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Research Article

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Use of Plant Growth-Promoting Bacteria and Humic Acids for Phytostabilization of Acid-Generating Mining Wastes

Svetlana Bratkova[1*], Katerina Nikolova[1], Petya Genova[1], Anatoliy Angelov[1]

[1] *University of mining and geology, Sofia 1700, Boyan Kamenov str., Bulgaria*

[*]Corresponding author

Svetlana Bratkova, University of mining and geology, Sofia 1700, Boyan Kamenov str., Bulgaria.

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Abstract

An interdisciplinary study was conducted on the effect of application of plant growth-promoting bacteria and humic acids in the reclamation of acid-generating mining wastes through a vegetation cover. The drainage water from the mining waste was characterized by a pH of 3.58 and high concentrations of sulfate, copper, manganese, and zinc. Strains of the Bacillus and Pseudomonas genera and humic substances produced by biotransformation from lignite were applied. The usage of plant growth-promoting bacteria and humic acid in the reclamation of acid-generating mining wastes produced several beneficial effects. The combined application resulted in a significant decrease in Cu, Fe, Zn and sulfate concentrations in a variety of drainage water samples, due to the suppression of pyrite oxidation. Both plant growth-promoting bacteria and humic acid improve plant growth, when used separately. The highest yield of fresh (between 22% and 43 %) and dry biomass of plants (between 31% and 41 %) was observed after combining both treatments, but the effect depended strongly on the dose of application. Yields of fresh and dry biomass in the combined application increased by treating plants with 0.42 g/kg humic acids, but decreased significantly when applying humic acids in a concentration of 0.84 g/kg soil. The treatment with a microbial consortium and humic substances enhanced significantly the uptake of nitrogen, phosphorus, and potassium by the plants. Furthermore, the addition of Bacillus and Pseudomonas bacteria in combination with humic acids to poor soil for reclamation reduced the Cu and Zn uptake.

Keywords: Reclamation; Plant Growth-Promoting Bacteria; Humic Acid; Phytoremediation

Introduction

Metallic ore extraction is associated with generating a great amount of mining waste. Mining waste dumps usually consist of overburden rocks, off-balance sulfide ores, and low-grade raw material [1]. These high-volume wastes sometimes contain significant levels of heavy metals and other toxic substances [2, 3]. Additionally, mining waste is also known to cause numerous environmental problems including air, water and soil pollution, toxicity, geo-ecological disasters, and loss of biodiversity [4].

Worldwide, one of the most prominent environmental problems in areas with mining waste is the generation of acidic mine drainage (AMD). AMDs are characterised by their high acidity, and typically contain Fe, Cu, Zn, Cd, Pb, Al, and Mn, as well as high concentrations of sulfate; whereby, the low pH of the generated drainage enhances the dissolution of heavy metals in water. The AMD chemistry depends on a number of factors: the mineralogical and chemical composition, the geotechnical properties of the mineral wastes, the chemolithotrophic bacterial activity, and the climatic conditions in the particular area of interest. Acid rock drainage mechanisms involve the oxidation of sulfide minerals in the presence of oxygen and water with the participation of sulfur and iron-oxidising bacteria [5, 6].

Closure technologies for mining dumps must be cost-effective, limit or reduce the infiltration of precipitation and require little or no maintenance. Numerous technologies have been developed to control AMD [7]. These methods have been classified into five major groups: physical barriers, bacterial inhibition, chemical barriers (or passivation), electrochemical protection, and desulfurisation [8].

In practice, dry cover is used often in the remediation of mining waste, because the layer with low filtration coefficient restricts the inflow of water into the mineral waste, as well as the oxygen supply. Materials used for dry cover can be composed either of soil, or a soil substitute. The soil used in reclamation technologies is often characterised by a low content of biogenic elements (nitrogen, phosphorus, and potassium) [9]. The low content of basic nutrients, the high heavy metal content in waste dump material, and the water deficit are important factors affecting the process of plant species installation. Current approaches to reclamation involve ameliorative and adaptive strategies to allow vegetation development [10].

The use of humic acid for mining waste remediation has been the subject of numerous studies [8,, 11]. Humic acid contains phenol and carboxylic acid groups. Due to the deprotonation of OH/OOH of these functional groups, humic substances are a preferred method for reclamation of mining wastes. The carboxyl groups in humic substances play a very important role in the sorption of heavy metals, such as Pb^{2+}, Cu^{2+} Cd^{2+}, Ni^{2+} and Zn^{2+}, as removal of metals depends on the pH of contaminated water [12]. Complexation reactions of humic substances not only prevent precipitation of metal ions, but they also reduce metals' toxicity. The stability constant of heavy metal complexes with humic acids increases with the increase in pH and the decrease in ionic strength [13].

