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WPŁYW FOSFOROWEGO NAWOZU MINERALNEGO WZBOGACONEGO MIKROBIOLOGICZNIE NA AKTYWNOŚĆ I BIORÓŻNORODNOŚĆ MIKROORGANIZMÓW GLEBOWYCH

THE INFLUENCE OF MICROBIOLOGICALLY ENRICHED PHOSPHORUS MINERAL FERTILIZER ON THE ACTIVITY AND BIODIVERSITY OF SOIL MICROORGANISMS

Rozprawa doktorska

Doctoral thesis

Rozprawa doktorska przygotowana pod kierunkiem

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oraz

Promotora pomocniczego: dr Agaty Gryty

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OŚWIADCZENIE PROMOTORA ROZPRAWY

Oświadczam, że niniejsza rozprawa została przygotowana pod moim kierunkiem i stwierdzam, że spełnia ona warunki do przedstawienia jej w postępowaniu o nadanie stopnia naukowego

Data..... Podpis promotora rozprawy.....

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Data..... Podpis promotora pomocniczego rozprawy.....

OŚWIADCZENIE AUTORA ROZPRAWY

Świadomy odpowiedzialności prawnej oświadczam, że niniejsza rozprawa została napisana przeze mnie samodzielnie i nie zawiera treści uzyskanych w sposób niezgodny z obowiązującymi przepisami.

Oświadcza również, że przedstawiona rozprawa nie była wcześniej przedmiotem procedur związanych z uzyskaniem stopnia naukowego w wyższej uczelni.

Oświadczam ponadto, że niniejsza rozprawa jest identyczna z załączoną wersją elektroniczną.

Data..... Podpis autora rozprawy.....

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Badania do rozprawy doktorskiej zostały wykonane w ramach projektu "Opracowanie technologii innowacyjnych nawozów mineralnych wzbogaconych mikrobiologicznie" (BIO-FERTIL) (BIOSTRATEG3/347464/5/NCBR/2017) zrealizowanego w ramach strategicznego programu badań naukowych i prac rozwojowych "Środowisko naturalne, rolnictwo i leśnictwo" - BIOSTRATEG, współfinansowanego przez Narodowe Centrum Badań i Rozwoju (NCBiR).

Streszczenie

Nieustanny wzrost populacji świata, a co za tym idzie silna intensyfikacja i chemizacja rolnictwa przyczyniają się do niepokojących przekształceń w agroekosystemach, czego skutkiem jest obniżenie produktywności i jakości gleb uprawnych. Nie ulega wątpliwości, że nawozy sztuczne są istotnym źródłem składników mineralnych niezbędnych roślinom uprawnym do prawidłowego wykształcenia cech morfologicznych, jednak nadmierna i nierozsądna aplikacja wspomnianych preparatów przyczynia się do zanieczyszczenia środowiska naturalnego i spadku bioróżnorodności wśród mikrobiomu i mykobiomu glebowego. Mikroorganizmy stanowią elementarny składnik ekosystemu, wyznaczając kierunek procesów zachodzących w glebie, a tym samym kształtując dostępność składników pokarmowych i dbając o zachowanie równowagi ekologicznej. Ze względu na nieoceniony wkład zbiorowisk bakterii, archeonów i grzybów w funkcjonowanie agroekosystemów, wzrasta zainteresowanie oceną wpływu różnych praktyk rolniczych na bioróżnorodność i aktywność mikroorganizmów zasiedlających gleby uprawne. Współczesna wizja ekologicznego i zrównoważonego rolnictwa łączy status mikrobiomu i mykobiomu z ogólną jakością gleb uprawnych, a bogata bioróżnorodność pozwala na utrzymanie potencjału produkcyjnego gleby na odpowiednio wysokim poziomie. Ma to szczególnie ważne znaczenie w przypadku gleb zdegradowanych, w których doszło do zaburzenia homeostazy i obniżenia zawartości składników mineralnych.

Jednym z rozwiązań wychodzącym naprzeciw problemom wynikającym z intensywnej działalności rolniczej człowieka jest wykorzystanie szczepów pożytecznych mikroorganizmów w formie bionawozów. Bionawozy stanowią przyjazną dla środowiska alternatywę lub suplementację dla tradycyjnych nawozów mineralnych, a coraz liczniejsze doniesienia potwierdzają pozytywny wpływ aplikacji tego typu preparatów na właściwości mikrobiologiczne gleb uprawnych, co jest nierozerwalnie związane z poprawą zdrowia gleby. Wykorzystanie bionawozów zawierających pożyteczne mikroorganizmy pozostaje także w zgodzie z założeniami zrównoważonego rolnictwa, a także z polityką nakazującą ograniczenie ilości agrochemikaliów aplikowanych do gleby.

Biorąc pod uwagę rosnące zainteresowanie bionawozami i rozwój zrównoważonych praktyk zarządzania glebą, w niniejszej rozprawie podjęto badania dotyczące wpływu innowacyjnego fosforowego nawozu mineralnego wzbogaconego szczepami pożytecznych bakterii na parametry mikrobiologiczne gleb zdegradowanych (aktywność enzymatyczną oraz różnorodność funkcjonalną i genetyczną zbiorowisk bakterii, archeonów i grzybów). Dwuletnie doświadczenia polowe prowadzono w latach 2018-2019 na dwóch różnych typach gleb, pod uprawą kukurydzy. W doświadczeniach polowych zastosowano dawkę optymalną nawozu bez wzbogacenia mikrobiologicznego (FC), dawkę optymalną wzbogaconą mikrobiologicznie (FA100) oraz dawkę zredukowaną o 40% zawierającą mikroorganizmy (FA60). Próbki gleby do badań pobierano trzykrotnie w obu latach trwania doświadczenia: przed aplikacją (bio)nawozów (kwiecień), tydzień po aplikacji (bio)nawozów (czerwiec) oraz po zbiorach kukurydzy (październik).

Aktywność enzymatyczną gleby oceniono na podstawie zmian aktywności proteazy, ureazy, fosfatazy kwaśnej oraz β-glukozydazy. Badania różnorodności funkcjonalnej obejmowały określenie zdolności katabolicznych zbiorowisk mikroorganizmów glebowych do utylizacji substratów węglowych rozmieszczonych na płytkach BIOLOG^{TM} ECO i FF. Różnorodność genetyczną zbadano na podstawie analizy polimorfizmu długości terminalnych fragmentów restrykcyjnych (ang. terminal restriction fragment length polymorphism - t-RFLP) oraz sekwencjonowania następnej generacji (ang. Next Generation Sequencing - NGS). Przeprowadzone badania dotyczyły zarówno natychmiastowej odpowiedzi mikroorganizmów po aplikacji fosforowego bionawozu, jak i sezonowych zmian w statusie mikrobiomu i mykobiomu w glebach nawożonych fosforowym bionawozem.

Przeprowadzone badania wykazały pozytywny wpływ fosforowego bionawozu na parametry mikrobiologiczne gleb zdegradowanych. Aplikacja fosforowego bionawozu spowodowała wzrost aktywności enzymatycznej oraz pozwoliła na utrzymanie tego efektu w czasie. Zaobserwowano także zmiany w stopniu utylizacji źródeł węgla należących do różnych grup związków i poszerzenie zdolności katabolicznych zbiorowisk mikroorganizmów glebowych. Fosforowy bionawóz stymulował występowanie mikroorganizmów promujących wzrost i rozwój roślin oraz zwiększył różnorodność genetyczną i liczbę funkcjonalnych operacyjnych jednostek taksonomicznych związanych z procesami metabolicznymi i komórkowymi. Odnotowano także wzrost plonu kukurydzy i zawartości dostępnego fosforu w glebie. Uzyskane wyniki świadczą o kompleksowym działaniu fosforowego bionawozu na środowisko glebowe i potwierdzają, że może być on efektywnie wykorzystywany w rolnictwie zrównoważonym i regeneracyjnym.

Słowa kluczowe: bionawozy, zrównoważone rolnictwo, mikroorganizmy glebowe, gleba zdegradowana, aktywność enzymatyczna gleby, bioróżnorodność mikroorganizmów glebowych, rolnictwo regeneracyjne

Abstract

The incessant growth of the world's population and the consequent strong intensification and chemization of agriculture contribute to disturbing shifts in agroecosystems, resulting in a decline in the productivity and quality of arable soils. It is beyond doubt that synthetic fertilizers are an important source of nutrients indispensable for the proper development of plants, however, excessive and inappropriate application of such preparations leads to environmental pollution and a loss of biodiversity within soil microbiome and mycobiome. Microorganisms are an integral component of the terrestrial ecosystems, directing the soil processes and thus shaping the availability of mineral compounds and ensuring of the ecological balance. Owing to the invaluable contribution of bacterial, archaeal and fungal communities to the functioning of agroecosystems, a growing interest is emerging in the evaluation of the impact of different agricultural practices on the biodiversity and activity of microorganisms inhabiting arable soils. The contemporary vision of ecological and sustainable agriculture links the status of the soil microbiome and mycobiome with the general soil quality, and the rich biodiversity allows for the maintenance of soil's productive potential at a sufficiently high level. This is of particular importance in case of degraded soils with disrupted homeostasis and reduced mineral content.

One of the approaches to overcome the issues arising from the intensive human agricultural activity is the exploitation of strains of beneficial microorganisms in the biofertilizers form. Biofertilizers represent an environmentally friendly alternative or supplement to traditional mineral fertilizers, and an increasing number of reports confirm the favourable impact of such formulations on the microbiological parameters of arable soils, which is inextricably linked to improved soil health. The employment of biofertilizers containing microorganisms also remains in line with the principles of sustainable agriculture, as well as with the policy requiring a reduction in the quantity of agrochemicals applied to the soil.

With an eye to the growing interest towards biofertilizers and the development of sustainable soil management practices, the research which was undertaken in this thesis concerned the effect of an innovative phosphorus mineral fertilizer enriched with strains of beneficial bacteria on microbiological parameters of degraded soils (including enzymatic activity and functional and genetic diversity of bacterial, archaeal and fungal communities). Two-year field experiments were conducted in 2018-2019 on two different soil types, under maize cultivation. The field experiments included the following fertilization treatments: an optimal dose of fertilizer without microbial enrichment (FC), an optimal dose containing microorganisms (FA100) and a 40% reduced dose of fertilizer containing microorganisms (FA60). Soil samples were taken three times in both years of the experiments: before (bio)fertilizers application (April), one week after (bio)fertilizers application (June) and after maize harvest (October).

Soil enzymatic activity was assessed based on the variations in the protease, urease, acid phosphatase and β-glucosidase activities. Investigation of functional diversity involved

the determination of the catabolic potential of soil microbial communities to utilize carbon substrates distributed on BIOLOG^{TM} ECO and FF plates. Genetic diversity was analyzed using terminal restriction fragment length polymorphism (t-RFLP) and Next Generation Sequencing (NGS). The performed research referred to both the immediate microbial response and seasonal changes in the status of the soil microbiome and mycobiome after phosphorus biofertilizer application.

The research showed a positive effect of the phosphorus biofertilizer on microbiological parameters of degraded soils. Application of the phosphorus biofertilizer increased soil enzymatic activity and maintained this effect over time. Changes in the utilization rates of carbon sources belonging to different groups and expansion of the catabolic capacity of soil microbial communities were also observed. The phosphorus biofertilizer stimulated the occurrence of microorganisms promoting plant growth and development and increased the genetic diversity and the number of operational taxonomic units associated with metabolic and cellular processes. There was also an increment in maize yield and in the content of phytoavailable phosphorus in the soil. The obtained results demonstrate the comprehensive impact of phosphorus biofertilizer on the soil environment and confirm its potential for effective implementation on sustainable and regenerative agriculture.

Keywords: biofertilizers, sustainable agriculture, soil microorganisms, degraded soil, soil enzymatic activity, soil microbial diversity, regenerative agriculture

Spis treści

Lista publikacji stanowiących podstawę rozprawy doktorskiej

Niniejsza rozprawa doktorska oparta jest na cyklu trzech publikacji na temat:

"Wpływ fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie na aktywność i bioróżnorodność mikroorganizmów glebowych":

P.1: **Mącik, M.**, Gryta, A., Frąc M., 2020. Biofertilizers in agriculture: An overview of concepts, strategies and effects on soil microorganisms. *Advances in Agronomy* 162, 31-87. Wskaźnik Impact Factor: 6,919.

Indywidualny wkład: przegląd literatury dotyczący bionawozów stosowanych w rolnictwie i mechanizmów ich działania, przygotowanie manuskryptu

P.2: **Mącik, M.**, Gryta, A., Sas-Paszt, L., Frąc, M., 2020. The Status of Soil Microbiome as Affected by the Application of Phosphorus Biofertilizer: Fertilizer Enriched with Beneficial Bacterial Strains. *International Journal of Molecular Sciences* 21, *8003*. Wskaźnik Impact Factor: 5,924; punktacja MNiSW: 140 punktów.

Indywidualny wkład: istotny udział w przeprowadzeniu badań bioróżnorodności zbiorowisk mikroorganizmów glebowych, analiza uzyskanych wyników, statystyczne i graficzne opracowanie wyników, przygotowanie manuskryptu

P.3: **Mącik, M.**, Gryta, A., Sas-Paszt, L., Frąc, M., 2022. Composition, activity and diversity of bacterial and fungal communities responses to inputs of phosphorus fertilizer enriched with beneficial microbes in degraded Brunic Arenosol. *Land Degradation & Development* 33(6), 844-865. Wskaźnik Impact Factor: 4,377; punktacja MNiSW: 200 punktów.

Indywidualny wkład: istotny udział w przeprowadzeniu badań bioróżnorodności zbiorowisk mikroorganizmów glebowych, analiza uzyskanych wyników, statystyczne i graficzne opracowanie wyników, przygotowanie manuskryptu

Uzupełnieniem wyników badań przedstawionych w wyżej wymienionych publikacjach jest analiza sezonowych zmian w statusie mikrobiomu i mykobiomu zasiedlającego glebę zdegradowaną chemicznie typu Abruptic Luvisol po aplikacji fosforowego bionawozu. Wyniki zostały przygotowane do publikacji i przedstawione jako badania uzupełniające niniejszej rozprawy doktorskiej, obejmujące manuskrypt artykułu naukowego zatytułowanego:

P.4: **Mateusz Mącik**, Agata Gryta, Lidia Sas-Paszt, Magdalena Frąc, New insight into the soil bacterial and fungal microbiome after phosphorus biofertilizer application as important driver of regenerative agriculture including biodiversity loss reversing and soil health restoration.

1. Wprowadzenie

1.1. Problematyka

Degradacja oraz ogólny spadek żyzności i jakości gleb uprawnych wywołane intensywną antropopresją należą do największych problemów, z którymi mierzy się współczesne rolnictwo (Gomiero, 2016). Silna intensyfikacja i chemizacja zabiegów agrotechnicznych pociąga za sobą negatywne zmiany w strukturze i we właściwościach gleby takie jak zakwaszenie, spadek zawartości materii organicznej, wypłukiwanie substancji mineralnych, zanieczyszczenie metalami ciężkimi, zasolenie oraz zagęszczenie (Kopittke i in., 2019; Shah i in., 2017). Nie ulega wątpliwości, że do pogorszenia właściwości fizycznych, chemicznych i biologicznych gleb przyczynia się nadmierna i nierozsądna aplikacja nawozów mineralnych oraz chemicznych środków ochrony roślin (Wu i in., 2020). Różnego rodzaju nawozy mineralne (azotowe, potasowe, fosforowe, wapniowe) są powszechnie stosowane w uprawach roślin, szczególnie w rejonach, w których gleby są ubogie w podstawowe składniki odżywcze, i przynoszą wymierne korzyści dla rolników, jednak w sytuacji nadużycia mogą spowodować efekty odwrotne do zamierzonych i przyspieszyć procesy związane z pogorszeniem stanu gleb (Asaye i in., 2022; Lin i in., 2019; Yousaf i in., 2017). Postępująca z coraz większą szybkością degradacja ziem uprawnych obniża długoterminową zdolność gleb do zapewniania społeczeństwu podstawowych funkcji obejmujących dostarczanie żywności, regulację klimatu oraz przepływ materii i energii w ekosystemie oraz zaburza stabilność niszy ekologicznej, stwarzając niekorzystne warunki dla wielu gatunków roślin i zwierząt (Dominati i in., 2014; Steinhoff-Knopp i in., 2021). Kwestią o zasadniczym znaczeniu jest zrozumienie, że dalsze obniżanie jakości gleb, przy jednoczesnym braku wdrożenia technik mających na celu poprawę życia biologicznego i parametrów fizyko-chemicznych, może prowadzić do utraty wymienionych usług ekosystemowych, a tym samym do zwiększenia kosztów gospodarczych związanych z produkcją rolną (Lal, 2014; Sharafatmandrad i Khosravi Mashizi, 2021). Nieuwzględnienie znaczenia gleby w coraz bardziej uprzemysłowionym rolnictwie może przynieść niewątpliwie poważne konsekwencje takie jak utrata terenów rolniczych czy zmniejszenie konkurencyjności danego regionu na rynkach krajowych i zagranicznych (Timmis i Ramos, 2021).

Rozważając negatywne implikacje działalności rolniczej człowieka nie należy zapominać, że intensywna i nieadekwatna do rzeczywistych wymagań i możliwości

eksploatacja gleb uprawnych wiążę się również ze spadkiem bioróżnorodności wśród mikroorganizmów zasiedlających agroekosystemy (Sünnemann i in., 2021; Tibbett i in., 2020). Jak powszechnie wiadomo, złożone i dynamiczne społeczności bakterii, archeonów oraz grzybów kształtują środowisko glebowe, kierując procesami rozkładu materii organicznej oraz obiegu pierwiastków, dbając tym samym o zachowanie równowagi ekologicznej w glebie (Zheng i in., 2019). Uważa się, że wysoka bioróżnorodność wśród mikrobiomu i mykobiomu jest gwarantem zachowania wysokiej jakości gleb o zwiększonej odporności na perturbacje środowiskowe (Jiao i in., 2019). Natomiast gleby charakteryzujące się niską bioróżnorodnością cierpią z powodu zakłócenia podstawowych procesów niezbędnych do zachowania ciągłości funkcjonowania ekosystemów, co w dłuższej perspektywie może prowadzić do ich destabilizacji (Wagg i in., 2021). Dlatego też niezwykle ważne jest utrzymanie bioróżnorodności wśród mikroorganizmów glebowych, zarówno tych powszechnie występujących, jak i bardziej wyspecjalizowanych, na odpowiednio wysokim poziome, dającym gwarancję na sprawny i efektywny przebieg procesów mikrobiologicznych w środowisku glebowym. Niektóre procesy glebowe (np. rozkład materii organicznej) są realizowane przez większą liczbę grup mikroorganizmów, podczas gdy inne (np. degradacja ksenobiotyków czy magazynowanie materii organicznej) przebiegają przy udziale mniej licznych, wyspecjalizowanych grup, które są w większym stopniu narażone na stresowe sytuacje środowiskowe (Bertola i in., 2021; Xun i in., 2019).

Biorąc pod uwagę fakt, że gleba stanowi niezwykle heterogeniczne i często nieprzyjazne środowisko, mikroorganizmy wykształciły szereg mechanizmów decydujących o ich sukcesie ewolucyjnym (Granato i in., 2019; Li i in., 2021). Część z tych adaptacji można rozpatrywać jako uzdolnienia pozytywnie wpływające na wzrost i rozwój roślin uprawnych oraz ogólny stan gleb, np.: synteza antybiotyków i substancji skierowanych przeciwko patogenom, formowanie biofilmu, wiązanie azotu atmosferycznego, uruchamianie składników mineralnych z trudno rozpuszczalnych źródeł, synteza hormonów roślinnych oraz bioremediacja zanieczyszczeń pochodzenia organicznego i nieorganicznego (Chandran i in., 2021). Mikroorganizmy są również bardzo wrażliwe na zmiany w środowisku i charakteryzują się szybką odpowiedzią w obliczu czynników zewnętrznych, dlatego też status mikrobiomu i mykobiomu glebowego uważany jest za wiarygodny bioindykator ogólnego zdrowia gleby (Hermans i in., 2020; Qiu i in., 2021). Wysoka czułość mikroorganizmów sprawiła, że popularne stało się monitorowanie zmian w parametrach mikrobiologicznych

gleb uprawnych pod wpływem różnych praktyk agrotechnicznych (Wang i in., 2019). Badania takie pozwalają na lepsze zrozumienie procesów zachodzących w glebie oraz mogą przynieść odpowiedź na pytanie o zasadność danego zabiegu jako potencjalnej metody poprawiającej parametry mikrobiologiczne gleb uprawnych i zdegradowanych. Istotne jest kontrolowanie nie tylko natychmiastowej odpowiedzi zbiorowisk mikroorganizmów glebowych, ale również śledzenie sezonowych zmian w zbiorowiskach mikroorganizmów glebowych, ponieważ umożliwia to oszacowanie stabilności agroekosystemów w obliczu czynników zewnętrznych takich jak nawożenie (Lacerda-Júnior i in., 2019).

Szerokie spektrum uzdolnień mikroorganizmów w kierunku poprawy jakości plonów i zdrowia gleb sprawiło, że wykorzystanie ich w formie bionawozów stało się niezwykle ważne w sektorze rolniczym opierającym się w coraz większym stopniu na powiązaniach agroekosystemów z różnorodnością mikroorganizmów glebowych (Daniel i in., 2022; Suman i in., 2022). Bionawozy zawierające szczepy pożytecznych bakterii i grzybów stanowią bezpieczną dla środowiska alternatywę lub uzupełnienie dla tradycyjnych nawozów mineralnych i pozwalają na ograniczenie problemów związanych z nadmierną chemizacją rolnictwa i degradacją agroekosystemów (Seenivasagan i Babalola, 2021). Koncepcja bionawozów jako strategii pozwalającej na zmniejszenie zależności od sztucznych nawozów zyskuje coraz większą popularność, szczególnie w obszarach zrównoważonego i ekologicznego rolnictwa (Mitter i in., 2021). Co więcej, zastosowanie tego typu preparatów wpisuje się także w ideę rolnictwa regeneracyjnego, które ma na celu utrzymanie wysokiego poziomu produkcji rolnej, zwiększenie różnorodności biologicznej, ze szczególnym naciskiem na bioróżnorodność gleb oraz wzmocnienie usług ekosystemowych takich jak sekwestracja dwutlenku węgla (EASAC, 2022). Uważa się, że wzrost bioróżnorodności wywołany przejściem z konwencjonalnych na zrównoważone metody zarządzania glebą pozwala na kreowanie stabilniejszego mikrobiomu i mykobiomu, co warunkuje utrzymanie gleb o wysokim poziomie żyzności, pozwalającym na zaspokojenie wymagań pokarmowych roślin uprawnych (Reyes-Sánchez i in., IUSS, 2022). Wdrożenie nawozów mikrobiologicznych do praktyk rolniczych pozostaje również w zgodzie ze współczesną polityką nakazującą ograniczenie ilości agrochemikaliów aplikowanych do gleby. Europejski Zielony Ład oraz Unijna Strategia na Rzecz Bioróżnorodności do 2030 roku zakładają wykorzystanie 25% powierzchni gruntów rolnych UE pod rolnictwo ekologiczne oraz zmniejszenie wykorzystania nawozów mineralnych o 20% (European Commission, 2020).

1.2. Znaczenie fosforu

Fosfor, obok azotu i potasu, należy do najważniejszych makroelementów determinujących prawidłowy wzrost i rozwój roślin, a co za tym idzie jakość i ilość plonów (Jiaying i in., 2022). Fosfor uczestniczy w wielu podstawowych procesach w komórkach roślinnych, takich jak podział komórki, fotosynteza, oddychanie, magazynowanie energii, formowanie błon komórkowych czy transport związków mineralnych. Jest także integralnym elementem ATP, NADPH, kwasów nukleinowych, białek, fosfolipidów i koenzymów (Carstensen i in., 2018; Mehta i in., 2021; Siedliska i in., 2021). Niedobory fosforu w komórkach roślinnych mogą prowadzić do spadku wydajności procesu fotosyntezy, obniżenia suchej masy korzeni i części nadziemnych oraz zmniejszenia zawartości chlorofilu, co wpływa negatywnie na plonowanie (Meng i in., 2021; Ulloa i in., 2021).

Fosfor w glebach występuje w formach organicznych oraz mineralnych. Niestety, tylko około 5% całkowitej ilości fosforu glebowego dostępna jest dla roślin i może być przez nie przyswojona w postaci ortofosforanów (Hallama i in., 2021). Dostępność fosforu warunkowana jest w dużej mierze odczynem gleby (Penn i Camberato, 2019). Uważa się, że dostępność fosforu w glebie jest maksymalna w zakresie pH od 4,5 do 6,5, ponieważ takie warunki minimalizują stopień wiązania tego pierwiastka przez wapń, aluminium i żelazo. W glebach o pH > 6,5 nieorganiczny fosfor tworzy wiązania z jonami wapnia, natomiast w glebie o niskich wartościach pH fosfor jest unieruchomiony poprzez wiązanie z jonami żelaza, manganu i glinu lub z ich wodorotlenkami i w tych formach pozostaje niedostępny dla roślin (Bouray i in., 2021; Schaller i in., 2019). Z drugiej strony Barrow i in. (2020) utrzymują, że optymalna wartość pH dla przyswajania fosforu jest niższa i plasuje się w okolicach 5,5. Nie zmienia to jednak faktu, że dostępność fosforu w glebach jest niska, szczególnie w glebach zdegradowanych, w których doszło do zaburzenia równowagi biologicznej i spadku bioróżnorodności. Gleby zdegradowane, poza niską zawartością substancji mineralnych, charakteryzują się zazwyczaj kwaśnym odczynem, co dodatkowo utrudnia przyswajanie mikro- i makroelementów przez rośliny ze względu na niską pojemność wymiany kationów oraz wysoką zdolność adsorpcji anionów (da Fonseca i in., 2021).

Najpopularniejszym sposobem przeciwdziałania niedoborom P w agroekosystemach jest aplikacja fosforowych nawozów mineralnych, które pozwalają utrzymać produkcję roślinną na poziomie spełniającym stale rosnące zapotrzebowania żywieniowe świata (Ros i

in., 2020). Szacuje się, że do roku 2050 globalne zapotrzebowanie na nawozy fosforowe wyniesie 22-27 milionów ton/rok dla gruntów uprawnych oraz 4-12 milionów ton/rok w przypadku użytków zielonych (Bindraban i in., 2020). Po aplikacji nawozów mineralnych, tylko niewielka część (ok. 30-40%) zawartych w nich substancji jest dostępna dla roślin i może być przez nie zaabsorbowana. Reszta pozostaje unieruchomiona w glebie lub jest tracona w efekcie wypłukiwania lub erozji i może przedostawać się do zbiorników wodnych powodując eutrofizację (Conijn i in., 2018; Kumar i in., 2022). Wzmożone nawożenie, niedostosowane do zawartości składników mineralnych w glebach i wymagań żywieniowych roślin uprawnych przyczynia się także do zagęszczenia gleby, co pociąga za sobą obniżenie ilości plonów (Liu i in., 2021b).

W dobie rosnącego zainteresowania zrównoważonymi praktykami zarządzania glebą, zwiększanie dostępności P w glebie, bez negatywnego wpływu na środowisko, może stać się możliwe dzięki implementacji bionawozów bazujących na mikroorganizmach zdolnych do uruchamiania fosforu z trudno rozpuszczalnych źródeł. Szczepy bakterii i grzybów mogą pozytywnie wpływać na zawartość dostępnego P w glebach uprawnych dzięki zwiększaniu rozpuszczalności mineralnych związków P oraz mineralizacji organicznych źródeł tego pierwiastka. Wśród bakterii zdolnych do uruchamiania P w glebie można wyróżnić przedstawicieli *Pseudomonas* spp., *Bacillus* spp., *Paenibacillus* spp., *Burkholderia* spp., *Azotobacter* spp., *Serratia* spp., *Rhizobium* spp., *Azospirillum* spp., *Pantoea* spp., *Arthrobacter* spp., *Rhodococcus* spp., *Klebsiella* spp. i *Enterobacter* spp. (Alori i in., 2017; Bargaz i in., 2021; Etesami i in., 2021). Grzyby wykazujące podobne uzdolnienia obejmują reprezentantów *Aspergillus* spp., *Mortierella* spp., *Oidiodendron* spp., *Paecilomyces* spp., *Penicillium* spp, *Trichoderma* spp., *Saccharomyces* spp., *Rhizopus* spp., *Glomus* spp., *Alternaria* spp. i *Yarrowia* spp. (Alori i in., 2017).

1.3. Wykorzystanie bionawozów w odniesieniu do zmian klimatu

W ostatnich latach coraz częściej mówi się o przybierających na sile zmianach klimatu i związanych z tym zjawiskiem zagrożeń dla ekosystemów takich jak susza, zasolenie, wzrost temperatury, ekstremalne zjawiska pogodowe, niedobór składników mineralnych oraz rozprzestrzenianie się patogenów roślinnych (Shah i in., 2021; Song i in., 2022; Velásquez i in., 2018). Ocieplenie klimatu przyczynia się do wydłużenia okresu wegetacyjnego oraz zmian w cyklach rozwojowych roślin wywołanych zacieraniem się granic pomiędzy naturalnie następującymi po sobie porami roku (Liu i in., 2021a; Wang i in., 2021a).

Intensywne opady deszczu mogą wymywać z pól uprawnych nawozy i pozostałości sztucznych pestycydów indukując tym samym eutrofizację zbiorników wodnych (Lu i in., 2019), a rozwój i rozprzestrzenianie się inwazyjnych gatunków bakterii, grzybów i owadów generuje straty w plonach i może negatywnie wpływać na zdrowie ludzi (Velásquez i in., 2018). Na skutek podnoszenia się poziomu mórz istnieje także ryzyko zalania nisko położonych ziem uprawnych (Roy i in., 2022). Aktualne prognozy przewidują, że stresy abiotyczne mogą spowodować straty w światowej produkcji rolnej sięgające 50%, w zależności od regionu (Fadiji i in., 2022).

Z jednej strony, rolnictwo stanowi sektor niezwykle podatny na stresowe sytuacje wywołane zmianami klimatu, z drugiej jednak nieracjonalna gospodarka nawozami mineralnymi przyczynia się do wzrostu efektu cieplarnianego poprzez emisję gazów szklarniowych (CH_4, N_2O, CO_2). Szacuje się, że globalne rolnictwo jest odpowiedzialne za emisję ~21-37% całkowitej ilości gazów cieplarnianych uwalnianych do atmosfery (Lynch i in., 2021; Mosongo i in., 2022; Nelson i in., 2014). Długoterminową strategią poprawy jakości plonów w obliczu presji związanej ze zmianami klimatu jest wdrożenie do praktyk rolniczych bionawozów bazujących na szczepach pożytecznych bakterii i grzybów. Mikroorganizmy mogą łagodzić negatywne skutki zmian klimatu dzięki mechanizmom takim jak synteza enzymów degradujących reaktywne formy tlenu (katalaza, dysmutaza ponadtlenkowa, peroksydaza asparaginianowa, peroksydaza gwajakolowa), synteza deaminazy kwasu 1-aminocyklopropano-1-karboksylowego (ACC) i kwasu indolilooctowego (IAA), aktywacja białek szoku termicznego, antagonizm przeciwko bakteriom promującym nukleację lodu, synteza lotnych związków organicznych, produkcja egzopolisacharydów i biofilmów, zwiększanie zdolności korzeni do absorpcji związków mineralnych przez grzyby mykoryzowe, akumulacja aminowaksów, synteza glomaliny, synteza kwasu abscysynowego (ABA) oraz akumulacja osmoprotektantów (prolina, betaina) (Fadiji i in., 2022; Fiodor i in., 2021).

Ograniczanie emisji gazów cieplarnianych i kontrolowanie ich stężenia w atmosferze również może być możliwe dzięki wykorzystaniu pożytecznych mikroorganizmów. Zostało opisane, że zastosowanie preparatów mikrobiologicznych obniżyło emisję metanu i tlenku azotu w doświadczeniach polowych (Akiyama i in., 2016; Kantachote i in., 2016). Aplikacja nawozów azotowych powoduje zwiększenie emisji N_2O do atmosfery; efekt ten może być zredukowany dzięki wykorzystaniu mikroorganizmów zdolnych do biologicznego wiązania

azotu takich jak m.in. *Rhizobium* spp., *Azotobacter* spp. czy *Azospirillum* spp. (Aasfar i in., 2021; Wen i in., 2021). Gleba stanowi również potencjalny rezerwuar CO_2. Stosowanie bionawozów może zwiększyć sekwestrację dwutlenku węgla w glebie, co przynosi jednocześnie dwie korzyści: spadek stężenia CO_2 w atmosferze oraz zwiększenie zawartości węgla organicznego w glebie (Gayathri i in., 2021). Wsród mikroorganizmów zdolnych do łagodzenia stresów indukowanych przez zmiany klimatu można wyróżnić przedstawicieli *Paraburkholderia* spp., *Pseudomonas* spp., *Pantoea spp*., *Bacillus* spp., *Azospirillum* spp., *Paenibacillus* spp., *Burkholderia* spp., *Streptomyces* spp., *Achromobacter* spp., *Citrobacter* spp., *Glomus* spp., *Acinetobacter* spp. i *Klebsiella* spp. (Fadiji i in., 2022).

2. Hipoteza badawcza i cele rozprawy doktorskiej

Hipoteza badawcza

Ze względu na ciągle aktualny i wymagający badań temat bionawozów i ich wykorzystania w zrównoważonym rolnictwie postawiono następującą hipotezę badawczą: fosforowy nawóz mineralny wzbogacony mikrobiologicznie ma pozytywny wpływ na bioróżnorodność mikroorganizmów i aktywność enzymatyczną gleb zdegradowanych i może stanowić uzupełnienie lub alternatywę dla tradycyjnych nawozów mineralnych.

Cel rozprawy doktorskiej

Celem rozprawy doktorskiej było określenie wpływu innowacyjnego fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie na aktywność i różnorodność funkcjonalną i genetyczną zbiorowisk mikroorganizmów zasiedlających gleby zdegradowane. W związku z tym postawiono następujące cele szczegółowe:

- określenie aktywności wybranych enzymów glebowych: proteazy, ureazy, fosfatazy kwaśnej oraz β-glukozydazy,
- określenie różnorodności funkcjonalnej mikroorganizmów glebowych na podstawie analizy profilu metabolicznego zbiorowisk mikroorganizmów przy zastosowaniu płytek metabolicznych BIOLOG^{TM} (ECO oraz FF),
- określenie różnorodności genetycznej zbiorowisk bakterii, archeonów oraz grzybów na podstawie analizy polimorfizmu długości terminalnych fragmentów restrykcyjnych (t-RFLP),
- analiza metataksonomiczna, z zastosowaniem sekwencjonowania następnej generacji (Next Generation Sequencing – NGS), zbiorowisk bakterii i grzybów występujących w glebie poddanej działaniu fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie.

Badania wpływu fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie na zbiorowiska mikroorganizmów glebowych obejmowały zarówno natychmiastową odpowiedź bakterii, archeonów i grzybów bezpośrednio po aplikacji bionawozu, jak również sezonowe zmiany w statusie mikrobiomu i mykobiomu zasiedlającego gleby zdegradowane chemicznie.

3. Materiały i metody

3.1. Doświadczenia polowe

Doświadczenia polowe prowadzono w latach 2018-2019 na dwóch różnych typach gleb, pod uprawą kukurydzy (odmiana P9241, FAO: K280, Z270, PIONEER). Pierwsze stanowisko doświadczalne zostało zlokalizowane w miejscowości Biszcza, w województwie lubelskim (50°43′N, 22°60′E), na wysokości 211 m n.p.m. Gleba, sklasyfikowana jako bielicowa wytworzona z piasku słabogliniastego (Brunic Arenosol - BA), została zdegradowana chemicznie w wyniku nieprawidłowej uprawy i nawożenia i charakteryzowała się bardzo niską zawartością potasu (2,9 mg 100 g^{-1}) i magnezu (1,2 mg 100 g^{-1}), wysoką zawartością fosforu (17,4 mg 100 g^{-1}) oraz zakwaszeniem (pH_{KCl} 4,8). Drugie stanowisko doświadczalne zostało zlokalizowane w miejscowości Basznia, w województwie podkarpackim (50°15′ N, 23°26′ E), na wysokości 230 m n.p.m. Typ gleby określono jako bielicowa wytworzona z pyłu (Abruptic Luvisol - AL), a do jej chemicznej degradacji doszło w wyniku działalności byłej kopalni siarki położonej w pobliżu stanowiska doświadczalnego. Gleba typu AL charakteryzowała się bardzo niską zawartością fosforu (4,8 mg 100 g^{-1}) oraz potasu (5,3 mg 100 g^{-1}), niską zawartością magnezu (3,6 mg 100 g^{-1}) oraz zakwaszeniem (pH_{KCl} 4,9).

W doświadczeniach polowych zastosowano fosforowy nawóz mineralny SUPER FOS DAR 40 (Grupa Azoty, Puławy), azotowy nawóz mineralny PULREA PUŁAWSKI MOCZNIK 46N (Grupa Azoty, Puławy) oraz sól potasową (Bialchem). Dawki nawozów obliczono w oparciu o zasobność gleby w składniki mineralne oraz wymagania żywieniowe kukurydzy. Dokładne dawki zaaplikowanych nawozów przedstawiono w Tabeli 1. Fosforowy nawóz mineralny został wzbogacony trzema szczepami pożytecznych bakterii: *Paenibacillus polymyxa* (CHT114AB), *Bacillus amyloliquefaciens* (AF75BB) oraz *Bacillus* sp. (CZP4/4), które zostały wyselekcjonowane z kolekcji SYMBIOBANK Instytutu Ogrodnictwa w Skierniewicach. Granule nawozu zostały otoczone mieszanką wyżej wymienionych szczepów w proporcji 1:1:1 (Borowik i in., 2019). Gotowe do użycia bionawozy zostały wytworzone i dostarczone do badań przez Instytut Nowych Syntez Chemicznych w Puławach. W doświadczeniu polowym uwzględniono następujące warianty nawożenia: FC - dawka optymalna fosforowego nawozu bez wzbogacenia mikrobiologicznego (wariant kontrolny), FA100 - dawka optymalna nawozu zawierająca mikroorganizmy oraz FA60 - dawka nawozu

zredukowana o 40% zawierająca mikroorganizmy. Każdy wariant nawożenia składał się z trzech poletek doświadczalnych o wymiarach 10 x 15 m. Próbki gleby do badań pobierano trzykrotnie w obu latach doświadczenia w następujących terminach: przed aplikacją (bio)nawozów (kwiecień 2018/2019), bezpośrednio po aplikacji (bio)nawozów (czerwiec 2018/2019) oraz po zbiorach kukurydzy (październik 2018/2019). Próbki gleby pobrano z pięciu losowo wybranych miejsc w obrębie każdego poletka z głębokości 0-25 cm i uśredniono przez intensywne mieszanie, a następnie przetransportowano do laboratorium i przesiano przez 2 mm sito w celu usunięcia zanieczyszczeń takich jak kamienie lub korzenie roślin. Oczyszczone próbki gleby zostały natychmiast wykorzystane do badań (aktywność enzymatyczna gleby) lub przechowywane w odpowiednich warunkach (-4°C do analiz z wykorzystaniem systemu BIOLOG^{TM} lub -80°C do izolacji DNA). Etapy prowadzonych badań przedstawiono na Rysunku 1.

Tabela 1. Dawki nawozów zastosowane w doświadczeniu polowym w latach 2018-2019. Dawki zaaplikowanych (bio)nawozów zostały obliczone oddzielnie da każdego typu gleby i podane w przeliczeniu na hektar.

Wariant nawożenia	Brunic Arenosol (BA)	Abruptic Luvisol (AL)
Dawka optymalna (FC)	125 kg fosforowego nawozu mineralnego; 365 kg mocznika; 290 kg soli potasowej	150 kg fosforowego nawozu mineralnego; 360 kg mocznika; 284 kg soli potasowej
Dawka optymalna wzbogacona mikrobiologicznie (FA100)	156,25 kg fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie; 365 kg mocznika; 290 kg soli potasowej	187,5 kg fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie; 360 kg mocznika; 284 kg soli potasowej
Dawka zredukowana 40% wzbogacona mikrobiologicznie (FA60)	93,75 kg fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie; 365 kg mocznika; 290 kg soli potasowej	112,5 kg fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie; 360 kg mocznika; 284 kg soli potasowej

3.2. Aktywność enzymatyczna gleby

Aktywność ureazy oznaczono metodą Zantua i Bremnera (1977) z użyciem mocznika jako substratu. Próbki gleby inkubowano w temperaturze 37°C przez 18 godzin, a stężenie amoniaku określono metodą Nesslera przez pomiar absorbancji przy długości fali 410 nm. Aktywność proteazy oznaczono metodą Ladda i Butlera (1972) zmodyfikowaną przez Alefa i Nannipieriego (1995). Próbki gleby inkubowano w temperaturze 50°C przez godzinę z roztworem kazeinianu sodu w buforze Tris-HCl o pH równym 8,1. Stężenie tyrozyny określono spektrofotometrycznie przy długości fali 578 nm. Aktywność β-glukozydazy oznaczono przy użyciu metody zaproponowanej przez Eivazi and Tabatabai (1988) zmodyfikowanej przez Alefa i Nannipieriego (1995). Metoda ta polega na kolorymetrycznym

oznaczeniu stężenia p-nitrofenolu (PNP) przy długości fali 400 nm, po inkubacji próbek gleby z roztworem p-nitrofenolu-β-D-glukopiranozydu (PNG) w temperaturze 37°C przez godzinę. Aktywność fosfatazy kwaśnej oznaczono na podstawie metody Tabatabai i Bremnera (1969). Próbki gleby inkubowano z roztworem p-nitrofenylofosforanu w buforze Tris-HCl (pH=6,5) w temperaturze 37°C przez godzinę, a następnie stężenie uwolnionego PNP zmierzono spektrofotometrycznie przy długości fali 400 nm. Aktywności poszczególnych enzymów zostały przeliczone w odniesieniu do suchej masy gleby określonej metodą wagową za pomocą wagosuszarki przez wysuszenie gleby w temperaturze105°C.

3.3. Zawartość przyswajalnego fosforu i plon kukurydzy

Zawartość przyswajalnego fosforu (P_2O_5) została określona według metody Egnera-Riehma zgodnie ze standardem PN-R-04023 (1996). Plon kukurydzy, po zbiorach, określono jako wagę wszystkich roślin z każdego poletka doświadczalnego w danym wariancie nawożenia w przeliczeniu na 1 ha.

3.4. Analizy z wykorzystaniem systemu BIOLOG™

Różnorodność funkcjonalną zbiorowisk mikroorganizmów glebowych oznaczono za pomocą systemu BIOLOG™ oraz płytek metabolicznych ECO i FF opłaszczonych substratami węglowymi należącymi do różnych grup związków chemicznych. Ogólna aktywność metaboliczna zbiorowisk mikroorganizmów została zbadana przy użyciu płytek ECO, natomiast aktywność zbiorowisk grzybów określono przy użyciu płytek FF. Płytki ECO zawierają zestaw 31 substratów węglowych w trzech powtórzeniach, płytki FF obejmują natomiast 95 różnych związków węgla. Na obu rodzajach płytek znajdują się również dołki kontrolne zawierające wodę zamiast związku węgla.

W celu przygotowania zawiesiny gleby, do 99 cm^3 sterylnej wody peptonowej wprowadzono 1 g świeżej próbki gleby. Zawiesinę wytrząsano w temperaturze pokojowej przez 20 minut a następnie inkubowano w temperaturze 4°C przez 30 minut (Gryta i in., 2014). Tak przygotowaną zawiesiną inokulowano płytki BIOLOG, przy czym ilość inokulum wynosiła, odpowiednio, 120 µl i 100 µl, dla płytek ECO i FF. Płytki inkubowano przez 10 dni w temperaturze 24°C, a pomiarów absorbancji dokonywano co 24 godziny przy długościach fali 590/750 nm (płytki ECO) i 490/750 nm (płytki FF).

3.5. Izolacja DNA

Izolację materiału genetycznego mikroorganizmów przeprowadzono bezpośrednio z 0,5 g próbek gleby przy użyciu zestawu do ekstrakcji DNA z gleby i osadów FastDNA SPIN Kit for Feces (MP Biomedicals, Solon, OH) zgodnie z protokołem producenta. Stężenie wyizolowanego DNA określono spektrofotometrycznie przy długości fali 260 nm, natomiast czystość materiału genetycznego przy użyciu wartości współczynników 260/230 i 260/280 obliczonych na podstawie pomiaru absorbancji przy długościach fali 230, 260 i 280 nm (NanoDrop 2000/2000c Thermo Scientific, West Palm Beach, FL). Następnie wyizolowane DNA przechowywano w temperaturze -20°C do dalszych analiz.

3.6. Analiza polimorfizmu długości terminalnych fragmentów restrykcyjnych typu multiplex (M-tRFLP)

Analiza M-tRFLP składała się z następujących etapów: reakcja PCR (łańcuchowa reakcja polimerazy) typu multiplex z fluorescencyjnie wyznakowanymi starterami specyficznymi dla danej grupy mikroorganizmów, trawienie amplikonów enzymem restrykcyjnym a następnie rozdział i detekcja uzyskanych fragmentów w analizatorze genetycznym.

W reakcji PCR bakteryjny region 16S rDNA został zamplifikowany z użyciem pary starterów 63f (5′-AGGCCTAACACATGCAAGTC-3′) (Marchesi i in., 1998) oraz 1087r (5′-HEX-CTCGTTGCGGGACTTACCCC-3′) (Hauben i in., 1997), do amplifikacji regionu ITS1 genomu grzybów wykorzystano startery ITS1F (5′-6-FAM-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes i Bruns, 1993) i ITS4R (5′-TCCTCCGCTTATTGATATGC-3′) (White i in., 1990), natomiast w przypadku archeonów zastosowano startery Ar3F (5′-TTCCGGTTGATCCTGCCGGA-3′) (Jurgens i Saano, 1999) i Ar9R (5′-ROX-CCCGCCAATTCCTTTAAGTTTC-3′) (Jurgens i in., 1997). Skład mieszaniny reakcyjnej o objętości 30 µl był następujący: 15 µl RedTaq®ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO, USA), 4 ng matrycowego DNA oraz startery rozcieńczone do stężenia 10 µM: 0,5 µl dla bakterii oraz 1 µl w przypadku archeonów i grzybów. Reakcję PCR przeprowadzano w termocyklerze gradientowym (Veriti 96 well Fast Thermal Cycler, Applied Biosystems) w następujących warunkach: denaturacja wstępna (95°C, 5 minut), 30 cykli obejmujących denaturację (95°C, 30 sekund), przyłączanie starterów (55°C, 30 sekund) i elongację (72°C, 60 sekund), a następnie elongacja końcowa

(72°C, 10 minut). Oceny jakościowej produktów amplifikacji dokonano w 2% żelu agarozowym wybarwionym barwnikiem SimplySafe (EURx, Gdańsk, Poland). Elektroforezę prowadzono w buforze TAE przez 40 minut przy napięciu 110V. Wielkość amplikonów (bakterie - 1000 pz, archeony - 900 pz, grzyby - 600 pz) określono przy użyciu markera masowego. W kolejnym kroku produkty reakcji PCR oczyszczono przy użyciu zestawu EXOSAP (zawierającego egzonukleazę I i alkaliczną fosfatazę), a następnie na kolumnach Performa® DTR (Dye Terminator Removal) Gel Filtration Cartridges (EdgeBio, San Jose, CA, USA) zgodnie z protokołami producentów. Oczyszczone amplikony poddano trawieniu enzymem restrykcyjnym HaeIII. Mieszanina restrykcyjna o objętości 13 µl zawierała 50 ng DNA, wodę, 0,6 µl buforu Tango, 0,6 µl enzymu HaeIII (10 U/µl) oraz 0,6 µl buforu ONE zawierającego surowiczą albuminę wołową. Restrykcję prowadzono w temperaturze 37°C przez 2 godziny, a inaktywację enzymu w temperaturze 80°C przez 20 minut. Następnie przygotowano mieszaninę reakcyjną, zawierającą 1 µl produktu restrykcji, 9 µl dejonizowanego formamidu i 0,5 µl markera wielkości GS-600LIZ (Applied Biosystems, Foster City, CA, USA), którą w kolejnym etapie naniesiono na 96-dołkową płytkę. Płytkę zdenaturowano (94°C, 3 minuty) i schłodzono na lodzie. Rozdział i detekcję fluorescencyjnie wyznakowanych terminalnych fragmentów restrykcyjnych (T-RFs) wykonano przez elektroforezę kapilarną w analizatorze genetycznym ABI 3130 (Applied Biosystems, Foster City, CA, USA) (Gryta i Frąc, 2020). Uzyskane wyniki poddano analizie za pomocą programu GeneMapper v. 4.0. Fragmenty restrykcyjne mniejsze niż 50 par zasad i stanowiące mniej niż 1% całkowitej powierzchni w obrębie danej próbki usunięto z analizy.

3.7. Sekwencjonowanie następnej generacji (Next Generation Sequencing - NGS)

Sekwencjonowanie następnej generacji zostało przeprowadzone w Genomedzie S.A. w Warszawie na platformie MiSeq Illumina. Metataksonomiczną analizę zbiorowisk mikroorganizmów glebowych wykonano na podstawie hiperzmiennego regionu V3-V4 16S rDNA (bakterie i archeony) oraz regionu ITS1 (grzyby). Do amplifikacji regionu V3-V4 16S rDNA użyto starterów 341F (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′) oraz 785R (5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′) (Klindworth i in., 2013). Natomiast starterów ITS1FI2 (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GAA CCW GCG GAR GGA TCA-3′) i 5.8S (5′-GTC TCG TGG GCT CGG

AGA TGT GTA TAA GAG ACA GCG CTG CGT TCT TCA TCG-3′) użyto do amplifikacji regionu ITS1 (Schmidt i in., 2013; Vilgalys Mycology Lab, 1992). Mieszanina reakcyjna o objętości 25 µl zawierała 10 ng matrycowego DNA, startery rozcieńczone do stężenia 10 µM oraz polimerazę DNA Q5® Hot Start High-Fidelity 2X Master Mix (NEB Inc., Ipswich, MA, USA). Amplifikację przeprowadzono w następujących warunkach: denaturacja wstępna (98°C, 30 sekund), 25-35 cykli obejmujących denaturację (98°C, 5-10 sekund), przyłączanie starterów (50-72°C, 10-30 sekund) i elongacja (72°C, 20-30 sekund), a następnie elongacja końcowa (72°C, 2 minuty). Sekwencjonowanie przeprowadzono w technologii odczytów sparowanych (ang. paired-end) 2 x 250 par zasad.

3.8. Analizy statystyczne i bioinformatyczne

Stopień utylizacji poszczególnych substratów węglowych na płytkach ECO i FF przedstawiono w postaci map cieplnych. Sezonowe zmiany w profilach katabolicznych zbiorowisk mikroorganizmów glebowych wyrażono za pomocą analizy głównych składowych (ang. principle component analysis - PCA). Potencjał metaboliczny mikroorganizmów wyrażono także jako stosunek OD 590 nm/OD 750 nm (ECO) oraz OD 490 nm/OD 750 nm (Pinzari i in., 2016).

Współczynnik Jaccarda (J) policzono z następującego wzoru $J = N_{AB}/(N_A + N_B - N_{AB})$, w którym poszczególne zmienne oznaczały: N_A - elementy występujące w zbiorze A, N_B - elementy występujące w zbiorze B, N_{AB} - elementy wspólne dla zbiorów A i B (López i in., 2019). Do wyliczenia współczynnika Sorensena (QS) zastosowano wzór $QS = 2C/(A + B)$, gdzie zmienne obejmowały: A - elementy występujące w zbiorze A, B - elementy występujące w zbiorze B, C-elementy wspólne dla zbiorów A i B (Walitang i in., 2019).

Mapy cieplne ilustrujące zmiany w liczbie i względnej obfitości terminalnych fragmentów restrykcyjnych (T-RFs) zostały wygenerowane przy użyciu narzędzia matrix2png (Pavlidis i Noble, 2003). Identyfikacji mikroorganizmów na podstawie wielkości wybranych T-RFs i bibliotek sekwencji DNA dokonano za pomocą aplikacji TRiFLe (Junier i in., 2008). Diagramy Venna skonstruowano przy użyciu narzędzi online http://bioinformatics.psb.ugent.be/webtools/Venn/ oraz https://bioinfogp.cnb.csic.es/tools/venny/ (Venny 2.1.0). Dendrogramy analizy skupień sporządzono w oparciu o względną obfitość T-RFs przy użyciu algorytmu Warda i odległości euklidesowej z zaznaczonymi kryteriami Sneatha (33% oraz 66%).

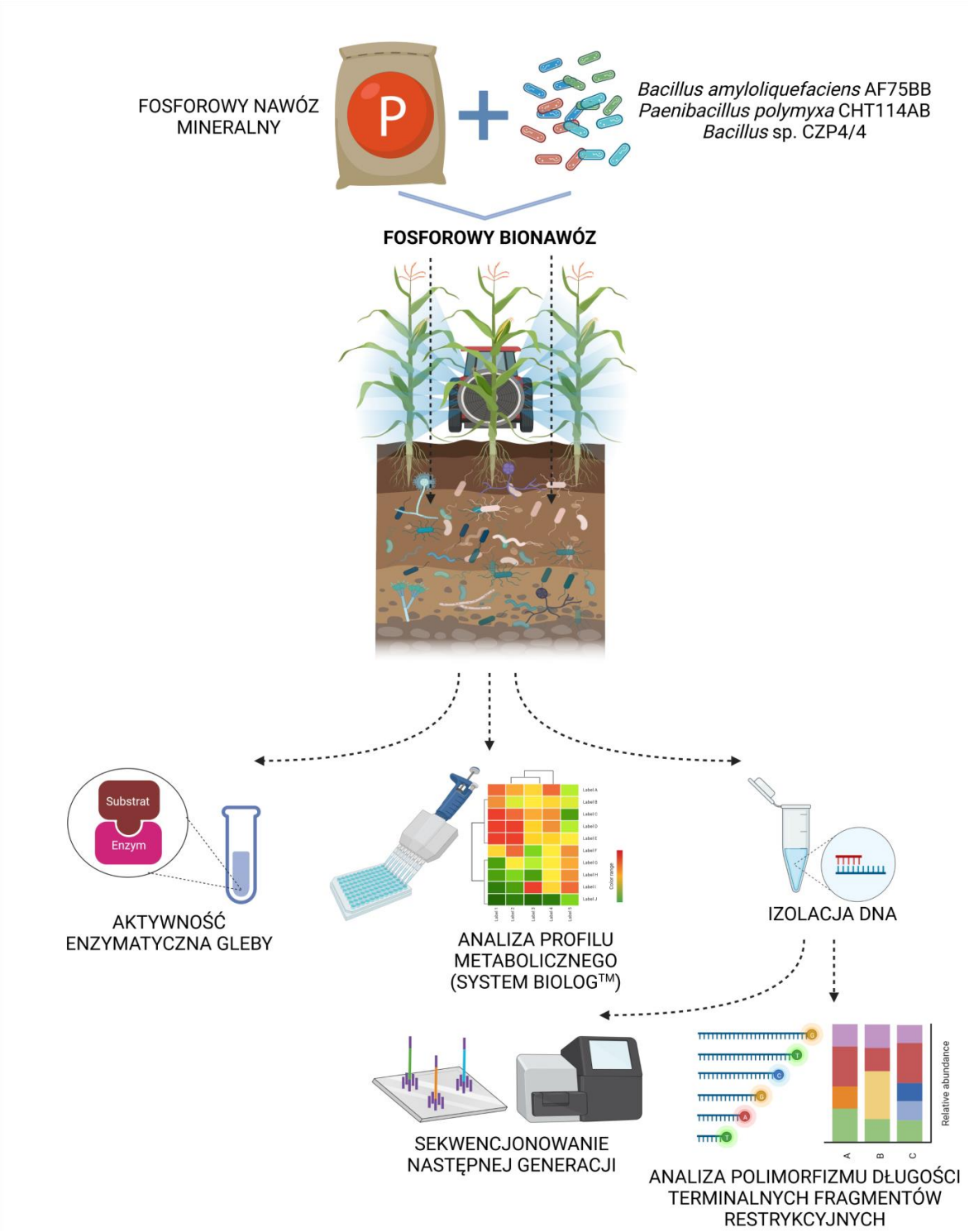
Automatyczną wstępną analizę danych sekwencjonowania następnej generacji polegającą na demultipleksowaniu i generowaniu plików fastq, przeprowadzono na platformie Miseq przy użyciu oprogramowania MiSeq Reporter (MSR) w wersji 2.6 (Illumina Inc., San Diego, CA). Analizę bioinformatyczną pozwalającą na przypisanie odczytów do odpowiednich poziomów taksonomicznych przeprowadzono za pomocą oprogramowania Quantitative Insights into Microbial Ecology (QIIME) (Caporaso i in., 2010) w oparciu o algorytm uCLUST (Edgar, 2010) i bazę danych GreenGenes v.13.8 (16S V3-V4) (DeSantis i in., 2006) oraz algorytm BLAST (Altschul i in., 1990) i bazę danych UNITE v.8 (ITS1) (Kõljalg i in., 2013; Nilsson i in., 2019).

Profil funkcjonalny zbiorowisk bakterii został wygenerowany z danych 16S rDNA przy użyciu narzędzi PICRUSt oraz PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille i in., 2013; Douglas i in., 2020), natomiast klasy genów funkcjonalnych określono za pomocą bazy danych KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa i Goto, 2000). Bazę danych FUNGuild wykorzystano z kolei do przypisania cech ekologicznych grzybów do operacyjnych jednostek taksonomicznych (ang. operational taxonomic units - OTUs) uzyskanych z sekwencjonowania regionu ITS1 rDNA (Nguyen i in., 2016).

Analiza beta różnorodności zbiorowisk mikroorganizmów glebowych obejmowała analizę głównych współrzędnych (ang. principle coordinate analysis - PCoA), konstrukcję dendrogramów metodą średnich połączeń (ang. unweighted pair group method with arithmetic mean - UPGMA) oraz niemetryczne skalowanie wielowymiarowe (ang. non-metric multidimensional scaling - NMDS).

Statystycznie istotne różnice pomiędzy wariantami nawożenia dla analizowanych parametrów (aktywność enzymatyczna gleby, plon kukurydzy, zawartość przyswajalnego fosforu, liczba operacyjnych jednostek taksonomicznych, AWCD (ang. Average Well Color Development), AWDD (ang. Average Well Density Development), wskaźniki bioróżnorodności Richness i Shannona) określono z wykorzystaniem odpowiednich testów statystycznych. Weryfikację założeń analizy wariancji (ANOVA) obejmujących normalność rozkładu oraz jednorodność wariancji przeprowadzono odpowiednio z użyciem testu Shapiro-Wilka oraz testu Levene'a. Przy spełnieniu założenia normalności rozkładu, ale braku jednorodności wariancji zastosowano test F-Welcha, a następnie test post-hoc Tukey'a.

Natomiast przy braku normalności rozkładu zastosowano test nieparametryczny Kruskala-Wallisa i test Dunna. Wszystkie analizy statystyczne wykonano z użyciem programów Statistica v. 13.1 (StatSoft Inc., Tulsa, OK) oraz RStudio v. 1.0.5.999 (R Core Team, Wiedeń, Austria).



Rys. 1. Etapy prowadzonych badań. Rysunek wykonano w programie BioRender.

4. Omówienie wyników przedstawionych w publikacjach i badaniach uzupełniających

W publikacjach P.1, P.2, P.3 oraz w badaniach uzupełniających obejmujących manuskrypt publikacji P.4, podjęto zagadnienie wykorzystania bionawozów we współczesnym rolnictwie oraz scharakteryzowano wpływ innowacyjnego fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie na aktywność i bioróżnorodność mikroorganizmów zasiedlających gleby zdegradowane. W dwuletnim doświadczeniu polowym prowadzonym w latach 2018-2019 na dwóch typach gleb zdegradowanych (Brunic Arenosol- BA i Abruptic Luvisol -AL) uwzględniono trzy warianty nawożenia: dawkę optymalną bez wzbogacenia mikrobiologicznego (FC), dawkę optymalną wzbogaconą mikrobiologicznie (FA100) oraz dawkę zredukowaną o 40% wzbogaconą mikrobiologicznie (FA60).

