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Microbiological soil activity after application of combined nitrogen fertilizers and *Bacillus subtilis*

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Abstract. *The main groups of soil microorganisms were determined in the experimental combination of ammonium fertilizers, microbial fertilizer containing Bacillus subtilis and molasses. In the cultivation of a nutrient medium for non-spore-forming bacteria and bacilli, the best variant in terms of the quantity of bacilli is the combination of ammonium nitrate (45%), ammonium polyphosphate (45%), ammonium thiosulfate (5%), molasses (5%) and 0.15% Bacillus subtilis, followed by the variant with 0.30% Bacillus subtilis and the lowest quantity of bacilli is in the variant with 0.50% Bacillus subtilis, with the same amounts of other additives. After fertilization with the variants, the quantity of bacilli and the biogenicity of the soil are the highest in the variant with 0.30% Bacillus subtilis, followed by the variant with 0.15% Bacillus subtilis and the lowest biogenicity of the soil is in the variant with 0.50% Bacillus subtilis. While in the first case the development of microorganisms is in a controlled environment, in the second case a complex of environmental factors exerts influence. The highest percentage share in the composition of the total microflora is occupied by non-spore-forming bacteria, followed by bacilli and mold fungi, and the least represented are actinomycetes. In the variant: liquid nitrogen fertilizer (45%) + liquid nitrogen-phosphorus fertilizer, ammonium polyphosphate (45%) + liquid nitrogen-sulfur fertilizer, ammonium thiosulfate (5%) + molasses (5%) + microbial fertilizer containing Bacillus subtilis (0.30%), a rearrangement in the composition of the total microflora was established - mold fungi were more numerous than bacilli. In the same variant, the values of catalase, cellulase and urease are the highest, following the trends for the total quantity of microorganisms and are strongly dependent on it.*

Keywords: compound fertilizers, soil microflora, Bacillus subtilis, catalase, cellulase, urease.

Introduction

The quantity, composition and activity of soil microorganisms play a key role in the cycling of substances, soil health and plant nutrition. In agrogenic, arable soils, the soil microbiome can be controlled to some extent by applied agrogenic measures. Some studies have found that fertilization, applied in norm, generally increases the quantity and activity of soil microorganisms and is an important factor in maintaining sustainability in agroecosystems and increasing crop yields (Plamenov et al., 2016; Yankova et al., 2016; Malcheva et al., 2018; Malcheva et al., 2019a,b; Malcheva et al., 2020). Excessive or incorrect fertilization can have a negative effect on the activity of soil microorganisms, which can also affect plant development. The total quantity of soil microorganisms increased with liquid nitrogen fertilizer (UAN) fertilization and decreased with urea and ammonium nitrate fertilization compared to the unfertilized control, and it is interesting that the mineralization activity with urea and ammonium

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nitrate fertilization was the highest, which indicates that the quantity of microorganisms is not the only prerequisite for their activity (Malcheva et al., 2019b). Therefore, the results are contradictory - fertilization stimulates the growth of microbial populations (Khonje et al., 1989), also of microbial biomass with two-year nitrogen fertilization (Zhang et al., 2005), while other authors found the opposite trend with nitrogen fertilization - a decrease in microbial biomass (Sarathchandra et al., 2001); inorganic fertilizers reduce the quantity of microorganisms and microbial biomass, which is explained by the rates of applied fertilizers (Nakhro and Dkhar, 2010) - at a high rate of nitrogen fertilization (200 kgN/ha) microbial biomass, as well as microbial functional diversity, significantly decreases, and increases when fertilizing with nitrogen fertilizer at a lower rate (50 kgN/ha), compared to a control unfertilized sample (He et al., 2012). The total soil microflora increases when using microbial fertilizers, to a greater extent when using preparations containing a complex of microorganisms (bacteria, actinomycetes, mold fungi) than when applying a fertilizer containing a single bacterial species (*Bacillus* sp.), but when using the microbial fertilizer with only *Bacillus* sp., the mineralization activity of the soil is higher (Malcheva, 2021a).

