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Assessing the impact of biofertilizer on soil microbial dynamics and metabolic activity in a controlled maize pot-grown experiment

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Abstract

Biofertilizers, consisting of carefully selected microorganisms across various species and genera, exhibit distinct features that enhance soil fertility and promote plant growth. Embracing the principles of eco-friendly agriculture, the use of biofertilizers emerges as a pivotal strategy for sustainable farming, contributing to environmental preservation and the overall health and biodiversity of the soil. In this study, a commercially available biofertilizer, containing a specialized strain of *Priestia megatherium* with nitrogen-fixing capabilities, was employed alongside chemical fertilizers at two different doses (30 and 40 mg per kg of soil). The primary objective was to evaluate the impact of biofertilizer on the metabolic activity and structure of microbial communities in a short-term experiment involving potted maize plants, utilizing the BIOLOG® EcoPlates technique. Parameters such as average well-color development (AWCD) and substrate utilization across six guilds (SAWCD) were assessed to gauge microbial metabolic activity. Additionally, functional indexes, including Shannon diversity, Shannon evenness, and Simpson diversity, were calculated as indicators of soil microbial community functionality. While statistically significant differences in AWCD among the studied variants were not observed, all estimated functional indexes consistently revealed heightened microbial diversity and evenness following the application of biofertilizer. This noteworthy finding, achieved within a relatively short period of plant cultivation, underscores the necessity for further research to explore the biofertilizer's enduring effects on soil communities, both in controlled laboratory environments and under real-world field conditions.

Keywords: Biofertilizer, BIOLOG® EcoPlate, functional indexes, metabolic activity, microbial communities, *Priestia megaterium*.

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Introduction

Biofertilizers contain living microorganisms which can affect plant growth and productivity by activating processes in the soil and in the plant. The use of biofertilizers is considered an eco-friendly approach, which complies with the aims set in the “farm to fork” strategy of the EU for 20% reduction of mineral fertilisers (Kurniawati et al., 2023). The biofertilizers usually contain either a single strain or a combination of different strains and species belonging to the genera *Azotobacter* spp., *Rhizobium* spp. and *Azospirillum* spp., and mycorrhizal fungi (AMF). The list of species applied as biofertilizers is constantly expanding and currently included strains from the genera *Aeromonas*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Klebsiella*, *Pseudomonas*, and *Serratia*. The microbial biofertilizers can be applied in the soil, on the leaves or seeds. One of the main goals of their application is towards increased microbial activity, diversity of beneficial microorganisms, and change in the ratio between different species which can boost soil processes and organic matter turnover (Bargaz et al., 2018; Vessey, 2003). Despite the expanding interest on the application of AMF and beneficial bacteria their mechanisms of action are not fully revealed (Backer et al., 2018; Ramakrishna et al., 2019). The effectiveness of biofertilizers would depend on the properties of the

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selected species and strains but the combined use of biofertilizers and the mineral fertilisers would require both laboratory and field data in order to establish the optimal ratio, to optimize the way of application and to reveal the possible mechanisms of interactions between microorganisms and plants. The justification of such approach should also take into account financial resources, available machinery and other intrinsic factors (Bargaz et al., 2018).

The primary objective of this study was to investigate the impact of biofertilizer on the metabolic activity of soil microorganisms and alterations in the structure of soil microbial communities. This investigation was conducted through a short-term laboratory experiment involving potted maize plants, carefully controlled to maintain constant conditions

Material and Methods

Maize variety

In the study was used maize hybrid - Kneja 307. The plants were grown in a climate-controlled chamber set at the following conditions: a photosynthetic photon flux density (PPFD) - 200 µmol m^{-2} s^{-1}, an average temperature - 25°C ± 1°C, an average relative humidity – 65% and 13/11 hours day/night photoperiod. After one-month cultivation, the plants were taken off from the pots and plant material and soil was used for further analyses.

Soil

The soil used in the pot experiment, 0 to 10 cm depth, was collected from the Experimental field of the Agricultural University-Plovdiv, Bulgaria. The soil type was classified as silty clay loam (mollic fluvisols) - 19.9% sand, 46.9% silt, 33.2% clay with 2.66% organic matter and high lime content (Table 1).

