Use Of Plant Growth-Promoting Bacteria And Humic Acids For Phytostabilization of Acid-Generating Mining Wastes

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Abstract

Generation of acidic mine drainage is a major environmental problem in areas with mining waste. The cost-effective method for reclaiming of acid-generating mining wastes is a vegetation cover. Using plant growth-promoting bacteria (PGPR) and humic acid in remediation have several beneficial effects. The application of both humic substances and PGPR resulted in a decrease in Cu, Fe, Zn and sulfate concentrations in a variety of drainage water samples. Both PGPR and humic acid improve plant growth when used separately, however, the combination of both treatments has the most positive effect on fresh biomass yield – between 22% and 43 % and dry biomass of plants – between 31% and 41 %. Furthermore, addition of *Bacillus* and *Pseudomonas* bacteria, in combination with humic acids, to poor soil for reclamation improved grass mineral nutrition and reduced Cu and Zn uptake. The treatment with PGPR and humic acids significantly increased the uptake of nitrogen, phosphorus and potassium by plants.

Highlights

- The need of cost-effective technology for recultivation requires phytoremediation with humates and bacteria application.
- The proposed treatment results in a decrease in Cu, Fe and Zn concentrations in leachates from sulfur-rich mining waste.
- The mineral nutrition of grass vegetation, grown on poor soil, is improved by treatment with Humic acids in combination with PGPR from genera *Bacillus* and *Pseudomonas*.

Introduction

Metallic ore extraction is associated with generation of great amount of mining waste. Mining waste dumps usually consist of overburden rocks, off-balance sulfide ores and low grade row material (Jordan and Alessandro 2004). These high-volume wastes sometimes contain significant levels of heavy metals and other toxic substances (Druschel et al. 2004; Dold 2014). Additionally, mining waste is also known to cause numerous environmental issues including soil erosion, air, water and soil pollution, toxicity, geo-ecological disasters, loss of biodiversity, and eventually loss of economic wealth (Wong 2003).

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 and high concentrations of sulfate; whereby, the low pH of generated drainage enhances the dissolution of heavy metals in water. The AMD chemistry depends on a number of factors: mineralogical and chemical composition, geotechnical properties of the mineral wastes, 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 (Sand et al. 2001; Schippers and Sand 1999).

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 (Park et al. 2019). These methods are classed into five major groups: physical barriers, bacterial inhibition, chemical barriers (or passivation), electrochemical protection and desulfurisation (Sahoo et al. 2013).

In practice, dry cover is preferably used in the remediation of mining waste as it contains of a layer with low filtration coefficient which restricts the oxygen supply and limits water percolation into the mineral waste. Materials used for dry cover can either be composed of soil or a soil substitute. The soil used in reclamation technologies is often characterised with low content of biogenic elements (nitrogen, phosphorus and potassium) (Sheoran et al. 2008). 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 for reclamation involve ameliorative and adaptive strategies to allow vegetation development (Tordo et al. 2000).

The use of humic acid for mining waste remediation has been the subject of numerous studies (Sahoo et al. 2013; Tapia et al. 2019). Humic acid is a preferred method due to the phenol and carboxylic acid functional groups it contains, which allow the deprotonation of OH/OOH. The carboxyl groups in humic substances play a very important role in 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 (Bogush and Voronin 2011). Complexation reactions of humic substances not only prevent precipitation of metal ions, but also reduce metals toxicity. The stability constant of heavy metal complexes with humic acids increases with increasing pH and decreasing ionic strength (Violante et al. 2010).

Furthermore, humic acid has the potential of passivating pyrite, thus reducing the rate of pyrite oxidation and the generation of acidic drainage water (Aćai et al. 2009). Commercially available humic substances are usually applied in soil to promote plant growth and improve the physical properties of soil, such as aggregation, aeration, permeability, water holding capacity, ions transport and availability through pH buffering (Canellas et al. 2015). 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 (Choudhary et al. 2017). 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 (Turan et al. 2007; Avis et al. 2008; Souza et al. 2015; Joseph et al. 2007; Krishnaveni 2010; Li and Jiang 2017). 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 easy assimilated using bacterial phosphatases, increase of nitrate assimilation, production of siderophores chelating the iron into 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 (Sheoran et al. 2010).

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

Materials And Methods

Experimental design

The *in vivo* pot tests were performed in 18 dm³ containers. The mining waste was placed at the bottom of the pots, forming a 25 cm layer. A layer of soil (10 cm) was laid on top of the waste. According to preliminary information, the mining waste contained the following minerals: quartz – 33%, albite – 30%, microcline – 13%, muscovite – 12%, clinocllore – 6%, calcite – 2% and pyrite – 4%. The soil was taken from a soil depot of a real mining object, located in the region of Srednogie. The soil had a pH (H₂O) - 5,65 and 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, respectively 0.98% and 0.196%. 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 is not contaminated with heavy metals and arsenic.