4.1. Publikacja P.1 (Biofertilizers in agriculture: An overview on concepts, strategies and effects on soil microorganisms)

Mącik, M., Gryta, A., Frąc M., 2020. Biofertilizers in agriculture: An overview of concepts, strategies and effects on soil microorganisms. *Advances in Agronomy* 162, 31-87.

W publikacji przeglądowej P.1 przedstawiono koncepcje i strategie wykorzystania bionawozów we współczesnym rolnictwie oraz podkreślono rolę nawozów mikrobiologicznych jako efektywnych zamienników lub suplementów tradycyjnych nawozów mineralnych w odniesieniu do negatywnych skutków związanych z nadmierną intensyfikacją i chemizacją rolnictwa. Scharakteryzowano bionawozy jako przyjazne środowisku preparaty, których implementacja pozostaje w zgodzie z zasadami zrównoważonego i ekologicznego rolnictwa, a także z regulacjami prawnymi dotyczącymi ograniczenia ilości agrochemikaliów aplikowanych do gleby. Podkreślono szerokie spektrum działania bionawozów w kierunku poprawy wzrostu i rozwoju roślin, jakości gleb uprawnych oraz struktury zbiorowisk mikroorganizmów glebowych. Przedstawiono także jak zmieniało się pojęcie "bionawóz" wraz z rozwojem wiedzy na temat interakcji między mikrobiomem glebowym i rośliną.

W publikacji P.1 scharakteryzowano najpopularniejsze nośniki bionawozów: nośniki stałe oraz preparaty płynne, a także przedstawiono nowe możliwości w produkcji środków mikrobiologicznych: immobilizację i enkapsulację komórek mikroorganizmów, wykorzystanie biofilmów bakteryjnych i grzybowych, nanonawozy oraz preparaty suszone w

złożu fluidalnym (ang. fluidized bed dried formulations - FDB). Przedstawiono także proces produkcji bionawozów począwszy od wyboru odpowiednich mikroorganizmów do pakowania gotowych preparatów. Zaprezentowano również wymagania jakościowe jakie muszą spełnić bionawozy, aby zostać dopuszczone do użytku oraz najczęściej stosowane metody aplikacji preparatów mikrobiologicznych: na nasiona, na sadzonki oraz doglebowo.

Dokonano również klasyfikacji i charakterystyki bionawozów na podstawie mechanizmu ich działania: wiązanie azotu, udostępnianie fosforu, stymulowanie wzrostu roślin, udostępnianie potasu, cynku, siarki i krzemu. W obrębie każdego mechanizmu przedstawiono mikroorganizmy zdolne do przeprowadzenia opisywanych procesów oraz podano przykłady komercyjnych preparatów stosowanych na świecie, a także scharakteryzowano globalny rynek bionawozów. Zademonstrowano także najbardziej popularne techniki wykorzystywane do analizy zmian w populacjach mikroorganizmów glebowych: elektroforeza w gradiencie czynnika denaturującego (DGGE), analiza polimorfizmu długości terminalnych fragmentów restrykcyjnych (t-RFLP), analiza restrykcyjna amplifikowanego rybosomalnego DNA (ARDRA), analiza polimorfizmu konformacji pojedynczej nici (SSCP) oraz profilowanie metaboliczne z wykorzystaniem systemu BIOLOG™.

4.2. Publikacja P.2 (The Status of Soil Microbiome as Affected by the Application of Phosphorus Biofertilizer: Fertilizer Enriched with Beneficial Bacterial Strains)

Mącik, M., Gryta, A., Sas-Paszt, L., Frąc, M., 2020. The Status of Soil Microbiome as Affected by the Application of Phosphorus Biofertilizer: Fertilizer Enriched with Beneficial Bacterial Strains. *International Journal of Molecular Sciences* 21, *8003*.

W publikacji P.2 przedstawiono wyniki badań dotyczących natychmiastowej odpowiedzi zbiorowisk mikroorganizmów glebowych po aplikacji fosforowego bionawozu w dwóch różnych typach gleb zdegradowanych - Brunic Arenosol (BA) oraz Abruptic Luvisol (AL). Do badań wykorzystano próbki gleby pobrane tydzień po aplikacji (bio)nawozów w sezonie letnim w 2018 roku. Potwierdzono pozytywny wpływ fosforowego bionawozu na parametry mikrobiologiczne gleb zdegradowanych, obejmujące aktywność enzymatyczną oraz różnorodność funkcjonalną i genetyczną bakterii, archeonów i grzybów.

Gleba typu AL charakteryzowała się wyższą aktywnością enzymatyczną w porównaniu do gleby typu BA. Zaobserwowano wzrost aktywności β-glukozydazy, proteazy oraz ureazy w obu typach gleb w wariantach FA100 i FA60 w porównaniu do gleby kontrolnej, co może oznaczać wzrost dostępności składników pokarmowych dla roślin i wydajniejszy obieg materii. Jeśli chodzi natomiast o fosfatazę kwaśną, to jej aktywność utrzymywała się na podobnym poziomie we wszystkich wariantach nawożenia w obu typach gleb, bez istotnych statystycznie różnic. Szczególnie ważny jest wzrost aktywności enzymatycznej w wariancie FA60, ponieważ daje to szansę na redukcję dawki nawozu mineralnego przy jednoczesnym pozytywnym wpływie na procesy enzymatyczne zachodzące w glebie.

Analiza profili metabolicznych zbiorowisk bakterii i grzybów wykazała wzrost zużycia substratów należących do różnych grup związków chemicznych (polimey, aminokwasy, kwasy karboksylowe, węglowodany, aminy). Zwiększenie wartości współczynnika "Richness" (liczba metabolizowanych substratów) w wybranych wariantach FA100 i FA60 może być związane z poszerzeniem zdolności metabolicznych mikroorganizmów. O wzroście ogólnej aktywności metabolicznej świadczy również wzrost wartości wskaźników AWCD i AWDD. W glebie typu AL obserwowano także wzrost utylizacji fosforanu D-glukozy oraz fosforanu D,L-α-glicerolu, związków mogących stanowić źródło fosforu.

Analiza różnorodności genetycznej przy użyciu metody M-tRFLP wykazała wzrost liczby terminalnych fragmentów restrykcyjnych (T-RFs) w wariantach FA100 i FA60 wśród zbiorowisk bakterii, archeonów i grzybów. Obecność T-RFs o tej samej wielkości w wariantach FC, FA100 i FA60 może wskazywać na obecność powszechnie występujących bakterii, archeonów i grzybów, niezależnie od sposobu nawożenia, natomiast zmiany we względnej obfitości poszczególnych T-RFs mogą być związane z wrażliwością mikroorganizmów na dany sposób nawożenia.

Metataksonomiczna analiza przy pomocy sekwencjonowania następnej generacji ujawniła różnice w strukturze populacji mikroorganizmów glebowych na poziomie typu, klasy i rzędu (alfa różnorodność). Na poziomie typu badane gleby były zdominowane przez Actinobacteriota (25.49–31.68%), Proteobacteria (24.78–28.59%) Acidobacteria (9,60–14,39%) i Chloroflexi (6,52–12,18%) oraz przez Ascomycota (33,70–56,15%),

Basidiomycota (10,17–17,90%) i Zygomycota (9,24–14,23%). Przedstawiciele Proteobacteria i Acidobacteria występują powszechnie w wielu typach gleb, przy czym Proteobacteria stanowią najbardziej zróżnicowany typ bakterii pod względem filogenetycznym. Wysoka względna obfitość Ascomycota może wynikać z tego, że mikroorganizmy należące do tego typu są najliczniej występującymi grzybami w środowiskach lądowych. Aplikacja fosforowego bionawozu stymulowała występowanie niektórych grup mikroorganizmów (Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Rhizobiales, Sordariomycetes, Agaricomycetes, Hypocreales). Jeśli chodzi o archeony to ich względna obfitość była niska i wynosiła 0,03-0,09% w zależności od typu gleby i wariantu nawożenia. W zbiorowiskach archeonów dominowały mikroorganizmy należące do typu Crenarchaeota (0,02–0,07%). Natomiast analiza beta różnorodności wykazała wyraźne grupowanie wariantów nawożenia w zależności od typu gleby. Odseparowanie wariantów FA100 i FA60 od gleby kontrolnej wynika z różnic w składzie populacji zbiorowisk mikroorganizmów w danym wariancie nawożenia.

Sekwencjonowanie następnej generacji umożliwiło także wgląd w profil funkcjonalny zbiorowisk bakterii. Większość (~55%) zidentyfikowanych funkcjonalnych OTU została przypisana do klasy "Metabolizm", zgodnie z modułem KEGG, co sugeruje, że procesy metaboliczne różnych związków zachodzą z największą intensywnością w zbiorowiskach bakterii. Gleba typu AL charakteryzowała się wyższą liczbą sekwencji, natomiast w glebie typu BA zaobserwowano wzrost liczby funkcjonalnych OTU przypisanych do głównych klas KEGG w wariantach FA100 i FA60. W glebie typu BA w wariantach FA100 i FA60 wzrosła także liczba sekwencji przypisanych do metabolizmu węglowodanów, aminokwasów, lipidów i ksenobiotyków, transdukcji sygnałów, translacji i transportu membranowego. Natomiast w glebie typu AL w wariancie FA100 po aplikacji fosforowego bionawozu wzrosła liczba OTU związanych z transportem membranowym, metabolizmem energetycznym oraz metabolizmem kofaktorów i witamin. W glebie typu BA w wariantach FA100 i FA60 wzrosła również liczba sekwencji związanych ze szlakami przemian fosforu: fosforylacją oksydacyjną, szlakiem pentozofosforanowym, metabolizmem glicerofosfolipidów, fosforanu inozytolu, fosfonianów i fosfinianów oraz systemem sygnalizacji fosfatydyloinozytolu. Ponadto, w wariancie FA100 zwiększyła się liczba OTU przypisanych do systemu fosfotransferazy. Analiza profilu funkcjonalnego zbiorowisk grzybów ujawniła natomiast, że w badanych glebach dominowały dwa poziomy troficzne: saprotrof (21,00-35,98%) oraz

saprotrof-symbiotrof (6,84-16,34%). W glebie typu BA w wariantach FA100 i FA60 istotnemu obniżeniu uległa liczba patotrofów oraz zwiększyła się liczba saprotrofów-symbiotrofów, natomiast w glebie typu AL zaobserwowano zwiększenie liczby OTU przypisanych do saprotrofów oraz symbiotrofów. Grzyby saprotroficzne stanowią istotny element środowiska glebowego, gdyż uczestniczą w dekompozycji martwej materii organicznej, więc zwiększenie ich liczby w glebie może być powiązane z wydajniejszym obiegiem materii i pierwiastków w ekosystemie.

Uzyskane wyniki wskazują na pozytywny wpływ fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie na zbiorowiska mikroorganizmów glebowych, ich bioróżnorodność i funkcjonalność bezpośrednio po aplikacji bionawozu.

4.3. Publikacja P.3 (Composition, activity and diversity of bacterial and fungal communities responses to inputs of phosphorus fertilizer enriched with beneficial microbes in degraded Brunic Arenosol)

Mącik, M., Gryta, A., Sas-Paszt, L., Frąc, M., 2022. Composition, activity and diversity of bacterial and fungal communities responses to inputs of phosphorus fertilizer enriched with beneficial microbes in degraded Brunic Arenosol. *Land Degradation & Development* 33(6), 844-865.

W publikacji P.3 przedstawiono sezonowe zmiany w statusie mikrobiomu i mykobiomu zasiedlającego glebę typu Brunic Arenosol po aplikacji fosforowego bionawozu. Do badań wykorzystano próbki gleby pobrane po zbiorach kukurydzy na jesieni w 2018 roku (ang. autumn 2018 - A18), po aplikacji (bio)nawozów w sezonie letnim 2019 roku (ang. summer 2019 - S19) oraz po zbiorach kukurydzy na jesieni 2019 roku (ang. autumn 2019 - A19).

Najwyższą aktywność proteazy odnotowano w wariancie FA100(S19). Jeśli chodzi o sezonowe zmiany aktywności proteazy, to w roku 2019 zaobserwowano obniżenie aktywności tego enzymu pomiędzy odpowiadającymi kontrolami i wariantami FA100 oraz zwiększenie między wariantami FA60. W przypadku ureazy najwyższą aktywność zanotowano w wariantach FA100(A18) and FA100(A19). W drugim roku doświadczenia polowego wystąpiła tendencja wzrostowa aktywności ureazy pomiędzy wariantami FA100. Aktywność fosfatazy kwaśnej utrzymywała się na wyższym poziomie w wariantach FA100 i FA60 we wszystkich analizowanych terminach. W S19 i A19 zaobserwowano zwiększenie

aktywności fosfatazy wraz ze zmniejszeniem dawki nawozu mineralnego. Natomiast najwyższą aktywność β-glukozydazy odnotowano w wariantach FA100(A19) i FA60(A19), ze statystycznie istotnymi różnicami w porównaniu do gleby kontrolnej. Wahania aktywności badanych enzymów w publikacji P.3 mogą wynikać z faktu, że parametr ten jest podatny na zmiany w ciągu 2 lat od zadziałania określonego czynnika (Dick i Kandeler, 2005). Analizując aktywności enzymatyczne ze wszystkich trzech terminów jednocześnie stwierdzono, że aktywności proteazy, ureazy, β-glukozydazy oraz fosfatazy kwaśnej utrzymywały się na wyższym poziomie w wariantach FA100 i FA60, co potwierdza pozytywny wpływ aplikacji fosforowego bionawozu na glebowe procesy enzymatyczne.

W obu latach doświadczenia polowego w wariantach FA100 i FA60 odnotowano zwiększenie plonu kukurydzy w porównaniu z glebą kontrolną, bez istotnych statystycznie różnic pomiędzy poszczególnymi wariantami. Warto zwrócić uwagę, że największe plony zaobserwowano w wariancie, w którym zaaplikowano dawkę zredukowaną nawozu zawierającą mikroorganizmy. Aplikacja fosforowego bionawozu w czerwcu 2019 roku spowodowała również zwiększenie zawartości dostępnego fosforu w glebie o 2,52% w porównaniu do wariantu kontrolnego. W terminach jesiennych obserwowano zmniejszenie zawartości P_2O_5 w wariantach FA100 i FA60, aczkolwiek spadek ten może być związany ze wzrostem plonu kukurydzy we wspomnianych wariantach i zużyciem składników mineralnych.

Analiza PCA wskazała na wyraźne grupowanie wariantów nawożenia w zależności od terminu poboru próbek gleby, zarówno w przypadku płytek ECO jak i FF. Sezonowa odpowiedź mikroorganizmów na aplikację bionawozu może być związana między innymi z nasileniem procesu fotosyntezy w sezonie letnim i uwalnianiem produktów tego procesu do gleby, a także ze zmianą preferowanych źródeł węgla w zależności od pory roku. O osiągnięciu równowagi metabolicznej wśród zbiorowisk mikroorganizmów glebowych i adaptacji do nowego sposobu nawożenia może świadczyć zgrupowanie wszystkich trzech wariantów nawożenia razem w analizach ECO i FF w obrębie terminu A19.

Sezonowe zmiany w strukturze zbiorowisk mikroorganizmów glebowych ujawniła także analiza t-RFLP. Bakterie i grzyby charakteryzowały się wyższą liczbą terminalnych fragmentów restrykcyjnych (T-RFs) w terminach jesiennych, natomiast najwyższą liczbę T-RFs w zbiorowiskach archeonów odnotowano bezpośrednio po aplikacji fosforowego

bionawozu w sezonie letnim w 2019 roku. Mniejsze wahania w liczbie T-RFs dla archeonów mogą sugerować, że ta grupa jest bardziej odporna na zmianę warunków środowiska. Identyfikacja na podstawie wielkości wybranych T-RFs przy pomocy narzędzia TRiFLe ujawniła obecność mikroorganizmów ważnych dla prawidłowego funkcjonowania gleby, np: *Nitrososphaera* spp. (archeony utleniające amoniak), *Ferroplasma* spp. (archeony uczestniczące w obiegu żelaza), *Rhizobium* spp. (wiązanie azotu atmosferycznego), *Lysobacter* spp. (biokontrola fitopatogenów grzybowych), *Cortinarius* spp., *Conocybe* spp. (saprotrofy), *Solicoccozyma* spp. (bioremediacja). Niektóre ze zidentyfikowanych rodzajów zostały również scharakteryzowane pod kątem uruchamiania fosforu z trudno dostępnych źródeł, np: *Lysobacter* spp., *Pseudomonas* spp., *Burkholderia* spp., *Pantoea* spp., oraz *Trichoderma* spp..

Na podstawie wyników z sekwencjowania następnej generacji przeanalizowano strukturę zbiorowisk mikroorganizmów na poziomie rzędu. W zbiorowiskach bakterii i archeonów wzrosła liczba rzędów w wariantach FA100 (181) i FA60 (180) w porównaniu do FC (178), co sugeruje pozytywny wpływ bionawozu na bioróżnorodność mikrobiomu. Badana gleba była zdominowana przez bakterie należące do Actinomycetales (12,69–19,08%), Rhizobiales (6,06–8,04%), Xanthomonadales (3,16–6,43%), Rhodospirillales (4,40–5,49%), Acidobacteriales (4,03–7,05%) i Solirubrobacterales (4,19–5,15%). Bakterie należące do rzędu Actinomycetales wykazują cechy promujące wzrost i rozwój roślin, np: udostępnianie fosforu, synteza sideroforów i hamowanie aktywności fitopatogenów. Po aplikacji bionawozu w sezonie letnim S19 zwiększeniu uległa względna obfitość Actinomycetales, Rhizobiales, Xanthomonadales, Sphingomonadales, Burkholderiales i Bacillales w porównaniu do terminów jesiennych. Warto zwrócić uwagę, że w obrębie wymienionych rzędów istnieją pożyteczne gatunki mikroorganizmów, więc zwiększenie ich liczebności może oznaczać zapewnienie dogodnych warunków dla rozwoju kukurydzy oraz poprawę stanu mikrobiologicznego i jakości gleby. Natomiast bakterie należące do Rhodospirillales, Acidobacteriales, Acidimicrobiales, Thermogemmatisporales, Solibacterales, Myxococcales i Gemmatales miały większy procentowy udział w kreowaniu struktury zbiorowisk bakterii w próbkach gleby pobranych po zbiorach kukurydzy na jesieni 2018 i 2019. W profilu funkcjonalnym zbiorowisk bakterii najwyższą liczbą sekwencji przypisanych do głównych klas i podklas KEGG charakteryzowały się warianty FA100-FA60(A19).

W zbiorowiskach grzybów zidentyfikowano 120 rzędów w wariantach FC i FA100 oraz 115 w wariancie FA60. W glebie typu BA dominowały Eurotiales (13,19–23,56%), Hypocreales (7,99–14,01%) oraz Mortierellales (4,22–7,88%). Wśród Eurotiales można wyróżnić wiele gatunków saprotroficznych, Hypocreales obejmują szeroki zakres entomopatogenów, natomiast niektóre gatunki Mortierellales uczestniczą w obiegu fosforu. W próbkach gleby zebranych w sezonie letnim w 2019 roku zaobserwowano wyższą względną obfitość grzybów z rodzajów Mortierellaes, Sordariales, Filobasidiales i Agaricales, natomiast Eurotiales, Chaetothyriales, Umbelopsidales i Pleosporales występowały liczniej w terminach jesiennych. Analiza profilu funkcjonalnego zbiorowisk grzybów wykazała, że dominującymi poziomami troficznymi były saprotrofy (29,12–39,57%), następnie saprotrofy-symbiotrofy (5,93–9,23%), patotrofy-saprotrofy-symbiotrofy (5,78–8,91%) i patotrofy (3,20–6,87%). W wariantach FA100(S19-A19) i FA60(S19-A19) uległa zmniejszeniu względna obfitość patotrofów w porównaniu do odpowiadających kontroli, natomiast w wariantach FA100(A18) i FA100-FA60(A19) zanotowano wzrost liczebności saprotrofów-symbiotrofów, co wskazuje na pozytywne oddziaływanie bionawozu na zbiorowiska grzybów.

Analiza beta różnorodności wykazała grupowanie wariantów nawożenia w zależności od terminu poboru próbek gleby, wskazując na różnice w składzie populacji bakterii i grzybów w poszczególnych terminach. Dendrogramy UPGMA pokazały, że w zbiorowiskach bakterii termin S19 był wyraźnie oddzielony od terminów jesiennych, natomiast w zbiorowiskach grzybów próbki pobrane w terminach S19 i A19 utworzyły jedną grupę, odseparowaną od terminu A18.

4.4. Badania uzupełniające (Określenie sezonowych zmian w statusie mikrobiomu i mykobiomu zasiedlającego glebę typu Abruptic Luvisol po aplikacji fosforowego bionawozu)

Mateusz Mącik, Agata Gryta, Lidia Sas-Paszt, Magdalena Frąc, New insight into the soil bacterial and fungal microbiome after phosphorus biofertilizer application as important driver of regenerative agriculture including biodiversity loss reversing and soil health restoration.

4.4.1. Wstęp

Uzupełnieniem badań przedstawionych w niniejszej rozprawie było określenie sezonowych zmian w statusie mikrobiomu i mykobiomu zasiedlającego glebę typu Abruptic Luvisol po aplikacji fosforowego bionawozu. Do badań wykorzystano próbki gleby pobrane

przed aplikacją (bio)nawozów w kwietniu 2018 i 2019 (ang. April 2018 - A18 i April 2019 - A19), po aplikacji (bio)nawozów w czerwcu 2019 (ang. June 2019 - J19) oraz po zbiorach kukurydzy w październiku 2018 i 2019 (ang. October 2018 - O18 i October 2019 - O19). Szczegółowe omówienie wyników dla próbek pobranych w czerwcu 2018 (J18) przedstawiono w publikacji Mącik i in. (2020), aczkolwiek na potrzeby badań uzupełniających analiza wyników sekwencjonowania następnej generacji została przeprowadzona całościowo (wyłączając terminy A18 i A19) z uwzględnieniem terminu J18.

4.4.2. Materiały i metody

Materiały i metody użyte do realizacji badań uzupełniających zostały opisane w rozdziale 3 niniejszej rozprawy.

4.4.3. Wyniki i dyskusja

4.4.3.1. Aktywność enzymatyczna gleby, zawartość przyswajalnego fosforu oraz plon kukurydzy

Bezpośrednio po aplikacji (bio)nawozów w czerwcu 2019 zaobserwowano wzrost aktywności proteazy, ureazy i fosfatazy kwaśnej w wariantach FA100 i FA60 oraz wzrost aktywności β-glukozydazy w wariancie FA100 w porównaniu do gleby kontrolnej. Podobny wpływ bionawozów został scharakteryzowany przez inne zespoły. Aktywność fosfatazy kwaśnej wzrosła w glebie inokulowanej *Azotobacter chroococcum*, *Acetobacter diazotrophicus* i *Aspergillus awamori* (Srivastava i Singh, 2022), podwyższoną aktywność ureazy odnotowano po aplikacji preparatu zawierającego *Frankia casuarinae* CcI3 (Qi i in., 2022), zastosowanie *Bradyrhizobium* sp. istotnie poprawiło aktywność proteaz w sezonach wegetacyjnych w 2018 i 2019 roku (El-Sawah i in., 2021), natomiast tendencję wzrostową aktywności β-glukozydazy odnotowano w przypadku biopreparatów TripleN®, Rhizosum N® i Rhizosum PK® w uprawie pszenicy zwyczajnej (Dal Cortivo i in., 2020). Nasilenie glebowych procesów enzymatycznych może być sprzężone z wydajniejszym rozkładem różnych związków, co przekłada się na wzrost dostępności składników mineralnych (Chaudhary i in., 2022). Dodatkowo, wzrost aktywności fosfatazy kwaśnej może wspomagać przyswajanie fosforu ze źródeł pochodzenia organicznego (El-Sawah i in., 2021).

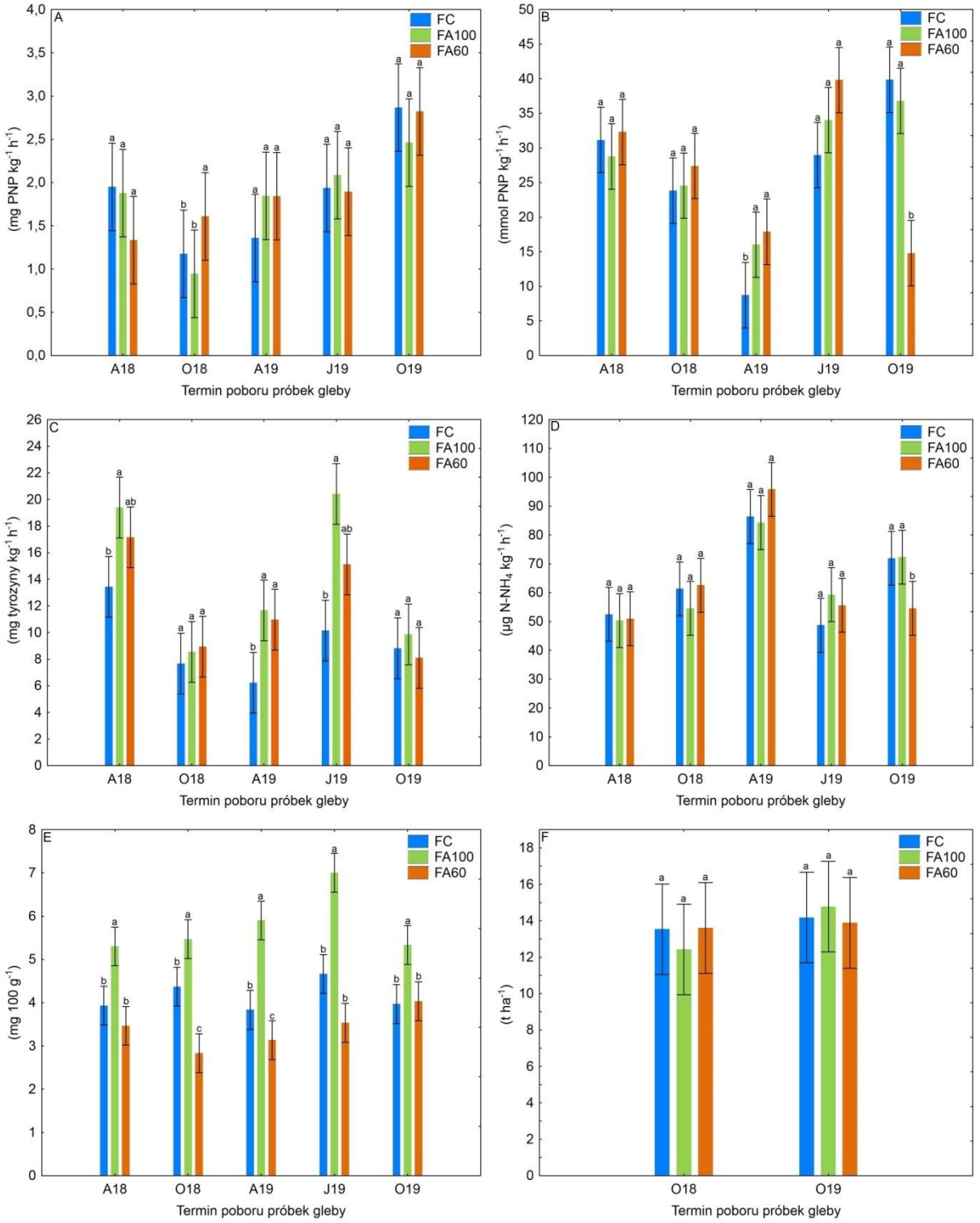
Najwyższą aktywnością β-glukozydazy charakteryzowały się próbki gleby zebrane w październiku 2019. Analiza sezonowych zmian aktywności β-glukozydazy wykazała wahania

pomiędzy poszczególnymi terminami poboru próbek gleby. W wariancie FA60 aktywność enzymatyczna wzrosła o 112,03% pomiędzy terminami A18 i O19, natomiast w wariancie FA100 o 158,95% pomiędzy terminami O18 i O19. Obserwowany przyrost sugeruje, że aplikacja fosforowego bionawozu poprawia badany parametr oraz umożliwia utrzymanie tego efektu w czasie. Aktywność β-glukozydazy wzrosła także pomiędzy odpowiadającymi wariantami wraz z kolejnymi terminami poboru próbek gleby w roku 2019 (Rys. 2A). Najwyższe aktywności fosfatazy kwaśnej zanotowano dla próbek gleby zebranych w czerwcu 2019 (warianty FA100 i FA60) oraz październiku 2019 (warianty FC i FA100). W obrębie terminów O18, A19 oraz J19 zaobserwowano zwiększenie aktywności fosfatazy kwaśnej wraz z redukcją dawki mineralnego nawozu (Rys. 2B). Aktywność proteazy utrzymywała się na wyższym poziomie we wszystkich wariantach FA100 i FA60 w trakcie trwania doświadczenia, z wyjątkiem wariantu FA60(O19). Zaobserwowano trend wzrostowy w aktywności proteazy pomiędzy terminami O18 i J19 dla wariantów FA100 i FA60. Stosunkowo wysokie wartości aktywności proteazy zanotowano dla próbek gleby pobranych w kwietniu 2018 (A18). (Rys. 2C). Wysoka aktywność enzymatyczna w próbkach gleby pobranych w kwietniu 2018 i kwietniu 2019 może sygnalizować o nasileniu przemian chemicznych zachodzących w glebie wraz z początkiem sezonu wegetacyjnego. Najwyższą aktywnością ureazy charakteryzowały się próbki zebrane w kwietniu 2019 roku. W obrębie terminów A18, A19 i O19 aktywność ureazy utrzymywała się na podobnym poziomie pomiędzy wariantami FC i FA100 (Rys. 2D).

Zawartość przyswajalnego fosforu (P_2O_5) utrzymywała się na wyższym poziomie w wariancie FA100 przez cały okres doświadczenia przy czym różnice były istotne statystycznie w porównaniu do wariantów FC i FA60. Bezpośrednio po aplikacji (bio)nawozów w czerwcu 2019 roku, zawartość P_2O_5 w wariancie FA100 zwiększyła się o 49,89% w porównaniu do wariantu kontrolnego. Zwiększoną zawartość dostępnego fosforu w glebie obserwowano również po inokulacji *Paenibacillus* sp. B1 (Li i in., 2020), *B. licheniformis* i *B. amyloliquefaciens* (Wang i in., 2021b) oraz *Bacillus sp*. SD01N-014 (Liu i in., 2018). Wzrost poziomu P może wynikać z udostępniania tego pierwiastka z trudno dostępnych źródeł przez mikroorganizmy dostarczone do gleby wraz z bionawozem. Analizując zmiany w dostępności P_2O_5 w czasie całego okresu doświadczalnego zaobserwowano zwiększenie zawartości fosforu między terminami A18 i J19 dla wariantu FA100 (wzrost o 32,08%) oraz między terminami O18 i O19 dla wariantu FA60 (wzrost o 42,35%). Co więcej, na koniec

doświadczenia w październiku 2019 (O19) zawartość P była wyższa w wariantach FA100 i FA60 w porównaniu do analogicznych wariantów z kwietnia 2018 (A18) (Rys. 2E). Stopniowy wzrost zawartości fosforu w wariantach FA100 i FA60 w trakcie trwania doświadczenia polowego wskazuje, że zastosowanie fosforowego bionawozu sprzyja mikrobiologicznemu udostępnianiu tego pierwiastka z trudno dostępnych źródeł i jego akumulacji w glebie.

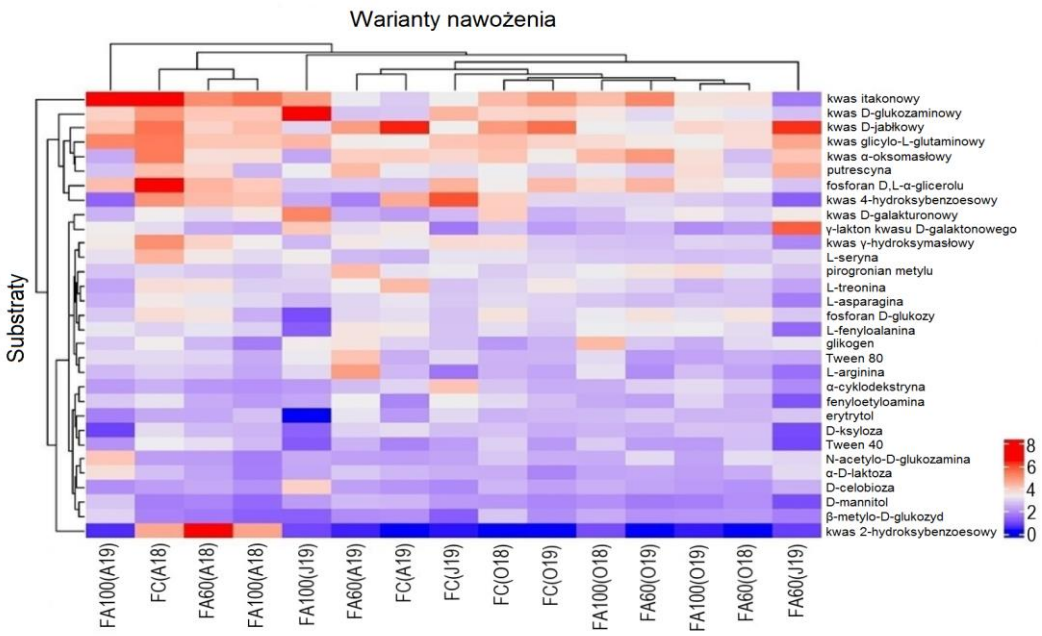
Aplikacja fosforowego bionawozu zwiększyła także średni plon kukurydzy w wariancie FA60 w roku 2018 o 0,49% oraz w wariancie FA100 w roku 2019 o 4,32% w porównaniu do gleby kontrolnej (Rys. 2F). Według analizy przeprowadzonej przez Schmidta i Gaudina (2018) plon kukurydzy wzrósł średnio o 15,3% w doświadczeniach polowych, w których zastosowano bionawozy Uzyskanie wyższego plonu może być związane ze zwiększoną aktywnością enzymatyczną gleby i lepszą dostępnością składników pokarmowych, w tym aminokwasów i węglowodanów, które mogą być przyswojone i wykorzystane przez rośliny (Moe, 2013).



Rys. 2. Zmiany w aktywności enzymatycznej gleby, zawartości przyswajalnego fosforu oraz plonie kukurydzy w glebie typu Abruptic Luvisol poddanej działaniu fosforowego bionawozu. (A) - aktywność β-glukozydazy, (B) - aktywność fosfatazy kwaśnej, (C) - aktywność proteazy, (D) - aktywność ureazy, (E) - zawartość przyswajalnego fosforu (P_2O_5), (F) - plon kukurydzy. Objaśnienia: FC - dawka optymalna bez wzbogacenia mikrobiologicznego, FA100 - dawka optymalna wzbogacona mikrobiologicznie, FA60 - dawka zredukowana o 40% wzbogacona mikrobiologicznie, A18 - kwiecień 2018, O18 - październik 2018, A19 - kwiecień 2019, J19 - czerwiec 2019, O19 - październik 2019, PNP - p-nitrofenol. Pionowe słupki oznaczają 0,95 przedziały ufności. Różne litery nad przedziałami ufności oznaczają różnice istotne statystycznie ($p < 0,05$) obliczone dla każdego terminu oddzielnie.

4.4.3.2. Potencjał metaboliczny zbiorowisk mikroorganizmów glebowych

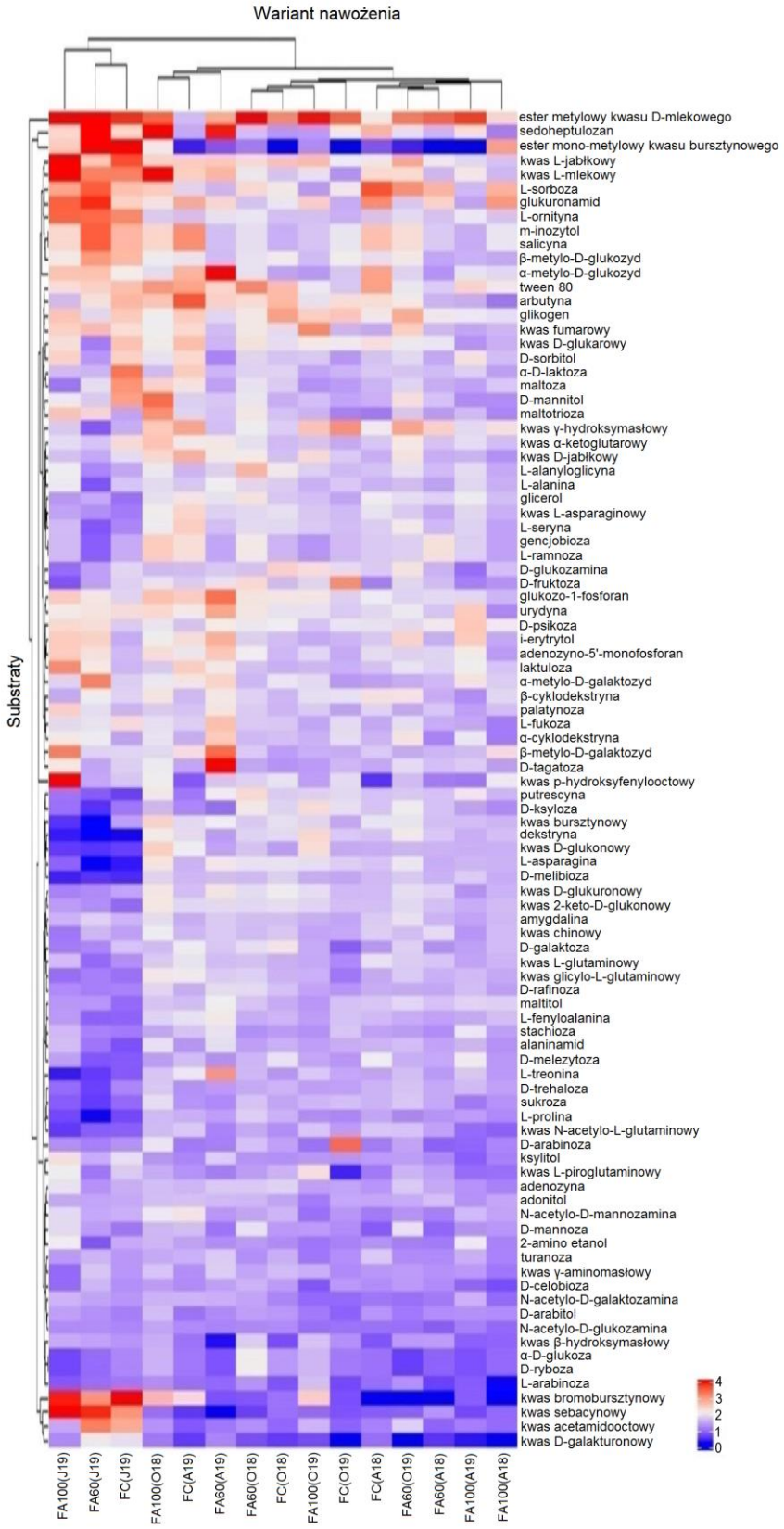
Potencjał metaboliczny zbiorowisk bakterii glebowych został wyrażony jako stosunek wartości utylizacji substratów węglowych (OD 590 nm) zamieszczonych na płytkach BIOLOG^{TM} ECO do przyrostu biomasy (OD 750 nm). Aplikacja fosforowego bionawozu obniżyła wartość stosunku OD590/OD750 dla D-ksylozy, fosforanu D,L-α-glicerolu, kwasu γ-hydroksymasłowego, L-fenyloalaniny, tweenu 40, α-cyklodekstryny i fenyloetyloaminy w wariantach FA100 i FA60. Spadek w wartości stosunku OD590/OD750 zaobserwowano także dla kwasu α-oksomasłowego, D-jabłkowego, i-erytrytolu, fosforanu D-glukozy w wariancie FA100(J19) oraz dla kwasu itakonowego, kwasu D-glukozaminowego, tweenu 80, L-asparaginy, D-mannitolu i L-treoniny w wariancie FA60(J19). Na początku doświadczenia polowego w kwietniu 2018 relatywnie wysokie wartości OD590/OD750 zanotowano dla kwasu 2-hydroksybenzoesowego, kwasu 4-hydroksybenzoesowego, kwasu D-glukozaminowego, kwasu glicylo-L-glutaminowego, L-seryny i kwasu γ-hydroksymasłowego, jednak wartości wspomnianego stosunku uległy obniżeniu w ostatnim terminie poboru próbek gleby w październiku 2019 roku. Do związków węgla, które nie spowodowały stresu substratowego w żadnym wariancie nawożenia należały β-metylo-D-glukozyd, D-mannitol, tween 40, D-ksyloza, i-erytrytol i fenyloetyloamina (Rys. 3).



Rys. 3. Stosunek wartości zużycia substratu (OD 590 nm) do przyrostu biomasy (OD 750 nm) określający teoretyczny potencjał metaboliczny zbiorowisk bakterii glebowych. Stosunek >4 wskazuje na niewielki przyrost biomasy przy jednoczesnej intensywnej utylizacji danego źródła węgla (stres substratowy). Stosunek <4 wskazuje na zachowanie równowagi pomiędzy zużyciem substratu a przyrostem biomasy. Objaśnienia jak na Rysunku 2.

Potencjał metaboliczny zbiorowisk grzybów został wyrażony jako stosunek wartości utylizacji substratów węglowych (OD 490 nm) zamieszczonych na płytkach BIOLOG^{TM} FF do przyrostu biomasy (OD 750 nm). Bezpośrednio po aplikacji fosforowego bionawozu w czerwcu 2019 roku, wartość stosunku OD490/OD750 uległa obniżeniu dla α-D-laktozy, maltozy, D-mannitolu, kwasu α-ketoglutarowego, L-fukozy, D-fruktozy i D-galaktozy w wariantach FA100 i FA60. Wartość współczynnika stresu substratowego została zredukowana także dla kwasu acetamidooctowego w wariancie FA100(J19) oraz dla arbutyny, kwasu D-glukarowego i D-sorbitolu w wariancie FA60(J19). Do substratów węglowych nie powodujących stresu substratowego należały L-arabinoza, D-ryboza, α-D-glukoza, D-arabitol, N-acetylo-D-galaktozamina, 2-aminoetanol, D-mannoza, ksylitol, D-melezytoza, stachioza, maltitol, D-rafinoza, N-acetylo-D-glukozamina, D-celobioza, turanoza, L-prolina, kwas N-acetyloglutaminowy, D-mannoza, adonitol i adenozyna. Względnie niskie wartości stosunku OD490/OD750 zanotowano w terminie J19 w przypadku dekstryny, kwasu D-glukonowego, L-asparaginy, D-melibiozy, putrescyny, L-treoniny i D-trehalozy w porównaniu do innych terminów poboru próbek gleby (Rys. 4).

Według Pinzari i in. (2016) niższe wartości stosunku OD590/OD750 i OD490/OD750 wskazują na bardziej dynamiczny wzrost mikroorganizmów idący w parze z niewielkim wykorzystaniem substratów węglowych. Z kolei wyższe wartości wspomnianych stosunków sygnalizują stresową sytuację metaboliczną, kiedy przyrost biomasy jest niewielki przy intensywnej utylizacji źródeł węgla. Uzyskane wyniki wykazały, że fosforowy bionawóz może łagodzić stres metaboliczny w przypadku niektórych substratów należących do różnych grup związków (węglowodany, kwasy karboksylowe, polimery, aminokwasy), zwiększając tym samym efektywność katabolicznę mikroorganizmów glebowych, co może wiązać się także z większą odpornością na różne stresy środowiskowe. Zmiana ta może wynikać z odmiennej aktywności pożytecznych mikroorganizmów dostarczonych wraz z bionawozem w porównaniu do rdzennego mikrobiomu i mykobiomu. Aplikacja bionawozu nie tylko doraźnie łagodzi efekt stresu metabolicznego, ale wykazuje także przedłużone działanie, co można zauważyć w przypadku kwasów karboksylowych na płytkach ECO. Natomiast brak stresowej sytuacji metabolicznej w obrębie niektórych grup substratów (np. węglowodanów) wskazuje, że pewne związki mogą być efektywnie metabolizowane niezależnie warunków środowiska i mogą stanowić preferowane źródło węgla.



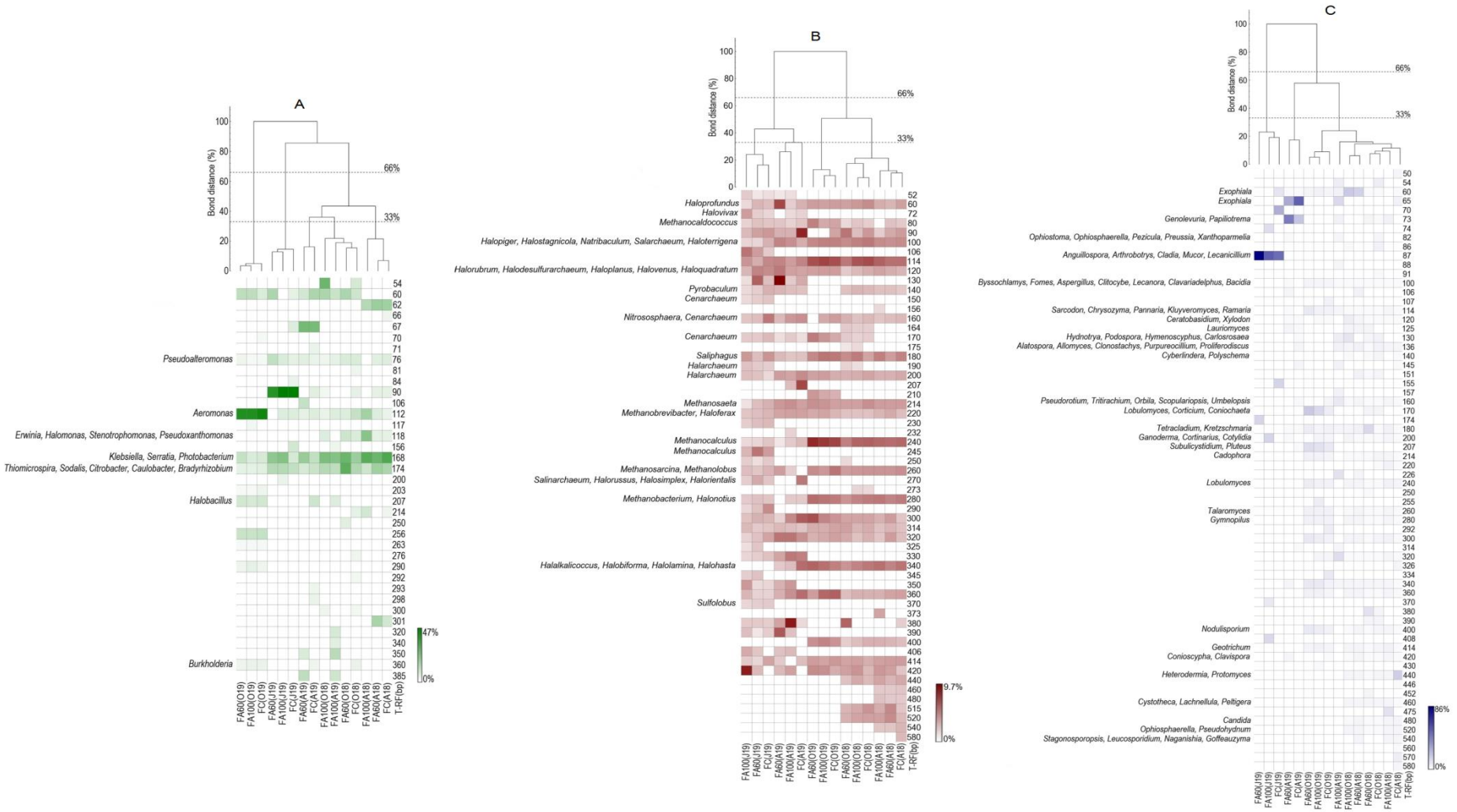
Rys. 4. Stosunek wartości zużycia substratu (OD 490 nm) do przyrostu biomasy (OD 750 nm) określający teoretyczny potencjał metaboliczny zbiorowisk grzybów glebowych. Stosunek >2 wskazuje na niewielki przyrost biomasy przy jednoczesnej intensywnej utylizacji danego źródła węgla (stres substratowy). Stosunek <2 wskazuje na zachowanie równowagi pomiędzy zużyciem substratu a przyrostem biomasy. Objaśnienia jak na Rysunku 2.

4.4.3.3. Charakterystyka różnorodności genetycznej zbiorowisk mikroorganizmów przy pomocy analizy polimorfizmu długości terminalnych fragmentów restrykcyjnych typu multiplex

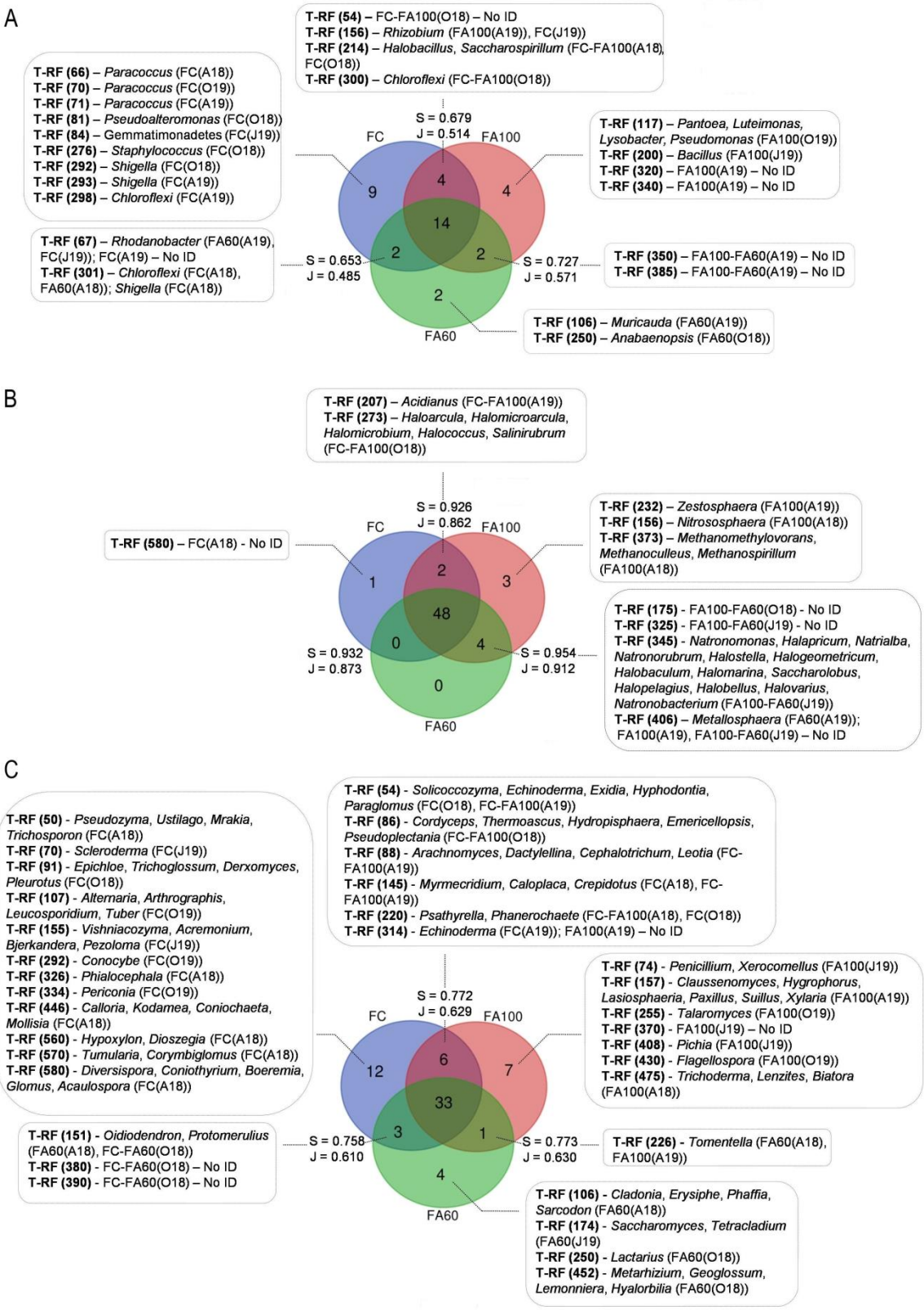
W wyniku analizy polimorfizmu długości terminalnych fragmentów restrykcyjnych (t-RFLP) otrzymano profile restrykcyjne zbiorowisk bakterii, archeonów i grzybów różniące się w obrębie danej grupy liczbą terminalnych fragmentów restrykcyjnych (T-RFs), ich wielkością oraz względną obfitością (Rys. 5A-C). Po zastosowaniu bionawozu w czerwcu 2019 roku liczba T-RFs zmniejszyła się, a następnie wzrosła w październiku 2019 w obrębie zbiorowisk bakterii i grzybów. Podobny efekt uzyskali Zhang i in. (2010), gdzie liczba T-RFs uległa obniżeniu w przypadku zbiorowisk bakterii w glebie inokulowanej *Rhizobium leguminosarum*. Natomiast wzrost liczby T-RFs między terminami J19 i O19 sugeruje, że pozytywny efekt bionawozu na bioróżnorodność staje się widoczny po pewnym czasie.

Wśród ogólnej puli T-RFs wyróżniono fragmenty wspólne ("mikrobiom rdzeniowy") oraz unikalne ("mikrobiom satelitarny") dla poszczególnych sposobów nawożenia, a ich rozkład zaprezentowano w postaci diagramów Venna. Najmniejszą liczbę T-RFs obserwowano w zbiorowiskach bakterii (Rys. 6A), przy czym mikrobiom rdzeniowy stanowił 37,83% całej puli fragmentów. Liczba T-RFs była wyższa w wariancie FA100 niż FA60 (FC-29, FA100-24, FA60-20). Największą liczbą "rdzeniowych" i jednocześnie najmniejszą "satelitarnych" T-RFs charakteryzowały się zbiorowiska archeonów (Rys. 6B), co może wskazywać na stabilność tych mikroorganizmów w obliczu nowego sposobu nawożenia. Jednocześnie, jedynie dla tej grupy całkowita liczba T-RFs uległa zwiększeniu w wariantach FA100 i FA60 w porównaniu z glebą kontrolną (FC-51, FA100-57, FA60-52). Zbiorowiska grzybów prezentowały największą liczbę T-RFs unikalnych dla danego sposobu nawożenia (Rys. 6C). Podobny trend co w przypadku bakterii zaobserwowano również dla grzybów, a mianowicie w wariancie FA100 liczba T-RFs była wyższa niż w wariancie FA60 (FC-54, FA100-47, FA60-41). Uzyskane wyniki sugerują, że preferowanym sposobem nawożenia jest zastosowanie dawki optymalnej fosforowego nawozu wzbogaconej mikrobiologicznie. Struktura zbiorowisk mikroorganizmów pod względem liczby wspólnych T-RFs była bardziej podobna pomiędzy wariantami FA100-FA60, na co wskazują najwyższe wartości współczynników Jaccarda i Sorensena.

Identyfikacja na podstawie wielkości wybranych T-RFs wykazała obecność mikroorganizmów należących do różnych rodzajów, w tym mikroorganizmów ważnych z punktu widzenia zachowania równowagi ekologicznej w glebie i poprawy jej jakości, np: *Pseudomonas* spp. (synteza fitohormonów) (Egamberdieva i in., 2017), *Bacillus* spp. (łagodzenie skutków stresów biotycznych i abiotycznych) (Radhakrishnan i in., 2017), *Bradyrhizobium* spp. (wiązanie azotu atmosferycznego) (de Matos i in., 2021), *Burkholderia* spp. (bioremediacja) (Morya i in., 2020), *Cordyceps* spp. i *Metarhizium* spp. (entomopatogeny) (Bogdányi i in., 2019; Vongsangnak i in., 2017), *Suillus* spp. i *Paraglomus* spp. (grzyby mykoryzowe) (Błaszkowski i in., 2012; Pérez-Pazos i in., 2021) oraz *Trichoderma* spp. i *Penicillium* spp. (grzyby promujące wzrost i rozwój roślin) (Radhakrishnan i in., 2014; Zhang i in., 2018). Identyfikacja mikroorganizmów w obrębie T-RFs sklasyfikowanych jako "mikrobiom rdzeniowy" została przedstawiona na mapach cieplnych (Rys. 5A-C).



Rys. 5. Mapy cieplne ilustrujące liczbę terminalnych fragmentów restrykcyjnych (T-RFs), ich wielkość oraz względną obfitość dla zbiorowisk bakterii (A), archeonów (B) oraz grzybów (C). Na mapach cieplnych przedstawiono identyfikację mikroorganizmów na podstawie wielkości T-RFs dla fragmentów sklasyfikowanych jako "mikrobiom rdzeniowy". Objaśnienia jak na Rysunku 2.



Rys. 6. Diagramy Venna ilustrujące rozkład oraz liczbę wspólnych i unikalnych terminalnych fragmentów restrykcyjnych (T-RFs) dla zbiorowisk bakterii (A), archeonów (B) i grzybów (C) oraz identyfikację mikroorganizmów na podstawie wielkości wybranych T-RFs. Objaśnienia jak na Rysunku 2. J - współczynnik Jaccarda, S - współczynnik Sorensena.

4.4.3.4. Charakterystyka różnorodności genetycznej zbiorowisk mikroorganizmów przy pomocy sekwencjonowania następnej generacji

Analiza metataksonomiczna ujawniła różnice w strukturze zbiorowisk bakterii i archeonów w glebie poddanej działaniu fosforowego bionawozu. Na poziomie typu badana gleba była zdominowana przez Actinobacteriota (25,43%-36,21%), Proteobacteria (20,56%-26,59%), Acidobacteriota (12,38%-16,55%) i Chloroflexi (5,81%-8,37%) (Rys. 7A). Przedstawiciele Actinobacteriota i Proteobacteria występują powszechnie w glebach (Gao i in., 2019; Zhang i in., 2019) , podczas gdy Acidobacteriota zasiedlają środowiska o niskim pH i ubogie w składniki mineralne (Wang i in., 2018). Warto wspomnieć również, że Acidobacteriota uczestniczą w rozkładzie materii organicznej i w obiegu pierwiastków (Kalam i in., 2020). Dominujące klasy obejmowały Alphaproteobacteria (12,74%-15,52%), Actinobacteria (10,58%-16,33%), Gammaproteobacteria (6,92%-11,83%) i Thermoleophilia (9,37%-12,38%) (Rys. 7B). Natomiast jeśli chodzi o rzędy to przeważały Rhizobiales (7,64%-9,58%), następnie Gaiellales (5,85%-7,94%), Burkholderiales (5,09%-7,54%), Vicinamibacteriales (3,16%-6,31%), Gemmatimonadales (3,25%-4,48%) i Solirubrobacterales (3,00%-4,16%) (Rys. 7C). W wybranych wariantach, w których zastosowano bionawozy stwierdzono zwiększoną względną obfitość bakterii należących do różnych poziomów taksonomicznych: Acidobacteriota, Gammaproteobacteria, Vicinamibacteria, Burkholderiales i Vicinamibacterales (warianty FA100 i FA60 we wszystkich terminach), Blastocatellia (FA100(O18, J18, O19) i FA60(J18, J19)) i Rhizobiales (FA60(J18) i FA100(O18)). Wzrost liczebności Blastocatellia zanotowano także w glebie nawożonej preparatem zawierającym bakterie *Ensifer fredii* (Pongsilp i Nimnoi, 2020). Zmiany w strukturze zbiorowisk bakterii mogą wynikać z konkurencji o niszę ekologiczną, zaburzeń równowagi wynikających z wprowadzenia do gleby aktywnych mikroorganizmów oraz oddziaływań pomiędzy rdzennym mikrobiomem a bakteriami zawartymi w bionawozie.

