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Original Research

Bioremediation Potential Assessment of Plant Growth-Promoting Autochthonous Bacteria: a Lignite Mine Case Study

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Abstract

Coal and lignite play a major energy supply role in many European countries, including Bosnia and Herzegovina. Yet mining activities are a heavy source of ecosystem contamination, posing significant environmental threats. The primary goal of this study was to isolate and identify autochthonous lignite mine spoil bacteria and evaluate their potential in bioremediation of these polluted soils. Two *Bacillus* species, *Bacillus simplex* and a *Bacillus cereus* group member, were identified using conventional, molecular, and bioinformatics approaches. This represents, to our knowledge, the first microbial characterization of mine overburden in Bosnia and Herzegovina. A co-inoculum of autochthonous bacterial populations was used to treat unvegetated as well as oat- and lettuce-vegetated lignite overburden samples. Our results illustrate the potential of recovered native species to enrich soil fertility and productivity through plant growth promotion.

Keywords: *Bacillus* spp., soil, bioremediation, lignite spoil

Introduction

Around 40% of power generated globally is based on hard coal and lignite[1], yet mining activities produce a high volume of mine overburden and tailings [1], causing soil errosion, heavy metal contamination, and acid mine drainage that leads to contamination of adjecent waters and agricultural land, thus posing severe environmental and health risks in vast areas surrounding mines [2]. While traditional chemical approaches to restorting these contaminated soils are not sufficiently efficient [3, 4], phytoremediation is a cost-effective, non-invasive strategy with promising results in the field [reviewed in 5]. However, given that the success of this approach

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is considerably constrained by a slow remediation rate, varying field factors, and root depth [5], substantial research efforts have focused on utilizing microorganisms [6-8] and plant-microbe interactions [9-11] to restore mine tailings and overburden.

In the present study, we investigated overburden soil (OS) from the Kakanj lignite mine, a significant coal production plant providing main fuels for power generation in Bosnia and Herzegovina (BiH) at 44°7.912 N 18°5.84 E [12]. Yet given that two thirds of Kakanj's production comes from surface mining, OS generation and disposal are of great concern. The main idea behind our approach was to identify and characterize mine OS autochtonous bacterial populations and assess their potential in restoring soil productivity and fertility, as measured by two plant species: lettuce (*Lactuca sativa* L.) and oat (*Avena sativa* L.).

Experimental Procedures

Chemical and Microbiological Characterization of OS and Soil Samples

OS samples (0-20 cm) collected from Kakanj in in July 2011 and control soil (S) samples (0-15 cm) collected from land under corn (Pancevo, Serbia) were used to create independent composite OS and S samples in the laboratory, which were chemically analyzed. Basic chemical properties of OS samples were determined according to [13]. Micronutrients and heavy metals were determined by atomic absorption spectroscopy (AAS), while organic pollutants were determined by gas chromatography-mass spectrometry (GC/MSD) and gas chromatography-flame ionization detector (GC/FID). OS samples were diluted in serial dilutions and plated (in triplicates) to ascertain the total number of microorganisms on 0.1x tryptic soy agar (TSA), ammonifying bacteria on meat peptone agar (MPA), *Azotobacter* sp. and oligonitrophilic bacteria on Fyodorov agar, fungi on rose bengal streptomycin agar, and actinomycetes on starch-ammonia agar. Soil was dried at 105ºC for 2 h and the number of microorganisms was estimated as CFU/g dry soil.

Dehydrogenase (DHA) activity was determined by the method of Casida et al. [14] and reported as μg TPF/g/h.

Identification of Bacteria from OS Samples

13k and 19k MPA colony and cell morphology were determined by a light microscope (Leica DMSL, Germany), while biochemical identification was conducted by API CH using the APIWEB system v. 1.1.0 (bioMerieux, Inc., France). Partial 16S rRNA gene was amplified from both isolates using total extracted DNA (DNeasy Blood & Tissue Kit, QIAGEN), universal 16S rDNA primers (27F 5'GAGAGTTTGATCCTGGCTCAG3' and 1523R 5'AGGAGGTGATCCAGCCG3'), and KAPATaq polymerase (KAPA Biosystems). Amplified products were purified (QIAquick PCR Purification Kit, QIAGEN) and sequenced (Macrogen Inc., Seul, South Korea). Sequences were deposited in NCBI GenBank database under accession numbers KF494373 for isolate 13k and KF494374 for isolate 19k.

