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Bacterial ACC Deaminase Activity in Promoting Plant Growth on Areas Contaminated with Heavy Metals

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ABSTRACT

The objective of this study was to explore the possible improvement of plant growth using the activity of the bacterial enzyme ACC (1-aminocyclopropane-1-carboxylate) deaminase (endophytes and rhizobacteria). The beneficial effect of ACC deaminase activity was tested on the plants growing under stress conditions (high concentrations of heavy metals: cadmium, lead, zinc in the soil). The bacteria were isolated from three plants species: *Festuca rubra* L., *Agrostis capillaris* L., *Arabidopsis thaliana* L. Heynh, acquired from the area contaminated with heavy metals. The strains with the highest ACC deaminase activity were used to prepare a bacterial consortium and inoculate the plants. It has been shown that inoculation of plants with ACC producing bacteria has a positive effect on their growth under stress conditions. The bacterial entophytes strains showed a higher activity of ACC deaminase, which resulted in a higher biomass growth of inoculated plants. The PGPB bacteria may limit the toxicity of harmful ions and thus the increase the adaptive properties of plants. Moreover, it was discovered that the bacteria mainly belonging to genus *Bacillus* and *Pseudomonas* had the highest ACC deaminase activity in the environment contaminated with multiple heavy metals. The use of selected microorganisms and plants will provide results in an increasing efficiency of phytoremediation.

Keywords: ACC deaminase, endophytes, heavy metals, plant growth promoting bacteria (PGPB)

INTRODUCTION

Heavy metals are natural components of the Earth's crust. Beside their natural occurrence, most environmental contamination and human exposure result from the anthropogenic activities. Migration of these contaminants into non-contaminated areas as dust or leachates through the soil and spreading of heavy metals containing sewage sludge are a few examples of events contributing towards the contamination of the environment (Kacprzak *et al.* 2014, Kanclerz *et al*. 2016).

Some trace elements, including heavy metals, such as Zn, Cu, Co, Ni and Mn are necessary micronutrients for plant growth, while others have an unknown biological function, such as Cd, Pb, and Hg (Wong-Villareal *et al.* 2016). Excessive level of heavy metal pollution in soil has a harmful effect on biological systems (Salihaj *et al*. 2016). All metals are toxic at higher concentrations, because they cause oxidative stress by formation of free radicals. Another reason why metals may be toxic is that they can replace essential metals in pigments or enzymes, thereby disrupting their function. The hazardous trace elements, especially highly bioavailable ones (Grobelak and Napora, 2015) create unsuitable land for plant growth and destroy the biodiversity (Hadi *et al.* 2014). As a consequence of heavy metal stress, the plant growth is significantly lower than it would be in their absence. Moreover, during its life, a plant is also subjected to a number of non-lethal stresses that limit the plant growth. Certain soil bacteria

can help plants to either avoid or partially overcome a variety of environmental factors. These bacteria improve the plant growth, enhance the root development, and thus increase the plant tolerance to metal stress. Some PGPB (Plant Growth Promoting Bacteria) promote the plant growth by lowering the level of ethylene (Ma *et al.* 2009). Ethylene is a plant growth hormone produced endogenously by plants. It is also produced in soil through a variety of biotic and abiotic mechanisms. Under stress conditions, the production of ethylene is accelerated substantially, which adversely affects the root growth and consequently the growth of the plant as a whole organism. Some plant growth promoting bacteria (PGPB) contain an important enzyme, 1-aminocyclopropane-1-carboxylate deaminase (ACCD) that regulates the ethylene production by metabolizing ACC (precursor of ethylene biosynthesis) into alpha-ketobutyrate and ammonia, which can be used up by the plants (Saleem *et al.* 2007). Ethylene biosynthesis takes place in several stages. The first is the conversion of methionine to S-adenosyl-L-methionine, which is transformed by the ACC synthase into 1-aminocyklopropano-1-carboxylic acid. The last step is to convert ACC using ACC oxidase into ethylene. The ACC synthase activity may be increased due to the high concentration of indole-3-acetic acid. As a result, the concentration of 1-aminocyklopropano-1-carboxylic acid, which is a precursor of ethylene, increases in the plant. Inactivation of 1-aminocyklopropano-1-carboxylic acid prevents ethylene biosynthesis, thereby reducing its concentration in the plant. In contrast, ammonia, one of the products of degradation of ACC, is a nitrogen source for the bacteria. With bacterial ACC deaminase, the growth of plant biomass is stimulated and improves their resistance for functioning under environmental stress conditions. (Saleem *et al.* 2007) PGPB rhizosphere bacteria metabolizing ethylene are applied to protect plants against stress factors, such as floods, the presence of organic toxins, heavy metals, as well as high soil salinity (Glick, 2014).