Some authors have found that when fertilizing with mineral and organic fertilizers alone, as well as with combined fertilization with mineral and organic fertilizers, the main share in the composition of the soil microflora is occupied by bacteria (non-spore-forming to a higher extent than spore-forming bacteria - bacilli), followed by actinomycetes and molds, and with combined fertilization, the soil microflora increases to a higher extent (Plamenov et al., 2016; Yankova et al., 2016; Malcheva et al., 2018; Malcheva et al., 2019a,b; Malcheva et al., 2020). Bacteria also dominate when fertilizing with microbial fertilizers (Malcheva, 2021a). Cellulase and catalase activities generally increase after fertilization (Yankova et al., 2016; Malcheva et al., 2018; Malcheva et al., 2019a,b; Malcheva et al., 2020). Combined microbial fertilizers increase the activity of the enzymes amylase, cellulase and invertase to a greater extent than microbial fertilizer containing a single type of bacillus, while for catalase the trend is the opposite (Malcheva, 2021a). The results are also contradictory. The application of combined fertilization (organic and inorganic) increases the activity of dehydrogenase, alkaline phosphatase, cellulase, cellobiase and urease compared to soils fertilized with organic fertilizers alone (Gaind and Nain, 2012). According to other authors, organic fertilizers increase the activity of dehydrogenase and catalase compared to the use of inorganic fertilizers (Samuel et al., 2008). Other studies have found increased soil enzyme activity with combined application of organic and inorganic fertilizers (Mandal et al., 2007; Garg and Bahl, 2008).

The aim of the scientific research was to determine the influence of combined fertilization with variants containing inorganic combined nitrogen fertilizers, microbial fertilizer containing *Bacillus subtilis* and an organic preparation - molasses, i.e. mineral-microbial-organic fertilization, on the quantity, composition and enzymatic activity of different groups of soil microorganisms involved in the decomposition of organic matter in soils and providing plant nutrition. Studies using newly created microbial preparations, especially in combination with inorganic and organic fertilizers, have been relatively poorly studied, and the results are contradictory. The hypothesis, the proof of the scientific assumption for the use of microbial fertilizers in combination with other preparations in order to increase soil fertility and crop yield, implies the conduct of many scientific studies in this area, which is the task set by the presented scientific experiment, as an opportunity for practical application in agriculture.

Material and methods

Cultures were grown on meat peptone agar to determine the quantity of *Bacillus* in the following variants:

► Variant 1 (V1): liquid nitrogen fertilizer UAN (45%) + liquid nitrogen-phosphorus fertilizer, ammonium polyphosphate APP (45%) + liquid nitrogen-sulfur fertilizer, ammonium thiosulfate ATS (5%) + molasses MEL (5%) + microbial fertilizer containing *Bacillus subtilis* (0.15%);

► Variant 2 (V2): UAN (45%) + APP (45%) + ATS (5%) + MEL (5%) + *Bacillus subtilis* (0.30%);

► Variant 3 (V3): UAN (45%) + APP (45%) + ATS (5%) + MEL (5%) + *Bacillus subtilis* (0.50%).

Cultivation of the cultures in a thermostat at 25^{0}C for 48 hours.

Potted variants were planted (plastic containers with a capacity of 1.0 l (1 kg of soil), outdoors with agrogenic alluvial-meadow soil, fertilized once with the indicated liquid variants, dose 50 ml/l. After application of fertilizer products: ammonium nitrate, ammonium polyphosphate, ammonium thiosulfate, microbial fertilizer containing *Bacillus subtilis* and molasses according to the indicated variants, and control (without application of fertilizer products - V0), microbiological analyses were carried out (two weeks after fertilization with periodic moistening and stirring) by the limiting dilution method and subsequent cultures on solid nutrient media: mesopeptone agar for non-spore-forming bacteria and bacilli, Čapek-Dox agar for micromycetes (mold fungi) and starch-ammonia agar for actinomycetes and bacteria that absorb mineral nitrogen (Gushterov et al., 1977; Mishustin and Emtsev, 1989). Cultivation took place in a thermostat at 25^{0}C - for 48 hours for bacteria and at 25^{0}C - for 10 days for actinomycetes and micromycetes (molds). Total microflora and mineralization coefficients were determined using computational methods (Mishustin and Runov, 1957; Malcheva and Naskova, 2018; Nustorova and Malcheva, 2020).