DNA sequence similarity searches were performed using the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu). Reference *Bacillus* 16S rDNA sequences were aligned and used to construct the phylogenetic tree using the maximum parsimony method, with statistical support obtained by generating 500 bootstrap replicates in MEGA 6 [15].

Greenhouse Cultivation

Lactuca sativa L. (10 seeds/cup) and *Avena sativa* L. (5 seeds/cup), previously sterilized in 30% H_2O_2 for 20 s, were seeded at 1 cm depth in 5 substrates: 100% OS, 75% OS, 50% OS, 25% OS, and control (100% S) in 10 replicates, five of which were co-inoculated with 13k/19k isolates (10%, w/w). The inoculum was prepared as follows: nutrient broth was inoculated with 13k and 19k pure cultures, incubated in an orbital shaker (30ºC, 150 rpm) for 24 h, and 10^8 cells/0.01M phosphate buffer were used to inoculate the substrates. Plants were allowed to germinate for 25 days, removed from the substrate, and used to assess the total number of microorganisms (CFU/g dry soil), germination rate (%), and shoot and root dry biomass (g/substrate), following natural drying, as well as oven drying at 60ºC for 120 min.

Statistical Analyses

Obtained results were subjected to analysis of variance (ANOVA) using Statistica software (StatSoft, US). Mean values of data were compared by Fisher's LSD test at significance level $p = 0.05$.

Results and Discussion

Chemical and Microbial Characteristics of Mine Overburden

Kakanj OS samples were found to be acidic (pH_{KCl} 5.23), with elevated organic C content (15.28% vs. 1.86%) and an enhanced C/N ratio (31.4:1 vs. 8.5:1; Table 1). The dominant form of nitrogen is ammonia (23.8 mg/kg), resulting from microbial mineralization of organic matter, while nitrification activity at pH 5.23 is low (Table 1). Analysis of microelements and heavy metals [16] revealed that OS material is deficient in P (30.0 mg/kg) and Mg (268.0 mg/kg), and rich in Fe (3.69 mg/kg), Ca (3372.0 mg/kg), Cd (0.70 mg/kg), and Ni (167.80 mg/kg). Bioavailability of toxic metals such as Ni, Pb, and Cd has been shown to increase at pH 5 [17].

The vast majority (~90%) of total microorganisms ($12.19x10^4$ CFU/g) were contributed by oligonitrophilic ($7.45x10^4$ CFU/g) and ammonifying ($3.39x10^4$ CFU/g)

Table 1. Chemical properties of overburden soil (OS) and soil (S) samples.

	pH (H_2O)	pH (KCl)	$CaCO_3$ (%)	C_{org} (%)	N_{tot} (%)	P_2O_5 mg/kg	K_2O mg/kg	NH_4 mg/kg	NO_3 mg/kg
OS	5.96	5.23	0.0	15.28	0.486	30.0	129.0	23.8	8.4
S	8.21	7.72	3.9	1.86	0.218	200.0	128.0	3.5	45.5
	Total Fe	Total Mn	Total Cd	Total Co	Total Cr	Total Ni	Total Pb	Total PAHs	Hydrocarbons C_{10}-C_{40}
OS*	3.69	268.0	0.70	24.86	84.97	167.80	23.90	0.291	1263.6

*Total microelement, heavy metal, and organic pollutant content in overburden soil are given in mg/kg

Table 2. Dehydrogenase activity (DHA) in various overburden (OS) samples (x10^{-5} μg TPF/g/h).

	Unvegetated OS	Non-inoculated oat	Inoculated oat	Non-inoculated lettuce	Inoculated lettuce
Mean	0.24	0.63	0.97	0.20	0.76
SE	0.02	0.13	0.09	0.03	0.06

bacteria, while fungi and actinomycetes were present in small amounts (1.15 x10^4 and 0.2 x10^4 CFU/g, respectively; Fig. 1). Our results coincide with findings that oligonitrophilic bacteria dominate in nitrogen-deficient soils [18]. *Azotobacter sp*. were not detected (Fig. 1), illustrating sensitivity to soil acidity, given that they are typically found in soil pH 7.1-9.0 [19, 20]. As a bioindicator of soil fertility [21, 22], OS DHA activity was determined to be 0.24x10^{-5} μg TPF/g/h (Table 2).

