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Original Research

The Influence of Biopreparation on Seed Germination and Growth

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Abstract

We studied a landfill where toxic sludge derived from copper ore production is deposited. These types of landfills pose a threat to the surrounding environment because of the complete lack of vegetation and dust emission. Thus, the problem refers to the future recultivation of these areas. In order to improve the quality of the affected environment, thus allowing plants development, we applied biopreparations containing biosurfactants and microorganisms for the following plant species: *Zea mays*, *Lupinus luteus*, *Pisum sativum*, *Avena sativa*, and *Sinapsis alba*. We studied their influence on both germination and plant growth. It was found that applying biosurfactants before germination stimulates all studied seeds. This effective technique could be used for the reclamation of soil contaminated by post-flotation waste. However, the usefulness of biosurfactants for further plant growth was not proved.

Keywords: biopreparation, biosurfactants, germination, copper, post-flotation waste

Introduction

The pre-sowing seed stimulation is aimed at accelerating the power and energy of germination (up to 90%). Treatment of this type consists of covering the seed surface with special substances protecting against the attack of microorganisms or increasing the availability of biogenic substances. The aim of the stimulation is to increase plant resistance to illnesses of viral, bacterial, or fungal origin and to deliver the initial dose of fertilizers for germination and first growing stage.

In order to preserve seeds against illnesses a chemical or physical (temperature, light) disinfection is usually applied. Such a treatment could have a damaging effect on seeds, such as shoots dying or lack of germination.

More and more attention is now being devoted to alternative methods, where the addition of biopreparation, created on the basis of natural substances of biological origin, is used for seed stimulation. One such method uses biosurfactants, i.e. compounds that lower the surface tension of a liquid, mainly of microbiological origin. Their application not only allows the seed protection against microorganisms, but simultaneously facilitates an uptake of biogenic substances by plants, e.g. phosphorus [1]. Moreover, these methods can be applied along with chemical plant protection products contributing to increase their solubility and level of pulverization [2]. The high biodegradability as well as temperature stability and small sensibility to changes of

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environmental pH are an undoubted advantage of surface-active agents produced by microorganisms [3].

Biosurfactants are most often non-toxic compounds. They do not accumulate in tissues of living organisms, thus they do not pose an ecological threat [4]. They are applied in small amounts and do not gather in the environment [3].

The aim of our studies was to assess the impact of bio-preparations containing biosurfactants and microorganisms on germination and growth of plants predicted to the revitalization of soil contaminated by post-flotation sludge. The waste came from the process of copper ore enrichment. The main issue arising from the processing of ore refers to a quantity of toxic sludge, which is deposited within special areas called landfills situated within the area of Nowe Zagłębie. Landfills are characterized by complete lack of vegetation. Huge masses of dust containing heavy metals are blown away from these areas [5, 6].

Experimental Procedures

Postflotation sediments from the "Wartowice" tailing pond contain lots of calcium oxides, silicon, aluminium, and magnesium (CaO, SiO_2, Al_2O_3, MgO). Cu, Pb, and Zn concentrations in soil remain below soil quality standards for arable lands, and in the light of IUNG guidelines correspond to 0-II, 0-I, and 0 contamination levels, respectively (Table 1) [7, 8].

Granulometric composition of deposits with the majority of fraction with 0.06 mm of diameter accelerate dust dispersion into bordering areas and influences flora growth (Table 2). In summary, this type of sludge is characterized by many disadvantageous properties for plant growth such as:

- extremely bad air-water conditions
- a small number of assimilable elements for plants
- the relatively large content of heavy metals (lead and copper)
- deficiency in organic substances
- relatively high pH (8.0-8.5)
- the possibility of smaller areas of tailing ponds being flooded by the surrounding water [7].

