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PHYTOREMEDIATION OF HEAVY METALS IN CONTAMINATED SOIL THROUGH COMPOSTS AND USEFUL MICROORGANISMS IN VEGETABLE PLANTS

ABSTRACT

OF DISSERTATION FOR AWARDING THE EDUCATIONAL AND SCIENTIFIC DEGREE "DOCTOR"

Professional field 4.4. EARTH SCIENCES
Scientific specialty "Ecology and ecosystems' protection"

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The PhD thesis is written on 200 pages and contains 29 tables and 61 figures. The literature index includes 508 sources, 2 in Cyrillic and 506 in Latin.

The PhD thesis was discussed and directed for the defence at the extended meeting of the Department of Microbiology and Environmental Biotechnology, Faculty of Plant Protection and Agroecology, Agricultural University - Plovdiv, held on May 09, 2022.

The specialised scientific jury:
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The defence of the dissertation will take place on …….2022 from ……..h in hall № …., Faculty of Plant Protection and Agroecology at the Agricultural University - Plovdiv. The materials related to the dissertation are available to those interested on the website of AU-Plovdiv (http://www.au-plovdiv.bg).

I. INTRODUCTION

Heavy metals are one of the most toxic and persistent pollutants in agriculture, along with pesticides. The continuing pollution of environmental components with them is difficult to control, providing for the pursuit of the global economy, the right to increase trade, welfare, standards and quality of life, and production, intended for consumption. The aim and mission of the European Union's overall policy are to ensure compliance with the requirements of levelling the playing field in the emerging single transnational market. This will produce and consume the cleanest possible goods and services, sparing renewable and non-renewable natural resources and caring for populations and natural resources.

The monitoring of soils and lands in our country is carried out at three levels by 15 accredited regional laboratories of the EEA. Observations at the first level (I level - large-scale monitoring) are carried out in a uniform network of 16x16 km at 397 points every five years and are aimed at identifying the current state of soils on several critical indicators: 9 heavy metals and metalloids, total nitrogen, organic hydrogen, phosphorus, pH active reaction of the soil, nitrate nitrogen, electrical conductivity, total carbon and persistent organic pollutants - 16 PAH, 6 PCB, 15-chlorine organic pollutants, bulk density.

As a result of the expert opinions, measures are taken following the adequacy and timeliness of the current situation. Phytoremediation is the most distinct and beneficial approach related to the purification of the environment from stratification and the high concentration of heavy metals. Among the advantages of the method is the ability to be applied on-site at the time of the study. Among the shortcomings, we can point out the lack of financial resources, space and time constraints (phytoremediation in our country is episodic and one-sided), the need for a comprehensive approach in the field, which involves a number of disciplines such as chemistry, physiology, ecology, microbiology and others.

II. PURPOSE AND TASKS

This dissertation aims to explore the possibilities for improving the growth and development of vegetable plants grown on soil contaminated with heavy metals using an environmental friendly approach.

For the practical realisation of the goal, several main tasks are assigned:

1. Isolation of microorganisms tolerant to heavy metals;
2. Selection of isolates capable of stimulating the growth of vegetable crops;
3. Species identification of isolates;
4. Study of the development of vegetable crops on contaminated soil using compost and selected populations of beneficial microorganisms;
5. Study of the behaviour of heavy metals in contaminated soil with the application of compost and appropriate populations of beneficial microorganisms;
6. Study of the changes in microbial communities in the rhizosphere and non-rhizosphere of the used vegetable plants.

III. MATERIAL AND METHODS

3.1. Isolation of plant growth-promoting bacteria.

In order to achieve optimal results and accuracy of empirical research in connection with the isolation of microorganisms that promote the growth of vegetable plants, soil samples were taken from the rhizosphere of the following plant species: family *Lamiaceae* - *Lamium amplexycoule*, family *Brasicaceae* - *Capsella bursa pastoris*, family *Geraniacea* - *Geranium molle*, family

Valerianaceae – Valerianella sp. and family *Asteraceae - Cirsium sp,* from the area of KCM - Plovdiv.

After their transfer to the Department of Microbiology and Environmental Biotechnology laboratory, decimal dilutions in sterile distilled water were made according to standard methods. Sowings were performed on an agar nutrient medium containing a concentration of Cd, 2 mg kg^{-1}, Pb, 10 mg kg^{-1} and Zn, 50 mg kg^{-1}.

Morphological characteristics of the isolates are studied by standard methods (Sapundjieva et al., 2010). The production of indoleacetic acid and siderophores was tested by the methods described by Belimov et al. (2005) and Perez-Miranda et al. (2007).

3.2. Tolerance to heavy metals (Pb, Cd, Zn).

Various soluble salts of heavy metals were added to freshly prepared agar flasks to achieve the appropriate concentrations: Pb, from 50 to 1100 mg kg^{-1}; Zn, from 100 to 300 mg kg^{-1} and Cd, from 5 to 100 mg kg^{-1}. This was done after preparing sterile stock solutions (sterilised by filtration through pores 0.22 μm). The isolates were inoculated into food broth flasks (100 ml) overnight and then plated on agar plates containing the appropriate concentrations. Maximum tolerance to heavy metals was determined based on growth after incubation at 28 ° C for 24-48 hours**.**

3.3. Pot trials.

For the experiment, soil contaminated with heavy metals from the area of KCM - Plovdiv and compost from the company "Biovet" Peshtera AD, as well as spinach seeds *Spinacea oleracea* L. variety "Matador" were used. The selection is not random - given the particular and specific influence of soil characteristics and especially soil acidity on the mobility of heavy metals and the possibility of their absorption by plants, the region of KCM-Plovdiv is exceptionally grateful for research.

3.3.1. First trail

The experiment was set in three treatments with three replicates, as follows (Table 1):

Table 1. Experimental staging.

	Treatments
1	Contaminated soil (100%)
2	Contaminated soil (75%) + compost (25%, v/ v)
3	Contaminated soil + *P. fluorescens*

The soil was previously cleaned of plant debris and stones. Plastic boxes with a capacity of 6000 cm^3 were used in the experiment. In the first and third treatments the soil was placed directly into the boxes, while in the second it was pre-mixed with compost in a ratio of 3:1 (v: v) and stayed for 14 days.

The seeds were sown in two rows along the length of the boxes, and those of the third treatment were pre-treated with a suspension of *Pseudomonas fluorescens* biotype F. In the course of experimental studies, the tolerance of this bacteria to Pb, Cd, Zn and its properties as a promoter of plant growth, releasing indoleacetic acid and siderophores in the middle was confirmed again.

This bacteria was not found to possess the enzyme ACC deaminase. A population of *P. fluorescens* was introduced into the soil twice during the growing season. The application was carried out in a concentration of 10^4 c.f.u./cm^3 of soil.

3.3.2. Second trial.

The second experiment used soil contaminated with heavy metals from the area of KCM - Plovdiv and compost from the company "Biovet "Peshtera AD, as well as spinach seeds (*Spinacea oleracea* L.) variety "Matador ", radishes (*Raphanus sativus*) variety "Regal" and peas *(Pisum sativum*) variety "Ran 1 ". The experiment was carried out in the vegetation house in three treatments with three replicates, as follows:

Table 2. Second experimental statement.

	Treatments
1	Contaminated soil (100%)
2	Contaminated soil (75%) + compost (25%, v/v)

3	Contaminated soil (75%) + compost (25%, v/v) + *P. fluorescens*

For the needs and completeness of the results of the experiment during the vegetation period of the plants, the length of the leaves of spinach, radish and pea plants, the length of their leaf stalk and the diameter of the leaf rosette were gradually measured.

A number of biometric, physiological (chlorophyll fluorescence intensities), enzymological (dehydrogenase and ß-glucosidase activity) and chemical (pH, EC and mobile forms of heavy metals in soil; accumulation of heavy metals in plant tissues) were performed.

3.3.3. Third trial.

This final experiment was based on soil contaminated with heavy metals from KCM - Plovdiv, compost from "Biovet "Peshtera AD, spinach seeds (*Spinacea oleracea* L.) variety "Matador "and various bacterial isolates, including combinations of them. The ratio of soil and compost in all treatments was 75%:25% (v: v).

The experiment was conducted in the vegetation house of the department and was set in ten treatments with three replicates, as follows:

Table 3. Third experimental setup.

	Treatments
1	Contaminated soil
2	Contaminated soil + compost
3	Contaminated soil + compost + isolates №32, 32‘, 41, 44
4	Contaminated soil + compost + isolates №32, 32‘,44, 44‘
5	Contaminated soil + compost + isolate №32
6	Contaminated soil + compost + isolate №32‘
7	Contaminated soil + compost + isolate №41
8	Contaminated soil + compost + isolate №41‘
9	Contaminated soil + compost + isolate №44
10	Contaminated soil + compost + isolate №44‘

The soil was pre-cleaned of plant debris and stones, and plastic pots with a capacity of 4000 cm^3 were used to carry out the experiment.

In previous experiments, these populations were shown to be tolerant to Pb, Cd, Zn, as well as confirmed to promote plant development by releasing indoleacetic acid and siderophores in the medium. A population of different bacterial isolates was introduced into the soil twice during the growing season. The application was made in a concentration of 10^6 c.f.u./cm^3 of soil.

During the vegetation of the plants, periodic measurements of the length of the leaves, the length of the leaf stalk and the diameter of the leaf rosette were resorted to. In the second half of June, the plants were harvested and subjected to a number of studies (biometric, physiological and chemical), explained in detail later in this paper.

3.4. Statistical data processing.

The data was processed using the "SPSS" program for Windows. Variation analysis (three treatments) was used to assess the accumulation of Pb, Zn and Cd in different plant organs in spinach, radish and peas, determining the mean values and standard error (deviation). Comparative multidirectional analysis according to Duncan (1955) and hierarchical cluster analysis using the method of Euclidean distance between groups are applied.

IV. RESULTS AND DISCUSSION

4.1. Isolation of microorganisms tolerant to heavy metals.

As a result of the initial steps for isolation of microorganisms from the rhizosphere of wild plants from the polluted area of KCM-Plovdiv, a collection of 35 isolates was formed. In addition, the determination of their tolerance was performed in relation to their ability to grow in an environment containing different concentrations of Cd, Pb or Zn. The data are presented in table 4. The results show tolerance in bacteria isolated from the rhizosphere of the following plant species growing freely in the area of KCM - Plovdiv: *Lamium amplexycoule*, *Capsella bursa pastoris*, *Geranium molle*, *Valerianella sp*. And *Cirsium sp*. As can be seen from the table, isolates № 22 and 32 show tolerance at all concentrations (1100 mg Pb kg^{-1}, 100 mg Zn kg^{-1}, 100 mg Cd kg^{-1}). Isolates 31' and 44 show the same tolerance as isolates 22 and 32 with respect to Pb and Zn, but lower with respect to Cd - 70 mg kg^{-1}. Isolates 12, 31, show high tolerance to Pb (1100 mg kg^{-1}), but lower to Zn and Cd - 100 mg kg^{-1} and 70 mg kg^{-1}, respectively. Isolate 11 (Pb - 500 mg kg^{-1}, Zn - 0 mg kg^{-1} and Cd - 20 mg kg^{-1}) showed the lowest tolerance to the tested metals.

Table 4. Tolerance of isolates to heavy metals.

Isolates	Pb	Zn (mg kg^{-1})	Cd
11	500	0	20
12	1100	100	70
13	1100	100	20
14	600	100	20
21	200	0	70
21'	500	100	70
22	1100	200	100
23	600	0	20
31	1100	100	70
31'	1100	200	70
32	1100	100	100
32'	700	100	20
41	500	200	20
41'	500	100	20
43	600	0	20
44	1100	200	70
44'	800	0	70
P. fluorescens	900	100	20

4.2. Characterisation of isolates.

4.2.1. Produce of indoleacetic acid.

The production of IAA from the isolates in the control treatment without tryptophan was significantly less pronounced compared to tryptophan treatments, suggesting its need for indoleacetic acid formation (Fig. 1).

