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Use of overburden waste for London plane (*Platanus* × *acerifolia*) growth: the role of plant growth promoting microbial consortia

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Overburden waste dumps represent a huge threat to environmental quality. The reduction of their negative impact can be achieved by vegetation cover establishment. Usually, this action is complicated due to site-specific characteristics, such as nutrient deficiency, elevated metal concentration, low pH value, lack of moisture and lack of organic matter. Establishment of vegetation can be facilitated by inoculation with plant growth promoting bacteria (PGPB) which improve the physicochemical and biological properties of degraded substrates and make them more hospitable for plants. In this study we selected several strains based on the ability to produce ammonia, indole-3-acetic acid, siderophores and lytic enzymes, and to solubilize inorganic phosphates. This selection resulted in microbial consortia consisting of *Serratia liquefaciens* Z-I ARV, *Ensifer adhaerens* 10_ ARV, *Bacillus amyloliquefaciens* D5 ARV and *Pseudomonas putida* P1 ARV. The effects of PGPB consortia on one-year-old London plane (*Platanus* × *acerifolia* [Aiton] Willd.) seedlings replanted into overburden waste from Kolubara Mine Basin were examined. After seven months, inoculated seedlings were 32% higher with 45% wider root collar diameter and over 80% higher total dry biomass compared to uninoculated seedlings grown in Kolubara's overburden. Inoculation resulted in higher amounts of total soluble proteins, higher chlorophyll and epidermal flavonoids content and higher total antioxidative capacity in the leaves. This study represents a successful search for effective PGPB strains and shows that microbial consortia have an important role in enhancing the growth of seedlings in nutrient deficient and degraded substrates such as overburden waste from open-pit coal mines. Positive response of London plane seedlings suggest that inoculation may help widening the opus of species for reforestation of post mining areas and speed up natural succession processes and recovery of degraded landscapes.

Keywords: Plant Growth Promoting Bacteria, London Plane, Overburden Waste, Revegetation

Introduction

Soils can be disturbed by a wide range of factors concerning unfavorable agricultural management, industry, mining activities, etc. Surface mining exerts long-term negative impact on the environment, destroying large areas of natural landscapes. During coal exploitation geological layers above and around the ore body are disturbed, and piled up in mixtures forming overburden deposits. The disposal of overburden is made non-selectively, resulting in new relief forms (Ristović et al. 2010). The image of open-pits destructive character is visible only 50 km southwest of Belgrade (Serbia) at Kolubara Mine Basin (Lazarevac district, Serbia). Currently, at this location, mining activity occupies over 5730 ha while overburden waste dumps cover 3395 ha. Even though large areas of overburden dumps are an environmental issue, causing erosion, water and air pollution, recultivation has been carried out on only 882 ha (Report on the state of environment in Branch MB Kolubara, Lazarevac - 2015).

Revegetation of overburden waste dumps is a worldwide problem, and establishment of vegetation cover on such foundations is complicated due to a numerous problems such as nutrient deficiency, elevated metal concentration, low pH value, lack of moisture, soil forming materials and of organic matter, high heterogeneity of substrate, disturbed soil hydrology and topography (Ristović et al. 2010, Karličić et al. 2016). At impoverished mine sites, enrichment with organic matter (cover crops, mulch, compost, hay) and encouragement of symbiotic relationships between plants and soil microbes makes a difference between life and death (Tredici 2007). In addition to already mentioned approaches, inoculation with plant growth promoting bacteria (PGPB) is emerging as a promising technique (Ribeiro & Cardoso 2012, Karličić et al. 2016).

PGPB reside in the rhizosphere, root surface, and plant inner tissues (Glick 2012) and stimulate plant growth through a variety of mechanisms. PGPB directly affect

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plant growth by improving nutrient assimilation through fixation of atmospheric nitrogen, siderophores production, solubilization of phosphorus, and/or other unavailable forms of nutrients (Glick 2012). Production and/or suppression of growth regulating hormones (auxins, gibberellins, cytokinins) is another effective direct mechanism for plant growth enhancement. Indirect mechanisms include antagonism against phytopathogens, niche competition, and increased disease resistance (Glick 2012).

Plant growth promoting bacteria improve physicochemical and biological properties of poor, degraded substrates and make them more suitable for plants (Grandlic 2008, Karličić et al. 2016) with simultaneous increase of plant survival and seedling quality, especially in soils with weak microbial activity (Chanway 1997, Dominguez-Nuñez et al. 2015). Positive outcomes of PGPB application are enhancement of seedlings emergence, faster plant growth, higher biomass production (Ribeiro & Cardoso 2012), increase of root length, and branching, increased leaf area, and chlorophyll content, and higher resistance to abiotic stresses (Glick 2012) as well as to pests or diseases (Cawoy et al. 2014).