Identification of Autochthonous Mine Overburden Bacteria

Unlike the majority of previous research on plant growth promoting (PGP) microorganisms, which focused on Gram negative bacteria [23, 24], we decided to explore ammonifying bacteria. Macroscopic investigation of MPA plates resulted in two types of morphologically distinguishable colonies: 13k and 19k. Isolate 13k colonies were large (2-6 mm in diameter), white-yellowish in color, circular to irregular shape, and moist (data not shown). Isolate 19k was depicted by 1-3 mm in diameter, white-yellowish, irregularly shaped colonies spread across agar (data not shown). Microscopic examination of both 13k and 19k revealed Gram-positive, spore-forming, rod-shaped bacteria without capsule (data not shown). The APIWEB technique, following incubation on API 50CH, suggested that 13k and 19k isolates exhibit the highest similarity to *Bacillus thuringiensis* and *Paenibacillus alvei*, respectively. Given constraints of the technique, molecular characterization was also performed.

Comparisons of 13k and 19k 16S rDNA sequence against a RDP database placed both isolates in the genus *Bacillus*, which agrees with the data of others examining mine waste [25]. Isolate 13k exhibited 100% identity with *B. simplex* isolates, but also with a *B. muralis* and a

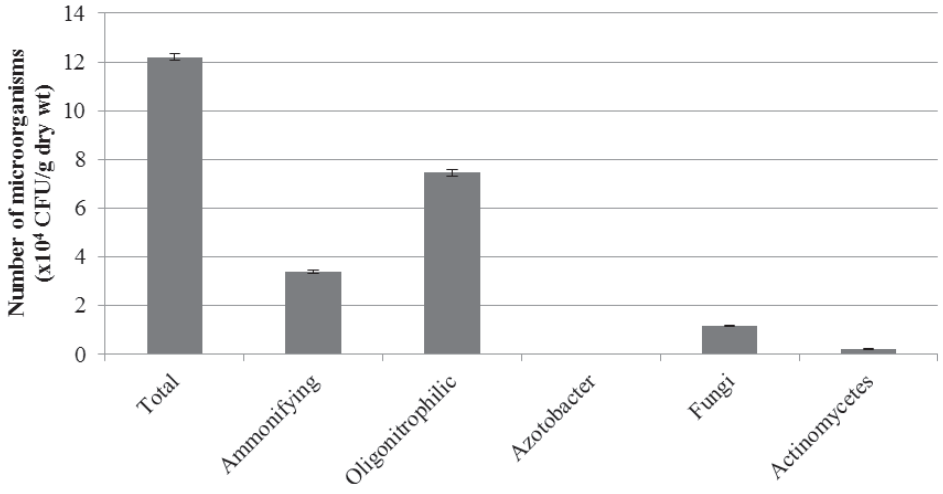

Fig. 1. Mean number of total microorganisms, ammonifying bacteria, oligonitrophilic bacteria, Azotobacter, fungi, and Actinomycetes in unvegetated mine overburden soil (OS) samples.

Brevibacterium sp. isolate (data not shown). A proposed set of distinguishing biochemical characteristics between *B. simplex* and *B. muralis* [26] suggested that 13k behaves as *B. simplex*. Phylogenetic analysis of soil *Bacillus sp.* representatives revealed that isolate 13k grouped with *B. simplex* (Fig. 2). Isolate 19K showed 100% identity with *B. thuringiensis* and *B. cereus* isolates (data not shown), while it grouped within a *B. cereus* group [27, 28] in the phylogenetic analysis (Fig. 2).

Effect of 13k/19k Co-Inoculation on Overburden Bacterial Community

In order to stimulate microbial abundance and diversity, we seeded pure and soil-assisted OS samples with oat (*Avena sativa* L.) and lettuce (*Lactuca sativa* L.). Lettuce was chosen due to its known sensitivity to environmental factors, such as increased heavy metal and salt concentrations [29], while fast-growing and easily managed, oat has been selected by many authors as a useful test for detection of toxic substances [30]. The total number of microorganisms in pure OS samples increased from $12.2x10^4$ CFU/g (Fig. 1) in an unvegetated sample to $2.45x10^6$ and $2.02x10^6$ CFU/g in oat- and lettuce-seeded OS, respectively (Fig. 3). The total number of microorganisms continued to increase with increase in soil content, where 50:50 OS-to-S ratio mimicked the microbial abundance of fertile soil (Fig. 3). Our data show that root exudates significantly affect microbial abundance in soil, as reported elsewhere [31].