Soil moisture was determined on a moisture balance, DBS model, a dry weight coefficient was calculated to recalculate the results for absolutely dry soil.

Catalase activity was determined by titration mangano-metric method (Khaziev, 1976).

Cellulase activity was determined by placing three sterile filter strips on moistened soil in petri dishes. Cultivation in a thermostat, periodic humidification and recording of the percentage of degraded area every 15 days with a grid standard (Khaziev, 1976).

The activity of the urease enzyme was determined spectrophotometrically (Kandeler and Gerber, 1988).

The correlation analysis was developed using the MS Excel 2010 program.

Results and discussion

The quantity of bacilli after cultivation in a thermostat was the highest in option 1 (ammonium nitrate (45%), ammonium polyphosphate (45%), ammonium thiosulfate (5%), molasses (5%) and 0.15% *Bacillus subtilis*) (Figure 1).

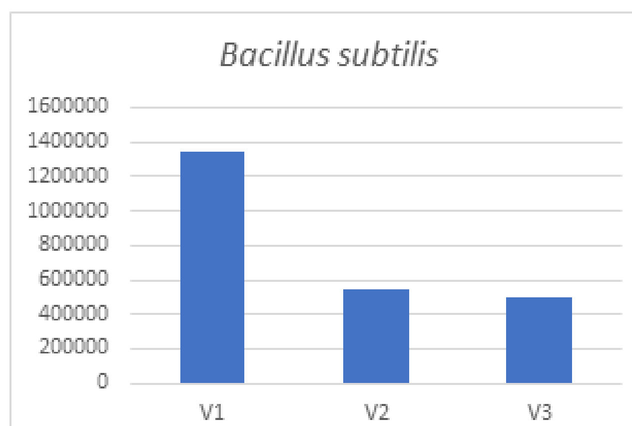

Figure 1. Quantity of *Bacillus* (CFU/ml)

Increasing the added quantity of *Bacillus subtilis* from 0.15% to 50% did not increase their growth on the nutrient medium. However, fertilizing the soils with the variants changed the result (Table 1).

Table 1. Quantity and composition of soil microflora (CFU/g)

Variants	Total micro-flora	Non-spore-forming bacteria	Bacillus	Actino-mycetes	Micro-mycetes	Bacteria assimilating mineral nitrogen	Minera-lization coefficient
V0	1033200	751000	242200	20000	20000	2082000	2.096
V1	1400000	955000	385000	20000	40000	4675000	3.489
V2	2485000	1210000	565000	40000	670000	4525000	2.549
V3	1055000	585000	345000	40000	85000	3162500	3.401

The application of the additives increases biogenicity of the soil compared to the control, without fertilization. The values of the total microflora have the following descending order: V2 > V1 > V3 > V0. The same trend is established for the bacilli. Under controlled conditions, the best development of bacilli was found in V1 (0.15% *Bacillus subtilis*), while under changing outdoor conditions, a higher quantity of *Bacillus subtilis* is necessary for better development - V2 (0.30% *Bacillus subtilis*). However, adding a higher amount is not the only prerequisite for better development. Just as the quantity of microorganisms is not an independent factor for their activity - the mineralization coefficient in V1 and V3 is higher than in V2, despite the fact that in V2 the total microflora is the highest. In principle, a complex of factors has an influence: soil temperature and humidity, pH, mechanical composition, soil type, type of vegetation and other factors. The mineralization activity in the fertilized variants is higher than in the control.

The main share in the composition of the total microflora is occupied by non-spore-forming bacteria. Non-spore-forming bacteria dominate over the micellar groups of microorganisms in other studies after fertilization (Plamenov et al., 2016; Yankova et al., 2016; Malcheva et al., 2018; Malcheva et al., 2019a,b; Malcheva et al., 2020; Malcheva, 2021a). In V1, V3 and the control, bacilli follow and the micellar groups of microorganisms - actinomycetes and molds - are less represented. In V2, a rearrangement in the composition of the total microflora is established - the quantity of micromycetes (molds) is higher than that of bacilli. The percentage distribution in the composition of the total microflora for each variant is presented in the following Figures 2 to 5.