Table 1. Physicochemical characteristics of bulk soil

Soil parameters	Value	Units
Nitrogen	7.94	ppm
Phosphorus (P_2O_5)	214.60	ppm
Potassium (K_2O)	423.50	ppm
$CaCO_3$	8.75	g/kg
Exchangeable cations (ca+mg)	16.20	meq/100 g
Electrical conductivity	108.10	µS/cm
Soil organic matter	2.66	%
pH	8.80	logarithmic units

Mineral fertilizer and biofertilizer

Ammonium nitrate was used as mineral fertilizer. The biofertilizer is a product available on the market under commercial name NUPTAK (Daymsa, Spain) and is comprised of a selected strain of gram-positive, spore-forming rod bacterium - *Priestia megatherium CB2001.*

Experimental design

The experiment was comprised of four treatments (Table 2), with five replicates (pots) with 4 plants per pot. The collected bulk soil was sieved, split in two and half of it was sprayed with a freshly prepared suspension of biofertilizer in a dose of 0.33 mg per kg soil. The other soil was sprayed with the same quantity of tap water and all soil samples were thoroughly mixed. The biofertilizer inoculated soil and the control (uninoculated soil) were further split in two and were fertilized either with 30 or with 45 mg ammonium nitrate per kg soil, respectively. After fertilization the soil samples were mixed with perlite at 3:1 volume ratio and each pot was filled with 1 kg of soil.

Table 2. Experimental variants - descriptions and abbreviations

Variants abbreviation	Variants description	
	Mineral fertilizer - ammonium nitrate, mg kg^{-1}	Biofertilizer – Priestia megaterium, mg kg^{-1}
30 ppm N	30	-
30 ppm N+PM	30	0.33
45 ppm N	45	-
45 ppm N+PM	45	0.33

The rizhospheric soil was carefully brushed from the plant roots and was used for metabolic analysis. The other soil in the pots was used for soil respiration and physicochemical analyses.

Soil analyses

Available mineralized forms of nitrogen (ammonium and nitrate) were determined by modified Kjeldahl method. Soil phosphorus analysis was conducted Egner-Riehm DL method according to Egner and Riehm (1955). The applied method, with slight modification, includes $SnCl_2$ dissolved in hydrosulfuric acid as an indicator and measurement of wavelength at 700 nm. The analysis for available potassium was done with 5 g of soil sample dissolved in 50 ml of 2N HCl acid and the suspension was shaken and filtered before reading on a flame photometer.

Soil respiration

The soil respiration was determined according to Isermeyer method described by Alef (1995).

Metabolic activity of microbial soil communities

Metabolic activity of soil communities was assessed using the 96-well Eco MicroPlates™ of BIOLOG® (Biolog Inc., USA). Each EcoPlate is comprised of 31 different substrates organized in the following guilds - carbohydrates (ten substrates), carboxylic acids (seven substrates), polymers (four substrates), amino acids (six substrates), amines (two substrates) and phenolic compounds (two substrates). The EcoPlate contains three replicates of guilds.

One gram of the rhizospheric soil was suspended in 9 ml sterile distilled water, thoroughly mixed and left to settle for 5 min, after which a 10^{-3} dilution was prepared. The inoculation of Biolog® EcoPlates was done with 150 µl and plates were incubated at 25±1°C. The plates were read spectrophotometrically immediately after inoculation and consequently at 24 hour intervals for 7 days (168h) with the MicroStation™ Reader provided by the BIOLOG® System. The calculations for average well-color development (AWCD) and separately for each guilds were based on the optical density (OD) measured at 590 nm and 750 nm according to the procedure described by Sofo and Ricciuti (2019) except the formula for AWCD which was according to Huang et al. (2012) as follows:

$$\mathrm{AWCD} = \sum (C_i - R)/31$$

where R is the control well (water) and C_i is the value of carbon substrate well

Functional indexes

The wells with an average OD ≤0.250 were not taken as a positive response for substrates utilization according to Sofo and Ricciuti (2019) and was set to zero in calculation. Shannon richness was calculate by the following formula:

$$H' = -\sum(P_i \times lnP_i),$$

where $P_i = (C_i - R)/\sum C_i - R)$

The Shannon evenness index (E) which was derived from the Shannon index used the formula:

$$E = H'/lnS$$

where S was the substrate richness (the number of wells which showed positive threshold OD≥0.250)

Simpson diversity index (D) was calculated according to Ge et al. (2018):

$$D = 1 - \sum P_i^2,$$

where P_i is the same as in the Shannon index calculation.