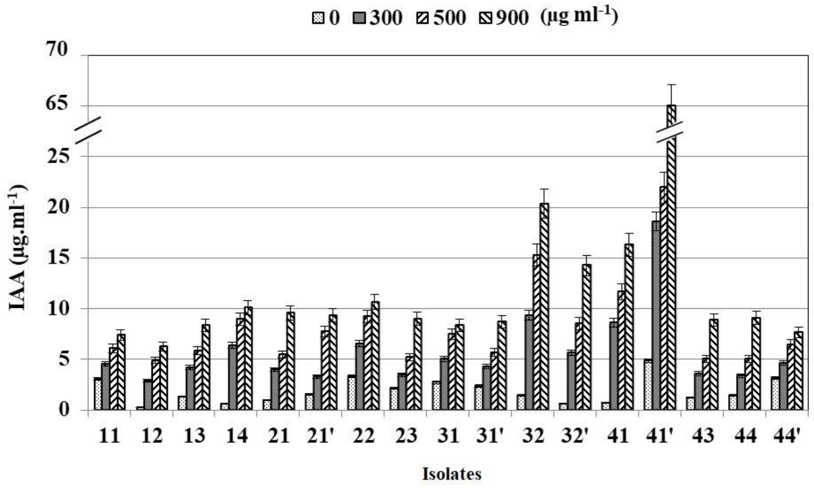


Figure 1. IAA production from isolates 14, 21, 21`, 22, 32, 32`, 41, 41`, 44, 44` in the presence of different amounts of tryptophan.

When tryptophan was introduced into the medium, the production of IAA increased, and a direct relationship was found between the increase in tryptophan concentration and the production of IAA. Thus, at 900 µg ml$^{-1,}$ tryptophan isolate № 41' (*Bacillus thuringiensis*) forms 64.84 µg IAA mg^{-1} DW. Isolates 32, 32' (*P. putida*) and 41 (*Pseudomonas fluorescens*) produced 20.38 µg, 14.28 µg and 16.33 µg IAA mg^{-1}, respectively. These values suggest a strong positive effect on the formation of plant roots in real conditions.

4.2.2. Production of siderophores.

Our study revealed a change in colour of the area around microbial colonies from blue to yellow-orange after 30 min from the application. After application of the coating medium, it was found that isolates 22, 31, 31', 32, 32', 41, 41', 43, 44, 44' produced siderophores (Fig. 2).

Figure 2. Productiontion of siderophores from isolates when applying CAS medium.

4.2.3. Identification of isolates

4.2.3.1. Metabolic identification through the Biolog system.

After the metabolic identification, the following results were found: isolates 32 and 32` are the species *Pseudomonas putida*; isolates 41 and 44'are *Pseudomonas fluorescens*; isolate 41` is *Bacillus thuringiensis,* and isolate 44 is *Pseudomonas synxantha*. They were identified as non-spore-forming, while isolates 23, 31, 41' were identified as *Bacillus thuringiensis* and are spore-forming.

4.2.3.2. Molecular identification by 16S rDNA sequence analysis.

As a result of the molecular identification of the best isolates, the following results were obtained with a high degree of probability:

1. Isolate 41 *’Bacillus cereus* - 99.56%
2. 190_50955_Isolate 32 *’Pseudomonas putida* - 99.53%
3. 191_50956_Isolate 44' *Pseudomonas putida*
4. 192_ 50957_32B_*Pseudomonas fluorescens* 16S ribosomal RNA, partial sequence - 99.48%
5. 193_50958_ 44B *Pseudomonas fluorescens* 16S ribosomal RNA, partial sequence 880 bp - 97.87%
6. 195_ 50959_ 41B *Pseudomonas fluorescens* 16S ribosomal RNA, partial sequence 944 bp - 98.52%

The nucleotide sequences of the isolates were registered in the NCBI gene bank with the following numbers (Table 5).

Table 5. Correspondance of isolates to their sequences and the number in GenBank of NCBI

Isoleates	Sequences	Numbers
41’	SUB11494244 seq1	ON557708
32’	SUB11494244 seq	ON557709
44’	SUB11494244 seq3	ON557710
32	SUB11494244 seq4	ON557711
44	SUB11494244 seq5	ON557712
41	SUB11494244 seq6	ON557713

The evolutionary history is presented in fig. 3.

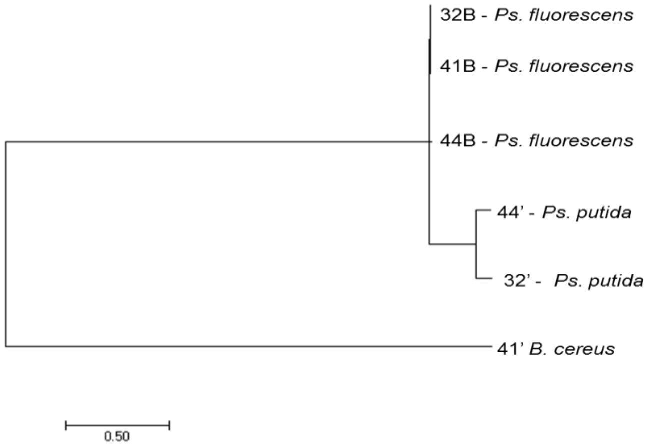


Figure 3. Molecular phylogenetic analysis by the method of the highest probability.

The identification of isolates was not unique to all. In some isolates there was a discrepancy in the result, which is attributed to insufficient performance of the methodology of the Biolog system, but also to its imperfection (Table 6).

Table 6. Comparison of the results of metabolic and genomic identification of isolates.

Isolates	Biolog	DNA
32	*Pseudomonas putida*	*Pseudomonas fluorescens*
32'	*Pseudomonas putida*	*Pseudomonas putida*
41	*Pseudomonas fluorescens*	*Pseudomonas fluorescens*
44'	*Pseudomonas fluorescens*	*Pseudomonas fluorescens*
41'	*Bacillus thuringiensis/cereus*	*Bacillus cereus*
44	*Pseudomonas synxantha*	*Pseudomonas fluorescens*

4.2.4. Testing the ability of isolates to stimulate seed germination and development of young plants.

Table 7 presents data on the newly formed roots' length and their weight with the seeds. The indicators are better for isolates 41 and 32. At the same time, these indicators are higher than those in control, by 22% and 43%, respectively.

Table 7. Root length and fresh mass of pea seeds.

Treatment	Root length (cm)	Fresh mass (g)
Control	5.8±0.5	2.9±0.2
41	7.1±0.6	3.2±0.2
32	8.3±0.7	3.6±0.3
44	5.6±0.5	2.8±0.2
41'	6.2±0.4	3.1±0.3

Figures 4 to 6 present the influence of the populations of isolates, applied alone, on three types of vegetables – spinach, radish and peas. These species were not selected randomly, as they are used as test crops to test the effect of chemical and biological agents in agriculture. The effect was different in different isolates but is consistent for the same isolate in different cultures.

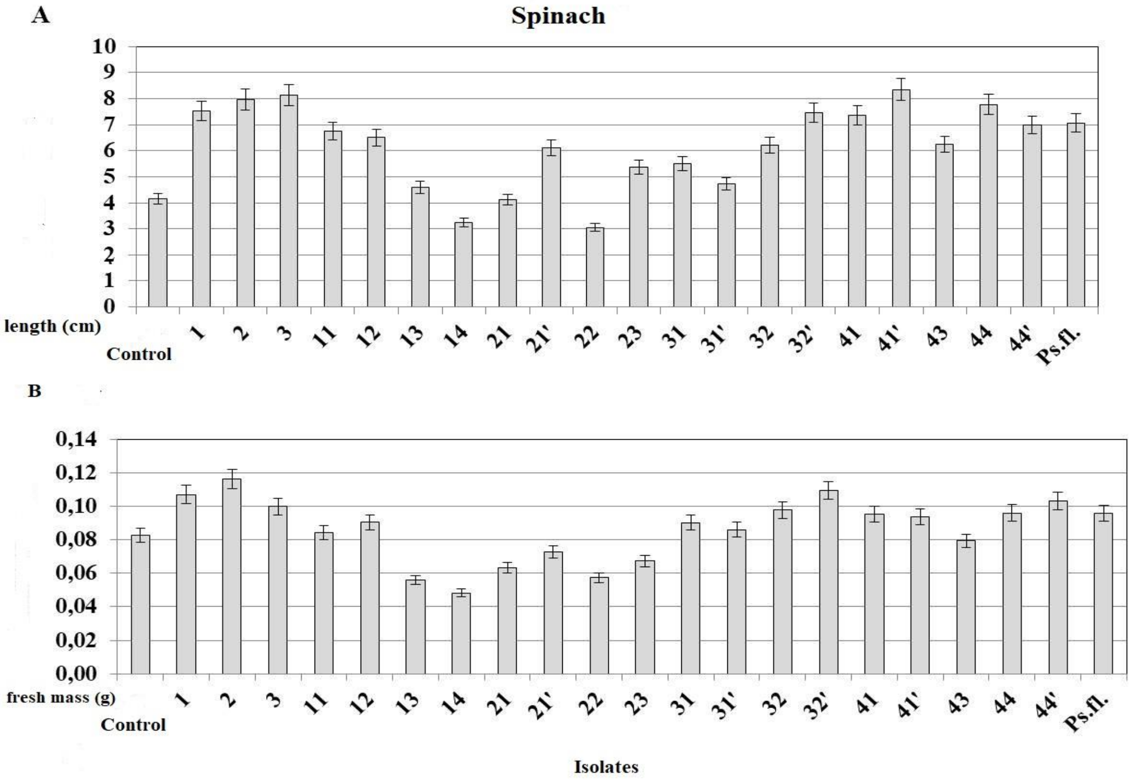


Figure 4. Length (A) and fresh mass (B) of young spinach plants after ten days of cultivation with the introduction of populations of different isolates tested separately. The data show the average of 10 replicates and the standard error.

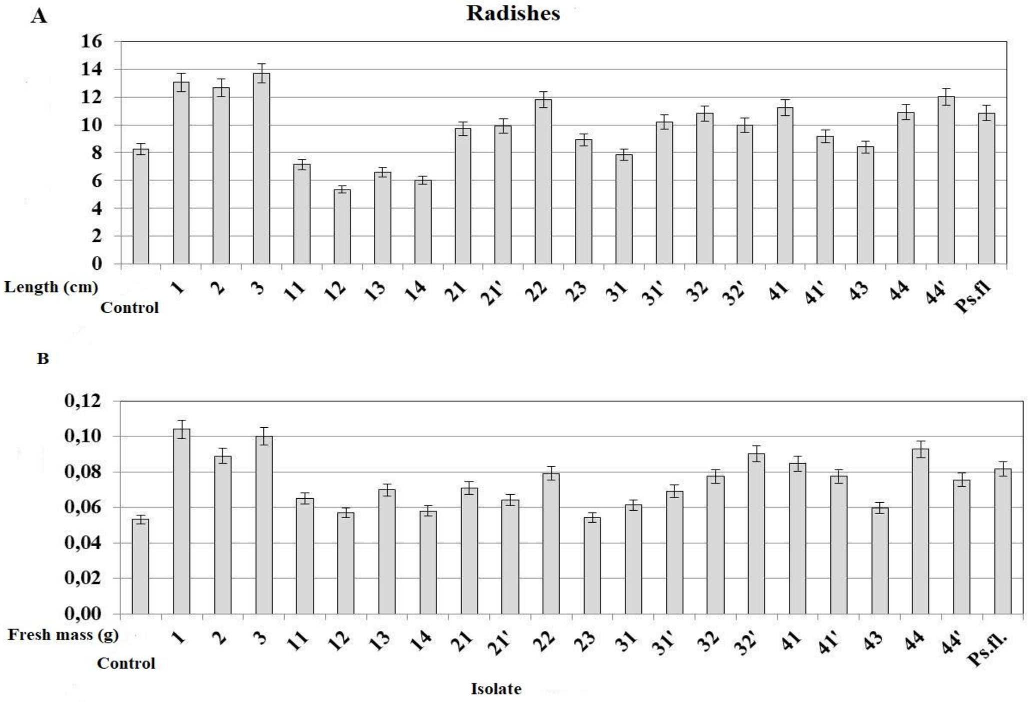


Figure 5. Length (A) and fresh mass (B) of young radish plants after ten days of cultivation with the introduction of populations of different isolates tested separately. The data show the average of 10 replicates and the standard error.