Analiza głównych współrzędnych (PCoA), oparta na odległościach Braya-Curtisa, wykazała wyraźne grupowanie próbek w zależności od sposobu nawożenia i terminu poboru gleby. Próbki pobrane bezpośrednio po aplikacji (bio)nawozów (J18, J19) były odseparowane od próbek pobranych po zbiorach kukurydzy (O18, O19). Co więcej, analiza PCoA wskazała na oddzielenie wariantów kontrolnych od wariantów FA100 i FA60 nie tylko w obrębie J18 i J19, ale także O19. Warianty FC(O18) i FA100(O18) tworzyły jedną grupę, natomiast druga grupa obejmowała warianty FA100(O18) oraz FA100-FA60(O19) (Rys. 7D). Z drugiej

strony, rozkład próbek w kołowym diagramie UPGMA ujawnił, że głównym czynnikiem grupującym był sposób nawożenia. Warianty FA100 i FA60 ze wszystkich terminów (z wyjątkiem FA60(O18)) uformowały jedną grupę, w obrębie której warianty FA100 były oddzielone od wariantów FA60 (Rys. 7F).

O wzroście bioróżnorodności wśród zbiorowisk bakterii może świadczyć zwiększenie wartości wskaźnika Shannona (H) w wariantach FA60(J18), FA100-FA60(O18), FA100-FA60(J19) i FA100(O19) w porównaniu z odpowiadającymi kontrolami (Rys. 7E). Zwiększenie wartości wskaźnika Shannona odnotowano także w glebie nawożonej bionawozami zawierającymi *Bacillus subtilis* (Wang i in., 2021b) oraz *Trichoderma* sp. (Zhang i in., 2022). Wzrost bioróżnorodności może oznaczać wykształcenie mikrobiomu wykazującego większą odporność na stresy środowiskowe (Pang et al., 2017).

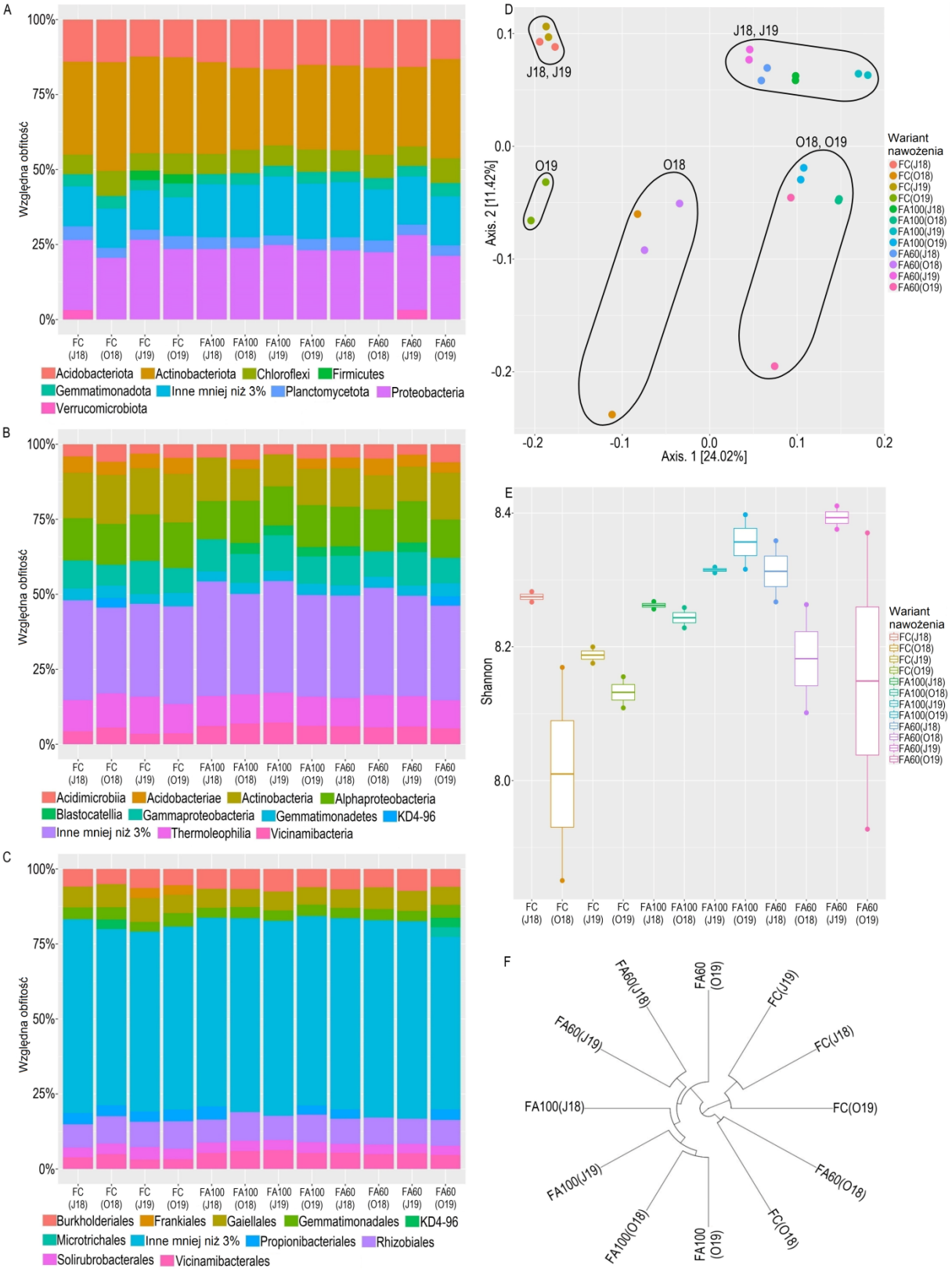
W przewidywanym profilu funkcjonalnym zbiorowisk bakterii najwyższa liczba sekwencji została przypisana do następujących klas funkcjonalnych: metabolizmu (~81,09%), w dalszej kolejności do procesowania informacji genetycznej (~12,08%), procesów komórkowych (~4,12%) oraz procesowania informacji środowiskowej (~2,04%). Ścieżki związane z układami organizmów oraz chorobami ludzkimi reprezentowały najmniej liczne główne klasy KEGG i stanowiły odpowiednio ~0,38% i ~0,30% wszystkich zidentyfikowanych sekwencji (Rys. 8A). Wysoka liczba OTU związanych ze szlakami metabolicznymi różnych związków może sugerować, że procesy te są fundamentalne dla prawidłowego działania ekosystemu glebowego i mogą stanowić wskaźnik stanu gleby (Qi i in., 2022), podobnie jak procesy związane z procesowaniem informacji genetycznej.

Aplikacja fosforowego bionawozu spowodowała wzrost liczby sekwencji w wariancie FA100(J18), natomiast w wariantach FA100(J19) i FA60(J19) zaobserwowano obniżenie liczby funkcjonalnych operacyjnych jednostek taksonomicznych w porównaniu z glebą kontrolną. Niemniej jednak, bezpośrednio po zastosowaniu bionawozów w czerwcu 2019 roku, liczba OTU uległa zwiększeniu względem próbek pobranych w czerwcu 2018 i październiku 2018 roku. W wariantach FA100 i FA60 w październiku 2018 i październiku 2019 odnotowano tendencję wzrostową w liczbie sekwencji związanych z głównymi klasami KEGG na tle wariantów kontrolnych. Ponadto, warianty FA100(O18) i FA100(O19) wykazywały odpowiednio o około 150% i 250% większą liczbę OTU niż odpowiadające im warianty kontrolne FC(O18) i FC(O19). Liczba funkcjonalnych OTU utrzymywała się na

podobnym poziomie pomiędzy wariantami FA100(J18)-FA100(O18) oraz FA100(J19)-FA100(O19). Ogółem, całkowita liczba sekwencji była wyższa w roku 2019 niż w roku 2018 (Rys. 8B). Metabolizm stanowił najbardziej zróżnicowaną główną klasę KEGG i obejmował 11 podklas, z największą liczbą OTU przypisanych do procesów związanych z przemianami aminokwasów i węglowodanów, następnie kofaktorów i witamin oraz terpenoidów i poliketydów. Stosunkowo wiele sekwencji przypisano także do biodegradacji i metabolizmu ksenobiotyków. Z kolei ścieżki powiązane z biotransformacjami nukleotydów i glikanów charakteryzowały się najniższą liczbą OTU (Rys. 8C). Ważne szlaki, z punktu widzenia poprawy jakości i zdrowia gleb, stanowią procesy związane z degradacją ksenobiotyków, związków powodujących zanieczyszczenie gleb oraz szlaki syntezy metabolitów wtórnych takich jak antybiotyki (Malla i in., 2018; Pirttilä i in., 2021), które mogą poszerzać antagonistyczne uzdolnienia mikroorganizmów skierowane przeciwko fitopatogenom. Biorąc pod uwagę pozostałe główne klasy KEGG, procesy komórkowe były zdominowane przez sekwencje związane ze wzrostem i śmiercią komórki oraz z jej ruchliwością, procesowanie informacji środowiskowej obejmowało głównie OTU przypisane do transportu membranowego, natomiast OTU związane z fałdowaniem, sortowaniem i degradacją, replikacją i naprawą oraz translacją były najliczniejsze w obrębie procesowania informacji genetycznej. Dla każdej podklasy liczba funkcjonalnych sekwencji uległa zwiększeniu w wariantach FA100(O18-O19) i FA60(O18-O19) w porównaniu do analogicznych kontroli (Rys. 9). W innych badaniach, po aplikacji bionawozów, obserwowano wzrost liczby sekwencji związanych z transportem i metabolizmem lipidów, ruchliwością komórki, syntezą metabolitów wtórnych (Qi i in., 2022), metabolizmem i degradacją ksenobiotyków (Wang i in., 2021b), transdukcją sygnału oraz metabolizmem nukleotydów (Tian i in., 2022).

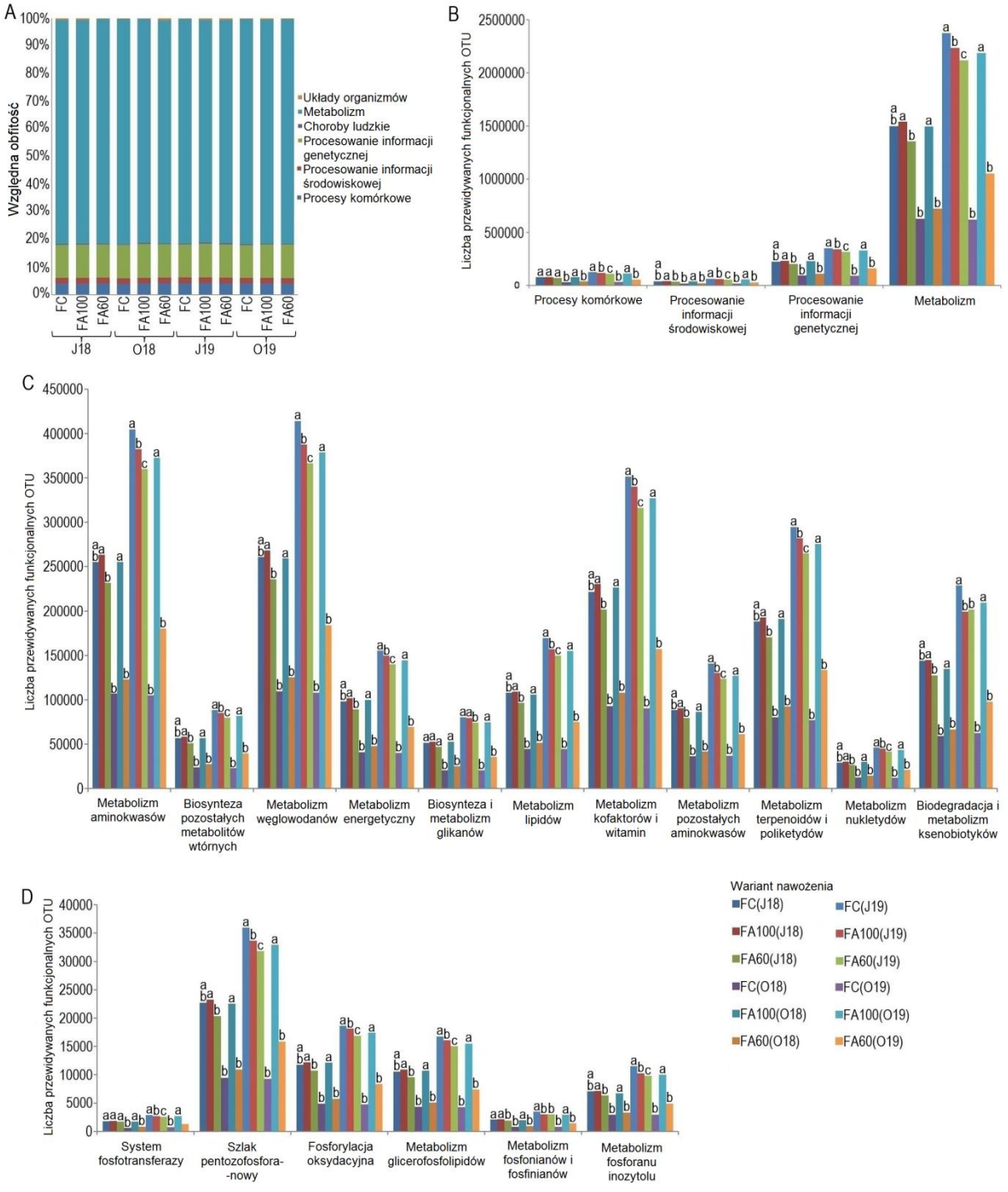
Aplikacja fosforowego bionawozu miała również wpływ na procesy biologiczne związków fosforu. Wyróżniono sześć szlaków biochemicznych związanych z fosforem, które pełnią istotne funkcje dla prawidłowego działania komórek: system fosfotransferazy (transport węglowodanów) (Erni, 2013), szlak pentozofosforanowy (synteza NADPH) (Rytter i in., 2021), fosforylacja oksydacyjna (synteza ATP) (Huang i in., 2019), metabolizm glicerofosfolipidów (formowanie membran komórkowych) (Kondakova i in., 2015), metabolizm fosfonianów i fosfinianów (związki mogące stanowić źródło fosforu) (Tapia-Torres i in., 2016) oraz metabolizm fosforanu inozytolu. Podobnie jak w przypadku głównych klas i podklas KEGG, liczba funkcjonalnych OTU związanych z przemianami fosforu

utrzymywała się na wyższym poziomie w wariantach FA100 i FA60 w terminach jesiennych (O18 i O19) (Rys. 8D).

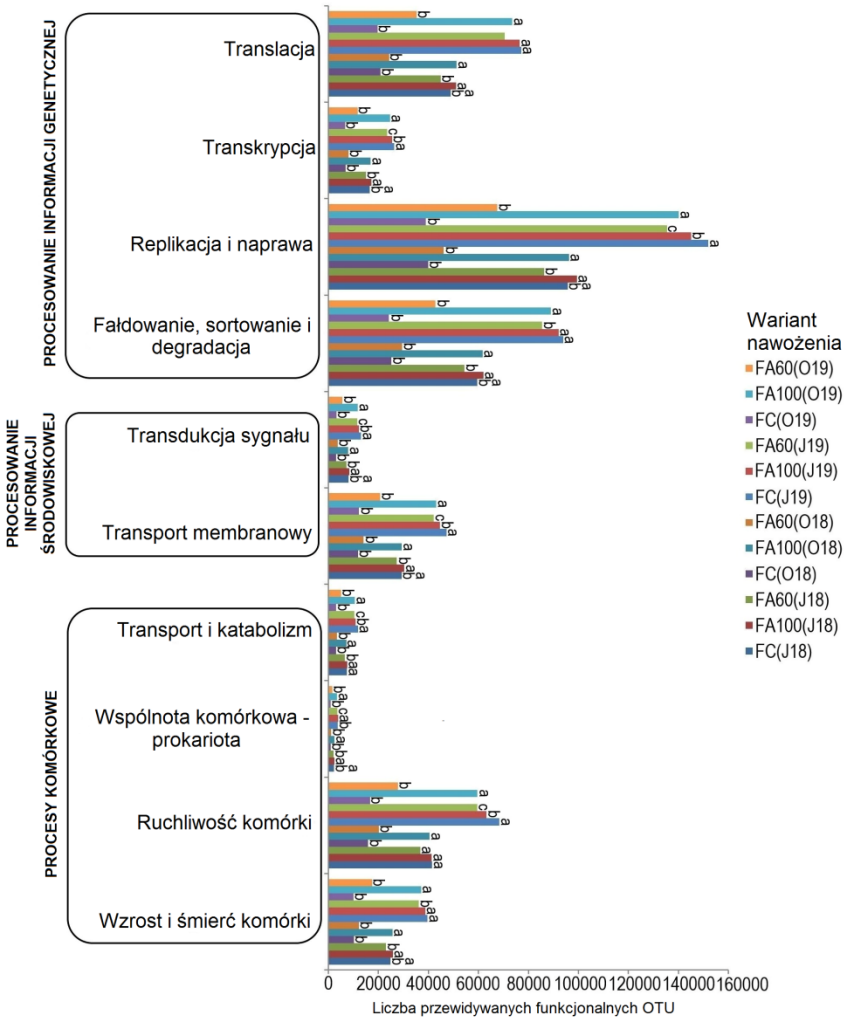


Rys. 7. Wpływ fosforowego bionawozu na różnorodność genetyczną zbiorowisk bakterii i archeonów. (A) - względna obfitość dominujących typów bakterii, (B) - względna obfitość dominujących klas bakterii, (C) - względna obfitość dominujących rzędów bakterii, (D) - analiza głównych współrzędnych (PCoA) na podstawie odległości Braya-Curtisa, (E) - wskaźnik bioróżnorodności Shannona, (F) - dendrogram UPGMA skonstruowany na podstawie odległości Braya-Curtisa. Objaśnienia: FC - dawka optymalna bez wzbogacenia mikrobiologicznego, FA100 - dawka optymalna wzbogacona

mikrobiologicznie, FA60 - dawka zredukowana o 40% wzbogacona mikrobiologicznie, J18 - czerwiec 2018, O18 - październik 2018, J19 - czerwiec 2019, O19 - październik 2019.



Rys. 8. Przewidywany profil funkcjonalny zbiorowisk bakterii. (A) - względna obfitość sekwencji związanych z głównymi klasami KEGG, (B) - liczba funkcjonalnych operacyjnych jednostek taksonomicznych przypisanych do głównych klas KEGG, (C) - liczba funkcjonalnych operacyjnych jednostek taksonomicznych przypisanych do procesów metabolicznych związków należących do różnych grup, (D) - liczba funkcjonalnych operacyjnych jednostek taksonomicznych przypisanych do procesów związanych z przemianami fosforu. Różne litery oznaczają różnice istotne statystycznie ($p < 0,05$) obliczone dla każdego terminu oddzielnie. Objaśnienia jak na Rysunku 7.



Rys. 9. Liczba przewidywanych funkcjonalnych operacyjnych jednostek taksonomicznych przypisanych do szlaków związanych z procesowaniem informacji genetycznej, procesowaniem informacji środowiskowej oraz procesami komórkowymi. Różne litery oznaczają różnice istotne statystycznie ($p < 0,05$) obliczone dla każdego terminu oddzielnie. Objaśnienia jak na Rysunku 7.

Strukturę zbiorowisk grzybów analizowano, podobnie jak w przypadku bakterii i archeonów, na trzech poziomach taksonomicznych. Na poziomie typu dominowały Ascomycota (46,63%-66,86%), Mortierellomycota (8,16%-21,95%) i Basidiomycota (>3,00%-22,92%) (Rys. 10A). Ascomycota uczestniczą w rozkładzie materii organicznej (Ma i in., 2013), Basidiomycota tworzą symbiotyczne oddziaływania z roślinami (Wang i in., 2022), a przedstawiciele Mortierellomycota mogą uruchamiać fosfor z trudno rozpuszczalnych źródeł (Ozimek i Hanaka, 2021). Najliczniejszą klasę stanowiły Sordariomycetes (20,48%-39,26%), następnie Mortierellomycetes (8,16%-21,95%), Dothideomycetes (3,63%-16,91%), Agaricomycetes (>3,00%-21,45%) i Eurotiomycetes (3,32%-11,43%) (Rys. 10B). Na poziomie rzędu dominowały grzyby należące do Hypocreales (11,31%-23,75%), Mortierellales (8,16%-21,95%), Sordariales (3,64%-14,30%)

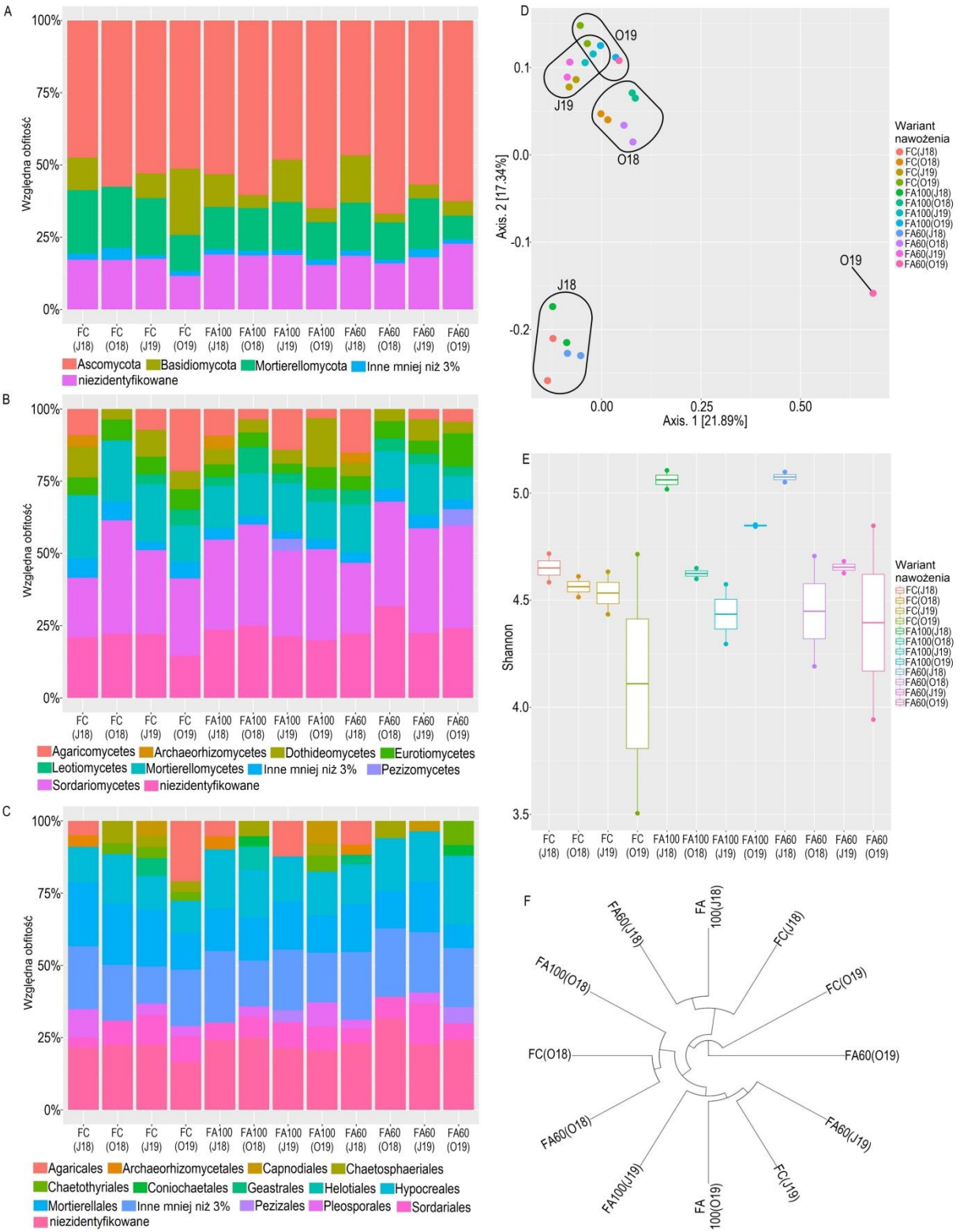
i Agaricales (>3,00%-20,91%) (Rys. 10C). W wybranych wariantach, w których zastosowano bionawozy uległa zwiększeniu względna obfitość Ascomycota ((FA100(J18), FA100-FA60(O18), FA60(J19) i FA100-FA60(O19)), Basidiomycota ((FA100-FA60(J18), FA100-FA60(O18) i FA100(J19)), Agaricomycetes ((FA100-FA60(J18), FA100(O18, J19)), Eurotiomycetes ((FA100-FA60(O19)), Leotiomycetes ((FA100-FA60(J18, O18)), Pezizomycetes ((FA100(J19), FA60(O19)), Sordariomycetes ((FA100-FA60(J18, J19, O19), Agaricales ((FA100-FA60(J18), FA100(J19)), Coniochaetales ((FA100(O18), FA60(O19)), Helotiales ((FA100(O18)), Hypocreales ((FA100-FA60(J18), FA60(O18), FA100-FA60(J19, O19)), Pezizales ((FA100(J19), FA60(O19)), Pleosporales ((FA100(O19)) i Sordariales ((FA100-FA60(J18), FA60(J19)). Zmiany we względnej obfitości zbiorowisk grzybów na różnych poziomach taksonomicznych mogą wynikać z różnego tempa rozwoju mikroorganizmów oraz związanego z tym zjawiskiem wyczerpania składników mineralnych (Yin i in., 2022). Wzrost względnej obfitości Ascomycota w terminach O18 i O19 może wynikać z nagromadzenia materii organicznej w glebie i ze zwiększonej aktywności grzybów zaangażowanych w jej dekompozycję. Zwiększoną liczebność grzybów Ascomycota zanotowano także w glebie nawożonej preparatem zawierającym bakterie *Bacillus* spp. (Cao i in., 2022). Fosforowy bionawóz stymulował występowanie niektórych taksonów, wśród których można wyróżnić mikroorganizmy wykazujące pozytywny wpływ na jakość i zdrowie gleb, np: Helotiales (grzyby saprofityczne i mykoryzowe) (Wang i in., 2006), Sordariales (saprotrofy oraz mikroorganizmy wykazujące aktywność przeciwgrzybiczą) (Luo i in., 2022) oraz Agaricales (saprotrofy syntetyzujące enzymy hydrolityczne rozkładające lignocelulozę) (Ruiz-Dueñas i in., 2020).

Analiza PCoA wykazała grupowanie próbek w zależności od terminu poboru gleby, z wyraźnym wyodrębnieniem terminu J18 od O18, J19 i O19. Próbki pobrane w 2019 roku (J19 i O19) były położone stosunkowo blisko siebie w płaszczyźnie PCoA (Rys. 10D). Taki układ próbek może wskazywać, że fosforowy bionawóz silniej oddziaływał na zbiorowiska grzybów podczas pierwszej aplikacji bionawozu, a w następnych terminach mikroorganizmy dostosowały się do nowego sposobu nawożenia. Grupowanie próbek w zależności od terminu potwierdziło się również na podstawie analizy dendrogramu kołowego UPGMA, w którym próbki gleby zebrane w terminach J18, O18 i J19 tworzyły trzy wyraźnie oddzielone skupienia. Co więcej, większość próbek zebranych w następujących po sobie terminach O18, J19 i O19 tworzyła jedno zgrupowanie (Rys. 10F).

W obrębie zbiorowisk grzybów wartość wskaźnika bioróżnorodności Shannona wzrosła w wariantach FA100-FA60(J18), FA100(O18), FA60(J19) i FA100-FA60(O19) w porównaniu do odpowiadających kontroli. Podobny efekt zaobserwował Zhao i in. (2022), po inokulacji nasion soi bakterią *Bradyrhizobium japonicum* 5038 oraz mieszanką szczepów *Bradyrhizobium japonicum* 5038 and *Bacillus aryabhattai* MB35-5. Najwyższe wartości wskaźnika H odnotowano w wariantach FA100(J18) i FA60(J18) (Rys. 10E).

Analiza profilu funkcjonalnego zbiorowisk grzybów przy pomocy narzędzia FUNGuild wykazała, że w glebie typu Abruptic Luvisol dominację przedstawicieli następujących poziomów troficznych: "patotrof-saprotrof-symbiotrof" (15,79%-44,58%), "saprotrof" (22,25%-38,91%) oraz "saprotrof-symbiotrof" (18,72%-33,52%) (Rys. 11A). W wybranych wariantach, w których zaaplikowano fosforowy bionawóz, w porównaniu z glebą kontrolną zwiększyła się względna obfitość mikroorganizmów zaliczanych do "saprotrofów" (FA100(J18), FA60(O18) i FA100(O19), "saprotrofów-symbiotrofów" (FA100-FA60(O19)) i "symbiotrofów" (FA100-FA60(J18), FA100-FA60(O18), FA60(J19) i FA60(O19)). Jednocześnie w wariantach FA100(J19) i FA60(O19) odnotowano obniżenie liczebności grzybów zaklasyfikowanych do poziomu troficznego "patotrof". Głębszy wgląd w profil funkcjonalny ujawnił, że zastosowanie nawozu wzbogaconego mikrobiologicznie zwiększyło względną obfitość niektórych grup ekologicznych (gildii). Były to między innymi saprotrofy rozkładające obornik (FA60(J18), FA60(O18), FA100-FA60(J19)), niezdefiniowane saprotrofy (FA100(J18), FA60(O18), FA100(O19)) oraz saprotrofy zdolne do wzrostu jednocześnie na oborniku i martwej materii roślinnej (FA100(J19), FA100-FA60(O19)) oraz na martwej materii roślinnej i drewnie ((FA60(J18), FA60(O18), FA100(O19)). Zaobserwowano także wzrost względnej obfitości grzybów zdolnych do przeprowadzenia mykoryzy arbuskularnej (FA60(O18), FA60(J19), FA60(O19)), gatunków ektomykoryzowych (FA100(J18), FA60(J18), FA60(J19)), endofitów (FA100(J18)), gatunków ektomykoryzowych wykazujących właściwości saprotroficzne (FA100(O19)) oraz patogenów zwierzęcych ((FA100(O18)) (Rys. 11C). Patogeny roślinne były liczniejsze we wszystkich analizowanych wariantach FA100 i FA60, z wyjątkiem FA100(J19) i FA60(O19), jednak ich względna obfitość zmniejszyła się pomiędzy odpowiadającymi wariantami FA100 i FA60 w roku 2018 oraz pomiędzy analogicznymi wariantami FA60 w roku 2019. Pomimo, że fosforowy bionawóz stymulował występowanie fitopatogenów, zakładamy, że efekt ten może być kontrolowany dzięki obecności mikroorganizmów antagonistycznych oraz

zwiększeniu bioróżnorodności mikrobiomu i mykobiomu. Analiza głównych składowych w oparciu o liczbę operacyjnych jednostek taksonomicznych wskazała na grupowanie wariantów nawożenia w zależności od terminu poboru próbek gleby (Rys. 11B).



Rys. 10. Wpływ fosforowego bionawozu na różnorodność genetyczną zbiorowisk grzybów. (A) - względna obfitość dominujących typów grzybów, (B) - względna obfitość dominujących klas grzybów, (C) - względna obfitość dominujących rzędów grzybów, (D) - analiza głównych współrzędnych (PCoA) na podstawie odległości Braya-Curtisa, (E) - wskaźnik bioróżnorodności Shannona, (F) - dendrogram UPGMA skonstruowany na podstawie odległości Braya-Curtisa. Objaśnienia jak na Rysunku 7.

Rys. 11. Profil funkcjonalny zbiorowisk grzybów uzyskany przy pomocy narzędzia FUNGuild. (A) - względna obfitość poziomów troficznych, (B) - analiza głównych składowych na podstawie liczby operacyjnych jednostek taksonomicznych przypisanych do poszczególnych poziomów troficznych, (C) - względna obfitość grup ekologicznych grzybów (gildii). W analizie uwzględniono gildie, których względna obfitość była ≥0,5%. Objaśnienia jak Rysunku 7.

5. Podsumowanie i wnioski

Nieustanna i silna eksploatacja gleb uprawnych i wynikające z tego zagrożenia dla bioróżnorodności agroekosystemów sprawiły, że poszukiwane są coraz to nowe metody mające na celu ograniczenie negatywnych skutków rolniczej działalności człowieka i jednocześnie pozostające w zgodzie z zasadami zrównoważonego rolnictwa. Mikroorganizmy glebowe prezentują szereg uzdolnień, dzięki którym ich wykorzystanie w formie bionawozów wydaje się atrakcyjnym rozwiązaniem dla utrzymania wysokiej bioróżnorodności i poprawy stanu gleb uprawnych i zdegradowanych. Mimo szerokich możliwości oferowanych przez szczepy pożytecznych bakterii i grzybów, tylko część z nich została wykorzystana w formie preparatów mikrobiologicznych ze względu na ograniczenia obejmujące niską przeżywalność mikroorganizmów w glebie, brak materiałów do produkcji bionawozów oraz niedostępność przepisów określających wymagania jakościowe gotowych produktów. Wymienione czynniki sprawiają, że temat bionawozów i ich implementacji w praktykach rolniczych pozostaje tematem aktualnym i wymagającym wielokierunkowych badań, które pozwolą na dalszy rozwój zrównoważonych metod zarządzania glebą opartych na powiązaniach jakości gleb z jakością mikrobiomów. Dlatego też badania zaproponowane w niniejszej rozprawie doktorskiej miały na celu dostarczenie informacji o korzyściach płynących z aplikacji innowacyjnego fosforowego nawozu mineralnego wzbogaconego szczepami pożytecznych bakterii na bioróżnorodność mikrobiologiczną dwóch różnych typów gleb zdegradowanych.

Na podstawie wyników przeprowadzonych badań można sformułować następujące wnioski:

- Aplikacja fosforowego bionawozu powoduje zmiany w różnorodności funkcjonalnej i genetycznej mikroorganizmów oraz w aktywności enzymatycznej gleby.
- Biorąc pod uwagę analizowane parametry nie stwierdzono negatywnego wpływu fosforowego bionawozu na zbiorowiska mikroorganizmów zasiedlające zdegradowane gleby typu Brunic Arenosol oraz Abruptic Luvisol.
- Zarówno dawka optymalna wzbogacona mikrobiologicznie (FA100), jak i dawka zredukowana o 40% zawierająca mikroorganizmy (FA60) przynoszą wymierne korzyści po ich zastosowaniu, wpływając na poprawę życia biologicznego w glebach zdegradowanych.

- Na różnorodność genetyczną i funkcjonalną zbiorowisk mikroorganizmów ma wpływ nie tylko sposób nawożenia, ale również termin poboru próbek gleby.
- Fosforowy bionawóz oddziałuje pozytywnie nie tylko na zbiorowiska mikroorganizmów, ale również na ilość dostępnego fosforu oraz plonowanie kukurydzy, co świadczy o jego kompleksowym działaniu.
- Aplikacja fosforowego bionawozu zwiększa aktywność enzymatyczną gleby oraz pozwala na utrzymanie tego efektu w czasie.
- Fosforowy bionawóz stymuluje występowanie mikroorganizmów wykazujących pożyteczne uzdolnienia w kierunku wzrostu i rozwoju roślin oraz poprawy jakości gleb zdegradowanych.
- Aplikacja fosforowego bionawozu zwiększa potencjał metaboliczny zbiorowisk mikroorganizmów glebowych.
- Aplikacja fosforowego bionawozu zwiększa liczbę operacyjnych jednostek taksonomicznych związanych z procesami komórkowymi i metabolizmem substancji należących do różnych grup związków chemicznych.
- Fosforowy nawóz mineralny wzbogacony mikrobiologicznie jest efektywnym środkiem do poprawy parametrów mikrobiologicznych gleb zdegradowanych i może być wykorzystywany w zrównoważonym rolnictwie.

6. Tekst publikacji P.1

CHAPTER TWO

Biofertilizers in agriculture: An overview on concepts, strategies and effects on soil microorganisms

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Contents

Abstract

Biofertilizer is a substance containing live microorganisms which exhibit beneficial properties toward plant growth and development. Various mechanisms are used by microbial strains in order to enhance nutrient uptake, improve soil fertility and increase crop

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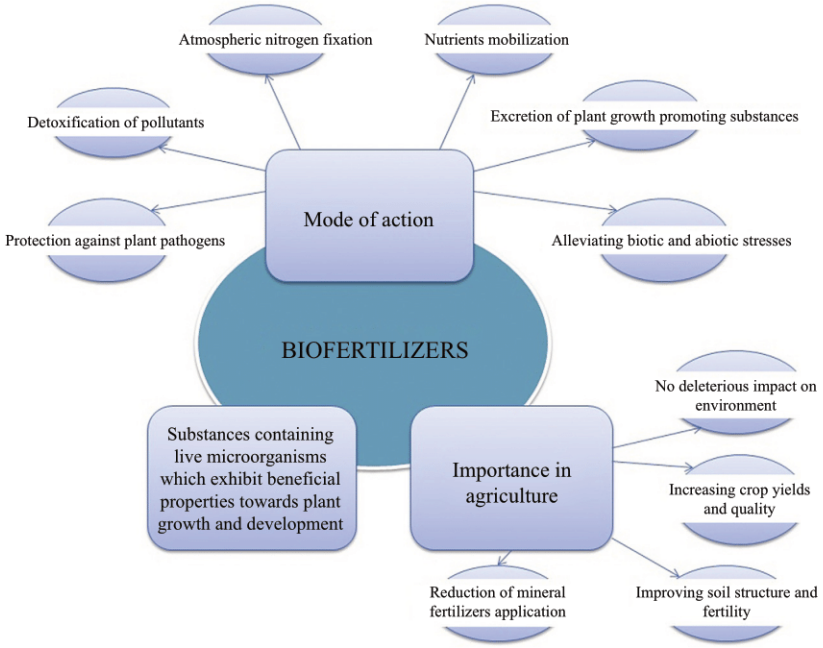
yields such as nitrogen fixation, potassium and phosphorus solubilization, excretion of phytohormones, production of substances suppressing phytopathogens, guarding plants from abiotic and biotic stresses and detoxification of belowground pollutants. Taking into consideration growing consumption requirements on Earth and hazards arising from the excessive use of chemical fertilizers and pesticides, biofertilizers are thought to be a promising and non-toxic alternative to synthetic agro-chemicals, including fungal control and minimization of mycotoxins contamination. The implementation of microbial inoculants is considered to overcome the shortcomings associated with chemical-based farming techniques, therefore research into widespread use of biofertilizers is one of the mainstream in scientific work for the development of sustainable agriculture.

1. Introduction



Currently, the global population is still increasing and it is estimated that around 2050 it will reach approximately 9.7 billion people living in the world (Ehrlich and Harte, 2015). This rapid growth is inextricably associated with intensive industrialization, urbanization and agricultural production. Due to the expanding global population, traditional agriculture is indispensable to meet the nutritional requirements of humanity, that is predicted to

achieve 321 million tons of food grain by 2020, and make countries self-sufficient in food production (Gizaki et al., 2015; Mahanty et al., 2016; Santos et al., 2012). However, the conventional farming methods mainly based on the widespread use of synthetic fertilizers and pesticides for plant nutrition and disease treatment (Vasile et al., 2015). Judicious dosage of these chemical inputs have an incontrovertible advantages not only for the plant growth, crop production and quality, but also for the farmers' income. Unfortunately, the increased use of artificial supplies may pose a considerable threat to the natural environment by contaminating water, air and soil (Rahman and Zhang, 2018). The mindless application of agro-chemicals and lack of biodegradation ability lead to their belowground accumulation, which result in unfavorable change of soil parameters in matters of its structure, fertility and water holding capacity (Savci, 2012). The excessive application of synthetic fertilizers is also closely linked to the eutrophication of water resources, greenhouse effect (Goenadi et al., 2018; Liu et al., 2014; Youseff and Eissa, 2014) and the toxic build-up of heavy metals such as arsenic, cadmium and plumbum (Atafar et al., 2010). What is worth mentioning, long-continued use of mineral fertilizers may lead to the decline of nutrients content in soil and make the crops more susceptible to various diseases (Aktar et al., 2009). Organic farming is an alternative to conventional agriculture and helps reduce dependence on artificial plant protection inputs in crop production. It combines ecofriendly agronomic practices and enables, in assumption, food production, which is deprived of contaminations (e.g., potentially toxic trace elements, leftovers of plant agents) and ensures maintenance high quality and biodiversity of soils. The use of chemical fertilizers and harmful pesticides is excluded (Niggli, 2015). Nowadays, the production and consumption of ecological food is still growing and organic agriculture is one of the fastest developing branches of agriculture (Brenes-Muñoz et al., 2016). The area of organic agricultural land has recorded impressive growth from 11 million hectares in 1999 to 57.8 million hectares in 2016 (Willer and Lernoud, 2018).

The aforementioned hazardous effects of overloading soil with synthetic agro-chemicals and raising consumers' awareness toward protection of natural environment and human health contributed to exploration of solutions which would be equally efficient but would not endanger terrestrial ecosystems (Geiger et al., 2010). Introduction of environmentally friendly agricultural techniques is supported by recent European Union policies (EU Regulation 2018/848). A agenda to reduce water pollution arising from nitrates from agricultural sources and to prevent further pollution

has been also implemented (Council Directive 91/676/EEC, 1991). One of the ways for sustainable farming to minimize problems arising from the excessive mineral fertilization is application of biofertilizers containing strains of beneficial microorganisms (Mishra and Dash, 2014). These microbial strains have a promising potential in agriculture as they improve plant growth and development through the increasing native nutrient (N, P, K, S, Zn) bioavailability and production substances which exhibit antibacterial and/or antifungal activity, and thus can be an alternative in minimization of mycotoxins contamination (Toyota and Watanabe, 2013). Furthermore, application of biofertilizers is known to enhance the activity of indigenous soil microorganisms and accelerate microbial processes occurring in the soil (Raja, 2013), release plant growth stimulants, protect plants from abiotic and biotic stresses, improve soil quality in terms of its biological, chemical and physical properties, convert complex chemical compounds into easy assimilable forms (El-Lattief, 2016) and detoxify of pollutants presented in soil, such as heavy metals (Siddiquee et al., 2013), 1,4-dichlorobenzene (Pant et al., 2016), pentachlorophenol (Singh et al., 2009), atrazine (Pelcastre et al., 2013) or pesticide mixtures (Fragoeiro and Magan, 2008). Biofertilizers comprise an inexpensive, environmentally friendly, and renewable source of plant nutrients in comparison with synthetic chemicals, therefore they gain worldwide popularity and importance in crop production (Swapna et al., 2016). The purpose of this review is to summarize the beneficial impact of rhizosphere microorganisms on agriculturally important plants and to emphasize the role of biofertilizers in sustainable agriculture.

2. Definitions of biofertilizers and their role in agriculture

Biofertilizer is a term that can be interpreted in various ways (El-Ghamry et al., 2018). It is not difficult to find definitions identifying biofertilizer as a seaweed extracts, composed urban wastes, microbial mixtures with unidentified constituents or mineral fertilizer products enriched with organic compounds. Interestingly, the scientific research papers present a very broad interpretation of this term, representing everything from green manures, through animal manures, to plant extracts (Vessey, 2003).

The concept of biofertilizer has changed along with the state of knowledge about associations occurring between the soil microorganisms and plants. According to Okon and Labandera-Gonzalez (1994) substance

which improve exploitation of nutrients presented in soil, but do not replace them (like mineral fertilizers) should not be determined as biofertilizer, but as inoculant. In 2003 Vessey determined biofertilizer as "a substance which contains living microorganisms, which, when applied to plant surfaces, seeds or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of essential nutrients to the host plant." This definition of biofertilizer does not comply with the above mentioned definition proposed by Okon and Labandera-Gonzalez (1994) and is a contraction of the term "biological fertilizer". Later, in 2005, Fuentes-Ramirez and Caballero-Mellado defined a biofertilizer as "a product that contains living microorganisms, which exert direct or indirect beneficial effects on plant growth and crop field through different mechanisms." With reference to their definition, products containing beneficial microorganisms, used for suppressing phytopathogens, could also be determined as biofertilizers, but these are rather called as biopesticides (Fuentes-Ramirez and Caballero-Mellado, 2005). Likewise, microorganisms enhancing plant growth by synthesis of phytohormones are considered as phytostimulators or bioenhancers, while those that possess the ability of biodegradation organic pollutants are referred to rhizoremediators (Somers et al., 2004). Therefore, not all microbial inoculants should be identified directly as biofertilizers (Bhattacharyya and Jha, 2012).

From the scientific point of view biofertilizer is an individual microorganism exerting plant growth promotion properties, but in the agronomical context this term pertain to product composed of beneficial strain(s), which are useful regarding nutrient mobilization, included in a carrier, possessing features that allow its storage at the time specified by the producer, and ready to effective application to the soil or plant. In this angle, biofertilizer can also enable addition of substances that contribute to the improvement of microorganisms' activity. Term "biofertilizer" should not be used interchangeably not only with terms such as plant or animal manure, intercrop or fertilizers referring to combination of mineral and organic compounds, but also with biostimulants derived from microorganisms (products based on dead microbial cells or extracts of microbial origin) (Malusá and Vassilev, 2014; Reddy, 2014).

The main role of biofertilizers application is promoting plant growth without deleterious side effects for environment and increasing harvest yields (Mishra et al., 2013). According to study conducted by Schütz et al. (2018), inoculation with biofertilizers increased crop yield averagely by 16.2% in comparison with non-inoculated controls. Microbial biofertilizers plays a

crucial role in maintaining soil fertility on the appropriate level and improving its structure by influencing the aggregation of the soil particles (Rashid et al., 2015). They also contribute to the better plant-water relation (Xiang et al., 2012), provide protection against drought, make plants less prone to some soil-borne diseases, including causing by fungi that additionally produce mycotoxins (Simarmata et al., 2016) and reduce the incidence of insect pests (Dey et al., 2014). Even though biofertilizers are a commercially promising approach in sustainable agriculture, there are few drawbacks making them less competitive, such as limited shelf life, lack of the suitable materials for production, increased sensitivity to high temperature and difficulties connected with the storage and transportation (Patil and Solanki, 2016). Apart from that, microbial fertilizers require higher amounts to provide plants with enough nutrient content, their effectiveness depends on the soil conditions prevailing in the application zone and results of their action are noticeable after prolonged use (Jangid et al., 2012). However, new technologies are being developed to overcome the disadvantages associated with the application of biofertilizers in agricultural ecosystems (García-Fraile et al., 2015).

3. Types of bioformulations

Appropriate formulation is one of the key factors for biofertilizers containing living microbial cells, affecting the quality of biological agent. Formulation is determined as a process during which selected microbial strain is unified with carrier (Bargaz et al., 2018). Formulated product consists of active ingredient established in a suitable carrier, frequently with additives that are responsible for stabilization and protection of microorganisms during storage and transportation (Namasivayam et al., 2014). A good formulation provide effective introduction of microorganisms at the target site and enhance their activity so as to attain the maximal profits after inoculation to the host plant. It is compulsory that the formulation should maintain stability during production and distribution. Moreover, it also ought to be simple to deal with and use by the farmers, must deliver microorganisms in the most suitable way and form and protect them from harsh environmental factors in order to maintain their good physiological state as long as possible (Nehra and Choudhary, 2015). Cost-effectiveness is another meaningful aspect that should be taken into account when choosing the formulated product (Xavier et al., 2004).

On the basis of physical nature and material used as a carrier there are many types of biofertilizers available on the worldwide market: solid

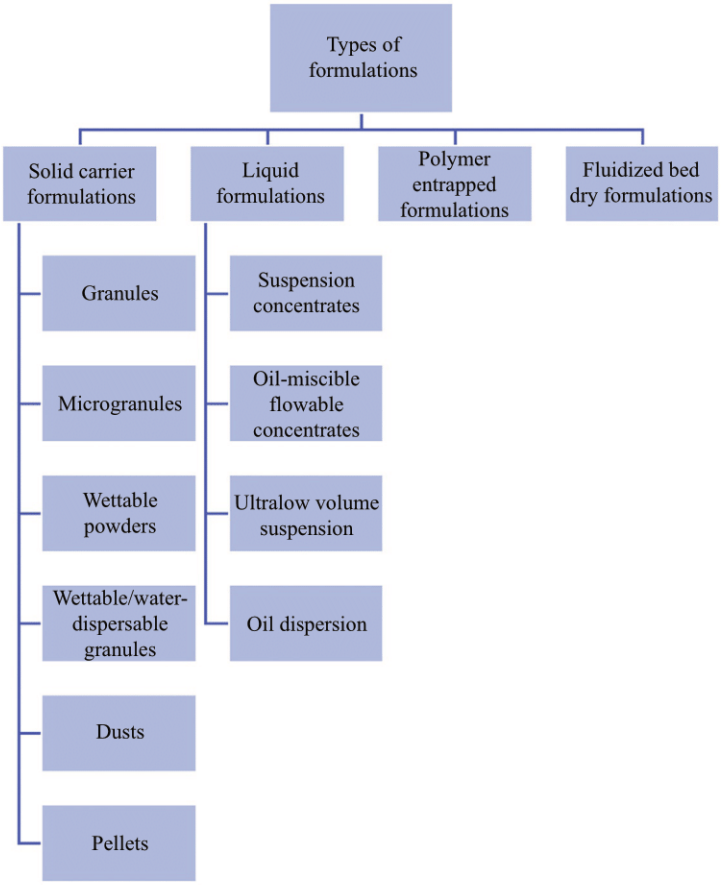


Fig. 1 Types of formulations of biofertilizers.

carrier-based bioformulations, liquid formulations, polymer entrapped formulations and fluidized bed dried formulations (Brahmaprakash et al., 2017). Fig. 1 presents types of formulations used during biofertilizers development.

3.1 Solid-carrier based bioformulations

In solid-carrier based biofertilizers microorganisms are binded with delivery vehicle which is responsible for transferring microbial strains from laboratory to rhizosphere (Patil et al., 2013). Carrier is a delivery material that carry microorganisms in sufficient quantities and keep them in good physiological state under specific conditions. Generally, after the application, beneficial strains presented in biofertilizer have to compete with the indigenous soil

microorganisms for energy source, essential nutrients and habitable space. Materials such as carriers play a significant role in maintaining high survivability of microorganisms and increase the effectiveness of the biofertilizers. Taking into account the high variability of microenvironment prevailing in the soil, they prevent microbial populations from declining by creating a temporary protective surface and providing substrates that promote microbial growth (Arora et al., 2010; Balasubramanian and Karthickumar, 2017).

Due to the fact that carrier constitute the major part of ready-to-use product (by volume or weight) and provide target microorganisms with conducive conditions to their survival, it should meet as many of the characteristics decisive its usability (Siddiq et al., 2018). According to Malusá et al. (2012) a good quality carrier must be non-toxic either to inoculated microbial strains or to the plants, easy to process and free of lump-forming materials and easy to sterilize, e.g., by autoclaving or gamma-irradiation. Furthermore, it must show a suitable physicochemical properties: a high water holding capacity, good pH buffering capability, easy adjustable pH and suitability for as many microbial strains as possible (Zayed, 2016). It has been stated that the carrier must be characterized by the high organic matter content and its structure must permit effortless gas exchange, especially oxygen (El-Fattah et al., 2013). A good carrier also should be economically sustainable; that suggest reasonable costs and availability in adequate amounts (Tabassam et al., 2015). Besides that, carrier ought to guarantee a sufficient shelf life of the commercial biofertilizer (at least 2–3 months in room temperature), addition of nutrients and allow easy handling by the farmers (Bashan et al., 2014).

No one single-carrier possess all these features. The aforementioned properties determine only the potential for a high-quality carrier, but eventually, selection of delivery material must be based on individual properties of microorganisms, physical form of final product, method of planting and equipment used for plant cultivation and, obviously, allowable costs (El-Fattah et al., 2013; Stamenković et al., 2018).

Materials used as a carriers have a various origin (organic, inorganic or may be synthesized from specific molecules) and can be divided into six categories: (I) soils—peat, coal, clays, lignite, (II) plant waste materials—composts, farmyard manure, soybean meal, wheat bran, press mud, (III) inert materials—vermiculite, perlite, bentonite, polyacrylamide gels, alginate beads, (IV) plain lyophilized microbial cultures and oil dried bacteria which enable later incorporation into a solid carrier or can be used as such,

(V) liquid inoculants containing some additives improving properties of final product and (VI) capsule-based carriers—cells in capsules and pelleted spores (Sahu and Brahmaprakash, 2016).

Solid formulations include granules, microgranules, wettable powders, wettable/water dispersible granules, dusts and pellets. They possess certain advantages, e.g., they are cheap, easy to produce and their production does not require high financial outlays (Mishra and Arora, 2016).

Granules are dry particles which consist of microbial active ingredient, a binder, a carrier and are divided on the basis of particle size into coarse particles (100–1000 μm in size) and microgranules (60–100 μm in size). Granules ought to meet the following characteristics: they should be non-dusty and non-caking and they should possess the ability to disintegration in order to release active ingredient in the soil. Granules are easy to store, easy in application and they are non-inhalable thus they are safer to handle. This type of bioformulation is mainly used in soil treatment (Mishra and Arora, 2016; Xavier et al., 2004).

Wettable powders are thought to be the one of the oldest type of bioformulations. They include technical powder (50–80%), filler (15–45%), dispersant (1–10%) and surfactant (3–5%). Due to the fact that they are readily miscible with water, they can be suspended in a liquid carrier just before application on the field (Brar et al., 2006).

Wettable/water-dispersible granules, also determined as a dry flowables, are a solid, non-dusty, free-flowing granular formulation which, when added to water, disperses or dissolves quickly. They have been developed as an improved, safer and commercially more appealing alternative to wettable powders. Similarly to wettable powders, they contain wetting and dispersing agents, but at higher concentrations in comparison with wettable powders (Hazra et al., 2017).

3.2 Liquid bioformulations

Taking into account the disadvantages of carrier-based formulations such as low shelf-life, sensitivity to temperature changes, risk of contamination and relatively low cell counts, liquid formulations has been developed as an alternative to carrier-based biofertilizers. These types of biofertilizers are also determined as flowable or aqueous suspensions and based on broth-cultures, mineral oils, organic oils or oil-in-water suspensions (Bharti et al., 2017). Typically, liquid formulations consists of 10–40% microbial organisms, 1–3% suspender ingredient, 1–5% dispersant, 3–8% surfactant

and 35–65% carrier liquid (water or oil) (Yadav and Chandra, 2014). Liquid biofertilizers are more attractive than carrier-based inoculants due to some characteristics which include longer shelf life (18–24 months), no contamination, easy handling and application and addition of ingredients which improve growth and survival of the microbial strains (Surendra Gopal and Baby, 2016). Furthermore, higher microbial density allow lower dosage of inoculant to obtain the same effects as with the formulations based on solid carriers (Schulz and Thelen, 2008). Unfortunately, liquid formulations have some limits which constrict their common use, even in developed countries. They are deprived of solid-carrier protection and may lose viability shortly after inoculation. Some specific conditions (cool temperatures) are required for long-time storage and, what was reported, liquid biofertilizers exhibit increased sensitivity to environmental stresses (John et al., 2011). Addition of components such as sucrose, glycerol, gum arabic, polyvinylpyrrolidone (PVP) may contribute to the improvement of survivability of microorganisms in liquid formulations (Kaur et al., 2018) due to the mechanisms including inactivation of the toxic substances, a better adhesion of inoculants to the seeds or boosting of the strains survival during storage and under various environmental conditions. These additives are also responsible for stabilization of the biofertilizer after inoculation and seed planting (Tittabutr et al., 2007). According to Lee et al. (2016) liquid inoculant containing *Rhodopseudomonas palustris* strain PS3 improves soil quality and increases Chinese cabbage yield up 40% over control. This bacterium has been reported to detoxify pollutants such as hydrogen sulfide in a paddy rhizosphere (Kornochalert et al., 2014).

Liquid bioformulations encompass the following types: suspension concentrates, oil-miscible flowable concentrate, ultralow volume suspension and oil dispersion (Mishra and Arora, 2016). Suspension concentrates are a solid active ingredient dispersed in water, which should be diluted with water before application. When compared to wettable powders, they have benefits such as absence of dust, ease to use and high effectiveness (Tadros, 2013). Oil-miscible flowable concentrate is a suspension containing active ingredient(s) dispersed in a fluid. Before use, its dilution in an organic liquid must be prepared. Ultralow volume suspension referred to liquid formulations which are ready to use by ultralow volume equipment (aerial or ground equipment generating extremely fine spray) (de Faria and Wraight, 2007; Singh and Merchant, 2013). Oil dispersion is a formulation containing active ingredient suspended in water-immiscible solvent or oil. Oil is known to evaporate much less therefore may remain in contact with target plants for greater time (Mishra and Arora, 2016).

3.3 New possibilities in bioformulations

The significant progress achieved in formulation improvement has led to new possibilities on the field of microorganisms entrapment and immobilization processes that are considered to be promising in agriculture (Herrmann and Lesueur, 2013). Immobilization is a biotechnological tool referring to the various ways of cell attachment or entrapment into a matrix (Vassilev et al., 2017). Flocculation, adsorption on surfaces, covalent binding to carriers, cross-linking of cells, and encapsulation in a polymer gel are widespread methods due to which plant beneficial microorganisms become immobilized (Abdelmajeed et al., 2012). However, encapsulation is regarded as the most attractive approach for constructing carriers of microbial strains in biofertilizers technology (Sivakumar et al., 2014). During this process microbial cells are being covered with protective shell or entrapped within suitable polymeric materials (Sathvika et al., 2018) to beads creation, which are permeable to nutrients, gases, and metabolites (John et al., 2011). Taking into consideration the size of polymeric bead produced, two types of encapsulation techniques have been distinguished: microencapsulation (bead size extending from 1 to 1000 μm, generally less than 200 μm) and macroencapsulation (bead size ranging from few millimeters to centimeters) (Nordstierna et al., 2010).

Generally, encapsulation of beneficial microorganisms seems to be more advantageous than free cell formulations (Young et al., 2006). This is the outcome of nutritive capsule surrounding microbial strains, which allow on gradual release of bacteria in the soil, improve their physiological activity and reduce contamination risk during storage and transport (Schoebitz et al., 2013). Encapsulation can also significantly increase the viability of plant beneficial microorganisms due to providing protection from adverse environmental factors. Consequently, increase in viability is connected with the longer shelf life of the product (Mortazavian et al., 2007). Moreover, encapsulated formulations can be stored even at room temperatures or at 4 °C for comparatively long periods. A study conducted by He et al. (2015) showed that approximately 88.9% of *Raoultella planticola* Rs-2, encapsulated with sodium bentonite and alginate, survived after 6 months of storage. Encapsulation was also used to immobilize strains such as *Klebsiella oxytoca* (Wu et al., 2011), *Azospirillum brasilense* (Trejo et al., 2012), *Bacillus subtilis* (Tu et al., 2016) and *Mesorhizobium* spp. (Alvarez et al., 2010).

In encapsulation, the most frequently used materials are polymers: naturally occurring (polysaccharides, proteinaceous material) as well as synthetic

(polyacrylamide, polyurethane). In terms of chemical structure, the polymers may be homo-, hetero-, or co-polymers. Polymers which are commonly used in the bioencapsulation include agar, starch, chitosan, alginate, gellan gum, gelatin, carrageenan, xanthan gum, milk proteins (casein, whey protein), polyacrylamide and polyvinyl alcohol (Rathore et al., 2013). During selection of the appropriate material, its chemical composition, molecular weight and ability to interact with other components should be taken into account (de Vos et al., 2009). Alginate and polyacrylamide has been used widely as a materials for encapsulation (Flores-Félix et al., 2019) nevertheless alginate gained more popularity, due to the toxicity of polyacrylamide (Date, 2001). Various kinds of cells may be encapsulated such as bacteria, fungal cells or small hyphal segments, therefore this procedure is a promising in production of inoculants based on single or multiple strains, e.g., phosphorus solubilizing biofertilizers—Arbuscular Mycorrhizal Fungi (AMF) or rhizobia—AMF (John et al., 2011).

Recently, a new promising approaches have been found in development of inoculants based on plant beneficial microorganisms. One of them assumes use of microbial biofilms as a potential carrier (Malusá et al., 2012). Biofilm is a complex structure made up of microbial cells embedded in the self-produced matrix which exert adhesion both to biotic and abiotic surfaces and helps microorganisms to alleviate the adverse environmental conditions (Parween et al., 2017a,b). Three main types of biofilm may occur in the soil: bacterial, fungal and fungal-bacterial biofilms. Bacterial, as well as, fungal biofilms may be formed on abiotic surfaces, however in formation of fungal-bacterial biofilm, fungi behave as the biotic surface (Frey-Klett et al., 2011; Guennoc et al., 2018). The major part of microorganisms associated with plant roots are capable of forming biofilm and it has been found that this structure exhibit plant growth promoting properties and enhance nutrient uptake in crops such as rice, wheat, cotton, mung bean, soybean, tomato, leguminous crops and spices (Bidyarani et al., 2016; Castiblanco and Sundin, 2016). The beneficial bacterial biofilms can also be used for protozoan grazing on bacteria and stimulate of bacteria to produce secondary metabolites in order to eliminate phytopathogenic microorganisms and decrease plant diseases (Müller et al., 2013; Weidner et al., 2017). It was also reported that biofilmed-based rhizobium inoculant allow microorganisms to survive at high salinity conditions (400 mM NaCl) by 10^5-fold in comparison with monocultures (Seneviratne et al., 2008). In soybean inoculated with biofilm fungal-rhizobium inoculants, increased nitrogen fixation has been observed as compared to plants inoculated with traditional

rhizobium biofertilizer (Parween et al., 2017a,b). Using PGPR with biofilm forming ability seem to be an interesting strategy to produce and development new form of bioinoculants.