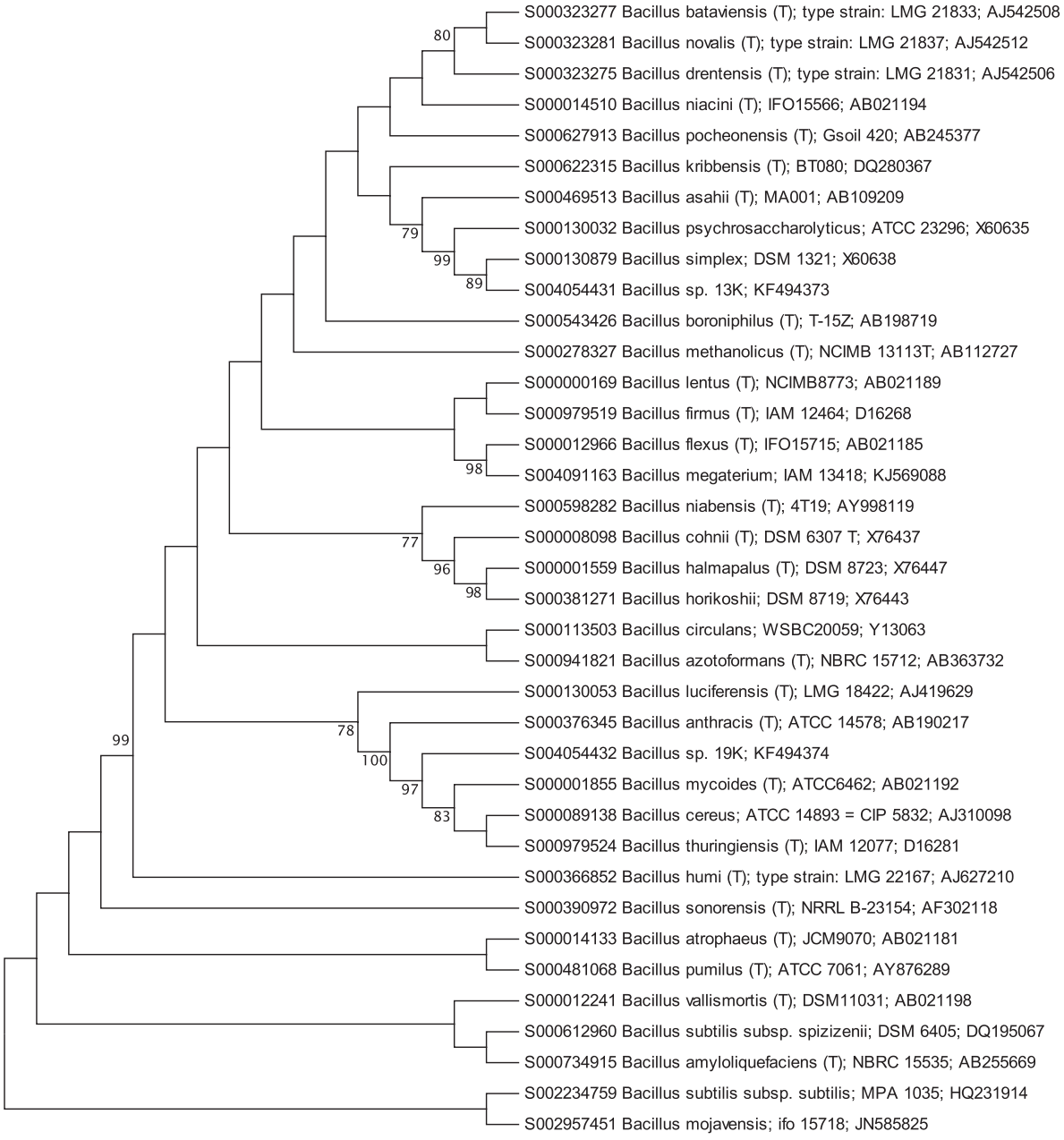

Fig. 2. A 16S rRNA gene phylogeny placing isolates 13k and 19k in soil *Bacillus spp*. Bootstrap values above the cut-off (75%) are shown.