A better understanding of the characteristics of heavy metal resistance and ACC deaminase-producing PGPB bacteria is needed for the development of efficient phytoremediation systems.

The present study was carried out to understand the mechanism of metal stress decrease in plants: *F. rubra* L. and *B. napus* L. by inoculation of ACC deaminase – producing metal resistant bacteria. In this study, the ACCD activity was analyzed for two groups of bacterial isolates: endophytic and rhizosphere. The aim of the study was to assess the effect of inoculation of plants with selected consortia on promoting the plant growth on the land contaminated with heavy metals. The purpose of the study was to find bacteria in soils with over 50-year history of toxic trace elements contamination, which exhibit very high ACC deaminase activity. Moreover, the aim of the study was to assess the bacteria genus characterized by the highest AAC deaminase activity in multiple heavy metal contaminated environment.

MATERIALS AND METHODS

Donor bacteria

The bacteria strains were isolated from: *Festuca rubra* L. (K), *Agrostis capillaris* L. (M). and *Arabidopsis thaliana* L. Heynh (R). The donor plants species were selected because they were characterized by high resistance in a contaminated environment. The three plants species were common in the barren area surrounding a zinc smelter, with toxic trace elements contamination (Pb, Cd, Zn). The endophytes were applied under phytotron conditions to inoculate fescue grass (phytostabilisation potential) and rape (cruciferous plant family similar to *Arabidopsis,* agricultural and phytoextraction potential). The plants were derived from heavy metal contaminated soil from the areas adjacent to the zinc smelter located in the central part of Silesia (Poland). The root samples were collected from plants. The roots were washed, cut and treated with 70% ethanol. In this study, different disinfection times were used to isolate rhizobacteria (disinfection time 0, only sterile distilled water was used) and endophytic bacteria (10 min disinfection followed by rinsing to eliminate ethanol) (Grobelak *et al.* 2014). The disinfected roots were tested for surface sterility via incubation on a solid nutrient agar medium for 3 days at 28°C. The roots were homogenized, serially diluted and placed in differentiating media: Congo-Red agar (CRA) or nitrogen-free base (NFb) media to isolate free-living diazotrophic bacteria, in Luria agar (LA) to isolate the nutritionally demanding bacteria, and Yeast-Extract-Manitol agar (YEMA) to isolate *Rhizobiaceae* bacteria. Next, bacteria were incubated at 30°C for 2 (CRA, NFb and LA) or 7 days (YEMA) to isolate bacteria. A total of 14 rhizosphere isolates and 38 endophytic isolates were collected. Donor bacteria were kept in 40% glycerol at –20°C for

maintenance (Sgroy *et al.* 2009). Isolated bacteria were tested for 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity., Only 6 isolates with the highest activity of ACCD were selected for the further study.

DNA isolation and 16S rRNA gene analysis

The selected endophytic bacteria and rhizobacteria were cultured in an LB medium at 28°C for 24 h. Then, 1.5 ml of the LB medium with bacteria was transferred into the Eppendorf tubes and centrifuged at 45 000 g for 5 minutes at 21°C. The genomic DNA was extracted from the pellet using the GeneMATRIX Bacterial Genomic DNA Kit (EurX, Poland). The 16SrDNA genes were amplified by PCR using the universal sequencing primer 518F as the forward primer (5 -CCA GCA GCC GCG GTA ATACG-3) and the 800R as the reverse primer (5 -TAC CAG GGT ATC TAATCC-3). The amplification was performed using a thermal cycler (Eppendorf). The PCR fragments were frozen and stored at –20°C. The PCR products were separated using 1.5% agarose gel electrophoresis and Sanger sequenced. The 16S rDNA sequences were compared with registered sequences in the Gen Bank database (Fig. 1) using NCBI.