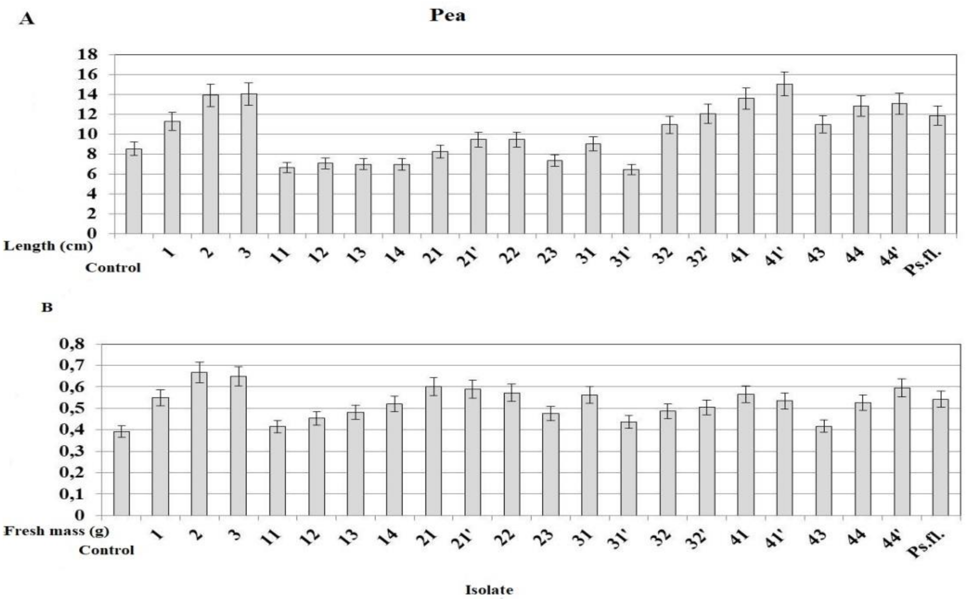


Figure 6. Length (A) and fresh weight (B) of young pea plants after ten days of cultivation with the introduction of populations of different isolates tested separately. The data show the average of 10 replicates and the standard error.

4.3. Study of changes in the soil-rhizosphere-plant system - first attempt.

4.3.1. Biometric indicators of spinach plants.

The rate of development of the different parts of the spinach is shown in fig. 7-9.

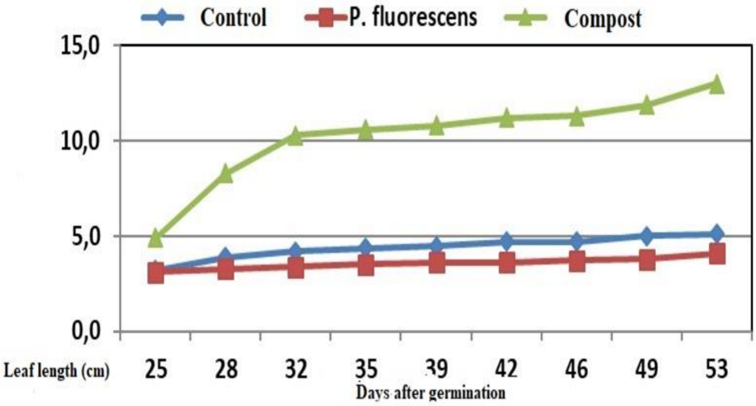


Figure 7. Dynamics of increase in leaf length of spinach plants in different treatments of the experiment (average of three replicates). The standard error is within 5%.

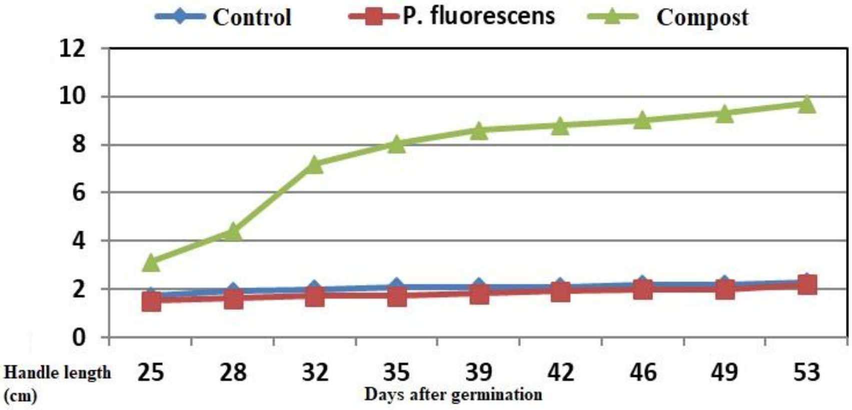


Figure 8. Dynamics of increase in the length of the stalk of spinach leaves in different treatments of the experiment (the average of three replicates). The standard error is within 5%.

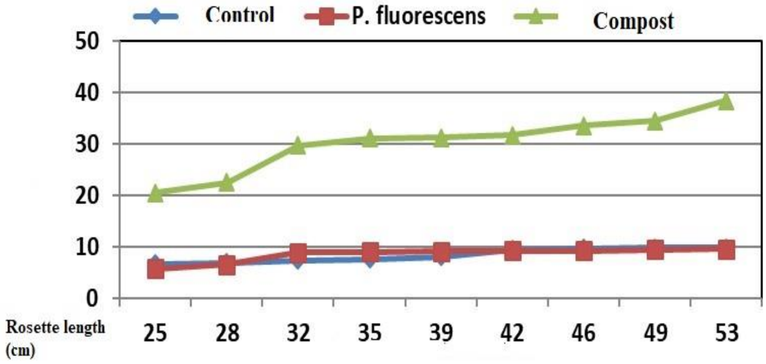


Figure 9. Dynamics of spinach leaf rosette growth in different treatments of the experiment (average of three replicates). The standard error is within 5%.

There is a noticeable difference in performance between treatments 1 and 2 and the compost treatment. A considerable improvement in spinach plant biometric indicators was found, confirming our thesis about the beneficial effects of compost and bacterial activity on vegetable crops. These results can be demonstrated both by the subsequent experiments, the quantitative and qualitative results derived from them, and by the plants' measured weights of the aboveground mass (Fig. 10).

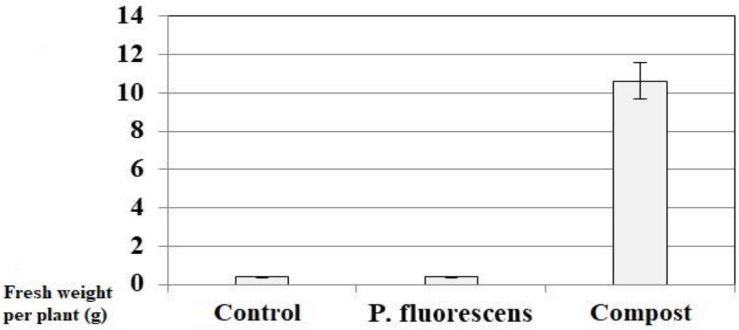


Figure 10. Weight of a spinach plant in different treatments of the first vascular experiment. The data express the mean of three replicates and the standard error.

The difference between the control and the third treatment, in which compost is imported, is extremely large. In the second treatment, there is no statistically significant change in the fresh weight of spinach in terms of control, which leads to the conclusion that it does not have the same reflex as compost.

The above results are confirmed by the pictures of the experimental trial at the end of the experiment, presented in fig. 11.

Figure 11. Pictures of plants at the end of the experiment.

4.3.2. Accumulation of heavy metals in plants.

The accumulation in the plants of the heavy metals present in the soil is presented in fig. 12. The concentration of Cd was higher in the root, with the highest values found in control - 51 mg kg^{-1}, and the lowest in the treatment with imported compost - 18.4 mg kg^{-1}. In Pb it was found that its accumulation in the spinach root is within the statistical error in the first and second treatments (160 and 149 mg kg^{-1}), and the lowest in the compost treatment - 62 mg kg^{-1}, which is a decrease of 61% and 58%, respectively. The situation with Zn was similar, but the difference with the compost treatment was lower (38% and 33%, respectively). This is because zinc is an essential element for the growth and development of plants, especially spinach.

On the other hand, the accumulation of these heavy metals in the spinach leaf mass has maintained a similar trend as in the root. The lowest Cd accumulation was found in the compost treatment (7.67 mg kg^{-1}) and the highest in the imported bacterial population of *P. fluorescens.*

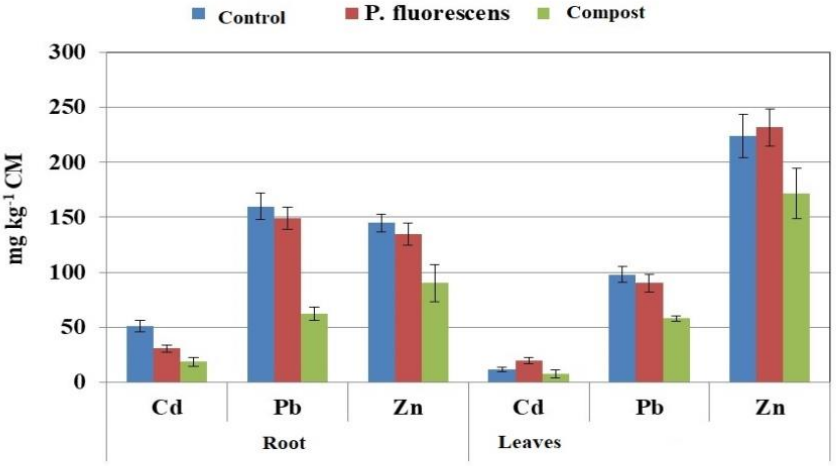


Figure 12. Accumulation of Cd, Pb and Zn in spinach root and leaves. The data show the mean and standard error (n = 3).

Concerning Pb in leaf mass, again, the lowest concentration was found in the compost treatment (58 mg kg^{-1}), while in the other two treatments, the values were within the standard error (98 and 90 mg kg^{-1}, respectively). No difference was found between the first two options - the control and the option with introducing a bacterial population (224 and 232 mg kg^{-1}, respectively). The concentrations of Zn in the leaves are significantly lower - 172 mg kg^{-1}, which is a 23-26% reduction.

In addition, spinach was analysed in terms of the accumulation of elements in the plant in different treatments of experiment 1 (Figs. 22 and 23). BF showed some differences in the various elements in the no-amendments treatment. While it was similar in Cd and Zn (~ 0.5), it was significantly lower in Pb (0.16). However, the BF is significantly below 1, which indicates a low potential for targeted extraction of heavy metals under the conditions of this experiment (Fig. 13). The introduction of the beneficial bacterial population resulted in a 70% increase in BF. When applying the compost, a decrease in BF was observed in the three tested heavy metals (Cd, Pb and Zn) compared to the control by 32.7%, 40.7% and 25.2%, respectively. As a result, we can say that in this case, the application of compost stabilises the soil and reduces the accumulation of elements in spinach.

4.3.3. Analysis of microbial communities in the soil.

4.3.3.1. Dehydrogenase activity.

Figure 14 presents the enzyme's activity at the end of the experiment in non-rhizosphere and rhizosphere soil. There is a clear significant difference in the values of enzyme concentrations between the two soils. At the same time, the first two treatments had a concentration within the standard error while applying the compost. After stabilising it with the soil, the activity of the enzyme increased. At the same time, the concentrations in the rhizosphere of the control treatment were 91.4% higher than in the soil far from the rhizosphere.

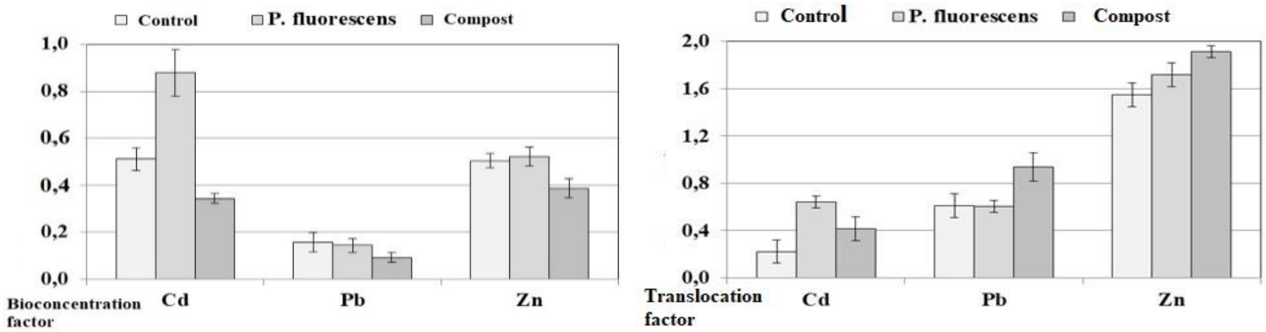


Figure 13. Bioconcentration factor (A) and translocation factor (B) of Cd, Pb and Zn accumulation in spinach leaves. The data show the mean and standard error (n = 3).

Also, the introduction of *P. fluorescens*' population and the development of spinach led to an increase in dehydrogenase activity of rhizosphere by 125.9%. The highest values and a significant increase in the concentration of the enzyme was observed in the third treatment, 122.5%. After 60 days of vegetation, the highest activity was found in the compost application treatment, which was 30.3% higher than the control treatment and 21.4% higher than in the second treatment.