Data analysis

AWCD values calculations and the graph visualization was done with Microsoft Excel using the three replicates on each ecoplate as independent measurements (n=3). In order to compare functional indexes of the different treatments one-way analysis of variance (ANOVA) was performed with SPSS program (IBM, ver. 26) with the level of significance $p<0.05$.

Results and Discussion

Effect of mineral fertilization and biofertilizer on soil parameters

The application of mineral fertilizer and biofertilizer affected the soil pH – a slight decrease from 8.80 to 8.45. Additionally, the level of available phosphorus oxide has increased after treatments which could be explained by solubilisation of the fixed phosphorus which correspond to the phosphorus solubilization activity assigned to the strain present in the biofertilizer according to information provided by the manufacturer. The increase of available phosphorus in the soil is often associated with the presence of phosphorus solubilizing microorganisms (Mehnaz, 2016). The analysis showed a higher level (8%) of ammonium in the soil treated with the lower dose of mineral fertilizer supplemented with biofertilizer – 30 ppm N+PM when compared to the non-supplemented with a biofertizer variant – 30 ppm N. However, there was not significant difference of available ammonium in the soil between 45 ppm N and 45 ppm N+PM treatment. The potassium (K_2O) level

declined from 423.5 ppm (Table 1) to 345 ppm due to its utilization by plants and the used chemical fertilizer does not provide potassium. The soil used in the current experiment, showed a relatively low nitrogen content which also declined for all variants at the end of plant cultivation. The study (data not shown) also included assessment of plant biometrics, photosynthetic activity, antiradical activity and analyses of leaf and root mineral content. In general, analyses of estimated parameters did not reveal statistically significant differences between experimental treatments which can be explained by the short duration of the performed pot experiment (Table 3).

Table 3. Mineral content of soil at the end of plant cultivation

Variant	pH	Mineral content, ppm			
		P_2O_5	K_2O	NH_4	NO_3
30 ppm N	8.52	296.4	326.0	2.93	5.65
30 ppm N+PM	8.43	300.1	346.0	3.19	5.68
45 ppm N	8.47	282.8	346.6	3.83	4.56
45 ppm N+PM	8.40	302.6	346.0	3.92	8.49

The inoculated with biofertilizer soils showed higher respiration rate (Table 2) in comparison to soils without biofertilizer.

Table 4. Soil respiration (mg CO_2/g dry soil/24 h)

Variants	Soil respiration
30 ppm N	0.062^{b} ±0.012
30 ppm N+PM	0.096^{ab} ±0.012
45 ppm N	0.078^{b} ±0.015
45 ppm N+PM	0.129^{a} ±0.021

Legend: The soil respiration is presented as mean ± SD, n=3; the different superscript letters indicate statistical difference at $p<0.05$

The average well-color development (AWCD) (Figure 1) which was used to assess the metabolic activity of soil microorganisms revealed a presence of a lag phase till 24th hour and highest activity between 48th hour and 96th hour; after that the changes in the optical density were moderate. There was not significant difference between different variants till 96th hour, however, the variants 30 ppm N+PM and 45 ppm N showed higher activity in comparison to other variants. Similar observation, which included a lag phase and a gradual increase in the metabolic activity of microorganisms, was made by Ge at al. (2018). The OD change could be assigned predominantly to bacterial activity because the reading of the EcoPlate started from 24th hour which is insufficient time for fungi development and their contribution to the color in the wells could be ignored (Figure 1). In fact, either in natural or in experimental conditions, if there is no inhibitory factors that could suppress the growth and metabolic activity of microorganisms the curve of AWCD usually keeps a typical sigmoid shape (Stefanowicz, 2006, Lima et al., 2015).