On the Fifth of 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 solution was performed three times for 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 genera *Bacillus*, respectively *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 dynamically at a temperature of 30 °C on medium containing: 25.0 g glucose, 3.0 g (NH₄)₂SO₄, 1.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 1 g yeast extract, 1.0 g peptone (per liter). For the purpose of this experiment, the liquid microbial cultures from the the above mentioned strains were mixed in equal quantities.

The humic substances were produced from lignite, obtained from Stanyantsi mine, Southwestern Bulgaria. The biotransformation of lignite was carried out by fermentation with microfungi *Trichoderma harzianum* and *T. viride* (Chakalov et al. 2012). The extract 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

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 (V/V) (Lapakko 2002). The water-soluble fraction of sulfate was determined using a spectrophotometric approach (with BaCl_2 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 $\text{NH}_2\text{OH}\cdot\text{HCl}$ 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) (He et al., 2013; Khoeurn et al. 2019). 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 plants

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

After grinding, the plant samples were digested through the use of H_2SO_4 , HNO_3 and HClO_4 (Plank 1992). Concentration of Ca, Mg, K, P, Fe, Mn, Cu and Zn was measured by ICP-spectroscopy. Total nitrogen content in dry biomass was determined by Kjeldahl digestion (ISO 11261).

Results And Discussion

Analysis of 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 concentration of 1.94 g/l.

Concentration and 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

The total concentration of Cu in the mining waste was 958.3 mg/kg. Distribution of copper between exchangeable, Fe - Mn oxyhydroxides, organic matter and 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 (Dold 2003), and it can be concluded that 23.1% of copper is in a water-soluble fraction the form of water-soluble fraction (e.g., chalcantite ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)) and as Cu, which may be released in the exchangeable fraction from vermiculite-type mixed-layer mineral in the mining sample. Twenty-five percent of copper was incorporated in iron-phases. In oxidizing conditions performed by a H_2O_2 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 and twenty-five percent of copper 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 residual fraction as sphalerite accounting for 73.6% of total Zn. Schaidler et al., 2007, found that relatively labile and bioaccessible mineral phases of Zn increased with decreasing particle size of mine waste.

The mining waste in this study contained pyrite at 4%. Iron was mainly distributed in two fractions, residual (86% of 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 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 had 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 (Chotpantarat et al. 2015; Soltani et al. 2017).

The presence of minerals such as albite, microcline, muscovite, clinocllore 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 exchangeable fraction.

The total concentration of As in the mining waste was 252.1 mg/kg. Sequential fractionation of As showed that 25% of total arsenic was distributed in the Fe and Mn oxides fraction. The arsenate and arsenite ions had an affinity to be sorbed from formed ferrihydroxides 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), sulfate and heavy metals concentrations in the effluents are presented in Table 2. Treatment with PGPR or/and humic substances led to a slight increase in pH of the effluents. 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 reduce the activity of the hemolytrophic microflora. These findings are 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. The suppression of oxidation of sulfide minerals in mining waste was more significant by the combination treatment with bacteria and humic acids, where the sulfate concentration is lowest.

The highest concentrations of Cu, Fe and Zn were determined in effluents of 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 effluent of 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 microbial oxidation of copper and zinc minerals, distributed in sulfide fractions. In studies on long-term acid generation and heavy metal leaching from the tailings, Khoern et al. (2019) 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 7.28 ± 0.34 mg/l was found only in the case of separate humic acid treatment (Table 2). In combination treatment with PGPR and humic acids, the lowest concentrations of all heavy metals in effluents were recorded.

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
SO ₄ ²⁻ , 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 application of humic substances to mine tailings significantly decreased Cu leaching, due to the formation of organomineral complexes (Tapia et al. 2019). According to Chotpantarat et al. (2015), the addition of humic acid strongly inhibited the bioavailability of Cu and Pb, whilst slightly decreasing the mobility factor of Co, Cr, and Zn but slightly increasing that for Mn and Ni, depending on the dose. However, humic substances can form both soluble and insoluble complexes with heavy metals which can increase or decrease metal mobility (Violante et al. 2010). Wang and Mulligan (2009) reported that humic acid could enhance the mobilization of arsenic and heavy metals from the mine tailings under alkaline conditions (pH 11). 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.

Metal–mineral–microbe interactions have also been the subject of a number of studies (Gadd 2010; Kong and Glick 2017). 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 (Karthiga and Natarajan 2015; Kong and Glick 2017; Li et al. 2017; Ashraf et al. 2017).