ACCD activity

The bacteria isolates were propagated in an LB liquid medium (to obtain OD600 = 1) and inoculated (1 ml) into 9 ml of M9 medium (5.8 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.25 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 % glucose, 0.3 µg/ml biotin). The isolates were incubated (3 days, 30°C). Afterwards, the cell suspension was centrifuged, washed in 0.1 M Tris-HCl (pH 7.5) and centrifuged again. For the centrifuged bacterial pellet, 4 ml of the modified M9 medium, enriched with ACC (5 mM) was added. The Eppendorf tubes were incubated in a laboratory shaker (3 days, 30°C). Afterwards, the bacterial suspension was centrifuged (10 min; 10 000 g), then washed twice in 0.1 M Tris-HCl, (pH 7.5) and centrifuged again. The bacterial pellet was diluted in 400 ml of 0.1 M Tris-HCl (pH 8.5). Subsequently, the 20 ml of toluene (5 %) was added and vortexed at the highest speed for 30 seconds. Then, 200 ml of cell extract was added with 20 ml of 0.5 M ACC. The samples were incubated at 30°C for 30 min. After the incubation period, the 1 ml of 0.56 N HCl was added and centrifuged (5 min; 10 000 g). Next, 800 ml HCl (0.56 N) was added to 1 ml of the supernatant, then vortexed and 300 ml 2.4-dinitrophenylhydrazine (0.2%) was added. The samples were incubated (30°C; 30 min) and then 2 ml NaOH (2 N) was added and vortexed. The absorbance of the prepared solution was measured at 540 nm using a scectrophotometer. The calibration curve was made on α-ketobutyrate (KB) in a concentration of 0.1 M to 1 M. The enzyme activity was expressed in mM/h of on α-ketobutyrate (KB) (Sgroy *et al.* 2009). Three replicates were used

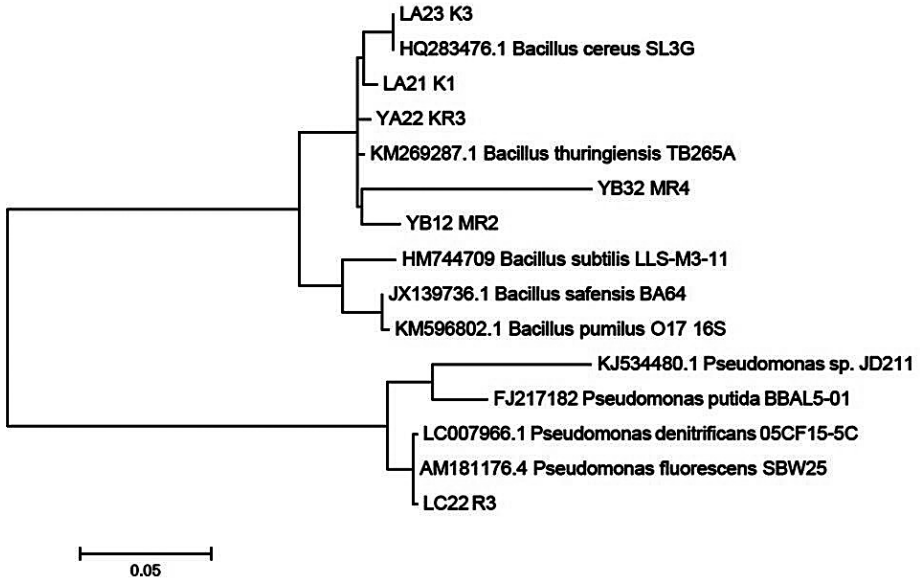


Fig. 1. Phylogenetic tree with two distinct clades corresponding to *Pseudomonas* sp. (endophytic strain R3) and *Bacillus* sp. (endophytic K1 strain and K3 strain identified as *B. cereus* and rhizobacteria strains: MR4, MR2 and KR3 identified as *B. thuringiensis*)

for all study samples. A mathematical analysis of the data received was made in the Excel 2007. An alteration of ACC concentration in samples was obtained by normalization to the average value. Data was also analyzed using the method of one-way analysis of variance (ANOVA).