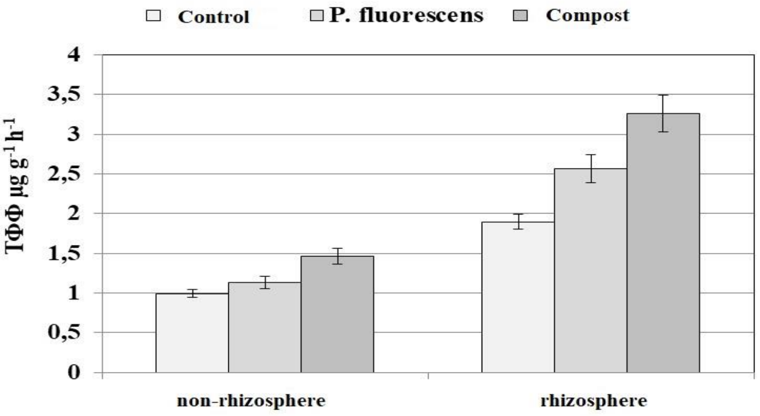


Figure 14. Soil dehydrogenase activity expressed as μg TPF g^{-1} soil h^{-1}. The data show the average of three replicates and the standard error.

4.3.3.2. β-glucosidase activity.

The values of β-glucosidase activity in the different treatments are relatively low before seed germination. The difference is due to the stabilisation of compost with soil for 14 days, which implies partial activation of these enzymes (Fig. 15). At the same time, the introduction of the *P. fluorescens* population also increased the values of this enzyme. At the end of spinach development, the values were significantly higher due to the interaction of plants and microbial populations in the rhizosphere. In the control treatment, the increase of the indicator is 7.2 times, while in the second and third, the increase is 5.6 and 5.5 times, respectively. However, they are expected to be the highest in the treatment with imported compost and the lowest in the one with only contaminated soil. This is due to the more pronounced toxicity of heavy metals in the treatment with soil only, but also due to the introduction of organic-rich compost, with a higher concentration of available elements. The activity of β-glucosidase in rhizosphere of spinach grown in a soil-compost mixture was 20.9% higher than in the treatment with the application of bacterial population and 71% compared to the control.

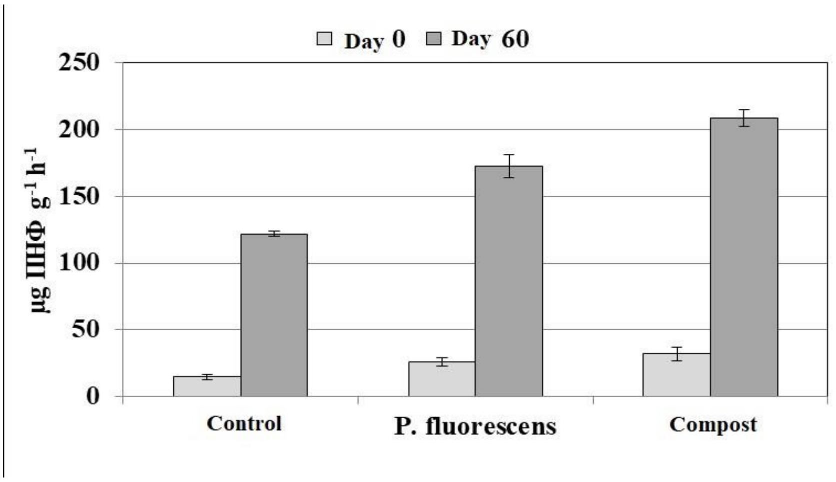


Figure 15. Soil β-glucosidase activity under experimental conditions. The data show the mean of three replicates and the standard error (n = 3).

4.4. Study of changes in the soil-rhizosphere-plant system - second attempt.

4.4.1. Nitrogen and phosphorus content in non-rhizosphere and rhizosphere soil

The incorporation of compost or compost and a beneficial bacterial population into the soil led to an increase in the content of nitrogen and phosphorus in it. The data in Table 8 can also demonstrate this.

Table 8. Nitrogen and phosphorus content in non-rhizosphere and rhizosphere soil at the end of the experiment in different treatments. The data show the average of three replicates.

Treatments	**Nitrogen,%**	**Phosphorus,mg kg^{-1}**
Non-rhizosphere soil		
B-1	0,23	593,69
B-2	0,39	2205,59
B-3	0,26	1730,64

Treatments	**Nitrogen,%**	**Phosphorus, mg kg^{-1}**
Radish		
B-1	0,22	598,56
B-2	0,44	2026,67
B-3	0,41	2547,17
Spinach		
B-1	0,19	667,69
B-2	0,27	1041,8
B-3	0,39	1824,17
Peas		
B-1	0,16	459,50
B-2	0,24	561,15
B-3	0,38	748,21

Our results show that the total nitrogen content is affected by applying compost or compost and *P. fluorescence*. Their addition maintains the nitrogen content in three vegetable crops. With regard to radish, the nitrogen content of the second and third treatment is 200% and 186%, respectively, higher than treatment 1 (soil without amendments). When sleeping, the nitrogen content of the second (compost) and third treatment (compost + bacterial population) is 126% and 205% higher than in the first treatment. The situation is similar with peas - 150% and 237%, respectively, with high nitrogen content in the second and third treatment in the first place.

The phosphorus content of the soil was influenced by the addition of compost and compost and bacterial population, with the lowest content in the control treatment in both non-rhizosphere and rhizosphere soils in all three vegetable crops. Concerning the non-rhizosphere soil, the phosphorus content in the second and third treatments compared to the first is 3.71 and 2.92 times higher, respectively. In radish, the phosphorus content in the second and third treatments is 3.39 and 4.26 times higher than in the control treatment, and in spinach - 1.56 and 2.73 times higher, respectively, the phosphorus content in the second and third treatments option compared to the first one. The situation is similar with peas - 1.22 and 1.62 times higher phosphorus content in the second and third versions compared to the first.

The high concentration of available nutrients, which in the compost and compost treatments and the bacterial population is many times higher than in the control treatment, can be explained by three mechanisms: 1) release of nitrogen and phosphorus, which are already present in the available form in compost; 2) mobilisation of nitrogen and phosphorus during the experiment due to the addition of compost and the population of *P. fluorescence*; 3) increased microbial activity as a result of the addition of compost and bacterial population.

4.4.2. Bioavailable fractions of Pb, Zn and Cd (ISO 14870).

Table 9 presents the bioavailable soil concentrations of Pb, Cd and Zn in non-rhizosphere soil. In contrast, the rhizosphere concentrations of radish, spinach and peas at the end of the experiment are shown in Table. 9.

Table 9. Bioavailable forms of heavy metals presented in the soil solution of non-rhizosphere soil with or without the introduction of compost or *P. fluorescens* biotype F. The mean values of three replicates and the standard error are shown.

	Bioavailable metals (mg kg^{-1})		
	Pb	Zn	Cd
When betting	16.4	158.1	8.15
1	16.1±2.1	172±5.1	7.85±0.5
2	16.3±1.8	154±7.9	7.6±0.3
3	16.6±1.3	153±4.6	7.3±0.4

The available forms of Zn at the end of the parallel experiment without plants (non-rhizosphere soil) showed some changes compared to its beginning. The main changes were observed in control, where concentration increased by 8.8% over the experimental period, while in the compost treatments, there was no statistically significant difference. Concerning Pb, no difference was found between the concentrations in the three treatments and the initial one. On the other hand, mobile forms of Cd decreased in the compost and compost and bacterial populations by 6.8% and 10.4%, respectively.

Considering the changes in the bioavailable concentrations of heavy metals at the end of the experiment in different vegetable plants, the strongly reduced concentrations of Zn in all radish treatments are impressive compared to the initial values and the other plants. While the initial concentration was 158.1 mg kg^{-1}, the concentration in control was 38% lower and those in the second and third treatments were 49.5% and 56.9% lower, respectively. In contrast, bioavailable Zn in the spinach rhizosphere was lower only in the third treatment (4.7%) and in peas in the second and third (12.2% and 10.6%). The values were within the standard error for the other plants and treatments.

4.4.3. Extraction of heavy metals.

Table 10 shows the average values of Pb, Zn and Cd accumulation in the studied plants' leaves, stems and roots, estimated by the Duncan method. The highest content of lead in radishes was found in the plants of treatment 1 for all studied organs - roots, stems and leaves. The accumulation data in treatments 2 and 3 put these treatments in one group, as the values are not statistically different but are significantly lower than those in treatment 1. In spinach, the accumulation of this element was again the most significant in the first treatment. In the leaves, it was more than twice as high as in treatments 2 and 3. In the case of peas, the lowest accumulation in the whole plant was found in the third treatment, while in the case of seeds, no accumulation of lead was found.

Regarding the accumulation of Zn in plants, the data are divergent. Zinc is extracted from the soil as an essential element for plants to carry out life processes. The strongest accumulation was found in spinach - more than twice as high in the leaves in treatment three compared to the same treatment in radish and peas. In all three crops, the results for the accumulation of Zn in the leaves are one-way - the reduction of the accumulation is performed from treatment 1 to treatment 3 and the results are statistically significant. At the roots, the trend is the same, but again it should be noted that the highest accumulation values showed spinach. The lowest concentrations of Zn were found in the seeds in the different treatments.

The accumulation of Cd in the plant parts kept the direction described for Zn. The strongest accumulation was found in spinach and the weakest in peas. There was a clear pattern of less accumulation in the compost or compost and the bacterial population treatments. Cadmium was not detected in the seeds.

BF for the three plant species is presented in Table 12. For Cd in control, BF was greater than one and increased in the following order: radish>spinach>peas (1.49>2.07>2.5). At the same time, it decreased in all plants from the first to the third treatment. In radish and spinach, thanks to the addition of amendments, the BF decreased below 1, which is a suitable condition for using the approach to stabilise Cd-contaminated soils. Pb and Zn BF were significantly below 1.

TF for Cd in the control treatment of the three plants decreased in the same sequence as in BF. In addition, the incorporation of amendments led to a reduction in accumulation of the element from treatment 1 to treatment 3. For the other elements, no clear general trend was found. The most significant point was the increase in Zn TF in spinach with the addition of amendments, probably due to its role in biochemical processes.

Table 10. Accumulation of heavy metals in plant tissues in radish, spinach and peas grown on contaminated soil, with or without application of compost or *Pseudomonas fluorescens* biotype F. (mean of three replicates analysed by Duncan's multidirectional comparative analysis).

	Cd (mg kg^{-1})				Pb (mg kg^{-1})				Zn (mg kg^{-1})			
Radish	Leaves	Stems	Roots	Seeds	Leaves	Stems	Roots	Seeds	Leaves	Stems	Roots	Seeds
Treatment 1	33,2^{a}	24,4^{a}	27,9^{a}	-	64,4^{a}	20,7^{a}	27,8^{a}	-	176,7^{a}	139,1^{a}	100,3^{a}	-
Treatment 2	21,3^{b}	15,2^{b}	13,4^{b}	-	26,3^{b}	11,9^{b}	11,4^{b}	-	135,3^{b}	90,4^{b}	66,6^{b}	-
Treatment 3	19,6^{b}	14,1^{b}	16,1^{b}	-	22,9^{b}	12,4^{b}	10,1^{b}	-	113,3^{c}	122,5^{a}	61,4^{b}	-
Spinach												
Treatment 1	46,1^{a}	42,6^{a}	44,2^{a}	-	104,2^{a}	41,1^{a}	64,9^{a}	-	307,5^{a}	202,3^{a}	319,4^{a}	-
Treatment 2	20,1^{b}	28,4^{b}	22,5^{c}	-	54,9^{c}	32,7^{ab}	11,8^{c}	-	278,9^{b}	136,9^{b}	106,9^{b}	-
Treatment 3	19,5^{b}	19,4^{c}	33,6^{b}	-	70,5^{b}	31,9^{b}	24,7^{b}	-	266,7^{c}	120,1^{c}	108,1^{b}	-
Pea												
Treatment 1	55,7^{a}	18,4^{a}	87,6^{a}	0	3,9^{a}	5,1^{a}	61,6^{a}	0	156,8^{a}	104,7^{a}	207,3^{a}	66,9^{a}
Treatment 2	40,1^{b}	12,8^{b}	77,7^{b}	0	2,5^{b}	3,9^{b}	19,2^{b}	0	145,2^{b}	97,2^{b}	191,2^{b}	53,5^{c}
Treatment 3	30,2^{c}	11,5^{b}	59,1^{c}	0	1,9^{c}	3,1^{b}	11,4^{b}	0	112,2^{c}	93,1^{b}	164,4^{c}	60,3^{b}

Table 11. Bioconcentration factor and translocation factor of Cd, Pb and Zn in radish, spinach and peas calculated on the basis of leaves and stems.