After more than a century of research, beneficial effects of PGPB are now exploited in agriculture, horticulture, forestry and environmental restoration (Lucy et al. 2004). Studying the interactions between PGPB and angiosperms/gymnosperms started during 1980s and 1990s (Chanway 1997); however, up to now, the opus of tested species cover a very few genera, such as *Pinus* sp., *Tsuga* sp., *Pseudotsuga* sp., *Quercus* sp., *Eucalyptus* sp. etc. (Rodriguez-Barrueco et al. 1991, Ribeiro & Cardoso 2012).

In previous attempts, Kolubara Mine landscapes were reforested with Black pine, Scots pine, European larch, European ash, Small-leaved lime, and Black locust (Karličić et al. 2016). Recultivation was mainly conducted with pioneer species, even though some of them were not the most desirable for our climate conditions (*e.g.*, Black locust). Through their beneficial activity on soil and plants, PGPB may help widening the opus of species for reforestation of overburden areas by providing the space for more demanding ones. This assumption was tested in the present study and London plane (*Platanus* × *acerifolia* [Aiton] Willd.) was used as test plant. The ornamental values of plane trees make them a valuable part of urban landscapes all over the world. The members of *Platanus* spp. are well known as street trees but also are suitable for phytoremediation purposes and reforestation of post-mined land (Skousen & Zipper 2014, Kang et al. 2016). Interestingly, even though London plane is one of the most frequent species in the public open space, so far there have been no studies dedicated to London plane - PGPB interactions.

Our starting hypothesis was that the overburden waste enriched with PGPB may represent a suitable substrate for London plane growth. For this purpose the search for the most effective PGPB strains was conducted. The final confirmation of PGPB beneficial influence was obtained through *in vivo* experiments. Monitoring the influence on London plane growth and performances in overburden waste confirmed the success of the search.

Material and methods

Collection of bacterial isolates

Forty four isolates of soil bacteria from the Laboratory for Microbial Ecology, Faculty of Agriculture, University of Belgrade (Serbia) represented the starting point of the experiment and were tested for the presence of plant growth promoting features.

Plant growth-promoting (PGP) features

Ammonia (NH_3) production was tested by growing isolates in 10 ml peptone water. After 48-72 h at 28 °C, Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow color was a positive test for ammonia production (Cappuccino & Sherman 1992).

Assay for indoleacetic acid (IAA) production was conducted using the colorimetric method (Patten & Glick 2002). The isolates were grown in 10 ml of minimal salt media supplemented with 100µg ml^{-1} of l-tryptophan (Sigma Aldrich, USA). Absorption was read after 48 h at 530 nm with a spectrophotometer (T70 UV/VIS Spectromer, PG Instruments LTD, UK). The amount of IAA was determined using a calibration curve of indole-3-acetic acid (Sigma Aldrich, USA) in the 1-100 µg ml^{-1} range.

Siderophore production was detected on the Chrome azurol S (Fluka, USA) agar medium (Alexander & Zuberer 1991). Chrome azurol S agar plates were spot inoculated with test organisms and incubated at 28 °C for 48-72 h. Development of yellow-orange halo zones around colonies was considered as positive result for siderophore production.

Phosphate solubilizing activity was tested on National Botanical Research Institute's phosphate growth medium (NBRIP – Nautiyal 1999). Strains were incubated for 14 days at 30 °C, and afterwards the presence of halo zones around colonies were used to indicate their phosphate solubilization capability. P solubilization index was calculated using the following formula (eqn. 1):

$$SI = \frac{Ø_{(colony)} + Ø_{(halozone)}}{Ø_{(colony)}} \quad (1)$$

Qualitative determination of lipase, N-acetyl-β-glucosaminidase and β-glucosidase was performed by API ZYM kits according to the manufacturer's protocol (Bio Mereux, France). The API strips were inoculated with 24-h-old cultures, and incubated at 30 °C for 4 h. The evaluation of the activity was carried out by comparing the colored reaction with the manufacturer's color chart. Protease production was determined using skim milk agar (Chaiharn et al. 2008). The agar plates were spot inoculated with test organisms and incubated at 30 °C for 5 days. The presence of a clear zone around the colonies indicated protease activity. The presence of cellulase was determined using carboxymethyl cellulose (CMC) agar method (Angsana et al. 2009). The agar plates were spot inoculated and incubated at 30 °C for 48 h. Plates were flooded with 0.1% congo red solution and were destained with 1M NaCl solution after 10 minutes. The appearance of clearing zones around colonies indicated cellulase activity.

Hydrogen cyanide (HCN) production was determined using nutrient broth amended with glycine (4.4 g l^{-1}). Bacteria were streaked on agar plate. Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with parafilm and incubated at 28 °C for 4 days. Development of orange to red color indicated HCN production.