Bacilli are spore-forming bacteria and are more resistant to adverse environmental conditions. *Bacillus subtilis* increases the solubility of phosphorus and potassium, and is also a nitrogen fixer (https://amiti.org/product/graundfiks-fosfor-kali-mikro-elemnti; https://www.agrocalculator.bg/razresheni-torove/bisolbifit-bisolbifit-tor-podobritel-na-pochvata/).

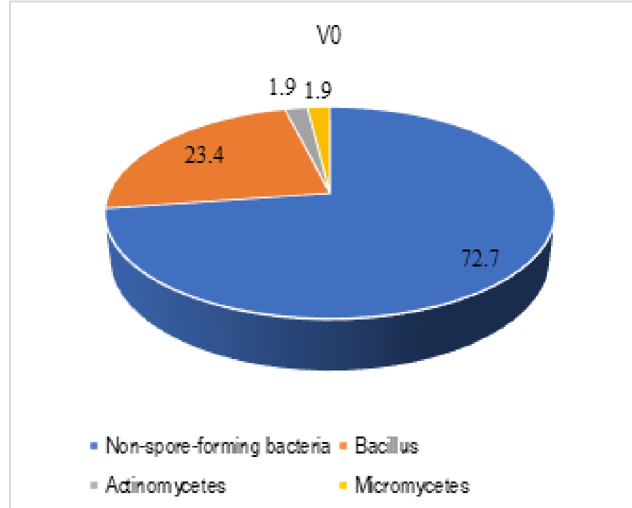

Figure 2. Percentage distribution of microorganisms in the composition of the total microflora in V0

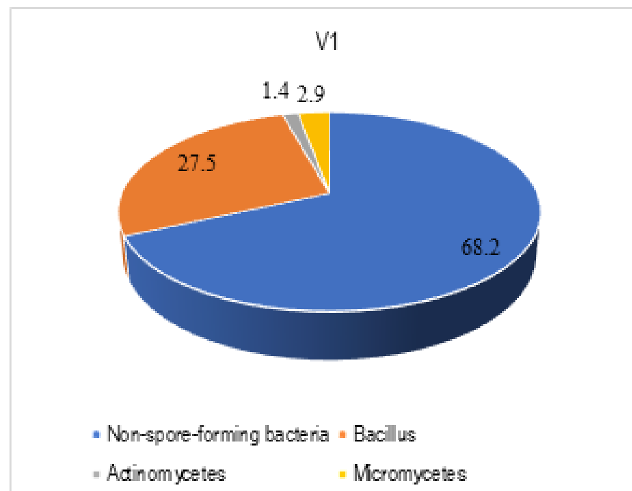

Figure 3. Percentage distribution of microorganisms in the composition of the total microflora in V1

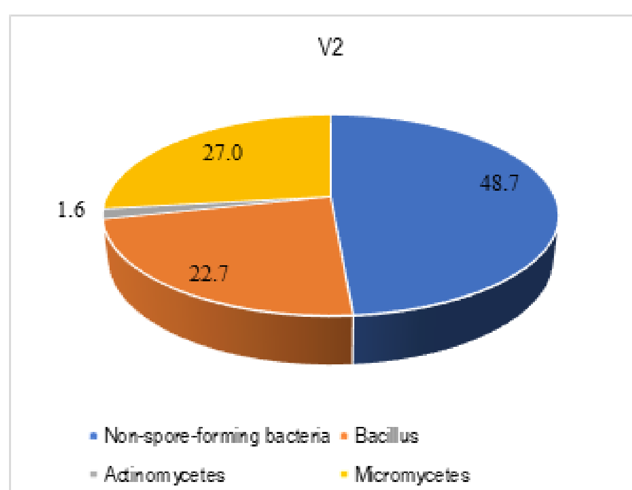

Figure 4. Percentage distribution of microorganisms in the composition of the total microflora in V2