Preparation of the bacterial consortium

In the experiment, two consortia were used. All bacterial strains were tested in plates on antagonistic activity between the strains. On the basis of the ACC deaminase activity, three isolates with the highest ACCD activity were selected for studying the effects of the strains on plant growth (Fig. 2). The first consortium consisted of three strains of rhizospheric isolates and a second consortium comprised three strains of endophytic isolates. The next phase was to immobilize the selected consortia of sodium alginates solution with a concentration of 2% w/v of LB medium (Luria Bertani) (0.8 g with 40 ml of double distilled H_2O) and calcium chloride solution (2.5% w/v). The sodium alginate was mixed with a suspension of bacteria (bacteria were propagated in LB medium, OD600 nm=1, then centrifuged) and stirred until homogeneous. The suspension of bacteria was transferred into the cylinder and added dropwise to a solution of calcium chloride. It was obtained in the form of a bed of beads with a diameter of 1.5–3 mm. Then, the conditioning time was applied (10 minutes) and beds were filtered on a Buchner funnel (Shin *et al.* 2007). The next phase of the experiment was the application of consortia to the trace elements contamination soil (Grobelak and Napora, 2015). The soil (Pb 9045–1209.7 mg/kg d.w; Cd 8.57–16.8 mg/kg d.w; Zn 773–1100 mg/kg d.w.) was sieved through a sieve with 1 mm mesh size. Due to the low pH of the soil, the lime fertilizer (0.8% weight) was added. Three replicates (pots) of each treatment were prepared. The concentration of bacterial cells calculated for OD600 nm=1.0 was determined for $60\times10^{7.6}$ to $10^{8.2}$ cells per pot (Reed and Glick, 2005). The control soil was also amended with alginate without the bacteria. The seeds were sown directly in the contaminated soil after mixing the soil with alginate beds (with or without bacteria). The experiment was carried out in the growth chamber under controlled conditions, optimal for the growth and development of plants (temperature: day 21°C, night 18°C, humidity approx. 85%). In this study, two plant species were used: *B. napus* L. and grass used in phytoremediation – *F. rubra* L. A plant growth experiment was conducted for 3 months (pot volume 2 l). Then, the obtained biomass gain was determined.

RESULTS AND DISCUSSION

In order to classify the different unknown bacteria**,** the rhizobia phylogenies based on the 16S rRNA gene were presented (Fig. 1). The 16S rRNA sequence obtained in this way was compared using BLAST tool with nucleotide database. It was found that six sequences with accurate