Molecular identification of isolates

Isolates that showed the most prominent plant growth promoting activities were molecularly identified by sequencing the *gyrB* gene. Genomic DNA was prepared by using ZR Soil Microbe DNA MiniPrep (Zymo Research, USA). The amplification of *gyrB* gene was performed with a thermal cycler (Kyratec, Australia, Model: SC300T) using the primer sets presented in Tab. 1.

The reaction mixtures (50 µl) contained 0.2 mM of each dNTP (Kapa Biosystems, UK), 1 µM of each primer, 0.5 U Robust HotStart DNA Polymerase (Kapa Biosys-

Tab. 1 - Primer pairs used for PCR amplification of *gyrB* gene fragments (R=A or G; Y=C or T; M=A or C; N=any).

Primers	Sequence (5'-3')	Isolate
gyr-320 (fw)	TAARTTYGAYGAYAACTCYTAYAAAGT	Z-I ARV
rgyr-1260 (rv)	CMCCYTCCACCARGTAMAGTTC	(Dauga 2002)
UP-1E (fw)	CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA	10_ARV, D5 ARV
modUP2r (rv)	TGTAAAACGACGGCCAGTCCRTCNACRTCNGCRTCNGTCAT	(Yamamoto et al. 2000)
UP-1E (fw)	CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA	P1 ARV
APrU (rv)	TGTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA	(Yamamoto et al. 2000)

tems, UK) and 20 ng of DNA template. PCR reactions were performed as previously described by Dauga (2002) and Yamamoto et al. (2000). PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Germany), and sequenced with the primers presented in Tab. 2.

PCR products were sequenced on ABI 3730XL Sequencer (Macrogen Inc., Seoul, Korea) in both directions. Alignment of obtained sequences was performed using the Clustal W 2.0 algorithm and MEGA5 software. The BLAST database of National Centre for Biotechnology Information (NCBI – http://www.ncbi.nlm.nih.gov) was used to compare the sequence of bacteria with those of known bacterial species in the existing database. Sequences were deposited in the GenBank database (Accession Numbers: KT265088, KT265086, KT265087, and KT265089, for Z-I ARV, 10_ARV, D5 ARV, and P1 ARV strains, respectively).

Tab. 2 - Primer pairs used for sequencing (R=A or G; Y=C or T; M=A or C).

Primers	Sequence (5'-3')	Isolate
gyr-320 (fw)	TAARTTYGAYGAYAACTCYTAYAAAGT	Z-I ARV
rgyr-1260 (rv)	CMCCYTCCACCARGTAMAGTTC	
M13R-pUC (fw)	CAGGAAACAGCTATGAC	10_ARV, D5 ARV, P1 ARV
M13-FP (rv)	TGTAAAACGACGGCCAGT	

Substrates and plant material

Substrates used for plant cultivation were Floradur® Plant Universal (FloraGard, Germany) and overburden waste from Kolubara Basin (Serbia). Floradur® Plant Universal contained: 50% white peat, 30% black peat, and 20% compost, with pH 5.8. Analyses of overburden were performed at the beginning of the experiment, before planting. Samples were taken from 20 different spots according to standard soil sampling principles. Obtained amount was homogenized and successive splitting by cone and quarter technique was applied. Overburden was composed of 43.2% clay, 52.7% silt, and 4.1% sand (determined with soil texture triangle according to Rowell 1994). Previous analyses of overburden waste showed neutral pH value, low content of nitrogen, humus (0.15%), and of organic carbon (Karličić et al. 2016). Prior to use, overburden waste was air-dried, ground, and sieved through a 2 mm diameter sieve.

One-year-old bare root seedlings of *Platanus x acerifolia* Aiton (Willd.) with mean height of 19.55 cm and mean collar diameter of 0.29 cm were obtained from the Nursery of forest and ornamental plants Vikumak (Idoš, Serbia).

Microbial consortia inoculum preparation

Inocula were prepared from four separately propagated isolates. Three bacterial strains (*Serratia liquefaciens* Z-I ARV, *Bacillus amyloliquefaciens* D5 ARV and *Psedomonas putida* P1 ARV) were grown in nutrient broth aerobically at 28 ± 2 °C / 48 h / 100 rpm (Biosan, Latvia). *Ensifer adhaerens* 10_ARV was grown in Fjodorov medium (Anderson 1958) at 28 ± 2 °C / 72 h / 100 rpm. The bacterial suspensions were centrifuged at 6000 × *g* for 10 minutes (5804 R, Eppendorf, Germany) and diluted in sterile distilled water to adjust bacterial cell density to 10^8 CFU mL^{-1}. Bacterial strain-specific inocula were mixed together to form a consortia inoculum in the 1:1:1:2 ratio (*Serratia liquefaciens* Z-I ARV: *Bacillus amyloliquefaciens* D5 ARV: *Psedomonas putida* P1 ARV: *Ensifer adhaerens* 10_ARV).

