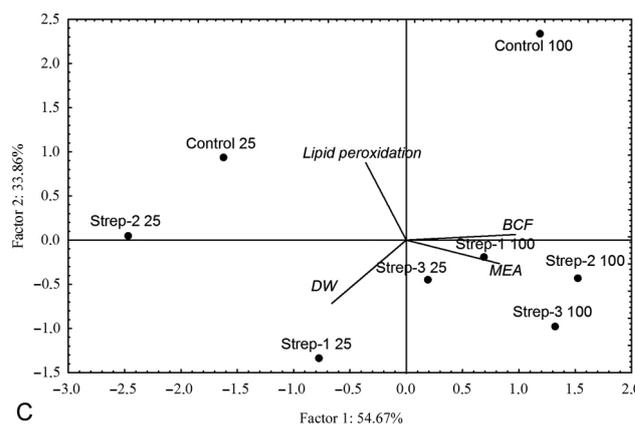
Fig. 4. The Principle Component Analysis (PCA) for the biomass production (DW), phytoextraction efficiency (MEA, BCF, Ti, MER) as well as lipid peroxidation level in the leaves (A), shoots (B) and roots (C) of inoculated and non-inoculated control of *S. viminalis* clones

Discussion

Decrease in plant biomass associated with accumulation of elevated amounts of Cd^{2+} in the plant organs were noted by e.g., Lunáčková et al. (2003), Sinha and Mukherjee (2008), Hattab et al. (2009) and Wei et al. (2012). The same phenomena was found in this study with *S. viminalis* growing in Cd^{2+} supplemented MS media without bacterial inoculation. In this study, bacterial inoculation in *S. viminalis* resulted in the overall increase in plant growth and biomass in Cd^{2+} supplemented MS media. Similar results were received by Dimkpa et al. (2009b) showing increased biomass of sunflower (*Helianthus annuus* L.) after inoculation with SPB *S. acidiscabies* E13. Many other examples of SPB strains promoting plant growth in the presence of Cd^{2+} can be found (e.g., Belimov et al., 2005; Madhaiyan et al., 2007; Kuffner et al., 2008). The main explanation for this enhancement in growth could be the increased Fe solubility and nutrient availability in plants. Most of the previous studies with SPB inoculations were conducted using Cd^{2+} contaminated soils; consequently the stimulation of plant growth was mainly due to the increased supply of appropriate amounts of Fe required by plants (Burd et al., 1998, 2000). The reduced Fe uptake under Cd^{2+} exposure in plants is primarily a result of inhibition of Fe^{3+} reductase activity, which is related to increasing levels of soluble Fe in the soil (Solti et al., 2011; Savvas et al., 2013). However, the role of Fe^{3+} reductase in increasing Fe availability under *in vitro* conditions is less important than in soil, because most of the Fe pool in the culture medium is present in a readily soluble form. Hence, this mechanism may not fully explain the stimulation of plant growth observed in our work. Moreover, Cd^{2+} ions may also cause a decrease in the uptake of other micro- and macro-elements important for plant development, such as Zn, Mn or Ca (Jiang et al., 2004; Suzuki et al., 2005; Wang et al., 2007). Unlike Fe, the reduction in the uptake of micro- and macronutrients takes place by competition with Cd^{2+} ions in transporter systems for absorption by the roots (Nedjimi & Daoud, 2009; Astolfi et al., 2012; Sipos et al., 2013), which may also be important under *in vitro* conditions. Thus, the stimulation of *S. viminalis* growth with different *Streptomyces* sp. inoculation observed in this experiment can be largely explained by the decrease in competition occurring in the transporter systems between Cd^{2+} ions and other elements (e.g., Zn^{2+}, Mn^{2+} and Ca^{2+}) resulting from Cd^{2+} complexation by siderophores.