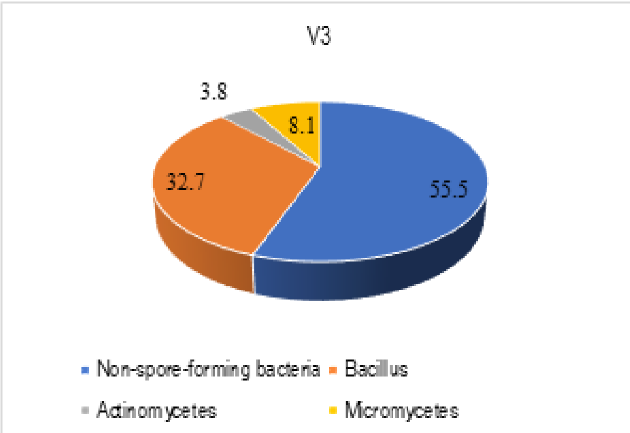

Figure 5. Percentage distribution of microorganisms in the composition of the total microflora in V3

Catalase activity of soil microorganisms is higher in the fertilizer variants compared to the control (Figure 6).

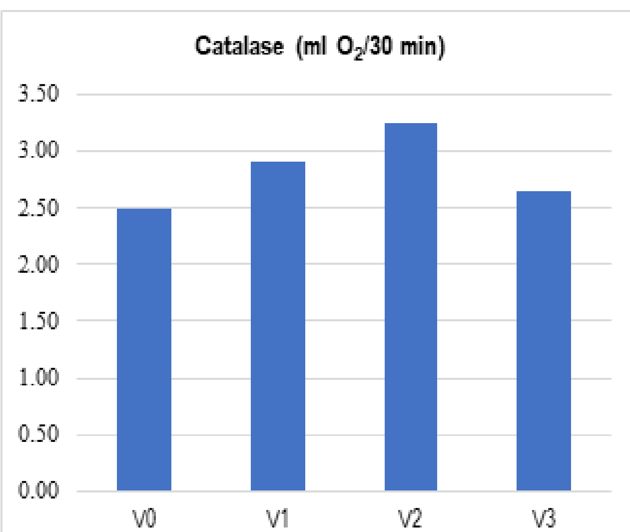

Figure 6. Catalase activity (ml O_2/30 min)

Catalase activity is the highest in the variant with the highest quantity of microorganisms and an average percentage of *Bacillus subtilis* addition - V2, followed by the variant with 0.15% of this species (V1) and the lowest catalase activity is when adding the highest quantity of *Bacillus subtilis* - 0.50% (V3). Catalase is a respiratory enzyme of biological and mineral origin, also depends on soil conditions, as there is catalase of plant origin as well.

Cellulase activity was recorded in dynamization for three months. The results are presented from the reading on the 90th day (Figure 7).

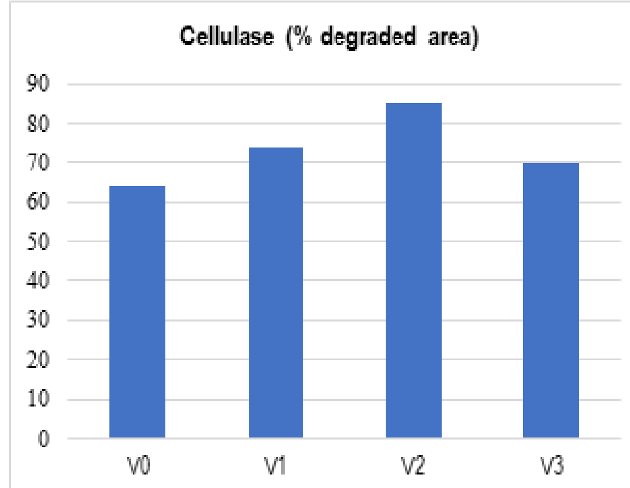

Figure 7. Cellulase activity (% degraded area)

Cellulose degradation also occurs at the highest level in V2 (0.30% *Bacillus subtilis*), which correlates with the highest total microflora, but not with mineralization activity. In the fertilized variants, the enzyme values are higher compared to the control.

Cellulase and catalase activities generally increase after fertilization (mineral, microbial, organic, combined), and fertilization rates should not be exceeded (Yankova et al., 2016; Naskova et al., 2016; Malcheva et al., 2018; Malcheva et al., 2019b; Malcheva et al., 2020; Malcheva, 2021a).