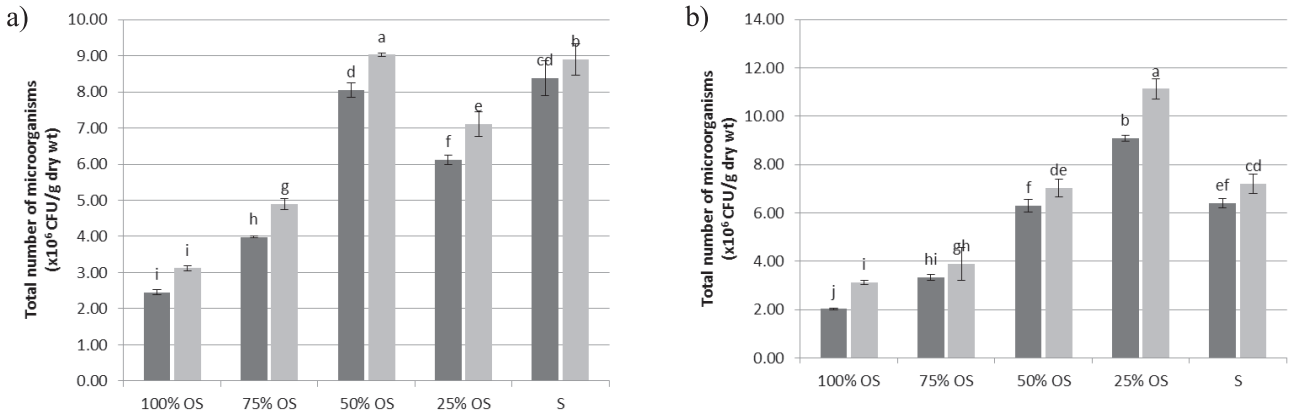

Fig. 3. Mean total number of microorganisms in oat (a) and lettuce (b), non-inoculated and inoculated overburden (OS) substrates. Means depicted with the same letter do not significantly differ according to Fisher's LSD test (p = 0.05).

Given that members of the *Bacillus* genus exhibit PGP activities [24, 32], we used 13k/19k to co-inoculate the seeded substrates. The co-inoculum caused an increase in the total number of microorganisms in 100% OS from $2.45x10^6$ to $3.12x10^6$ CFU/g (oat) and from $2.02x10^6$ to $3.12x10^6$ CFU/g (lettuce) (Fig. 3). These stimulating effects of autochthonous *Bacillus* species on overall microbial activity in pure OS samples were also mirrored in DHA activity levels (Table 2). The same trend was observed upon the addition of soil, while 50:50 OS-to-S ratio mimicked the microbial abundance of inoculated fertile soil (Fig. 3).

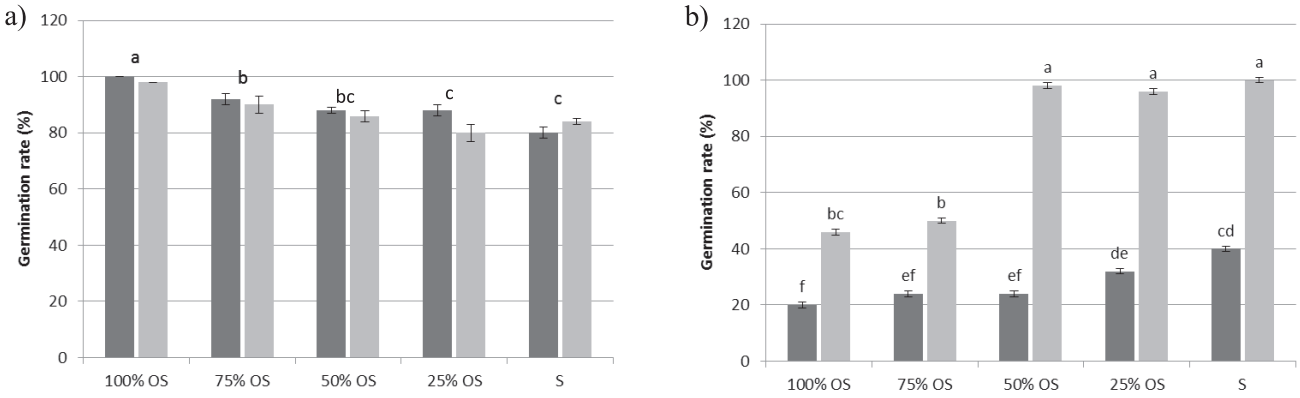

Fig. 4. Oat (a) and letuce (b) germination rates (%) in non-inoculated and inoculated overburden soil (OS) substrates. Means depicted with the same letter do not significantly differ according to Fisher's LSD test (p = 0.05).