In vivo trials

The experiment was based on three treatments: (a) seedlings replanted into commercial substrate, Floradur® Plant Universal (FS); (b) seedlings replanted into overburden (O); (c) seedlings replanted into overburden and inoculated with PGPB consortia (OI). All seedlings were placed in 2.5 dm^3 volume of substrate in 3 dm^3 polyethylene bags during the period of dormancy. Inoculation was conducted two times, at the beginning of the growing season (March) and 12 weeks later. Each seedling in the OI treatment received 100 ml of inoculum prepared as described above. Seedlings in the other two treatments (FS and O) received 100 ml of distilled water. Plants were grown until the beginning of October. The temperature conditions in Belgrade during the experiment are presented in Tab. 3. The trial was performed outdoors at a location of the Faculty of Agriculture in Belgrade (Serbia) and was arranged as a completely randomized split-plot design (n = 20 seedlings per treatment).

Plant growth measurements and leaf analyses

Seedling height and root collar diameter were recorded two times. The first measurement was conducted 18 weeks after the first inoculation (July) and the second in October. At the end of the experimental period (October), shoot and root dry weights were also recorded. Plants from each treatment were grouped and dried at 65 °C until constant weight.

For biochemical analyses, fully developed, green and vital mid-shoot leaves without visible signs of senescence were sampled in the beginning of October (harvest period) and ground in liquid nitrogen. Soluble proteins were extracted in 100 mM potassium-phosphate buffer (pH 6.5) with 0.1% Triton X-100 (w/v), 5% insoluble polyvinylpyrrolidone (PVP) and 1 mM phenylmethylsulfonyl fluoride (PMSF), according to Vidović et al. (2015).

Following centrifugation at 16,000 × *g* (5415 R, Eppendorf, Germany) for 10 min at 4 °C, protein content in the soluble fraction was determined using bovine serum albumin as standard (Bradford 1976). For determination of total soluble phenolics content, leaf samples were homogenized in liquid nitrogen and extracted in methanol with 0.1% HCl (Vidović et al. 2015). Following centrifugation, the supernatants were separated and the content of total soluble phenolics was determined using the Folin-Ciocalteu reagent, as described by Morina et al. (2008). The concentrations of phenolics in methanolic extracts were calculated using gallic acid as a standard (Singleton & Rossi 1965). Total antioxidative capacity of methanolic extracts was measured using 2.2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ABTS assay, as described by Morina et al. (2008). For simultaneous *in vivo* assessment of total leaf chlorophyll and epidermal flavonoids content, optical leaf-clip meter Dualex4Scientific (Force-A, Orsay, France) was used, as described in Cerović et al. (2012).

Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) followed by LSD tests ($p < 0.05$) and Independent Two Sample *t*-test. The analyses were conducted using the software package SPSS v. 22 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Overburden waste dumps represent huge areas of unproductive land with erosion susceptibility and hazardous influence on soil, water, and air quality. These problems are usually solved through vegetation development (Ristović et al. 2010).

Tab. 3 - Daily maximum and daily minimum temperature range during the outdoor experiment (data for Belgrade, Serbia, obtained from the Hydrometeorological Service of Serbian Republic).

Period	Daily maximum temperature range (°C)	Daily minimum temperature range (°C)
February (dormant season)	-0.3 → 23.4	-6.4 → 8.8
Spring (March, April, May)	8.4 → 29.7	1.5 → 17.8
Summer (June, July, August)	18.0 → 34.8	11.0 → 22.9
September	15.8 → 28.3	8.0 → 20.0

Tab. 4 - Plant growth promoting attributes of isolated strains. (NH_3): ammonia; (IAA): indoleacetic acid; (Sid): siderophores; (PSI): phosphate solubilization index; (+) presence of the feature; (-) absence of the feature. (*): selected strains.