	Cd		**Pb**		**Zn**	
	BF	**TF**	**BF**	**TF**	**BF**	**TF**
Radish						
Treatment 1	1,5	1,2	0,1	2,3	0,4	1,76
Treatment 2	1,0	1,6	0,04	2,3	0,3	2,0
Treatment 3	0,9	1,2	0,03	2,3	0,26	1,85
Spinach						
Treatment 1	2,1	1,0	0,2	1,6	0,7	1,0
Treatment 2	0,9	0,9	0,09	4,7	0,6	2,6
Treatment 3	0,9	0,6	0,11	2,9	0,6	2,47
Pea						
Treatment 1	2,5	0,6	0,01	0,06	0,35	0,75
Treatment 2	1,5	0,5	0,004	0,13	0,33	0,76
Treatment 3	1,5	0,5	0,003	0,17	0,25	0,68

4.4.4. Biometric indicators of spinach, peas and radish plants.

In addition to the above information, we can report that in the next stages of the study of spinach plant development in treatments 2 and 3, a larger leaf rosette was formed, and they had a faster rate of growth and development compared to these from treatment 1 (Fig. 16).

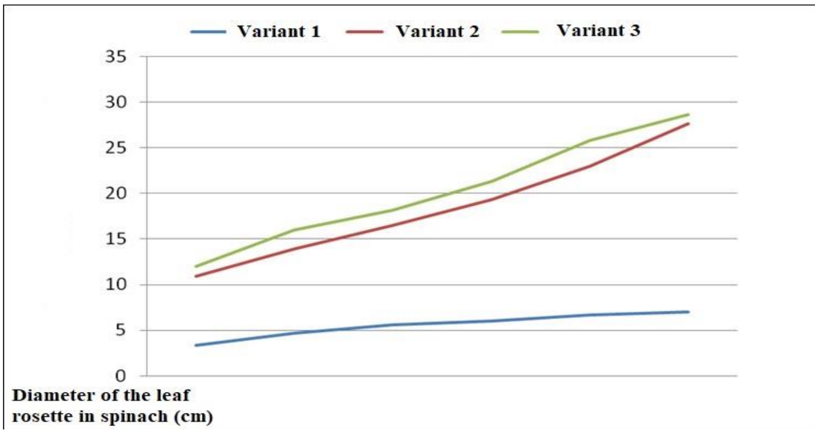


Figure 16. Diameter of the leaf rosette in spinach (cm).

The above is probably due to the inhibitory effect of heavy metals, inhibiting the development of the root system and the absorption of nutrients. However, the plants from the studied treatments accumulated more biomass and formed a larger leaf rosette compared to the control. In it, the diameter of the leaf rosette reached only 6.5 cm, and in the plants of treatment three and treatment two, respectively, 28.1 cm and 26.9 cm. The situation was similar to radish, which is a typical representative of root crops (Fig. 17). Their shorter vegetation period and faster growth and development rate showed even greater differentiation between the studied options. The plants of treatment two and treatment three managed to overcome the inhibitory effect of heavy metals and accumulate more biomass (up to 11.2 g per plant), forming a larger root crop than the control. In the latter, a root crop weighing only 0.76 g is developed.

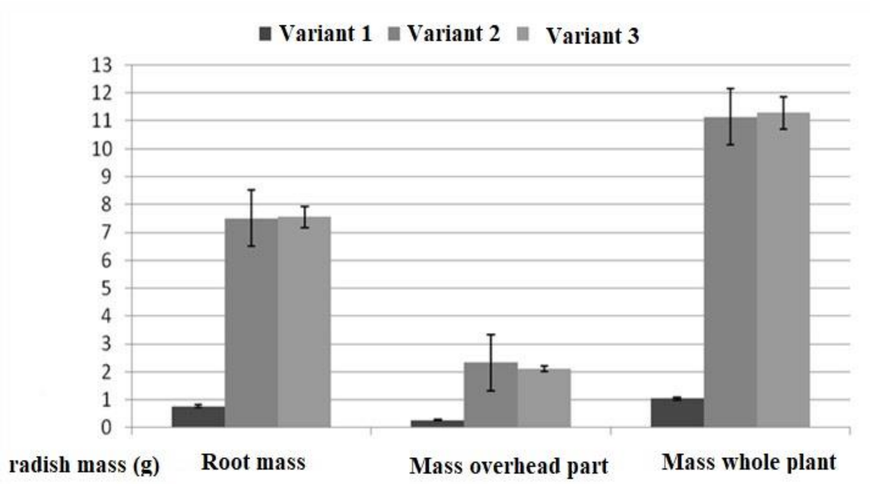


Figure 17. Mass of root crop, aboveground part and whole radish plant, measured at the end of the second vascular experiment. The data show the average of three replicates and the standard error.

In the case of pea plants, there wasn't as much differentiation between the studied treatments as in the case of radish. This is most likely due, on the one hand, to the species and varietal characteristics of peas and, on the other hand, to the potential influence of nitrogen-fixing bacteria, especially tuberous bacteria, thanks to which pea plants have partially compensated for heavy metals. However, the plants of treatments 2 and 3 accumulated more biomass and reached a higher height than the control (Fig. 18).

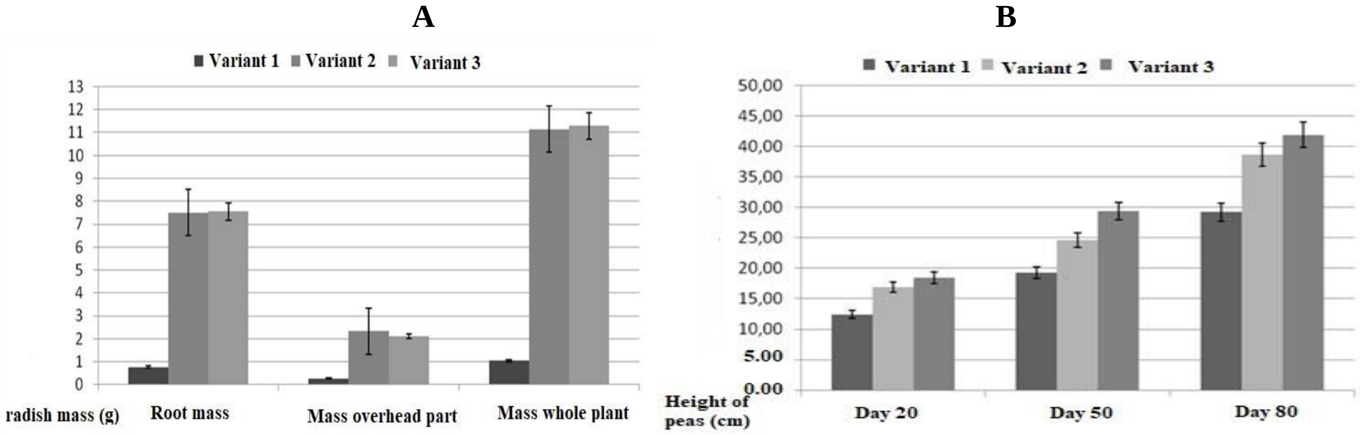


Figure 18. Fresh mass (A) and pea height (B) (cm) measured during a second vessel experiment. The data show the average of three replicates and standard error.

Figure 19. Pictures of radish, pea and spinach plants at the end of the experiment.

In general, the presence of heavy metals has had a strong suppressive effect on vegetable plants when tested alone. This effect was particularly pronounced in spinach and radish, where higher growth inhibition was observed, while compost application alone or in combination with the tolerant beneficial bacteria *Pseudomonas fluorescens* biotype F had an outstanding result in overcoming toxicity.

4.4.5. Analysis of microbial communities in the soil.

4.4.5.1. Dehydrogenase activity.

Figures 20 show the dehydrogenase activity in the non-rhizosphere and rhizosphere of plants before sowing, on the 30th and 60th day of the experiment.

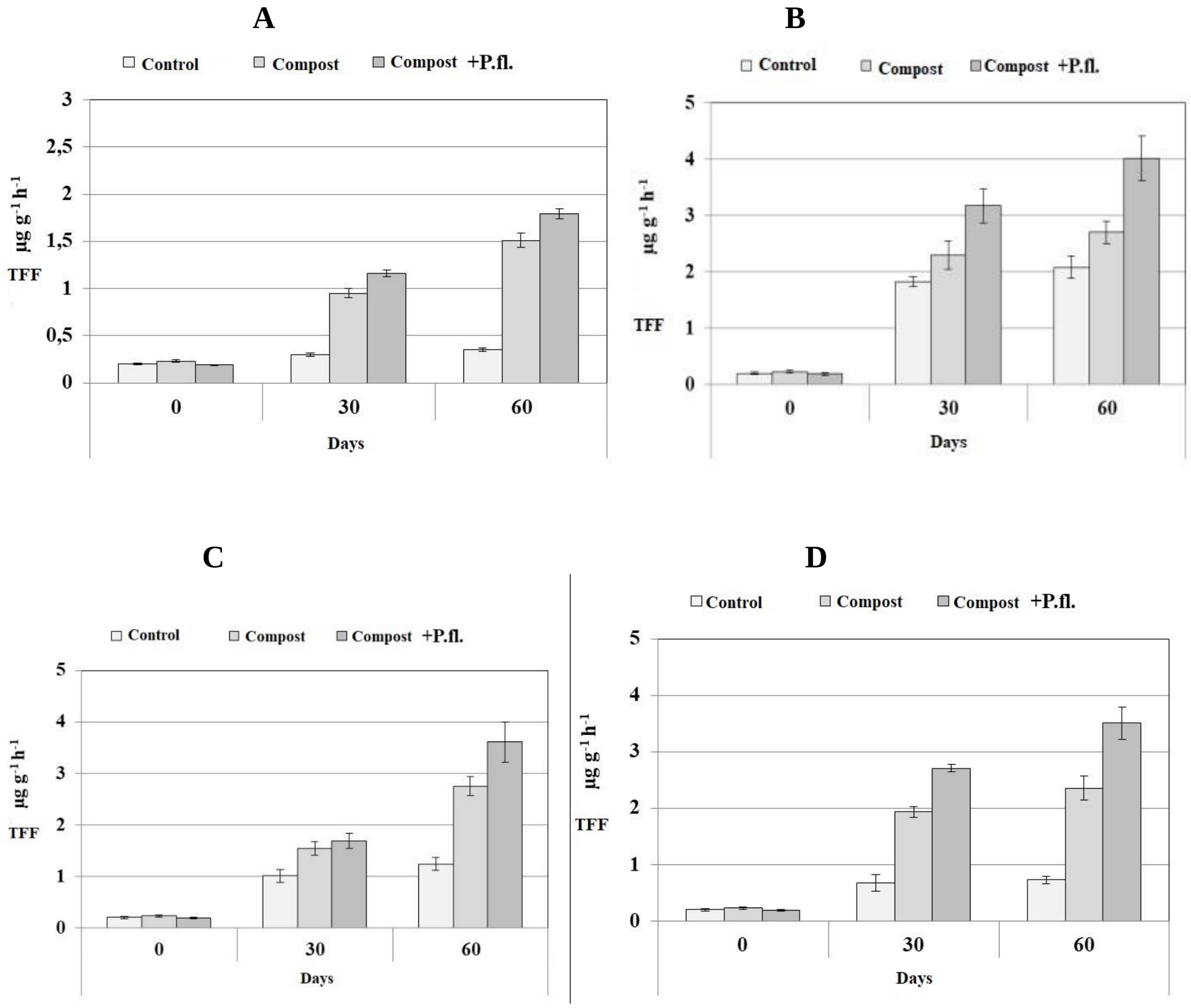


Figure 20. Dehydrogenase activity in non-rhizosphere soil (A), radish rhizosphere (B), spinach (C) and peas (D), expressed as µg TPF g-1 h-1. The data show the average of three replicates and the standard error.

The results show increased activity of dehydrogenases in the rhizosphere soil of the three crops compared to non-rhizosphere soil. There was also a tendency to increase this activity when adding amendments (compost with or without the bacterial population). This was evident in both rhizosphere and non-rhizosphere soils. The most significant increase in dehydrogenase activity was observed in the third treatment in all three cultures.

In this sense, we can conclude that the redox processes performed by microorganisms in the soil are enhanced in the rhizosphere than in the non-rhizosphere of spinach, radish and peas when grown on soils contaminated with heavy metals. In addition, applying compost with or without a bacterial population tolerant to the present heavy metals leads to a further increase in dehydrogenase activity due to the increased concentration of organic matter and beneficial bacterial population.

4.4.5.2. β - glucosidase activity.

According to the conducted research (Fig. 21), the β-glucosidase activity is very similar in the rhizosphere and non-rhizosphere soil, especially in the treatments with amendments. On the other hand, there was significantly less activity in the first treatment compared to the second and third, which was well expressed in all three vegetable crops. The most significant increase of enzyme β-glucosidase activity was observed in spinach and radish on the 30th and 60th days in the 2nd and 3rd treatments. No significant difference was found between the last two options on this indicator.