The urease activity of the soil is presented in Figure 8.

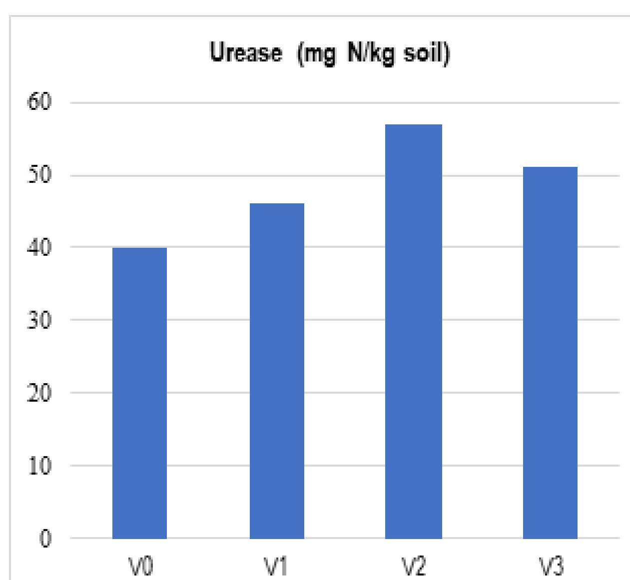

Figure 8. Urease activity (mg N/kg soil)

Urease activity is higher in the fertilized variants compared to the control. In the variants with additives, the enzyme values are the highest in V2 (0.30% *Bacillus subtilis*), after V1 (0.15% *Bacillus subtilis*) and V3 (0.50% *Bacillus subtilis*), which correlates with the established quantity of bacilli and total microflora in the samples. Microbial biomass nitrogen and urease and protease activities increased with the addition of vermiculite, mineral fertilization and liming in a vegetation experiment with *Lolium perenne* L. on regenerated substrates from a humus depot, tailings pond and mine, with microbial biomass nitrogen correlating strongly with urease activity and moderately with protease activity (Malcheva, 2021b).

The studied enzyme activities are sensitive indicators of changes occurring in soils and for assessing microbial nutrient needs, and depend on a complex of factors - quantity, composition and activity of microorganisms, temperature, humidity, pH, mechanical composition of soils, soil type, vegetation type and other factors (Malcheva, 2012; Gaind and Nain, 2012; Wang et al., 2015; Yankova et al., 2016; Naskova et al., 2016; Li et al., 2017; Malcheva et al., 2018; Malcheva et al., 2019b; Malcheva, 2020; Malcheva et al., 2021a,b).

The best variant in terms of quantity of microorganisms and enzyme activities is V2 (0.30% *Bacillus subtilis*), and in terms of mineralization coefficient - V1 (0.15% *Bacillus subtilis*). Therefore, the addition of the highest quantity of *Bacillus subtilis* is not an independent factor for its development, as well as for the development and activity of the entire soil microbiome. Both with mineral fertilizers and with microbial ones, it is advisable to observe the fertilization norms. The dependences of mineralization and enzyme activity on the quantity and composition of the soil microflora are presented by correlation analysis (Table 2).

Table 2. Correlation coefficients

Indi-cators	Total microflora	Non-spore-forming bacteria	Bacillus	Actino-mycetes	Micro-mycetes	Bacteria assimi-lating mineral N	Minerali-zation coeffi-cient	Cata-lase	Cellu-lase	Urease
Total micro-flora	1									
Non-spore-forming bacteria	0.92	1								
Bacillus	0.95	0.82	1							
Actino-mycetes	0.47	0.10	0.61	1						
Micro-mycetes	0.96	0.79	0.91	0.64	1					
Bacteria assimi-lating mineral N	0.68	0.72	0.81	0.22	0.51	1				
Minerali-zation coeffi-cient	-0.18	-0.21	0.12	0.16	-0.27	0.53	1			
Cata-lase	0.95	0.90	0.98	0.44	0.87	0.87	0.10	1		
Cellu-lase	0.95	0.85	1.00	0.55	0.90	0.83	0.12	0.99	1	
Urease	0.78	0.51	0.90	0.88	0.83	0.64	0.25	0.81	0.88	1

High correlation is established between the enzyme activities and the total microflora. The dependence between the enzymes cellulase and catalase and the quantity of non-spore-forming bacteria is very high, while urease depends significantly on this group of microorganisms. Catalase depends moderately, cellulase significantly, and urease highly on the values of actinomycetes in the soils. The dependence between the mineralization coefficient and the bacteria assimilating mineral nitrogen is significant, and between the mineralization coefficient and the enzymes it is weak.