In order to obtain more detailed information about specific metabolic activity of bacteria the AWCD was also calculated separately for the six guilds of substrates (amino acids, carbohydrates, carboxylic acids, amines, polymers, and phenolic compounds) provided by the Biolog® EcoPlate.

The utilization of amino acids (Figure 2) showed a distinct dynamics for different variants, especially after the 96th hour of incubation, despite the lack of statistical difference. After 72nd hour of inoculation until the end of the incubation period the variant 30 ppm N + PM showed a higher metabolic activity in comparison to the other variants.

The utilization of amines, on the contrary to the utilization of all other guilds, was more active only for the variants that were not supplemented with biofertilizer and there was a clear difference between variants in the created graph (Figure 3). Ge et al. (2018) observed the low utilization of amines and considered that among all substrates provided in the Ecoplate these substrates were less preferable. However, the authors reported an optical density of 1.600 on the 7th day of incubation, and in the current experiment the maximum values for the same period was only 1.097 for variant 30 ppm N and this value was comparable with the value of carbohydrates utilization (1.193). The observed mean values were accompanied with a notable standard deviation which restrained any reliable conclusions about microbial metabolism of amines in the variants.

There was not significant difference of carboxylic acid utilization between variants (Figure 4). However, the utilization of this guild of ten different carboxylic acids was detected, even at a relatively low level, on the 24th hour measurement only for the two variants that were treated with biofertilizer. According to some authors utilization of carboxylic acids could be considered as the most representative substrate guild of the Ecoplate and they associated the better utilization of carboxylic acids to improved plant health. The authors found that the higher carboxylic acids utilization of microbial communities was related to the lower level of damages to the trees which were objects of the study (Cai et al., 2010).