Furthermore, humic acid has the potential of passivating pyrite, thus reducing the rate of pyrite oxidation and the generation of acidic drainage water [14].

Commercially available humic substances are usually applied in soil to promote plant growth and improve the physical properties of the soil, such as aggregation, aeration, permeability, water holding capacity, ions transport, and availability through pH buffering [15]. However, humic acid is insoluble at a pH below 2, therefore, it may not be as effective in highly acidic conditions.

On the other hand, the interaction between plants and rhizosphere microorganisms plays an important role in their development [16]. The rhizosphere is a habitat for microorganisms from different systematical groups, whereby *Bacillus* and *Pseudomonas* were the most predominant genera to be isolated from rhizosphere samples [17, 18, 19, 20]. The bacteria that enhance plant growth and protect plants from disease are known as plant growth-promoting bacteria (PGPB). PGPB stimulate growth via direct and indirect mechanisms, such as nitrogen fixation of atmospheric nitrogen, modification of soil organic carbon, transformation of poorly soluble phosphorous compounds to easily assimilate using bacterial phosphatases, increase in nitrate assimilation, production of siderophores chelating the iron into a plant bioavailable form, synthesis of physiologically active substances (vitamins, enzymes, and phytohormones), variations in root cell membrane permeability, protection against stressful environmental factors, phytopatogenes, etc. Due to this, a whole process of biological reclamation of mining waste can be promoted by the use of PGPB, with the purpose of enhancing plant mineral nutrition. Productivity of poor soil used for reclamation can also be increased by adding various natural amendments, including sewage sludge and animal manures, as these amendments stimulate microbial activity [21].

Metal–mineral–microbe interactions have also been the subject of a number of studies [22, 23]. Microorganisms have a variety of properties that lead to the mobilization or immobilization of metals, due to the changes in metal speciation, toxicity, and mobility. Solubilization mechanisms are related to the production of siderophores, excreted metabolites (amino acids, phenolic compounds, and organic acids) with metal-complexing properties, chemical oxidation, or reduction. On the other hand, processes such as biosorption to cell walls, formation of biopolymers, such as proteins, nucleic acids, and extracellular polysaccharides, intracellular accumulation, or precipitation of metal in and/or around microbial cells are involved in the immobilization of metals. The variety of mechanisms that mobilize or immobilize metals makes a number of soil microorganisms attractive for bioremediation of contaminated mining sites [24, 23, 25].

There are currently a lot of studies on the effects of PGPR in combination with humic substances in the field of sustainable agriculture [26, 27, 28]. According to the inoculation of crops of potato produces significant increases in potato growth, tuber yields, and quality. reported that the use of humic acid-based bio-stimulants in combination with PGPB in seed treatment increased the initial performance of maize and its productive potential [29, 30].

The objectives of this study were to investigate the effect of the applied PGPB or/and humic acids on three aspects of the biological reclamation of acid-generating mining waste: 1) the chemical composition of drainage water generated from mineral waste; 2) the improvement of vegetation of grass mixture on soils with insufficient nutrient content; and 3) the assimilation of biogenic elements and heavy metals by plants.

Materials and Methods

Experimental design

The *in vivo* pot tests were performed in containers (18 x 25 x 40 cm) with a volume of 18 dm3. The mining waste weighing 25 kg was placed at the bottom of the pots, forming a 25 cm layer. 5.2 kg of soil was placed on the mining waste and the layer was 10 cm high. According to preliminary information, the mining waste contained the following minerals: quartz (33%), albite (30%), microcline (13%), muscovite (12%), clinochlore (6%), calcite (2%), and pyrite (4%). The soil was taken from a soil depot of a real mining object, located in the region of Srednogorie. The soil had a pH (H_2O) 5.65 and a pH (KCl) 4.39, which defined it as medium acidic. The soil was classified as sandy clay loam, and the humus and Kjeldahl-N content were lower: 0.98% and 0.196%, respectively. According to the legal framework (Art. 5 of Regulation № 3 of August 1, 2008, on the rules on the permitted content of hazardous substances in soils), the soil was not contaminated with heavy metals and arsenic.