Another important factor determining the suitable growth of plants is the level of phytohormones, primarily indole-3-acetic acid (IAA), which is responsible for the development and growth of plant biomass (Glick, 2012). The negative impact of HMs on the synthesis of IAA and destruction of the molecule itself, resulting in the inhibition of plant growth is well documented (Seneviratne & Vithanage, 2015). For instance, oxidative degradation of IAA was reported in pea leaves (*Pisum sativum* L.) in the presence of elevated Cd^{2+} and Cu^{2+} concentrations (Chaoui & El Ferjani, 2005; Potters et al., 2007). It is known that the presence of siderophores significantly reduces the degradation of IAA molecules (Dimkpa, 2009a, 2009b). Siderophores can inhibit the formation of free radicals by binding HMs, thus preventing the degradation of auxins, can lower reactive oxygen species (ROS) production and act as a protecting agent of antioxidant systems, e.g. by stimulating peroxidase activity (Rajkumar et al., 2009; Iqbal & Ahemad, 2015; Seneviratne & Vithanage, 2015). This supports the results in our experiment of enhanced *S. viminalis* growth after inoculation, which may be partly explained by the protective role of *Streptomyces* strains in maintaining the appropriate level of endogenous auxins in Cd^{2+} treated plants (the tested strains did not demonstrate the ability to synthesize IAA itself) (Rajkumar et al., 2009; Iqbal & Ahemad, 2015; Seneviratne & Vithanage, 2015). Increase of dry weight in variants of *S. viminalis* inoculated with bacterial strains under *in vitro* conditions is consistent with the results obtained previously in the pot experiment by Złoch et al. (2017). This pot experiment was designed using four types of soils with different levels of heavy metals contamination and the same *Streptomyces* sp. strains (used in the present study) for inoculation of *S. dasyclados* L. These bacterial strains significantly increased dry weight of *S. dasyclados* L. regardless of the type of soil and level of HMs contamination (Złoch et al., 2017). Contrary to *in vitro* results described in this work, strain Strep-1 characterized by the highest siderophores secretion capacity (Złoch et al., 2016), showed a greater effect compared to the other two strains, indicating that the level of bacterial siderophores is more important in soil conditions. Such a phenomenon most likely results from the synergic action of many mechanisms of plant growth stimulation *via* siderophores secretion taking place in the soil, which was described in detail in the review paper by Hrynkiewicz et al. (2018).

Biomass increase is an important factor in the process of HMs phytoextraction (Rajkumar & Freitas, 2008; De Maria et al., 2011), however, efficiency of the HMs uptake and accumulation in the plant organs is considered as the most important parameter in evaluating the usefulness of the specific plant-microorganism system (Wu et al., 2011). There are still two conflicting theories in current literature pertaining to whether the synthesis of bacterial siderophores increases or decreases the availability of HMs (Seneviratne & Vithanage, 2015). After inoculation with SPB strains, both the increase and decrease in HMs uptake

was observed, depending on the plant species, bacterial strains and tested metals (Rajkumar et al., 2010). Sinha and Mukherjee (2008) reported significant stimulation of pumpkin and mustard plant growth as a result of inoculation with *P. aeruginosa* KUCd1 strain under Cd^{2+} presence. The authors explain that besides the ability to synthesize siderophores, *P. aeruginosa* KUCd1 also accumulated metal intracellularly thus reducing Cd^{2+} accumulation in plant biomass, which consequently reduced the availability of Cd^{2+} in the soil. Similarly, in a previously quoted study by Dimkpa et al. (2009a, 2009b) showed that the SPB *S. acidiscabies* E13 strain stimulated the growth of *Vigna unguiculata* by reducing Ni^{2+} accumulation in plant biomass. On the other hand, the same strain simultaneously stimulated growth of *Helianthus annus* and increased Cd^{2+} accumulation in the shoots of the tested plants. Due to the difficulty of simultaneous monitoring of siderophores, as well as their ability to complex metals in soil solution, Ferret et al. (2015) proposed the need to use simplified systems to select the optimal plant-microorganism system. These requirements can be met by *in vitro* experiments that allow the analysis of the effects of toxic substances under strictly controlled conditions (Harms, 1992). In this view, our study employs and demonstrates the importance of using *in vitro* experiments and possibility to predict the results in natural conditions.