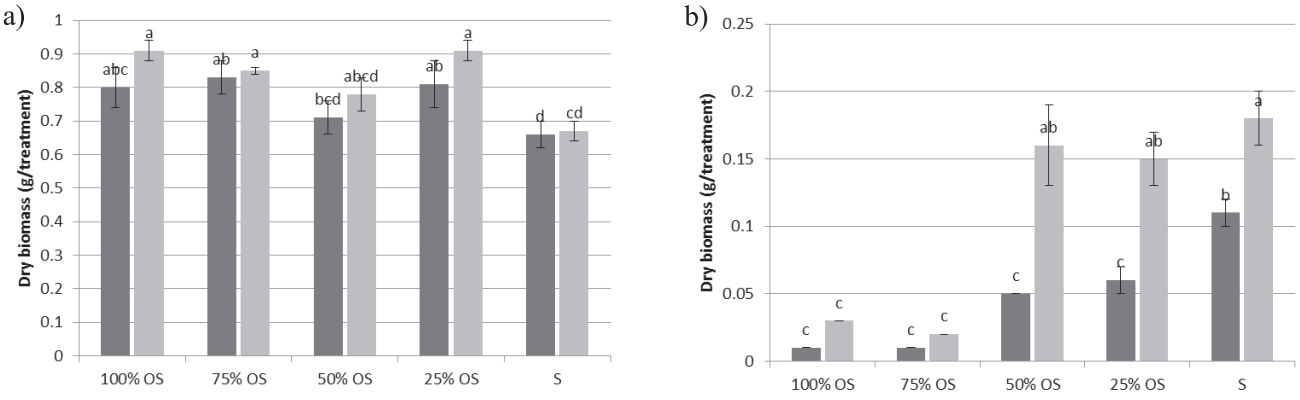

Fig. 5. Oat (a) and letuce (b) dry biomass in non-inoculated and inoculated overburden soil (OS) substrates. Means depicted with the same letter do not significantly differ according to Fisher's LSD test (p = 0.05).

Effect of 13k and 19k Co-Inoculation on Oat and Lettuce Growth Parameters

The addition of soil alone, as well as co-inoculation of substrates with 13k/19k, did not significantly alter oat's germination rate (Fig. 4a) and dry biomass (Fig. 5a). For instance, oat germination rate in 100% OS was 100% and 98% in non-inoculated and inoculated pots, respectively (Fig. 4a), while dry biomass was 0.8 g/substrate and 0.91 g/substrate (Fig. 5a). Our results illustrate that *A. sativa*'s growth is not impaired in soil contaminated by metals and organic pollutants. This unresponsiveness to soil toxicity has also been seen by others, in studies of growth parameters and biochemical assays, performed on various soil types [33-35]. Given that oat's phytoextraction abilities allow removal of contaminants from soil, our results speak in favor of *A. sativa*'s potential for bioremediation of lignite mine OS through re-vegetation. Yet this approach yields biomass accumulated with contaminants that could pose a significant health risk for humans, as well as domestic and wild animals.

With this constraint in mind, we also tested *Lactuca sativa* L. growth under varying conditions. Lettuce non-inoculated 50% OS germination rate was 24%, compared to 40% in S, while non-inoculated 50% OS dry biomass was 0.05 g/substrate, compared to 0.11 g/substrate in S (Figs 4b and 5b). Thus the addition of soil alone did not notably improve lettuce growth parameters and they never reached the levels seen in S (Figs 4b and 5b). Co-inoculation with 13k/19k, on the other hand, significantly increased lettuce germination in all substrate types, in comparison to respective non-inoculated substrates (Fig. 4b). The level of germination in fertile soil and a significant stimulation of lettuce dry biomass were achieved in 50:50 OS-to-S ratio (Figs 4 and 5).

Conclusions

We have isolated and identified two lignite OS native bacteria, *B. simplex* and a *B. cereus* group member. To our knowledge, this represents the first microbial characterization of mine waste in BiH. Previous research has demonstrated the ability of *B. simplex* to accumulate metals, such as Cd, Co, Ni, and Sr [36], as well as to promote plant growth by altering plant root architecture, i.e., by stimulating the emergence of more lateral roots [24]. Studies have also shown PGP activities of *B. cereus* [37] and other group members, such as *B. thuringiensis* [38], as well as the potential role of *B. thuringiensis* in biodegradation of organophosphorus in contaminated soil [39]. Our results suggest that autochthonous *B. simplex* and *B. cereus* group bacteria are candidates for the bioremediation of lignite mine waste. We acknowledge that soil-assisted strategies are not always economically feasible and complicate infrastructure. Thus we are further exploring the potential roles of other previously reported PGP *Bacillus* spp., such as *B. subtilis* [40], *B. amyloliquefaciens* [41], *B. pumilus* [10], *B. licheniformis* [42], and *B. megaterium* [43], plus other autochthonous bacterial species, alone or together with re-vegetation, in bioremediation of lignite mine waste.

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