Biosurfactant and bacterial preparations were used for processing seeds. Biosurfactant so-called biocomplex came from *Pseudomonas* sp. PS-17 from Lviv Department of Physical-Organic Chemistry Institute, National Academy of Sciences of Ukraine, Lviv, Ukraine. It contained rhamnolipids and egzopolysaccharides [9] and was characterized by a low surface tension (29.5 mNm) and interfacial tension (0.17 mNm; n-heptan), and by the ability to emulsify (E24 = 85%). Biocomplex solution of 0.05 g/l was used for seed stimulation. The bacterial preparation was a suspension of bacteria strain, which was able to bind the atmospheric nitrogen from *Azotobacter* UK genus. Bacteria came from the collection of Ukrainian Academy of Sciences. The number of bacteria in suspension was $38 \cdot 10^6$ CFU/ml.

Five species of plants were used in our studies: maize (*Zea mays*), lupine (*Lupinus luteus*), pea (*Pisum sativum*), mustard (*Sinapsis alba*), and oat (*Avena sativa*). In order to study the seeds' ability to germinate, we determined the energy and power of seed germination. The energy of the germination means the number of germinated seeds in the initial period in relation to the number of planted seeds expressed as a percent. It provides the information about the vitality of seeds and their ability for quick germination. The power of germination means the number of germinated seeds in both times, expressed in percent in relation to planted seeds. It describes the number of seeds that have an ability to germinate even with a certain delay. The time required to determine the power of the germination should be long enough to allow germinating of all seeds that are capable of doing so. The time of germination is different for particular species and also depends on seed size.

The experiment was designated as follows: Petri dishes were filled with cotton layer and filter paper moistened with distilled water. We used 50 seeds of each species for the

Table 1. The avarage chemical composition of post-flotation sludge according to Mizera [7].

Compound	Content [%]	Compound	Content [%]
Na_2O	0.107	As_2O_5	0.008158
Li_2O	0.0118	SeO_2	0.000052
K_2O	3.25	MoO_3	0.001103
CaO	22.75	CrO_3	0.0132
MgO	3.96	WO_3	0.000011
SO_3	0.462	CdO	0.0000406
B_2O_3	0.1004	PbO	0.00396
ZnO	0.0082	CoO	0.002866
P_2O_5	0.1749	NiO	0.00287
Al_2O_3	11.74	BeO	0.001836
Fe_2O_3	2.58	V_2O_5	0.01835
SiO_2	53.9	CuO	0.22199
Mn_2O_5	0.268	Ag_2O	0.00218
SrO	0.0542	TiO_2	0.124
BaO	0.0212	ZrO_2	0.00217
SnO_2	0.000684	HgO	0.00019
TlO_2	0.0001843	UO_3	0.000898

Table 2. Average granulometric composition of post flotation waste in the studied area (according to PN-R04033).

Granulometric class [%]			
>2.0 mm	0.05 -2.0 mm	0.002-0.05 mm	<0.002 mm
0	1-13	63-85	5 – 35

experiment. We did not add any biostimulants at this stage. Seeds germinated at 20ºC. After three days all germinating seeds were counted in order to estimate the energy of the germination. We also counted all germinated seeds after 5-10 days to determine the germination strength of seeds. The experiment was conducted in greenhouse L/D 16:8, with temperature varying from 20ºC to 22ºC. Plants grew in vases (14 cm of diameter). We planted 12 seeds in one vase (*Zea mays*, *Lupinus luteus*, *Pisum sativum*, *Avena sativa*) with the exeption of *Sinapsis alba* (25 seeds). We put 100 g of coarse-grained grit and drain at the bottom of every vase. All vases were filled up with 475 g of post-flotation sludge and 25 g of uncontaminated sand.

Seed stimulation was conducted in three variants:

- Variant 1 (PS): five species (*Zea mays*, *Lupinus luteus*, *Pisum sativum*, *Avena sativa*, and *Sinapsis alba*) were used. Seeds of these plants were stimulated through one hour with biopreparation containing the biosurfactant (concentration 0.05 g/l). In order to mix it up, 4% of biopreparation (in relation to seeds mass) was added.
- Variant 2 (AB): only three species were planted: *Zea mays*, *Sinapsis alba*, and *Avena sativa*. The seeds were placed in nutrient (for one hour) containing nitric bacteria *Azotobacter* sp. before planting.
- Variant 3 (PS+AB): the same species were used as in variant 2, but biosurfactant was added into the liquid nutrient with *Azotobacter* sp. (the concentration of 0.05 g per 1 litre of nutrient).