In the conducted experiment, inoculation with selected *Streptomyces* strains in *S. viminalis* resulted in several-fold increase in the Cd^{2+} phytoextraction efficiency parameters in above-ground parts (leaves, shoots) in relation to the non-inoculated ones, regardless of the Cd^{2+} concentration. In the case of leaves and shoots, the highest values of MEA, BCF, Ti and MER coefficients were noted for strain Strep-1 which was previously characterized by Złoch et al. (2016) as the highest siderophores secreting strain under increasing cadmium concentrations. Strep-3 was also reported as the strain with the lowest ability to synthesize siderophores and in the present study it has the lowest values for phytoextraction efficiency in comparison to the other 2 strains. Considering that both strains belong to the same bacterial genus (*Streptomyces*) and differ only in the ability to synthesize siderophores (Złoch et al., 2016), suggest that the difference in Cd^{2+} phytoextraction by *S. viminalis* L. under *in vitro* conditions can be linked to the siderophores secretion capacity of the strains. The same effect was noted in the pot experiment (Złoch et al., 2017) wherein the strain Strep-1 demonstrated considerably higher ability for Cd^{2+} accumulation and the other investigated HMs (Zn and Pb) in comparison to the other tested strains, particularly Strep-3 had the lowest effect on the HMs uptake. Similar observations were made by Kumar et al. (2008), while investigating the effect of metalotolerant *Enterobacter*

sp. NBRI K28 and its mutant (*Enterobacter* sp. NBRI K28 SD1) strains both characterized by overproduction of siderophores, on the level of metal accumulation (Zn, Ni, and Cr) in *Brassica juncea*. Both the inoculated strains enhanced plant growth and HMs phytoextraction from contaminated soil. Moreover, the mutant strain exerted a greater impact on metal accumulation than the wild one. Both the above mentioned studies indicate that increased secretion of bacterial siderophores can protect plants against toxic effects of HMs, without lowering the level of metal accumulation in plant biomass. This hypothesis was confirmed in the results of previous studies carried out by Dimkpa et al. (2009b), where inoculation with SPB *S. acidiscabies* E13 strain increased Cd^{2+} accumulation in *H. annus* shoots as much as EDTA addition, simultaneously stimulating biomass growth of above-ground parts.

The measurement of changes of the HMs content in plant biomass provides valuable information on the efficiency of metal uptake by the plant as a result of microbial inoculation. However, this is not sufficient to assess the effectiveness of the designed phytoextraction process. To achieve a reliable evaluation of the phytoextraction efficiency, it is necessary to determine the BCF value (concentration of metal accumulated in plant organs up to its content in the substrate) and the Ti parameter (the distribution of accumulated metal between the roots and above-ground parts: leaves and stems) in plants. According to many authors, plants that demonstrate high phytoextraction potential should not only accumulate higher concentrations of metals in biomass in relation to concentrations measured in the substrate (BCF > 1), but also have a high level of their transport from roots to above-ground parts (Ti > 1 or 100 in the case of percentages) (Mejáre & Bülow, 2001; Sakakibara et al., 2011; Shabani & Sayadi, 2012). This is particularly important because the collection of roots is much more problematic than the leaves and shoots (Tangahu et al., 2011). Lampis et al. (2015) investigated the influence of SPB strains: *Pseudomonas* sp. P1III2 and *Delftia* sp. P2III5 on the efficiency of As phytoextraction from soil by *Pteris vittata*. They measured the changes in As content in plant biomass, and found that inoculation caused an eight-fold and triple increase of BCF and PE (phytoextraction efficiency coefficient) values compared to control plants, respectively.

Our experiment is the first report that incorporates many phytoextraction efficiency parameters (BCF, Ti, MER, MEA, AF) for evaluation of Cd^{2+} accumulation rate of *S. viminalis* L. plants inoculated with bacterial strains under *in vitro* conditions. In general, inoculation of *S. viminalis* L. with *Streptomyces* sp. strains increased both BCF and Ti values. Although inoculation did not influence BCF values in roots,

but significantly increased metal extraction amount (MEA), which may be explained by stimulation of root biomass yield. Considering, that calculated Cd^{2+} BCF values for SPB inoculated plants ranged from 7.12 to 72.47, and measured metal concentrations in leaves (25 μM Cd^{2+}) and stems (100 μM Cd^{2+}) exceeded ~1.2–3.0 times those found in roots, it can be concluded that in accordance with the adopted criteria, used in the experiment, the plant-microorganism system demonstrates a high potential for Cd^{2+} phytoextraction. The phytoextraction potential demonstrated in this work, confirms the results of the earlier performed pot experiment in which the investigated SPB strains increased ~1.3–3.5 times the Cd^{2+} BCF values in above-ground parts of *S. dasyclados* L. (Złoch et al., 2017). Moreover, most of the accumulated Cd^{2+} was translocated to the leaves as indicated by the Ti levels reaching the highest values for variants inoculated with strain Strep-1 – from 123.02 to 155.84. Contrary to the *in vitro* experiment, under soil conditions shoots played only transport role for Cd^{2+} since measured metal concentrations, were 4–10 times lower compared to that found in the roots, regardless contamination level and the microbial strain used (Złoch et al., 2017).