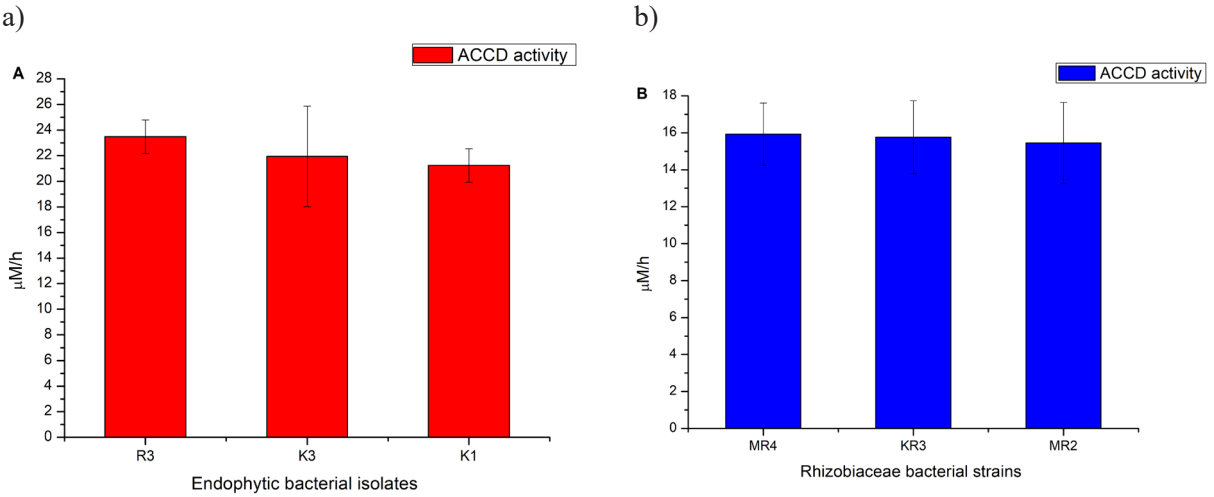


Fig. 2a. ACCD activity of (A) endophytic strains: R3 (23 mM/h); K3 (21.94 mM/h); K1 (21.23 mM/h). The results were shown as mean with SD

Fig. 2b. ACCD activity of rhizospheric strains: MR4 (15.92 mM/h); KR3 (15.76 mM/h); MR2 (15.45 mM/h). The results shown as mean with SD

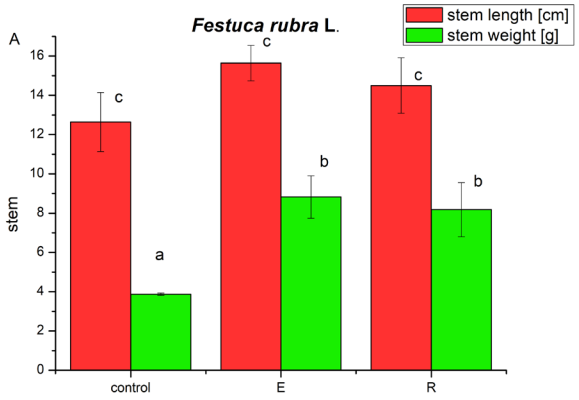
species annotation showing the highest sequence homology proportion and query coverage with lowest the lowest E values were used to develop Neighbor Joining (NJ) tree using MEGA 4.0 for multiple sequence alignment. Phylogenetic tree analysis showed that strains have maximum similarity with: *R3(Pseudomonas sp.), K3 (B. cereus), K1 (Bacillus sp.)* and rhizobacteria strains*: MR4 (Bacillus sp.); KR3 (B. thuringiensis.); MR2 (Bacillus sp.)*. In the biochemical tests, 38 endophytic isolates and 14 isolates of rhizobacteria were tested for the ACCD activity. It was found that 85% of endophytic isolates produced ACC-deaminase. The highest activity of ACC deaminase was observed for R3 isolate (23.49 μM/h); 79% of rhizobacteria isolates produced ACC-deaminase. For these strains, the activity was high and was recorded at the level of (15.92 μM/h) for MR4.

The effect of plant growth promotion was found for the consortia of three isolates. These results are in agreement with the studies where the inoculation effect of the plant growth-promoting bacteria (PGPB) on the growth of plants was determined (Rashid *et al.* 2011). The consortium of bacteria is capable of rapid colonization of the roots during the growing season. It is known that microorganisms isolated from the environment, contaminated with heavy metals, often exhibit tolerance to multiple pollutants because they have adapted to such conditions (Pal *et al.* 2005). According to Glick (2014), ACC may act as a source of nitrogen for some soil bacteria. Recent studies suggest that the mechanism is more complicated and the ACC deaminase activity may be more important in stress minimizing, than in competition among other rhisospheric microorganisms. The investigated soil was also very poor in microorganisms.