Seeds were stimulated for 1 hour before planting. The fertilization of plants was different depending on the applied variant. 10 ml of NH_4NO_3 was added into 0.5 kg of base in variant 1, which corresponded to the dose of 10 mg of N and 20 ml of K_2HPO_4 (20 mg of P), $MgSO_4 \times 7H_2O$ (10 mg of Mg).

In variants 2 and 3 the dose of nitrogen was reduced to 2.5 mg of N per 0.5 kg of the base. Additionally, in these two variants, after filling the vases, chopped straw (3 g) in the surface layer (2-2, 5 cm) was applied as an organic compound.

For each variant a different type of control was prepared:

- five species (*Zea mays*, *Lupinus luteus*, *Pisum sativum*, *Avena sativa*, and *Sinapsis alba*) were used for control of variant 1. What is more, 10 ml of NH_4NO_3 was added into 0.5 kg of base in control of variant 1, which corresponded to the dose of 10 mg of N and 20 ml of K_2HPO_4 (20 mg of P) , $MgSO_4 \times 7H_2O$ (10 mg of Mg).
- three species (*Zea mays*, *Sinapsis alba*, and *Avena sativa*) were planted in controls of variant 2 and 3. Additionally the nitrogen dose was diminished to 2.5 mg per 0.5kg of the base.

An addition of straw was not applied in control vases.

All vases were filled with the base, watered after three days, and then seeds were planted. The experiment was conducted in four repetitions.

In variant 1, before planting seeds, the base was hydrated with distilled water (100 ml) using the drain.

In variants 2 and 3, 50 ml of the distilled water was added by drain, but 2% solution of glucose as a nutrient for bacteria was poured evenly onto medium surface.

The hydration of control vases was similar to variant 1. 14 days after plant germination, an additional feeding was applied for variant 1 and its control: we introduced $Ca(NO_3)_2$ – 5 mg of nitrogen on 10 ml of base for the vase.

The experiment was conducted for 40 days, applying everyday watering. After 40 days plants were removed from the soil, measured, and weighed.

Dehydrogenase activity of base was also determined according to the methodology given by Show and Burns [10]. In the method a principle was applied that the redox dye 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) as the acceptor of hydrogen and electrons (of bigger affinity than oxygen) in the water solution is colourless. After accepting hydrogen and electrons from dehydrogenases, INT is reduced to colorful (red) triphenyl formazone (INTF), the color intensity is a measure of the dehydrogenase activity of studied medium.

The achieved results were statistically analyzed with t-test, one-way ANOVA. In order to assess the impact of various experimental factors, Tukey test was used (StatSoft package, Inc. (2010), STATISTICA version 9.1, www.statsoft.com).

The mean plant height and mass was compared with regard to the control in all studied variants (1, 2, 3) and their combinations: variant 0, where the influence of fertilization by applying different doses of nitrogen was assessed. For that purpose, we did the statistical analyses for mean control for variant 1 in relation to the control for variants 2 and 3. The obtained results allowed for the assessment of the impact of used biopreparations for the stimulation of the growth of all species used in the experiment.

Results

The analysis of the influence of stimulation with biopreparations on the germination ability of examined plants was the first stage of conducted studies. Before the stimulation the potential power and energy of the germination was determined. Fig. 1 presents the achieved results. We assessed the germination energy for all studied plants from 72 to 100%, and the power from 94 to 100%.