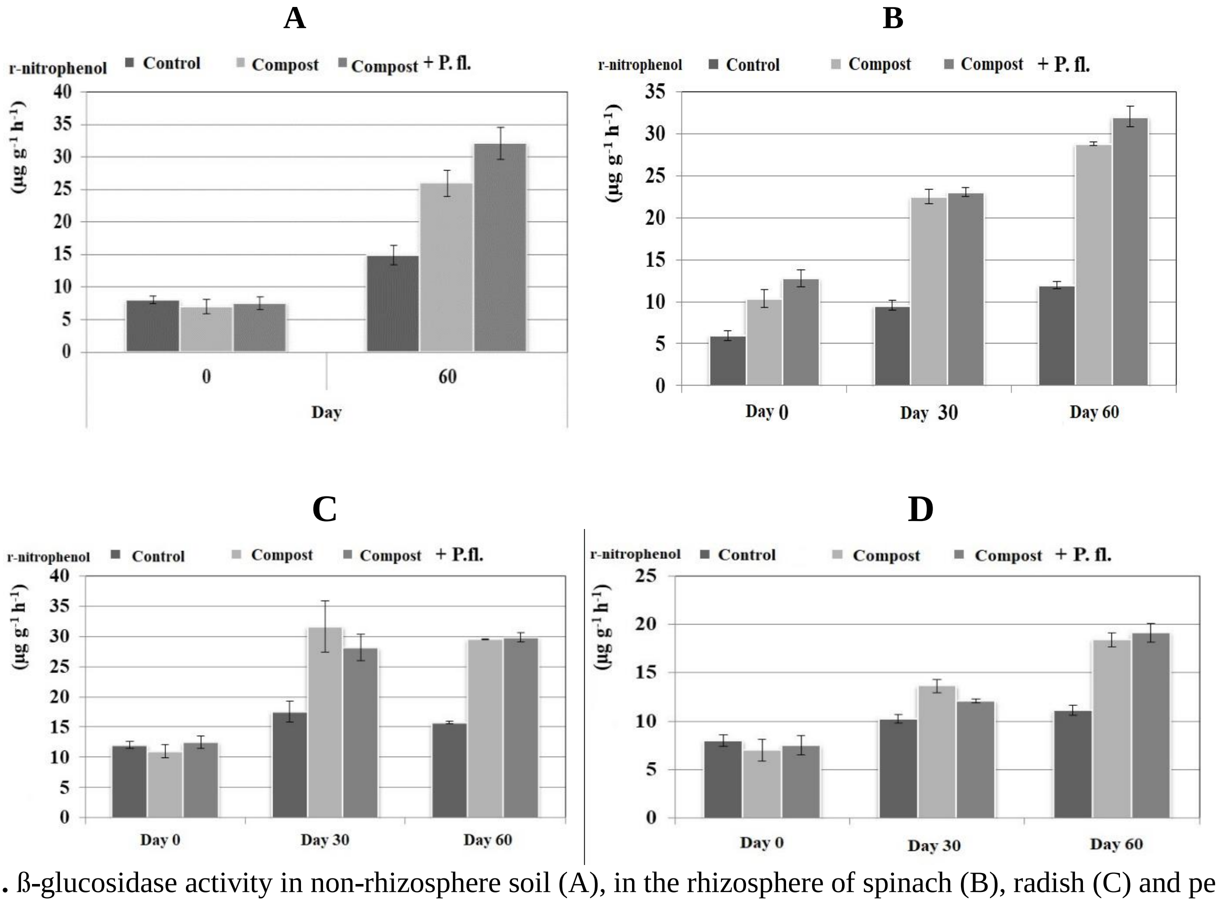


Figure 21. ß-glucosidase activity in non-rhizosphere soil (A), in the rhizosphere of spinach (B), radish (C) and pea (D) plants, expressed as μg p-nitrophenol g^{-1} h^{-1}. The data show the average of three replicates and the standard error.

4.5. Study of changes in the soil-rhizosphere-plant system - third attempt.

4.5.1. Bioavailable fractions of Pb, Zn and Cd.

Table 12 presents information on bioavailable fractions of heavy metals in soil. It is clear that the bioavailable forms of lead are mostly reduced in treatments 9 and 10, while in 3, 4 and 8 the values are similar. The concentrations in the first treatment are the strongest, but this is not valid for all three elements. Although no significant differences in values have been found for statistical science, applying compost and other amendments leads to a reduction in fractions and, hence, their adverse effects on soil and plant species.

Table 12. Bioavailable forms of heavy metals presented in the soil solution of the rhizosphere in spinach grown on contaminated soil with the introduction of compost and/or populations of bacterial isolates. The data show the average of three replicates and the standard.

	Bioavailable metals (mg kg^{-1})		
	Cd	**Pb**	**Zn**
B-1	4.2±0.5	12.0±0.4	50.0±4
B-2	3.55±0.7	12.25±1.7	57.5±3
B-3	3.1±0.3	10.4±2	43.75±2
B-4	3.0±0.9	10.15±1.8	43.8±2.5
B-5	3.65±0.7	10.95±2.1	52.5±5
B-6	4.4±0.3	12.0±1.2	66.25±4.2
B-7	3.2±0.6	11.55±2.3	56.25±4.9
B-8	3.65±0.7	10.45±1.2	43.75±8
B-9	2.7±0.3	10.35±2.3	37.5±9.1
B-10	3.5±0.6	10.3±1.7	41.25±7.1

4.5.2. Extraction of heavy metals.

Studies of the heavy metals in the soil (Pb, Zn and Cd) were performed in the various organs. The experiment's final results, which were achieved by the method of Duncan (Table 13) and BF (Table 14), are presented in tabular form.

The average values obtained show the following: in the group with the highest Cd content with statistically proven differences, the plants of treatment 1 for all studied organs - roots, stems and leaves. In the group with the lowest Cd content in roots, stems and leaves are the plants from treatments 2, 3 and 4, with statistically significant differences compared to treatment 1. In addition, the concentration of metals is almost the same in both roots. It stems from all treatments, which leads to the conclusion that cadmium is absorbed equally well and evenly by the underground organs and the aboveground accent leaves.

Regarding the accumulation of Pb in plants, the data show that the group with the highest Pb content is treatment 1, with proven differences for all studied organs. The lowest content of Pb for all studied organs was found in treatments 2, 3 and 4 with statistically significant differences compared to treatment 1. In all treatments, lead is accumalated primarily in the leaves and equally in the stems and roots.

The accumulation of Zn in plant organs retained the direction described for Cd and Pb. The leaves are best saturated with the element, and the metal is least present in the second and third treatments, in the leaves and roots. The group with the highest content of Zn includes the plants of treatment 1 for all studied organs - roots, stems and leaves with proven differences. Treatments 2 and 3 falls into the group with the lowest Zn content for all tested organs with statistically significant differences compared to treatment 1.

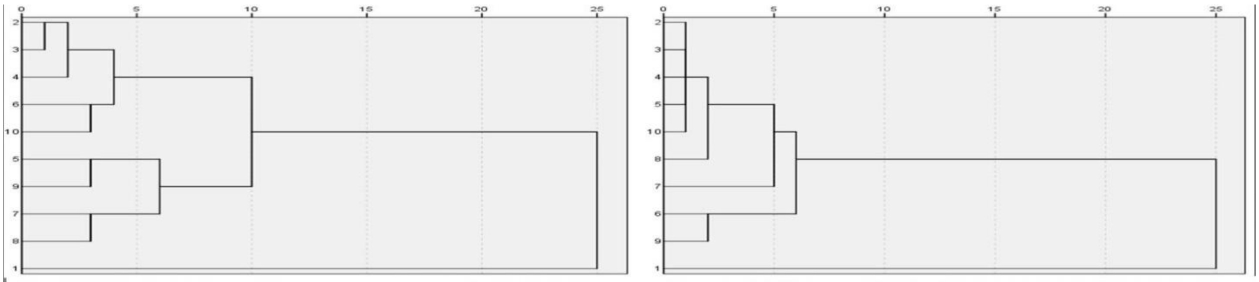
Table 13. Accumulation of heavy metals in plant organs in spinach grown on contaminated soil with the introduction of compost or compost and the corresponding bacterial population or combination thereof. The data represent the mean of three replicates analysed by Duncan's multidirectional comparative analysis.

	Cd (mg kg^{-1})			Pb (mg kg^{-1})			Zn (mg kg^{-1})		
Spinach	Leaves	Stems	Roots	Leaves	Stems	Roots	Leaves	Stems	Roots
Treatment 1	30.4 a	28.1 a	24.2 a	60.7 a	24.2 ab	26.9 a	296.9 a	133.8 a	125.8 a
Treatment 2	9.4 c	12.3 d	12.3 d	33.3 d	14.3 cd	8.2 e	224.5 f	88.7 e	90.2 f
Treatment 3	14.6 bc	12.5 cd	12.8 cd	32.8d	10.5 d	8.9 de	227.9 f	91.9 e	86.1 f
Treatment 4	10.4 c	12.7 cd	12.7 cd	31.8 d	14.1 cd	9.5 de	232.1 ef	106.2 d	95.1 def
Treatment 5	12.5 bc	14.3 bcd	14.3 bcd	56.5 a	16.6 bcd	11.3 cde	254.4 cd	106.4 d	110.6 cd
Treatment 6	27.3a	16.2 bcd	16.2 bcd	48.3 b	20.1 abc	13.0 cde	230.5 ef	112.8 cd	92.3 ef
Treatment 7	14.4 bc	20.6 bc	20.6 bc	44.5 bc	21.7 abc	12.7 cde	264.4 c	126.4 ab	108.3 cde
Treatment 8	16.7 bc	10.5 d	10.4 d	49.1 b	20.4 abc	20.3 b	282.5 b	120.8 bc	115.1 bc
Treatment 9	21.4 ab	16.7 bcd	16.7 bcd	48.5 b	14.6 cd	16.3 bc	242.4 de	104.3 d	129.5 ab
Treatment 10	40.3 c	20.3 bcd	13.6 bcd	44.3 c	20.3 abc	13.6 cd	242.6 de	120.5 bc	98.4 cdef

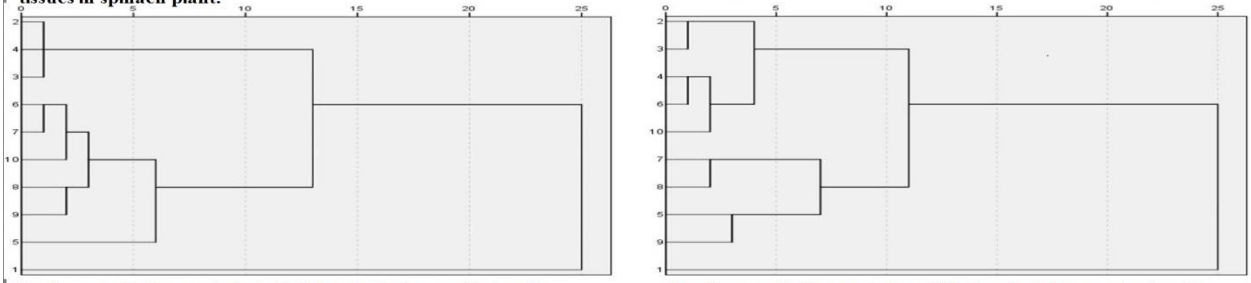
Table 14. Cd, Pb and Zn bioconcentration factor in spinach.

Spinach	Cd	Pb	Zn
Treatment 1	1.36	0.10	0.67
Treatment 2	0.42	0.05	0.51
Treatment 3	0.65	0.05	0.51
Treatment 4	0.47	0.05	0.52
Treatment 5	0.56	0.09	0.57
Treatment 6	1.22	0.08	0.52
Treatment 7	0.65	0.07	0.60
Treatment 8	0.75	0.08	0.64
Treatment 9	0.96	0.08	0.55
Treatment 10	1.81	0.07	0.55

The monitoring of the processes of accumulation and distribution of heavy metals could be illustrated by grouping the treatments in clusters (Fig. 22).



Dendogram 1. Accumulation of heavy metals (Pb, Zn, Cd) in plant tissues in spinach plant. **Dendogram 2. Accumulation of Cd in plant tissues of spinach.**



Dendogram 3. Accumulation of Pb in plant tissues of spinach. **Dendogram 4. Accumulation of Zn in plant tissues of spinach.**

Figure 22. Dendrograms of the accumulation of heavy metals (Pb, Zn, Cd) in plant organs in spinach grown on contaminated soil with compost and various bacterial isolates, as well as a combination of them.

Spinach grown on contaminated soil is affected by Cd, Pb and Zn and accumulates them at different rates and concentrations in various organs and when applying compost and bacterial isolates, as well as a combination of the latter. On the dendrogram, the treatments are grouped in 2 clusters concerning each studied element.