On 5 May 2018, five pots were planted with a universal grass mix (5 g/pot). The composition of the grass mixture was, as follows:

Lolium perenne rosemary (30%), *Lolium perenne esquire* (25%), *Festuca rubra casanova* (25%), and *Festuca rubra gondolin* (20%).

The experiment scheme included 5 variants of treatment: 1 – Control; 2 – Treatment with rhizospheric microflora (PGPR); 3 – Treatment with humic acids (HA); 4 – Combined treatment: rhizospheric microflora + humic acids (PGPR + HA); and 5 – Combined treatment: rhizospheric microflora + humic acids in a double dose (2xPGPR + 2xHA).

The treatment with one liter of the solution was performed three times over three consecutive months. The different solutions, containing rhizosphere microflora (2 ml/l), humic acids (2 ml/l), or rhizosphere microflora + humic acids (2 ml/l and 4 ml/l each for the relevant variant) have been applied two times in the first week of every month. The plants were grown under natural climatic conditions and they were watered when periods of drought occurred.

Microbial Strains and Humic Substances

The isolates used in the study were obtained from the rhizosphere of wild plants. Four of the strains belong to the *Bacillus* genera: *B. subtilis* CI R1, *B. amyloliquefaciens* CI R2, *B. megaterium* AM1, and B. simplex AM3. The other three strains were *Pseudomonas* chlororaphis 1S4, *Ps. fluorescens* AM2, and *Ps. arsenicoxydans* AM4.

The microbial strains were cultivated on a medium containing 25.0 g glucose, 3.0 g $(NH_4)_2SO_4$, 1.0 g KH2PO4, 0.5 g $MgSO_4.7H_2O$, 1 g yeast extract, and 1.0 g peptone (per liter). For the purpose of the experiment, the pure cultures were cultured on the above-described culture medium dynamically at 30 °C for 48 h. After the cultivation, the pure cultures were diluted with distilled water to a final concentration of 10^8 cells/ml, and were mixed in equal quantities.

The humic substances were isolated from lignite obtained from Stanyantsi mine, Southwestern Bulgaria. The biotransformation of lignite was carried out under the conditions of solid-phase static fermentation with Trichoderma harzianum and T. viride [31]. The extract thus obtained had the following composition: total organic C (12.9 %), humic acid (10.87%), and fulvic acid (1.22%).

Study Methods

Chemical Analysis of Mining Waste and Effluents

The mining waste samples were air dried, powdered, sieved, and stored in polyethylene packets for laboratory study. The paste pH was determined in a 1:1 weight ratio [32]. The water-soluble fraction of the sulfate was determined using a spectrophotometric approach (with BaCl2 as reagent) at a 420 nm wavelength. A subsequent extraction procedure was used to determine the most soluble phases in the mining waste. The procedure consisted of extracting the metals in five forms: (1) exchangeable fraction (1 M $MgCl_2$); (2) carbonate-bound (1 M HOAc); (3) Fe–Mn oxide fraction (0.04 M $NH_2OH.HCl$ in 25% HOAc); (4) organic bound and secondary sulfides (30% H_2O_2, 0.02 HNO_3 and 3.4 M NH_4OAc); and (5) residual fraction (HNO_3-HCl digestion) [33, 34]. The concentration of heavy metals and As were measured by ICP-spectroscopy. Samples of effluents were taken weekly, and pH, electrical conductivity (EC), and redox potential were measured. Concentrations of Fe, Mn, Cu, Zn, As and sulfate were determined twice a month.

Analysis of The Plants

The weight of the fresh biomass of the plants was determined in early June, August, and October. The dry weight of the aboveground biomass was determined after drying the biomass at 80 o C for 24 hours.

After grinding, the plant samples were digested through use of H_2SO_4, HNO_3, and $HClO_4$ [35]. The concentrations of Ca, Mg, K, P, Fe, Mn, Cu and Zn were measured by means of ICP-spectroscopy. The total nitrogen content in the dry biomass was determined by Kjeldahl digestion (ISO 11261). The statistical analysis of the weight of the fresh and the dry biomass as % to control was performed using the Stratigraphics Centurion software.

Results and Discussion

Analysis of The Mining Waste

The common test used to assess the presence of soluble acid salts in mining wastes is paste pH. The mining waste used in this study has a paste pH of 3.23 and a water-soluble sulfate concentration of 1.94 g/l. The concentrations and the relative distributions of heavy metals and As in mining waste are shown in Table 1 and Fig 1.