Isolates	Production			PSI
	NH_3	IAA (µg ml^{-1})	Sid	
3N ARV	-	1.7 ± 0.3	+	-
6N ARV	+	0.4 ± 0.1	-	-
7N ARV	+	0.8 ± 0.1	-	2.33 ± 0.45
8N ARV	+	0.8 ± 0.2	-	-
15N ARV	+	0.4 ± 0.1	-	-
19 D ARV	+	0.5 ± 0.02	-	-
Z-I1 ARV	+	9.6 ± 0.2	+	2.25 ± 0.32
Z-I ARV*	+	8.4 ± 0.3	+	2.60 ± 0.30
Z-H ARV	+	0.5 ± 0.1	-	-
CSP ARV	-	2.6 ± 0.3	-	-
G1SP ARV	+	2.0 ± 0.2	-	-
GSP ARV	-	1.8 ± 0.3	-	-
2TSB ARV	+	0.8 ± 0.1	-	-
O3 ARV	+	1.1 ± 0.2	-	-
2T ARV	+	10.5 ± 0.5	-	-
F ARV	+	0.4 ± 0.1	-	-
333 ARV	-	26.4 ± 0.5	+	2.11 ± 0.22
10_ARV*	+	44.5 ± 0.5	+	2.33 ± 0.30
NV1 ARV	+	4.0 ± 0.2	-	2.11 ± 0.25
NV2 ARV	+	5.6 ± 0.4	+	-
NV3 ARV	+	3.8 ± 0.4	+	2.14 ± 0.15
NV4 ARV	+	4.0 ± 0.3	+	-
NV5 ARV	+	8.0 ± 0.4	-	1.50 ± 0.15
NV6 ARV	+	2.4 ± 0.3	-	-
NV7 ARV	+	1.1 ± 0.2	-	-
4 ARV	+	1.4 ± 0.2	-	-
8 ARV	+	0.4 ± 0.1	-	2.22 ± 0.25
D1 ARV	+	0.8 ± 0.1	-	-
D2 ARV	+	1.1 ± 0.15	-	-
D3 ARV	+	0.5 ± 0.1	-	2.27 ± 0.28
D5 ARV*	+	1.5 ± 0.3	+	-
D6 ARV	+	1.8 ± 0.2	-	-
P1 ARV*	+	1.2 ± 0.1	+	2.70 ± 0.25
T1 ARV	-	1.4 ± 0.1	-	-
T6 ARV	+	4.0 ± 0.2	+	-
T10 ARV	-	1.4 ± 0.3	-	2.50 ± 0.30

One of the promising ways for raising the effectiveness of overburden revitalization is the application of PGPB, and this has been successfully achieved in the present study. After series of biochemical tests, isolates with the most prominent PGP features were identified as *Serratia liquefaciens* Z-I ARV (KT265088), *Ensifer adhaerens* 10_ARV (KT265086), *Bacillus amyloliquefaciens* D5 ARV (KT265087) and *Psedomonas putida* P1 ARV (KT265089). These four strains were selected from the collection of 44 soil isolates which were tested for the presence of the most frequent PGPB features (Tab. 4).

The results of biochemical test have shown that NH_3 was produced by 33 isolates (75%), whereas 36 isolates (82%) were characterized as IAA producers. Such results were expected since these two features are usual among beneficial soil bacteria (Gamalero & Glick 2011). The results of IAA production distinguished *Ensifer adhaerens* 10_ARV which produced considerably higher amounts compared to others (44.5 µg ml^{-1}). Similar findings on *E. adhaerens* have been noted by Kaur et al. (2014). Indole-3-acetic acid is the most common form of auxins (Gamalero & Glick 2011) which affect plant cell division, extension, and differentiation, increase the rate of root development and nodulation, initiate lateral and adventitious root formation and stimulate seed and tuber germination. Metabolic processes such as photosynthesis, biosynthesis of various metabolites, and resistance to stressful conditions are modulated by auxins (Glick 2012). Accordingly, IAA indirectly increases water and nutrient supplies leading to higher root exudation and biomass production (Gamalero & Glick 2011, Glick 2012).

The production of siderophores was expressed by 11 isolates. This characteristic of PGPB is very important since the amount of available iron in soil is limited (Gamalero & Glick 2011). To survive with a restricted supply of iron, bacteria produce low-molecular weight compounds (siderophores) with high affinity for Fe^{+3} (Gamalero & Glick 2011). PGPB siderophores raise Fe supply to plants and lower down the Fe amounts available for plant pathogens (Gamalero & Glick 2011). In addition, siderophores form stable complexes with Al, Cd, Cu, Ga, In, Pb and Zn and are important for reducing the level of plant stress caused by high concentration of heavy metals (Gamalero & Glick 2011).

Solubilization of phosphates is another crucial feature of PGPB which provides more $H_2PO_4^-$ and HPO_4^{2-} for plants (Gamalero & Glick 2011). Slightly higher number of isolates showed positive result in phosphate solubilization testing (27%) compared to siderophores production. *Serratia liquefaciens* Z-I ARV and *Pseudomonas putida* P1 ARV expressed the highest P solubilization ability. *Ensifer adhaerens* 10_ARV was also capable to solubilize inorganic phosphates (Tab. 4). This characteristic was already recorded among other representatives of these three bacterial species (Jha et al. 2009, Singh & Prakash 2012, Kaur et al. 2014).