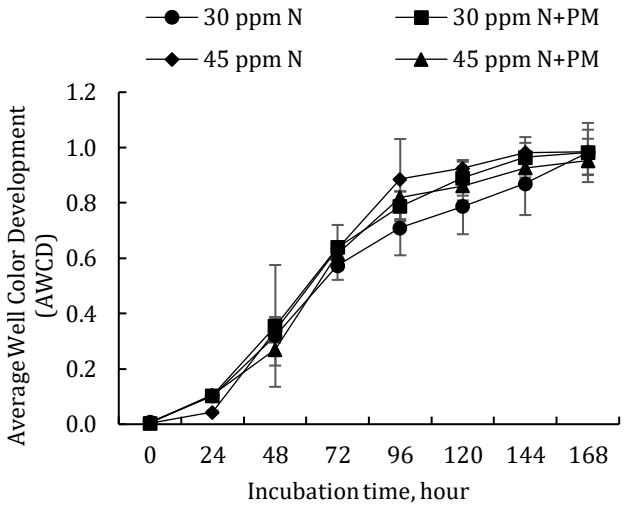

Figure 1. Microbial metabolic activity in the Biolog® EcoPlate

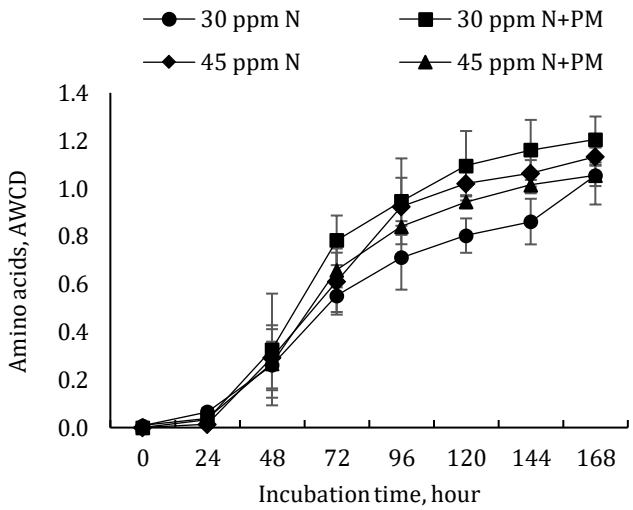

Figure 2. Dynamics of amino acids utilization in the Biolog® EcoPlate

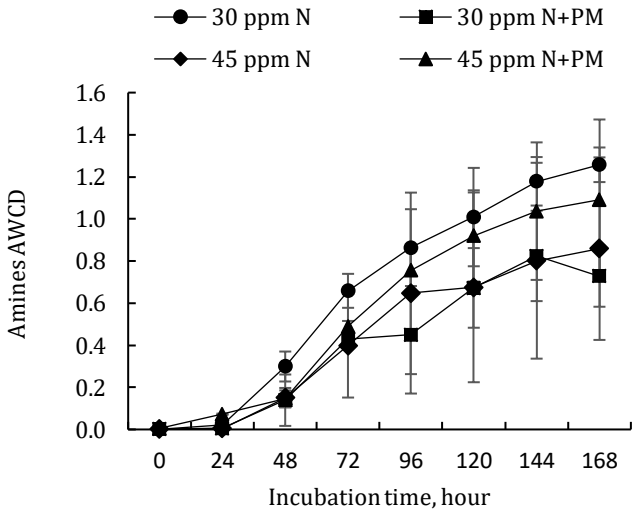

Figure 3. Dynamics of amines utilization in the Biolog® EcoPlate

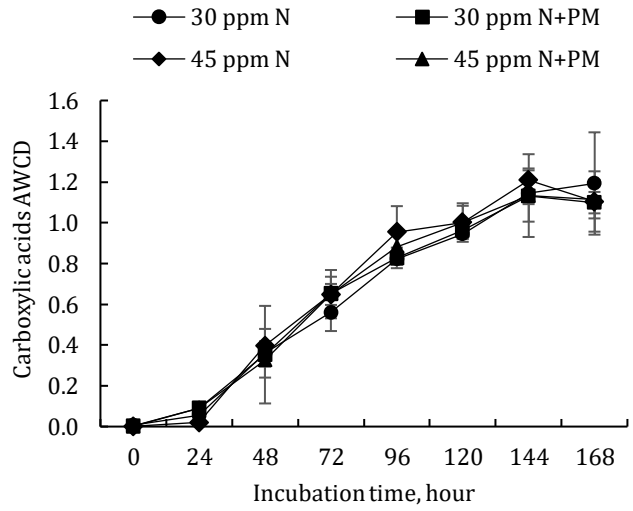

Figure 4. Dynamics of carboxylic acids utilization in the Biolog® EcoPlate

The carbohydrates were utilized (Figure 5) intensively up to 72nd hour. The variant 40 ppm N showed a relatively low value (0.104) at the 24th hour but reached OD of 0.487 at the measurement on 48th hour. After 120th hour until the end of the incubation the variants 30 ppm N+Priestia and 45 ppm N showed a higher metabolic activity in comparison to the other variants. The optical density varied between 0.716 to 0.800 on 72nd hour, but after that the curve tend to flat and at the end of the incubation the values ranged between 0.889 and 1.006 and there was no significant difference between variants.

In comparison to the other substrates, utilization of polymers began slowly and very uniformly among the variants and the same uniformity remained up to 96th hour despite that the curve became steep after 48th. Only after 120th hour the different variants could be distinguished one from another (Figure 6). In general, the utilization of polymers is not a very common characteristic for bacterial metabolism. This is especially true for the included in the *Ecoplate cyclodextrin*, wells (E 1-5-9), which metabolization was related to the existence of a relatively rare metabolic path in bacterial cells. The metabolic path has been described in the cells of hyperthermophilic archaea such as *Thermococcus* sp., *Pyrococcus furiosus* and *Archaeoglobus fulgidus* and other studies found it only in a few species of mesophilic bacteria such as *Klebsiella oxytoca* and *Bacillus subtilis* (Centeno-Leija et al., 2022). As a result the polymer utilization is assigned mainly to some bacteria with a relatively low abundance in the soil.

Xiao et al. (2022) used in their study *Rhodopseudomonas palustris* and *Bacillus subtilis* as microbial inoculants and found that they not only positively influenced the rice yield (17.73%) but there was also a synergy

between inoculants. Additionally, the inoculants have an influence on the structure of microbial communities and this effect concerned predominantly the rare species than the typical ones. The changes in the rare species authors explained with the increase of beneficial microorganisms in the soil and with the intensification of some essential soil processes (Xiao et al., 2022).