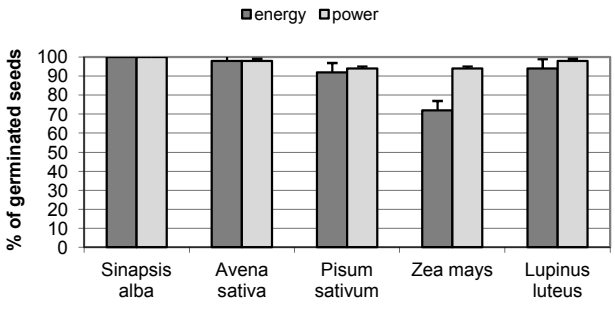

Fig. 1. The results obtained for all studied species of plants concerning power and energy of germination. Seeds were tested without any addition of biostimulants. Means (columns) and SEs (error bars) for four replicates.

The germination ability of seeds subjected to stimulation with biopreparations (variants 1, 2, 3) was also studied. It was shown that stimulated seeds with biosurfactant (variant 1, PS) had germinated better than in test vases (Fig. 2) ($t = 3.44$, $p < 0.05$). The number of germinated seeds was higher by 4-6% in comparison with seeds germinated in test vases for all plants used in this experiment. Similar results were obtained when bacterial biopreparation (variant 2, AB) was applied (Fig. 3) ($t = 3.16$, $p < 0.05$). Improvement of the germination capacity of 14% for *Zea mays* and 2% for *Avena sativa* was observed. The only exception was *Sinapsis alba*, in this case the application of the biopreparation (variant 2, AB) did not influence its germination.

It is astonishing, but the ability of seed germination stimulated with biopreparation containing biosurfactant and bacteria (variant 3, PS+AB) was reduced for all examined species of plants from 6 to 13% (Fig. 3) ($t = 4.9$, $p < 0.05$).

An assessment of the impact of applied biopreparations (3 variants) for the stimulation of plant growth was the second research stage. For that purpose, after 40 days of the experiment, biomass and height of every plant was determined. Table 3 shows the obtained results.

We can assume that biostimulation affects the height and masses of plants compared with control specimens. The impact of stimulation with biosurfactant (variant 1, PS) on growth of studied plants in the experience (*Zea mays*, *Lupinus luteus*, *Pisum sativum*, *Sinapsis alba*, and *Avena sativa*) was not revealed. Both types (control and vases) did not differ significantly regarding their height and mass ($t=0.47$, $p < 0.05$).

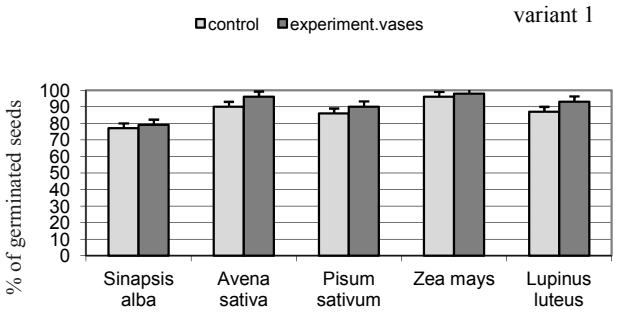

Fig. 2. The results obtained for variant 1(PS) of the experiment concerning germination percentage (±SE).

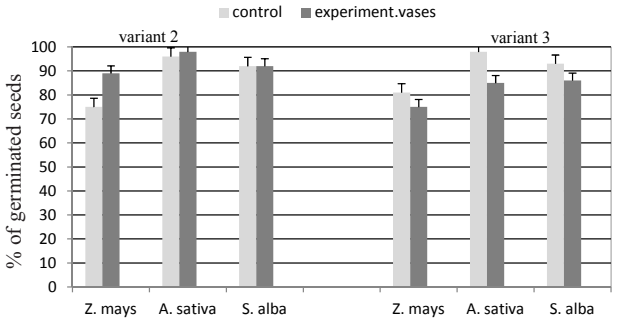

Fig. 3. The results obtained for both variants: 2 (AB) and 3 (PS+AB) concerning germination percentage (±SE).