Platanus × *acerifolia* is very tolerant to numerous abiotic stresses such as limited root space, unfavourable soil conditions, drought, air pollution, urban climate (Mimet et al. 2009) and exhibits high accumulation capacity of heavy metals (Ivanová et al. 2007, Kang et al. 2016). Those features make plane trees suitable for urban, surface mine areas (Skousen & Zipper 2014) and phytoremediation activities (Kang et al. 2016). On the other side, a number of pests and pathogens can threaten this genus causing considerable damage. Some of the most destructive are canker stain caused by *Ceratocystis fimbriata* Ell.et. Halsted f.sp. *platani*, anthracnose by *Apiognomonia veneta* (Sacc. et Speg.), Hohn, powdery mildew by *Microsphera platani* (Howe) and trunk rot by *Phytophthora cinnamomi* Rands (Pilotti et al. 2014). Considering the prevalence of plane trees in urban areas all over the world it is important to develop suitable measures to prevent or restrict the pathogens. However, up to now, the application of phytosanitary measures, chemical treatments and biological control measures were not particularly efficient (Panconesi 1999, Pilotti et al. 2015). A genetic approach with the aim of selecting resistant plane genotypes is believed to be an effective way of disease control (Pilotti et al. 2015). Breeding programs with American sycamore (*Platanus occidentalis* L.) and oriental plane tree (*Platanus orientalis* L.) resulted in cultivars "Columbia" and "Liberty", which are highly resistant to *Apiognomonia veneta* (Sacc. et Speg.), Hohn (Kowalski 2013). In addition, Vigouroux & Olivier (2004) and Pilotti et al. (2009) produced genotypes resistant to *Ceratocystis fimbriata* Ell.et. Halsted f.sp. *platan*.

However, the selection for resistant genotypes is very extensive while large-scale usage of a small number of clones can be detrimental for plane trees biodiversity (Pilotti et al. 2015). This emphasizes the need

for more intensive selection activities and new approaches which may include biological control. PGPB with biocontrol features already represent an effective measure against numerous plant pathogens (Glick 2012, Cawoy et al. 2014). The mechanisms employed by biocontrol bacteria are diverse and include antibiotics, siderophores and lytic enzymes production which are all effective against *Botrytis cinerea, Fusarium* spp., *Phytophthora* spp., *Rhizoctonia solani, Pythium ultimum,* etc. (Glick 2012). PGPB are also capable to activate induced systemic resistance (ISR), and prepare plant defense mechanisms for potential pathogen attack. ISR is not pathogen-specific and it can effectively suppress diseases caused by different plant pathogens (Glick 2012). All those mechanisms secure wide spectra of PGPB action. Combination of resistant genotypes or genotypes with certain level of resistance with PGPB may represent a new approach in reducing the incidence or severity of plane diseases. Also, PGPB with biocontrol function can be applied through soil introduction or foliar application which is effective for suppression of foliar diseases (Meena 2014). Genetic manipulation with potentially effective biocontrol agent may result in superior biocontrol strains, and offer even more promising results (Gamalero & Glick 2011).

In our study, the biocontrol potential of the isolates was estimated trough production of several lytic enzymes (lipase, N-acetyl-*β*-glucosaminidase, *β*-glucosidase, protease, cellulase), which are capable of damaging fungal cell-walls (Glick 2012, Cawoy et al. 2014). These tests revealed that only ten isolates produce substances which are known to have antifungal activity (Tab. 5). *Serratia liquefaciens* Z-I ARV, *Pseudomonas putida* P1 ARV and *Bacillus amyloliquefaciens* D5 ARV were capable to produce lytic enzymes. These species have already been recognized as effective biocontrol agents (Whiteman & Stewart 1998, Cawoy et al. 2014, Sharma et al. 2014). The only isolate that stood up after screening of PGP direct mechanisms but did not express any indirect mechanism was *Ensifer adhaerens* 10_ARV.

New tendencies in bacterial inoculum application emphasize the ecological advantages of mixed populations over single strain inocula (Gamalero & Glick 2011). After series of *in vitro* tests, inoculum consisting of *Serratia liquefaciens* Z-I ARV, *Ensifer adhaerens* 10_ARV, *Bacillus amyloliquefaciens* D5 ARV and *Pseudomonas putida* P1 ARV was used for *in vivo* experiments aimed at confirming the PGPB potential of selected isolates under field conditions. *S. liquefaciens* Z-I ARV was selected based on high P solubilization index and the ability to perform all four direct mechanisms and produce several lytic enzymes. *E. adhaerens* 10_ARV showed the highest IAA production. *B. amyloliquefaciens* D5 ARV showed ability to use several direct mechanisms and was the only isolate that produced cellulase. *P. putida* P1 ARV stood up with the highest P solubilization index among tested isolates.

Tab. 5 - The presence of lytic enzymes. (Na*β*): N-acetyl-*β*-glucosaminidase; (*β*-glu): *β*-glucosidase; (HCN): hydrogen cyanide; (+) presence of the feature; (-) absence of the feature; (*): selected strains.