Table 1: Concentration of Heavy Metals and As In Mining Waste

Fraction	Cu	Zn	Fe	Mn	Cd	Al	As
	mg/kg						
Exchangeable	221.3	55.6	21.75	161.25	<0.1	222.15	<0.1
Carbonates	25	0.4	6.55	8.55	<0.1	10.8	<0.1
Fe and Mn oxides	249.8	18.4	5350	98.3	<0.1	2506	57.45
Organic matter and secondary sulfides	220	44.4	638.5	5	<0.1	1501	18.85
Residual	242.2	332.0	36925	206.95	<0.1	15155	175.6
Total	958.3	450.8	42941.8	480.05	<0.5	19394.95	252.1

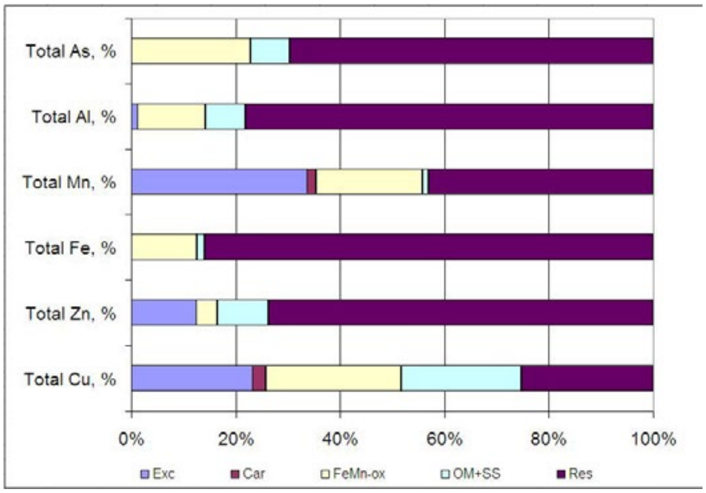

Figure 1: Relative distributions of As, Al, Mn, Fe, Zn and Cu in mining waste, according to the sequential extraction procedure: Exc – exchangeable; Car – carbonates; FeMn-ox – Fe and Mn oxides; OM+SS – organic matter + secondary sulfides; Res – residual

The total concentration of Cu in the mining waste was 958.3 mg/kg. Distribution of copper between the exchangeable, the Fe - Mn oxyhydroxides, the organic matter, and the residual fraction was approximately the same (ranging from 23–26%). The results obtained can be interpreted according to a sequential extraction procedure adapted for geochemical studies of copper sulfide mine waste [36], and it can be concluded that 23.1% of copper is in the form of a water-soluble fraction (e.g., chalcanthite (CuSO4.5H2O)) and as Cu, which may be released in the exchangeable fraction from the vermiculite-type mixed-layer mineral in the mining sample. Twenty-five percent of the copper was incorporated in iron-phases. In oxidizing conditions performed by a H2O2 leach, one quarter of the total copper was dissolved. It can be concluded that part of the copper in the mining waste is in the form of supergene Cu-sulfides, such as covellite and chalcocite–digenite, while twenty-five percent of it is in the residual fraction.

The content of total zinc in the mining waste was 451 mg/kg. Distribution of zinc between exchangeable and organic matter fractions was 16.7% and 13.4 %, respectively. The major part of Zn was found in the residual fraction as sphalerite, accounting for 73.6% of the total Zn.

The mining waste in this study contained pyrite at 4%. Iron was mainly distributed in two fractions: residual (86% of the iron content) or Fe and Mn oxides (dissolved schwertmannite, ferrihydrite, Mn-hydroxides, secondary jarosite, as well as goethite formed acid mine drainage), which contained 12.5% of the iron content. High pyrite content in mining waste and low paste pH values are a prerequisite for the growth and activity of iron-oxidizing hemolithotrophic bacteria, such as *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*.

Unlike iron, manganese was present in high concentrations in the exchangeable fraction (33.6%). Distribution of manganese in Fe and Mn oxides and residual fractions was 20.4 and 43%, respectively. A number of authors have found that Mn has the higher mobility potential to be released from the mining waste, since its content in the exchangeable fraction in much of the research is high [37, 38].

The presence of minerals, such as albite, microcline, muscovite, and clinochlore, in the studied mining waste is a premise for the significant fraction of total aluminum to be in the residual fraction (78%). 13% of Al was in the composition of the Fe and Mn oxides fraction, and only 1% was distributed in the exchangeable fraction.