The first cluster is the most numerous, while the second is represented only by treatment 1. On dendrograms 1 and 4, two subgroups represent the first cluster. The first includes 2, 3, 4, 6 and 10 treatments, and the second - 5, 7, 8 and 9. Treatments 2 and 3 form a subgroup with at least remote units.

From the dendrogram two, based on the accumulation of Cd, it can be seen that the treatments are also grouped in 2 clusters - the most numerous cluster is the one containing treatments 2, 3, 4, 5, 8 and 10. The second subgroup is represented by treatment 7, and the third - 6 and 9.

The treatments in terms of Pb accumulation in plant organs in spinach are also grouped in two clusters. Dendrogram 3 shows that the first cluster is the most numerous and is presented in 3 groups. The first group includes treatments 2, 3 and 4; the second subgroup is represented by treatments 6, 7, 8, 9 and 10. In the third subgroup, only treatment five is positioned.

4.5.3. Biometric indicators of spinach plants.

Biometric studies make it possible to quantify changes in the growth of spinach plants. Significant differences in the dry weight of the plants were observed in the different treatments (Fig. 23). The highest biomass was formed in treatments 2 to 6, and the lowest in treatments 7 to 10. The plants in control without compost (B-1) had lower biomass than those from B-2 to B-6. The application of compost contributed to the increase of plant biomass by 66.8% (B-2). The addition of isolates 32 and 32' did not significantly affect this indicator (B-5 and B-6, respectively), while the combination of isolates in B-3 and B-4 had a positive, statistically proven effect on plants (13.3 % and 9.6%, respectively).

At the opposite pole were the plants from treatments B-7 to B-10. The addition of the respective populations resulted in significant inhibition of spinach growth compared to compost control (two isolates of *P. fluorescens* and one of *Bacillus thuringiensis* and *P. synxantha*). This may also be due to heavy metal accumulation and bacterial populations combined.

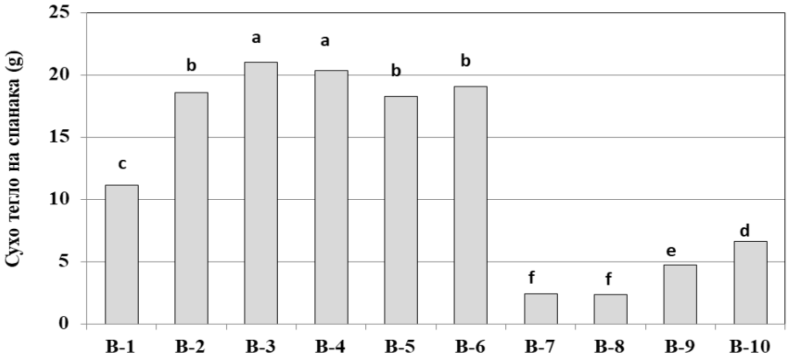


Figure 23. Dry weight of spinach plants in different treatments of the experiment. The data represent the mean of three replicates analysed by Duncan's multidirectional comparative analysis.

Abundant information about the growth and development of vegetable crops considered the work, give us fig. 24. In summary, the difference in leaf length, leaf stalk, leaf rosette diameter and number of leaves can be traced in dynamics on the 15th, 40th and 60th days. It can be seen that the length of the leaves is the largest in treatments 2 to 6 at the end of the reporting period. Again, applying compost with or without the addition of bacterial populations has a positive effect on the development of spinach as a test culture.

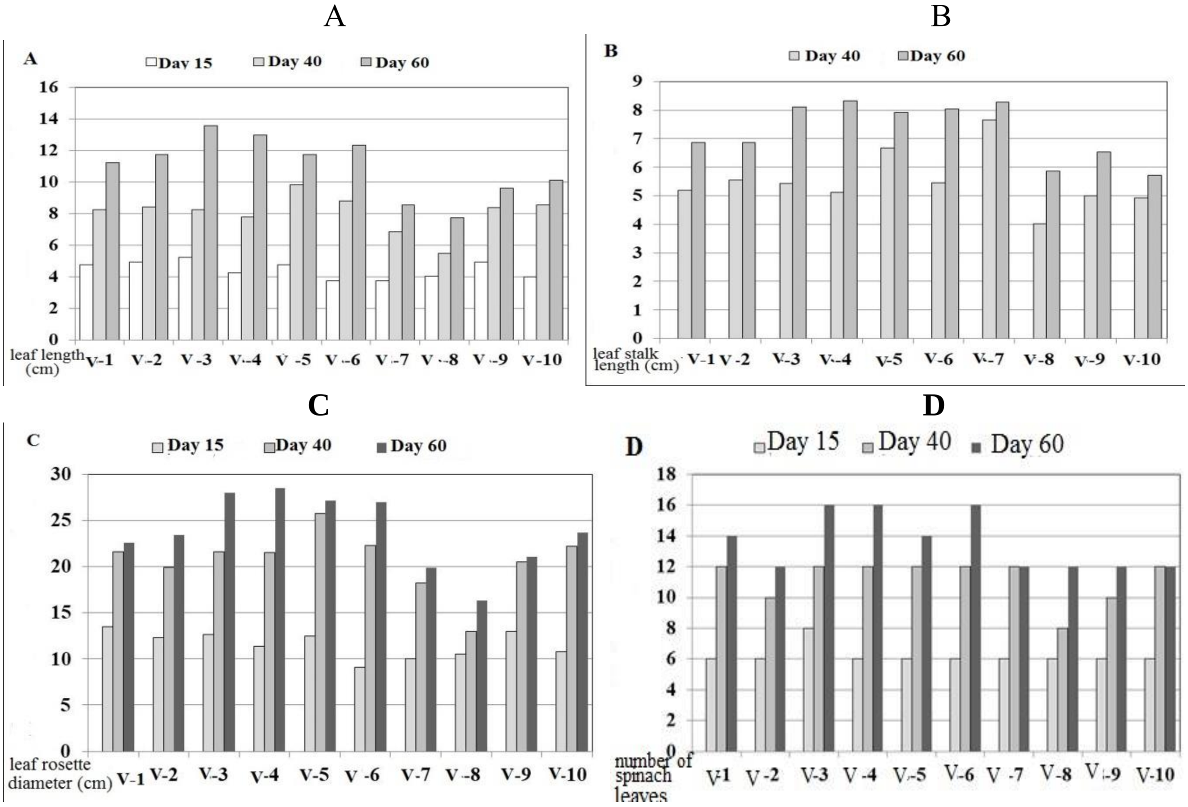


Figure 24. Leaf length, leaf stalk length, leaf rosette diameter and number of spinach leaves grown on contaminated soil with or without compost and/or bacterial populations

The length of the leaf stalk is the largest in treatments 3 to 7 and the smallest in treatments 8 and 10 for the last registration period. Regarding leaf rosette length, the first four treatments with added bacterial populations are the most prominent, and the number of leaves is highest in treatments 3, 4 and 6. The latter, together with the weight of the plants, is one of the most the biometric parameters indicative of their development.

Some fluctuations in growth dynamics are characterised by the length of the stalk and leaves in the second moment of measurement - April 26.

Figure 25. Picture of the plants from the experimental setup at the end of the experiment

4.5.4. Analysis of the development of microbial communities in the soil.

4.5.4.1. Dehydrogenase activity.

In a relatively similar way is the issue of dehydrogenase activity in the rhizosphere and non-rhizosphere of spinach plants, which was monitored in 10 treatments three times within 60 days (Fig. 26). In both areas, the activity of these enzymes increased throughout the experiment, with the highest being at the end of the 60th day. In the non-rhizosphere, the values were lower than in the rhizosphere, and as a rule in control (B-1) they were lower than in the treatments with added compost.

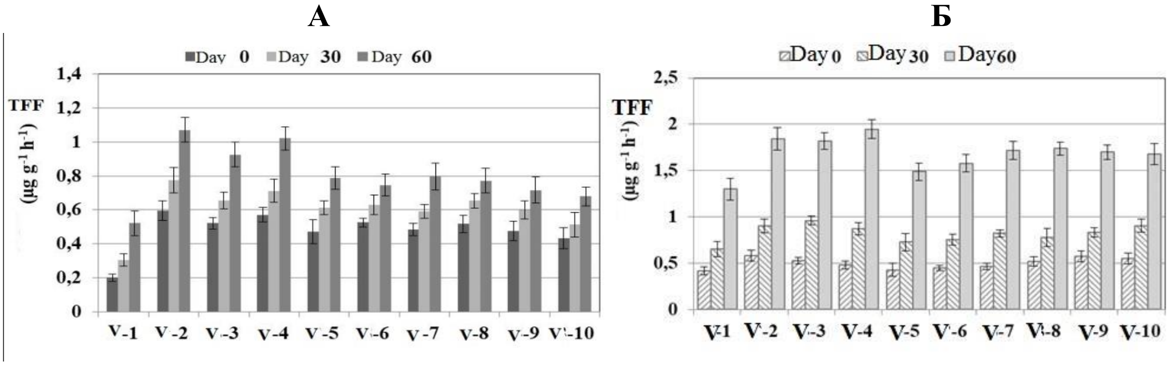


Figure 26. Dehydrogenase activity in non-rhizosphere (A) and rhizosphere soil (B) of spinach, expressed as µg TPF g^{-1} soil h^{-1}. The data show the average of three replicates and the standard error.

The diagrams support the above statements about the activity of dehydrogenases primarily in the rhizosphere soil of spinach and less in the non-rhizosphere. Again, we can assume that the hypothetical tendency to increase this activity by incorporating bacterial populations will become a fact. The most significant increase in dehydrogenase activity was observed in the second, third and fourth treatments, both in the rhizosphere and the non-rhizosphere.

It can be concluded that the redox processes performed by microorganisms in the soil are more enhanced in the rhizosphere than in the non-rhizosphere of spinach when grown on soils contaminated with heavy metals. The quantitative expression of the increased activity is about 80-110%. In addition, the incorporation of compost and bacterial populations tolerant to the presented heavy metals leads to a further increase in dehydrogenase activity.

4.5.4.2. β - glucosidase activity.

Later, in a study of β-glucosidase activity in spinach in 10 treatments and non-rhizosphere soil (Fig. 27), it was observed that it was higher in rhizosphere soil than in non-rhizosphere soil. However, the difference between the two soil types is smaller than in the case of dehydrogenase activity, 70-75%.

In the treatments with and without added isolates, this enzyme activity is increased, which is most evident in treatments 2 (compost), 3 (combination of *Pseudomonas putida*, *Pseudomonas fluorescense* and *Pseudomonas synxantha*) and 4 (combination of *Pseudomonas putida*, *Pseudomonas synxantha Pseudomonas fluorescence*). In the fifth and sixth treatments (*Pseudomonas putida* isolates), β-glucosidase activity was reduced to control values (without amendments).

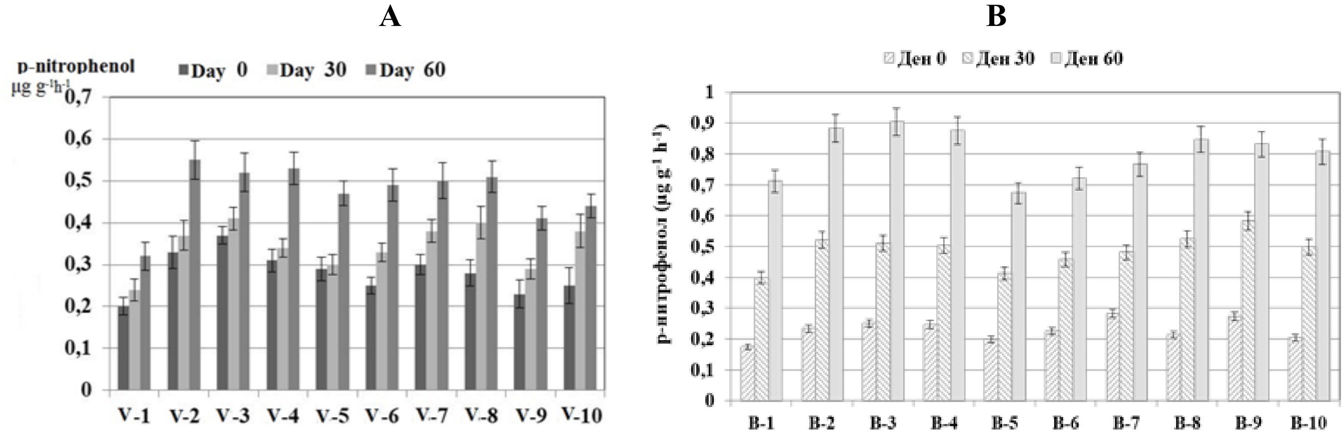


Figure 27. ß-glucosidase activity in non-rhizosphere and rhizosphere soil, expressed as µg p-nitrophenol g^{-1} soil. The data show the average of three replicates and the standard error.