Isolates	Lytic enzymes				
	lipase	Na*β*	*β*-glu	protease	cellulase
7N ARV	-	-	-	+	-
Z-I ARV*	+	+	+	+	-
G1SP ARV	-	+	-	-	-
333 ARV	+	+	+	+	-
NV4 ARV	-	-	-	+	-
NV5 ARV	-	-	-	+	-
7 ARV	+	-	-	-	-
D5 ARV*	-	-	-	+	+
D6 ARV	+	-	-	-	-
P1 ARV*	-	+	+	-	-

Tab. 6 - The height and root collar diameter of *Platanus* × *acerifolia* seedlings. (OI): seedlings re-planted into overburden and inoculated with PGPB consortia; (O): seedlings re-planted into overburden; (FS): seedlings re-planted into commercial substrate (Floradur® Plant Universal). The first measurement was in July, and the second measurement was in October. Mean values and standard errors (n=20) are reported. Values in the same column with different letters differ significantly ($p<0.05$) according to the LSD test.

Treatment	Seedling height (cm)			Root collar diameter (cm)		
	Start	1st meas.	2nd meas.	Start	1st meas.	2nd meas.
OI	19.98 ± 4.31^a	37.00 ± 5.11^b	42.60 ± 4.88^b	0.30 ± 0.05^a	0.57 ± 0.07^b	0.68 ± 0.07^b
O	19.33 ± 3.55^a	28.43 ± 5.03^a	32.33 ± 4.65^a	0.27 ± 0.07^a	0.40 ± 0.08^a	0.47 ± 0.08^a
FS	19.35 ± 4.12^a	39.55 ± 6.05^b	47.43 ± 5.98^c	0.30 ± 0.04^a	0.71 ± 0.15^c	0.77 ± 0.13^c
$LSD_{0.05}$		3.105			0.056	

Tab. 7 - Roots and shoots dry biomasses of *Platanus* × *acerifolia* seedlings. (OI): seedlings re-planted into overburden and inoculated with PGPB consortia; (O): seedlings re-planted into overburden; (FS): seedlings re-planted into commercial substrate (Floradur® Plant Universal); RDB: root dry biomass; SDB: shoot dry biomass. Mean values and standard errors (n=20) are reported. Values in the same column with different letters differ significantly ($p<0.05$) according to the LSD test.

Treatment	RDB (g)	SDB (g)
OI	2.35 ± 0.94^b	4.34 ± 0.65^b
O	1.23 ± 0.91^a	2.47 ± 0.98^a
FS	3.73 ± 2.69^c	7.28 ± 3.46^c
$LSD_{0.05}$	1.091	1.336

Plant-bacteria interactions are under strong influence of abiotic factors and indigenous microflora. Those two factors are the most common cause of PGPB failure in uncontrolled environment (Karličić et al. 2016) and *in vivo* testing is crucial for proper assessment of PGPB potential.

The effects of applied treatments on height and root collar diameter are presented in Tab. 6. At the start of the experiment, the seedlings had similar features (height and root collar diameter). After 12 weeks inoculated seedlings (OI) had similar height to those grown in the commercial substrate (FS), but were significantly higher compared to uninoculated overburden seedlings (O). At the end of the experiment the FS seedlings showed the highest growth increment, followed by OI seedlings, while O treatment seedlings were the smallest. Comparison of overburden seedlings revealed positive effect of inoculation on height increment.

Measurements of root collar diameter conducted in July put the treatments in the following order, FS>OI>O and this trend was maintained until the end of the experiment. In addition, positive effect of inoculation on seedlings width was observed when comparing OI and O treatments.

At the end of the experiment, seedlings were uprooted; roots and shoots were separated and dried until constant weight. Tab. 7 shows roots and shoots dry biomass of *Platanus* × *acerifolia* seedlings. The highest biomass production was noted in FS treatment. The OI treatment was intermediate as it yielded significantly higher root and shoot biomass than the O treatment, suggesting that inoculation had a positive influence on seedlings biomass production.

Tab. 8 - Fitness condition of *Platanus* × *acerifolia* overburden seedlings. (OI): seedlings re-planted into overburden and inoculated with PGPB consortia; (O): seedlings re-planted into overburden. Mean values and standard errors (n=12) are reported. Different letters in the same column indicate significant differences ($p<0.05$) after Independent Two Sample *t*-test.