The total concentration of As in the mining waste was 252.1 mg/kg. Sequential fractionation of As showed that 25% of the total arsenic was distributed in the Fe and Mn oxides fraction. The arsenate and arsenite ions had an affinity to be sorbed from formed ferric hydroxides having a highly developed specific surface. In oxidizing conditions, 5% of the arsenic was dissolved. Most of the toxic elements (69.7%) were found in the residual fraction.

Analysis of the effluents

Data on the measured pH, redox potential, electrical conductivity (EC), and the concentrations of sulfate and heavy metals in the effluents are presented in Table 2.

Table 2: General Parameters Measured At The Effluents From The In Vivo Pot Tests

Parameters	Control	PGPR	HA	PGPR + HA	2xPGPR + 2xHA
pH	3.58±0.26	3.65±0.31	3.78±0.31	4.02±0.22	4.13±0.25
Eh, mV	364±24	319±17	335±18	337±21	325±24
EC, mS/cm	2.457±0.39	2.050±0.24	2.032±0.23	1.411±0.36	1.375±0.31
SO42-, g/l	1.76±0.22	1.49±0.14	1.27±0.20	0.63±0.32	0.55±0.29
As, mg/l	<0.01	<0.01	<0.01	<0.01	<0.01
Cu, mg/l	14.65±0.35	4.02±0.13	2.17±0.09	1.19±0.11	1.59±0.07
Fe, mg/l	0.64±0.12	0.29±0.05	0.24±0.14	0.05±0.04	0.09±0.03
Mn, mg/l	5.79±1.21	2.11±0.75	7.28±0.34	0.64±0.14	0.58±0.18
Zn, mg/l	2.57±0.73	0.64±0.21	1.08±0.32	0.61±0.29	0.56±0.17

The treatment with PGPR or/and humic substances led to a slight increase in the effluents' pH. The Eh values in all five cases ranged from 301 to 388 mV, with the highest values being established in the control (364±24 mV). The highest EC values were found in the first effluents in all variants. Over time, the electrical conductivity of the effluents decreased. From the obtained results it can be concluded that the applied treatment approaches reduced the activity of the hemolytotrophic microflora. These findings were also supported by data on the concentration of sulfate in the effluents. The highest concentrations of sulfate in the leachates were established in the control (1.76±0.22 g/l). The concentrations of sulfates in cases of separate treatments of PGPR and humic acids were 1.49±0.14 and 1.27±0.20 g/l, respectively. [14] Studied passivation of pyrite surface by adsorption of humic acids by the method of inverse liquid chromatography. According to the authors, humic acids inhibit completely the electrochemical activity of the pyrite surface. Treatment with the studied microbial consortium results also in inhibition of pyrite oxidation by the secreted microbial metabolites. [39] Reported that soluble microbial products from cell growth and decay formed complexes with ferric iron, thus inhibiting its participation in the oxidation of pyrite. We found that the application of humic acids and bacteria exerted a synergistic effect on the suppression of pyrite oxidation. The lowest concentrations of sulfate in the leachates (between 0.55 and 0.63 g/l) were established in the cases of combined treatment with PGPB and humic substances.

The highest concentrations of Cu, Fe and Zn were determined in the effluents of the control treatment yielding values of 14.3–15 (mean 14.65 mg/l), 0.52–0.76 (mean 0.64 mg/l) and 1.84–3 (mean 2.57 mg/l), respectively. The concentration of manganese in the effluent of the control treatment was 5.79±1.21 mg/l. The high concentrations of copper, manganese and zinc in the leachate were due to the high proportion of these heavy metals in the easy-soluble exchangeable fraction (Fig. 1) and the microbial oxidation of the copper and zinc minerals distributed in the sulfide fractions. In studies on long-term acid generation and heavy metal leaching from the tailings, Khoeurn et al. (2019) [34] proved the involvement of these two mechanisms in different phases of contaminated water generation from mining waste.

We found that the application of both humic substances and PGPR resulted in a decrease in Cu, Fe and Zn concentrations in leachates. An increase in the concentration of manganese 28±0.34 mg/l was found only in the case of separate humic acid treatment (Table 2). In the combination treatment with PGPR and humic acids, the lowest concentrations of all heavy metals in the effluents were recorded. Humic substances can form both soluble and insoluble complexes with heavy metals which can increase or decrease metal mobility [13]. According to Chotpantarat et al. [37], the addition of humic acid inhibited strongly the bioavailability of Cu and Pb, whilst decreasing slightly the mobility factor of Co, Cr, and Zn, and increasing slightly the same factor for Mn and Ni, depending on the dose. The application of humic substances to mine tailings significantly decreased Cu leaching, due to the formation of organomineral complexes [39, 11]. Wang and Mulligan reported that humic acid could enhance the mobilization of arsenic and heavy metals from the mine tailings under alkaline conditions (pH 11) [40].