Another technology that could set new trends in carrier-based microbial bioformulations is bionanotechnology (Mishra et al., 2017). This technique is based on the employing nanoparticles with at least one dimension in the size of 100 nm or less, made of inorganic or organic materials. The whole microbial cells may be integrated inside nanostructures, what can have different applications, not only in agriculture (Veronica et al., 2015). Due to the nanotechnology there is a possibility to encapsulate nutrients by nanomaterials or they can be delivered in the form of emulsion (Chhipa, 2017). In this type of fertilizers nutrients can be released in a controlled and gradual way (slowly or quickly) under the influence of environmental factors such as temperature, moisture and acidity or alkalinity of the soil (El-Ghamry et al., 2018). There are some examples of nanofertilizers application in agricultural ecosystems. It has been reported that maize, treated with TiO_2 nanoparticles showed increased growth; titanium nanoparticles contributed to the increased light absorption and photo energy transmission (Dağhan, 2018). In different case, SiO_2 and TiO_2 nanoparticles have been found to enhance the activity of nitrate reductase in soybean; the plant absorption capacity has been also intensified (Khan and Rizvi, 2017). It has been noticed that nonoformulations may enhance the stability of biofertilizers. The addition of hydrophobic nanoparticles made of silica to the formulation containing fungal strain *Lagenidium giganteum* minimize the desiccation of the mycelium. The physical properties of the formulation are also improved and microbial cells maintain viability and activity after 12 weeks of storage at room temperature (Vandergheynst et al., 2007).

Application of nanotechnology is still getting popularity and importance in agriculture in certain categories: improving production rates and yield, applications of nanofertilizers and nanopesticides, nanosensors, treatment of agricultural waste with the use of nano-based technology and increasing the efficiency of resource utilization. Nanomaterials have also exhibited promise effect in plant protection using for development of nanoformulations against plant pathogens (Sharma et al., 2016). Nanofertilization is thought to be more effective as compared to traditional fertilizers owing to the fact that nutrients delivered in this way may be absorbed by plants more efficiently (Sekhon, 2014).

Taking into consideration substantial factors in biofertilizer quality such as reduction of contaminations and limited shelf life of the products,

fluidized bed dried (FDB) formulations have been developed (Sahu et al., 2018). Fluid bed dryer is a dryer in which solid particles or granules are suspended against gravity in an upward flowing warm or hot air stream that creates a fluidized condition (Brahmaprakash and Sahu, 2012). Microbial agents can be sprayed onto blowing carriers before drying or may be delivered as dry mass and then coated with a protective shell in a fluidized bed. When process of drying is finished, the moisture content of bioinoculant is reduced to a level indisposing contaminations to grow and outcompete the target microbial cells. Very low water activity is the reason why constituent microorganisms cannot interact and due to it, nearly constant number of cells may be delivered to the rhizosphere. An example of FDB formulation, the *Pseudomonas fluorescens* with mix of vermiculite and EB™ (clay and wood particles) as solid carriers has been developed (Berninger et al., 2018; Sahu and Brahmaprakash, 2016).

The FDB inoculant formulation has a several advantages such as limitation of decline in number of microbial cells, deprivation of contaminants, possibility of changing the drying temperature according to need, certain ingredients may be mixed and dried and, what it is worth mentioning, ambient temperature is used for the drying process (Sahu and Brahmaprakash, 2016).

4. Production of biofertilizers

The development and production of a successful biofertilizer is a multistep procedure including the following stages: (I) selection of a suitable culture and isolation of effective microbes, (II) determining the properties of selected microorganisms on a proper medium with the appropriate growth conditions, (III) up-scaling of microbial biomass, (IV) selection of a carrier, (V) formulation of the bioinoculant, (VI) field studies, (VII) large scale experiments and production at the industrial level, (VIII) establishment of a quality control, storage and transportation system (Shaikh and Sayyed, 2015; Stamenković et al., 2018). For the obtaining of a high quality biofertilizer, each of above-mentioned steps is important and must be carried out under strictly defined conditions (Mohod et al., 2015). Microorganisms used as biofertilizers should possess certain characteristics, which determine their usefulness and effectiveness. These properties include high rhizosphere competence, promoting plant growth and development by various mechanisms or by secretion of biologically active substances and compatibility with indigenous rhizobacteria inhabiting soil.

They also should be easy for mass multiplication, exhibit wide scope of action and should not pose threat to natural environment (Nakkeeran et al., 2005). Large scale growth of the selected microbes takes place on a selected medium which should be inexpensive, easily available and provide all fundamental nutrients indispensable for obtaining microbial strains in adequate amount (Glick, 2015). This step in biofertilizers production is achieved due to liquid, semisolid and solid state fermentation techniques. It has been documented that for the maximum growth of microorganisms, various types of chemically defined media (CDM) are used, because they allow for modification the ratio of substances influencing proliferation of microbial strains (Stamenković et al., 2018). Then, selection of a high quality carrier must be done, based on the properties of the used microbial strains and the preferred form of the final product. The next step includes impregnation of carrier with fully grown microbial broth or immobilization of grown cells in order to obtain liquid formulations. Before being commercialized, biofertilizer must undergo a number of greenhouse tests or field experiments and fulfill certain requirements that will confirm: (i) lack of eco-toxicological effects and (ii) possession of beneficial impact in terms of promoting plant growth and increasing crop yields. Then, registration and regulatory approval of the biofertilizer must be done (Backer et al., 2018; Bashan et al., 2014). Formulated biofertilizers are packed and each package should be marked with the following information: the name of the product, microbial strain(s) contained in it, plants for which is intended, name and address of the manufacturer, date of production, the expiry date, instructions and recommendations for application (Bhattacharjee and Dey, 2014; García-Fraile et al., 2015).

5. Quality of biofertilizers

The quality of the biofertilizers is the parameter influencing not only positive or negative reception by the end users, farmers, but also the progress of a whole biofertilizers industry, so it has be supervised at all production stages (Sethi and Adhikary, 2012). Unfortunately, in the European Union and USA the production standards and quality parameters are not unambiguously established. In countries where biofertilization has become more popular, their use is better regulated. For example, in China the quality is determined on the basis of eight parameters: amount of living cells, carbon and water content, pH, size of carrier (for solid biofertilizers), appearance, contamination and the expiry period. Nevertheless, the microbial density

remains the most important factor according to Chinese standard. This parameter has been defined for the seven groups of microorganisms: rhizobia, fast and slow growing, nitrogen fixing bacteria, phosphorus solubilizing bacteria, for organic and inorganic phosphorus, silicate solubilizing bacteria and multi strain consortia. The amount of used living cells depends on the kind of bacteria and ranges between $>0.5 \times 10^9$ cfu mL^{-1} or $>1.5 \times 10^9$ cfu mL^{-1} in the case of liquid biofertilizers and between $>0.1 \times 10^9$ cfu g^{-1} or $>0.3 \times 10^9$ cfu g^{-1} when it comes to solid products. Organic matter content should make up at least 20% of the biofertilizer, regardless of its physical form and the validity of the product cannot be shorter than 6 months. In India, standards are established for seven quality parameters: the physical form, the minimum count of viable cells, the level of contamination, the particle size in case of carrier based materials, the water content, pH and the efficiency character. In India, standards are set for four groups of microorganisms: *Rhizobium* sp., *Azotobacter* sp., *Azospirillum* sp., phosphate solubilizing bacteria and mycorrhizal biofertilizers. In bacterial biofertilizers the minimum viable cells for solid carrier is 5×10^7 cfu g^{-1} and 1×10^8 cfu mL^{-1} for liquid carrier. In case of biofertilizers based on mycorrhizal fungi, 1 g of ready-to-use product must contain at least 100 viable propagules (Sekar et al., 2016; Suh et al., 2006).

6. Application of biofertilizers

Biofertilizers can be applied on seeds, seedlings or directly to the soil (Chen, 2006). Each technique has advantages and disadvantages, taking into consideration the properties of inoculant, type of crops, environmental conditions and constraints arising from technical background of farmers (Mahmood et al., 2016). However, there are some precautions which should be taken before application, e.g., direct exposure to sunlight and keeping used solution overnight is not good. Biofertilizers should also be stored at appropriate temperature, no below 0 °C and over 35 °C (Muraleedharan et al., 2010).

Seed treatment remains the most common practice of applying biofertilizers due to its simplicity and small amount of product required for inoculation (Asif et al., 2018). Inoculant can be applied to the seeds in one of the following ways: dusting, slurry and seed coating (Malusá and Ciesielska, 2012). With dusting, dry seeds are mixed directly with the inoculant. This method may result in weak adherence of microorganisms to the seeds, therefore is thought to be least effective. With slurry, bioinoculant is

mixed with wetted seeds or directly with water and then with seeds. Alternatively, the seeds may be left in the slurry for night (Malusá and Ciesielska, 2012; Muraleedharan et al., 2010). Due to the fact that each seed must be coated with the appropriate number of microorganisms adhesives such as gum arabic, carboxy methyl cellulose, sucrose solutions, vegetable oils and non-toxic commercial products are used (Bashan et al., 2014). If the biofertilizer does not contain adhesives, it advisable to add 25% solution of molasses or 1% milk powder to the suspension. In last method, seed coating, seeds are mixed with the slurry prepared from the inoculant and then are coated with finely ground inorganic inert materials such as lime, clay, rock phosphate, charcoal, dolomite, calcium carbonate or talc. As a result of this process, microorganisms are protected from adverse environmental conditions and from the harmful impact of chemical fertilizers and pesticides (Malusá and Ciesielska, 2012). Seed treatment may be conducted with bacteria belonging to the following genus: *Rhizobium*, *Azotobacter*, *Azospirillum*, phosphorus solubilizing microorganisms (PSM) and also with the consortium of microorganisms. In this case, seeds are firstly coated with *Rhizobium* sp., *Azotobacter* sp. or *Azospirillum* sp. and then, PSM inoculant is added as the outer layer thus the higher number of viable microbial cells can be maintained (Brahmaprakash et al., 2017).

Soil inoculation is recommended in case of introducing a large population of microbial strains directly to the soil. In this technique, carrier material in form of granules (0.5–1.5 mm) is preferred and granular forms of peat, perlite, talcum powder or soil aggregates are commonly used. Generally, soil inoculation consist in placing granules in the seedbed under, above, as well as alongside the seed. In case of the liquid forms of biofertilizers, seeds in the furrows can be sprayed with the inoculants; there is also a possibility to use hydroponic systems. Soil treatment enables control of the location and the application rate of the inoculant, protects inoculants from the harmful impact of pesticides and fungicides and avoids damage to the seed coats. The risk of losing some part of the inoculant while using seeding machinery is minimized. The soil inoculation increases chance of contacting seeds with the higher concentration of biofertilizer in comparison with seed treatment. On the other hand, there are some disadvantages associated with this method, mostly technical, e.g., specialized equipment, larger quantities of biofertilizer which imposes more storage area and transport. These factors are simultaneously connected with higher financial outlays. Generally, soil inoculation with granules has been implemented in developed countries, where advanced machinery and accessories for fertilization are used (Bashan et al., 2014; Deaker et al., 2004).

7. Types of biofertilizers and their working principles

Biofertilizers used in organic agriculture are classified into different types on the basis of group of microorganisms they contain (Itelima et al., 2018). The different types of these preparations include:

7.1 Nitrogen fixing biofertilizers (NFB)

Nitrogen is one of the most essential nutrient for plant growth and development and plays a crucial role in crop production (Thilakarathna et al., 2016). It is a key macronutrient in all enzymatic reactions in plants' cells and is a vital constituent of the chlorophyll molecules (Wagner, 2011). Atmosphere consists of many gases, but nitrogen remains the most abundant gas of its composition (do Figueiredo et al., 2013). Despite the fact that the atmosphere is the largest reservoir of this element on Earth, dinitrogen gas is unavailable for plants and animals. This is the outcome of triple bond occurs between the atoms in nitrogen molecule, which makes it chemically stable and difficult to break. In order for atmospheric nitrogen to be effectively used, it must be reduced to plant-usable forms; this process is referred as biological nitrogen fixation (BNF) (Galloway et al., 2003; Tairo and Ndakidemi, 2013). BNF is a conversion of inactive atmospheric nitrogen to metabolically useful form, for example, ammonia, by nitrogen-fixing microorganisms, catalyzed by a complex metaloenzyme, called nitrogenase (Vicente and Dean, 2017). Microorganisms which are capable of nitrogen fixation are divided into symbiotic and non-symbiotic. Symbiotic organisms, mostly bacteria from the *Rhizobiaceae* family, form mutualistic relationships with host, leguminous plants. On the other hand, BNF is non-symbiotic when is conducted by free living and endophytic microorganisms such as *Azotobacter* sp., *Azospirillum* sp. and *Cyanobacteria* sp. (García-Fraile et al., 2015). The overall reaction of biological nitrogen fixation can be schematically presented followed by Biswas and Gresshoff (2014):

$$N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

7.1.1 Symbiotic nitrogen fixing bacteria

The best known bacteria which are capable of nitrogen fixing belong to the family *Rhizobiaceae* (α-proteobacteria) and include the following genera:

Rhizobium, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium* and *Allorhizobium,* commonly called rhizobia (Patel and Sinha, 2011). Rhizobia interact into mutualistic associations with the leguminous plants, inducing formation of special structures on their roots, called nodules, in which nitrogen fixation process occurs. Inside nodules bacteria differentiate into bacteroids, i.e., forms that are capable of converting atmospheric nitrogen to ammonium, which is afterward transported to plant cells and effectively metabolized (Shridhar, 2012). After the application of rhizobial biofertilizer, it has been noticed that pulse crop yields increased due to symbiosis between microorganisms and host plants. *Rhizobium* strains are able to fix 15–20 kg N ha^{-1} and increase crop yield up to 20% in case of legume plants (Mitra, 2019). The ability to fix atmospheric nitrogen differs considerably among rhizobial strains and host plant species, therefore, during biofertilizer production the compatibility between organisms must be taken into account. It has been estimated that due to the microbial activity of rhizobia, 126–319 kg N ha^{-1} is fixed in case of groundnut, 33–643 kg N ha^{-1} in soybean, 77–92 kg N ha^{-1} in pigeon pea, 25–100 kg N ha^{-1} in green gram and 125–143 kg N ha^{-1} in black gram (Gopalakrishnan et al., 2014). The agriculturally important plants which form relationships with rhizobial strains include *Trifolium* sp., *Pisum sativum*, *Phaseolus vulgaris*, *Medicago sativa*, *Lotus corniculatus*, *Glycine* max and *Cicer arietinum* (Verma et al., 2010).

The beneficial effects of symbiotic nitrogen fixing bacteria application has been documented in the literature. A study conducted by Wani et al. (2008) showed that inoculation chickpea with *Mesorhizobium* sp. strain increased the plants dry matter, number of nodules, seed yield and grain protein. There have been shifts in nitrogen content in roots and shoots, which increased, by 46% and 40%, respectively, as compared to non-inoculated controls. In addition, the plant growth promoting properties of rhizobial strains have been investigated in case of soybean, cowpea, faba bean, pigeon pea, lentil, common bean and pea (Aremu et al., 2017). It has been reported that rhizobacterial genera, e.g., *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium* are able to promote legumes growth and provide nitrogen to the plants in metal-contaminated soils (Bramhachari et al., 2018). Representatives from *Rhizobiaceae* family were also found to produce compounds such as siderophores, indoleacetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Wdowiak-Wróbel et al., 2017). According to Ahmad et al. (2013) *Rhizobium* strains exerting ACC-deaminase activity contribute to the improvement of growth, physiology and quality of mung bean under salt-affected conditions.

Another microorganisms which are involved in symbiotic relationships are N_2-fixers from the genus *Frankia*. *Frankia* include a free-living Gram-positive soil-borne bacteria which participate in associations with actinorhizal plants (Mus et al., 2016). Similarly to rhizobial microorganisms, *Frankia* induce formation of nodules in which nitrogen is fixed, but leguminous and actinorhizal nodules show differences in morphology, anatomy, origin and functioning (Hocher et al., 2009). Microbial strains from this genus possess the ability to infect and nodulate eight families of actinorhizal plants (primarily woody plants) (Santi et al., 2013), which are used for wood production, land reclamation, for timber and fuel wood production, for windbreaks and for shelterbelts along coastlines and deserts (Gentili and Jumpponen, 2006). Some of these plants are used for the rehabilitation of degraded ecosystems. Inoculation with *Frankia* strains may increase the fertility of degraded soils and improve the beneficial impact of the actinorhizal trees during their plantation in arid environments (Diagne et al., 2013). According to Sayed (2011) *Casuarina* trees, which are agriculturally important in South Africa, Senegal, China and India, inoculated with *Frankia* showed increased growth and biomass.

7.1.2 Free-living non-photosynthetic nitrogen fixing bacteria

Despite the fact that the rhizosphere is the habitat for many genera and species of bacteria capable of nitrogen fixing, mainly microorganisms belonging to *Azotobacter* and *Azospirillum* genera have been investigated as a potential biofertilizers in cereal and legume crops (Gupta et al., 2016). Microorganisms from the *Azotobacter* genus are a free-living, aerobic bacteria, which colonize mostly neutral and alkaline soils and are capable of fixing averagely 20 kg N ha^{-1}/year (Bag et al., 2017; Mahanty et al., 2016). Six species from the *Azotobacter* genus, i.e., *A. armeniacus*, *A. beijerinckii*, *A. chroococcum*, *A. nigricans*, *A. paspali*, *A. vinelandii* play a crucial role in nitrogen fixation and their application as biofertilizers is advantageous for non-legume crops such as wheat, barley, oat, rice, sunflower, maize, line, beetroot, tobacco, tea, coffee and coconuts (Chandra et al., 2018; Wani et al., 2013). According to study conducted by Iwuagwu et al. (2013) application of *Azotobacter* sp. increased the plant height, stem base diameter, fresh and dry matter of maize seedlings. It has been documented that inoculation with *Azotobacter* sp. increased the dry matter of wheat, oat and clove by 10–23%, 13–19% and 14–27%, respectively, in comparison with non-inoculated controls (Sethi and Adhikary, 2012). *Azotobacter* sp. as biofertilizer not only increases the amount of nitrogen available for plants but also synthesizes

substances which are beneficial for plant growth and development (Gothandapani et al., 2017). These microorganisms are known to produce compounds such as gibberellins, auxins, cytokinins, substances like B vitamins, nicotinic and panthotenic acid and substances improving seed germination. It has been documented that seed inoculated with *Azotobacter* sp. exerted increased germination by 20–30%. *Azotobacter* sp. also protect plants against root pathogens, stimulate the activity of indigenous soil microorganisms and enhance the crop yield (Kader and Mian, 2003; Mahato and Kafle, 2018; Vikhe, 2014).

7.1.3 Free-living photosynthetic nitrogen fixing bacteria

Representatives of this nitrogen-fixing microorganisms are determined as cyanobacteria or blue-green algae (BGA). They are free-living oxygenic Gram-negative organisms, commonly occurring in ponds, lakes, water streams and rivers (Singh et al., 2016), which transform N_2 into nitrogenous and ammonium compounds. Most of BGA, from the genera such as *Nostoc*, *Anabaena*, *Aulosira*, *Cylindrospermum*, *Calothrix*, *Tolypothrix* and *Stigonema* are endowed with heterocyst, specialized thick-walled modified cells, which are known to be place of nitrogen fixation by nitrogenase (Kumar et al., 2010). The ability N_2-fixing has been noticed not only in heterocystous BGA, but also in non-heterocystous unicellular (*Aphanothece, Chroococcidiopsis*, *Dermocapsa*) and filamentous (*Oscillatoria*, *Schizothrix, Trichodesmium*) genera of cyanobacteria (Berrendero et al., 2016). Interestingly, cyanobacteria exert the plant growth promoting abilities as they capable of producing variety of substances, e.g., amino acids, polypeptides, vitamins and antibacterial/antifungal compounds (Rathod et al., 2018). Furthermore, BGA are able to solubilize the insoluble phosphate forms such as calcium phosphate, ferric phosphate, aluminum phosphate and hydroxyapatite in soil, due to which they improve the availability of indigenous phosphorus to the plants (Rai et al., 2019).

There are not many reports in the scientific literature about commercial products based on BGA, but cyanobacterial species *Anabaena variabilis*, *Nostoc muscorum*, *Aulosira fertissima*, *Tolypothrix tenuis* have been found as a potential biofertilizers (Kaushik, 2014). It has been documented that application of cyanobacteria enhance the N availability in the rice fields (Mishra and Pabbi, 2004). According to Singh et al. (2016), addition of cyanobacteria (in vitro) contributed to the improvement of plant shoot, root length and dry weight in wheat crops. A study conducted by Ismail and Abo-Hamad (2017) proved that *Anabaena* sp. treatment boosted the germination percentage,

fresh and dry mass weights, length of shoots and photosynthetic pigments content in barley and fenugreek. In India *Aulosira fertilissima* is thought to be the most active cyanobacteria in terms of nitrogen fixation in rice fields (Thingujam et al., 2016). Use of cyanobacteria in agriculture systems offers benefits such as enhancement mobility of essential nutrients, bioremediation of heavy metals and xenobiotics, protection of plants from phytopathogens and improving the physico-chemical soil properties. These microorganisms are able to fix 20–40 kg N ha^{-1}, therefore they seem to be a promising alternative to chemical nitrogen fertilizers (Issa et al., 2014; Singh et al., 2016).

7.1.4 Associative nitrogen fixing bacteria

Azospirillum sp. are another free-living aerobic bacteria that possess the ability of nitrogen fixing and is considered as important plant growth promoter of many cereals, not only in greenhouse but also in field trials (Vurukonda et al., 2016). Bacteria of the genus *Azospirillum* are non-nodule forming microorganisms and they are commonly found in the soils of tropical, subtropical and temperate ecosystems. They colonize the surface and the interior of roots, both in wild and agricultural plants and this kind of relationship is called a rhizosphere association (Gangwar et al., 2018). *Azospirillum* sp. is able to fix 20–40 kg N ha^{-1} and due to its inoculation yields increase averagely by 5–10%. The application of biofertilizers containing *Azospirillum* sp. strains is recommended in non-legume plants such as paddy, millets, oilseeds, sugarcane, banana, coconut, oil palm, cotton, chilly, lime, coffee, tea, spices and herbs (Pathak et al., 2018). *Azospirillum lipoferum* and *A. brasilense* have been reported to exert beneficial effects on the productivity of different crops (Cassán and Diaz-Zorita, 2016). It was estimated that up to 50% of the nitrogen content in sugarcane could be supplied by *Azospirillum* sp. nitrogen fixers (Sivasakthivelan and Saranraj, 2013). According to Omar et al. (2009) *A. brasilense* helps alleviate salt stress in barley crops. Bacteria from this genus are also well-known for excreting phytohormones which change morphological and physiological properties of inoculated plant roots (Atta et al., 2018).

7.2 Phosphorus biofertilizers

Similarly to nitrogen, phosphorus is the next indispensable macronutrient, required in highly assimilable forms, for maximizing crop production and plant growth and development. It is involved in enzyme pathways regulating plant metabolism, it takes part in photosynthesis and energy transfer and several other important processes (Sharma et al., 2013). Phosphorus deficiency

in young plants may lead to retarded growth. This underdevelopment result in reduction of the leaf size (Yeh et al., 2000). There are three forms of phosphorus occurring in soil: soil phosphate solution and insoluble phosphate, distinguish on organic and inorganic compounds. Phosphorus can be absorbed as $H_2PO_4^-$ and HPO_4^{2-} but, approximately 95–99% of soil phosphorus is presented in insoluble forms that cannot be utilized by plants (Sharma, 2011; Vijayabharathi et al., 2016). A common way to cope with phosphorus deficiency is an application of P-mineral fertilizers in the form of monocalcium phosphate or monopotassium phosphate (Sharon et al., 2016). Nevertheless, the long-lasting use of chemical inputs shows a severe environmental effects and may limit the amount of naturally occurring phosphorus. It has been reported that a great proportion on phosphorus in mineral fertilizers becomes unavailable for plants. This is the outcome of the formation of strong bonds between phosphorus with iron and aluminum in acidic pH; the similar situation occurs in alkaline soils where chemical bonds with magnesium and calcium are formed (Mehrvarz and Chaichi, 2008; Ranjan et al., 2013). Biofertilizers based on microbial strains exerting phosphate-solubilizing activity are a promising approach to increase the phosphorus bioavailability in agricultural soils and environmentally friendly implication to the use of P-mineral fertilizers (Barea, 2015).

Bacterial strains that have been found to increase the availability of soil phosphates for plants include *Pseudomonas* spp., *Agrobacterium* spp. and *Bacillus circulans*. Other bacteria that are capable of phosphorus solubilization include strains of the following genera: *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Kushneria*, *Paenibacillus*, *Ralstonia*, *Rhizobium*, *Rhodococcus*, *Serratia*, *Bradyrhizobium*, *Salmonella*, *Sinomonas*, and *Thiobacillus* (Alori et al., 2017; Elias et al., 2016).

Not only bacteria, but also fungi have been reported to be useful in terms of phosphorus solubilization. The noteworthy microbial fungi that exhibit important functions in mobilization of poorly available phosphorus include strains from the following genera: *Achrothcium*, *Alternaria*, *Arthrobotrys*, *Aspergillus*, *Cephalosporium*, *Cladosporium*, *Curvularia*, *Cunninghamella*, *Chaetomium*, *Fusarium*, *Glomus*, *Helminthosporium*, *Micromonospora*, *Mortierella*, *Myrothecium*, *Oidiodendron*, *Paecilomyces*, *Penicillium*, *Phoma*, *Pichia fermentans*, *Populospora*, *Pythium*, *Rhizoctonia*, *Rhizopus*, *Saccharomyces*, *Schizosaccharomyces*, *Schwanniomyces*, *Sclerotium*, *Torula*, *Trichoderma* and *Yarrowia* (Alori et al., 2017; Pal et al., 2015).

On the basis of the application of phosphate-solubilizing bacteria and phosphate mobilizing-microorganisms two types of phosphate biofertilizers have been distinguished:

7.2.1 Phosphorus solubilizing biofertilizers (PSB)

Microorganisms belonging to this group are bacterial and fungal strains capable of solubilizing insoluble phosphate compounds, e.g., tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock phosphate. A study conducted by the Ibarra-Galeana et al. (2017) proved that *Sinorhizobium meliloti*, *Bacillus flexus* and *Bacillus megaterium* are capable of solubilizing tricalcium phosphate and hydroxyapatite. Some other microbial species, such as *Aspergillus niger*, *Penicillium variable*, *Yarrowia lipolytica* and *Paecilomyces marquandii AA1* have been found to solubilize insoluble P compounds (Shrivastava et al., 2018).

The phosphorus solubilization mechanisms include secretion of low molecular weight organic acids (e.g., malic, succinic, fumaric, citric, glutonic, 2-ketoglutonic, etc.), secretion of mineral dissolving compounds, discharge of extracellular enzymes (biochemical phosphate mineralization) and release of phosphate during substrate degradation (biological phosphate mineralization) (Choudhary et al., 2018).

The solubilization of mineral P is possible due to organic acid production which contribute to lowering the pH in rhizosphere, chelation of the cations taking part in precipitation of P, forming soluble complexes with metal ions from insoluble P compounds (Ca-P, Al-P, Fe-P) and to competing with P for sorption sites on the soil (Shrivastava et al., 2018). These organic acids are a products of microbial metabolism and can directly dissolve the mineral phosphorus as an outcome of anion exchange of phosphate by acid anion or can chelate cations such as Fe, Al and Ca associated with P (Zaidi et al., 2009). Gluconic, oxalic, citric, lactic, tartaric and aspartic acid are the common organic acids synthesized by phosphorus solubilizing microorganisms (Ahmed and Shahab, 2009).

Another important process involved in increasing bioavailability of soil phosphorus is solubilization of organic P compounds. The efficiency of organic phosphorus mineralization is influenced by physicochemical and biochemical properties of these compounds. Some of them, e.g., nucleic acids, phospholipids and sugar phosphates are easily mineralized as compared to pythic acids, polyphosphates and phosphonates. Organic P compounds presented in soil, mainly associated with clay particles, are highly mineralized as a fraction of them is easily accessible for utilization by microorganisms (Shrivastava et al., 2018).

Some belowground microorganisms from the genera such as *Aspergillus*, *Penicillium*, *Mucor*, *Rhizopus*, *Bacillus*, *Pseudomonas* are capable of synthesizing phosphatases, enzymes which break phosphoester or phosphoanhydride

bonds and dephosphorylate organic P compounds (Mengel et al., 2001). It has been documented that microbial phosphatases are more efficient in terms of organic P compounds mineralization as compared to phosphatases produced by plants (Tarafdar et al., 2001). *Aspergillus niger* have been also found to produce phytase, an enzyme due to which utilization of phosphorus from phytates is possible (Neira-Vielma et al., 2018). Owing to this, plants can assimilate phosphorus directly from organic P compounds (Singh and Satyanarayana, 2011). Another microbial enzymes are phosphonates and C-P lyases which break the C-P bond in organophosphonates. These enzymes have been described in strains such as *Bacillus stearothermophilus*, *Bacillus licheniformis*, *Thermus thermophilus* and *Thermus caldophilus* (Nalini et al., 2015; Rodríguez et al., 2006). Yi et al. (2008) proved that some representatives of *Enterobacter* sp. (EnHy-401), *Arthrobacter* sp. (ArHy-505), and *Azotobacter* sp. (AzHy-510), producing significant amount of exopolysaccharides (EPS), are able to solubilize tricalcium phosphate. Beneficial impact of phosphorus solubilizing microorganisms have been documented in various crops like maize, lettuce, canola, chickpea, barley, common bean, sugarcane, wheat, mung bean and millet (Shrivastava et al., 2018).

7.2.2 Phosphorus mobilizing biofertilizers (PMB)

Microorganisms defined as P-mobilizers have the ability to increase the phosphorus uptake by mobilizing it from soil instead of solubilizing P compounds (Kumar et al., 2018). Mycorrhizal fungi are thought to be the most important P-mobilizers. They form a symbiotic association with vascular plants, among which there are some agriculturally important species such as maize, wheat, rice and potato. In this kind of relationships, fungi colonize the roots of host plants and form a way due to which plants can assimilate nutrients. Mycorrhizal fungi can be divided into two groups: endomycorrhizal and ectomycorrhizal (Barman et al., 2017; Frąc et al., 2018). In endomycorrhiza the hypha of fungi penetrate root cortical cells and invaginate the cell membrane. The most common type of endomycorrhiza is arbuscular-forming mycorrhiza where fungal hyphae form structures called arbuscules. The arbuscular mycorrhiza fungi (AMF) include the following genera: *Acaulospora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Sclerocystis*, *Scutellospora* (Sadhana, 2014). AMF produce long external hyphae which can give increased contact with soil phosphates as compared to non-mycorrhizal root systems, therefore they can increase P uptake by plants. What is worth mentioning, the small diameter of hyphae

allow for penetration of soil pores that cannot be exploited by roots. Recently, it has been reported that AMF hardly ever utilize organic P by themselves and may be incapable of synthesizing extracellular phosphatases. Nevertheless, phosphate solubilizing bacteria can colonize the fungal hyphae and draw energy from the hyphal exudates, simultaneously mineralize phosphorus compounds presented in the soil (Zhang et al., 2018). This interaction has been documented between *Glomus mosseae* and phosphatase-producing fungus *Aspergillus fumigatus* (Shrivastava et al., 2018) and it has been reported that dual inoculation with this strains increased shoot length, root dry weight and root length in wheat (Bhale et al., 2018). The positive effects of AMF inoculation on the growth parameters have been noticed in kidney bean, pepper, watermelon, muskmelon, onion, tomato, cucumber and asparagus (Chen et al., 2017). Mycorrhizae fungi may also enhance plant tolerance to salinity and drought, heavy metals, soil-borne pathogens and improve soil structure (Bi et al., 2018).

7.3 Plant growth promoting biofertilizers (PGPB)

Microbial representatives of this group enhance plant growth and development by production variety of agroactive substances such as siderophores, 1-aminocyclopropane-1-carboxylate deaminase (ACC), phytohormones, e.g., indolacetic acid (IAA), gibberellic acid (GA), volatile organic compounds (VOCs), antibiotics, cyanides and fungal cell-wall-degrading enzymes (Lugtenberg and Kamilova, 2009; Majeed et al., 2015). These compounds are synthesized in large quantities and have diverse beneficial effects on overall morphology of host plants (Gouda et al., 2018). Plant growth promoting rhizobacteria (PGPR) belongs to different genera, e.g., *Agrobacterium*, *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Actinoplanes*, *Azotobacter*, *Bacillus*, *Pseudomonas sp.*, *Rhizobium*, *Bradyrhizobium*, *Erwinia*, *Enterobacter*, *Amorphosporangium*, *Cellulomonas*, *Flavobacterium*, and *Xanthomonas* (Mohammadi and Sohrabi, 2012; Vejan et al., 2016). In last year's interest in actinomycetes, as a potential plant growth promotors, has raised due to their abilities to produce biologically active compounds (Anwar et al., 2016). Actinomycetes which display biocontrol traits against root pathogenic fungi include strains *Micromonospora* sp., *Streptomyces* spp., *Streptosporangium* sp., and *Thermobifida* sp. (Franco-Correa et al., 2010). What is worth mentioning, some of PGPR can exhibit bifunctional properties, either biofertilizers or biopesticides. For example, strains of *Burkholderia cepacia* have been observed with biocontrol properties of *Fusarium* sp. that can produce mycotoxins,

whereas they are capable of producing siderophores, which boost growth of maize under iron deficiency conditions (Bhattacharyya and Jha, 2012).

PGPR can be divided into two groups, depending on level of intimacy with plant roots: extracellular PGPR (ePGPR) existing in rhizosphere, on the rhizoplane or in the spaces between cells of root cortex and intracellular PGPR (iPGPR) which are located inside specialized structures of root cells, called nodules (Ahemad and Kibret, 2014).

Despite the fact that the individual group of microorganisms exhibit significant disparities among themselves, their working principles are similar (Mahanty et al., 2016). They can promote plant growth in two distinct ways: directly or indirectly. The direct mechanisms consists in supplying plants with compounds produced by microorganisms such as IAA, GA and siderophores as well as making the soil nutrients uptake more efficient by nitrogen fixation and micro and macroelements solubilization. On the other hand, the indirect way is related to limitation or complete removal of the detrimental effects of phytopathogens; this is possible due to the excretion of antibiotics, lytic enzymes and boosting induced systemic resistance (Beneduzi et al., 2012; Bhattacharyya and Jha, 2012).

It has been documented that bacterium *Paenibacillus polymyxa* significantly increased the biomass of pepper and elicited induced systemic resistance to bacterial pathogen *Xanthomonas axonopodis* pv. *vesicatoria* (Phi et al., 2010). Some species of *Paenibacillus* and *Bacillus* have been reported to promote root and shoot growth in rice plants (Beneduzi et al., 2008).

According to Rotaru (2015), increased acid phosphatase activity in the soil rhizosphere of soybean have been noticed after the application of *Pseudomonas fluorescens* and *Azotobacter chroococcum*.

According to Shaharoona et al. (2006) *Pseudomonas* sp. contributed to root elongation in maize. Some strains of *Pseudomonas* sp. have been noticed to enhance dehydrogenase activity and the nutrient uptake in soybean and wheat (Sharma et al., 2011). *Pseudomonas* strains were also found to produce antimicrobial metabolites such as pyrrolnitrin, pyoluteorin, phenazines and cyclic lipopeptides like viscosinamide, which exert toxic effects against a wide range of organisms (Flury et al., 2017). It was established that viscosinamide synthesized by *Pseudomonas fluorescens* DR54 shows biocontrol properties toward *Pythium ultimum* in sugar beet (Thrane et al., 2000). The research undertaken by Sachdev et al. (2009) confirmed that bacterium *Klebsiella pneumomiae* improved root and shoot development in wheat.

7.4 Potassium biofertilizers

Potassium is the third biogenic macronutrient essential for plant growth and metabolic processes occurring in plants' cells. It is involved in photosynthesis, enzyme activation, protein synthesis and sugar degradation (Basak and Biswas, 2009). This macronutrient is presented in soil in the following forms: mineral K, non-exchangeable K, exchangeable K and solution K. Total amount of K in soil is within the range from 0.04% to 3%. However, most of the soil K (90–98%) is mineral K, thus becomes unavailable directly for plants uptake (Etesami et al., 2017). Various groups of microorganisms such as bacteria, fungi and actinomycetes were found to increase potassium availability due to the production a number of organic acids (citric, oxalic, tartaric, succinic, coumaric, syringic and malic acid), polysaccharides, acidolysis, complexolysis, chelation and exchange reactions (Etesami et al., 2017; Kumar et al., 2018) The mechanisms involved in K solubilization include transforming unavailable K into available form by creating metal-organic complexes with Si^{4+} ion to bring the K into soil solution, dissolution of feldspar caused by decreased pH, mineral structural cation released by the attack of H^+ derived from organic acids, release K from orthoclase by carboxylic acids and capsular polysaccharide synthesized by *Bacillus mucilaginosus* and *Bacillus edaphicus*. It has been also documented that biofilms help to solubilize K from biotite and anorthite (Das and Pradhan, 2016). Microorganisms which have been reported with K solubilization activity include *Bacillus circulans*, *Bacillus edaphicusand*, *Burkholderia* sp., *Acidithiobacillus ferrooxidans*, *Arthrobacter* sp., *Paenibacillus mucilaginosus*, *P. frequentans*, *Cladosporium* sp., *Aminobacter* sp., *Sphingomonas* sp., *Paenibacillus glucanolyticus*, and *Enterobacter hormaechei* (Meena et al., 2016). Bacterium *Frateuria aurantia* is thought to be an effective K-mobilizer and is used in commercial biofertilizer such as Symbion-K, Biosol-K and K Sol B® (Mishra and Arora, 2016).

A study conducted by Prajapati et al. (2013) showed that bacterium *Enterobacter hormaechei* significantly improved potassium and chlorophyll content in okra. Arbuscular mycorrhizal fungi are known to increase the availability of K by releasing protons, H^+, CO_2 and organic acids like malate, citrate and oxalate. According to Wu et al. (2005) dual inoculation with *G. intraradices* and *G. mosseae* increased K uptake in maize. Potassium solubilizing microorganisms (KSM) have been reported to exert beneficial effects on growth of cotton, rape, pepper, cucumber, khella, sorghum, wheat, tomato, chili, sudan grass and tobacco (Meena et al., 2016).

Biofertilizers containing KSM are a promising tool in agriculture as they increase K uptake by plants, improve soil fertility and crop quality, enhance activity of indigenous soil microorganisms and minimize the use of mineral K fertilizers (Bashir et al., 2017).

7.5 Zinc solubilizing biofertilizers (ZSB)

Zinc is the essential trace element, required in low concentrations (5–100 mg kg^{-1}), for optimum plant growth and development. In plants' cells it takes part in carbohydrate and auxin metabolism, behaves as an anti-oxidant and is involved in energy transfer reactions. Zinc deficiency may contribute to the chlorosis, reduction of leaf size, increasing vulnerability to heat and light stress and fungal phytopathogens (Alloway, 2008; Goteti et al., 2013; Gurmani et al., 2012). The occurrence of zinc deficiency in the crops is the outcome of low solubility of this element, rather than a low total amount of Zn in the soil. Application of zinc mineral fertilizers is a common way to increase its content but overloading soil with chemical inputs may pose a threat to the natural environment and is connected with high financial outlays (Gontia-Mishra et al., 2017). Compared with artificial fertilizers, microorganisms solubilizing sparingly soluble zinc compounds are potential alternate (Hafeez et al., 2013). Various microbial strains demonstrate abilities to improve the bioavailability of zinc presented in soil. PGPR that have been reported to show improved growth and zinc content on inoculated plants include *Pseudomonas* sp. and *Rhizobium* sp. strains, *Bacillus aryabhattai*, *Azospirillum* sp., *Bacillus* sp. and *Thiobacillus thioxidans*. Some studies proved that bacteria *Gluconacetobacter diazotrophicus*, *Burkholderia cenocepacia*, *Serratia liquefaciens* and *S. marcescens* are able to solubilize zinc on a lab scale (Kamran et al., 2017; Vyas and Meena, 2018). The zinc solubilization is possible due to two main mechanisms, depending on the soil pH. First takes place in acidic soils and is based on cation exchange. The second one occurs by means of chemisorption of Zn on $CaCO_3$ when $ZnCaCO_3$ is formed. Other mechanisms involved in Zn solubilization include synthesis of siderophores (Saravanan et al., 2011) and organic acids such as gluconate or its derivative, 2-ketogluconate. Some *Bacillus* sp. strains are capable of synthesizing a number of organic acids, e.g., ferulic acid, cinnamic acid, caffeic acid, chlorogenic acid and gallic acid in a liquid medium. These compounds contribute to the acidifying of microenvironment nearby rhizosphere and owing to this, micronutrient such as zinc becomes assimilable for plants

(Hussain et al., 2018; Rawat et al., 2018). Interestingly, it has been documented that with one unit increase in pH, the availability of Zn decreases 100 times (Zeb et al., 2018).

The study conducted by Hussain et al. (2015) showed that strain *Bacillus* sp. AZ6 has a positive impact on growth parameters of maize (shoot length, root length, fresh and dry shoot and root biomass) in comparison with plants cultivated without inoculation of zinc solubilizing bacteria. It was observed that plants inoculated with this strain were up to 59% longer than control shots. According to the research, *Bacillus* sp. AZ6 has a beneficial effect on photosynthetic rate of maize, which increased by 90% as compared to uninoculated plants. It has also been reported that strain *Bacillus aryabhattai* improve the zinc uptake, which result in better growth of the maize (Mumtaz et al., 2017). Growth promotion of rice and increased zinc uptake in grain after inoculation with Zn solubilizing bacteria have been characterized by researchers (Vaid et al., 2014).

7.6 Sulfur oxidizing biofertilizers (SOB)

Sulfur is thought to be the fourth major plant nutrient after nitrogen, phosphorus and potassium (Anjum et al., 2015). It is a substantial component of some aminoacids (cysteine, cystine and methionine) and plant enzyme systems. Sulfur deficiency in plants may contribute to the impaired nitrogen metabolism, chlorosis, low oil percentage and low yield (Saha et al., 2018). Sulfur in soil exists in organic as well as inorganic forms, but plants are able to absorb only inorganic sulfur compounds, especially sulfate ion (SO_4^{2-}); however, sulfur content in this form is less than 5% of total S in the soil (Wilhelm Scherer, 2009).

In order to sulfur be uptaken by plants, it must undergo a number of chemical transformations from immobile to easily assimilable forms (Vidyalakshmi et al., 2009). The soil microorganisms which are capable of oxidizing sulfur are classified to bacterial genera (*Xanthobacter, Alcaligenes, Bacillus, Pseudomonas*) and species (*Thiobacillus ferrooxidans*, *T. denitrificans*, *T. thiooxidans*, *T. thioparus,*), fungi (*Fusarium* sp., *Aspergillus* sp., *Penicillium* sp.) and actinomycetes (*Streptomyces* sp.), but the most active sulfate oxiders remains bacteria (Banerjee et al., 2006; Vidyalakshmi et al., 2009). The microbial activity is connected with the following sulfur transformations: mineralization (high molecular weight of S compounds are disintegrated into smaller units, which can be afterward converted into sulfates), immobilization (microbial transformation of inorganic S compounds to organic S compounds), oxidation (this process refers to the oxidation of elemental

S or compounds such as hydrogen sulfide, sulfite and thiosulfate to SO_4^{2-}, conducted by chemoautotrophic and photosynthetic bacteria). Some microorganisms, e.g., *Desulfovibrio* sp. and *Desulfotomaculum* sp. are capable of reducing SO_4^{2-} to H_2S, but due to the fact that this process contributes to reducing the quantity of sulfate ion in the soil for plant nutrition, it is not beneficial in terms of agricultural productivity (Choudhary et al., 2018; Khan and Mazid, 2011).

Some plant growth promoting effects of sulfur oxidizing microorganisms have been noticed by researchers. In 2014 Pujar et al. (2014) determined that the application of graded levels of sulfur enriched with SOB (*Thiobacillus thiooxidans*) has a positive impact of some properties of sunflower (*Helianthus annuus* L.) in comparison with non-inoculated control and with plants cultivated only with the sulfur source. The study showed that *Thiobacillus thiooxidans* increased plant height, dry matter, head diameter and seed weight of the sunflower. According to Mohamed et al. (2014), onion inoculated with *Thiobacillus* sp. exerted increased height, yield and nitrogen uptake as compared to plants grown without SOB treatment. It has been documented that garlic plants cultivated with the addition of sulfur oxidizing bacteria considerably increased growth parameters including plant height, neck and bulb diameter and fresh and dry mass of leaves and bulb (Youssif et al., 2015). Generally, biofertilizers containing sulfur oxidizing microorganisms are recommended for oilseeds crops, onion, oat, cauliflower, ginger and garlic (Santra et al., 2018).

7.7 Silicate solubilizing biofertilizers (SSB)

Despite the fact that the silicon is the second most abundant element in Earth's crust, plant-usable forms of Si are limited due to it is mainly present in sparingly soluble forms which cannot be readily assimilated by roots. Silicon is not considered as essential nutrient in plants, but exhibits some beneficial effects on their growth and development. Silicon boosts plant resistance to biotic (fungi, nematodes, viruses) and abiotic stress (salinity, heating, UV-B), decreases content of cadmium and arsenic in edible plant parts and contribute to the increase the biomass of food crops. It also influence the uptake and distribution of macronutrients in plants (Frew et al., 2018; Greger and Landberg, 2015; Greger et al., 2018). Silicate solubilizing bacteria are thought to play an important role in transforming inaccessible forms of silicates into compounds that plants are capable of assimilation (Naureen et al., 2015).

According to Kang et al. (2017) strain *Burkholderia eburnea* CS4-2, in combination with insoluble silica, promoted some growth properties of japonica rice, including shoot length, root length, shoot fresh weight, root fresh weight and chlorophyll content in comparison with the control and plant cultivated only with insoluble silica source.

The research conducted with *Bacillus* sp. showed that the bacterium has a positive impact on the structure and functions of photosynthetic apparatus of *Brassica juncea*. Application of SSB enriched biofertilizer to clay substrate has led to increase the thickness of mesophyllic layer, the number of mesophyll cells, the plastid material volume and the photosynthetic pigment content. There have also been shifts in content of nitrogen, phosphorus and potassium in the leaves of examined plant. In comparison with control, the total amount of aforementioned biogenic elements increased, respectively, by 18, 20 and 25% (Maleva et al., 2017).

8. Impact on soil microorganisms

The application of biofertilizer not only influences the physicochemical soil properties but also structure and functions of soil microorganisms (Javoreková et al., 2015). The effects of introduction of plant growth promoting microorganisms in the rhizosphere vary significantly between indigenous populations. Certain groups of microbes may be enhanced, other may be inhibited and in some cases, application of PGPR does not show any changes in microbial populations (Castro-Sowinski et al., 2007). Techniques which are used to analyze shifts in microbial communities under the influence of biofertilizers include denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (t-RFLP), amplified ribosomal DNA restriction analysis (ARDRA), single strand conformation polymorphism (SSCP) and the community level physiological profiling (CLPP) with the use of BIOLOG® plates (Javoreková et al., 2015; Rastogi and Sani, 2011). Trabelsi et al. (2011, 2012), using the t-RFLP technique, have proved that field inoculation with rhizobial strains (*Rhizobium gallicum* 8a3 and *Sinorhizobium meliloti* 4H41) significantly affected structure and diversity of α- and γ-proteobacteria together with *Firmicutes* and *Actinobacteria*. The number of TRFs was considerably higher in the inoculated treatments. It has been noticed that inoculation with both above mentioned strains was less effective than inoculation only with one strain. Inoculation with two *Azospirillum brasilense* strains (40 and 42 M) have been found to change the CLPP profiles of the microorganisms associated with

rice (de Salamone et al., 2010). Changes in CLPP profiles have been also found in case of faba-bean inoculated with *Rhizobium leguminosarum* bv. *viciae* (Siczek and Lipiec, 2016). Using the SSCP technique, it has been reported that inoculation *Medicago sativa* with *Sinorhizobium meliloti* L33 increased the population of α-proteobacteria and decreased γ-proteobacteria in the rhizosphere of inoculated plant (Wang et al., 2018). According to study conducted by Zhang et al. (2017), probiotic strain *Stenotrophomonas acidaminiphila* BJ1 increased the bacterial population in the chlorothalonil-contaminated rhizosphere of *Vicia faba*. However, the effects of microbial inoculation on soil microorganisms have been characterized by researchers (Trabelsi and Mhamdi, 2013).

9. Global biofertilizers market

The commercial use of biofertilizers has begun more than 120 years ago, when "Nitragin" was registered for plant inoculation with *Rhizobium* sp. Bioinoculants based on the rhizobial strains are now available on the agricultural market for more than 100 years (O'Callaghan, 2016). According to Verma et al. (2019), approximately 5% of total fertilizer market constitutes biofertilizers and more than 150 products based on microbial strains is registered for agricultural purposes. Some of the biofertilizers commonly used around the world are presented in Table 1. According to Owen et al. (2014) rhizobial bioinoculants are the most popular microbial inoculants in last few years and constitute approximately 79% of the worldwide demand. Phosphate solubilizing biofertilizers share ~15% of global market and other inoculants, such as mycorrhizal fungi, make up ~7%. It has been documented that in 2017, global biofertilizer market reached USD 1263.4 million and is expected to be worth 2304.2 million by 2023 with a cumulative annual growth rate (CAGR) of 10.1% during the forecast period of 2018–2023. The enormous growth of the biological fertilizers market is supported by growing demand for organic food around the world. Taking into consideration geography, the worldwide biofertilizers market is divided into North America, Europe, Asia-Pacific, Latin America, Middle East and Africa. Among these sectors, Asia-Pacific is has the fastest growing market for biofertilizers. This stems from the fact that countries such as India and China are characterized by the large population, vast landscapes and increasing economies (Biofertilizers Market, 2018).

Table 1 Types of biofertilizers used in agriculture in different continents of the world.

Types of biofertilizers	Strain	Name of biofertilizer	Manufacturer's continent	References
Nitrogen fixing biofertilizers	*Azotobacter chroococcum*	Azotovit	Europe	Mishra and Arora (2016) Mikhailouskaya and Bogdevitch (2009) García-Fraile et al.(2017) Mehnaz (2016) Klimek-Kopyra et al. (2018)
	Rhizobium spp.	Rizotorphin		
	Azospirillum brasilense	Azobacterin		
	Azotobacter vinelandii	Rhizosum N		
	Nitrogen fixing strain of *Azospirillum*	Rhizosum Aqua		
	Rhizobium leguminosarum and *Penicillium bilaii*	TagTeam		
	Rhizobium leguminosarum	Nitragina		
	Rhizobium leguminosarum biovar *viceae*, strain 1435	Nodulator XL	North America	García-Fraile et al. (2017)
	Bradyrhizobium japonicum	BiAgro 10	South America	Uribe et al. (2010)
	Azotobacter chrooococcum	Dimargon		
	Azospirillum, Rhizobium, Acetobacter, and *Azotobacter*	Symbion N	Asia	Mishra and Arora (2016) Chakdar and Pabbi (2020)
	Rhizobium and *Bradyrhizobium*	Mamezo		
	Two *Azospirillum* sp.	Bio N		

Phosphorus biofertilizers	*Bacillus megaterium*	Rhizosum P	Europe	Mehnaz (2016) García-Fraile et al. (2017) Saxena (2015)
	Azotobacter chroococcum and *Bacillus megaterium*	Phylazonit M		
	Penicillium bilaii	JumpStart		
	Bacillus megaterium	Bio Phos	Asia	Dash et al. (2017) Celador-Lera et al. (2018)
	Bacillus mucilaginosus and *Bacillus subtilis*	CBF		
	Pseudomonas striata, *Bacillus polymyxa* and *Bacillus megaterium*	P Sol B		Mehnaz (2016)
	Penicillium janthinellum	FOSFOSOL	South America	Uribe et al. (2010)
	Azorhizobium, *Azoarcus*, *Azospirillum*	Twin N	Australia	Adeleke et al. (2019)
	Pseudomonas fluorescens	FOSFORINA	North America	Uribe et al. (2010)
Plant growth promoting biofertilizers	*Pseudomonas chlorapsis*	Cedomon	Europe	Mehnaz (2016) Mikiciuk et al. (2019) Saeid (2019) https://totalhumus.pl/the-bacterbase/ https://www.procam.pl/landing/biogen-rewital-pro/ https://www.probiotics.pl/
	Pseudomonas chlorapsis	Cerall		
	Pseudomonas azotoformans	Amase		
	Composition of 18 species of *endo*- and ectomyccorhiza	RhizoBio		
	Streptomyces, *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Cellulomonas*, *Arthrobacter*, *Paenibacillus*, *Pseudonocardia*	BIOGEN REWITAL PRO+		
	Lactic acid bacteria, photosynthetic bacteria	EmFarma Plus		

Continued

Table 1 Types of biofertilizers used in agriculture in different continents of the world.—cont'd

Types of biofertilizers	Strain	Name of biofertilizer	Manufacturer's continent	References
	Rhizophagus irregularis, *Funneliformis mosseae*, *Claroideoglomus etunicatum*	Mykoflor		
	Bacillus velezensis strain Sp130AA, *Bacillus amyloliquefaciens* strain Sp130CC	BACTERBASE		
	Azospirillum brasilense, *Azotobacter chroococcum*, *Bacillus subtilis*, *Bacillus megaterium*, *Pseudomonas fluorescens*	BACTIM STARTER		
	Pseudomonas aurantiaca strain SR1	Liquid PSA	South America	Celador-Lera et al. (2018)
	Consortium of more than 30 microorganisms	Inogro	North America	Celador-Lera et al. (2018) Mehnaz (2016)
	Polymicrobial inoculant containing more than 30 microorganisms	SumaGrow		
	Azotobacter chroococcum and *Pseudomonas fluorescens*	Bio Gold	Asia	Minaxi et al. (2013) Mehnaz (2016)
	Chaetomium cupreum	BioKuprum		
	Trichoderma viride	Bio Vaccine		

Potassium biofertilizers	*Bacillus circulans*	Kaliplant	Europe	Mikhailouskaya and Bogdevitch (2009)
	Frateuria aurantia	K Sol B	Asia	Mehnaz (2016)
Zinc solubilizing biofertilizers	*Thiobacillus thiooxidans*	Zn Sol B	Asia	Mehnaz (2016)
Sulfur oxidizing biofertilizers	*Thiobacillus thiooxidans*	S Sol B	Asia	Mehnaz (2016)
	Delftia acidovorans and *Bradyrhizobium japonicum*	BioBoost	North America	Adesemoye et al. (2017)
Silicate solubilizing biofertilizers	Spores of *Bacillus* species	Si Sol B	Asia	Mehnaz (2016)

10. Conclusion

Biofertilizers comprise a promising tool in agricultural ecosystems as a supplementary, renewable and ecofriendly source of plant nutrients. As they have an ability to transform nutritionally important elements from non-usable to highly assimilable forms without deleterious effects on natural environment, they are an important component of Integrated Plant Nutrient System (IPNS) (Alley and Vanlauwe, 2009). Application of biological fertilizers is thought to be a key element in maintaining soil fertility and crop productivity on the sufficiently high level, indispensable to achieve sustainability of farming. Biofertilizers may also help mitigate pitfalls arising from the growing demand of global population for food and from the widespread chemicalization in agroecosystems. The changing approach to the agricultural practices makes biofertilizers a vital part of modern-day crop production and emphasizes significance of biological inoculants in forthcoming years. A number of rhizosphere microorganisms is known to exert multifarious plant growth promoting activities, but very few have been formulated in the form of biofertilizers. Therefore, new techniques allowing for their extended application are required to attain objectives of organic farming.

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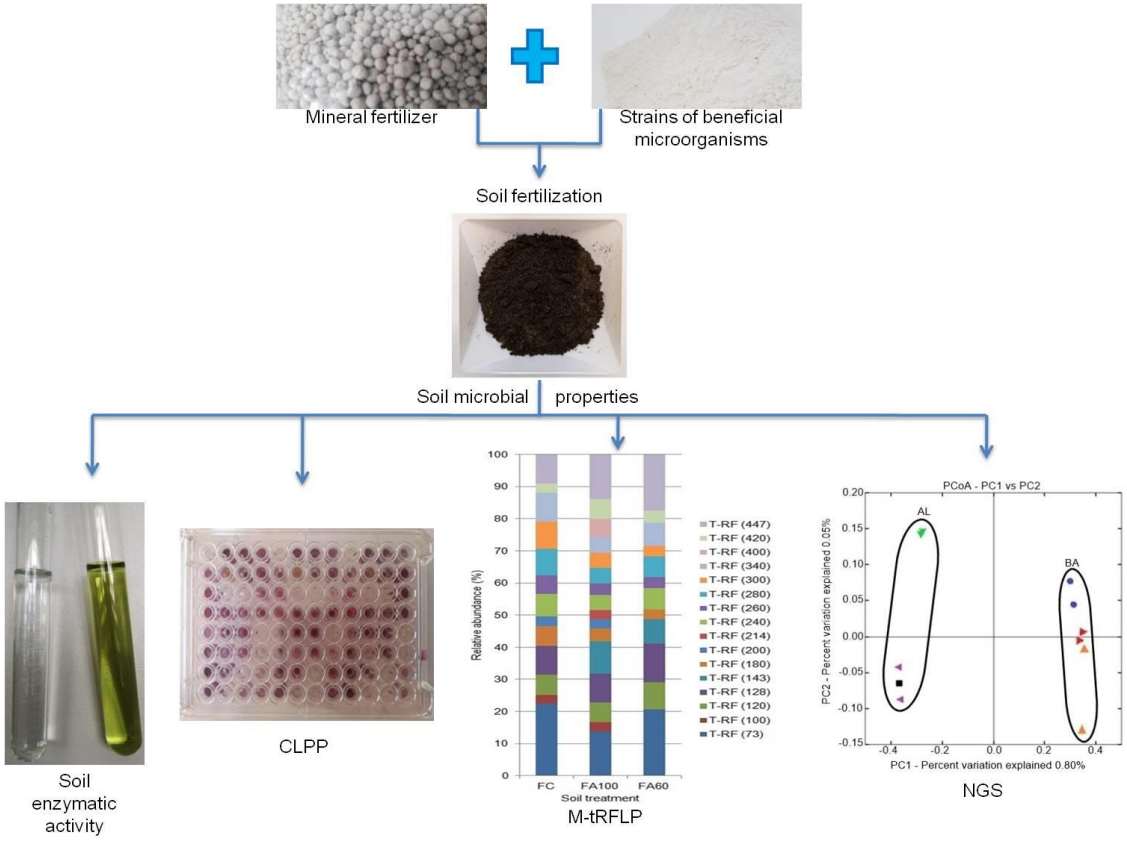
7. Tekst publikacji P.2



GRAPHICAL ABSTRACT

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The Status of Soil Microbiome as Affected by the Application of Phosphorus Biofertilizer: Fertilizer Enriched with Beneficial Bacterial Strains

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Abstract: Regarding the unfavourable changes in agroecosystems resulting from the excessive application of mineral fertilizers, biopreparations containing live microorganisms are gaining increasing attention. We assumed that the application of phosphorus mineral fertilizer enriched with strains of beneficial microorganisms contribute to favourable changes in enzymatic activity and in the genetic and functional diversity of microbial populations inhabiting degraded soils. Therefore, in field experiments conditions, the effects of phosphorus fertilizer enriched with bacterial strains on the status of soil microbiome in two chemically degraded soil types (Brunic Arenosol—BA and Abruptic Luvisol—AL) were investigated. The field experiments included treatments with an optimal dose of phosphorus fertilizer (without microorganisms—FC), optimal dose of phosphorus fertilizer enriched with microorganisms including *Paenibacillus polymyxa* strain CHT114AB, *Bacillus amyloliquefaciens* strain AF75BB and *Bacillus* sp. strain CZP4/4 (FA100) and a dose of phosphorus fertilizer reduced by 40% and enriched with the above-mentioned bacteria (FA60). The analyzes performed included: the determination of the activity of the soil enzymes (protease, urease, acid phosphomonoesterase, β-glucosidase), the assessment of the functional diversity of microorganisms with the application of BIOLOG^{TM} plates and the characterization of the genetic diversity of bacteria, archaea and fungi with multiplex terminal restriction fragment length polymorphism and next generation sequencing. The obtained results indicated that the application of phosphorus fertilizer enriched with microorganisms improved enzymatic activity, and the genetic and functional diversity of the soil microbial communities, however these effects were dependent on the soil type.