Analysis of plants

Data on fresh and dry weights of the aboveground biomass are presented in Table 3. This data suggests that the application of PGPR and humic acid improves plant growth on poor soil used for mine waste reclamation. The results of statistical analysis of fresh and dry weights of the aboveground biomass are presented graphically in Fig. 2 and Fig. 3 respectively.

Table 3 Fresh and dry weights of the aboveground biomass

Variant	07.June		13. August		26.October	
	Fresh	Dry	Fresh	Dry	Fresh	Dry
	weight, g	weight, g	weight, g	weight, g	weight, g	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

Treatment with only PGPR increased fresh aboveground biomass yield by 12%, 7% and 22% for different months of determination. Fresh biomass was 20 - 35 % above controls in the case of separate humic acid treatment. The highest results (22%, 43% and 37% above controls for different 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 in a double dose. respectively 15%, 25% and 19%. 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.

The application of PGPR separately has the effect of increasing the dry biomass by 16%, 9% and 17% respectively (Fig. 3). Treatment with only humic acid increased dry biomass yield by 40%, 33% and 25% over specific months. Dry biomass data also demonstrate 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 different months). Dry aboveground biomass was lower in the combined treatment in a double dose. Khaled and Fawy (2011) studied the effect of different levels of humic acids on plant growth and nutrient content. and reported that the dry weight and nutrients uptake were negatively affected by the application of higher dosage humic acids (4 g humus/kg). 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.

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

The application of PGPR impacted phosphorus uptake having the effect of increasing the phosphorus content by 2.2%. The application of humic acids affected the uptake of phosphorus with 12.9%. The highest assimilation of phosphorus from the grass (30.3% above control) is observed with combined treatment in a double dose.

This study found that treatment with PGPR and humic acids significantly increased the uptake of potassium by plants. Treatment with only PGPR increased K uptake to 49.5% and in the case of separate humic acid treatment K uptake was 81.3% above control. Combination of both treatment variants showed an increase in potassium assimilation of more than 100% compared to the control.

According to the analysis of results, the application of PGPR and humic acids also increased 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, respectively 31.4% and 21.3% above the control.

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 only in the case of humic acid treatment. The application of PGPR (both alone and in combination with humic acids) had as effect the decrease the Mn and Zn content. In all cases of treatment, the uptake of copper from the plants was reduced as accumulation was undetectable (Table 4).

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 results obtained for the effect of applied treatments on the aboveground biomass of grass and mineral elements uptake show the applicability of a combination of PGPR and humic acids in the reclamation of mining waste. Selected strains of bacteria belonging to the genera *Bacillus* and *Pseudomonas* 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 for increasing plant available phosphorus (Batkova et al. 2015). Also, these strains produce Indole-3-acetic acid. *Ps. chlororaphis* 1S4 completely inhibits the growth of three molds (*Aspergillus flavus*, *Penicillium claviforme* and *Rhizopus arrhizus*) (Georgieva et al. 2018). *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, amylase (data not published). 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 also reducing the uptake of Cu and Zn. The similar effects on *Lupinus luteus* inoculated with metal resistant PGPR have been reported by Dary et al. 2010. According to Tripathi et al. (2005) 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 PGPR may also alter metal bioavailability and increase plant metal uptake (Li and Ramakrishna 2011; Ashraf et al. 2017; Ren et al. 2019). The large number of studies on the effects of PGPR on different plants for the reclamation of mining sites indicate the possibility of their application in both phytoextraction and phytostabilization (Kong and Glick 2017). The combination of PGPR with humic substances has an even greater effect on plant growth and improving their mineral nutrition.

Conclusions

The cost-effective and environmentally sustainable method for stabilising and reclaiming of acid-generating mining wastes is a vegetation cover. The use of humic acid and PGPR for mining waste remediation has several positive effects in the reclamation of waste, characterized by a high content of copper and zinc in water-soluble and exchangeable fractions. The application of both humic substances and PGPR resulted in a decrease in Cu, Fe and Zn concentrations in leachates. Furthermore, both treatments improved plant growth when used separately, but their combination has the most positive effect on biomass yield. In conclusion, applying *Bacillus* and *Pseudomonas* in combination with 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.

Declarations

Data Availability

All data generated or analyzed during this study are included in this published article.

Conflict of interest

The authors report no conflicts of interest.

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Authors' contributions

Svetlana Bratkova and Katerina Nikolova contributed to the study conception and design. Material preparation, data collection and analysis were performed by Petya Genova and Anatoliy Angelov. The first draft of the manuscript was written by Svetlana Bratkova. All authors read and approved the final manuscript.

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Figures

Figure 1

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

Figure 2

Fresh weight of the aboveground biomass (AGB) in percentage towards the control at the different treatment variants.

Figure 3

Dry weight of the aboveground biomass (AGB) in percentage towards the control at the different treatment variants.