Metal immobilizing PGPR and their application to reduce the availability of metals have also been the subject of a number of studies [41, 42, 43, 44]. Mechanisms by which metal-resistant bacteria might decrease the availability of metals can include interactions of metals with anionic functional groups [45], chelating the metal ions through the organic acids, extracellular polymers, and siderophores, transformation of poorly soluble phosphorous compounds to soluble phosphate by bacterial phosphatases [46]. The strains used in the present study have a number of properties that reduce the availability of metals. *B. subtilis* CI R1, *B. amyloliquefaciens* CI R2, *B. megaterium* AM1, *B. simplex* AM3, *Ps. fluorescens* AM2 and *Ps. arsenicoxydans* produce alkaline phosphatase and acid phosphatase. *B. subtilis* CI R1 and *B. amyloliquefaciens* CI R2 are also positive for starch hydrolysis and casein de-composition, and they produce acid from glucose, arabinose, and mannitol. B. megaterium AM1 and B. subtilis CI R1 have positive activity for siderophore production (data not published).

Analysis of The Plants

The data on the fresh and dry weights of the above-ground biomass is presented in Table 3. It suggests that the application of PGPR and humic acid improves plant growth on poor soil used for mine waste reclamation.

Table 3: Fresh and Dry Weights of The Aboveground Biomass

Variant	**07.June**		**13. August**		**26.October**	
	Fresh weight, g	Dry weight, g	Fresh weight, g	Dry weight, g	Fresh weight, g	Dry weight, g
Control	40.801	2.781	16.902	3.330	28.195	4.302
PGPR	45.502	3.228	18.076	3.637	34.304	5.044
HA	48.913	3.899	22.305	4.435	38.105	5.392
PGPR + HA	49.812	3.930	24.112	4.850	38.611	5.623
2xPGPR + 2xHA	47.113	3.458	21.092	4.362	33.413	5.071

The results of the statistical analysis of the fresh and dry weights of the above-ground biomass as % to control are presented graphically in Fig. 2 and Fig. 3, respectively. The box plots of each one of them comprise a dataset: median (line across box), mean value (small cross in the box), minimum and maximum values (lower and upper ends of the whisker, respectively). Since the P-value of the F-test is less than 0.05, there is a statistically significant difference between the means of the 5 variables at the 5% significance level.

Treatment with only PGPR increased the fresh above-ground biomass yield by 12%, 7% and 22% for the different months of determination. Fresh biomass was 20–35% above controls in the case of separate humic acid treatment. The one-month dose of humic acid applied in this variant was 0.42 g/kg soil. The highest results (22%, 43% and 37% above controls for the various months) were obtained by treating the plants with a combination of PGPR and humic acid applied at a dose of 2 ml/l. The fresh biomass of plants was lower in the combined treatment with a double dose: 15%, 25%, and 19 %, respectively. In this variant, the humic acid was applied at a concentration of 0.84 g/kg soil per month.

The application of PGPR alone had the effect of increasing the dry biomass by 16%, 9% and 17%, respectively (Fig. 3). Treatment with humic acid only increased the dry biomass yield by 40%, 33%, and 25% over the various months. Dry biomass data demonstrates also that the combination of PGPR and humic acid at a dose of 2 ml/l has the greatest positive effect on plant growth (41%, 46% and 31% above controls for the different months). Dry above-ground biomass was lower in the combined treatment with a double dose, at which the concentration of humic acid was 0.84 g/kg soil per month.

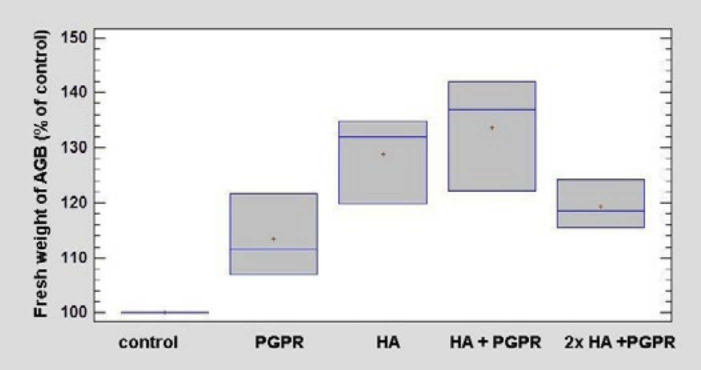

Figure 2: Fresh Weight of The Above-ground Biomass (Agb) In Percentage Versus The Control At The Different Treatment Variants.