Keywords: biofertilizers; soil microorganisms; degraded soils; sustainable agriculture; biodiversity

1. Introduction

Biodiversity is one of the key factors which determine the productivity and stability of ecosystems [1]. The maintenance of a high degree of variability among living organisms in terrestrial ecosystems is indisputably important due to the fact that the natural environment provides the human population with food and regulates changes to the climate [2]. From an agricultural point of view, the proper functioning of ecosystems is necessary to meet the expanding nutritional requirements of the human population [3]. In agroecosystems, particular emphasis is placed on the biodiversity of soil-inhabiting microorganisms due to their multi-faceted involvement in belowground biochemical processes. Complex communities of bacteria, fungi and archaea are responsible for the circulation of biogenic elements (*C*, *N*, *P*), organic matter decomposition and improving the status of degraded soils [4,5]. In studies focused on the symbiotic interactions between microorganisms and their

host plants and the ability of soil microbiota to produce substances that adversely affect soil-borne phytopathogens, it was found that they have a positive impact on the health and development of agriculturally important plants [6]. All of these factors contribute to improved soil quality and fertility which is a crucial element in the proper functioning of agroecosystems and in the maintenance of plant production at a sufficiently high level [4].

In order to provide plants with the proper conditions for growth and development, mineral fertilizers are commonly used, which replace the nutrients present in the soil. The application of synthetic fertilizers is especially popular in regions where soils are poor in basic nutritional elements [7]. Phosphorus, along with nitrogen and potassium, is one of the most important elements in crop production, therefore traditional agriculture is heavily reliant on the intensive application of *N*, *P* and K fertilizers [8,9]. Taking into consideration the fact that phosphorus is often present in the soil in forms which are not easily accessible to plants, the application of balanced amounts of phosphorus mineral fertilizers is a determinant in the achievement of optimum yields [10,11]. Phosphorus is a vital element of compounds such as adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), uridine triphosphate (UTP) and nucleic acids. It is also involved in biochemical processes responsible for the maintenance of the proper functioning of plant cells, cell division, and the activation or inactivation of intracellular enzymes and the development of the morphological structures of plants such as the roots, stalk and stem, therefore P is required at all stages of plant development [12]. Unfortunately, the non-judicious and excessive application of mineral fertilizers contributes to some undesirable effects such as lowering soil fertility and the inefficient use of substances provided with the applied agrochemicals [13]. Overloading soil with chemical fertilizers may pose a threat to the natural environment and result in the eutrophication of water resources, global warming and also in the depletion of biodiversity in agricultural soils [14,15]. Through an acknowledgement of the negative aspects of the use of traditional fertilizers, research concerning organic farming and agricultural techniques as well as substances that will contribute to efficient plant production and, at the same time, will not cause adverse changes to the environment are currently underway. Moreover, the European Green Deal policy initiatives and The EU Biodiversity Strategy for 2030 includes targets to reduce the use of fertilizers by 20% and making 25% of EU agriculture organic by the year 2030 [16]. One of the assumptions of modern agriculture is the exploitation of beneficial microorganisms which could improve the uptake of nutrients by plants and promote plant growth [17]. Biofertilizers, which contain live microbial cells, comprise an innovative, economically attractive and ecofriendly alternative to chemical fertilizers and therefore at the present time they are gaining more popularity as a tool for crop production [18]. Several bacterial species exert plant growth promoting properties and may be used as a microbial inoculants [19]. The application of bacteria from the *Bacillus* and *Paenibacillus* genera in biofertilizers is of particular importance due to their abilities including increasing the bioavailability of nutrients, boosting plant disease resistance and synthesis of plant hormones and substances directed against phytopathogens [20,21]. The phosphorus mineral fertilizer used in this experiment was enriched with strains of beneficial bacteria: *Paenibacillus polymyxa*, *Bacillus amyloliquefaciens* and *Bacillus* sp. Moreover, *Paenibacillus polymyxa* was found to exhibit phosphorus solubilization properties due to gluconic acid production and an ability for phosphonates degradation [22]. What is worth mentioning, *Paenibacillus polymyxa* is able to synthesize exopolysaccharide involved in biofilm formation which interact with plant roots and protect them from various adverse factors and take part in nutrients acquisition [23]. A study conducted by Vinci et al. [24] showed that soil inoculation with the strain of *Bacillus amyloliquefaciens* combined with compost increased the growth and nutrient exploitation of maize. It was also documented that strain of *Bacillus amyloliquefaciens* help to tolerate salt stress and can cause increase of chlorophyll content in maize seedlings [25]. Strains which belong to *Bacillus* sp. were also found to exhibit phosphorus solubilization properties through the production of organic acids and the enhancement of acid phosphatases activity [26]. Some *Bacillus* species, through the synthesis of enzymes degrading fungal cell walls (chitinase) are known to suppress *Fusarium* spp. infections in

crops [27]. Taking into account the aforementioned beneficial properties of *Bacillus* and *Paenibacillus* strains, their implementation in agriculture in form of biofertilizers seems justified.

The intensification of traditional agricultural methods has led to alarming changes in agroecosystems and biofertilizers seem to be a promising approach to bridging the gap between the desire to achieve increased crop yields and the maintenance of a suitable ecological balance and a high degree of biodiversity [28]. Intensive research with biofertilizers has proven their positive impact on soil microbiological parameters and on the morphological characteristics of plants, it has been established that they pose a viable option for farmers and biofertilizers manufacturers [29]. An improved understanding of the powerful contribution of microorganisms to the agricultural production system will lead to an increase in the level of interest toward biofertilizers and the gradual reduction in the application of mineral fertilizers [30,31]. However, the development and application of conventional mineral fertilizers enriched with microorganisms is a completely new and innovative approach, which may allow for a reduction in the adverse effects of mineral fertilizers on soil environment biodiversity by decreasing the need for their use due to the addition of beneficial microorganisms to fertilizers.

Therefore, the aim of the study was to determine the immediate effects of phosphorus fertilizer enriched with beneficial bacterial strains (biofertilizer) on the status of the microbiome, biodiversity and enzymatic activity of chemically degraded soil under maize cultivation.

2. Results

2.1. Enzymatic Activity

The application of phosphorus mineral fertilizer enriched with microorganisms contributed to changes in soil enzymatic activity in both soil types as compared to the control treatments. The activities of four soil enzymes showed various trends across the treatments (Figure 1). In general terms, the soil enzyme activities remained at a higher level in the AL soil type as opposed to the BA soil. A significant increase in β-glucosidase activity was observed in the BA soil type for FA100 and FA60 treatments as compared to the control treatment. A similar trend was observed in AL soil where β-glucosidase activity also remained at a higher level for treatments in which microbiologically enriched fertilizer was applied, with a statistically significant change occurring for the FA60 treatment. The results obtained for acid phosphomonoesterase showed that the enzyme activities remained at a similar level with no statistically significant changes across treatments for both soil types. An enhancement of protease activity was noted for the FA100 treatment in BA soil and for the FA100 and FA60 treatments in the case of AL soil. Urease activity was significantly higher for the FA100 and FA60 treatments as compared with the control for BA soil, but in the case of AL soil results showed that enzyme activities remained at a similar level for all treatments.

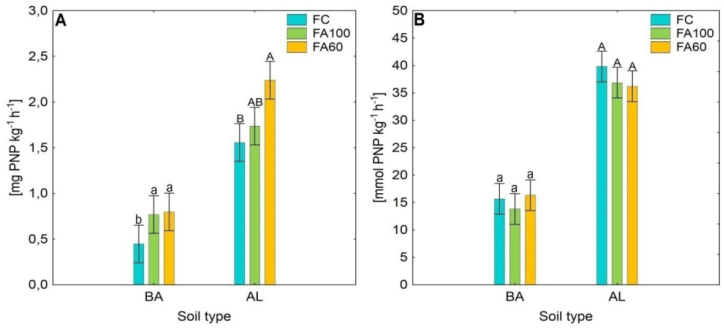


Figure 1. *Cont.*

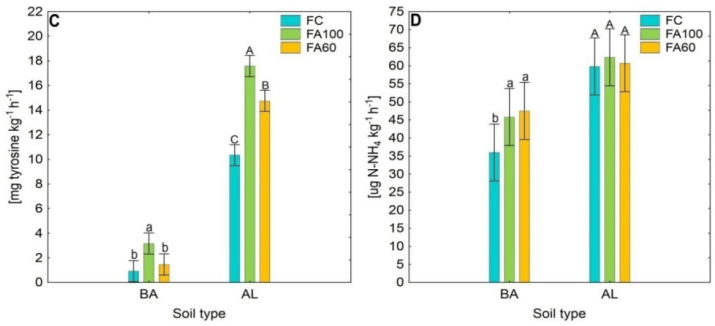


Figure 1. Changes in enzymes activity in soil as influenced by the application of phosphorus mineral fertilizer at an optimal dose (FC), at an optimal dose enriched with beneficial bacterial strains (FA100) and at a 40% reduced dose enriched with beneficial bacterial strains (FA60). (**A**)—the activity of β-glucosidase, (**B**)—the activity of acid phosphomonoesterase, (**C**)—the activity of protease, (**D**)—the activity of urease. Vertical bars denote 0.95 confidence intervals. Different letters indicate significant differences ($p < 0.05$). The significant differences were calculated separately for each soil type by post hoc Tukey tests after F-Welch test for β-glucosidase (**A**) and after ANOVA for the other enzymes (**B**—**D**). Different lowercase letters indicate significant differences within BA while uppercase letters within AL soil type. Explanation: BA—Brunic Arenosol, AL—Abruptic Luvisol, PNP—*p*-nitrophenol.

2.2. Community Level Physiological Profiles (CLPP)

2.2.1. Metabolic Potential of the Soil Bacterial Community

BIOLOG ECO plates were used to evaluate the capability of soil bacterial communities to utilize different carbon sources. In order to distinguish between individual C-substrates utilized to the largest and smallest extent, a multivariate statistical analysis was performed: the cluster analysis included the grouping of objects and features (Figure 2).

For BA soil, a higher degree of substrate utilization was recorded for L-phenylalanine, D-mannitol, D-glucosaminic Acid, Ls, Tween 40 and L-arginine for the FA100 and FA60 treatments as compared with the control treatment. A high rate of putrescine, i-erythritol, *N*-acetyl-D-glucosamine, 4-hydroxybenzoic Acid, α-cyclodextrin and Tween 80 utilization was also observed for the FA60 treatment. The compounds metabolized at the lowest level across all treatments were D,L-α-glycerol phosphate, itaconic acid, 2-hydroxybenzoic acid and, in case of the FA60 treatment, phenylethylamine.

For the AL soil type, out of the 31 different *C*-compounds, the increased utilization of D-cellobiose, D-xylose, D-glucosaminic acid, itaconic acid, i-erythritol, D-mannitol, L-asparagine, D-glucose-1-phosphate, D-malic acid, D,L-α-glycerol phosphate, D-galacturonic acid, D-galactonic acid γ-lactone, α-ketobutyric acid and phenylethylamine was observed for the FA100 and FA60 treatments in contrast to the FC treatment. A high utilization rate was reported for β-methyl-D-glucoside, L-arginine, L-threonine, L-serine, Tween 40 and 4-hydroxybenzoic Acid for the FA100 treatment and for putrescine and glycogen for the FA60 treatment. It was also noted that 2-hydroxy- benzoic acid was metabolized in the lowest degree between all treatments and 4-hydroxybenzoic acid in the case of FA60 treatment. The AWCD and Richness indices were higher for the FA100 and FA60 treatments as compared to the control for the AL soil type. The increase in the Shannon and Richness indices were observed for the BA soil fertilized with FA100 (Table 1).

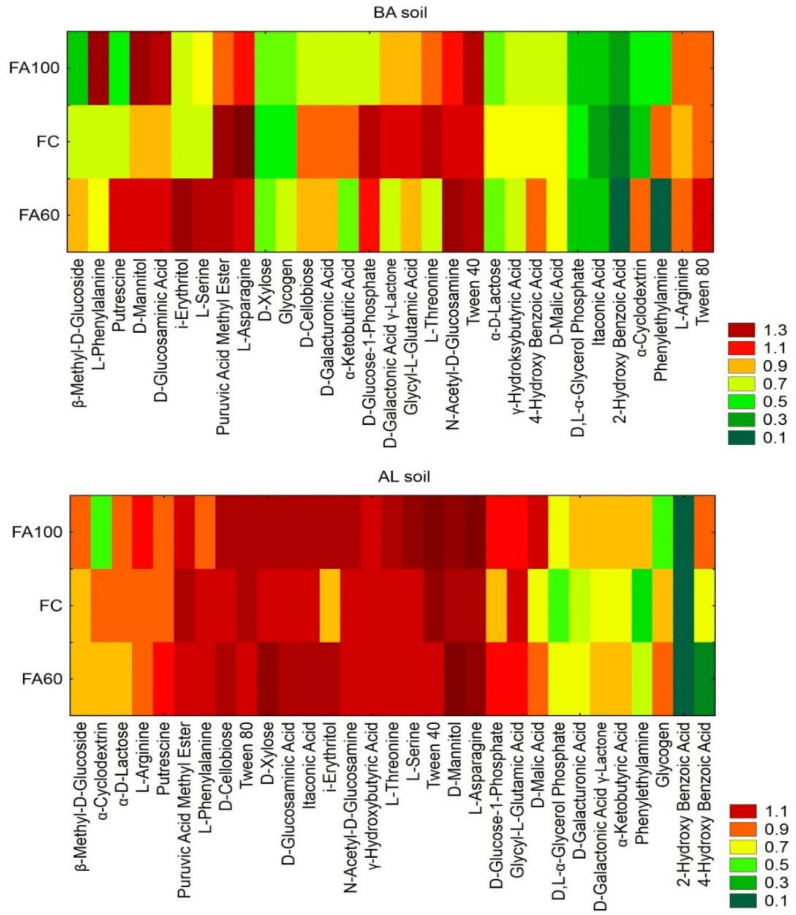


Figure 2. BIOLOG ECO^{TM} plate carbon substrates utilization intensity diagrams. Explanation: BA—Brunic Arenosol, AL—Abruptic Luvisol, FC—optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microorganisms, FA60—of fertilizer enriched with microorganisms (dose reduced by 40%).

Table 1. Average or median values of AWCD, AWDD, H, R indices and number of OTUs. Different letters indicate significant differences ($p < 0.05$) calculated separately for each soil type by ANOVA post hoc Tukey test (AWCD, ECO Plates R, AWDD, FF Plates R, NGS 16S H, t-RFLP archaea R, t-RFLP archaea H), F-Welch and post hoc Tukey test (ECO Plates H, FF Plates H) or Kruskal-Wallis and Dunn test (NGS 16S OTU, NGS ITS1 OTU, NGS ITS1 H, t-RFLP bacteria R, t-RFLP bacteria H, t-RFLP fungi R, t-RFLP fungi H).

		Soil Type/Treatment					
		BA			AL		
		FC	FA100	FA60	FC	FA100	FA60
ECO Plates	AWCD *	0.81 ± 0.08 a	0.70 ± 0.16 b	0.80 ± 0.03 ab	0.81 ± 0.32 a	0.91 ± 0.06 a	0.92 ± 0.13 a
	R *	20.00 ± 1.00 a	19.00 ± 3.00 a	19.00 ± 2.00 a	21.00 ± 3.00 a	21.00 ± 1.00 a	22.00 ± 0.00 a
	H *	2.84 ± 0.07 a	2.87 ± 0.07 a	2.74 ± 0.12 a	3.05 ± 0.11 a	2.92 ± 0.29 a	3.04 ± 0.02 a
FF Plates	AWDD *	0.55 ± 0.25 a	0.60 ± 0.09 a	0.51 ± 0.11 a	0.40 ± 0.17 a	0.47 ± 0.08 a	0.38 ± 0.06 a
	R *	52.00 ± 13.00 a	58.00 ± 9.00 a	56.00 ± 10.00 a	52.00 ± 13.00 a	58.00 ± 4.00 a	51.00 ± 6.00 a
	H *	3.82 ± 0.41 a	3.92 ± 0.16 a	3.88 ± 0.13 a	3.88 ± 0.14 a	3.94 ± 0.10 a	3.83 ± 0.15 a
NGS 16S	OUT **	2549 min 2507; max 2590 a	2744 min 2736; max 2750 a	2578 min 2565; max 2590 a	3037 min 2998; max 3976 a	2928 min 2925; max 2930 a	3038 min 3035; max 3040 a
	H *	8.78 ± 0.01 a	8.94 ± 0.01 a	8.79 ± 0.01 a	9.14 ± 0.02 a	9.12 ± 0.01 a	9.19 ± 0.01 a
NGS ITS1	OUT **	1234 min 1215; max 1251 a	1342 min 1325; max 1358 a	1338 min 1329; max 1345 a	1385 min 1296; max 1471 a	1411 min 1351; max 1470 a	1441 min 1424; max 1458 a
	H **	6.47 min 6.38; max 6.56 b	6.83 min 6.83; max 6.84 a	6.75 min 6.74; max 6.75 ab	7.23 min 7.16; max 7.30 a	7.51 min 7.45; max 7.57 a	7.61 min 7.50; max 7.71 a
t-RFLP bacteria	R **	3.00 min 2.96; max 3.00 b	7.00 min 4.98; max 8.96 ab	10.00 min 9.98; max 10.00 a	8.00 min 6.99; max 8.00 a	11.00 min 10.99; max 11.00 a	9.00 min 6.65; max 10.98 a
	H **	1.04 min 1.03; max 1.04 b	1.86 min 1.59; max 2.13 ab	2.21 min 2.20; max 2.22 a	1.86 min 1.84; max 1.87 a	2.02 min 2.00; max 2.04 a	1.91 min 1.72; max 2.09 a
t-RFLP archaea	R *	24.00 ± 1.00 a	24.00 ± 0.00 a	23.00 ± 2.00 a	26.00 ± 4.00 a	25.00 ± 3.00 a	30.00 ± 3.00 a
	H *	3.01 ± 0.03 ab	3.04 ± 0.00 a	2.93 ± 0.08 b	3.16 ± 0.17 a	3.10 ± 0.19 a	3.33 ± 0.04 a
t-RFLP fungi	R **	11.00 min 9.97; max 11.99 ab	16.00 min 15.98; max 16.00 a	10.00 min 7.99; max 11.01 b	19.00 min 11.96; max 26.00 a	25.00 min 19.90; max 28.93 a	29.00 min 26.98; max 29.92 a
	H **	2.26 min 2.20; max 2.32 a	2.62 min 2.58; max 2.65 a	2.11 min 1.88; max 2.32 a	2.69 min 2.16; max 3.20 a	2.93 min 2.71; max 3.15 a	3.15 min 3.11; max 3.17 a

Explanation: ±—standard deviation, min—minimal value, max—maximal value, BA—Brunic Arenosol, AL—Abruptic Luvisol, FC—optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microorganisms, FA60—fertilizer enriched with microorganisms (dose reduced by 40%), AWCD—Average Well Color Development, AWDD—Average Well Density Development, R—Richness, H—Shannon index, OUT—operational taxonomic units, *—average values, **—median values.

2.2.2. Growth Pattern of Soil Fungal Community on Different Carbon Sources

Research concerning the functional diversity of fungi allowed for the determination of the impact of biofertilizers on the growth pattern of filamentous fungi inhabiting the soil under maize cultivation. The results of a thorough assessment of fungal growth patterns as expressed by biomass production in the experimental treatments are shown in Figure 3.

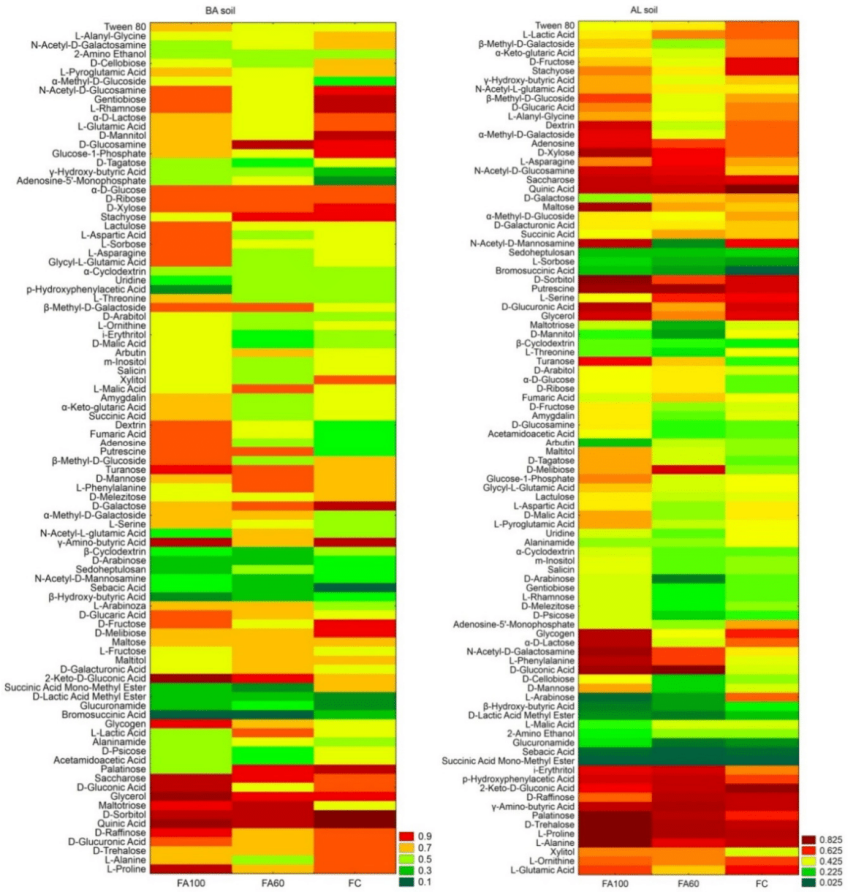


Figure 3. Diagrams of fungal growth intensity on the BIOLOG FF^{TM} plate carbon substrates. Explanation: BA—Brunic Arenosol, AL—Abruptic Luvisol, FC—optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microorganisms, FA60—fertilizer enriched with microorganisms (dose reduced by 40%).

In BA soil, it was found that fungal communities were able to grow on substrates such as β-methyl-D-galactoside, putrescine, turanose, L-arabinose, D-glucaric Acid, 2-keto-D-gluconic acid, glycogen, α-methyl-D-galactoside, maltotriose and saccharose at relatively high levels for the FA100 and FA60 treatments as compared to the control. A higher degree of biomass production was also recorded for C-compounds such as Tween 80, lactulose, L-aspartic Acid, L-sorbose, L-asparagine, glycyl-L-glutamic acid, L-threonine, dextrin, fumaric acid, adenosine, β-methyl-D-glucoside,

D-gluconic acid, D-raffinose, D-glucuronic Acid, L-proline, amygdalin, α-ketoglutaric acid, succinic acid and glycerol for the FA100 treatment. In the case of the FA60 treatment, it was reported that a higher level of growth was found on substrates such as D-glucosamine, arbutin, L-malic acid, D-mannose, L-phenylalanine, *N*-acetyl-L-glutamic acid, L-fructose, D-galacturonic acid and L-lactic acid. The lowest biomass production on substrates across all treatments included sebacic acid, D-lactic acid methyl ester and bromosuccinic acid.

For AL soil, out of 95 various *C*-compounds an increased turbidity for D-xylose, *N*-acetyl-D-glucosamine, putrescine, turanose, D-melibiose, *N*-acetyl-D-galactosamine, L-phenylalanine, D-gluconic acid, i-erythritol, *p*-hydroxyphenylacetic acid, palatinose, xylitol and adenosine was observed for the FA100 and FA60 treatments in comparison with the control soil. For the FA100 treatment an increased degree of growth was noted for β-methyl-D-glucoside, dextrin, α-methyl-D-galactoside, saccharose, maltose, *N*-acetyl-D-mannosamine, D-sorbitol, D-glucuronic acid, turanose, maltitol, D-tagatose, glucose-1-phosphate, glycyl-L-glutamic acid, D-malic acid, L-pyroglutamic acid, glycogen, α-D-lactose, D-mannose, palatinose, D-trehalose, L-proline and L-alanine, and for the FA60 treatment L-asparagine and fumaric acid utilization was reported. The lowest biomass production on substrates across all treatments comprised sedoheptulosan, L-sorbose, bromosuccinic acid, D-lactic acid methyl ester, glucuronamide, sebacic acid and succinic acid monomethyl ester. Increased AWDD values were observed for FA100 treatments in both soil types. Higher Richness and Shannon indices values were reported for the FA100 and FA60 treatments in BA soil and for FA100 treatment in AL soil (Table 1).

2.3. Terminal Restriction Fragment Length Polymorphism

As a result of analyses, the restriction profiles of the individual taxa of microorganisms, consisting of a pattern of DNA fragments of different lengths were obtained. The restriction profiles of individual taxa differed in the length of fragments as well as their relative abundance. The differences in the number of fragments within a particular group of microorganisms between the FC, FA100 and FA60 treatments were also recorded. Within the total pool of T-RFs, there were fragments presented for all experimental treatments and those that were only characteristic of a particular fertilization method.

The restriction profiles for a BA soil type are presented in the Figure 4. The bacterial restriction profile was characterized by the presence of 10 T-RFs of 63–206 bp, with the presence of each fragment reported in the FA60 treatment (Figure 4A). For the FC treatment, three restriction fragments were reported with a relative high abundance of 63 bp T-RF. The T-RF that only appeared in the FA60 treatment was a fragment of 173 bp. The differences in the relative abundance of 63, 113, 171 bp T-RFs across all treatments were also noted. According to the Venn diagrams, which were prepared in order to visualize which T-RFs were shared between particular treatments, 60% of the terminal restriction fragments obtained were common for treatments with applied biofertilizers and 30% of the total T-RFs number were shared across all treatments (Figure 5A).The presence of 24 restriction fragments of 60–518 bp was noted in the case of the archaeal restriction profile (Figure 4B), so it was characterized by the greatest variety of obtained T-RFs as compared with bacteria (Figure 4A) and fungi (Figure 4C). 100% of the obtained T-RFs were presented across all treatments with no specific fragments found for the individual fertilization method (Figure 5B). It was reported that the relative abundance of 90 bp T-RF increased in treatments where microbiologically enriched fertilizer was applied as compared to treatments where traditional mineral fertilizer was used. In the fungal restriction profile, 16 T-RFs of 73–447 bp were observed (Figure 4C) and 68.8% of them were common for all treatments (Figure 5C). All of the obtained T-RFs were found to be present in the FA100 treatment with the fragments of 214 and 400 bp specific for this fertilization method. The 73 bp fragment was the most abundant across all treatments. Compared to the FC, an increase in the relative abundance of the 447 bp T-RF in the FA100 and FA60 treatments was reported. The T-RF which only appeared in FA100 and FA60 was 143 bp.

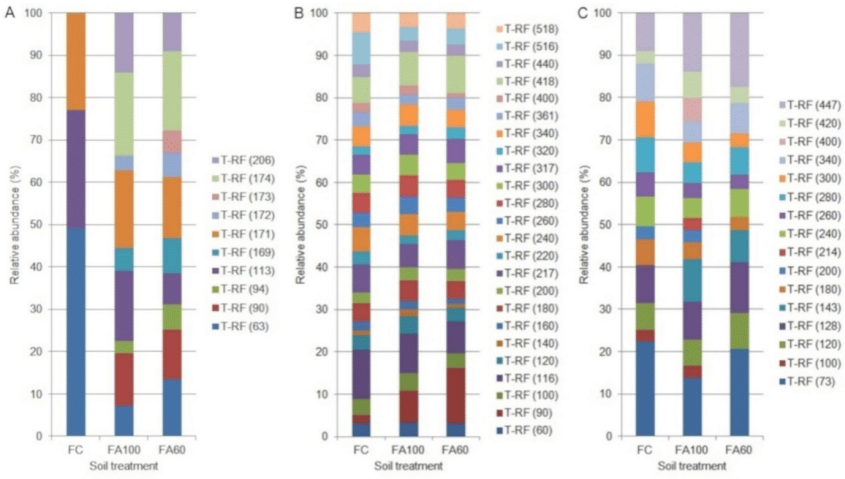


Figure 4. Relative abundance of the terminal restriction fragments obtained after HaeIII digestion for the bacterial (**A**), archaeal (**B**) and fungal (**C**) communities in Brunic Arenosol soil. Explanation: FC—optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microorganisms, FA60—fertilizer enriched with microorganisms (dose reduced by 40%).

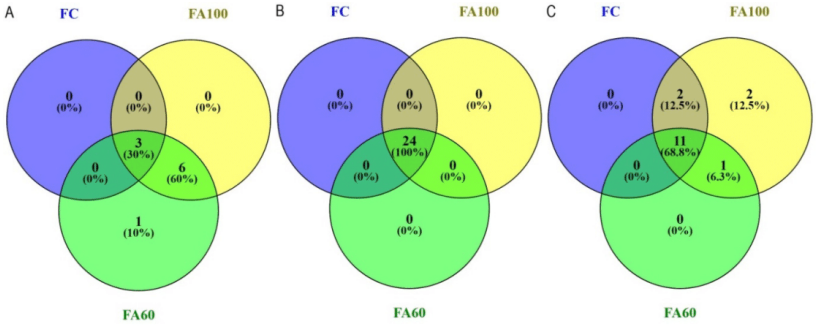


Figure 5. Venn diagrams showing the number of shared and unique terminal restriction fragments among bacteria (**A**), archaea (**B**) and fungi (**C**) communities in Brunic Arenosol soil. Explanation: FC—optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microorganisms, FA60—fertilizer enriched with microorganisms (dose reduced by 40%).

The predominant bacterial T-RF 63 in the control soil which is present in treatments with biofertilizers could be represented by *Rathayibacter* and *Caldicoprobacter* (Table S1), which was assessed based on a prediction approach used in silico analysis with TRiFLe software [32] and NCBI database (https://www.ncbi.nlm.nih.gov/) [33] The archaeal T-RF 90 present in all tested treatments (FC, FA100, FA60) which increases after biofertilizer application could be a representative of *Methanocaldococcus* and *Thaumarchaeote* based on in silico analysis mentioned above. Finally, the fungi connected with 447 T-RF may belong to the genus *Aspergillus* and *Pyrenochaetopsis* (Table S1).

Results from the AL soil type are presented in Figure 6. For the Abruptic Luvisol soil a greater variety of restriction fragment sizes was observed in comparison with Brunic Arenosol. In the bacterial restriction profile, the presence of 14 T-RFs with a size of 59–419 bp was noted, with the greatest degree of diversity characterized by the FA100 treatment, in which 13terminal DNA fragments were found

to be present (Figure 6A). Fragments unique to the treatments in which microbiologically enriched phosphorus mineral fertilizer was applied were T-RFs with the size of 206, 298, 341 and 361 bp and they constituted 28.5% of all T-RFs. In relative terms the most abundant T-RFs were 117 and 168 bp, with an increase in the abundance of the 168 bp fragment for the FA100 and FA60 treatments. It was also reported that 57.1% of the obtained T-RFs were common to all of the tested treatments (Figure 7A). In the case of archaea, a restriction profile consisting of 32 fragments of 60–539 bp (Figure 6B) was obtained and the majority (93.8%) of them were shared between all of the treatments (Figure 7B). FA100 was characterized by the greatest variety of T-RFs. For the same soil treatment, in relative terms the most abundant fragment was T-RF with a size of 360 bp. A fragment of 170 bp only appeared in the FA100 and FA60 treatments. The profile obtained for the fungal communities consisted of 31 restriction fragments with a size range of 61–560 bp (Figure 5C). All of these T-RFs were found in the FA100 and FA60 treatments, with fragments of 82, 172, 380, 390 and 560 bp with a size unique to them, which represents 16.1% of all the obtained T-RFs (Figure 7C). For the FA100 treatment an increase in the occurrence of 151 bp and 420 bp fragments was noted, while for the FA60 treatment a T-RF of 172 bp was found as compared to the control soil. An increase in the relative abundance of 130 bp T-RF in FA100 and FA60 was reported in comparison with FC. A terminal restriction fragment with a size of 61 bp was the most abundant in relative terms across the whole restriction profile.

Based on in silico analysis selected T-RFs were assigned to the following representatives of bacteria *Caloramator* (117 bp), *Pelosinus*, *Pandoraea* and *Burkholderia* (168 bp), archaea *Nitrososphaera* and *Euryarchaeotae* (360 bp) and fungi *Mucor* (200 bp), *Clonostachys* (420 bp) and *Penicillium* (82 bp) (Table S1).

Jaccard's coefficient index, based on the presence or absence of T-RFs and their relative abundance, was used to analyse similarities between the M-tRFLP profiles of soil microbial communities. The Jaccard coefficient values range from 0 to 1, where 1 indicates that the communities are identicals and 0 indicate no connections between them [34]. In this study it was reported that Jaccard's coefficient values were higher in bacterial and fungal profiles in both soil types between the FA100 and FA60 treatments (Table 2).

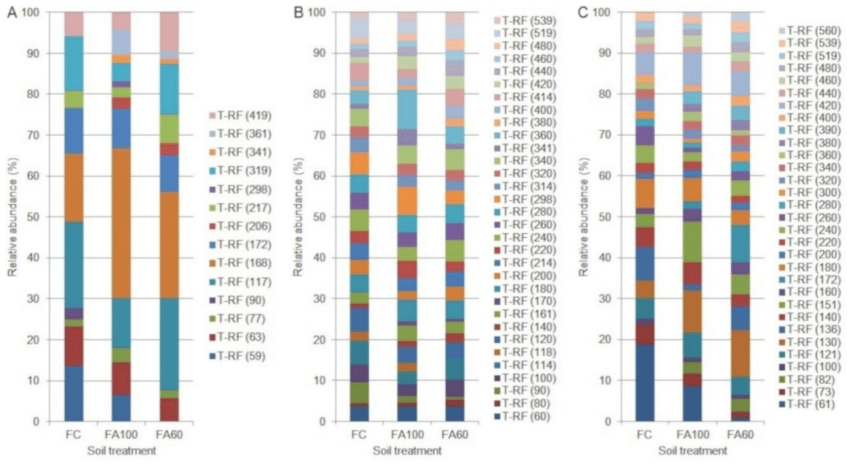


Figure 6. Relative abundance of the terminal restriction fragments obtained after HaeIII digestion for bacterial (**A**), archaeal (**B**) and fungal (**C**) communities in Abruptic Luvisol soil. Explanation: FC—optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microorganisms, FA60—fertilizer enriched with microorganisms (dose reduced by 40%).

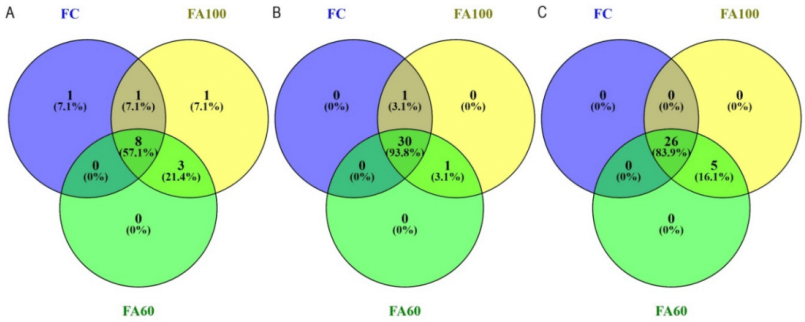


Figure 7. Venn diagrams showing the number of shared and unique terminal restriction fragments among bacteria (**A**), archaea (**B**) and fungi (**C**) communities in Abruptic Luvisol soil. Explanation: FC —optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microorganisms, FA60—fertilizer enriched with microorganisms (dose reduced by 40%).

Table 2. Jaccard's coefficient values *. Explanation: FC—optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microorganisms, FA60—fertilizer enriched with microorganisms (dose reduced by 40%).

	Soil Type					
	BrunicArenosol (BA)			Abruptic Luvisol (AL)		
Treatment	**Bacteria**	**Archaea**	**Fungi**	**Bacteria**	**Archaea**	**Fungi**
FC-FA100	0.33	1	0.81	0.64	0.97	0.84
FC-FA60	0.30	1	0.61	0.62	0.94	0.84
FA100-FA60	0.90	1	0.75	0.85	0.97	1

* Jaccard's coefficient was calculated based on the number of peaks; the peak was taken into account when was observed in at least two out of three replicates; no differences in the number of peaks between replicates were observed.

2.4. Next Generation Sequencing

2.4.1. Alpha Diversity

The soil microorganism community structure from both soil types was analysed through next generation sequencing. The results of NGS indicated differences in the composition of microbiomes between particular treatments (Figures 8 and 9).

The most abundant phyla among the tested soils were Actinobacteria (25.49–31.68%) and Proteobacteria (24.78–28.59%). The third and fourth most numerous phyla constituted Acidobacteria (9.60–14.39%) and Chloroflexi (6.52–12.18%), respectively (Figure 8A). It is worth noting that for the FA100 and FA60 treatments, in both soil types, the increased relative abundance of bacteria belonging to the Proteobacteria, Acidobacteria and Chloroflexi groups was reported, as compared to the control soil (FC).

The prominent classes in the bacterial metagenome in both soil types were Actinobacteria (12.81–16.79%), Alphaproteobacteria (13.46–16.59%) and Thermoleophilia (8.05–11.04%) (Figure 8B). Bacteria belonging to the Ktedonobacteria group were also characterized by a relatively high abundance (6.68–8.41%) as compared to other identified classes. In Brunic Arenosol soil it was observed that the relative abundance of Alphaproteobacteria was higher for the FA100 (15.59%) and FA60 (16.59%) treatments in comparison with FC (14.86%). A similar trend was noted in the case of Betaproteobacteria (FC-5.44%, FA100-6.41%, FA60-6.56%), Acidobacteria-6 (FC-3.87%, FA100-5.70%, FA60-5.49%) and Deltaproteobacteria (FC-3.71%, FA100-4.53%, FA60-5.06%) in Abruptic Luvisol soil.

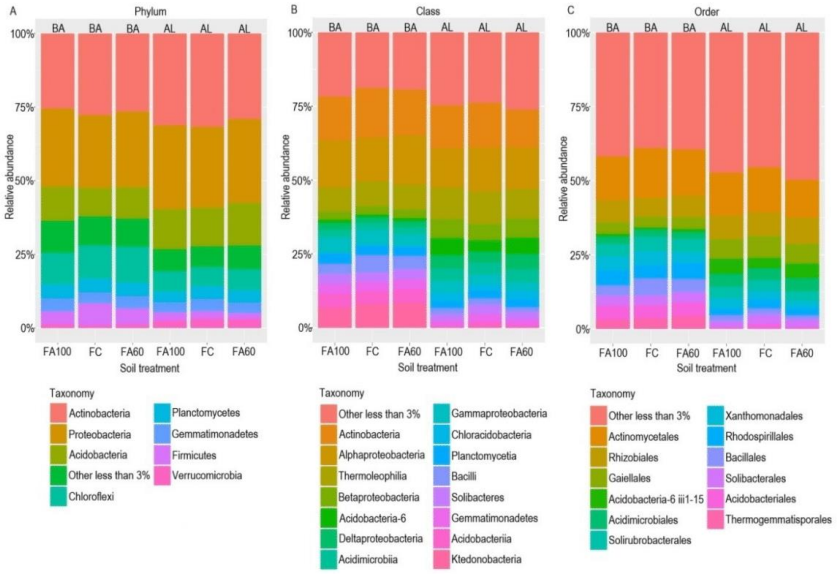


Figure 8. Distribution of bacterial phyla (**A**), classes (**B**) and orders (**C**) obtained by next generation sequencing of DNA extracted from soil samples. Explanation: BA—Brunic Arenosol, AL—Abruptic Luvisol, FC—optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microorganisms, FA60—fertilizer enriched with microorganisms (dose reduced by 40%).

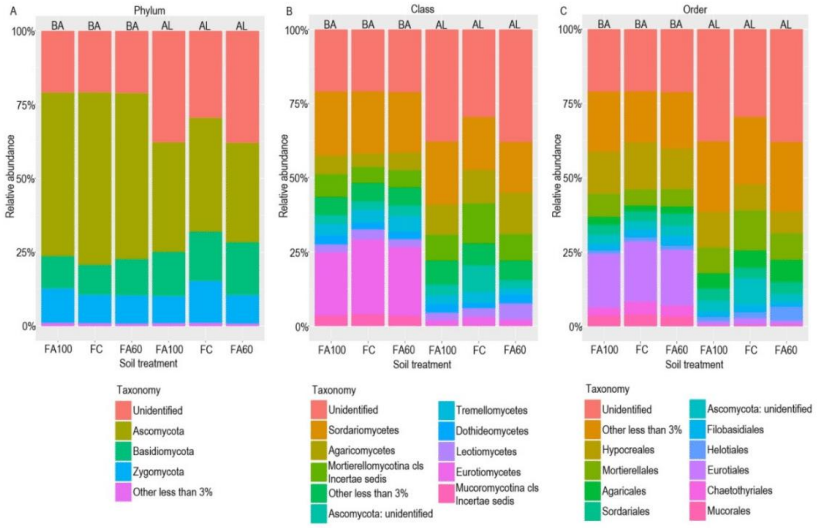


Figure 9. Distribution of fungal phyla (**A**), classes (**B**) and orders (**C**) obtained by next generation sequencing of DNA extracted from soil samples. Explanation: BA—Brunic Arenosol, AL—Abruptic Luvisol, FC—optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microorganisms, FA60—fertilizer enriched with microorganisms (dose reduced by 40%).

At the taxonomic order level, it was observed that the most abundant were members of Actinomycetales (12.81–16.79%) and Rhizobiales (6.37–8.80%) for both soil types (Figure 8C). The relatively high abundance of Gaiellales (6.63–7.32%) was also reported in AL soil. In Brunic Arenosol the increased relative abundance of Rhizobiales, Gaiellales and Rhodospirillales (FC-4.48%, FA100-5.13%, FA100-5.48%) was noted for treatments where microbiologically enriched fertilizer was applied, as compared to the control soil. The same trend was observed for Acidobacteria-6 iii1-15 (FC-3.43%, FA100-4.95%, FA60-4.81%) and for Rhizobiales (FC-8.09%, FA60-8.80%) in Abruptic Luvisol soil.

The dominant bacteria for particular treatments are shown in Table S2. 16s rDNA next generation sequencing revealed the presence of Archaea in the tested soils, however, their relative abundance was low and remained between 0.03–0.09%, depending on the soil type and fertilization treatment (Table S3). The archaeal community was mainly composed of Crenarchaeota at the phylum level (0.02–0.07%). The tested soils were almost devoid of Euryarchaeota (<0.00 to 0.01%) and Parvarchaeota (<0.00%). Among Crenarchaeota two classes were identified: marine benthic group archaea (MBGA) (<0.00–0.04%) and Thaumarchaeota (0.02–0.03%). Two classes belonging to the Euryarchaeota group were also identified, namely Methanomicrobia (<0.00%) and Thermoplasmata (>0.00–0.01%). At the order level, NRP-J were identified among MBGA with a relative abundance 0.00–0.04% and Cenarchaeales and Nitrososphaerales with a percentage share of 0.00–0.01% and 0.02%, respectively. The representatives of Methanomicrobia belonging to the Methanosarcina order comprised <0.00% in the archaeal community. In the Thermoplasmata class NGS revealed the presence of the E2 order with a relative abundance of >0.00–0.01%.

The fungal community was found to be comprised mainly of three phyla, with the dominance of Ascomycota (33.70–56.15%), followed by Basidiomycota (10.17–17.90%) and Zygomycota (9.24–14.23%) (Figure 9A). A relative increased in abundance was observed for Basidiomycota for the FA100 and FA60 treatments in case of BA soil and for the FA60 treatment in AL soil. In BA soil fungi belonging to the Zygomycota group were also characterized by a higher relative abundance for the FA60 treatment as compared to the control soil.

Among all of the identified classes, for BA soil the most numerous fungi were Sordariomycetes (20.41–21.56%) and Eurotiomycetes (21.27–23.20%). Sordariomycetes were also relatively abundant in AL soil (17.07–21.34%), followed by Agaricomycetes (10.14–14.01%) and Mortierellomycotina cls Incertae sedis (8.71–13.52%) (Figure 9B). When comparing particular treatments it was reported that the relative abundance of Sordariomycetes was higher for the FA100 treatments than for FC in both tested soils (FC_{BA}-20.94%, $FA100_{BA}$–21.56%, FC_{AL}-17.87%, $FA100_{AL}$-21.34%). A similar trend was noted for the Agaricomycetes (FC_{BA}-4.57%, $FA100_{BA}$-6.23%, $FA60_{BA}$-5.88%, FC_{AL}-11.26%, $FA60_{AL}$-14.01%). On the other hand, in the case of Mortierellomycotina cls Incertae sedis, which was one of the dominant classes in AL soil, a decrease in relative abundance was reported for treatments in which microbiologically enriched fertilizer was applied as compared to the control soil (FC_{AL}-13.52%, $FA100_{AL}$-8.71%, $FA60_{AL}$-8.92%).

A closer examination of the taxonomy of identified fungi revealed that the dominant orders in BA soil were Hypocreales (13.66–15.84%) and Eurotiales (18.12–20.27%) (Figure 9C). Hypocreales were also quite numerous in AL soil, with the increase in their relative abundance occurring for the FA100 treatment (FC-8.73%, FA100-11.99%). The second most abundant order in AL soil was Mortierellales, whose percentage share was higher in FC (13.52%) than in FA100 (8.71%) and FA60 (8.92%) treatments. Certain fungi which had a relative increase in abundance were found in treatments with applied biofertilizers and belonged to the Agaricales group (FC_{BA}-1.86%, $FA100_{BA}$-2.64%, $FA60_{BA}$-2.38%, FC_{AL}-5.79%, $FA60_{AL}$-7.46%) and the Sordariales group (FC_{BA}-3.24%, $FA100_{BA}$-3.41%, $FA60_{BA}$-3.99%, FC_{AL}-3.51%, $FA100_{AL}$-4.22%, $FA60_{AL}$-3.93%).

The dominant fungal species for particular treatments are shown in Table S4. The number of obtained OTUs are presented in Table 1. An increased number of operational taxonomic units was reported as a result of a 16S rDNA analysis in BA soil for the FA100 and FA60 treatments and also as a result of an ITS1 analysis for both soil types in treatments where microbiologically enriched fertilizer

was applied as compared to the control soil. Simultaneously, higher values of the Shannon index for the FA100 and FA60 treatments were noticed. However, there were no statistically significant changes between treatments in the aforementioned indices.

2.4.2. Beta Diversity

The Bray-Curtis dissimilarity distance was used for the construction of UPGMA dendrograms which indicate beta-diversity between the belowground microbial communities from the obtained soil treatments. The dendrogram showing the genetic relationships among the bacterial communities (Figure 10A) was based on a 16S rDNA nucleotide sequence analysis which indicated two main clusters which separately encompassed microorganisms inhabiting the AL and BA soil type, respectively. For both groups it was reported that bacteria from treatments with microbiologically enriched phosphorus mineral fertilizer formed individual clusters.

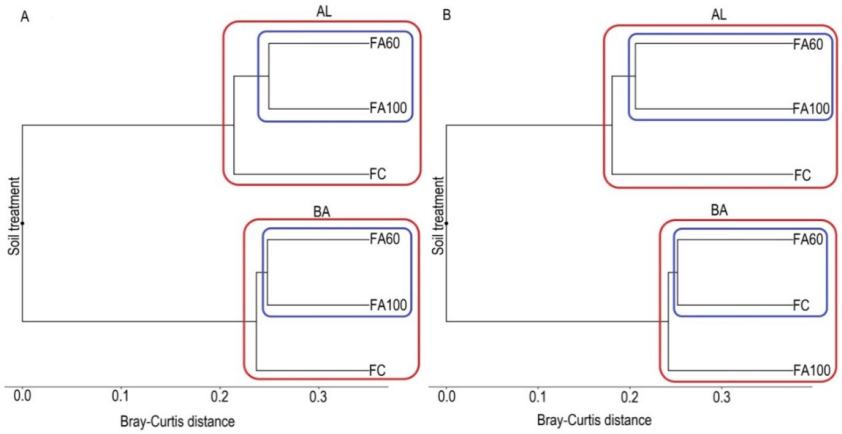


Figure 10. Cluster UPGMA dendrograms based on Bray-Curtis distances for soil bacterial (**A**) and fungal (**B**) communities. Explanation: FC—optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microorganisms, FA60—fertilizer enriched with microorganisms (dose reduced by 40%), BA—Brunic Arenosol, AL—Abruptic Luvisol.

The UPGMA dendrogram for fungal communities (Figure 10B) is based on ITS1 nucleotide sequence analysis and showed two separated clusters. One group included the treatments from the AL soil type and second treatments from the BA soil type. A further division in the first group comprised fungal communities from the FA60 and FA100 treatments. For the second group it was reported that microorganisms from the FA60 and FC treatments formed a separate cluster.

The principal coordinate analysis based on a Bray-Curtis dissimilarity showed evident clustering of treatments by soil type. Figure 11 indicates the distribution of beta-diversity between particular treatments. An analysis based on the 16S rDNA nucleotide sequence revealed two main clusters in which bacteria from the BA soil type are clearly separated from the bacterial communities in the AL soil type and the first two coordinates (PC1 and PC2) are explained, respectively, by an approximate 0.80% and 0.05% of the total variation in the bacteria communities (Figure 11A). For AL soil it was reported that the distribution of beta diversity had lower values between the FA100 and FA60 treatments when compared to the control soil. For the BA soil, beta diversity was higher within the controls in comparison with the FA100 and FA60 treatments.

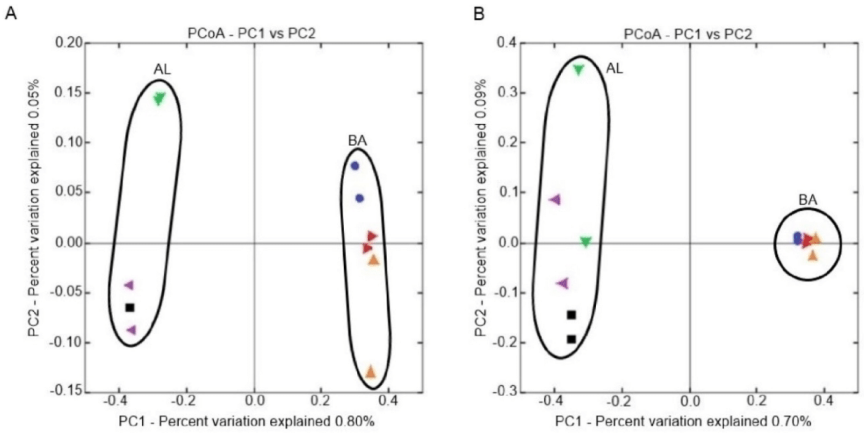


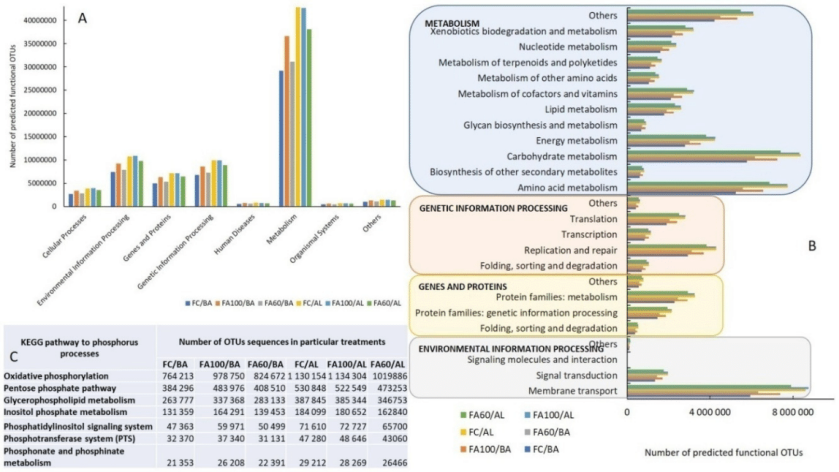
Figure 11. Principal coordinates analysis (PCoA) plots of the Bray-Curtis distances for soil bacterial (**A**) and fungal communities (**B**). Explanation: AL—Abruptic Luvisol, BA—Brunic Arenosol, green triangle—optimal dose of fertilizer AL, black square—optimal dose of fertilizer enriched with microorganisms AL, purple triangle-fertilizer enriched with microorganisms (dose reduced by 40%) AL, orange triangle—optimal dose of fertilizer BA, blue circle-optimal dose of fertilizer enriched with microorganisms BA, red triangle-fertilizer enriched with microorganisms (dose reduced by 40%) BA.

The sequencing of the ITS1 region showed a similar trend as in the case of 16S rDNA analysis, namely two different clusters in the fungal metagenome were distinguished (Figure 11B). One group comprised fungal communities from the BA soil type and a second fungi from the AL soil. PC1 and PC2 coordinates explained, respectively, 0.70% and 0.09% of the total variation. For the BA soil, it was observed that beta diversity had approximate values across all treatments. For the AL soil, the differences between the beta diversity values were lower within treatments in which microbiologically enriched fertilizer was applied in comparison with FC treatments.

2.4.3. Functional Prediction of the Bacterial Community

Bacterial function profiles predicted with the use of PICRUSt and based on the pathway database, KEGG, are presented in Figure 12. The majority of the predicted OTUs sequences annotated with the KEGG pathway in all tested treatments belonged to the following groups: metabolism group ~55%, environmental information processes ~14%, genetic information processes ~13%, genes and proteins ~9%, cellular processes ~5%, organismal systems ~1%, human diseases ~1% and other ~2%. For treatments with biofertilizers applied to BA soil a tendency to increase the sequences related to the main pathways group was observed, especially for treatment FA100. Such a tendency was not noted for AL soil (Figure 12A). Taking into account the results of the main KEGG classes, there were twelve pathways for metabolism, five for genetic information processing and four for genes and proteins and environmental information processing in this study (Figure 12B). In general, the number of sequences of particular KEGG classes were higher for AL in comparison with the BA soil type. However, for the BA soil the number of sequences of carbohydrate metabolism, amino acids metabolism, energy metabolism, lipid metabolism, xenobiotics biodegradation and metabolism increased for both treatments with biofertilizers (FA100, FA60) as compared to the control soil with mineral fertilization. A similar tendency was found for the sequences of translation, replication and repair, signal transduction and membrane transport. In contrast, for the AL soil type after the application of biofertilizers (FA100) only the sequences of membrane transport, energy metabolism and the metabolism of cofactors and vitamins

increased, whereas the rest of the predicted functions generally decreased compared to the control soil (Figure 12B).



C

KEGG pathway to phosphorus processes	Number of OTUs sequences in particular treatments					
	FC/BA	FA100/BA	FA60/BA	FC/AL	FA100/AL	FA60/AL
Oxidative phosphorylation	764 213	978 750	824 672	1 130 154	1 134 304	1019886
Pentose phosphate pathway	384 296	483 976	408 510	530 848	522 549	473253
Glycerophospholipid metabolism	263 777	337 368	283 133	387 845	385 344	346753
Inositol phosphate metabolism	131 359	164 291	139 453	184 099	180 652	162840
Phosphatidylinositol signaling system	47 363	59 971	50 499	71 610	72 727	65700
Phosphotransferase system (PTS)	32 370	37 340	31 131	47 280	48 646	43060
Phosphonate and phosphinate metabolism	21 353	26 208	22 391	29 212	28 269	26466

Figure 12. Variation in bacterial function profiles after biofertilizers application in Brunic Arenosol and Abruptic Luvisol soils analysed by PICRUSt. (**A**)—Biochemical metabolic pathways, (**B**)—Metabolism, (**C**)—Phosphorus processes based on KEGG function predictions. Explanation: BA—Brunic Arenosol, AL—Abruptic Luvisol, FC—optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microorganisms, FA60—fertilizer enriched with microorganisms (dose reduced by 40%).

There were some differences in the efficiency of bacterial function connected with phosphorus processes among the tested treatments for the BA soil type. The abundance of sequences assigned to oxidative phosphorylation, the pentose phosphate pathway, glycerophospholipid metabolism, inositol phosphate metabolism, the phosphatidylinositol signalling system, the phosphotransferase system, phosphonate and phosphinate metabolism increased after biofertilizer application as compared to the control soil with mineral fertilization (Figure 12C). In contrast, the genes related to these pathways decreased or were at the same level as in the control soil for treatments tested in Abruptic Luvisol.

2.4.4. Functional Guilds Prediction of the Fungal Community

Among the OTUs from the tested treatments of FC/BA, FA100/BA, FA60/BA, FC/AL, FA100/AL, FA60/AL, 65.98%, 66.53%, 66.44%, 52.69%, 49.68% and 50.22%, respectively were assigned to different functional groups, while the rest were unassigned. The functional groups found in all tested treatments included seven ecological guilds: pathotroph-saprotroph-symbiotroph, pathotroph-saprotroph, pathotroph-symbiotroph, pathotroph, saprotroph-symbiotroph, saprotroph, symbiotroph. The two ecological guilds saprotroph and saprotroph-symbiotroph were dominant for all tested treatments (Figure 13A). However, the OTUs counts of the saprotrophs, symbiotrophs and pathotrophs-saprotrophs in the Brunic Arenosol soil were approximately twice those identified in the Abruptic Luvisol soil (Figure 13C,G,H). Relative abundance and the total OTUs counts of pathotrophs in Brunic Arenosol soil after biofertilizers application (FA100, FA60) was significantly lower than those in the control soil (FC) (Figure 13A,E). No differences noteworthy in pathotrophs populations were found among the different treatments in Abruptic Luvisol (Figure 13E). However, a significantly higher proportion of symbiotrophs and pathotrophs-symbiotrophs in AL soil was identified for the FA100 treatment in comparison with the FC and FA60 treatments (Figure 13H,D).

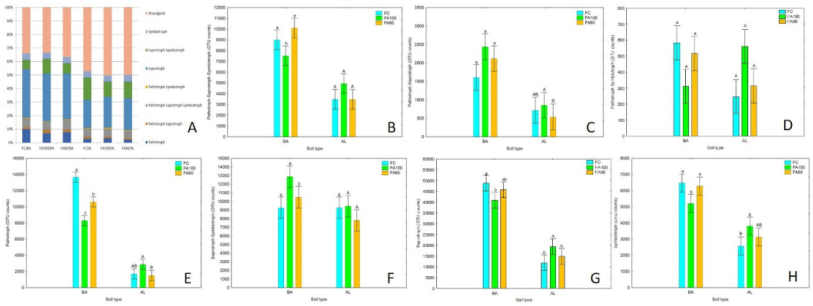


Figure 13. Relative abundance and OTUs counts for fungal functional groups (guilds) in two different soil types Brunic Arenosol and Abruptic Luvisol after biofertilizer application. (**A**)—fungal functional groups in tested soils and treatments, (**B**)—OTUs counts of pathotrophs-saprotrophs-symbiotrophs, (**C**)—OTUs counts of pathotrophs-saprotrophs, (**D**)—OTUs counts of pathotrophs-symbiotrophs, (**E**)—OTUs counts of pathotrophs, (**F**)—OTUs counts of saprotrophs-symbiotrophs, (**G**)—OTUs counts of saprotrophs, (**H**)—OTUs counts of symbiotrophs. Explanation: BA—Brunic Arenosol, AL—Abruptic Luvisol, FC—optimal dose of fertilizer, FA100—optimal dose of fertilizer enriched with microoganisms, FA60—fertilizer enriched with microorganisms (dose reduced by 40%). Vertical bars denote 0.95 confidence intervals. Different letters indicate significant differences ($p < 0.05$). The significant differences were calculated separately for each soil type by ANOVA and a post hoc analysis using the Tukey test (**B**,**E**,**F**,**H**) or Kruskal-Wallis and Dunn test (**C**,**D**,**G**). Different lowercase letters indicate significant differences within BA while uppercase letters within AL soil type.