The key factor affecting the success of the phytoremediation process is also the HMs phytotoxicity, which in the long term can lead to significant weakening of plant growth, and thus cause a decrease in the remediation performance of contaminated soils (Glick, 2003; Shin et al., 2012). It has been observed that the presence of Cd^{2+} ions may lead to the ROS production, inducing lipid peroxidation in plants (Mishra et al., 2006; Islam et al., 2008; Xu et al., 2011). The level of MDA is an indicator of the lipid peroxidation in plants and its high accumulation is related to oxidative stress (Cao et al., 2012). In this study, we demonstrated that inoculation with all tested SPB strains considerably decreased the level of MDA accumulation in leaves, stems, and roots of *S. viminalis* L.. Similar observation was noted by Dimpka et al. (2009a) in cowpea inoculated with *S. acidiscabies* E13 SPB strain under Ni^{2+} exposure. Another study on *Oudemansiella radicata* treated with the culture filtrates containing siderophores synthesized by 6 different SPB strains demonstrated a significant decline in MDA level within mycelium exposed to Cd^{2+} and Pb^{2+} (Cao et al., 2012). The above mentioned studies including our results indicate that under HMs contamination, the siderophores can potentially alleviate oxidative stress by binding to free ions of heavy metals. previously, we demonstrated, that the SPB inoculation caused a decline in both MDA and H_2O_2 levels within plant organs, reaching the lowest values in variants treated with strain Strep-1 by 9–22% (H_2O_2) and 25–40% (MDA) with respect to control ones (Złoch et al., 2017).

The correlation analysis including dry weight, phytoextraction efficiency ratios (MEA, BCF, Ti and MER), as well as the level of lipid peroxidation clearly indicates that the applied SPB strains stimulate plant growth and Cd^{2+} uptake, whereas uninoculated plants were characterized by lower dry weight and higher lipid peroxidation level. These observations confirm the idea of Rajkumar et al. (2009) stating that facilitated metal accumulation by plants due to microbiological synthesis of siderophores increases the effectiveness of phytoextraction processes of polluted soils. The results obtained in the course of *in vitro* studies confirm the high efficiency of the selected bacterial strains and are comparable to the microbial assisted phytoextraction of metalliferous soils performed in pot experiments (Złoch et al., 2017). This study also proved the significance of the statement by Watson et al. (2003), that *in vitro* cultures may be successfully used to predict the results of field trials.

Conclusions

The difference in Cd^{2+} phytoextraction by *S. viminalis* L. under *in vitro* conditions can be linked to the siderophores secretion capacity of the strains. The highest siderophores secreting strain Strep-1: Streptomyces sp. SIIB-Zn-R8 (characterized by Złoch et al., 2016) used in this study not only highlights the role of siderophores producing bacteria but demonstrates its application in increasing the phytoextraction efficiency of *S. viminalis* L. The accumulation factor for both 25 μM and 100 μM Cd^{2+} was the highest in the variants inoculated with Strep-1. Analysing the phytoextraction factor i.e. MER which represents the percentage of Cd^{2+} removed from the culture medium, revealed a several fold increase of Cd in the aboveground parts after *Streptomyces* sp. inoculation (specifically by strain Strep-1). In comparison to the pot experiment by Złoch et al. (2017), our observations in this study reveals the advantage of performing *in vitro* experiments that not only shortens the experiment duration and space, but also allows counteracting the problems in performing pot experiments with HM soil (e.g. the difficulty of simultaneous monitoring of siderophores as well as their ability to complex metals in soil solution). This established *in vitro* model system can prove to be a useful tool for prediction of Cd^{2+} phytoremediation efficiency simulating soil condition.

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