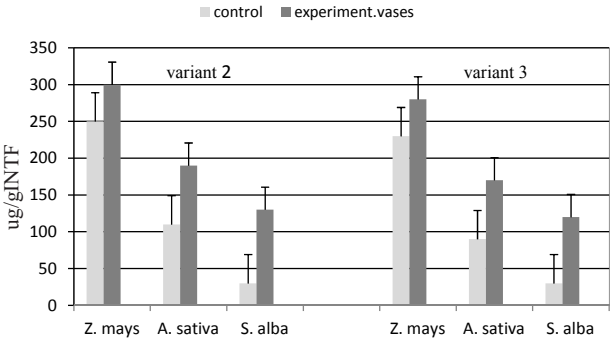

Fig. 4. The results obtained for both variants: 2 (AB) and 3 (PS+AB) concerning dehydrogenase activity of base (±SE).

In variant 2 (AB) the average height and mass of examined plants compared with control did not differ ($t = 0.44$, $p < 0.05$) except for *Avena sativa*, where the average plant height was lower than in test vases ($t = 27.14$, $p < 0.05$).

However, stimulation with the use of biopreparation containing biocomplex and *Azotobacter* sp. (variant 3, PS+AB) affected both height and mass of two species *Sinapsis alba* and *Avena sativa* – their height and mass were reduced significantly in relation to the control ($t = 5.04$, $p < 0.05$). The only exception was *Zea mays*, where the difference in comparison to the control was not revealed, both types did not differ significantly ($t = 1.17$, $p < 0.05$) .

Dehydrogenase activity of soil microorganisms was determined after the end of the experiment. Base samples taken from every vase in variant 2 (AB) and 3 PS+AB) were used for analysis. Fig. 4 presents the obtained results.

The dehydrogenase activity was higher in vases, where seeds were stimulated with bacterial biopreparation (variant 2,AB) and with the use of biopreparation containing biocomplex and *Azotobacter* sp. (variant 3, PS+AB) in comparison to the control ($t = 3.7$, $p < 0.05$). Such results suggest that bacteria kept their metabolic activity during the progress of the experiment.

The base of *Zea mays* had the highest dehydrogenase activity, the medium taken from *Sinapsis alba* had lower one and the lowest was this of *Avena sativa*. Such findings were observed in every examined variant (PS, AB, PS+AB) and controls. The higher activity was characterized by media where seeds had been processed earlier with biopreparation (variant 2, AB) compared to media with the biopreparation containing biocomplex and *Azotobacter* sp. (variant 3, PS+AB). The obtained results suggest that dehydrogenase activity depends on biopreparation type used for stimulation and on plant species chosen for the experience.

Discussion of Results

Nowadays, specially constructed biopreparations are more and more applicable in order to improve seed quality. Such treatment improves the plant germination particularly on a contaminated base. Additionally, many biopreparations stimulate the plant growth as they serve as a nutrient [1].

Table 3. An assessment of the impact of applied biopreparations (3 variants) for the stimulation of plant growth after 40 days of the experiment. The data concerning height and dry mass yield subjected were to one-way ANOVA, the differences among treatment were compared by Tukey's test using StatSoft, Inc. (2010). STATISTICA (data analysis software system), version 9.1. www.statsoft.com. in order to assess the impact of experimental factors. The same letters signify homogenous groups according to Tukey test p=0.05.