The pathotroph population was mainly dominated by animal pathogens, which in general were present in a higher percentage in the control soils in comparison with treatments amended with biofertilizers and plant pathogens with higher counts in soils enriched with phosphorus biofertilizers (Table S5). The symbiotrophs were mainly dominated by ectomycorrhizal fungi, which showed an upward tendency after biofertilizers application for both doses (FA100, FA60) in Abruptic Luvisol and after FA100 incorporation in Brunic Arenosol. Finally, the saprotrophs consisted principally of unidentified saprotrophs fungi, which had a lower relative abundance and OTUs counts after biofertilizer use in Brunic Arenosol and a higher one in Abruptic Luvisol as compared to the control soil with the addition of only mineral fertilizers without microorganisms (Table S5).

3. Discussion

Different fertilization methods, both organic and inorganic, are known to influence the activity and biodiversity of soil microorganisms. The introduction of various substances to the soil may change the composition of indigenous microbiota and modify processes taking place in the soil environment [35–37].

At present, agricultural lands fertilized with biofertilizers fit into the growing trend of organic farming [29,38]. Biofertilizers were found to have a positive impact on multifarious aspects associated with agriculture. The application of beneficial microorganisms is known to improve nutrient exploitation, soil quality and fertility, plant growth, response to biotic and abiotic stresses and enhance crop yield [17]. It has also been documented that biofertilizers contribute to shifts in the biodiversity of soil microbiota [39], changes in soil biological and enzymes activities [40,41] and to the reduction in belowground pollutants [42]. Providing plants with nutrients which are not readily available is thought to be one of the most important functions of biofertilizers. Such elements include, among others, phosphorus whose availability in soil is limited and moreover, the excessive application of P mineral fertilizers may further diminish its uptake by plants. Therefore, it is important to

implement microbial-based techniques that will improve the phosphorus availability and the efficiency of its uptake [43].

The activity of soil enzymes is thought to be an important indicator of soil quality and microbial community properties. Soil enzymes, produced by belowground microorganisms, are sensitive to various agronomic practices and changing environmental conditions, therefore an understanding of their role in soil functioning is necessary for the maintenance of an ecological balance in agricultural ecosystems [44,45]. In the presented study, we investigated the impact of phosphorus mineral fertilizer enriched with beneficial bacterial strains on the activity of four soil enzymes: β-glucosidase, protease, urease and acid phosphomonoesterase, these play a key role in substrates mineralization and in the biogeochemical cycles of carbon, nitrogen and phosphorus [46]. The obtained results showed that the application of biofertilizers did not adversely affected enzymes activity. For the treatments with applied biofertilizers an increase in enzymatic activity in relation to the control soil was noticeable. This indicates that the microorganisms contained in the fertilizers, in cooperation with indigenous microbiota, could metabolize compounds supplied to the soil more intensively, thereby providing nutrients for plant growth. The enhancement of enzymatic activity is of particular importance for FA60 treatments, because it presents the opportunity to reduce the dose of applied mineral fertilizer with a simultaneously advantageous effect on the intensity of biochemical processes occurring in the soil. The higher activity of β-glucosidase in FA100 and FA60 treatments, which has been proposed as an indicator of the soil organic matter (SOM) decomposition status, suggests an increased rate of SOM degradation [47]. As Lin et al. [48] noted, for soil amended with chemical fertilizers combined with organic manure, the activities of protease, urease, acid phosphatase and β-glucosidase remained at higher level as compared to the soil where only chemical fertilizers were used. The higher activity of β-glucosidade was also reported for soil in which wheat seeds were fertilized with the commercial products of Rhizosum N® and Rhizosum PK®. Rhizosum PK® is a biofertilizer containing i.a. *Bacillus megaterium* and *Frateuria aurantia* which were found to exhibit potassium-mobilizing properties [49–51]. A study conducted by Mengual et al. [52] showed that the introduction of microbial inoculants to the soil positively the affected activities of urease, protease and β-glucosidase with significant changes occurring in the case of urease and protease. The increased protease activity for FA100 and FA60 treatments may be associated with the capability of *Bacillus* sp. and *Paenibacillus* strains to produce heat stable proteases [53,54]. The limitations of phosphorus availability in soils are known to stimulate plant roots to secrete acid phosphatases which may contributed to an improvement in the aforementioned enzyme activity in AL soil in comparison with BA soil [55,56]. Proteases are enzymes that catalyze hydrolysis of peptide bonds in proteins, causing their breakdown into free amino acids [57]. In our study, increased protease activity was observed for the FA100 and FA60 treatments in both soil types, which may be associated with the increased availability of free amino acids in soils and their higher utilization rates in BIOLOG plates.

The functional diversity of soil microorganisms is another important parameter taken into account in assessing soil quality and fertility because it is connected with the activity of belowground microorganisms and their ability to adapt to soil environment modifications [58]. The BIOLOG system is widely used in environmental microbiology for the evaluation of the impact of various agricultural management practices [44,59], the contamination of soil and sewage sludge with heavy metals [60,61] and pesticides [62], the application of organic matter from organic wastes [63] and some stressing factors such as salinity, pH and heating [64] on the functional diversity of soil microbial communities.

The presented results of the metabolic profile of bacterial and growth intensity of fungal soil communities indicated differences in the functional diversity of soil microorganisms both between soil types and individual treatments. It is commonly known that, similarly to soil enzymatic activity, substrate use and the metabolic potential of soil microbes are an indicator of the intensity of biochemical processes occurring in the soil [65,66]. The highest level of metabolic diversity indices (AWCD, AWDD, H) were observed for the AL soil type which corresponds to increased enzymatic activities in the

aforementioned soil type as compared to the BA soil type. The enhancement of the general metabolism of soil microorganisms for the FA100 and FA60 treatments is also indicated by the increased AWCD values [67].

An assessment of the ability of soil microorganisms to utilize different carbon sources is a rapid method for testing the differences and similarities between particular treatments [60]. In this work, supplying soil with microorganisms contained in biofertilizers contributed to shifts in the degree of utilization of C-compounds. Live microorganisms applied directly to the soil may influence the activity of indigenous soil microbiota [68] and changes in the level of utilization of some C-compounds reflect the changes to the metabolic abilities of microbial communities. With the simultaneous increase in the utilization of some C-substrates, a decrease in the use of others was noted, but their low level indicates the possibility of microorganisms' adaptation to new conditions. According to Chaudhry et al. [69] higher functional diversity was observed for organically cultivated land as opposed to land management based on chemical fertilizers. The results obtained on ECO and FF plates showed that soil microorganisms are able to utilize various carbon sources. The increased utilization of particular polymers, amino acids, carbohydrates, carboxylic acids and amines for the FA100 and FA60 treatments suggest that the introduction of beneficial bacterial strains to the soil may enhance the metabolic properties of microorganisms towards certain compounds, which may constitute a source of essential nutrients, which are necessary not only for microorganisms but also for plants.

Through an analysis of utilization rates, it was reported that some amino acids and amines were metabolized more intensively in treatments with applied biofertilizers. Amino acids constitute not only a carbon, but also an important organic nitrogen source in soil [70]. The highest utilization rates of D-glucose-1-phosphate and D,L-α-glycerol phosphate, which may pose a phosphorus source for soil microbial communities, were also reported for AL soil. The increased utilization rate of the aforementioned compounds may be associated with the increased activity of microorganisms involved in the biochemical conversion of nitrogen and phosphorus. However, microorganisms more efficiently metabolize L-amino acids than their D-enantiomers [71] and our results suggest that the introduction of beneficial bacterial strains to the soil may accelerate processes responsible for L-amino acids utilization. These results are in accordance with the prediction results based on sequences obtained via NGS, where an increase in the sequences of amino acids metabolism under biofertilizers application into the soil was obtained.

It was observed that the utilization of polymers increased for the FA100 and FA60 treatments, as compared to the control treatment. According to Cheng et al. [72] Tween 80 was found to be easily biodegradable by soil indigenous microorganisms. It is also worth mentioning that microbial based techniques in combination with the addition of Tween 80 were successful in soil bioremediation from hydrophobic organic compounds. On the other hand, it was reported that the *Sphingomonas* strain in combination with Tween 80 was less effective in phenanthrene biodegradation. This may be explained by the fact that Tween 80 is the preferred carbon source over phenanthrene [73]. Moreover, a functional prediction of the bacterial community indicated that a higher number of sequences originated from xenobiotics biodegradation and metabolism in treatments with biofertilizers applied into BA soil and had no effect on AL soil, confirming that soil microbial community responses are dependent on soil type and properties.

It was described previously that carbohydrates comprise 70% of maize root exudates [74]. Considering the fact that oligo- and monosaccharides are preferred carbon source for microorganisms [75], it may be assumed that microbial strains inhabiting soil under maize cultivation have adapted their metabolic pathways in favour of carbohydrates utilization. The application of biofertilizers containing beneficial strains may stimulate indigenous microbiota to intensify the breakdown of carbohydrates. The increased metabolism of carbohydrates in FA100 and FA60 treatments may be connected with increased β-glucosidase activity in the aforementioned treatments [76]. Furthermore, the sequences related to carbohydrate metabolism increased for treatments with biofertilizers in the BA soil type.

Research concerning the diversity of soil microorganisms should also be based on an analysis of genetic diversity. In order to determine the structure of soil microorganisms, techniques based

on the genetic fingerprint of the microbial communities are commonly used [77]. One of the most effective methods described in the literature is the analysis of terminal restriction fragment lengths polymorphism (t-RFLP) [78]. In this study a modified t-RFLP method was used, namely multiplex t-RFLP, which enables the simultaneous analysis of bacterial, archaeal and fungal soil microbial populations [79]. The obtained T-RFs patterns indicate changes in the genetic diversity after the application of biofertilizers. The presence of restriction fragments of the same length for all treatments may indicate the existence of bacterial, archaeal and fungal species which are commonly found, regardless of the environmental conditions. Changes observed in their relative abundance may result from shifts that occurred after the introduction of the biofertilizer. In the FA100 and FA60 treatments an increase in the T-RFs number was observed compared to the control soil. A similar trend was noticed by Trabelsi et al. [80], where soil inoculation, under potato cultivation, with rhizobial strains, contributed to an increase in the number of T-RFs in bacterial populations. Jaccard's coefficient was analysed for each treatments and showed that microbial populations for the FA100 and FA60 treatments were more closely related to each other than to microorganisms in the control soil.

For a more detailed metataxonomic characterization of the soil microbial communities, next generation sequencing (NGS) was performed to identify representatives of different taxa present in soil amended with biofertilizers. The implementation of NGS in the field of environmental microbiology is very appealing for the exploration of soil microbial diversity due to the fact that it allows for an accurate and extensive analysis of multiple samples at the same time [81]. The metagenomic approach simplifies not only the study of phylogenetic relationships between microorganisms but it also determines their functionality in the soil environment [82].

Several studies have proven that fertilization strategies have an impact on the genetic diversity of soil microbial communities [83–85]. Our study revealed that the application of mineral fertilizer enriched with beneficial bacterial strains increased the number of 16S rDNA OTU's in BA soil and ITS1 OTU's for both soil types.

In the current study, Actinobacteria, Proteobacteria, Acidobacteria and Chloroflexi were the dominant phyla for all treatments in both soil types. Moreover, it was observed that for the FA100 and FA60 treatments, the relative abundance of Proteobacteria and Acidobacteria increased as compared with the control soil. Proteobacteria constitute the largest and the most diverse phylum in phylogenetic terms and along with Actinobacteria, their presence in the soil is connected with a high degree of carbon availability. The increased activity of β-glucosidase, which is involved in carbon cycling, in AL soil may be associated with the higher relative abundance of Actinobacteria and Proteobacteria in comparison with BA soil. At the same time, Acidobacteria is known to inhabit acidic and nutrient poor environments [86,87]. Generally, the members of the Proteobacteria and Acidobacteria group are commonly found in almost all soil types [88].

The results obtained with NGS showed that the relative abundance of potential nitrogen fixing strains increased for the FA100 and FA60 treatments, depending on soil type. The aforementioned nitrogen-fixing bacteria included Frankiaceae, Bradyrhizobiaceae and Rhodospirillaceae. Some of the members of the Frankiaceae group, especially those from the genus *Frankia* are known as a nitrogen-fixing symbiotic partners of actinorhizal plants [89]. The family of Bradyrhizobiaceae includes the genus *Bradyrhizobium*, which has members that are important microorganisms involved in biological nitrogen fixation (BNF) in legume plants. Due to possibility of using various nitrogen sources, bacteria from the Bradyrhizobiaceae family are vital constituents of the nitrogen cycle in the environment [90]. In considering the Rhodospirillaceae family, it is worth mentioning that one of the genus within Rhodospirillaceae is *Azospirillum*, which has representatives that exhibit plant growth promoting properties due to their N-fixing abilities [91].

Urease is an enzyme that catalyses the hydrolysis of urea to ammonia and carbon dioxide. Ammonia can then be oxidized to nitrates in the nitrification process, which may be conducted by ammonia oxidizing archaea (AOA) that belong to the Nitrososphaerales group in the Thaumarchaeota class [92]. Our results, which were obtained by both approaches NGS and t-RLFP, proved the occurrence

of the aforementioned archaea in tested soils. The next step is denitrification and it has been described that many members of *Rhodoplanes* have the possibility of initiating this process [93]. *Rhodoplanes* lead to chemotrophic growth using denitrification in anoxic conditions in the presence of nitrates [94]. The increased urease activity for the FA100 treatments may be associated with an increase in the relative abundance of *Rhodoplanes* in the aforementioned treatments.

Members of the Syntrophobacteraceae family are described as sulfate-reducing bacteria [95]. In comparing the studied soils, the highest percentage share of this family in the bacterial community was reported in AL soil which may be associated with the fact that the experimental site was located near a sulphur mine.

The increased relative abundance of bacteria from the Actinomycetales order was reported for the FA100 and FA60 treatments in BA soil. Actinomycetales perform important functions in soil such as the decomposition of organic matter, suppressing some phytopathogens and the degradation of complex compounds in dead plants, animals and fungal manure. One noteworthy aspect of the bacteria is the fact that some Actinomycetales exhibit phosphorus solubilization and mineralization properties due to the production of organic acids and mechanisms encompassing chelation, exchange reactions and formation of polymeric compounds. It was also found that Actinomycetales secrete phosphatases, both acid and alkaline, and phytases, enzymes which are involved in belowground phosphorus biotransformations [10].

In the case of fungal communities, three phyla were identified with the domination of Ascomycota across all treatments in both soil types followed by Basidiomycota and Zygomycota. The high relative abundance of Ascomycota in both soil types may result from the fact that Ascomycota are the most numerous fungi in terrestrial environments [96].

In both soil types one of the dominant orders was Mortierellales and among them it was possible to distinguish some species: *Mortierellahorticola*, *Mortierellaelongata*, *Mortierella humilis* and *Mortierella* sp. However, the beneficial impact of *Mortierella* sp. strains on soil and plant properties has been described by researchers [97–99]. In this study it was reported that the relative abundance of some Mortierellales representatives increased in treatments fertilized with phosphorus biofertilizer. This result is in agreement with Li et al. [100] who observed that the relative abundance of *Mortierella* increased in soil treated with organic amendments. It is worth mentioning that, Li et al. [100] observed that the soil inoculated with *M. elongata* was characterized by a higher β-glucosidase activity. Another factor worth noting is the fact that some members of the *Mortierella* sp. are able to dissolve inorganic phosphorus compounds due to the production of oxalic acids [101,102] and to co-operate with mycorrhizal fungi in P uptake. Another strain, *M. humilis* was found to synthesize enzymes involved in the degradation of xylans, paraffin, chitin and some saccharides like cellulose, lignin, galactose, fructose and mannose [103]. Taking into account the beneficial properties of *M. elongata* described in the aforementioned study, the application of microbiologically enriched phosphorus mineral fertilizer seems to be a favourable option in improving the quality of the soil microbiome.

The Brunic Arenosol fungi from the *Penicillium* genus were characterized by quite a high relative abundance as compared to other identified fungi. The plant growth promoting properties of the *Penicillium* species include the production of antibiotics and plant hormones, protection from salinity and the induction of plant resistance. *Penicillium* also exhibit phosphate solubilization capabilities [104,105]. The most dominant *Penicillium* species was *P. simplicissimum* and it is worth noting that the relative abundance of this fungus increased for the FA60 treatment as compared to the control soil. According to Sangale et al. [106] *P. simplicissimum* is capable of degrading polyethylene and can induce resistance against Cucumber mosaic virus in tobacco plants and in *Arabidopsis thaliana* [107].

The fungi identified across all treatments for both soil types was *Fusarium oxysporum* which is considered to be one of the most widespread of the phytopathogenic fungi [108,109]. In this study it was reported that the relative abundance of *F. oxysporum* decreased for the FA60 treatment in AL soil and also for the FA100 treatment in BA soil as compared to the control treatments. The results from Qiu et al. [110] showed that the soil fertilized with amino acid fertilizer in combination with

organic manure and strains of *Bacillus subtilis*, *Paenibacillus polymyxa* and *Trichoderma harzianum* was characterized by a lower relative abundance of *F. oxysporum* than soil devoid of the aforementioned beneficial microorganisms strains. According to Zhang et al. [111] *Paenibacillus polymyxa* is able to produce fusaricidin-type peptide antibiotics that suppress plant pathogenic fungi like *F. oxysporum*.

Isaria fumosorosea (formerly known as *Paecilomyces fumosoroseus*) is a fungi that appeared only in the FA100 treatment in the BA soil type at a relative abundance >1%, however in the FA60 treatment it was found that the relative abundance was higher than that found in the control soil. This microorganism is known as an entomopathogenic fungus and due to its various properties including a wide host range, the lack of a deleterious impact associated with the application of synthetic pesticides and the quality of remaining ecofriendly, it is thought to be a promising option as a biological agent against crop insects [112]. According to Kuźniar et al. [113] *Isaria fumosorosea* may be used as a bioinsecticide against the European corn borer (*Ostrinianubilalis* Hbn.) in sweet maize cultivation. Another identified enthomopathogenic fungus was *Metarhizium robertsii*, which was found to have a relative abundance that decreased in the FA100 and FA60 treatments in BA soil, however, it remained at a higher level in the FA60 than in the FA100 treatment. *Metarhizium* spp. strains were found to exert plant growth promoting properties encompassing the suppression of soil insects and plant pathogens, forming symbiotic relationships with plants and increasing nutrient uptake [114]. The results obtained from Sasan and Bidochka [115] showed that *Metarhizium robertsii* was able to establish close associations with the roots of switchgrass and haricot bean and to stimulate their growth and root hair proliferation.

The members of the Chaetothyriales sp., including the, *Cladophialophora* genus were identified in the BA soil type. These fungi are known as human pathogens, which may cause nervous system infections [116,117]. However, it was reported that in the FA100 and FA60 treatments the relative abundance of Chaetothyriales sp. decreased as compared to the control soil and, moreover, the relative abundance of *Cladophialophora* sp. in the FA100 and FA60 treatments remained below 1%. This may suggest that the beneficial strains contained in phosphorus biofertilizer exert antagonistic properties toward the Cladophialophora sp. species. A study conducted by Romero et al. [118] showed that the *Bacillus subtilis* strain may inhibit the growth of pathogenic fungus *Cladophialophora carrionii* in vitro.

NGS showed that the relative abundance of *Solicoccozyma terricola* increased in the FA60 treatment in BA soil as compared to the control. According to Stosiek et al. [119], the psychrotolerant strain *Solicoccozyma terricola* M 3.1.4. is capable of degrading glyphosate, which is commonly used in chemical pesticides; however, it was reported that glyphosate may pose a threat to living organisms. The application of the aforementioned fungus seems to be environmentally friendly tool in bioremediation and the introduction of beneficial microorganisms to the soil may positively affect the presence of other microbial strains useful in the field of ecology and agriculture.

The increased relative abundance of *Agrocybe pediades* and *Stropharia coronilla* was observed in the FA60 treatment and FA100 treatment in AL soil, respectively. These fungal strains are saprobic microorganisms [120,121] and, moreover, the strain of *Stropharia coronilla* was found to have the capability of metabolizing benzo[a]pyrene in a liquid culture [122].

Through cluster analysis, based on Bray-Curtis distances, we reported that the microbial communities from the FA100 and FA60 treatments were more similar to each other than those presented in the control soils. This suggests that the introduction of biofertilizers modified the composition of the microbial populations in both soil types. These results correspond to the results obtained with PCoA analysis, where a clustering of microorganisms from different soil types is clearly observed. This shows that for the tested treatments soil type and fertilization strategies are the main factors driving the structure of the microbial communities.

4. Materials and Methods

4.1. Study Site and Soil Sampling

Field experiments established in April 2018, were conducted on two different soil types, under maize cultivation (variety of P9241, FAO: K280, Z270, PIONEER, Warsaw, Poland). One field study was performed in Biszcza, South-East Poland (50°43′ N, 22°60′ E) on agricultural land that has been degraded due to inadequate cultivation and fertilization. The soil type was determined to be a Brunic Arenosol (BA) [41] with a pH_{KCl} of 4.8 and P, K, Mg contents of 174, 29 and 12 mg kg^{-1}, respectively. The altitude of the study was about 211 m above sea level. Another field experiment was established in Basznia, South-East Poland (50°15′ N, 23°26′ E). The soil was classified as a Abruptic Luvisol (AL) [41] and it was chemically degraded as a result of the sulphur mine located near the experiment site. The soil pH_{KCl} at the beginning of the study was 4.9 and the contents of P, K, Mg were, respectively, 48, 53 and 36 mg kg^{-1}. The experiment was located at an altitude of 230 m above sea level.

The doses of applied fertilizers were calculated based on the plant nutritional requirements and soil mineral content. The following mineral fertilizers were used in the study: phosphate mineral fertilizer SUPER FOS DAR 40 (Grupa Azoty, Puławy, Poland), nitrogen fertilizer PULREA PUŁAWSKI MOCZNIK 46N (Grupa Azoty, Puławy, Poland), granulated potassium salt (BIALCHEM, Poland). The microbial beneficial strains were provided by SYMBIOBANK and originated from the Research Institute of Horticulture in Skierniewice, Poland. In order to obtain biofertilizers, the phosphorus mineral fertilizer was enriched with *Paenibacillus polymyxa* strain CHT114AB, *Bacillus amyloliquefaciens* strain AF75BB and *Bacillus* sp. strain CZP4/4. The granules of fertilizer were coated with the mixture prepared in equal proportions 1:1:1 of each aforementioned strain [123]. The biofertilizers were prepared and provided by the Łukasiewicz Research Network–New Chemical Syntheses Institute (Puławy, Poland). A summary of the treatments that were conducted within both experiments is presented in Table 3.

Table 3. The treatments arrangements within the field experiments. The doses of applied; fertilizers/biofertilizers were calculated per ha separate for each soil type.

Treatment	Brunic Arenosol (BA)	Abruptic Luvisol (AL)
Optimal dose (FC)	125 kg phosphate mineral fertilizer; 365 kg urea; 290 potassium salt	150 kg phosphate mineral fertilizer; 360 kg urea; 284 kg potassium salt
Optimal dose enriched with microorganisms (FA100)	156.25 kg phosphate mineral fertilizer enriched with beneficial bacterial strains; 365 kg urea; 290 potassium salt	187.5 kg phosphate mineral fertilizer enriched with beneficial bacterial strains; 360 kg urea; 284 kg potassium salt
Dose reduced by 40% enriched with microorganisms (FA60)	93.75 kg phosphate mineral fertilizer enriched with beneficial strains; 365 kg urea; 290 potassium salt	112.5 kg phosphate mineral fertilizer enriched with beneficial bacterial strains; 360 kg urea; 284 kg potassium salt

Because of that much of the P applied to the soil as mineral fertilizers is bound to the soil, thereby creating a pool of residual P, or it is lost through leaching, runoff, or erosion and may contribute to the eutrophication of waterbodies [124], the research carried out included the application of a 40% reduced dose of phosphorus fertilizer enriched with beneficial bacteria strains which have the potential to activate phosphorus present in the soil. Each type of fertilization was arranged in 3 replications with 10 m × 15 m plots for both soil types. The soil sampling in this study was performed in June 2018, one week after fertilizer application. The soil samples were collected from the 0–25 cm layer from five sites within each plot and then averaged by homogenization and intensive mixing. The samples were transported afterwards under refrigeration at 4 °C to the laboratory and sieved through a 2 mm sieve. The soil samples were promptly used for measurements or stored (at 4 °C for CLPP and enzymatic activity or at −80 °C for DNA extraction).

4.2. Enzymatic Activities

Urease activity was determined according to the Zantua and Bremner [125] method with the use of a urea solution as a substrate, after 18-h soil incubation at 37 °C. The wavelength of urease activity was measured at 410 nm.

Protease activity was assessed using the Ladd and Butler [126] method as a modification of Alef and Nannipieri [127], after one hour of soil incubation at 50 °C with a Tris-HCl (pH 8.1) sodium caseinate solution as a substrate. The concentration of released tyrosine was measured spectrophotometrically at 578 nm.

β-Glucosidase activity was determined using the Eivazi and Tabatabai [128] method modified by Alef and Nannipieri [127]. This method is based on the determination of the p-nitrophenol (PNP) released after the incubation of soil with a p-nitrophenyl-*β*-d-glucoside (PNG) solution as a substrate, for 1 h at 37 °C. The enzymatic activity was measured colorimetrically at a wavelength of 400 nm.

The acid phosphomonoesterase activity was assessed according to the Tabatabai and Bremner method [129] after one-hour of soil incubation at 37 °C using a PNP in Tris-HCl buffer at pH 6.5. The concentration of released PNP was determined spectrophotometrically at a wavelength of 400 nm.

The results were calculated with reference to the oven-dry (105 °C) weight of soil.

4.3. Community Level Physiological Profiling (CLPP)

One way to determine functional diversity is community level physiological profiling (CLPP) with the application of plates (BIOLOG®, Hayward, CA, USA) coated with various carbon sources and dedicated to specific groups of microorganisms. As a result, a characteristic and unique utilization pattern of the C-substrates called "metabolic fingerprinting" is obtained [130,131]. For the evaluation of the catabolic abilities of soil bacterial and fungal communities, BIOLOG ECO and BIOLOG FF microplates were used, respectively [132]. The ECO microplate consists of a set of 31 different carbon sources plus a water control, in three replications, contained in 96-well microtiter plates [133]. The BIOLOG FF microplate consists of 95 various carbon sources and a non-C control distributed in 96-well microtiter plates [134].

The CLPP analysis using BIOLOG plates was prepared in the following way: 1 g of fresh soil was suspended in 99 mL of sterile saline peptone water and shaken for 20 min at 20 °C before being incubated for 30 min at 4 °C. Afterwards, each well of the BIOLOG ECO plate and FF plate was inoculated with a prepared suspension of 120 µL and 100 µL respectively, and incubated at 23 °C. Absorbance readings were performed at 590 nm in the case of ECO plates and at 750 nm as for FF plates with a BIOLOG MicroStation plate reader (Biolog®, Hayward, CA, USA) every 24 h for 216 incubation hours. Detailed procedures of the analyses were described by Wolińska et al. [135] and Gryta et al. [35].

4.4. DNA Extraction

Genomic DNA was extracted from 0.5 g of fresh soil samples using a FastDNA Spin Kit for Feces (MP Biomedicals, Solon, OH, USA) according to manufacturer protocol. The isolated DNA was then eluted in 50 µL of TES buffer. The quantity of extracted DNA was determined spectrophotometrically at a wavelength of 260 nm and the purity was determined using coefficients (260/230, 260/280) calculated on the basis of absorbance readings at 230, 260 and 280 nm (NanoDrop 2000/2000c, Thermo Scientific, West Palm Beach, FL, USA). The extracted DNA was then stored at −20 °C for further analyses comprising multiplex terminal restriction fragments length polymorphism (M-tRFLP) and next generation sequencing (NGS).

4.5. Multiplex Terminal Restriction Fragment Length Polymorphism (M-tRFLP)

In this work, the multiplex t-RFLP (M-tRFLP) fingerprinting method was used, which allowed for a simultaneous analysis of three microbial taxa: bacteria, archaea and fungi. The M-tRFLP reaction includes: DNA amplification with fluorescently labelled individual taxon-specific primers, digestion

using restriction endonuclease selected appropriately for all amplification products present in the reaction mixture and the separation of the restriction products using a genetic analyser [136].

The first step of the M-tRFLP analysis was the PCR reaction for all extracted DNA samples. For the bacterial community analysis, the PCR primer pair (63f/1087r) targets the 16S rDNA region was used and the primers were as follows: primer F: 5′-AGGCCTAACACATGCAAGTC-3′ [137] and primer R:5′-HEX-CTCGTTGCGGGACTTACCCC-3′ [79,138]. For the fungal community analyses, the PCR primers (ITS1F/ITS4R) were used to amplify the ITS1 spacer and the primers had the following sequences: primer F: 5′-6-FAM-CTTGGTCATTTAGAGGAAGTAA-3′ [139] and primer R: 5′-TCCTCCGCTTATTGATATGC-3′ [140]. For the archaeal community analyses the PCR primer pair (Ar3F/Ar9R) was used and the primers were as follows: primer F: 5′-TTCCGGTTGATCCTGCCGGA-3′ [141] and primer R: 5′-ROX-CCCGCCAATTCCTTTAAGTTTC-3′ [79,142]. Each PCR reaction mixture contained 15 µL of RedTaq®ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO, USA), 4 ng of genomic DNA, in the case of the bacterial mixture: 0.5 µLwas used and for the fungal and archaeal mixture: 1 µL each of the forward and reverse primers (diluted to a final concentration 10 µM) were used. The PCR reaction was performed with a gradient thermal cycler (Veriti 96 well Fast Thermal Cycler) with the following temperature cycle: 95 °C initial denaturation for 5 min, followed by 30 cycles at 95 °C denaturation for 30 s; 55 °C annealing for 30 s; 72 °C elongation for 60 s, and a final extension at 72 °C for 10 min. The presence and size of the amplification products (bacteria 1000 bp, archaea 900 bp and fungi 600 bp) were checked electrophoretically (110 V, 40 min) on a 2% agarose gel, which was stained with a SimplySafe (EURx, Gdańsk, Poland) solution and visualized with UV excitation. Subsequently, the PCR products were purified by using a mixture of thermo sensitive alkaline phosphatase and Exonuclease I (Exo-BAP-Mix, EURx) followed by incubation at 37 °C for 15 min and then at 80 °C for 15 min. After the Exo-BAP reaction, the amplification products were purified with Performa® DTR (Dye Terminator Removal) Gel Filtration Cartridges (EdgeBio, San Jose, CA, USA) according to the producer protocol. The amount and quality of DNA was determined by a spectrophotometer at a wavelength of 260 nm. The purified PCR products were then digested by endonuclease HaeIII (EURx, Gdańsk, Poland). The restriction mixture (13 µL) containing 5–10 µL of purified PCR products (about 50 ng of DNA), 0.6 µL of restriction enzyme (10 U/µL), 0.6 µL of ONE buffer containing bovine serum albumin (BSA) (EURx, Gdańsk, Poland) and water, was incubated at 37 °C for 2 h. Enzyme inactivation was performed by incubation at 80 °C for 20 min. Aliquots (1 µL) of the digest were mixed with 9 µL of deionized formamide and 0.5 µL of DNA fragment length standard GS-600LIZ (Applied Biosystems, Foster City, CA, USA). The restriction samples were applied on the plate in three replications for each sample. Then, the plate was denaturated at 94 °C for 3 min and chilled on ice. The sizes of the fluorescently labelled restriction fragments were determined by capillary electrophoresis using an automated ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The procedure was optimized and described in detail by Gryta and Frąc [79]. The obtained results were analyzed using GeneMapper v. 4.0. software (Applied Biosystems, Foster City, CA, USA). The analysis included restriction fragments larger than 50 bp and >1% of the total area within the sample. Based on in silico analysis using TRiFLe software [32] the terminal restriction fragments (t-RFs) were identified using The National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) [33] for 16S rDNA and ITS1 rDNA and *amo*A AOA Feifei-Liu reference database from the FunGene functional gene pipeline and repository [143].

4.6. Next Generation Sequencing (NGS)

Next generation sequencing was conducted at Genomed S.A. (Warsaw, Poland) on Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). A metataxonomic analyses of the prokaryotic (bacterial and archaeal) and eukaryotic (fungal) communities were performed based on the hypervariable region V3–V4 of the 16S rDNA gene and hypervariable region ITS1, respectively. The MiSeq platform (Illumina Inc., San Diego, CA, USA) was used to sequence the DNA of the microorganisms. The following primers: 341F (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA

CAG CCT ACG GGN GGC WGC AG-3′) and 785R (5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′) were used for the V3-V4 region of 16S rDNA [144]. Whereas, the primers ITS1FI2 (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GAA CCW GCG GAR GGA TCA-3′) and 5.8S (5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GCG CTG CGT TCT TCA TCG-3′) were used to amplify the ITS1 rDNA region [145,146]. In order to perform an amplification and prepare the libraries, the above-mentioned primers and Q5 Hot Start High-Fidelity 2× Master Mix were applied as recommended by the manufacturer (NEB Inc., Ipswich, MA, USA). The v2 Illumina kit was applied for sequencing using 2 × 250 bp pair-ended technology.

4.7. Statistical and Bioinformatics Analyses

All statistical analyses were performed with Statistica 13.1 software (StatSoft Inc., Tulsa, OK, USA). The C-compounds utilization patterns were prepared using heatmap graphs. The enzyme activities, Average Well Color Development (AWCD) [147], Average Well Density Development (AWDD) [148], Richness (the number of utilized carbon sources), number of OTU's (operational taxonomic units) and Shannon index (H) [149] differences between the treatments were assessed with appropriate statistical tests. The analysis of variance (ANOVA) regarding the soil type and applied fertilization and a post-hoc Tukey test were used for significant differences calculation when ANOVA assumptions were met. To verify whether ANOVA assumptions, including dataset normality and homoscedasticity of the variance were met, Shapiro-Wilk and by Levene tests were used, respectively. If normality assumption of a parametric test was violated, then Kruskal-Wallis and Dunn test was used instead. If normality of dataset was met but homoscedasticity of variance was violated, then F-Welch test with post hoc Tukey test was used instead.

Beta diversity between treatments was measured using Bray-Curtis distances and visualized in the form of UPGMA dendrograms and with the application of principal coordinate analysis (PCoA).

The Venn diagrams were constructed with the application of the Venny 2.1 online program [150].

Jaccard's coefficient index was calculated using the following formula:

$$Jaccard\ similarity\ between\ profile\ A\ and\ B\ = \frac{N_{AB}}{N_A + N_B - N_{AB}} \quad (1)$$

where N_{AB}—number of common T-RFs present in both profile A and B, N_A—number of T-RFs in profile A, N_B—number of T-RFs in profile B [151].

MiSeq Reporter (MSR) v. 2.6. software (Illumina Inc., San Diego, CA, USA) was used for the first preliminary processing of the data, including demultiplexing and the generation of fastq files. The Quantitative Insights into Microbial Ecology (QIIME) tool was used to elaborate the raw sequence reads [152]. The taxonomical classification of 16S V3–V4 OTUs was achieved using the uCLUST algorithm [153] and the GreenGenes v. 13_8 database [154], while a Basic Local Alignment Search (BLAST) [155] against the UNITE database [156] was used for the ITS1 region [157].

In order to predict the functional responses to the application of biofertilizers to two different soil types, PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, software) [158] was used to generate a functional profile from the 16S rDNA data. Prior to the metagenomes prediction, the OTUs of the 16SrDNA sequences were normalized using PICRUSt. PICRUSt and Kyoto Encyclopedia of Genes and Genomes (KEGG) [159] were used to prepare classes of functional genes according to the KEGG module, which were present in particular soil samples. Moreover, the FUNGuild online application was used to assign functional information including the ecological guild of fungi [160] to OTUs obtained in high-throughput sequencing datasets based on ITS1 rDNA.

5. Conclusions

In summary, we have analysed the impact of adding phosphorus mineral fertilizer enriched with strains of beneficial microorganisms on the soil microbiome status. The study encompassed genetic

and functional diversity and enzymatic activity. The introduction of live microorganisms to the soil enhanced the activity of the soil enzymes, modified metabolic pathways toward certain compounds and increased genetic diversity. It is noteworthy that no negative shifts in the microbial communities (based on analysing the relevant parameters) were observed after the application of biofertilizers. Of particular interest are the changes activated by introducing into the soil a reduced dose of mineral fertilizer, but microbiologically enriched. This creates the opportunity of limiting the doses of mineral fertilizers used in agriculture with a simultaneous beneficial impact on the environment. The results presented in this study show that biofertilization promotes the occurrence of microbial strains involved in the circulation of essential nutrients, the decomposition of organic matter and the eradication of potentially pathogenic organisms is also promoted. This is a very important effect, taking into account that the aforementioned properties contribute to the improvement of soil fertility and quality and, eventually to improved crop yields. A variation in bacterial function profiles after biofertilizer application was analyzed using predictive functional profiling of microbial communities based on 16S rDNA marker gene sequences which indicated that multiple metabolic pathways occurred in two different soil types. Biofertilizer application shifted the composition and functional ecological guilds in the soil fungal communities. In general, there was an upward tendency in the abundance of ectomycorrhizal fungi which dominated the symbiotrophs group after biofertilizers application. In contrast, there was a significant decrease in the relative abundance of pathotrophs in Brunic Arenosol soil after biofertilizer application, but no remarkable differences in pathotrophs were found among the different treatments in Abruptic Luvisol. Our work suggests that mineral fertilizers enriched with beneficial microbial strains may be an alternative to traditional chemical fertilizers leading to a reduction in the consumption of mineral fertilizers, which is in accordance with the latest policy initiatives and law regulation, such as The European Green Dealand EU Biodiversity Strategy for 2030. However, soil type and properties should be taken into consideration during biofertilizers use, due to the various responses of the tested microbial communities in different soils.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/21/8003/s1.

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7.1. Materiały uzupełniające publikacji P.2

Table S1. Predicted and measured T-RF size (bp) for assessment microbial composition in t-RFLP profile in two different soil types Brunic Arenosol and Abruptic Luvisol after biofertilizers application.

Bacterial species in Abruptic Luvisol	Predicted t-RF size (bp)	Experimental t-RF size (bp)	t-RF size present at the Figure (bp)	Distance	Average
Caloramator	120	121	117	2	2
Pelosinus	166	169	168	3	3
Pandoraea	166	169	168	3	3
Burkholderia	166	169	168	3	3
Archaeal species in Abruptic Luvisol	**Predicted t-RF size (bp)**	**Experimental t-RF size (bp)**	**t-RF size present at the Figure (bp)**	**Distance**	**Average**
Nitrososphaera	363	364	360	1	1
Euryarchaeote	363	364	360	1	1
Fungal species in Abruptic Luvisol	**Predicted t-RF size (bp)**	**Experimental t-RF size (bp)**	**t-RF size present at the Figure (bp)**	**Distance**	**Average**
Penicillium	79	81	82	3	3
Mucor	203	201	200	1	1
Clonostachys	420	425	420	5	5
Bacterial species in Brunic Arenosol	**Predicted t-RF size (bp)**	**Experimental t-RF size (bp)**	**t-RF size present at the Figure (bp)**	**Distance**	**Average**
Rathayibacter	58	65	63	7	7
Caldicoprobacter	57	65	63	8	8
Archaeal species in Brunic Arenosol	**Predicted t-RF size (bp)**	**Experimental t-RF size (bp)**	**t-RF size present at the Figure (bp)**	**Distance**	**Average**
Methanocaldococcus	83	88	90	5	5
Thaumarchaeote	83	88	90	5	5
Fungal species in Brunic Arenosol	**Predicted t-RF size (bp)**	**Experimental t-RF size (bp)**	**t-RF size present at the Figure (bp)**	**Distance**	**Average**
Aspergillus	435	446	447	11	11
Pyrenochaetopsis	433	446	447	13	13

Table S2. Dominant bacteria (relative abundance and total counts) in soils fertilized with phosphorus mineral fertilizer in optimal dose (FC), in optimal dose enriched with microorganisms (FA100) and in 40% reduced dose enriched with microorganisms (FA60). Explanation: BA - Brunic Arenosol, AL - Abruptic Luvisol.

					Relative abundance (%)						Total counts					
					BA			AL			BA			AL		
Phylum	Class	Order	Families	Genus	FA100	FC	FA60	FA100	FC	FA60	FA100	FC	FA60	FA100	FC	FA60
Acidobacteria	Acidobacteriia	Acidobacteriales	Acidobacteriaceae		1.36	4.45	4.51	0.10	1.02	0.81	798	774	663	59	174	42
			Koribacteraceae		2.17	2.80	3.69	0.31	1.89	1.60	1274	830	962	193	552	270
			Koribacteraceae	*Candidatus Koribacter*	0.90	0.78	1.14	0.06	0.32	0.08	530	382	588	39	194	46
	Acidobacteria-6	iii1-15			0.85	0.58	0.75	4.29	3.08	4.11	496	283	385	2638	1876	2256
	[Chloracidobacteria]	RB41			0.10	0.01	0.02	1.86	1.43	1.82	58	4	11	1144	869	1000
	DA052	Ellin6513			1.58	3.65	4.33	0.09	0.10	0.02	930	482	628	54	228	78
	Solibacteres	Solibacterales			0.99	0.79	0.87	0.90	1.12	1.05	582	386	450	554	680	574
		Solibacterales	Solibacteraceae		1.18	2.46	2.71	0.07	5.44	5.27	693	743	756	42	97	26
			Solibacteraceae	*Candidatus Solibacter*	1.24	2.43	2.70	1.01	1.70	1.60	728	444	574	622	1110	688
Actinobacteria	Acidimicrobiia	Acidimicrobiales			1.65	2.60	2.71	1.26	0.53	0.54	966	918	876	773	796	802
		Acidimicrobiales	C111		0.09	0.09	0.09	1.69	1.35	1.67	52	42	44	1040	824	916
			EB1017		0.69	0.50	0.60	1.35	1.37	1.29	406	245	306	832	836	706
	Actinobacteria	Actinomycetales			3.60	1.94	2.16	0.77	0.80	0.97	2115	2188	2319	472	622	444
		Actinomycetales	Frankiaceae		0.89	0.97	1.05	0.22	0.57	0.26	526	478	540	136	346	142
			Mycobacteriacea	*Mycobacterium*	1.34	1.75	2.40	1.81	2.54	2.29	784	644	680	1112	1220	1010
			Nocardioidaceae		0.29	0.35	0.26	3.25	2.32	2.13	170	174	134	1998	1414	1167
			Nocardioidaceae	*Nocardioides*	0.17	0.15	0.15	0.77	1.22	0.71	102	72	75	474	745	390
	MB-A2-108	0319-7L14			0.06	0.01	0.02	1.51	1.23	1.49	32	4	9	930	750	815
	Thermoleophilia	Gaiellales			1.12	1.69	1.87	1.28	0.91	0.49	660	530	632	785	1128	732
		Gaiellales	Gaiellaceae		2.75	2.65	2.43	5.36	0.36	0.26	1615	1208	1390	3300	3312	2892
		Solirubrobacterales			1.68	2.36	1.92	2.28	0.09	0.03	988	870	882	1400	1299	990
		Solirubrobacterales	Conexibacteraceae		2.08	1.77	1.72	0.23	2.13	1.81	1222	1300	1251	143	222	140
AD3	ABS-6				1.58	1.87	1.71	0.05	1.31	1.46	930	304	547	33	155	42
	JG37-AG-4				1.03	2.14	1.05	0.00	1.08	0.86	608	230	254	2	12	3
Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae		0.74	0.77	0.49	1.27	1.25	1.19	435	380	252	780	762	650
Chloroflexi	Ellin6529				0.73	0.44	0.59	2.69	2.34	2.87	428	216	302	1656	1422	1576
	Ktedonobacteria	Ktedonobacterales	Ktedonobacteraceae		1.41	0.98	1.22	0.03	0.37	0.14	826	696	814	19	84	29
		Thermogemmatisporales	Thermogemmatisporaceae		3.11	0.62	1.06	0.01	0.25	0.08	1827	1791	2224	7	60	10
		B12-WMSP1			0.87	1.14	0.92	0.00	0.01	0.00	509	562	475	0	8	0
		JG30-KF-AS9			0.89	1.47	1.03	0.05	0.16	0.06	522	722	527	34	96	32
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	0.68	1.15	0.83	0.47	0.41	0.39	402	562	429	292	247	212
Gemmatimonadetes	Gemmatimonadetes				1.35	1.57	1.65	0.67	0.77	0.43	796	579	696	410	570	392
	Gemm-1				0.85	0.43	0.59	1.45	1.36	1.35	502	214	302	894	825	742
Planctomycetes	Phycisphaerae	WD2101			1.17	1.42	1.58	0.89	0.14	0.05	686	613	712	549	747	518
	Planctomycetia	Gemmatales	Gemmataceae		1.39	1.24	1.18	0.60	0.89	0.71	818	609	608	366	544	392
Proteobacteria	Alphaproteobacteria	Ellin329			1.25	1.57	1.29	0.45	0.29	0.08	732	514	553	277	588	331
		Rhizobiales			0.38	0.36	0.41	0.81	0.75	1.96	222	176	212	498	457	1075
		Rhizobiales	Bradyrhizobiaceae		2.74	1.18	1.36	1.64	0.94	0.71	1612	1194	1386	1010	1036	880
			Hyphomicrobiaceae		0.29	0.24	0.27	1.22	1.23	1.10	172	118	140	750	747	602
			Hyphomicrobiaceae	*Rhodoplanes*	2.21	1.31	1.32	2.37	2.01	1.84	1299	862	1233	1457	1544	1256
		Rhodospirillales	Acetobacteraceae		1.49	1.05	1.08	0.42	0.97	0.60	876	771	848	257	466	238
			Rhodospirillaceae		3.50	0.90	1.12	1.56	1.83	1.26	2056	1376	1894	957	1148	875
		Sphingomonadales	Sphingomonadaceae	*Kaistobacter*	0.93	1.56	1.56	1.18	1.51	0.81	546	764	800	727	918	446
	Betaproteobacteria				0.11	0.04	0.07	1.00	0.69	1.01	64	22	34	618	421	556
	Betaproteobacteria	SC-I-84			0.47	0.45	0.57	1.17	1.03	1.12	274	223	292	718	628	612
	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae		2.41	1.51	1.47	1.14	0.16	0.05	1414	952	1111	702	488	531
			Xanthomonadaceae		1.59	1.25	1.39	1.16	1.23	0.94	932	1052	538	714	658	469
	Deltaproteobacteria	Myxococcales			1.19	1.05	0.97	1.31	1.48	1.60	700	514	500	804	898	880
		Syntrophobacterales	Syntrophobacteraceae		0.26	0.17	0.22	1.38	1.03	1.25	152	82	115	849	628	687
Verrucomicrobia	[Spartobacteria]	[Chthoniobacterales]	[Chthoniobacteraceae]	*DA101*	0.39	0.22	0.35	0.82	1.37	0.90	229	108	180	504	836	495
WPS-2					1.82	1.08	1.23	0.04	1.85	1.33	1068	1159	984	27	55	19

Table S3. Distribution of archaeal phyla, classes and orders obtained by next generation sequencing of DNA extracted from soil samples. Explanation: BA - Brunic Arenosol, AL - Abruptic Luvisol, FC - optimal dose of fertilizer, FA100 - optimal dose of fertilizer enriched with microorganisms, FA60 - dose reduced by 40% of fertilizer enriched with microorganisms.

				Relative abundance (%)						Total counts					
				BA			AL			BA			AL		
Kingdom	Phylum	Class	Order	FA100	FC	FA60	FA100	FC	FA60	FA100	FC	FA60	FA100	FC	FA60
Archaea				0.09	0.05	0.05	0.03	0.04	0.03	51	27	27	20	25	15
	Crenarchaeota			0.07	0.05	0.05	0.02	0.03	0.02	44	27	26	13	17	12
		MBGA		0.04	0.03	0.02	0.00	0.01	0.00	26	13	10	1	4	0
			NRP-J	0.04	0.03	0.02	0.00	0.01	0.00	26	13	10	1	4	0
		Thaumarchaeota		0.03	0.03	0.03	0.02	0.02	0.02	18	14	16	12	13	12
			Cenarchaeales	0.01	0.01	0.01	0.00	0.00	0.00	8	3	4	0	1	0
			Nitrososphaerales	0.02	0.02	0.02	0.02	0.02	0.02	10	11	13	12	12	12
	Euryarchaeota			0.01	0.00	0.00	0.01	0.01	0.00	6	1	1	8	8	3
		Methanomicrobia		0.00	0.00	0.00	0.00	0.00	0.00	2	1	1	2	1	0
			Methanosarcinales	0.00	0.00	0.00	0.00	0.00	0.00	2	1	1	2	1	0
		Thermoplasmata		0.01	0.00	0.00	0.01	0.01	0.00	5	0	1	6	7	3
			E2	0.01	0.00	0.00	0.01	0.01	0.00	5	0	1	6	7	3
	[Parvarchaeota]			0.00	0.00	0.00	0.00	0.00	0.00	1	0	0	0	0	1
		[Parvarchaea]		0.00	0.00	0.00	0.00	0.00	0.00	1	0	0	0	0	1
			YLA114	0.00	0.00	0.00	0.00	0.00	0.00	1	0	0	0	0	1

Table S4. Dominant fungal species (relative abundance and total counts) in soils fertilized with phosphorus mineral fertilizer in optimal dose (FC), in optimal dose enriched with microorganisms (FA100) and in 40% reduced dose enriched with microorganisms (FA60). Explanation: BA - Brunic Arenosol, AL - Abruptic Luvisol, ND - No Data.

						Relative abundance (%)				Total counts							
						BA			AL			BA			AL		
Phylum	Class	Order	Families	Genus	Species	FA100	FC	FA60	FA100	FC	FA60	FA100	FC	FA60	FA100	FC	FA60	Trophic mode (according to Funguild)	Guild (according to Funguild)
Ascomycota	Archaeorhizomycetes	Archaeorhizomycetales	Archaeorhizomycetaceae	*Archaeorhizomyces*	*Archaeorhizomyces sp.*	1.54	1.38	1.11	2.48	2.33	1.80	1 790	1 862	1 509	2 091	1 324	1 135	Saprotroph	Soil Saprotroph
	Dothideomycetes	Pleosporales	Pleosporales fam Incertae sedis	*Didymella*	*Didymella pelleae*	1.38	0.43	0.66	0.19	0.16	0.21	1 605	576	891	163	89	132	Pathotroph-Saprotroph	Animal Pathogen-Plant Pathogen-Undefined
	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Cladophialophora*	*Cladophialophora sp.*	0.51	1.42	0.80	0.01	0.01	0.00	595	1 929	1 081	8	5	3	Saprotroph	Undefined Saprotroph
			unidentified	*unidentified*	*Chaetothyriales sp.*	1.69	2.29	2.16	0.01	0.05	0.01	1 973	3 096	2 927	5	31	6	ND	ND
		Eurotiales	Trichocomaceae	*Penicillium*	*Penicillium adametzii*	0.31	2.55	0.03	0.01	0.00	0.00	366	3 458	35	5	0	0	Saprotroph	Undefined Saprotroph
					Penicillium annellidiae	1.48	2.01	0.58	0.16	0.12	0.13	1 727	2 728	790	133	66	79	Saprotroph	Undefined Saprotroph
					Penicillium atrovenetum	6.07	5.35	6.63	0.12	0.10	0.13	7 080	7 512	8 983	101	56	83	Saprotroph	Undefined Saprotroph
					Penicillium simplicissimum	8.01	9.04	9.74	0.04	0.09	0.03	9 340	12 236	13 198	32	54	17	Saprotroph	Wood Saprotroph
	Leotiomycetes	Helotiales	Rutstroemiaceae	*unidentified*	*Rutstroemiaceae sp.*	0.00	0.00	0.01	0.01	0.02	2.79	0	3	19	11	13	1 762	Saprotroph	Plant Saprotroph
		Leotiomycetes ord Incertae sedis	Leotiomycetes fam Incertae sedis	*Geomyces*	*Geomyces asperulatus*	0.83	1.06	0.54	0.10	0.16	0.13	967	1 438	727	83	91	84	Saprotroph	Soil Saprotroph
	Sordariomycetes	Chaetosphaeriales	Chaetosphaeriaceae	*Chaetosphaeria*	*Chaetosphaeria vermicularioides*	2.08	0.41	0.76	0.72	0.91	0.99	2 429	560	1 035	610	519	624	Saprotroph-Symbiotroph	Endophyte-Litter Saprotroph-Wood Saprotroph
		Coniochaetales	unidentified	*unidentified*	*Coniochaetales sp.*	0.39	0.33	0.49	0.87	0.81	1.57	456	443	661	731	462	992	ND	ND
		Hypocreales	Clavicipitaceae	*Metarhizium*	*Metarhizium robertsii*	4.13	8.03	5.41	0.02	0.01	0.02	4 821	10 867	7 331	14	4	10	Pathotroph	Animal Pathogen
			Cordycipitaceae	*Isaria*	*Isaria fumosorosea*	2.83	0.07	0.77	0.00	0.00	0.00	3 304	102	1 040	2	2	0	Saprotroph	Undefined Saprotroph
			Hypocreaceae	*Trichoderma*	*Trichoderma pubescens*	0.43	1.04	0.67	0.27	0.12	0.16	499	1 413	910	225	70	104	Symbiotroph	Endophyte
			Hypocreales fam Incertae sedis sp.	*unidentified*	*Hypocreales fam Incertae sedis sp.*	0.01	0.00	0.00	3.12	1.70	0.91	16	1	5	2 635	964	574	Saprotroph	Undefined Saprotroph
			Nectriaceae	*Fusarium*	*Fusarium oxysporum*	2.24	2.62	2.87	1.18	1.14	0.85							Pathotroph-Saprotroph-Symbiotroph	Animal Pathogen-Endophyte-Undefined Saprotroph
				unidentified	*Nectriaceae sp.*	1.14	0.75	0.82	0.60	0.40	0.31	1 324	1 016	1 108	508	228	198	Saprotroph	Undefined Saprotroph
			unidentified	*unidentified*	*Hypocreales sp.*	1.04	1.18	1.27	1.70	1.23	1.13	1 214	1 592	1 715	1 434	698	712	Saprotroph	Undefined Saprotroph
		Sordariales	Chaetomiaceae	*unidentified*	*Chaetomiaceae sp.*	1.26	0.36	1.08	0.78	0.52	0.59	1 464	481	1 462	660	298	372	Pathotroph-Saprotroph-Symbiotroph	Animal Pathogen-Dung
		Sordariomycetidae ord Incertae sedis	Apiosporaceae	*unidentified*	*Apiosporaceae sp.*	0.01	0.01	0.03	1.59	1.73	1.31	7	13	39	1 342	981	827	Saprotroph	Plant Saprotroph
	unidentified	unidentified	unidentified	*unidentified*	*Ascomycota sp.*	3.04	2.97	3.56	3.62	8.97	2.72	3 547	4 020	4 823	3 055	5 094	1 714	ND	ND
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	*unidentified*	*Hygrophoraceae sp.*	0.66	0.54	0.71	0.79	0.88	1.26	769	733	967	665	501	795	Saprotroph-Symbiotroph	Ectomycorrhizal-Undefined
			Strophariaceae	*Agrocybe*	*Agrocybe pediades*	0.13	0.00	0.47	0.32	0.53	2.18	157	6	634	267	299	1 372	Saprotroph	Dung Saprotroph-Soil Saprotroph
				Stropharia	*Stropharia coronilla*	0.00	0.00	0.00	1.29	0.01	0.04	0	0	0	1 090	5	26	Saprotroph	Soil Saprotroph
			unidentified	*unidentified*	*Agaricales sp.*	0.15	0.24	0.20	0.98	1.30	1.44	174	320	278	823	736	910	Pathotroph-Saprotroph-Symbiotroph	Bryophyte Parasite-Dung Saprotroph
		Geastrales	Geastriaceae	*Sphaerobolus*	*Sphaerobolus ingoldii*	0.09	0.00	0.00	0.74	0.78	1.49	105	1	0	629	441	938	Saprotroph	Undefined Saprotroph
		Trechisporales	unidentified	*unidentified*	*Trechisporales sp.*	0.66	0.58	1.32	0.06	0.06	0.06	777	782	1 790	51	31	40	ND	ND
	Tremellomycetes	Filobasidiales	Piskurozymaceae	*Solicoccozyma*	*Solicoccozyma terricola*	1.59	1.80	2.31	1.27	1.79	1.30	1 856	2 555	3 130	1 070	1 014	820	ND	ND
		Tremellales	unidentified	*unidentified*	*Tremellales sp.*	0.98	1.03	1.17	0.53	0.80	0.23	1 145	1 389	1 589	446	453	144	Pathotroph-Saprotroph-Symbiotroph	Fungal Parasite-Undefined ND
	unidentified	unidentified	unidentified	*unidentified*	*Basidiomycota sp.*	0.64	0.74	0.76	1.10	1.11	0.99	751	1 009	1 024	926	628	622		
Zygomycota	Mortierellomycotina_cls_Incertae_sedis	Mortierellales	Mortierellaceae	*Mortierella*	*Mortierella elongata*	1.56	1.44	1.51	1.11	2.82	1.01	1 821	1 949	2 044	936	1 603	638	Saprotroph-Symbiotroph	Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined
					Mortierella horticola	0.51	0.30	0.44	1.23	1.17	1.50	591	401	602	1 034	667	948	Saprotroph-Symbiotroph	Endophyte-Litter Saprotroph-Soil Saprotroph
					Mortierella humilis	2.00	1.22	1.56	0.38	0.64	0.36	2 338	1 655	2 109	320	362	226	Saprotroph-Symbiotroph	Endophyte-Litter Saprotroph-Soil Saprotroph
					Mortierella sp.	1.99	1.18	0.86	4.17	7.23	4.74	2 317	1 005	1 295	1 028	4 153	2 988	Saprotroph	Undefined Saprotroph
	Mucoromycotina cls Incertae sedis	Mucorales	Umbelopsidaceae	*Umbelopsis*	*Umbelopsis ramanniana*	3.26	3.60	3.15	0.01	0.07	0.01	3 804	4 871	4 263	8	41	4		

Table S5. Functional guilds for three main trophic modes: patotrophs, symbiotrophs and saprotrophsin two different soil types Brunic Arenosol (BA) and Abruptic Luvisol (AL). Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%).