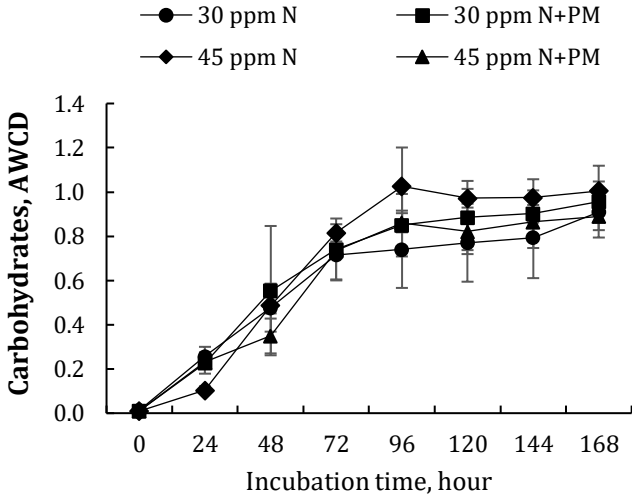

Figure 5. Dynamics of carbohydrates utilization in the Biolog® EcoPlate

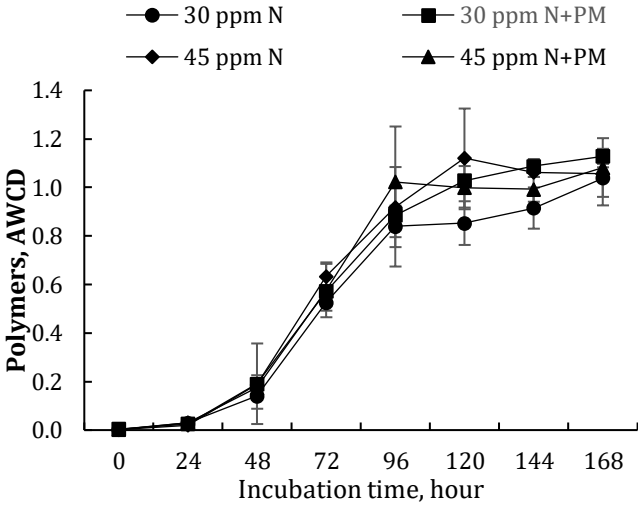

Figure 6. Dynamics of polymers utilization in the Biolog® EcoPlate

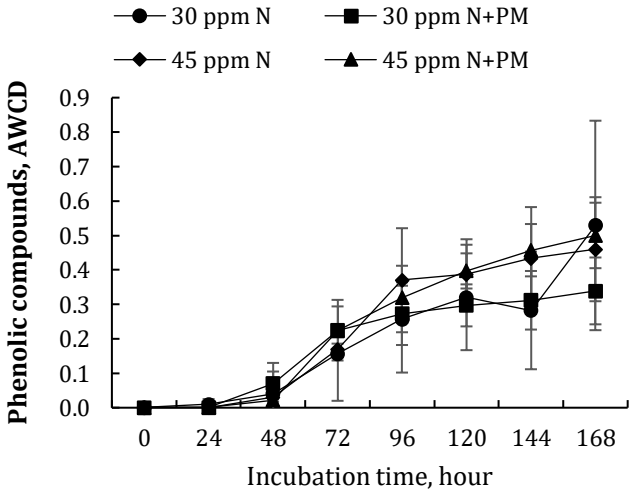

Figure 7. Dynamics of phenolic compounds utilization in the Biolog® EcoPlate

The utilization of phenolic compounds (2- hydroxy benzoic acid and 4- hydroxy benzoic acid) was lowest in comparison to all other guilds present in the Ecoplate. It was especially true for 2- hydroxy benzoic acid since the optical density in the corresponding wells (C 3-7-11) was either very low or the estimated values were even negative after correction with the control well which additionally contributed to the relatively larger standard deviation during calculations. At the end of the incubation period, the highest mean value was observed for the variant 30 ppm N - 0.529 with a standard deviation of 0.304.