The biochemical tests have shown that the endophytic bacteria strains have a greater potential for ACCD production (Fig. 2). The ACC deaminase has been proposed as an agent that plays an important role in the association process of plants and bacteria (Reiter *et al.* 2002). PGPB can significantly increase the growth of plants, especially under difficult conditions or the presence of heavy metals (Khan, 2005). This study has shown that plant inoculation with bacteria consortium (endophytic or rhizobacteria) significantly affected the weight and height of plants (Fig. 3–4).

The endophytic microbes residing inside the host plants, with ACC deaminase activity, can benefit the host plant by reducing stress and increasing the plant growth (Hardoim *et al.* 2008). In the study of Belimov *et al.* (2005), the isolated cadmium-resistant *V. paradoxus* from the rhizosphere of *B. juncea* promoted the plant growth. In general, the metal resistant rhizospheric bacteria have an exceptional ability to protect the host plants from metal toxicity by several possible mechanisms, the best known mechanism being the utilization of ACC by rhizospheric bacteria.

a)



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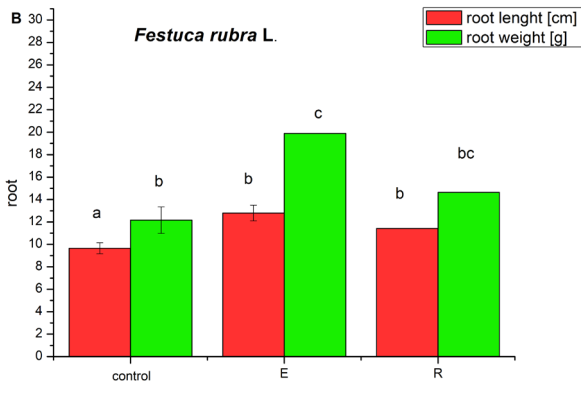
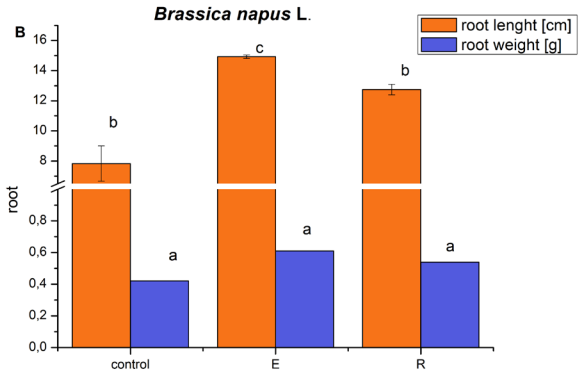


Fig. 3a. *F. rubra* L. – stem length and weight (control – plants not inoculated with the bacterial consortium; E – plants inoculated with the endophitic bacterial consortium; R – plants inoculated with the Rhizobiaceae bacterial consortium. The results were shown as means (SD). The means not sharing the same letter are statistically different ($p<0.05$)

Fig. 3b. *F. rubra* L. – roots length and weight (control – plants not inoculated with the bacterial consortium; E – plants inoculated with the endophitic bacterial consortium; R – plants inoculated with the Rhizobiaceae bacterial consortium. The results were shown as means (SD). The means not sharing the same letter are statistically different ($p<0.05$)

a)



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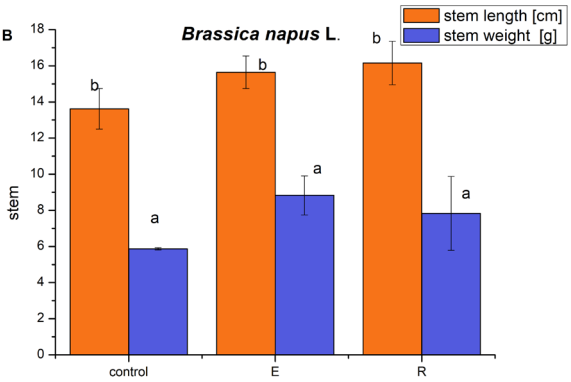

Fig. 4a. *B. napus* L. – stem length and weight (control – plants not inoculated with the bacterial consortium; E – plants inoculated with the endophitic bacterial consortium; R – plants inoculated with the rhizospheric consortium. The results were shown as means (SD). The means not sharing the same letter are statistically different ($p<0.05$)