Trophic mode	Treatments					
	FC/BA	FA100/BA	FA60/BA	FC/AL	FA100/AL	FA60/AL
	OTUs numbers					
Pathotroph	13,682	8,309	10,613	1,696	2,877	1,512

Functional guild	Treatments					
	FC/BA	FA100/BA	FA60/BA	FC/AL	FA100/AL	FA60/AL
	OTUs numbers					
Animal Parasite-Fungal Parasite	5	14	20	0	3	2
Animal pathogen	**11,494**	**5,676**	**8,261**	**632**	**830**	**393**
Endophyte-Plant Pathogen	0	1	2	0	0	0
Fungal Parasite	190	193	268	202	258	246
Fungal Parasite-Lichen Parasite	0	0	0	31	103	24
Litter Saprotroph-Plant Pathogen	0	0	1	0	0	2
Plant Pathogen	**2,101**	**2,584**	**2,157**	**1,004**	**2,018**	**1,072**
Plant Pathogen-Undefined Parasite-Undefined Saprotroph	2	0	17	4	1	5

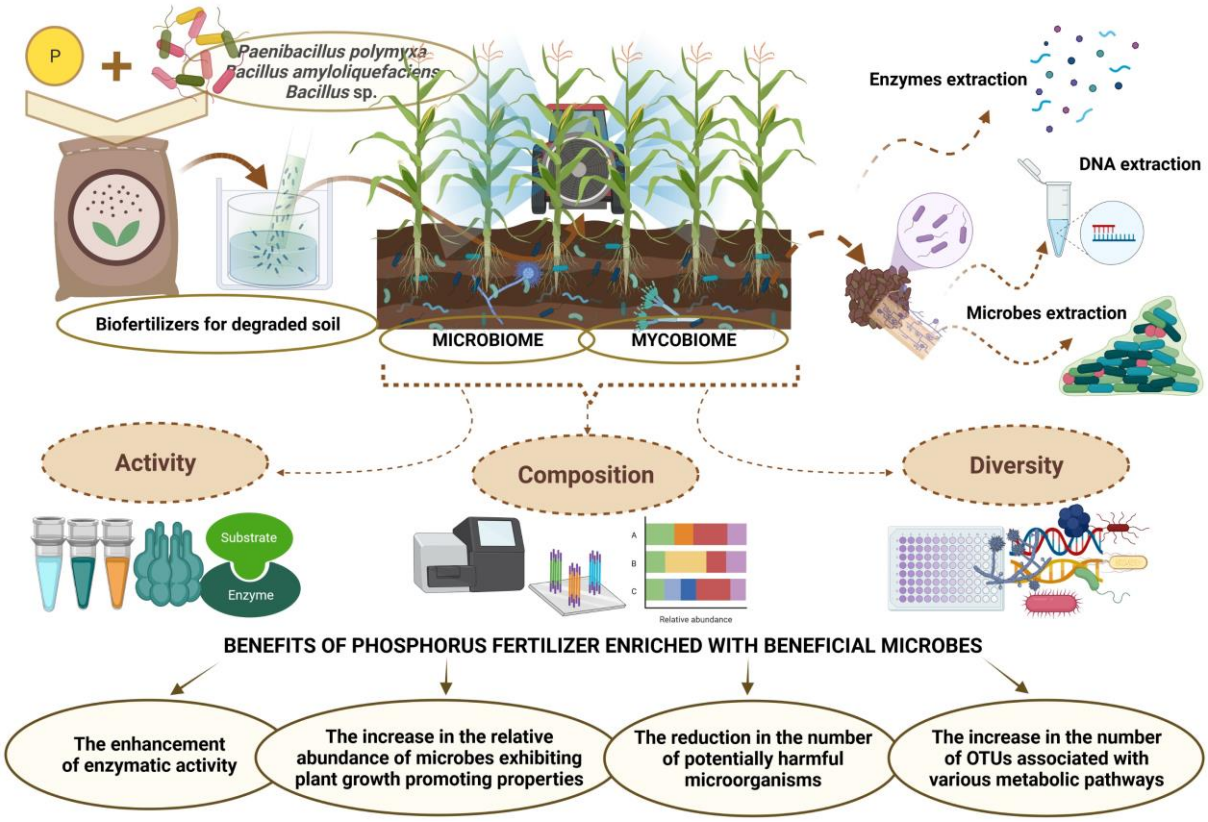
Trophic mode	Treatments					
	FC/BA	FA100/BA	FA60/BA	FC/AL	FA100/AL	FA60/AL
	OTUs numbers					
Saprotrophs	48,714	40,885	45,820	11,922	19,414	14,890

Functional guild	Treatments					
	FC/BA	FA100/BA	FA60/BA	FC/AL	FA100/AL	FA60/AL
	OTUs numbers					
Dung Saprotroph	164	289	67	158	152	145
Dung Saprotroph-Plant Saprotroph	423	239	429	166	693	753
Dung Saprotroph-Plant Saprotroph-Wood Saprotroph	8	1	1	20	253	312
Dung Saprotroph-Soil Saprotroph	127	129	411	25	80	36
Dung Saprotroph-Soil Saprotroph-Undefined Saprotroph	6	157	634	301	267	1,402
Dung Saprotroph-Undefined Saprotroph	1	42	4	2	4	5
Dung Saprotroph-Undefined Saprotroph-Wood Saprotroph	0	0	0	2	2	1
Dung Saprotroph-Wood Saprotroph	15	64	26	245	593	131
Endophyte-Litter Saprotroph-Undefined Saprotroph	0	0	0	0	0	0
Leaf Saprotroph	0	10	23	2	29	9
Leaf Saprotroph-Wood Saprotroph	0	0	4	1	5	1
Litter Saprotroph-Soil Saprotroph-Wood Saprotroph	15	12	11	10	13	14
Plant Saprotroph	25	11	83	1,164	1,412	2,608
Plant Saprotroph-Wood Saprotroph	3	12	18	24	7	3
Soil Saprotroph	3,356	2,832	2,303	1,506	3,352	1,306
Soil Saprotroph-Undefined Saprotroph	1	1	0	7	12	7
Undefined Saprotroph	**31,105**	**26,679**	**27,461**	**7,781**	**11,707**	**7,574**
Undefined Saprotroph-Wood Saprotroph	775	373	514	302	512	202
Wood Saprotroph	12,688	10,037	13,835	210	325	381

Trophic mode	Treatments					
	FC/BA	FA100/BA	FA60/BA	FC/AL	FA100/AL	FA60/AL
	OTUs numbers					
Symbiotrophs	6,482	5,212	6,288	2,571	3,816	3,134

Functional guild	Treatments					
	FC/BA	FA100/BA	FA60/BA	FC/AL	FA100/AL	FA60/AL
	OTUs numbers					
Animal Endosymbiont	3	2	2	2	0	0
Arbuscular Mycorrhizal	437	424	536	176	160	98
Ectomycorrhizal	**2,231**	**2,373**	**1,987**	**1,976**	**2,878**	**2,467**
Ectomycorrhizal-Orchid Mycorrhizal-Root Associated Biotroph	2	0	0	3	7	1
Endophyte	2,158	1,177	1,946	365	707	534
Epiphyte	0	0	0	0	3	3
Lichenized	1,637	1,236	1,818	49	63	32
Lichenized-Wood Saprotroph	15	0	0	0	0	0

8. Tekst publikacji P.3



GRAPHICAL ABSTRACT

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RESEARCH ARTICLE

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Composition, activity and diversity of bacterial and fungal communities responses to inputs of phosphorus fertilizer enriched with beneficial microbes in degraded Brunic Arenosol

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Abstract

Anthropogenic-induced deterioration of soil health remains a global problem, resulting in a diminished productivity of agroecosystems. In order to improve soil quality, we investigated the impact of phosphorus biofertilizer on the microbiological parameters of soil (type Brunic Arenosol) degraded as a result of inappropriate cultivation and fertilization, characterized by low pH and decrease in K and Mg content. Two-year field experiment included control treatment (FC) without microbial enrichment and FA100 (fertilizer amendment at optimal dose) and FA60 (fertilizer amendment at 40% reduced dose) treatments containing beneficial microorganisms. The results showed that the biofertilizer enhanced soil enzymatic activity (as expressed by the increased activities of urease, protease, acid phosphomonoesterase, and β-glucosidase), increased number of operational taxonomic units associated with metabolic processes (including phosphorus related pathways and degradation of xenobiotics), improved crop yield, increased bacterial diversity, and changed the quantity of phytoavailable phosphorus in the soil. Biofertilizer also stimulated the occurrence of plant growth promoting microorganisms involved in phosphorus biotransformations, decomposition of organic matter, nitrogen fixation, and protection plants against pathogens. Next generation sequencing (NGS) and Biolog analyses showed that the composition of soil microbiome was affected also by soil sampling time, suggesting seasonal variations in the preferred carbon sources and level of C-substrates utilization, and pointing the differences in the relative abundance of individual microbial groups at particular stages of the experiment. Taking into consideration the improvement of microbiological indicators of soil health, phosphorus biofertilizer seems to be effective approach to implement in sustainable agriculture linking soil microbiome quality with the general soil condition.

KEYWORDS
biofertilizer, degraded soil, phosphorus biotransformations, soil health, soil microbiome

1 | INTRODUCTION

It cannot be denied that the degradation of arable soils is an important issue influencing not only the quality and quantity of crop yields but also the functioning of whole agroecosystems (Gomiero, 2016). The intensive and unsustainable soil cultivation leads to severe alterations in its structure including a depletion of mineral components, acidification, accumulation of heavy metals or loss of organic matter (Lin

et al., 2019). These changes result in the gradual degradation of arable soils and decrease their production potential (Geng et al., 2019). Considering the application of mineral fertilizers, it should be considered that their excessive amounts remain not indifferent to a key element of soil environment architecture, namely complex communities of bacteria, archaea, and fungi (Geisseler & Scow, 2014). As described earlier, soil microorganisms play the foremost role in the basic biochemical transformations occurring below ground level and therefore their role in maintaining soil balance is invaluable (Hellequin et al., 2020; Lacerda-Júnior et al., 2019). Regarding the importance of soil microorganisms, the deterioration of soil health may be evidenced by microbiological indicators encompassing reduction of microorganisms activity (reflected by low soil enzymatic activity and poor efficiency of metabolic processes) and simplification of community structure (expressed through a small number of different taxa and low biodiversity among microorganisms performing specific roles) (Lee et al., 2020; Nunes et al., 2012; Zhang et al., 2017). It goes without saying that phosphorus is a key macronutrient which availability determines plant growth and development and, consequently, crop productivity. P is involved in enzymatic and metabolic processes (such as photosynthesis and cell division), formation of phospholipids and constitute the vital element of nucleic acids. Unfortunately, the majority of soil P (∼95%) is unavailable for plant uptake and, in addition, prolonged or inappropriate application of phosphorus mineral fertilizers may result in limitation of indigenous P (Hallama et al., 2021; Siedliska et al., 2021). With regard to the disadvantages of mineral fertilizers, the use of beneficial microorganisms capable of converting insoluble P-compounds into ortho-phosphates which can be uptaken by plants, seems to be an innovative and ecofriendly alternative or supplementation (Hallama et al., 2021; Kour et al., 2020). Unlike synthetic fertilizers, microbial based preparations do not pose a threat to the natural environment and their application remains in agreement with sustainable and organic agriculture principles which involve the limitation or complete exclusion of mineral fertilizers and artificial plant protection agents (Bhardwaj et al., 2014; Mącik, Gryta, & Frąc, 2020). It was reported that application of biofertilizers based on P-solubilizing microorganisms provides benefits including increase highly assimilable P compounds content (Alori et al., 2017), improve plant (e.g. maize) growth parameters (Zhao et al., 2014), enhance acid phosphatase activity (Heidari et al., 2019), boost nutrient (N, P, K, Mg, Fe) uptake (Chen et al., 2021), suppress phytopathogens (Mitra et al., 2020), and increase bacterial richness and diversity in the rhizosphere (Wang, Liu, et al., 2021). The phosphorus biofertilizer used in this study was enriched with the following beneficial bacterial strains: *Paenibacillus polymyxa* (CHT114AB), *Bacillus amyloliquefaciens* (AF75BB), and *Bacillus* sp. (CZP4/4). The aforementioned strains were selected with respect to their plant growth promoting properties. As described earlier, *Paenibacillus polymyxa* strains were found to suppress plant pathogens, for example, *Rhizooctonia solani*, *Fusarium oxysporum*, and *Botrytis cinerea* due to synthesis of antifungal metabolites such as fusaricidin. What is more, representatives of *P. polymyxa* carry phn genes, a gene cluster encoding proteins involved in bacterial conversion of phosphonates to phosphorus forms readily assimilated by plants (Li et al., 2020). The P solubilization properties were also reported among *Bacillus* spp. strains, which produce organic acids and phosphatases converting inorganic P-compounds into absorbable forms. Apart from increasing P uptake, *Bacillus* spp. also synthesize plant hormones (IAA, cytokinins) and siderophores chelating and reducing Fe^{3+} to Fe^{2+} ions (Radhakrishnan et al., 2017). Meanwhile, the microbial biofertilizer containing *Bacillus amyloliquefaciens* in combination with different P-based fertilizers enhanced the P uptake in maize leaves (Vinci et al., 2018).

Shifts in condition of the soil microorganisms under the influence of various substances or treatments are the subject of large-scale research since many years, including both short- and long-term studies. A result obtained from short-term study (from September 2018 to June 2019) showed that soil inoculated with microbial inoculant containing *B. subtilis* and *P. polymyxa* exhibited increased relative abundance of Acidobacteria, Actinobacteria and Chloroflexi, with a simultaneous decrease in Bacteroidetes, as compared to non-inoculated control. In the same study, the Chao1 index of bacterial diversity was significantly higher in soil amended with *B. subtilis* and *B. cereus* than in control soil (Chen et al., 2021). In long term study (2012–2016) it was reported that inoculation with *Burkholderia cepacia* ISOP5 increased the relative abundance of genes involved in P-solubilization and mineralization such as phoN (acid phosphatase), phnA (phosphonoacetate hydrolase), and phnFGHIJKLMNOP (the C-P lyase subunit). What is more, the increase in the relative abundance of genes associated with N metabolism was also observed in soil inoculated with the abovementioned strain (Wang, Peng, et al., 2021).

Owing to the fact that soil microbial communities are sensitive to environmental fluctuations, they may display seasonal responses depending on temperature, moisture, and indigenous microorganisms properties. Seasonal variations in the status of soil microbiome are also associated with the availability of carbon sources (Koranda et al., 2013). During growing season, soil microorganisms may demonstrate increased activity due to plant root exudates containing highly assimilable carbon sources such as carbohydrates and amino acids. On the other hand, low temperatures may result in reduced microbial activity leading to an accumulation of soil organic matter (SOM) (Badri & Vivanco, 2009; Canarini et al., 2019; Xu et al., 2021). Higher SOM content may then stimulate the fungal decomposition activity (Rousk & Bååth, 2007). The seasonal response is thought to be predictable as a result of the environmental signals preceding these changes, however responses may differ among ecosystems. On the other hand, the influence of various agrotechnical practices may interfere with the regularity of microbial responses arising from the succession of natural factors including temperature fluctuations and day/night length (Bleuven & Landry, 2016; Jia et al., 2020). Tracking seasonal changes in the status of soil microbiome is essential in order to estimate the stability of agroecosystems in the face of external factors such as fertilization. It is of particular importance for new farming techniques that were not formerly used (Lacerda-Júnior et al., 2019).

The contemporary concept of agriculture, ensuring high soil quality and fertility, is increasingly driven by the interrelationship of agroecosystems with soil microbial diversity. Maintaining a high

biodiversity among soil microbial communities, which is intended to stimulate crop resistance to pathogens, enhance nutrient uptake and improve microbiological indicators of soil health, is a promising strategy for sustainable soil management (Bertola et al., 2021; Hartman et al., 2018). This is particularly important for degraded soils owing to the fact that successful soil restoration is inextricably linked with the soil microorganisms welfare (Zhang et al., 2020). Even though soil fertilization with different organic amendments is widely described by researchers, the approach considering exploitation of traditional mineral fertilizers enriched with strains of beneficial microorganisms is still poorly recognized. Therefore, this study aimed determine the seasonal effects of innovative phosphorus mineral fertilizer enriched with strains of beneficial bacteria (*Paenibacillus polymyxa*—CHT114AB, *Bacillus amyloliquefaciens*—AF75BB, and *Bacillus* sp.—CZP4/4) on the biodiversity of soil microbiome and enzymatic activity of chemically degraded (very low content of K, Mg, and pH value) soil under maize cultivation. Because of an essential task of agricultural systems is to improve P recycling in the environment, for example, by decreasing the doses of phosphorus mineral fertilizers applied to the soil and incorporation of microbes that will be able to solubilize internal phosphorus present in the environment, we mainly wanted to evaluate the influence of tested treatments on soil mycobiome and microbiome as relevant soil health and quality indicators. Moreover, these types of results are missing or present as very fragmentary in literature, therefore the tested approach was innovative, especially for degraded soil. We hypothesize that the application of phosphorus biofertilizer will improve microbiological indicators of soil degradation and thus soil quality. Consequently, we assume that the obtained results will provide guidelines for sustainable soil management, based on the status of soil microbiome, and the phosphorus biofertilizer will find practical implementation in modern agriculture.

2 | MATERIALS AND METHODS

2.1 | Field experiment

The study was conducted over 2 years under field experiment conditions in 2018–2019, on agricultural land in Biszcza, Southeast Poland (50°43′N, 22°60′E). The study was located at the altitude of 211 m above sea level. The soil, classified as a Brunic Arenosol (BA), was degraded due to inadequate fertilization and cultivation, with the following physicochemical parameters: pH_{KCl} 4.8 and content of P_2O_5, K_20, and Mg of 17.4 mg 100 g^{-1} (high), 2.9 mg 100 g^{-1} (very low), 1.2 mg 100 g^{-1} (very low), respectively. The additional physicochemical soil properties were investigated and described in previous studies (Boguta et al., 2021; Pertile et al., 2021; Walkiewicz et al., 2020).

The field experiment was conducted according to the method of Mącik, Gryta, Sas-Paszt, & Frąc (2020). Soil was fertilized with the phosphate mineral fertilizer SUPER FOS DAR 40 (Grupa Azoty, Puławy, Poland), nitrogen fertilizer PULREA PUŁAWSKI MOCZNIK 46N (Grupa Azoty, Puławy, Poland) and granulated potassium salt (BIALCHEM, Poland). The experiment comprised of the following treatments: FC – optimal dose of fertilizer (control treatment), FA100—optimal dose of fertilizer enriched with microorganisms and FA60–40% reduced dose of fertilizer enriched with microorganisms. The field experiment was conducted under maize cultivation (variety of P9241, FAO: K280, Z270, PIONEER).

Phosphorus mineral fertilizer was enriched with the following bacterial strains: *Paenibacillus polymyxa* (CHT114AB), *Bacillus amyloliquefaciens* (AF75BB), and *Bacillus* sp. (CZP4/4), provided by the Research Institute of Horticulture in Skierniewice, Poland. The biofertilizers were prepared according to Borowik et al. (2019), namely granules of fertilizer were coated with the mixture containing 1:1:1 each aforementioned strain. The ready-to-use biofertilizers were produced and provided by the Łukasiewicz Research Network—New Chemical Syntheses Institute (Puławy, Poland).

Each fertilization treatment included three replications plots (10 × 15 m). The soil samples were taken in autumn 2018 (A18), summer 2019 (S19), and autumn 2019 (A19) at a depth of 0–25 cm from five random sites within each plot. Afterward, samples were delivered to the laboratory and passed through a 2 mm sieve in order to get rid of impurities such as stones or plant roots. The purified soil samples were then immediately used for measurements or stored (at 4°C for Biolog and enzymatic analyses or −80°C for DNA extraction).

2.2 | Weather conditions

Weather conditions were recorded using the meteorological station in Zamość (Poland) (50°70′N, 23°25′E), located ∼50 km of the experimental site. Total rainfall in 2018 and 2019 accounted for 439.14 and 525.47 mm, respectively, while average annual air temperature were 9.3 and 9.8°C, respectively. The highest monthly rainfall during the 2-year experiment was reported in July 2018 and May 2019. Considering the growing season (April–July), the average temperature during this period was 16.8°C and 15.4°C in 2018 and 2019, respectively. Analyzing weather conditions in soil sampling months (October 2018, June 2019, and October 2019) it was reported that monthly rainfall remained at similar level and accounted for, respectively, 28.97, 23.62, and 26.4 mm. The highest temperature was observed in June 2019 (20.9°C), whereas in October 2018 and October 2019 accounted for 9.7°C and 10.8°C, respectively (Figure S1). The weather conditions were obtained through online climate database tutiempo.net (Tutiempo, 2021).

2.3 | Soil enzymatic activity, assimilated phosphorus content, and maize yield

Protease activity was assessed using the Tris–HCl (pH 8.1) sodium caseinate as a substrate and determining the release of tyrosine after incubation for 1 hr at 50°C. The concentration of tyrosine was measured calorimetrically at a wavelength of 578 nm (Ladd & Butler, 1972 with modification of Alef & Nannipieri, 1995). For urease analysis, urea was used and after 18 hr incubation at 37°C, the concentration

of ammonia was measured at 410 nm (Zantua & Bremner, 1977). Acid phosphomonoesterase activity was determined by incubating (for 1 hr at 37°C) the soil samples with p-nitrophenyl phosphate and evaluating the released p-nitrophenol (PNP) spectrophotometrically at 400 nm (Tabatabai & Bremner, 1969). β-glucosidase activity (as determined by the PNP concentration (at 400 nm) after incubation with p-nitrophenol glucoside (PNG) for 1 hr at 37°C) was assessed according to the methods of Eivazi & Tabatabai (1988) with a modification developed by Alef & Nannipieri (1995). The enzymes activities were calculated based on the dry (105°C) weight of the soil. The assimilated phosphorus content (P_2O_5) was determined by the Egner-Riehm method according to Polish Standard PN-R-04023 (1996), using a Sherwood flame photometer, Genesys 6 spectrophotometer. After maize harvest at full maturity, its yield was assessed by weighing together all plants (including grains and straw) from each plot in particular treatments.

2.4 | Metabolic potential of soil microbial communities

The metabolic potential of soil bacterial and fungal communities was determined with the application of Biolog system using ECOplates and FFplates (Biolog Inc., Hayward, CA). The suspension containing 1 g of fresh soil and 99 ml of sterile saline peptone water was shaken for 20 min at 20°C and incubated for 30 min at 4°C (Gryta et al., 2014). Afterward, ECOplates and FFplates wells were inoculated with soil microorganisms suspension with the amount of 120 and 100 µl, respectively. After the inoculation, during 216 hr incubation period at 23°C, absorbance readings were taken every 24 hr interval at 590 nm (ECOplates) and 490 nm (FFplates).

2.5 | DNA extraction

Genomic DNA was extracted from a 0.5 g of soil sample using a FastDNA SPIN Kit for Feces (MP Biomedicals, Solon, OH) according to the manufacturer's protocol. The amount of DNA was determined spectrophotometrically at 260 nm (NanoDrop 2000/2000c Thermo Scientific, West Palm Beach, FL). The extracted DNA was then stored at −20°C and used for multiplex terminal restriction fragments length polymorphism (M-tRFLP) and next generation sequencing (NGS).

2.6 | Multiplex terminal restriction fragment length polymorphism (M-tRFLP)

The genetic diversity of soil bacterial, archaeal, and fungal communities was characterized by the multiplex terminal restriction fragment length polymorphism (M-tRFLP). M-tRFLP analysis included the following steps: multiplex PCR reaction (parameters of performed PCR are shown in Table S1), digestion of the obtained amplicons with restriction enzyme HaeIII and detection of separated terminal restriction fragments in genetic analyzer (ABI 3130). M-tRFLP was carried out as described in detail by Gryta & Frąc (2020) and Mącik, Gryta, Sas-Paszt, & Frąc (2020).

2.7 | Next generation sequencing (NGS)

The DNA sequencing was performed with the application of MiSeq platform (Illumina Inc., San Diego, CA) at Genomed S.A. (Warsaw, Poland). The PCR primer set 341F and 785R targeting the V3-V4 hypervariable region of 16S rDNA was used in bacterial metagenome analysis, whereas the primers ITS1FI2 and 5.8S were used to amplify the hypervariable fungal ITS1 rDNA region (Table S1). According to the manufacturer (NEB Inc., Ipswich, MA), the aforementioned primers and Q5 Hot Start High-Fidelity 2× Master Mix were applied to perform an amplification. The v2 Illumina kit was used for sequencing using 2 × 250 bp pair-ended technology.

2.8 | Statistical and bioinformatics analyses

Differences and similarities in carbon substrates utilization profiles between treatments were analyzed by principal component analysis (PCA) and by Jaccard's coefficient index. Jaccard's coefficient was calculated based on the number of utilized C-substrates between particular treatments; the substrate was considered when the absorbance value was greater or equal 0.25 (A ≥ 0.25). The average absorbance values from 216 hr incubation period were used. Jaccard's coefficient was calculated with the following formula: $J = N_{AB}/(N_A + N_B - N_{AB})$, where N_A-number of C-substrates utilized in profile 1, N_B-number of C-substrates utilized in profile 2, N_{AB}-number of C-substrates utilized in both profile 1 and 2 (López et al., 2019).

The relative abundance of particular terminal restriction fragments (T-RFs) was visualized as heatmaps with the matrix2png web interface (Pavlidis & Noble, 2003). TRiFLe tool was used for in silico identification of microorganisms, based on the size of the obtained T-RFs and defined nucleotide sequence sets (Junier et al., 2008). The Sorensen's similarity coefficient (QS) was calculated based on the number of shared and unique peaks between particular treatments, according to the formula: $QS = 2C/(A + B)$, where A-number of T-RFs in profile 1, B-number of T-RFs in profile 2, C-number of T-RFs common for both profile 1 and 2 (Walitang et al., 2019).

Beta diversity, based on Bray-Curtis distances was visualized using unweighted pair group method with arithmetic mean (UPGMA) clustering, principal coordinate analysis (PCoA), and non-metric multidimensional scaling (NMDS).

The bacterial functional traits, based on the 16S rDNA data, were assessed with PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) (Langille et al., 2013) software in cooperation with KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa & Goto, 2000) online database. In addition, based on PICRUSt/KEGG results, the analysis concerning metabolic pathways related with P biotransformations in particular treatment was

TABLE 1 Changes of soil enzymatic activity, P_2O_5 content and maize yield under the influence of phosphorus biofertilizer at an optimal dose (FC), at an optimal dose enriched with microorganisms (FA100) and at a 40% reduced dose enriched with microorganisms (FA60)

	Sampling time/treatment								
	A18			S19			A19		
	FC	FA100	FA60	FC	FA100	FA60	FC	FA100	FA60
Protease activity (mg tyrosine kg^{-1} hr^{-1})	0.48 ± 0.02 b	2.50 ± 0.15 a	2.22 ± 0.79 a	3.34 ± 1.16 a	4.20 ± 1.59 a	2.22 ± 0.71 a	1.71 ± 0.29 a	2.00 ± 0.51 a	2.48 ± 0.79 a
Urease activity (μg N-NH_4 kg^{-1} hr^{-1})	46.90 ± 0.10 ab	59.86 ± 2.76 a	40.29 ± 1.94 b	41.03 ± 4.44 a	40.76 ± 5.33 a	43.81 ± 18.69 a	33.83 ± 6.30 a	50.82 ± 6.92 a	43.82 ± 3.29 a
Acid phosphomonoesterase activity (mmol PNP kg^{-1} hr^{-1})	13.79 ± 2.14 a	20.20 ± 6.13 a	19.31 ± 4.16 a	14.22 ± 1.32 a	17.85 ± 10.67 a	26.71 ± 10.35 a	16.28 ± 3.50 a	17.85 ± 0.56 a	19.39 ± 2.44 a
β-glucosidase activity (mg PNP kg^{-1} hr^{-1})	0.65 ± 0.15 a	0.74 ± 0.20 a	0.59 ± 0.22 a	0.71 ± 0.22 a	0.48 ± 0.16 a	0.76 ± 0.08a	0.60 ± 0.08b	1.21 ± 0.30 a	1.05 ± 0.05 ab
P_2O_5 content (mg 100 g^{-1})	17.77 ± 0.35 a	16.57 ± 0.68 b	16.97 ± 0.31 ab	13.10 ± 0.96 a	13.43 ± 0.49 a	13.07 ± 0.64 a	15.27 ± 0.42 a	13.90 ± 0.17 b	13.70 ± 0.10 bc
maize yield (t ha^{-1})	24.56 ± 1.70 a	26.08 ± 1.63 a	27.60 ± 1.10 a	-	-	-	11.39 ± 3.74 a	11.72 ± 3.22 a	15.20 ± 0.63 a

Note: significant differences ($p < 0.05$) were calculated for each sampling time separately. Explanation: PNP-*p*-nitrophenol, A18-autumn 2018, S19-summer 2019, A19-autumn 2019. Different letters indicate statistically significant differences between treatments.

performed. The functional characterization of soil fungal communities was conducted using the FUNGuild online database (Nguyen et al., 2016).

The differences in enzyme activities and number of functional operational taxonomic units (OTUs) between particular treatments and sampling times were determined with statistical tests. The analysis of variances (ANOVA) regarding soil sampling time and fertilization method and a post hoc Tukey test were used to calculate significant differences when ANOVA assumptions were met (β-glucosidase, PICRUSt/KEGG analyses, maize yield). The verification of ANOVA assumptions, including dataset normality and homoscedasticity of the variance, was conducted using Shapiro–Wilk and Levene tests, respectively. F-Welch test with post hoc Tukey test were used when normality of dataset was maintained but variance was not homogenous (protease, acid phosphomonoesterase, P_2O_5 content). On the other hand, when the dataset normality was violated, Kruskal-Wallis and Dunn tests were used (urease).

The automatic preliminary analysis of the NGS data, consisting on demultiplexing and the generation of fastq files, was performed on the MiSeq platform using the MiSeq Reporter (MSR) version 2.6 software (Illumina Inc., San Diego, CA). Bioinformatic analysis providing classification of reads to species taxonomic level was performed with the Quantitative Insights into Microbial Ecology (QIIME) software (Caporaso et al., 2010) based on the uCLUST algorithm (Edgar, 2010) and the GreenGenes version 13_8 database (16S V3-V4 OTUs) (DeSantis et al., 2006) and BLAST algorithm (Altschul et al., 1990) and UNITE version 8 database (ITS1 region) (Kõljalg et al., 2013; Nilsson et al., 2019).

All statistical analyses were performed with Statistica version 13.1 software (StatSoft Inc., Tulsa, OK) and R version 1.0.5.999 software (R Core Team, 2018, Vienna, Austria).

3 | RESULTS

3.1 | Soil enzymatic activity, assimilated phosphorus content, and maize yield

The changes of soil enzymatic activity in response to application of biofertilizers and sampling time are shown in Table 1. The activities of protease, urease, acid phosphomonoesterase, and β-glucosidase varied between individual fertilization treatments in a particular year.

The highest protease activity across whole experiment was observed in FA100(S19). On the other hand, the topmost increments in protease activity were reported in FA100(A18) and FA60(A18) (by 415.78% and 358.95%, respectively) as compared to control. Concerning the seasonal variations in protease activity, 2019 witnessed a decrease in aforementioned enzyme activity between controls and between FA100 treatments and an increase between FA60 treatments. It was also observed that activity of protease remained at higher levels in FA100 and FA60 in both A18 and A19.

In case of the urease, the highest activity during 2-year experiment was recorded in FA100(A18) and FA100(A19). Simultaneously, a greater variations in urease activity were observed in aforementioned sampling times in comparison with S19. In 2019 there was an upward trend in the urease activity between FA100 treatments.

FA100 and FA60 were characterized by increased acid phosphomoesterase activity as compared to controls (by 9.69%–87.85% depending on fertilization method and sampling time) throughout whole experiment. In S19 and A19, an upward trend in aforementioned enzyme activity was observed along with the decrease in the mineral fertilizer dose. No statistically significant changes occurred across the experimental period.

The highest increase in β-glucosidase activity as compared with control was recorded in FA100(A19) and FA60(A19) (by 102.28% and 75.45%, respectively). However, a reduction in the activity of β-glucosidase was observed in FA60(A18) and in FA100(S19). Statistically significant changes were noticed in A19.

When analyzing enzymatic activities throughout whole experimental period it is clearly visible that protease, β-glucosidase, urease, and acid phosphomonoesterase activities remained at higher levels in FA100 and FA60. The highest activities of protease and urease were observed in FA100. The application of biofertilizer also increased the activity of acid phosphomonoesterase in FA60. On the other hand, β-glucosidase activities were similar in both FA100 and FA60 throughout whole experiment. The statistically significant differences between treatments were reported in case of acid phosphomonoesterase activity (Figure 1).

Application of phosphorus biofertilizer increased the content of phytoavailable P_2O_5 in FA100(S19) by 2.52% as compared to FC(S19). The second year of the field experiment witnessed a decline in the quantity of P_2O_5 in the soil. Both FC(A18) and FC(A19) were characterized by the higher P_2O_5 content as compared to corresponding FA100 and FA60 treatments, whereas in FA100 and FA60 in a particular sampling time the quantity of assimilable phosphorus forms remained at a similar level (Table 1).

In both year of the field experiment, application of phosphorus biofertilizer increased average maize yield in FA100 and FA60 treatments. In 2018, in FA100 and FA60 yield increased by 6.19% and 12.38%, while in 2019 by 2.90% and 33.45%, respectively (as compared with corresponding controls). No statistically significant differences occurred between particular treatments. Noteworthy is the fact that FA60 treatments were characterized by the higher average maize yield in comparison with FA100 treatments (Table 1).

3.2 | Metabolic potential of soil microbial communities

Based on the average absorbance values of C-substrates utilization in ECO and FF Biolog plates during 216 incubation hours, the PCA was performed. In most cases, treatments from particular sampling times were clustered separately. PCA grouped the samples collected in A19 together in both plate types. Moreover, in ECOplates two groups were formed: first contained FC(S19) and FA100(S19) and second consisted of FA60(A18) and FA100(A18). Similar trend was noticed in

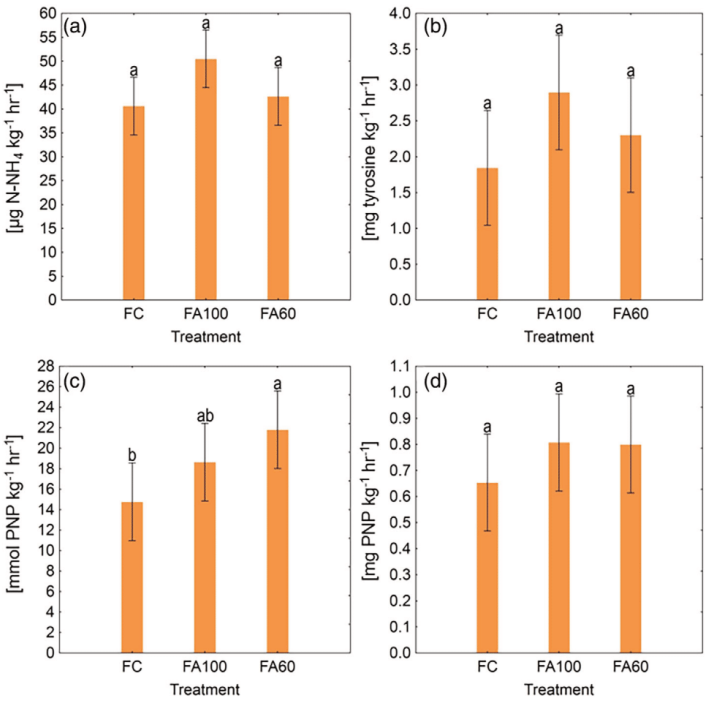

FIGURE 1 Changes in soil enzymatic activity in particular treatments throughout the experimental period. (a)-the activity of urease, (b)-the activity of protease, (c)-the activity of acid phosphomonoesterase, (d)-the activity of β-glucosidase. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), PNP-*p*-nitrophenol. Vertical bars denote 0.95 confidence intervals. Vertical bars denote 0.95 confidence intervals. Significant differences ($p < 0.05$) were calculated for all sampling times together [Colour figure can be viewed at wileyonlinelibrary.com]

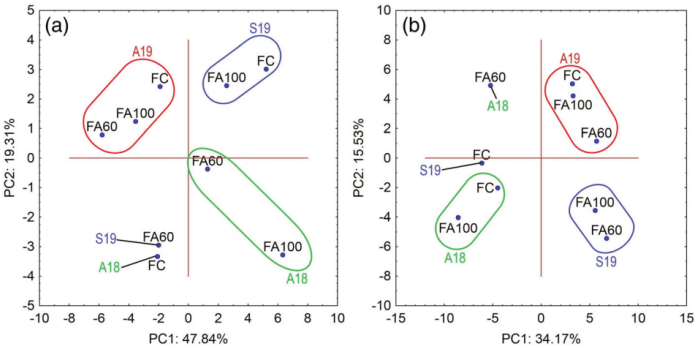

FIGURE 2 Principal component analysis (PCA) of C-substrate utilization patterns after 216 hr incubation of ECO (a) and FF (b) biolog plates. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), A18-autumn 2018, S19-summer 2019, A19-autumn 2019 [Colour figure can be viewed at wileyonlinelibrary.com]

FF plates where FA100(S19), FA60(S19), FC(A18), and FA100(A18) also formed separated clusters (Figure 2).

Jaccard's coefficient index, based on the absorbance values of utilized carbon substrates was calculated in order to point similarities between particular treatments. The high values of aforementioned index (0.95–1) indicate a strong resemblance of C-substrates utilization patterns. The most similar utilization profiles in ECO plates were observed between FA100(A19) and FA60(A19), and in the case of FF plates, between FC(S19) and FA100(S19) (Table 2).

3.3 | The multiplex terminal restriction fragment length polymorphism

Multiplex tRFLP fingerprinting profile showed that not only fertilization method, but also sampling time influenced the structure of soil microbiome. Soil samples taken at different times exhibited variations in the number of T-RFs, their size and relative abundance. In silico analysis with the TRiFLe software allowed to identify microbial genera based on the size of selected T-RFs. Our results showed that T-RFs

with different sizes could be represented by various microorganisms and, what is more, the same genera were identified within T-RFs which differ from each other by several base pairs.

Soil bacterial communities were characterized by the richness (number of obtained T-RFs) ranging from 4 to 15, with a simultaneous decline in samples collected in S19 (Figure 3a). However, the increase in the number of T-RFs were observed in FA60(A18) and FA100(S19) as compared to corresponding controls. In whole restriction profile there were no T-RFs common for all treatments and sampling times, nonetheless 170 bp appeared throughout the whole experiment, with the exception of FC(S19). Fragments with the highest relative abundance were 112 bp (~41–49% reported from samples collected in A19) and 128 bp (48.29%in FA60(S19)). It is worth mentioning that the relative abundance of 113 bp (assigned to *Lysobacter*, *Pseudomonas*, and *Pantoea*) increased in FA100(S19) (30.39%) as compared to FC(S19) (13.36%). The similar trend was also observed in case of 120 bp (assigned to the abovementioned microorganisms) which relative abundance in FC(A18), FA100(A18), and FA60(A18) accounted for 4.23%, 6.76%, and 6.55%, respectively. Comparing restriction profiles from particular sampling times it is clearly seen that both A18 and A19 exhibited increased diversity of obtained T-RFs and higher Sorensen coefficient values (Table 3). Analysis with TRiFLe revealed the presence of microorganisms assigned to selected DNA fragments and belonged to the following phyla: Proteobacteria (*Filomicrobium* (60 bp), *Paracoccus* (76 bp), *Rhizobium* (155 bp), *Burkholderia* (360/364 bp), *Salicola* (60 bp), *Proteus* (90/94/100 bp), *Klebsiella* (170 bp), *Shigella* (290 bp), Actinobacteria (*Micrococcus* [90/94 bp]), Bacteroidetes (*Proteiniphilum* (90/94 bp), *Flavobacterium* (170 bp), Firmicutes (*Clostridium* (170 bp), *Staphylococcus* (272 bp) Cyanobacteria [*Anabaenopsis* (256 bp]] and Gemmatimonadetes (74/80 bp).

TABLE 2 Jaccard's coefficient values

	PlateType/sampling time					
	ECOplate			FFplate		
Treatment	A18	S19	A19	A18	S19	A19
FC-FA100	0.80	0.90	0.97	0.93	0.95	0.92
FC-FA60	0.97	0.87	0.97	0.91	0.92	0.91
FA100-FA60	0.77	0.90	1.00	0.93	0.91	0.92

Note: explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), A18-autumn 2018, S19-summer 2019, A19-autumn 2019

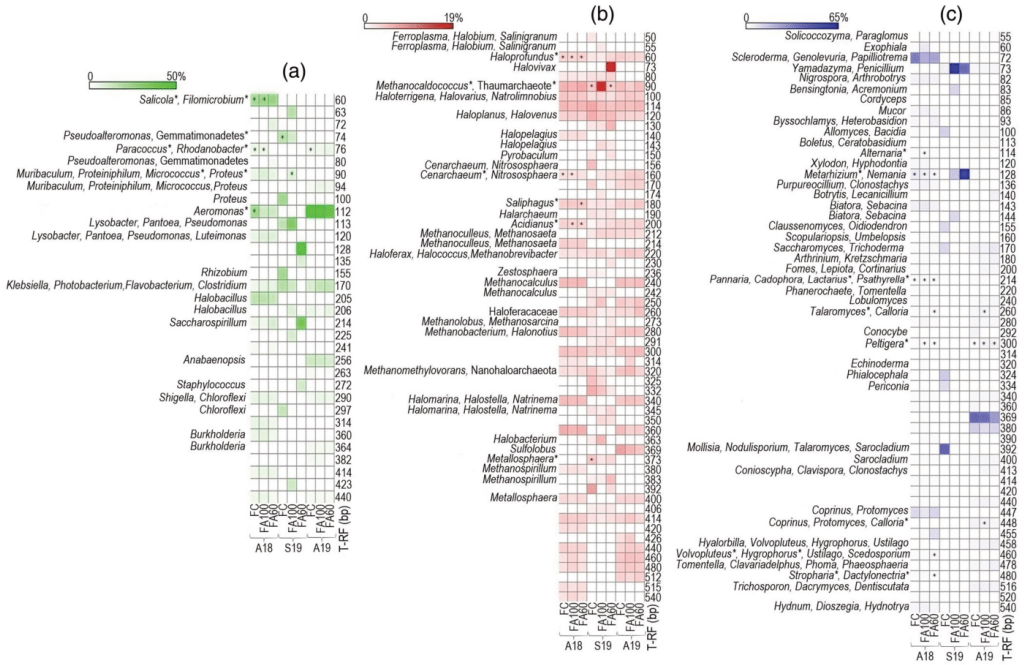

FIGURE 3 Heat maps presenting the number of terminal restriction fragments, their relative abundance and genera prediction in bacterial (a), archaeal (b) and fungal (c) communities. *The particular T-RF was present, but no identification of microorganisms occurred. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), A18-autumn 2018, S19-summer 2019, A19-autumn 2019 [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Sorensen's similarity coefficient values

	Microbial group/sampling time								
	Bacteria			Archaea			Fungi		
Treatment	A18	S19	A19	A18	S19	A19	A18	S19	A19
FC-FA100	0.963	0.429	0.957	1	0.841	0.981	0.971	0.182	0.870
FC-FA60	0.897	0	0.957	1	0.824	1	0.958	0.222	0.809
FA100-FA60	0.857	0	0.909	1	0.806	0.981	0.986	0.667	0.889

Note: explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), A18-autumn 2018, S19-summer 2019, A19-autumn 2019

In archaeal communities, richness ranged from 26 to 35, with the highest values reported in soil samples taken in S19 (33–35 T-RFs, depending on the treatment) (Figure 3b). The only treatment where the number of T-RFs was higher than the control was FA100(S19). In restriction profiles there were common T-RFs shared within all sampling times and treatments (60, 90, 100, 180, 200, 220, 320 bp) and some unique fragments which appeared only in treatments where biofertilizers were applied (55, 143, 174, 350 bp). The highest Sorensen coefficient values were noted in case of soil samples taken in A18 and A19 (Table 3). The predominant archaeal T-RFs were 73 bp (affiliated to *Halovivax*) and 90 bp (attributed to *Methanocaldococcus* and Thaumarchaeote) with the relative abundance accounted for 18.53% [in FA60(S19)] and 16.81% [in FA100(S19)], respectively. Identification with TRiFLe software showed that representatives of *Ferroplasma*, *Halobium*, *Salinigranum*, *Halopelagius*, *Cenarchaeum*, *Nitrososphaera*, *Methanoculleus*, *Methanosaeta*, and *Methanocalculus* could be assigned to different T-RFs depending on the treatment and sampling time. Our results proved that some archaea were found in all treatments during the experimental period, for example, *Haloterrigena*, *Halovarius*, *Natrolimnobius*, *Haloplanus*, and *Halovenus*. On the other hand, there were genera belonged to unique T-RFs, e.g. *Pyrobaculum* assigned to 150 bp and *Zestosphaera* connected with 236 bp.

In fungal communities, the number of T-RFs varied between 2 and 37 and similar to bacteria, A18 and A19 were characterized by increased richness and Sorensen coefficient values (Table 3) as compared to S19. The increase in number of T-RFs was observed in FA100(A18) and FA60(A18) as compared to FC(A18). Fragments that reached the highest relative abundance throughout the experimental period were 72 bp (32.52%in FC(A18)), 73 bp [64.46%in FA100(S19)], 128 bp [63.3%in FA60(S19)], 392 bp [41.54% in FC(S19)], and 369 bp (38.83%in [FC(A19)] (Figure 3c). There were no T-RFs common for all sampling times and treatments, however some of them appeared in FC, FA100 and FA60 in both A18 and A19, e.g. 180, 240, 260, 340, 380, and 400 bp. It was observed that that the relative abundance of 73 bp (assigned to *Penicillium*) increased in FA1009(S19) (64.46%), FA60(S19) (36.69%) and FA60(A19) (2.92%) as compared to FC(S19) (4.57%) and FC(A19) (2.78%). The increased relative abundance of 170 bp (affiliated to *Trichoderma*) was reported in FA60(A18) (2.08%), FA100(A19) (3.33%) and FA60(A19) (5.25%) in comparison with corresponding control treatments FC(A18) (1.76%) and FC(A19) (3.16%).

3.4 | Next generation sequencing (NGS)

3.4.1 | Bacterial and archaeal community composition and functional prediction

Among the 188 identified OTUs at the order level, 171 were shared between FA100, FA60, and FC across the all sampling times and constituted the core microbiome (Figure 4a). What is worth mentioning, FA100 and FA60 were characterized by the greater diversity than control (FC – 178, FA100–181, FA60–180 identified orders). The analysis of seasonal shifts within particular treatments showed that the core microbiome of FC (Figure 4d) consisted of higher number of identified orders as compared to FA100 and FA60. The most diverse treatments in terms of individual orders were FA100(A18), FA100(A19) (Figure 4b), and FA60(A19) (Figure 4c). The distribution of common and unique bacterial and archaeal orders in particular treatments and sampling times is presented in Table S2 and S3. Bacterial orders which were characteristic to FA100 included C20 (Chlorobi), Lactobacillales, S0208 (Chloroflexi), and VC38 (Chlorobi) while GMD14H09 (Proteobacteria) and TG3-1 (Fibrobacteres) were unique to FA60. Orders characteristic to both FA100 and FA60 included Rhodothermales, LD1-PB3 (Verrucomicrobia), Oceanospirillales, and SJA-36 (Acidobacteria) (Table S3). The bacteria which dominated treatments within all sampling times belonged to the following orders: Actinomycetales (12.69–19.08%), Rhizobiales (6.06–8.04%), Xanthomonadales (3.16–6.43%), Rhodospirillales (4.40–5.49%), Acidobacteriales (4.03–7.05%), and Solirubrobacterales (4.19–5.15%). The relative abundance of particular orders was dependent on the sampling time and fertilization method. In S19 the relative abundance of Actinomycetales, Rhizobiales, Xanthomonadales, Sphingomonadales, Burkholderiales, and Bacillales increased as compared to A18 and A19. On the other hand, the relative abundance of Rhodospirillales, Acidobacteriales, Acidimicrobiales, Thermogemmatisporales, Solibacterales, Myxococcales, and Gemmatales was higher in samples collected in autumn (Figure 4e).

Predictive functional profiling of the bacterial communities, based on the 16S rDNA data, was conducted with PICRUSt (Langille et al., 2013) and KEGG (http://www.kegg.jp/) and revealed that the majority of identified OTUs was assigned to "Metabolism" (∼54%) followed by "Environmental Information Processing" (∼15%), "Genetic Information Processing" (∼13%), "Genes and Proteins" (∼10%) and "Cellular Processes" (∼5%) (Figure S2). In A18 number of

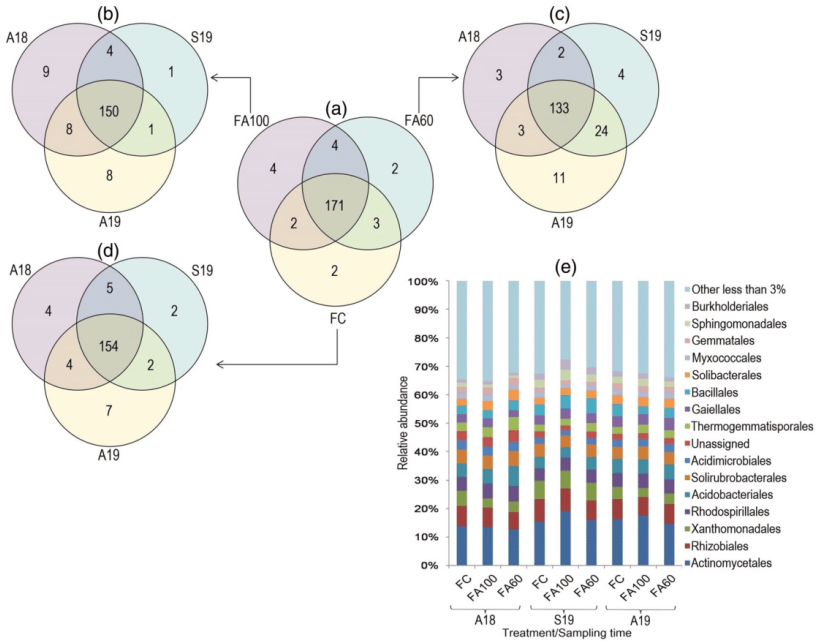

FIGURE 4 Venn diagrams showing the distribution of shared and unique bacterial and archaeal operational taxonomic units (OTUs) identified at the order level. (a)-treatments from all sampling times, (b)-seasonal variations in FA100 treatment, (c)-seasonal variations in FA60 treatment, (d)-seasonal variations in FC treatment, (e)-the relative abundance of dominant bacterial orders throughout the experimental period. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertizer enriched with microorganisms (dose reduced by 40%), A18-autumn 2018, S19-summer 2019, A19-autumn 2019 [Colour figure can be viewed at wileyonlinelibrary.com]

functional sequences in particular main KEGG classes remained at similar level in all treatments. The application of biofertilizers in S19 contributed to the increase in the number of OTUs assigned to each main KEGG class in FA100 and FA60, however, FC showed a relatively similar number of functional OTUs to samples taken in A18. An upward trend in the number of OTUs was also observed in A19 not only in FA100 and FA60, but also in controls as compared to treatments from S19. Throughout the experimental period, FA60(A19) was characterized by the greatest number of OTUs associated with each main KEGG class (Figure S3). The metabolism-related pathways encompassed 12 subclasses, of which amino acids and carbohydrates biotransformations displayed the highest number of OTUs. On the other hand, the lowest number of OTUs identified with metabolic processes was assigned to pathways associated with biosynthesis of other secondary metabolites and glycans. It was also observed that FA100 and FA60 in S19 and A19 were characterized by increased number of sequences connected with xenobiotics degradation and metabolism of terpenoids and polyketides (Figure S4). In-depth analysis of PICRUSt metabolic pathways revealed that some OTUs were assigned to P-related processes including glycerophospholipid metabolism, inositol phosphate metabolism, oxidative phosphorylation, pentose phosphate pathway, phosphatidylinositol signaling system, phosphonate and phosphinate metabolism, and phosphotransferase system (PTS). The application of phosphorus biofertilizer in S19 increased the number of functional OTUs associated with phosphorus processes in FA100 and FA60 treatments as compared to control soil and A18 samples. It is worth mentioning that the identical trend was maintained in soil samples collected in A19. Among P-related pathways, the highest number of OTUs was assigned to oxidative phosphorylation, followed by pentose phosphate pathway and glycerophospholipid metabolism. Analyzing variations throughout the experimental period, FA100(A19) and FA60(A19) were characterized by the highest number of functional OTUs associated with phosphorus biotransformations (Figure S5). Some seasonal variations in bacterial functional profiles were visualized with PCA plots, namely treatments from particular sampling times formed separated clusters (Figure S6).

3.4.2 | Fungal community composition and functional guilds prediction

The composition of soil fungal communities was analyzed, similarly as bacterial and archaeal communities, at the order level. Our data

showed that the core fungal microbiome, common for all treatments across the experimental period, consisted of 106 detected OTUs out of the total 131 defined orders (Figure 5a). Other identified OTUs were distributed among particular fertilization methods. The soil environment was dominated by representatives from the following orders: Eurotiales, Hypocreales and Mortierellales, which accounted for 13.19–23.56%, 7.99–14.01%, and 4.22–7.88%, respectively, according as sampling time and treatment (Figure 5e). In FC and FA100 the presence of 120 various fungal orders were reported, whereas for FA60 this number was 115. Regarding seasonal variations, a similar trend to the bacterial community was observed, namely core microbiome of FC encompassed higher number of orders than FA100 and FA60. What is more, A18 and A19 within each treatment were characterized by higher number of specific OTUs (Figure 5b–d). The distribution of common and unique fungal orders in particular treatments and sampling times is presented in Tables S4 and S5. Fungal orders specific for FA100 included Coryneliales, GS02 (Rozellomycota), Microstromatales, Rozellomycotina_ord_Incertae_sedis, and Teloschistales whereas Gomphales and Hyaloraphidiales were unique to FA60. Orders characteristic for both FA100 and FA60 encompassed Endogonales, Falcocladiales, Myriangiales, and Tilletiales (Table S5). The soil environment was dominated by representatives from the following orders: Eurotiales, Hypocreales, and Mortierellales, which accounted for 13.19–23.56%, 7.99–14.01%, and 4.22–7.88%, respectively, according as sampling time and treatment. Samples collected in S19 were characterized by the increased relative abundance of Mortierellaes, Sordariales, Filobasidiales, and Agaricales, whereas the relative abundance of Eurotiales, Chaetothyriales, Umbelopsidales, and Pleosporales remained higher in A18 and A19 samples. In case of Hypocreales, their contribution to the creation of soil fungal communities was higher in both S19 and A19 as compared to A18 (Figure 5e).

A functional prediction of the fungal community showed that the soil environment was dominated by the representatives of saprotrophs (29.12–39.57%), followed by saprotrophs-symbiotrophs (5.93–9.23%), pathotrophs-saprotrophs-symbiotrophs (5.78–8.91%), and pathotrophs (3.20–6.87%). It is worth mentioning that FA100 and FA60 in both S19 and A19 were characterized by the lower relative abundance of pathotrophs as compared to corresponding controls. At the same time, the increments in relative abundance of saprotrophs-symbiotrophs were observed in FA100(A18) and FA100-FA60(A19) (Figure S7). A deeper look in fungal ecological guilds revealed that the saprotrophs population was mainly dominated by undefined

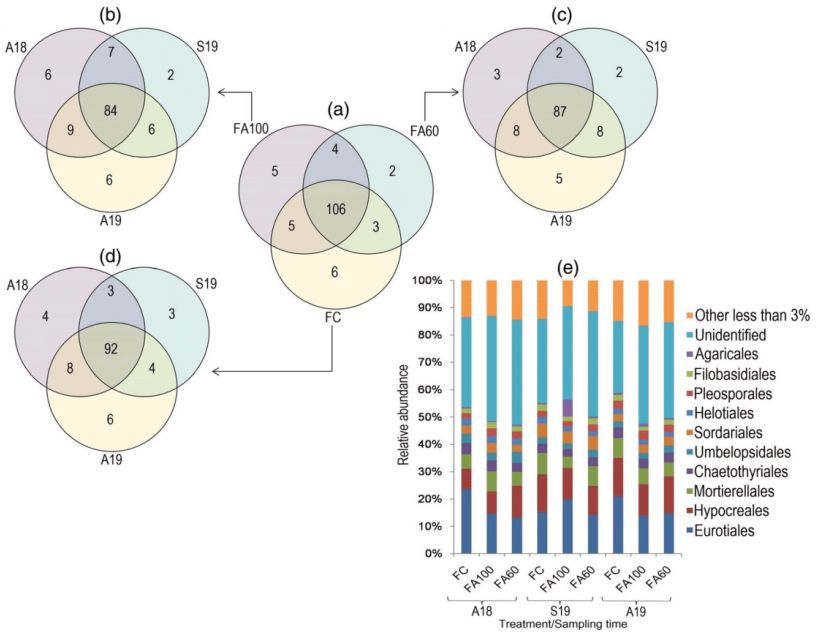

FIGURE 5 Venn diagrams showing the distribution of shared and unique fungal operational taxonomic units (OTUs) identified at the order level. (a)-treatments from all sampling times, (b)-seasonal variations in FA100 treatment, (c)-seasonal variations in FA60 treatment, (d)-seasonal variations in FC treatment, (e)-the relative abundance of dominant fungal orders throughout the experimental period. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertizer enriched with microorganisms (dose reduced by 40%), A18-autumn 2018, S19-summer 2019, A19-autumn 2019 [Colour figure can be viewed at wileyonlinelibrary.com]

saprotrophs which accounted for ~50–60% of the total aforementioned guild, whereas endophyte-litter saprotroph-soil saprotroph-undefined saprotroph was the dominant group among saprotrophs-symbiotrophs (7.13–13.72%). Pathotrophs were mainly represented by animal and plant pathogens, however, the relative abundance of animal pathogens decreased in FA100 and FA60 in both S19 and A19 in comparison with corresponding controls. Lower contribution of plant pathogens in fungal community structure was also observed in FA60(A18) and FA100(S19). The relative abundance of selected fungal ecological guilds, important in terms of soil health, increased in FA100 and FA60 treatments. Such changes included the following guilds: wood saprotroph (FA60(A18), FA100-FA60(A19)), dung saprotroph-ectomycorrhizal-litter saprotroph-undefined saprotroph (FA60(A19)), ectomycorrhizal-wood saprotroph (FA100(A19)), endophyte-litter saprotroph-wood saprotroph (FA100-FA60 across whole experimental period), and arbuscular mycorrhizal (FA100-FA60 (A18), FA60(S19), and FA60(A19) (Figure S8).

3.4.3 | Beta diversity of soil microbial communities

Visualization of the beta diversity distribution between treatments and sampling times was demonstrated by the PCoA plots and UPGMA dendrograms on the basis of Bray–Curtis dissimilarity index. In PCoA plots the clear separation of treatments, depending on the soil sampling time, for both bacteria (Figure 6a) and fungi (Figure 6b) was reported. Interestingly, in most cases it was possible to distinguish inner clusters in which controls would be isolated from FA100 and FA60.

UPGMA dendrogram for bacteria showed two well-defined clusters: one included S19 treatments and second one encompassed differentiated A19 and A18. In both S19 and A19, the FC was separated from FA100 and FA60 (Figure 6c). Similar trend, namely two main groups were distinguished in case of fungal communities, however, some differences in comparison with bacteria were noticed. First main cluster was composed by A18 treatments and second, including three sub-clusters, by S19 and A19 treatments. FA100 and FA60 treatments from particular sampling time were clustered together and were clearly isolated from controls. What is worth mentioning, there is a possibility to distinguish another inner cluster which would encompass FA100(A19), FA60(A19), FC(S19), and FC(A19) (Figure 6d).