After taking into account the values for optical density above 0.250 which were considered as a positive response towards substrates utilization the measurement taken on the 72nd hour was used for functional indexes calculation. The Shannon richness is related to species abundance and in the current study it has the higher values for variants supplemented with biofertilizer - 3.245 and 3.236 for variants 45 ppm + PM and 30 ppm N + PM, respectively (Table 3). Additionally, the Shannon evenness and Simpson diversity indices also showed higher values for biofertilizer-supplemented variants. However, none of the observed differences was statistically significant but the consistent trend across the calculated indexes could be considered as a clear indication that the biofertilizer has a positive effect on soil microbial communities. The effect of biofertilizer on microbial communities in the current study could be restrained to some extend due to the relatively short duration of the experiment, low organic matter content in the soil, high carbonate content or other factors. On the contrary to the presented results are findings of Roesti et al. (2006) who reported a significant modification in the structure of microbial communities after biofertilizer application.

Table 5. Metabolic functional diversity indices of soil samples treated with mineral fertilizer and Priestia megatherium-based biofertilizer

Variant	Indices		
	Shannon diversity (H´)	Shannon evenness (E)	Simpson diversity index (D)
30 ppm N	3.157 ± 0.202	0.962 ± 0.025	0.955 ± 0.006
30 ppm N+PM	3.236 ± 0.032	0.978 ± 0.007	0.958 ± 0.002
45 ppm N	3.181 ± 0.070	0.973 ± 0.007	0.955 ± 0.003
45 ppm N+PM	3.245 ± 0.082	0.978 ± 0.002	0.959 ± 0.003

Legend: Indexes are presented as: mean ± stand. deviation, n=3

Some studies did not find a significant effect of biofertilizer on soil microbial communities or the effect was very limited (Baldi et al., 2021, Wang et al., 2021). Other authors reported that biofertilizers did not provided the expected changes of the observed parameters or their effect was highly dependent on the applied doses (Al-Zubade et al., 2021, Siswanti and Riesty, 2021). Hou et al. (2023) used in their study three doses of mineral fertilizer (0, 200 and 400 kg N $ha^{-1}yr^{-1}$) and found that different doses of fertilizer affected variously microbial communities structure but fertilization at a moderate dose triggered higher diversity. Such data have practical importance because they offer information that can be used in the process for establishment of optimal ratio of mineral fertilizer and biofertilizer. The proper ratio would provide the expected positive effect of biofertilizer application along with the low input of mineral fertilizer. In their study Adesemoye et al. (2009) also tried to solve the question if the reduced doses of mineral fertilizer combined with a biofertilizer on tomato plants could provide the plant development comparable to those at optimal mineral fertilization and to what extend could be reduced fertilizer when it is supplemented with biofertilizer. The authors used biofertilizer that contained bacteria (*Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4), and arbuscular mycorrhizal fungi - AMF (*Glomus intraradices*). The results indicated that when the biofertilizer was added the reduction in the doses of mineral fertilizer could be up to 75% and the plant growth and yield was equivalent to the use of recommended doses of mineral fertilizer alone. When the doses of mineral fertilzer was further reduced below 75% the trend of positive effects were no longer convincing or consistent across the estimated parameters. According to the authors, when the doses of mineral fertilizer were reduced to 70% the combined use of both bacteria and AMF seemed mandatory. The results of the study showed the positive effect of biofertilizer but authors recommended furhter estimation of applied in the study microorganisms before they could become a part of the intergraded agricultural management (Adesemoye et al., 2009).

Conclusion

In conclusion, this study revealed notable shifts in soil composition and a favorable impact on soil microbial communities following the application of biofertilizer. The microbial metabolic activity demonstrated the ability of microorganisms to utilize EcoPlate substrates, displaying certain preferences for specific guilds. Although no statistically significant differences were observed among the variants at this stage, the consistent positive effects of the biofertilizer across estimated functional indexes suggest its potential benefits. This observation underscores the need for further research to explore the potential long-term effects and broader applications of biofertilizers in soil management practices.

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