Variants	*Sinapsis alba*		*Avena sativa*		*Zea mays*		*Pisum sativum*		*Lupinus luteus*	
	Height	Biomass	Height	Biomass	Height	Biomass (g)	Height	Biomass	Height	Biomass
	(cm)	(g)	(cm)	(g)	(cm)		(cm)	(g)	(cm)	(g)
Biosurfactant (v. 1. PS)	15.7±0.7bc	0.62±0.03b	25.8±0.6a	0.62±0.03c	46.6±0.9bc	4.8±0.3b	12.7±0.3*	2.0±0.1**	14.8±0.6**	3.5±0.2 **
Control	16.4±0.8c	0.63±0.03b	26.2±0.6a	0.61±0.03bc	49.3±1.1c	5±0.4b	12.3±0.3 **	1.8±0.1**	14.8±0.7 **	3.2±0.2 **
Azotobacter sp.(v. 2. AB)	11.4±0.6a*	0.24±0.01a	22.9±0.4bc	0.49±0.02ab	37.6±1.1a	2.4±0.2a	-	-	-	-
Control	13.0±0.7ab	0.27±0.02a	24.9±0.7ab	0.55±0.03abc	36.7±1.7a	2.6±0.3a	-	-	-	-
Biosurf.+ Azotobacter sp. (v. 3. PS+AB)	13.0±0.6ab	0.24±0.01a	20.8±0.5c	0.45±0.02a	39.9±1.6a	2.6±0.2a	-	-	-	-
Control	15.9±0.6c	0.31±0.01a	24.5±0.6ab	0.56±0.04abc	42.5±1.6ab	3.1±0.3a	-	-	-	-

Surface-active agents are surface-active compounds, being characterized by amphiphil structure of particles. The presence of both hydrophobic and hydrophilic parts causes molecules to gather on the border of two, not-mixing phases. Surfactants cause a drop of surface tension, and also generate and stabilize emulsion and foam [11]. The increased effectiveness of removing metals by solutions of anionic surface-active agents is connected with two mechanisms: desorption and complexing. Molecules of the surface-active compound accumulating on the border of solid phase and soil solution lower inter-phase tension and functioning of capillary forces and participate in metals binding. These processes increase significantly the content of metal ions in soil solution. Desorption is also accelerated by complexing of metal cations through molecules and micelles of biosurfactant occurring in the water phase. The bonds created between cations of heavy metal and negatively charged of biosurfactant parts are strong enough that the lavage completely removes these complexes [11-14].

Promising effects have been achieved when rhamnolipid, sophorolipid, and surfactin were applied. The reliability of these methods depends on several factors and requires earlier, experimentally selection of the lavage conditions (a choice of the type and the concentration biosurfactant especially) according to the type of pollutants and character of the soil. The level of removal depends also on the frequency of conducted extractions of ground. It was showed that single lavage of the soil with the 0.1% solution of surfactin in the 1% of NaOH allows to remove 25% of Cu, 6% of Zn, and 5% of Cd; however, repeating this activity five times allows to achieve a level of removal up to 70% of Cu, 25% of Zn, and 15% of Cd [11, 15]. The method that could significantly improve the effectiveness of soil lavage and facilitate the control of the whole process at the same time is the application of biosurfactants in the form of foam [11]. On the other hand, the ability of surfactive agents to create foams can cause exploitation problems in bioreactors which, in such cases, should be equipped with devices for nailing foam [16].

Surfactants of natural origin have been applied in many commercial branches so far [17, 18]. The successful attempts to apply biosurfactants in farming also have been achieved [19].

Adding biosurfactants before germination could have a beneficial effect on seeds as they exhibit antifungal, antibacterial, and antiviral properties [12]. Applying fertilizers which contain nitric bacteria from *Azotobacter* sp. genus is used universally when planting seeds of crop plants and tubers or vaccinating roots of seedlings. *Azotobacter* enriches plants into nitrogen as well as stimulates their height by producing height stimuli.

Conclusions

In the conducted experiments three types of biopreparations were applied and introduced altogether with seeds onto base containing waste after the flotation of the copper ore. It was found that only biosurfactants could be totally

useful for the revitalization of soil contaminated by post-flotation waste. The application of *Azotobacter* sp. improved the germination rate among three studied plants, too (with one exception: *Sinapsis alba*). Use of a combination of the two variants described above (biosurfactant + *Azotobacter* sp.) alone did not influence germination satisfactorily. What is more, a beneficial effect of stimulation with three studied variants (PS, AB, PS+AB) on further growth of plants on contaminated soil has not been observed too, however the unfavorable effect, when adding biosurfactants, has not been also revealed. Thus, it is necessary to establish whether supplementation during the experiment by the addition of biosurfactants could have a beneficial impact on plant development.

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