4.5.5. Changes in the metabolic profiles of soil microbial communities.

In our study, during the third experiment, the metabolic profiles of the spinach rhizosphere soil microbial communities were studied. We initially examined the absorption rate of all substrates, expressed as the mean of the colour response of the wells at the end of the study (120 h). It can be summarised that for the five days of the study, the highest rate of substrate uptake was observed in control treatment (B-1) and in treatment 7 (compost application and *P. fluorescens* population), and the lowest degree in option 4 (compost application and combination of 4 bacterial populations, Fig. 28).

In the initial stages of the study, the difference in the absorption of the studied substrates was significant between the different options, which was gradually overcome. At the end of the study, at 120 h, the difference in optical density between the highest (B-1) and the lowest value in the group of treatments (B-2) was below 10% (9.5%). The lowest absolute value was found for B-4, which was 72.9% of B-1.

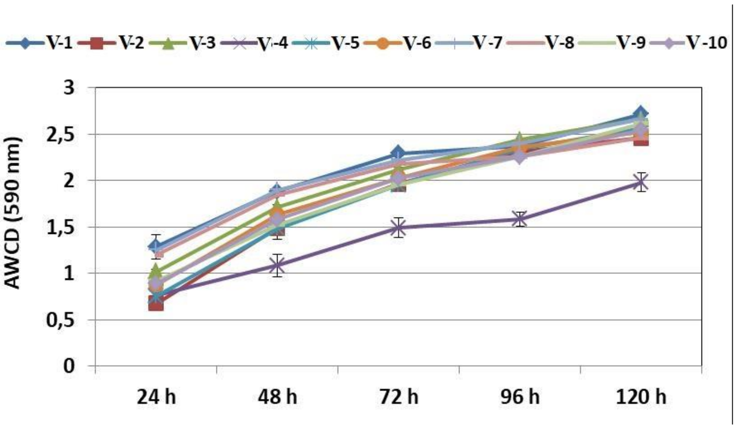


Figure 28. Dynamics in the absorption of different carbon sources of ecoplates at the end of the experiment at 590 nm. The results show the mean of three replicates ± standard deviation.

The Biolog system through ecoplates allows the grouping of carbon sources according to their characteristics into six groups: amino acids, amines and amides, carbohydrates, carboxylic acids, phenolic compounds and polymers. The assimilation of these groups of sources is presented separately in fig. 49 and 50. These compounds' highest degree of absorption was observed in control (B-1) with a total optical density at 590 nm of 15.76, and the lowest in B-4 (Fig. 28). In addition, the highest degree of absorption and the strongest colour reaction at 590 nm was found for amino acids and polymers and relatively lowest for phenolic compounds and amines and amides (Fig. 29). In all groups of sources, it turned out that the lowest absorption is in B-4. In terms of amino acids, most communities have shown a good ability to absorb them. The same was observed in case of the phenolic compounds, but such ability was lower in B-10. In the case of amines and amides, the most significant differentiation of the ability to use them was found. The highest use was observed in B-10, followed by B-1, B-3 and B-2.

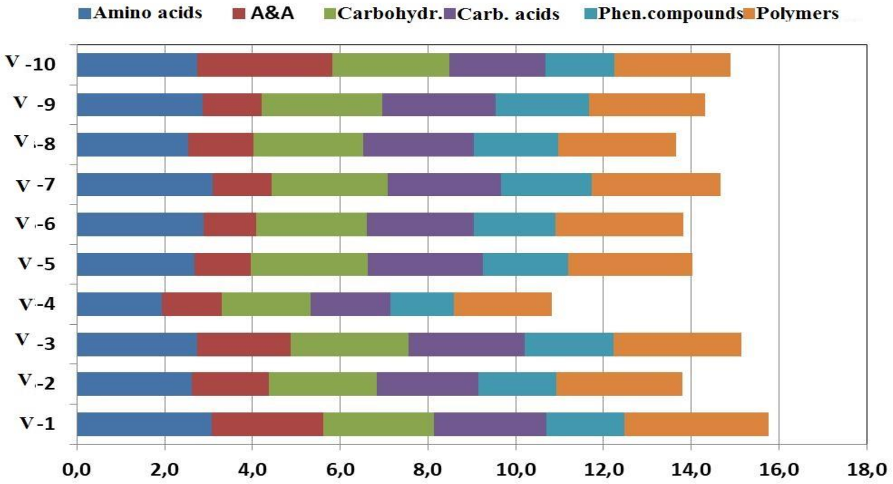


Figure 29. Absorption of different carbon sources from ecoplates and the weight of each of them expressed as a percentage of total activity at 590 nm. The results show the average of three replicates.

Table 15 presents the main indices of metabolic activity of soil microbial communities. In general, the values of the Shannon Diversity Index range from 2.49 to 3.29, which can be defined as a stable and balanced structure of habitat communities. However, in the control treatment, there is a noticeable decrease in this index, as well as the Pielou index.

Table 15. Main indices of metabolic activity of soil microbial communities in different treatments of the experiment. The results show the mean of three replicates ± standard error.

Treatments	**Shannon Diversity Index (*H'*)**	**Pielou uniformity index (E)**	**Functional wealth (R)**
1	2.49±0.1	0.76±0.01	26±0.2
2	3.15±0.1	0.97±0.01	26±2.0
3	2.92±0.1	0.97±0.00	29±0.2
4	2.94±0.05	0.98±0.01	27±1.8
5	2.98±0.07	0.98±0.01	28±0.6
6	3.25±0.1	0.99±0.00	26±0.4
7	3.28±0.1	0.98±0.01	28±0.5
8	3.26±0.1	0.98±0.00	28±0.3
9	3.22±0.1	0.99±0.00	26±1.1
10	3.14±0.1	0.98±0.00	27±0.5

V. CONCLUDING REMARKS

Present work has shown that the combined approach is suitable for studying terrains contaminated with heavy metals. The use of vegetable crops together with compost and populations of beneficial microorganisms is an environmental approach, combining recycling of biowaste and biotechnological methods, resulting in reduced mobility of the pollutant and stabilisation of the disturbed terrains.

The application of compost and beneficial populations of microorganisms affects the soil response, electrical conductivity, concentration of heavy metals and the degree of their absorption by plants, as well as a number of other indicators to judge the condition of rhizosphere and non-rhizosphere soil *in situ*. Through the practical implementation of tasks such as: isolation of microorganisms that stimulate the growth of vegetable plants, study the development of the same and contaminated soils by applying compost and the bacteria *P. fluorescens*, we were able to confirm the thesis that reducing the accumulation of heavy metals in plants with the help of an environmentally friendly approach is quite possible, even recommended.

Phytoremediation is one of the main approaches, methods and ways of extracting, retaining or immobilising soil pollutants based on the ability of plant species, through their root system, to contribute to the restoration of soil structure and composition and properties. In the dissertation, several studies were conducted with radish, peas and spinach, which showed the effectiveness of phytoremediation supported by the introduction of beneficial populations of microorganisms. The current biotechnological approach is an alternative to chemical methods that lead to the destruction of the soil structure and the humus layer.

The introduction of compost and beneficial bacterial populations into the soil makes it possible to control the impact of available concentrations of Pb, Zn and Cd on living organisms, particularly on plants, which results in the restoration of agroecosystems. An example in this respect is the studied variety of parameters of the development of the three vegetable crops. The improved condition of plants subjected to abiotic stress is entirely the result of the introduction of compost and populations of beneficial bacteria. An additional added value of this approach is to increase the concentration of organic matter in the soil, the condition of soil microbial communities and generally improve soil fertility. Therefore, future ways to deal with elevated concentrations of heavy metals in soils, especially in agricultural areas, should follow the specified environmentally friendly approach applying phytoremediation assisted by beneficial microbial populations.

VI.CONCLUSIONS

Based on the results obtained, the following more important conclusions can be drawn:

1. The ability of isolated beneficial bacterial populations to promote plant growth is directly dependent on their tolerance to heavy metals present in the soil and the presence of tryptophan in the medium due to its importance as a precursor in the production of indoleacetic acid.
2. At least two different approaches must be used in the species identification of bacterial isolates if molecular identification is not used.
3. The development of spinach, peas and radishes in soils contaminated with heavy metals reduces pH and electrical conductivity in the rhizosphere of plants.
4. The application of compost leads to an increase in the supply of soils with nitrogen, phosphorus and organic matter.
5. Compost has a stronger positive effect on the development of vegetable crops in conditions of contaminated soils compared to the independent introduction of bacterial populations.
6. The speed of electronic transport in the leaves increases significantly when compost is introduced into the soil.

7. The development of vegetable plants without amendments leads to the mobilisation of heavy metals' bioavailable concentrations, while compost's introduction significantly reduces them.

8. The application of compost leads to a significant reduction in the accumulation of heavy metals in the biomass of vegetable crops. The strongest accumulation is observed in spinach.

9. There is a direct relationship between the concentration of heavy metals in the soil and their accumulation in plants. It is most pronounced with regard to cadmium and to a lesser extent in relation to zinc and lead.

10. Spinach is a suitable plant species for detecting the presence of bioavailable heavy metals in agricultural soils. The concentration of heavy metals in the leaves is proportional to their concentration in the soil.

11. The approach used with compost and populations of beneficial bacteria is a type of phytostabilisation of soils contaminated with heavy metals.

12. Low concentrations of Cd and Pb lead to stimulation of microbial activity, while high ones significantly reduce it.

13. The activity of the enzymes dehydrogenase and β-glucosidase is significantly higher in the rhizosphere than in non-rhizosphere soil. The addition of compost and beneficial populations leads to its additional stimulation.

14. Microbial communities on soils contaminated with heavy metals are most active and well developed with added compost with *P. fluorescens* or a combination of isolates, using most optimally amino acids and polymers, and least amines and amides.

VII. CONTRIBUTION

7.1.Scientific contributions

- A comprehensive scientific study of the influence of compost and populations of beneficial bacteria on the development, growth and accumulation of heavy metals in spinach, peas and radish;
- For the first time in Bulgaria, a study of soil microbial communities was conducted based on their metabolic profile in the phytostabilisation of soils contaminated with heavy metals.

7.2.Scientific and applied contributions

- It was found that the application of compost in soil contaminated with heavy metals leads to an improvement in the overall condition of the studied vegetable plants;
- It has been proven that the addition of populations of beneficial bacteria and compost leads to improved soil health in both the rhizosphere and non-rhizosphere;
- It is demonstrated to improve the development of soil microbial communities and reduce the concentration of bioavailable fractions of heavy metals due to compost.
- The applicability of ecoplates as a suitable tool for characterising the metabolic capacity of microbial communities in a given soil habitat has been proven.

7.3. Applied contributions

- It has been proven that spinach can be successfully used as a test crop to detect heavy metal contamination of agricultural soils.
- The use of quality organic amendments in conjunction with populations of beneficial bacteria is an appropriate, promising approach to the phytostabilisation of soils contaminated with heavy metals.

VIII. LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

The results obtained in the study have been reported at current scientific forums and published in scientific journals:

1. Babrikova Iv., St. Shilev, T. Babrikov (2016). Reduce the accumulation of heavy metals in spinach grown on contaminated soil using compost and beneficial bacteria. Proceedings of "Ecology and Health" 09-10 June 2016, pp. 435-440, ISSN 2367-9530, http://hst.bg/bulgarian/conference.htm.(BG)

2. Babrikova I., S. Shilev, T. Babrikov. (2016). Compost and PGPR decrease heavy metal availability and toxicity to vegetables. In: (Filcheva, Stefanova, Ilieva eds.). 4th Nat. conf. of BHSS with Int. Participation. 8-10 September, 2016, Sofia, ISBN 978-619-90189-2-7, 285-294.

3. Shilev, S., Azaizeh, H., Vassilev, N., Georgiev, D., Babrikova I. 2019. Interactions in soil-microbe-plant system: adaptation to stressed agriculture. pp.131-171. In: Singh, D.P., Gupta, V.K., Prabha, R. (Eds.) Microbial Interventions in Agriculture and Environment, Volume 1: Research Trends, Priorities and Prospects. Springer Singapore. doi: 10.1007/978-981-13-8391-5.

4. Shilev S, Babrikova I, Babrikov T. 2020. Consortium of plant growth-promoting bacteria improves spinach (*Spinacea oleracea* L.) growth under heavy metal stress conditions. Journal of chemical technology and biotechnology, 96(4), pp. 932-939 https://doi.org/10.1002/jctb.6077.