Conclusion

Combined fertilization variants based on nitrogen fertilizers combined with phosphorus and sulfur, microbial fertilizer containing *Bacillus subtilis* and molasses were used. Fertilization increases the biogenicity of the soils. The quantity of bacilli and the total microflora of the studied soils are the highest in the variant with 0.30% *Bacillus subtilis*, followed by the variant with 0.15% *Bacillus subtilis* and the lowest biogenicity of the soil is in the variant with 0.50% *Bacillus subtilis*. Bacteria dominate over actinomycetes and mold fungi, and in V2: UAN (45%) + APP (45%) + ATS (5%) + MEL (5%) + *Bacillus subtilis* (0.30%) a partial rearrangement in the composition of the total microflora is established - the amount of mold groups is higher than that of bacilli. Fertilization increases the activity of the studied enzymes - cellulase, catalase and urease. In the same variant (V2) the quantity of microorganisms and enzyme activities increase the most, with the produced enzymes of microbial origin correlating strongly with the total quantity of microorganisms, mainly at the expense of bacilli, non-spore-forming bacteria and mold fungi in the case of cellulase and catalase, and bacilli, actinomycetes and mold fungi in the case of urease. The best variant in terms of the quantity of microorganisms and enzyme activities is V2 (0.30% *Bacillus subtilis*), and in terms of the mineralization coefficient - V1 (0.15% *Bacillus subtilis*). The studied microbiological and enzymatic indicators are sensitive biological and biochemical markers for changes occurring during combined fertilization with inorganic, organic and microbial products.

References

Gaind S and Nain L, 2012. Soil carbon dynamics in response to compost and poultry manure under rice (*Oryza sativa*)-wheat (*Triticum aestivum*) crop rotation. Indian Journal of Agricultural Sciences, 82, 410-415.

Garg S and Bahl GS, 2008. Phosphorus availability to maize as influenced by organic manures and fertilizer P associated phosphatase activity in soils. Bioresource Technology, 99, 5773-5777.

Gushterov G, Andonov P, Todorov T, Kominkov L and Gincheva-Starcheva M, 1977. Practical course in microbiology with virology, Science and Art, Sofia.

He Y, Qi Y, Dong Y, Xiao S, Peng Q, Liu X and Sun L, 2012. Effects of Nitrogen Fertilization on Soil Microbial Biomass and Community Functional Diversity in Temperate Grassland in Inner Mongolia, China. Clean – Soil, Air, Water, 41, 1216-1221.

Kandeler E and Gerber H, 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fertil Soils, 6, 68-72.

Khaziev F, 1976. Enzymatic activity of soils, Science, Moscow, (Ru).

Khonje DJ, Varsa EC and Klubek B, 1989. The acidulation effects of nitrogenous fertilizers on selected chemical and microbiological properties of soil. Journal Communications in Soil Science and Plant Analysis, 20, 1377-1395.

Malcheva B, 2012. Soil-microbiological indicators for establishing the status of anthropogenic soils on the territory of Sofia municipality. Thesis for PhD, University of Forestry, Sofia, Bulgaria, (Bg).

Malcheva B and Naskova P, 2018. Guide for laboratory exercises in Microbiology, University Publishing House at the Technical University of Varna, Varna, Bulgaria, (Bg).

Malcheva B, Naskova P, Plamenov D and Iliev Y, 2018. Study on impact of mineral fertilizers on biogenicity and enzymatic activity of soils with common wheat. International Journal of Advanced

Research, 6, 137-144.