Fig. 4b. *B. napus* L. – roots length and weight (control – plants not inoculated with the bacterial consortium; E – plants inoculated with the endophitic bacterial consortium; R – plants inoculated with the rhizospheric consortium. The results were shown as means (SD). The means not sharing the same letter are statistically different ($p<0.05$)

Dell'Amico *et al.* (2008) proved that the bacteria having the characteristics of producing ACC deaminase, may enhance the metal accumulation in plant tissue with concurrent stimulation of plant growth. Inoculation with endophytic isolates might have a significant potential for improving the phytoextraction efficiency in metal-contaminated soils (Rajkumar *et al.* 2009). Statistical analysis of the results (*F. rubra* L., *B. napus* L.) showed a significant impact on the plants inoculation (with the endophytic bacterial consortium and rhizobacteria). A one-way ANOVA test showed that the results are statistically significant for both the weight and length of the root and the stem of the tested plants. The highest increase in the weight and length of the stem (Fig. 3) for *F. rubra* L. was obtained after inoculation of the endophytic consortium. These results indicated that the activity of ACCD has an impact on the effective promotion of plant growth, the highest ACCD activity and the highest biomass. Similar pieces of evidence that endophytic bacteria promote plant growth were obtained from crop studies, including potato and clover (Bashan and Bashan**,** 2005). The highest increase in the weight and length of the root (Fig. 3) of *F. rubra* L. was also obtained after inoculation of the endophytic bacterial consortium. The increase in the above-ground parts (stems) was correlated with better root development (Fig. 5). The role of ACCD in decreasing the ethylene levels by the enzymatic hydrolysis of ACC into KB and ammonia has been presented as one of the major mechanisms of PGPB in promoting the root development and plant growth under metal stress conditions (Madhaiyan *et al.* 2006). The inoculation of rape (*B. napus* L.) (Fig. 4) with a bacteria consortium has an impact on its growth. For an increased stem length, a more pronounced effect was observed after inoculation with a rhizospheric consortium. For the stem weight of rape, higher results were noted after inoculation with endophytic consortium (statistically confirmed impact). As shown in Figure 4, inoculation of plants with a bacterial consortium resulted in the growth of root biomass. The endophytic consortium of bacteria was more effective in promoting root length. Bacterial strains utilizing ACC as a sole source of nitrogen use ACC and enhance the elongation of plant roots (Glick *et al.* 1998). This was also confirmed in this study. The bacteria used in this experiment improved the plant growth and enhanced the dense root system. The obtained results suggest an increase of plant tolerance to metals. In turn, well developed plants are more capable of restoring the plant cover the soils contaminated with trace elements.

CONCLUSIONS

In conclusion, the results indicate that inoculation with ACCD active PGPB may facilitate plant growth and thus increase the phytoremediation efficiency. It was found that the applied

bacteria improved the biomass of plants under very difficult soil conditions. PGPB containing ACCD activity could be helpful in sustaining plant growth and development under stress conditions by reducing the stress-induced ethylene production. Achieving plant biomass for such areas is very difficult and can have a very significant impact on profitability phytoremediation.

Plant inoculation was beneficial for plant growth. The conducted research confirmed that among (52) isolates, (6) have much higher ACCD activity. Our research confirmed that such isolates can be used to improve the plant growth in heavy metal contaminated areas. The conducted research showed that high bacterial ACCD activity has an impact on promotion of the growth of roots biomass. It was found that the bacteria screening in soils with over 50 years toxic trace elements contamination have very high ACC deaminase activity. Moreover, it was discovered that the bacteria mainly belonging to genus *Bacillus* and *Pseudomonas* had the highest AAC deaminase activity in the environment contaminated with multiple heavy metals.

Some bacteria can adapt to stress conditions and metal-resistant bacteria can survive in contaminated environment. The use of plant growth promoting bacteria in assisted phytoremediation of metal contaminated sites is necessary.

Acknowledgements

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