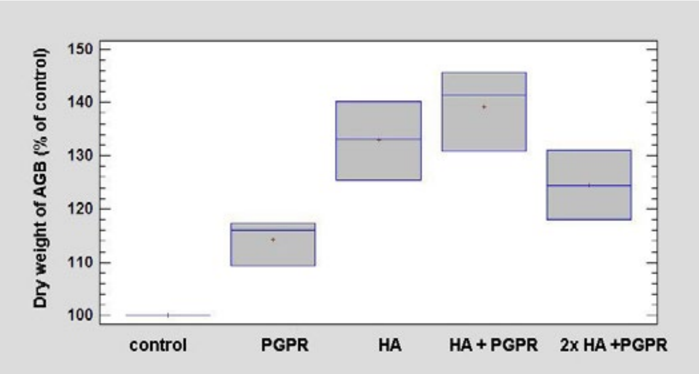

Figure 3: Dry weight of the above-ground biomass (agb) in percentage versus the control at the different treatment variants.

The use of PGPR or humic acid for mining waste remediation has been the subject of numerous studies [8, 11, 47, 23]. Plant growth-promoting bacteria can improve revegetation of mine tailings and increased biomass production by excretion of physiologically active substances (vitamins, enzymes, and phytohormones), production of siderophores and organic acids, fixation of atmospheric nitrogen, phosphorus mobilization, etc [25]. It should be noted that the strains used in the present study were selected considering their proven positive effects on the growth of different test plants (*Lepidium sativum L.* (Cress), *Solanum lycopersicum* (tomato) and *Cucumis sativus* (cucumber)) and/or conducted hypocotyl tests (data not published). Selected strains of bacteria belonging to the *Bacillus* and *Pseudomonas* genera have a number of properties that classify them as plant growth-promoting bacteria. *B. subtilis* CI R1 and *B. amyloliquefaciens* CI R2 are very effective in increasing plant available [48]. Also, these strains produce Indole-3-acetic acid. Ps. chlororaphis 1S4 inhibits completely the growth of three molds (*Aspergillus flavus, Penicillium claviforme,* and *Rhizopus arrhizus*) [49]. B. megaterium AM1, B. *simplex* AM3, *Ps. fluorescens* AM2, and *Ps. arsenicoxydans* AM4 have diverse lytic enzyme activities: esterase, esterase lipase, alkaline phosphatase, acid phosphatase, protease, and amylase (data not published). In our previous study, it was found that treatment with this microbial consortium led to an increase in the chlorophyll content, underground biomass, and the development of lateral roots of *Trifolium repens L.* (white clover) cultivated in soil poor in nutrients [50]. Humic substances are usually applied in soil to promote plant growth and reduce the toxicity of metals. The dose effects of the application of humic acids have been the subject of various studies [51, 52]. [53] examined the efficiency of various doses (0, 0.5, 1.0, 1.5, 2.0, and 2.5 g/kg) of humic acid of different origin (compost, manure, and coal) on the physical and chemical properties of saline soil and the growth and yield of rice. The same authors reported that the optimal dose for dry weight of hay was 1.0–1.5 g/kg for humic acid from coal and 1.5–2.0 g/kg for humic acid from compost. [54] Studied the effect of different levels of humic acids on plant growth and the nutrient content of corn and reported that the dry weight and nutrients' uptake were affected negatively by the application of humic acids in a higher dosage (4 g humus/kg). According to Atiyeh et al. [52], plant growth was increased by treatments of the plants with 50–500 mg/kg humic acids, but decreased significantly when the concentrations of humic acids exceeded 500–1000 mg/kg, due to the hormone-like activity of humic acids, or due to adsorbtion of plant growth hormones onto the humates. A similar negative effect on biomass yield when treated with 0.84 g/kg humic acids was found in the present study. The origin and composition of humic substances, the dose of application, pH, metal concentration, and speciation in mining waste are important factors that need to be taken into account in reclamation technologies.