Malcheva B, Yordanova M and Nustorova M, 2019a. Influence of composting on the microbiological activity of the soil, International Conference of the University of Agronomic Science and Veterinary Medicine of Bucharest, June 6-8 2019, Bucharest, Romania, Scientific Papers, series B, Horticulture, LXIII, 1, 621-625.

Malcheva B, Naskova P and Plamenov D, 2019b. Investigation of the influence of mineral nitrogen fertilizers on the microbiological and enzymic activity of soils with rapeseed. New Knowledge Journal of Science, 8-4, 80-90, (Bg).

Malcheva B, 2020. Soil-microbiological indicators for establishing the status of anthropogenic soils on the territory of Sofia Municipality, Gea-Print Publishing House, Varna, Bulgaria, (Bg).

Malcheva B, Petrova V, Yordanova M, Naskova P and Plamenov D, 2020. Influence of biochar and manure fertilization on the microbiological activity of agricultural soil, International Conference of the University of Agronomic Science and Veterinary Medicine of Bucharest, June 4-6 2020, Bucharest, Romania, Scientific papers, series B-Horticulture, LXIV, 2, 191-198.

Malcheva B, 2021a. Influence of microbial fertilizers on microbiological and enzymatic decomposition of carbohydrates in agrogenic soil. Bulgarian Journal of Crop Science, 58, 95-103, (Bg).

Malcheva B, 2021b. Microbial and enzyme decomposition of proteins to reclaimed substrates - vegetation experiment with *Lolium perenne*, International Conference of the University of Agronomic Science and Veterinary Medicine of Bucharest, June 3-5 2021, Bucharest, Romania, Scientific papers, series B-Horticulture, LXIV, 1, 104-111.

Mandal A, Patra AK, Singh D, Swarup A and Masto RE, 2007. Effect of long-term application of manure and fertilizer on biological and biochemical activities in soil during crop development stages. Bioresource Technology, 98, 3585-3592.

Mishustin E and Runov E, 1957. The success of the development of the principles of microbiological diagnosis of soil condition. Advances in Modern Biology, 44, 256-268, (Ru).

Mishustin F and Emtsev N, 1989. Microbiology. Kolos, Moskva, (Ru).

Nakhro N and Dkhar MS, 2010. Impact of Organic and Inorganic Fertilizers on Microbial Populations and Biomass Carbon in Paddy Field Soil. Journal of Agronomy, 9, 102-110.

Naskova P, Malcheva B, Yankova P and Plamenov D, 2016. Impact of the biological fertilizers on chemical indexes and enzyme activities of soils at cucumbers. International Research Journal of Natural and Applied Sciences, 3, 120-131.

Nustorova M and Malcheva B, 2020. Guide for laboratory exercises in Microbiology, Gea-Print Publishing House, Varna, Bulgaria, (Bg).

Plamenov D, Naskova P, Malcheva B and Iliev Y, 2016. Chemical and microbiological studies for determination the influence of fertilizers produced by “Agropolychim” AD on winter common wheat and oilseed rape. International Journal of Science and Research, 5, 1481-1486.

Samuel AD, Domuţa C, Ciobanu C and Şandor M, 2008. Field management effects on soil enzyme activities. Romanian Agricultural Research, 25, 61-68.

Sarathchandra SU, Ghani A, Yeates GW, Burch G and Cox NR, 2001. Effect of Nitrogen and Phosphate Fertilizers on Microbial and Nematode Diversity in Pasture Soils. Soil Biology and Biochemistry, 33, 953-964.

Wang RZ, Dorodnikov M, Yang S, Zhang YY, Filley TR, Turco RF, Zhang YG, Xu ZW, Li H and Jiang Y, 2015. Responses of enzymatic activities within soil aggregates to 9-year nitrogen and water addition in a semi-arid grassland. Soil Biology and Biochemistry, 81, 159–167.

Yankova P, Naskova P, Malcheva B and Plamenov D, 2016. Impact of the biological fertilizers on the microorganisms and the nutrient elements in the soil. International Journal of Current Research, 5, 39681-39686.

Zhang YD, Sun ZH and Shen YX, 2005. Effect of Fertilization on Soil Microorganism of Deteriorated Grassland in Dry-Hot Valley Region of Jinsha River. J. Soil Water Conserv., 19, 88-91.