Treatment	Σ proteins (mg gFW^{-1})	Σ phenolics µmol gallic acid eq. (gFW^{-1})	Σ chlorophyll (mg cm^{-2})	Σ antioxidative capacity µmol ascorbate eq. (gFW^{-1})	epidermal flavonoids (g cm^{-2})
OI	1.41 ± 0.32[b]	119.03 ± 9.69[a]	16.6 ± 1.06[b]	10.09 ± 2.01[b]	1.13 ± 0.161[b]
O	0.94 ± 0.11[a]	105.88 ± 13.07[a]	13.9 ± 0.94[a]	4.93 ± 1.10[a]	0.38 ± 0.286[a]

Comparison of inoculated and uninoculated overburden seedlings revealed positive effects of PGPB consortia on plant performances (Tab. 6, Tab. 7). Egamberdiyeva (2007) claimed that poor substrates are more suitable for exhibiting the full potential of PGPB, while in rich substrates plants are already well supplied. In our *in vivo* test one third of the seedlings were grown in commercial substrate with the aim of comparing performances of inoculated seedlings to those grown in nutrient sufficient substrate. At the end of the experiment, even though applied PGPB consortia enhanced London plane growth, their effects were not strong enough to parry seedlings grown in commercial substrate, suggesting that inoculation can compensate the lack of nutrients, but to a certain extent (Tab. 6, Tab. 7).

Seedlings grown in FS were 10% higher with 12% wider root collar diameter, 40% higher shoot dry biomass and 37% higher root dry biomass in comparison to OI seedlings. On the other hand, comparison of uninoculated and inoculated overburden seedlings showed that the presence of PGPB caused 32% increase in seedling height, 45% wider root collar diameter, 76% increase of shoot dry biomass and 91% of root dry biomass (Tab. 6, Tab. 7). Similar results were reported by Karlidag et al. (2007) where inoculation with *Bacillus* sp. and *Microbacterium* sp. induced 30% increase of apple shoot length. Rodriguez-Barrueco et al. (1991) reported 90% increment of oak seedlings biomass after inoculation with *Azospirillum brasilense*, while *Mesorhizobium* sp. increased White birch biomass by 60% (Sousa et al. 2015). Moreover, we have previously reported that inoculation with *Azotobacter chroococcum* and several *Bacillus* sp. strains resulted with 34% higher root dry biomass of Scots pine and 23% of Norway spruce seedlings (Karličić et al. 2016).

The data on PGPB effects on plant morphology are voluminous, and recent studies are more interested in physiological aspects of inoculation. In this manner efforts are directed towards revealing PGPB influences on mineral content of plants, chlorophyll content, and accumulation of secondary metabolites (Rojas-Tapias et al. 2012). At the end of our experiment, fitness condition of *Platanus* × *acerifolia* inoculated and uninoculated overburden seedlings were estimated through the content of total soluble proteins, total soluble phenolics, total chlorophyll, and several other parameters (Tab. 8). A significant influence of applied consortia on total soluble proteins content, chlorophyll content, total antioxidative capacity, and epidermal flavonoids content has been revealed. On the other hand, inoculation did not affect the total soluble phenolics content. Increase of leaf chlorophyll content, amount of total proteins, together with higher shoot growth and total dry matter, indicate an enhanced nutrient assimilation, as similarly observed by Mia et al. (2010). Increased total antioxidative capacity in OI treatment compared to O seedlings may be of great importance for stress amelioration (Rojas-Tapias et al. 2012), especially considering that the seedlings are intended for harsh environments, such as overburden waste dumps. Successful adaptation and growth of plants in inhospitable environments highly depends on species choice, seedling quality (Karličić et al. 2016), and the ability to recover the damaged root system shortly after replanting. The most critical moment is replanting from nurseries to final place. At this point, the presence of PGPB can be of great help considering their influence on transplant shock mitigation (Kloepper et al. 2004). Rapid development of new roots, increase of root growth, length and weight are commonly reported responses in trees to PGP bacteria inoculations (Karlidag et al. 2007, Sousa et al. 2015). These effects has also been confirmed in the present study where inoculation caused 91% increase of root dry biomass (Tab. 7), presumably by modulating endogenous plant mechanisms which regulate root development (Ribeiro & Cardoso 2012).

Literature data emphasize the validity of PGPB inoculation in revegetation projects of anthropogenically devastated areas. This has been further confirmed by the results obtained in the present study. All benefits that PGPB inoculation provides to London plane seedlings may be of great help in alleviating overburden waste dumps challenges. Also, such effects may be crucial for trees in urban areas considering their constant exposure to numerous stresses (Calfapietra et al. 2015). The main issue is finding the suitable bacteria strains that will be capable to express their beneficial effects in uncontrolled conditions. Considering the obtained results, our search has been successful, and the presence of the PGPB consortia in overburden waste raised its suitability for plant growth. Positive response of London plane seedlings suggests that inoculation may help widening the opus of species for reforestation of post-mining areas. This may be the proper measure that opens the door for more demanding species and speeds up natural succession processes and recovery of degraded landscapes.

Conclusion

PGPB used in our study were selected based on their PGP mechanisms. *In vivo* experiments with PGPB consortia confirmed their plant growth promoting nature through stimulating effects on London plane height, root collar diameter, total biomass production, and fitness. This study justifies the PGPB inoculation as a proper technique in mitigation of overburden waste dumps issues.

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