The applications of PGPR and humic acid had a significant effect on the uptake of biogenic and macro elements in plants growing on poor soil (content of the humus and Kjeldahl-are 0.98% and 0.196%, respectively). In all cases of treatment, the nitrogen content was higher than the control (Table 4), and the highest nitrogen uptake (10.3% above control) was obtained with the combined treatment with a double dose.

Table 4: Effect of Application of Pgpr and Humic Acid on Plant Nutrients and Pollutants Uptake

Treatments	N. %	P. mg/kg	K. mg/kg	Ca. mg/kg	Mg. mg/kg	Fe. mg/kg	Mn. mg/kg	Cu. mg/kg	Zn. mg/kg	As. mg/kg
Control	1.84	1870	10403	5937	2397	402	349	79.5	53.10	<5
PGPR	1.98	1911	15550	7643	2435	530	341	33.5	43.9	<5
HA	1.94	2111	18870	7801	2908	711	540	35.6	82.1	<5
PGPR + HA	1.99	2112	20898	6767	2496	588	275	25.7	34.9	<5
2xPGPR+ 2xHA	2.03	2436	20957	6402	2461	623	252	34.5	53.2	<5

The application of PGPR impacted phosphorus uptake, resulting in an increase in phosphorus content by 2.2%. The application of humic acids augmented the uptake of phosphorus by 12.9%. The highest assimilation of phosphorus from the grass (30.3% above control) was observed with the combined treatment with a double dose. This study found that treatment with PGPR and humic acids increased significantly the uptake of potassium by plants.

Treatment with PGPR only increased K uptake to 49.5%, and in the case of treatment with humic acid only, K uptake was 81.3% above control. The combination of both treatment variants showed an increase in potassium assimilation by more than 100% compared to the control.

According to the analysis of the results, the application of PGPR and humic acids increased also the uptake of Ca and Mg (Table 4). The highest uptake of Ca and Mg by plants was obtained in the case of humic acid treatment: 31.4% and 21.3% above the control, respectively. Similar positive effects of the same *Bacillus* and *Pseudomonas* strains and humic acids on plant growth and assimilation of nutrients have been found in our previous study in field experiments in the area of the Medet tailings pond, Bulgaria [55].

In the present study, the application of PGPR and humic acids increased the uptake of Fe from 31.8% to 76.9% for the different variants of treatment. An increase in Mn and Zn uptake (54.7 % and 54.6%) was found in the case of humic acid treatment. The application of PGPR (both alone and in combination with 0.42 g/kg humic acids) produced a decrease in Mn and Zn content. In all cases of treatment, the uptake of copper from the plants was reduced (Table 4).

The results for the effect of the applied treatments on the aboveground biomass of grass and on the uptake of mineral elements show the applicability of a combination of PGPR and humic acids in the reclamation of mining waste. The combination of these strains applied in poor soil for the reclamation of acid-generated mining waste has the effect of not only improving the mineral nutrition of the grass but reducing also the uptake of Cu and Zn. The similar effects on *Lupinus luteus* inoculated with metal resistant PGPR have been reported by [41]. According to Tripathi et al. [56], inoculation with a Pb- and Cd-tolerant *Pseudomonas putida* KNP9 strain increased plant growth but reduced the Pb and Cd uptake by *Phaseolus vulgaris.* However, it should be noted that PGPB may also alter metal bioavailability and increase plant metal uptake [57, 58]. The large number of studies on the effects of PGPR on different plants for the reclamation of mining sites indicates the possibility of their application in both phytoextraction and phytostabilization [23]. The combination of PGPB with humic substances has an even greater effect on plant growth and improves their mineral nutrition.

Conclusions

The use of humic acid and PGPR for mining waste remediation has several positive effects in the process of waste reclamation characterized by a high content of copper and zinc in the water-soluble and exchangeable fractions. The application of both humic substances and PGPR resulted in a decrease in sulfate, Cu, Fe and Zn concentrations in leachates, due to the suppression of pyrite oxidation. Furthermore, both treatments improved plant growth, when used separately, but their combination had the most positive effect on biomass yield, as plant growth depended strongly on the dosage of application of humic acids. With the combined application, the yields of the fresh and the dry biomass were increased by treating plants with 0.42 g/kg humic acids but decreased significantly with the application of humic acids in a concentration of 0.84 g/kg soil. In conclusion, applying *Bacillus* and *Pseudomonas* in combination with 0.42 g/kg humic acids in poor soil for the reclamation of acid-generated mining waste has the beneficial effect of improving the mineral nutrition of the grass and reducing the uptake of Cu and Zn [59, 60].

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