The relationships between soil microbial communities in particular treatments and sampling times were also investigated with the application of NMDS (Figure 7). In bacterial metagenome, a clear separation of A18 treatments from S19 and A19 along the axis 2 was noticed. Regarding the clustering along axis 1, all three controls remained at the higher part of axis 1, while FA60(A18) and FA60(S19)

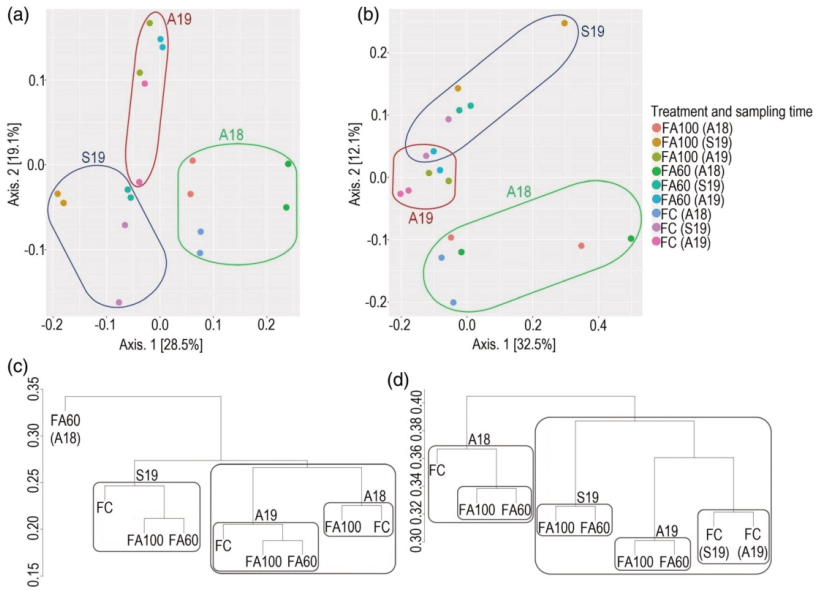

FIGURE 6 Principal coordinates analysis plots (a and b) and UPGMA dendrograms (c and d) based on the bray-Curtis distances for bacterial (a and c) and fungal (b and d) communities. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), A18-autumn 2018, S19-summer 2019, A19-autumn 2019 [Colour figure can be viewed at wileyonlinelibrary.com]

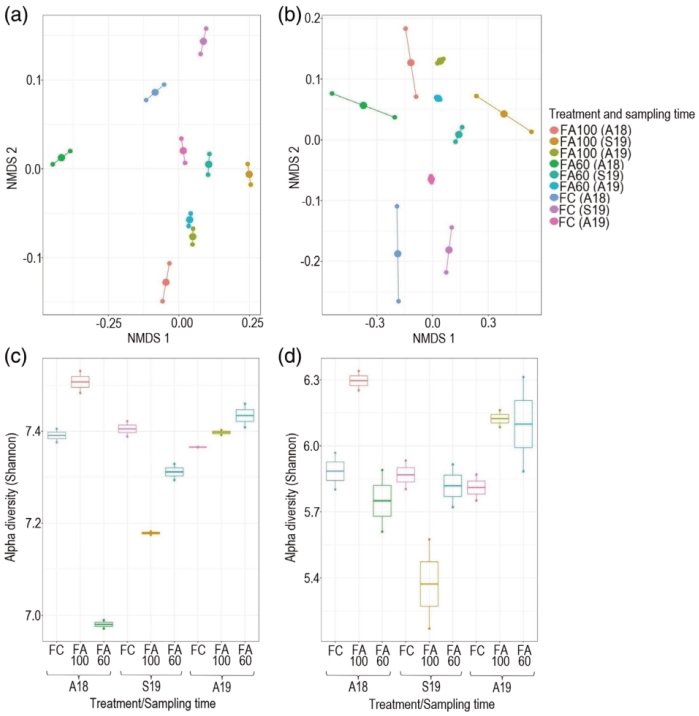

FIGURE 7 Nonmetric multidimensional scaling (NMDS) plots (a and b) and Shannon diversity (c and d) for soil bacterial (a and c) and fungal (b and d) communities. In (a) and (b) large dots represent centroids and small dots represent individual samples connect to the centroids. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), A18-autumn 2018, S19-summer 2019, A19-autumn 2019 [Colour figure can be viewed at wileyonlinelibrary.com]

were isolated from FA100(A18) and FA100(S19) and occupied the higher and the lower part of aforementioned axis, respectively. It was also noticed that individual replications of FA100(A18) treatments were the furthest apart in the ordination space; on the other hand points corresponding to FA60(A19) and FA100(A19) were relatively closely related (Figure 7a). In fungal communities the NMDS ordination plot showed a clear clustering of treatments along the two axes. Microbiologically enriched treatments were located at the higher part of axis 2, whereas the controls occupied the lower part of this axis. Similar trend as in bacterial communities was observed, namely A18 treatments were placed at the left side of axis 1, while the opposite extreme of this axis was positioned by S19 and A19 treatments (Figure 7b). The highest dispersion between individual replications was observed in case of A18 samples. Relatively far apart were placed points corresponding to FA100(S19) and FC(S19), however these distances were smaller as compared to A18 treatments. The closest points to each other in the ordination space were A19 samples.

The alpha diversity was expressed by the Shannon diversity index (H). The highest H values in bacterial communities were reached in FA100(A18) and FA60(A19) (Figure 7c), while in fungal metagenome in FA100(A18) and FA100-FA60(A19) (Figure 7d). Fungal communities were characterized by the higher variation in H as compared to bacterial communities, whereas H reached higher values in case of 16S rDNA data.

4 | DISCUSSION

Enzymatic activity is unquestionably one of the bioindicator of soil quality and reflects shifts in soil environment under the influence of various treatments, both organic and inorganic (Kwiatkowski et al., 2020). This study revealed variations in the activity of selected soil enzymes: protease, urease, acid phosphomonoesterase, and β-glucosidase. Analyzing the results in individual treatments throughout experimental period it was found that FA100 and FA60 were characterized by enhanced activity of protease, urease, acid phosphomonoesterase, and β-glucosidase. According to Mengual et al. (2014) degraded soil inoculated with beneficial bacterial strains (*Bacillus megaterium*, *Enterobacter* sp., *Bacillus thuringiensis*, and *Bacillus* sp.) and sugar beet was characterized by the increased dehydrogenase, protease, urease, and β-glucosidase activity. The fluctuations in the activity of investigated enzymes in this study may arise from the fact that this parameter is sensitive to changes in the soil environment within 2 years after the exposure to a specific factor (Dick & Kandeler, 2005). It is particularly noteworthy that in the FA100(A19) and FA60(A19) treatments, the activity of all analyzed enzymes remained at higher levels as compared to control treatment, which may indicate that some stability was achieved among the microbial communities and the pathways of N, P, and C compounds mineralization were adjusted to changes in underground environment caused by introduction to the soil microorganisms

combined with fertilizer granules. Adaptation of microorganisms to the new fertilization technique may also be supported by recording the highest protease, acid phosphomonoesterase, and β-glucosidase activities in second year of field experiment. The increased acid phosphomonoesterase activity maintained throughout the experimental period in FA100 and FA60 treatments may be explained as the introduction of a specific fertilizer combined with beneficial bacterial strains directed and stimulated soil microorganisms towards phosphorus metabolism. Enhanced acid phospomonoesterase, β-glucosidase, and protease activity may be also associated with the acceleration of metabolic processes as reflected by the increased number of functional OTUs (in FA100 and FA60) assigned to phosphorus, carbohydrates, and amino acids biotransformations.

It is commonly known that weather conditions are one of the factors affecting the status of soil microbiome (Furtak et al., 2020). In our study rainfall remained at similar level on each of the three sampling times which may suggest that it had no effect on soil microbiome properties. It is worth mentioning that S19 recorded a relatively high temperature, possibly stimulating the activity of soil microorganisms. According to Heidari et al. (2019) acid phosphatase activity increased in soil along with the temperature which may be supported by the enhanced activity of abovementioned enzyme in FA60(S19). Higher temperature may also stimulate metabolic processes and occurrence of particular microbial orders in S19. Nevertheless, it should be considered that the application of phosphorus biofertilizer affected soil microorganisms to a greater extent than weather conditions.

Application of phosphorus biofertilizer resulted in changes in the bioavailability of P in the soil. The increase in the quantity of available P in FA100(S19) remains in agreement with Wang, Liu, et al. (2021) who observed that soil samples inoculated with *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* were characterized by the higher content of assimilable phosphorus forms as compared to non-inoculated control. However, the decline in P_2O_5 content in particular FA100 and FA60 treatments may be related to enhanced acid phosphomonoesterase activity and more efficient exploitation of P compounds.

In meta-analysis conducted by two researchers (Schmidt & Gaudin 2018) it was found that the biofertilizers increased maize yield by an average 15.3% in field experiments and 18.4% in pot studies. Increased maize yield in FA100 and FA60 may be related with the enhanced soil enzymatic activity and hence with the higher nutrients availability. It is possible that the microorganisms provided in the biofertilizers accelerated the solubilization of phosphorus compounds, resulting in improved plant productivity. Furthermore, it was described that amino acids are plant growth stimulants and phosphorus biofertilizers, by increasing the number of OTUs associated with amino acids metabolism, may improve crop yield (Moe, 2013; Wang, Liu, et al., 2021). Increased maize yield in 2018 as compared to 2019 may be also related to higher P_2O_5 content in the soil. Simultaneously, a reduction in the quantity of available P may also be linked to the depletion of nutrients necessary for plant growth. Our study showed that the phosphorus biofertilizer application was more efficient in FA60 treatments, creating an opportunity to reduce mineral fertilizer doses along with achieving higher yields.

The metabolic potential of soil microorganisms toward C-substrates utilization showed a seasonal responses to an application of biofertilizers. In most cases, a clear clustering of treatments according to sampling time was observed. However, the apparent discrimination of FC and FA60 among A18 and S19 in PCA ordination space may indicate that both bacteria and fungi modify their metabolic pathways differently in response to biofertilizers application and this response depends on the stage of growing season. As Zhang, Tang, et al. (2010) described, soil bacteria associated with non-inoculated *P. tabulaeformis* seedlings were significantly separated from samples inoculated with ectomycorrhizal fungi in terms of functional profiles. The clear separation of S19 from A18 and A19 may be related to the intensification of photosynthesis in summer and the releasing of the products of this process to the soil. Simultaneously, the separation of FA60 may be connected with the competition for nutrients between indigenous microbiota and microorganisms provided with biofertilizers (Kandasamy et al., 2019). The fact that seasonality affected the metabolic properties of particular soil microbial communities is evidenced by the higher differences in Jaccard's coefficient reported in both ECO and FF plates in A18 and S19, respectively. As Koranda et al. (2013) mentioned, microbial communities differ in utilization of C-compounds depending on the season and the previously mentioned separation may be related to the fact that various chemical compounds were the preferred carbon sources in the particular treatments at different stages of the experiment. In winter, the preferred carbon source was cellulose, whereas utilization of glucose increased in summer. Nevertheless, the highest number of functional OTUs associated with amino acids and carbohydrates metabolism throughout the experimental period point that certain compounds are exploited primarily by microorganisms, regardless of sampling time and fertilization techniques. According to Jacoby et al. (2017) plants exudates are rich in sugars and amino acids and the high bioavailability of aforementioned components may be related to their enhanced metabolism in comparison with other compounds. On the other hand, the accomplishment of a metabolic balance and adjustment to a new fertilization practice among microbiome inhabiting studied soil may be confirmed by clustering of A19 treatments together in both ECO and FF plates and relatively high values and small differences of Jaccard's coefficient between FC, FA100, and FA60. Grouping all A19 treatments together may be also associated with the fact that FC(A19), FA100(S19), and FA60(S19) were characterized with the highest number of functional OTUs associated with metabolic processes. It is thought that soil microorganisms remain more responsive in warm season (Xu et al., 2021), however, our results showed that application of biofertilizer may prolong the activity of soil microbiome and moreover, enhance it despite autumn temperature decline. Seasonal variations in functional and metabolic profiles of soil microorganisms were also supported by the PCA grouping based on the number of functional OTUs assigned to a particular biochemical pathways.

Investigation of soil microorganisms biodiversity involves not only soil enzymatic activity and functional profiles, but also genetic variability. Our research showed that the application of biofertilizers affected the composition of soil microbiome, however, the response

depended on particular treatment, sampling time, and microbial group. The observed variations in genetic restriction profile referred to both the number of T-RFs in the restriction profiles and their relative abundance. The increase in the number of T-RFs in FA100 and FA60 may suggest the positive impact of applied biofertilizers on the genetic diversity of soil microorganisms. Similar results were observed by Trabelsi et al. (2011, 2012) and Kandasamy et al. (2019) where bacterial richness increased in soil inoculated with beneficial microorganisms. Soil inoculated with *Ensifer meliloti* 4H41, *Rhizobium gallicum* 8a3 and consortium of these strains exhibited the increased number of T-RFs, accounted for 51, 46, and 36, respectively, as compared to non-inoculated control (13 T-RFs) (Trabelsi et al., 2012). On the other hand, the decrease in the number of T-RFs in bacterial and fungal communities in S19 may be a result of sensitivity of microorganisms to a new fertilization regime. According to Zhang, Sun, et al. (2010) *Rhizobium* spp. inoculation lowered the bacterial richness in the rhizosphere of faba bean. In archaea community, the number of T-RFs remained at a relatively similar level during the whole experimental period, which may indicate greater stability of aforementioned group in the face of modified environmental conditions. The high Sorensen's coefficient values between treatments in A18 and A19 point to the close similarity in the distribution of terminal restriction fragments among soil bacteria, fungi, and archaea communities (Walitang et al., 2019). On the contrary, in S19 treatments lower values of above-mentioned index result from the presence of many unique T-RFs that were specific for a particular fertilization method.

The assignment of selected T-RFs to a various microbial genera with TRiFLe software revealed the presence of some ecologically important microorganisms. Among archaea, members of *Nitrososphaera* genus are ammonia oxidizing archaea (AOA) which are thought to be a key element in the nitrogen biogeochemical cycle (Mukhtar et al., 2019), *Ferroplasma* spp. species are involved in iron cycling (Golyshina, 2011) and representatives of *Methanocaldococcus* spp., *Methanosaeta* spp., *Methanosarcina* spp., and *Methanomethylovorans* spp. are well-described methanogens (Cha et al., 2013; Ferry, 2020; Mori et al., 2012; Susanti et al., 2019). Beneficial microorganisms among bacteria may be represented by strains of *Rhizobium* spp. and *Lysobacter* spp. Members of *Rhizobium* spp. genus are commonly known legume plants symbionts and nitrogen fixers (Lindström & Mousavi, 2020), whereas *Lysobacter* spp. was found to exert plant growth promoting properties and was considered as a biocontrol agent against *Fusarium graminearum* (Chen et al., 2020). It is worth emphasizing that the aforementioned microorganisms were also characterized in terms of phosphate dissolving abilities. *Lysobacter enzymogenes* LE16 increased water-soluble phosphorus content in the soil and boosted P uptake in lettuce seedling (Chen et al., 2019), *Burkholderia cepacia* SCAUK0330 solubilized phosphate from insoluble $Ca_3(PO_4)_2$ in a liquid medium (Zhao et al., 2014), *Pseudomonas aeruginosa* KR270346 and KR27034 increased the available P content in the soil and enhanced phosphatase activity (Linu et al., 2019), whereas *Pantoea agglomerans* IALR1325 synthesized extracellular enzymes (acid phosphatase and phytase) and dissolved $Ca_3(PO_4)_2$ and $Ca_5(PO_4)_3OH$ (Mei et al., 2021). The application of phosphorus biofertilizer in S19 increased the relative abundance of 113 bp and 120 bp T-RFs within which *Lysobacter* spp., *Pantoea* spp. and *Pseudomonas* spp. representatives were identified, what may suggest that the phosphorus biofertilizer promotes the occurrence of microorganisms involved in P solubilization. The identified bacterial genera were assigned to different phyla, confirming the high diversity among studied community. The analysis of fungal community showed that selected T-RFs may be attributed to strains that exhibit plant growth promoting properties (*Penicillium* spp.) (Naziya et al., 2020), saprotrophs involved in SOM decomposition (*Cortinarius* spp., *Conocybe* spp.) (Barnes et al., 2016; Müller et al., 2020) biocontrol agents (*Metarhizium* spp., *Trichoderma* spp., *Clonostachys* spp.) (Faria et al., 2017; Sun et al., 2020), plants and algae/cyanobacteria symbionts (*Boletus* spp., *Bacidia* spp.) (Malíček et al., 2018; Treindl & Leuchtmann, 2019) and microorganisms used in bioremediation (*Solicoccozyma* spp.) (Stosiek et al., 2019). Interestingly, representatives of *Penicillium* spp. and *Trichoderma* spp. were also found to exhibit P-solubilizing abilities. *Penicillium guanacastense* was able to solubilize phosphorus in microbiological media containing $AlPO_4$, $Ca_3(PO_4)_2$, and $FePO_4{\cdot}4H_20$, while *Trichoderma koningiopsis* synthesized organic acids to solubilize $Ca_3(PO_4)_2$ at high pH stress (Qiao et al., 2019; Tandon et al., 2020). The afore-described properties are significant for land rehabilitation based on microbial-derived products as they contribute to the improvement of nutrient availability, reduce the number of potentially harmful organisms and detoxify pollutants.

A broader analysis of the genetic diversity of microorganisms in soil fertilized with phosphorus biofertilizer was conducted using NGS and metataxonomic approach. Venn diagrams were drawn in order to expose the number and the seasonal distribution of OTUs identified at order level within microbial communities. The high number of OTUs between individual treatments indicates the presence of many commonly occurring microorganisms, regardless of fertilization regime. Particularly noteworthy is the fact the studied soil was dominated by Rhizobiales and Actinomycetales, some representatives of which were previously characterized for their beneficial features toward plant growth and development and improvement of soil quality. Rhizobiales, apart from nitrogen-fixing properties, synthesize plant hormones and improve nutrient uptake. Actinomycetales are involved in P solubilization in the soil, suppress pathogens such as *Pythium ultimum* and *Erwinia carotovora*, excrete siderophores, increase nutrient uptake and boost plant growth (AbdElgawad et al., 2020; Erlacher et al., 2015). Beneficial properties including synthesis of plant hormones (abscisic acid, gibberellins, and zeatin) were also found among *Sphingomonas* spp. which belong to the Sphingomonadales (Sun et al., 2021). Furthermore, the highest relative abundance of aforementioned orders was reported in FA100 in S19, which may suggest the beneficial impact of microbiologically enriched phosphorus biofertilizer. Taking into consideration fact that the relative abundance of these bacterial orders increased also in FC(S19) as compared to FC(A18), application of phosphorus biofertilizer in optimal dose may stimulate seasonal changes in bacterial communities. Simultaneously, the fungal community comprised mainly of Eurotiales, Hypocreales and Mortierellales. It is worth mentioning that most representatives of Eurotiales are

saprotrophs and exert high diversity in metabolic abilities (Geiser et al., 2006), Hypocreales encompasses wide range of entomopathogenic fungi (Barnett & Johnson, 2013) and some species within Mortierellales (e.g. *Mortierella globalpina*, *Mortierella elongata*) are involved in phosphorus cycling in soil (Mącik, Gryta, Sas-Paszt, & Frąc, 2020; Ozimek & Hanaka, 2021). The domination of saprotrophic Eurotiales remains in agreement with FUNGuild results which showed that saprotrophs was main ecological fungal guild throughout the experiment.

Considering the increase in the number of bacterial order level OTUs in FA100 and FA60, it can be assumed that the application of biofertilizers contribute to a favorable shift in the composition of microbiome. The obtained results remained in agreement with Shen et al. (2015) who observed increase in bacterial diversity in soil amended with biofertilizer in 2-year experiment. However, the positive impact of biofertilizers on genetic diversity of soil microorganisms have been described by the researchers. In study conducted by Dong et al. (2019) it was reported that application of biofertilizer increased bacterial diversity, increased the relative abundance of *Bacillus*, *Burkholderia*, *Rhizobium*, and *Streptomyces* which are considered as a potentially beneficial microorganism and lowered the relative abundance of *Fusarium*. A result obtained from Wang, Liu, et al. (2021) showed that soil amended with *Bacillus subtilis* was characterized by higher number of OTUs and increased Chao1 and Shannon indices as compared to non-inoculated control. Nevertheless, in case of fungi, total number of OTUs remained at the same amount in FC and FA100, with slight decrease in FA60, which may be associated with the less content of nutrients provided with the 40% reduced dose of mineral fertilizer. It is worth emphasizing that one the fungal orders specific for FA100 was Teloschistales, which comprise more than a 1000 lichenized species (Gaya et al., 2015). The analysis of the seasonal shifts in soil microbiome within particular treatments showed that the A18 and A19 treatments were characterized by the higher number of specifically occurring OTUs, which is inextricably linked with the greater genetic diversity. Furthermore, the greater seasonal variation in FA100 and FA60 may also be indicated by the lower number of OTUs constituting the core microbiome in the aforementioned treatments as compared to control. Overall, the relative abundance of identified orders was affected to greater extent than their number. A similar trend at the phylum and class level was observed in fungal communities in study conducted by Wen et al. (2020). The increase in the genetic diversity in soil microbiome may be also supported by the higher Shannon index values, especially in FA100-FA60(A19) as compared to control. Analyzing H values in soil microbiome it was reported that bacteria were characterized by the greater diversity than fungi, which is also confirmed by the number of identified OTUs at order level.

Predictive functional profiling of soil bacterial communities, based on the 16S rDNA data, revealed that the application of phosphorus biofertilizer affected all metabolic pathways identified with KEGG database, also biotransformations and processes associated with P-compounds. Improved energy metabolism was evidenced by, i.e., enhanced oxidative phosphorylation, which may indicate the increased ATP synthesis, main energy carrier in intracellular metabolic processes (Chen & Nielsen, 2019). The higher number of OTUs associated with lipid metabolism was linked with the enhanced glycerophospholipid metabolism. Gram negative bacteria cell wall membranes, which determines survival under stress conditions and the proper cell functioning, are mainly composed of phospholipids (Chi et al., 2020). Hence, the higher metabolism of abovementioned P-containing compounds may result in better adaptation of soil bacteria to not only environmental factors but also new fertilization regime (Srour et al., 2020). Another P-related pathways identified among 'Metabolism' main KEGG class included phosphonate and phosphinate metabolism, pentose phosphate pathway and inositol phosphate metabolism. Certain microorganisms (Planctomycetes, Cyanobacteria, and Firmicutes) were found to utilize phosphonates, thus increasing the amount of available P in the soil (Tapia-Torres et al., 2016) and, interestingly, representatives of Firmicutes (*Clostridium* spp., *Staphylococcus* spp.) and Cyanobacteria (*Anabaenopsis* spp.) were identified with TRiFLe software in this study. According to Bore et al. (2017) intensification of pentose phosphate pathway is inextricably linked with increased NADPH synthesis, a compound involved in protein and lipid biosynthesis. On the other hand, phosphorylated inositol forms may constitute the P source in the soil (Herrou & Crosson, 2013). P-associated processes were also found in 'Environmental Information Processing' and encompassed "Phosphotransferase system" (PTS) and "Phosphatidylinositol signaling system". PTS is a translocation system which combines carbohydrates (e g. glucose, fructose, lactose, mannitol) uptake with their phosphorylation. In general, PTS system provide bacteria with a favourable pathway to obtain sugars from the environment (Erni, 2013). On the other hand, phosphatidylinositol may be a signal mediator in salt stress response in mycobacteria (Morita et al., 2010).

Apart from phosphorus compounds transformations, an important aspect, in terms of improving soil health, is the removal of contaminants such as xenobiotics (Mishra et al., 2021) The higher number of sequences assigned to xenobiotics biodegradation and metabolism may indicate that phosphorus biofertilizer stimulate bacterial bioremediation potential toward specific substances. What is more, the high genetic diversity of soil microbiome inhabiting studied soil may be confirmed by the occurrence of some metabolic pathways. According to Xun et al. (2019) genes involved in 'Xenobiotics biodegradation and metabolism' and 'Metabolism of terpenoids and polyketides' are carried by a small group of soil microorganisms (Actinobacteria), hence biofertilizer may stimulate the activity of microbial groups demonstrating highly specialized functions.

Fungal functional profile, constructed based on the ITS data, revealed the presence of ecological guilds useful for soil health improvement. Saprotrophic fungi (involved in decomposition of substances including wood, dung, and plant litter) are known to increase nutrient content, improve water retention and participate in carbon cycling (Clocchiatti et al., 2020), whereas ectomycorrhizal and arbuscular mycorrhizal fungi stimulate rooting and provide plants with essential mineral components, including phosphorus (Frąc et al., 2018).

β-diversity expressed by PcoA, UPGMA, and NMDS analyses showed that the sampling time was main factor influencing the composition of soil microbiome. The distinct clustering of treatments according to sampling time was associated with the relative abundance of particular microbial groups. Based on the UPGMA clustering, bacterial communities were more similar to each other in A19 and A18, so application of biofertilizer may resulted in only temporary changes that did not persist over time. This may also be related to the rapid adaptation of the soil bacteria to the new fertilization regime and stimulation of the occurrence of selected microbial groups during warm season. On the other hand, changes in the relative abundance did not reflect variations in functional profiles, as the A19 treatments were associated with the highest number of functional OTUs. Analyzing shifts in the fungal communities, treatments were separated according to the year, so it may indicate that the introduction of biofertilizer resulted in variations in the relative abundance that persisted from summer to autumn, which may be also supported by PCoA analysis. This may also point to slower adjustment of fungal communities to agrotechnical procedures such as fertilization. Moreover, the distinction of FA100 and FA60 from FC showed that biofertilizer shaped the soil environment and microbial communities tried to adopt to the new conditions. Grouping treatments according to soil sampling time was also observed in study conducted by Yang et al. (2020), where soil samples (under wheat cultivation), inoculated with mixture of beneficial bacteria (including *Azospirillam brasilense*, *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus mucilaginosus*) formed clusters (in PCoA and UPGMA) encompassing samples collected in tillering period, turning green period, grain filling period, and maturity period, respectively.

In NMDS ordination space the close promixity of smaller points indicates that treatments are more similar to each other in the composition of microorganisms; simultaneously greater distances between points reflects greater variations in the biodiversity of bacterial and fungal communities (Galitskaya et al., 2021). In our study, the distances between smaller points corresponding to individual samples were relatively short within A19 treatments as compared to other sampling times, which may infer that some balance in the composition of the soil microbiome has been achieved. According to Wang, Liu, et al. (2021) microbial communities, faced with different fertilization techniques, may remain more stable in long-term studies as compared to short experimental period. Taking into consideration growing number of OTUs along with the sampling time, we hypothesize that the application of biofertilizers in long-term studies may induce changes in microbial communities at the gene level. The distinction of mineral fertilization from organically amended soil in NMDS plots was also reported in study conducted by Francioli et al. (2016).

5 | CONCLUSIONS

Unsustainable land management in agroecosystems may result in soil degradation and reduction in the quality of soil microbiome. In view of the importance of soil microorganisms for the proper functioning of arable lands, we proposed the application of phosphorus mineral fertilizer enriched with strains of beneficial bacteria and highlighted the improvement of microbiological indicators of soil health including soil enzymatic activity, occurrence of microorganisms exerting plant growth promoting properties and increased genetic diversity among microbial communities. We have evaluated the effect of phosphorus biofertilizer expressed by the enhanced maize yield and increased number of OTUs associated with phosphorus processes. We have also emphasized seasonal changes in the status of soil microbiome. Throughout the experimental period we have observed variations in soil environment leading to certain stability in the autumn 2019 which may be a result of adaptation of indigenous microbiota to new fertilization regime. Our results indicate that phosphorus biofertilizer is an ecofriendly and effective alternative to traditional mineral fertilizers and has potential applications in sustainable agriculture and soil management practices based on microbial-derived preparations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available within manuscript and supplementarry files.

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SUPPORTING INFORMATION

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8.1. Materiały uzupełniające publikacji P.3

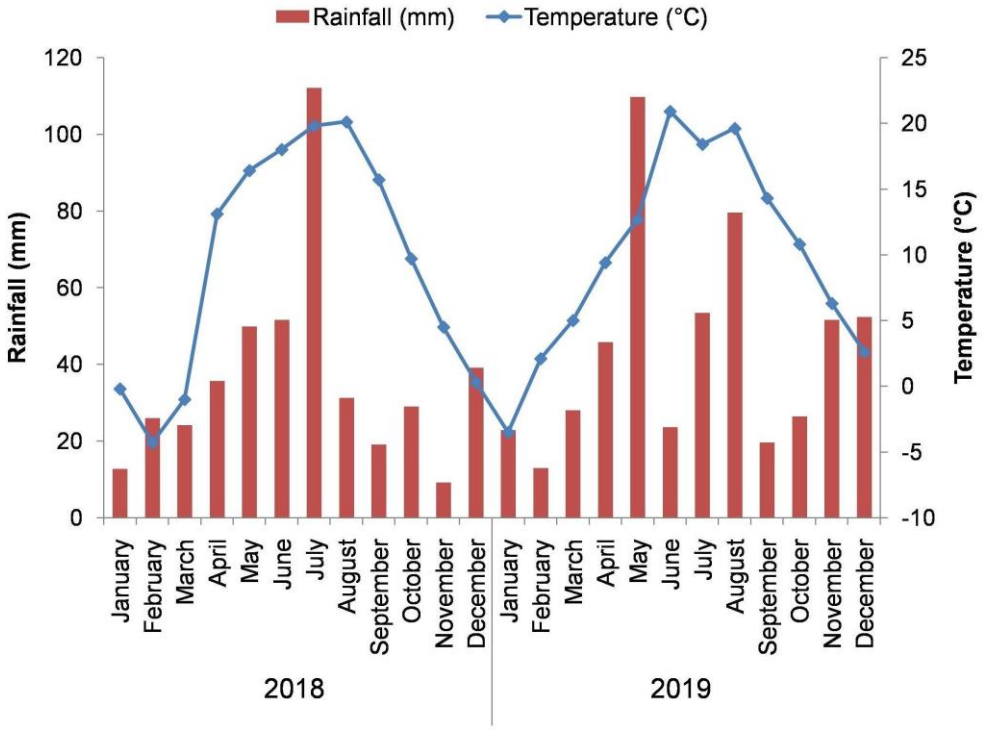

Figure S1. Weather conditions during 2-year field experiment in Biszcza.

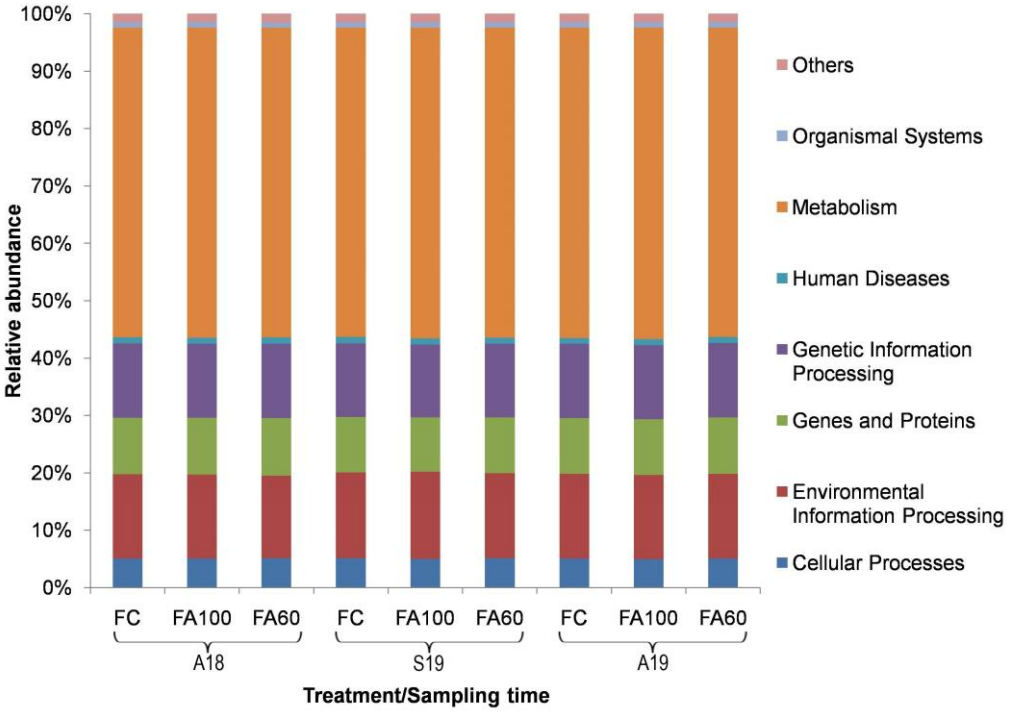

Figure S2. The relative abundance of main KEGG classes. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), A18-autumn 2018, S19-summer 2019, A19-autumn 2019.

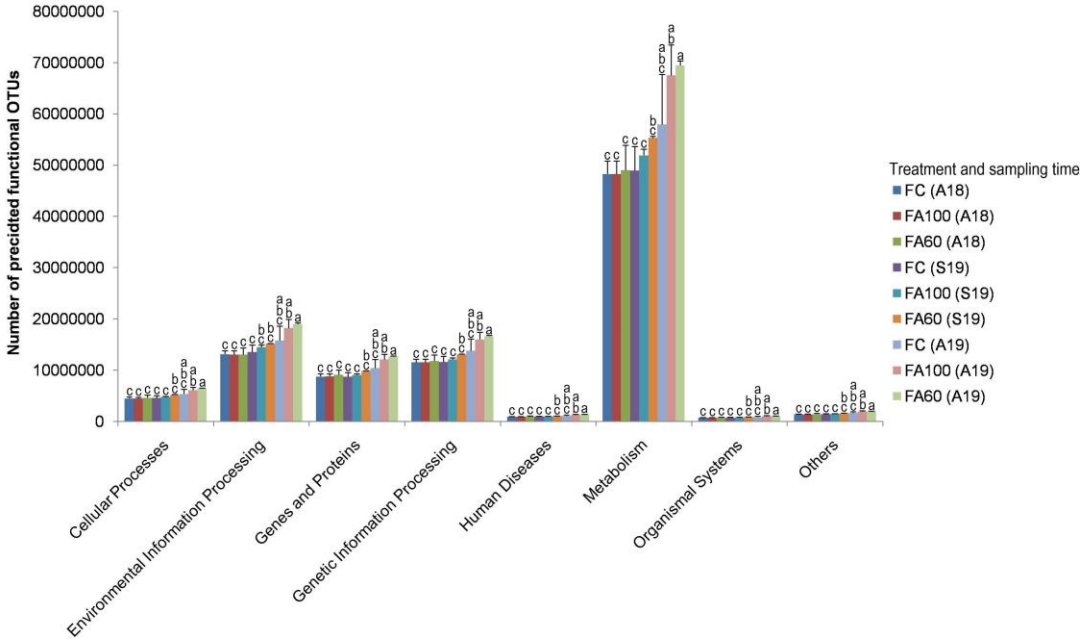

Figure S3. The number of functional operational taxonomic units (OTUs) associated with particular main KEGG classes. Explanation as in the Figure S2. The significant differences ($p < 0.05$) were calculated for all sampling times together within particular KEGG class.

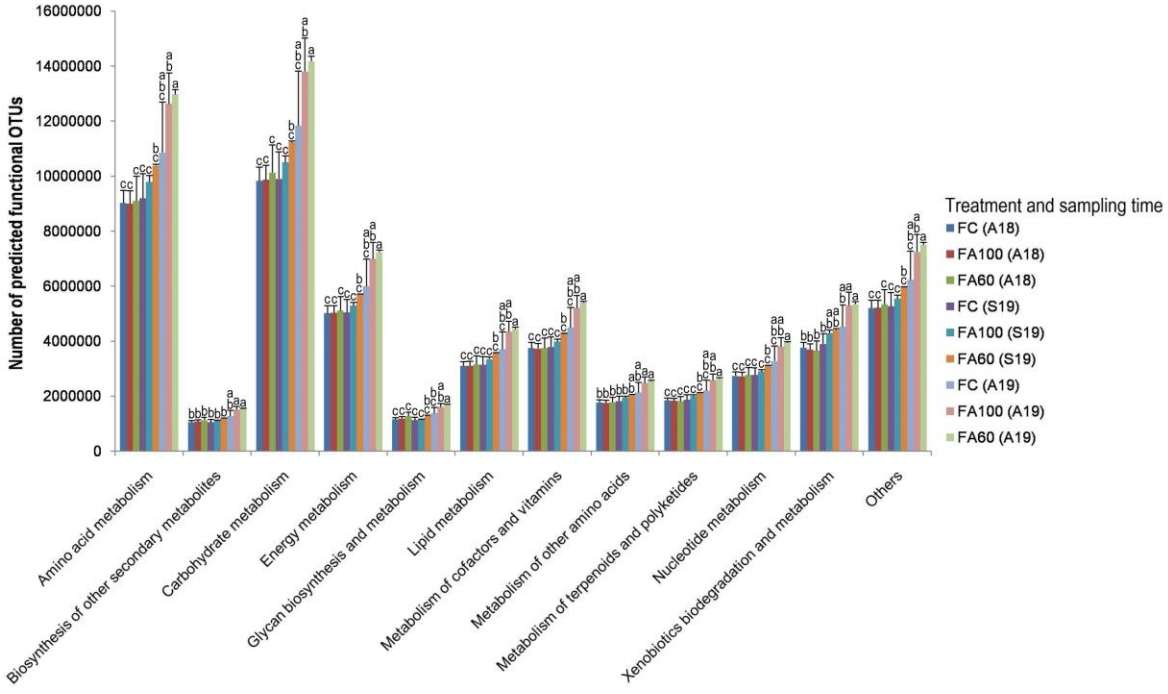

Figure S4. The number of functional operational taxonomic units associated with metabolism of different compounds. Explanation as in the Figure S2. The significant differences ($p < 0.05$) were calculated for all sampling times together within particular KEGG class.

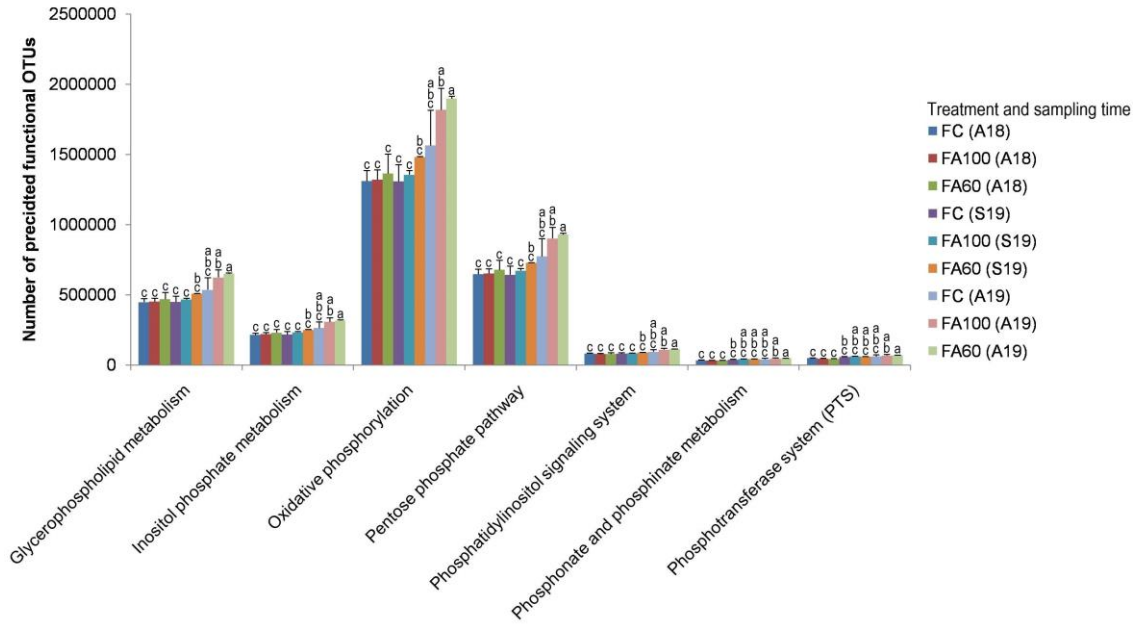

Figure S5. The number of functional operational taxonomic units associated with phosphorus processes. Explanation as in the Figure S2. The significant differences ($p < 0.05$) were calculated for all sampling times together within particular KEGG class.

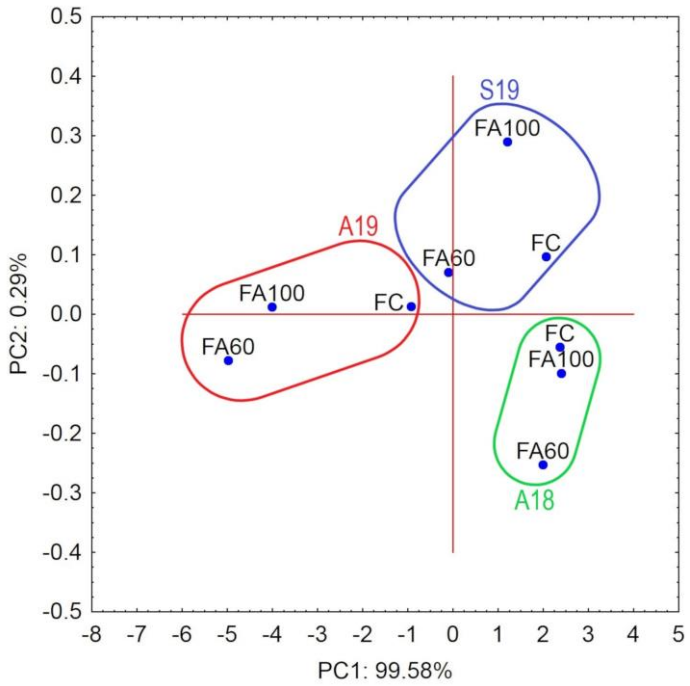

Figure S6. Principle component analysis (PCA) of functional operational taxonomic units associated with main KEGG classes. Explanation as in the Figure S2.

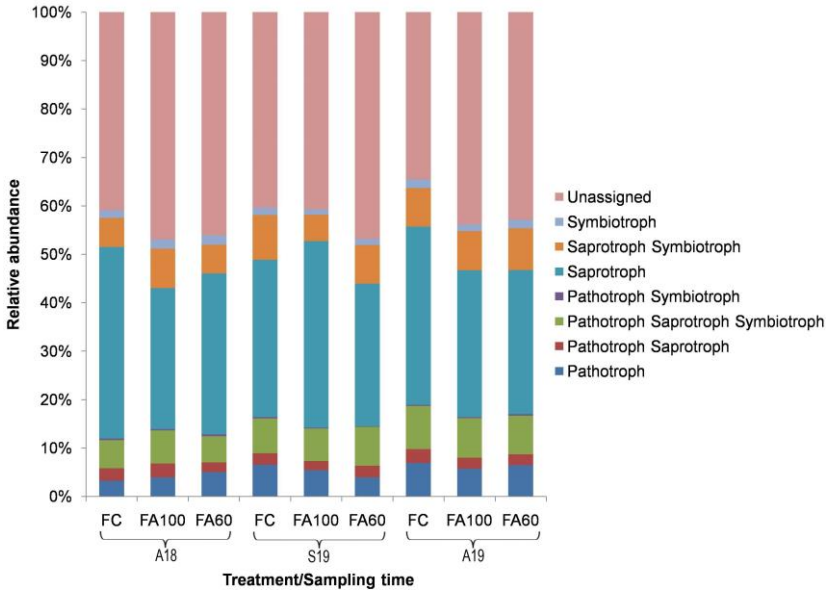

Figure S7. The relative abundance of fungal functional guilds. Explanation as in the Figure S2.

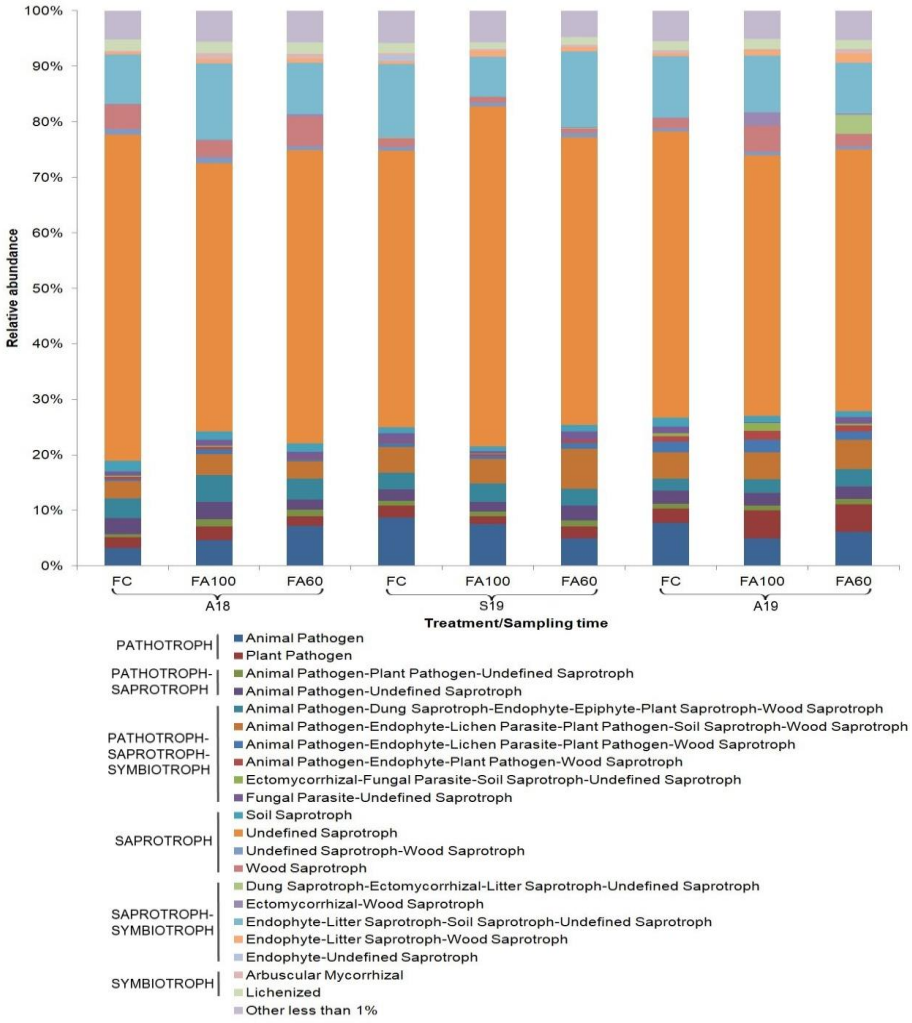

Figure S8. The relative abundance of fungal ecological guilds. OTUs with relative abundance equal or greater than 1% (in at least one treatment) were analyzed. Explanation as in the Figure S2.

Table S1. Primers used in M-tRFLP and NGS.

Group of microorganisms	Primers	Reaction mixture	PCR conditions
Bacteria - 16S rDNA	63f (5' AGGCCTAACACATGCAAGTC 3') and 1087r (5' HEX-CTGGTTGCGGGACTTACCCC 3') (Hauben et al., 1997; Marchesi et al., 1998)	4 ng of DNA template, 0,5 µl of 10 µM primers,15 µl of RedTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO,USA) and 12 µl of PCR grade water (in 30 µl)	95°C - 5 min; 30 cycles of 95°C - 30 s, 55°C - 30 s, 72°C - 1 min; 72°C - 10 min
Archaea - 16S rDNA	Ar3f (5' TTCCGGTTGATCCTGCCGGA 3') and Ar9r (5' ROX-CCCGCCAATTCCTTTAAGTTTC 3') (Jurgens & Saano, 1999; Jurgens et al., 1997)	4 ng of DNA template, 1 µl of 10 µM primers,15 µl of RedTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO,USA) and 11 µl of PCR grade water (in 30 µl)	
Fungi - ITS1F/ITS4R	ITS1F (5' 6-FAM-CTTGGTCATTTAGAGGAAGTAA 3') and ITS4R (5' TCCTCCGCTTATTGATATGC 3') (Gardes & Bruns, 1993; White et al., 1990)	4 ng of DNA template, 1 µl of 10 µM primers,15 µl of RedTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO,USA) and 11 µl of PCR grade water (in 30 µl)	
Bacteria - V3-V4 region of 16S rDNA	341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 785R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013)	10 ng of DNA template, 10 µM primers, Q5 Hot Start High-Fidelity 2x Master Mix (NEW ENGLAND BioLabs) (in 25 µl)	98°C - 30 s; 25-35 cycles of 98°C - 5-10 s, 50-72°C - 10-30s, 72°C - 20-30 s; 72°C - 2 min
Fungi - ITS1	ITS1Fl2 (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAACCWGCGGARGGATCA-3') and 5.8S (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGCTGCGTTCTTCATCG-3') (Schmidt et al., 2013; Vilgalys Mycology Lab, 1992)	10 ng of DNA template, 10 µM primers, Q5 Hot Start High-Fidelity 2x Master Mix (NEW ENGLAND BioLabs) (in 25 µl)	

Table S2. The seasonal distribution of common and unique bacterial and archaeal orders in soil fertilized with phosphorus mineral fertilizer in optimal dose (FC), in optimal dose enriched with microorganisms (FA100) and in 40% reduced dose enriched with microorganisms (FA60). Explanation: A18-autumn 2018, S19-summer 2019, A19-autumn 2019.

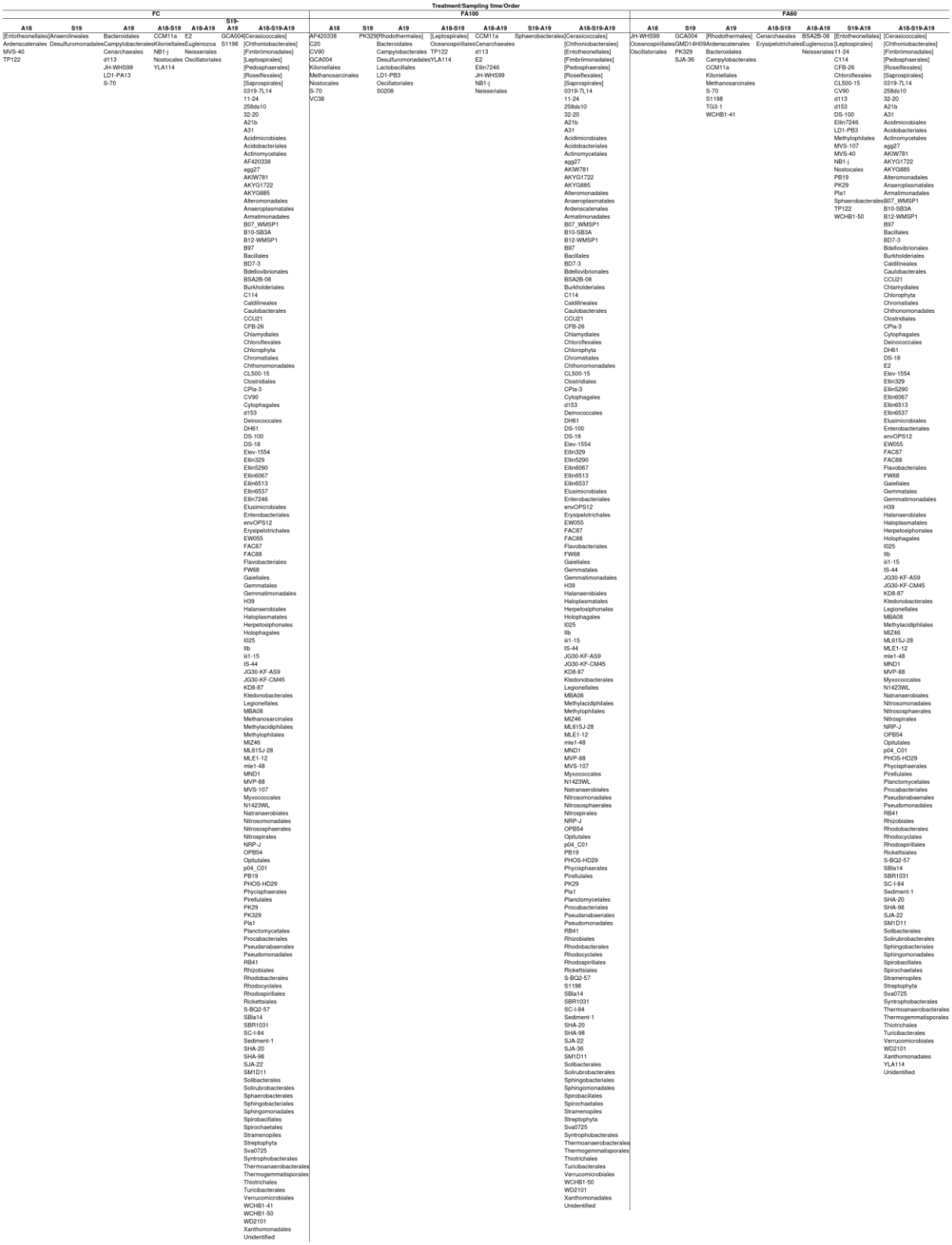

Table S3. The distribution of common and unique bacterial and archaeal orders in soil fertilized with phosphorus mineral fertilizer in optimal dose (FC), in optimal dose enriched with microorganisms (FA100) and in 40% reduced dose enriched with microorganisms (FA60).

Treatment/Order						
FA100	FA60	FC	FA100-FA60	FA100-FC	FA60-FC	FA100-FA60-FC
C20	GMD14H09	Anaerolineales	[Rhodothermales]	AF420338	Euglenozoa	[Cerasicoccales]
Lactobacillales	TG3-1	LD1-PA13	LD1-PB3	Desulfuromonadales	MVS-40	[Chthoniobacterales]
S0208			Oceanospirillales		WCHB1-41	[Entotheonellales]
VC38			SJA-36			[Fimbriimonadales]
						[Leptospirales]
						[Pedosphaerales]
						[Roseiflexales]
						[Saprospirales]
						0319-7L14
						11-24
						258ds10
						32-20
						A21b
						A31
						Acidimicrobiales
						Acidobacteriales
						Actinomycetales
						agg27
						AKIW781
						AKYG1722
						AKYG885
						Alteromonadales
						Anaeroplasmatales
						Ardenscatenales
						Armatimonadales
						B07_WMSP1
						B10-S83A
						B12-WMSP1
						B97
						Bacillales
						Bacteroidales
						BD7-3
						Bdellovibrionales
						BSA2B-08
						Burkholderiales
						C114
						Caldilineales
						Campylobacterales
						Caulobacterales
						CCM11a
						CCU21
						Cenarchaeales
						CFB-26
						Chlamydiales
						Chloroflexales
						Chlorophyta
						Chromatiales
						Chthonomonadales
						CL500-15
						Clostridiales
						CPla-3
						CV90
						Cytophagales
						d113
						d153
						Deinococcales
						DH61
						DS-100
						DS-18
						E2
						Elev-1554
						Ellin329
						Ellin5290
						Ellin6067
						Ellin6513
						Ellin6537
						Ellin7246
						Elusimicrobiales
						Enterobacteriales
						envOPS12
						Erysipelotrichales
						EW055
						FAC87
						FAC88
						Flavobacteriales
						FW68
						Gaiellales
						GCA004
						Gemmatales
						Gemmatimonadales
						H39
						Halanaerobiales
						Haloplasmatales
						Herpetosiphonales
						Holophagales
						I025
						IIb
						iii1-15
						IS-44
						JG30-KF-AS9
						JG30-KF-CM45
						JH-WHS99
						KD8-87
						Kiloniellales
						Ktedonobacterales
						Legionellales
						MBA08
						Methanosarcinales
						Methylacidiphilales
						Methylophilales
						MIZ46
						ML615J-28
						MLE1-12
						mle1-48
						MND1
						MVP-88
						MVS-107
						Myxococcales
						N1423WL
						Natranaerobiales
						NB1-j
						Neisseriales
						Nitrosomonadales
						Nitrosophaerales
						Nitrospirales
						Nostocales
						NRP-J
						OPB54
						Opitutales
						Oscillatoriales
						p04_C01
						PB19
						PHOS-HD29
						Phycisphaerales
						Pirellulales
						PK29
						PK329
						Pla1
						Planctomycetales
						Procabacteriales
						Pseudanabaenales
						Pseudomonadales
						RB41
						Rhizobiales
						Rhodobacterales
						Rhodocyclales
						Rhodospirillales
						Rickettsiales
						S-70
						S-BQ2-57
						S1198
						SBla14
						SBR1031
						SC-I-84
						Sediment-1
						SHA-20
						SHA-98
						SJA-22
						SM1D11
						Solibacterales
						Solirubrobacterales
						Sphaerobacterales
						Sphingobacteriales
						Sphingomonadales
						Spirobacillales
						Spirochaetales
						Stramenopiles
						Streptophyta
						Sva0725
						Syntrophobacterales
						Thermoanaerobacterales
						Thermogemmatisporales
						Thiotrichales
						TP122
						Turicibacterales
						Verrucomicrobiales
						WCHB1-50
						WD2101
						Xanthomonadales
						YLA114
						Unidentified

Table S4. The seasonal distribution of common and unique fungal orders in soil fertilized with phosphorus mineral fertilizer in optimal dose (FC), in optimal dose enriched with microorganisms (FA100) and in 40% reduced dose enriched with microorganisms (FA60). Explanation: A18-autumn 2018, S19-summer 2019, A19-autumn 2019.

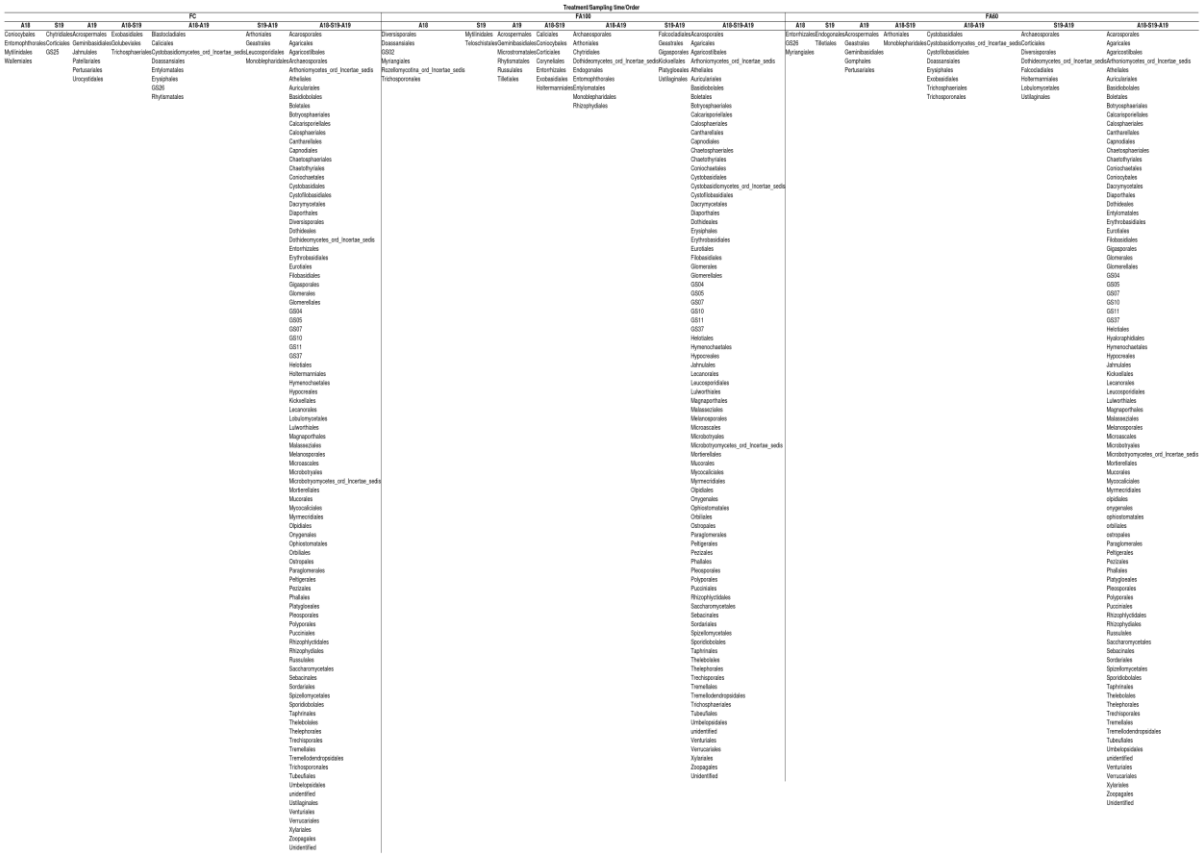

Table S5. The distribution of common and unique fungal orders in soil fertilized with phosphorus mineral fertilizer in optimal dose (FC), in optimal dose enriched with microorganisms (FA100) and in 40% reduced dose enriched with microorganisms (FA60).

Treatment/Order						
FA100	FA60	FC	FA100-FA60	FA100-FC	FA60-FC	FA100-FA60-FC
Coryneliales	Gomphales	Blastocladiales	Endogonales	Caliciales	GS26	Acarosporales
GS02	Hyaloraphidiales	Golubeviales	Falcocladiales	Chytridiales	Lobulomycetales	Acrospermales
Microstromatales		GS25	Myriangiales	Entomophthorales	Pertusariales	Agaricales
Rozellomycotina_ord_Incertae_sedis		Patellariales	Tilletiales	Mytilinidales		Agaricostilbales
Teloschistales		Urocystidales		Rhytismatales		Archaeosporales
		Wallemiales				Arthoniales
						Arthoniomycetes_ord_Incertae_sedis
						Atheliales
						Auriculariales
						Basidiobolales
						Boletales
						Botryosphaeriales
						Calcarisporiellales
						Calosphaeriales
						Cantharellales
						Capnodiales
						Chaetosphaeriales
						Chaetothyriales
						Coniochaetales
						Coniocybales
						Corticiales
						Cystobasidiales
						Cystobasidiomycetes_ord_Incertae_sedis
						Cystofilobasidiales
						Dacrymycetales
						Diaporthales
						Diversisporales
						Doassansiales
						Dothideales
						Dothideomycetes_ord_Incertae_sedis
						Entorrhizales
						Entylomatales
						Erysiphales
						Erythrobasidiales
						Eurotiales
						Exobasidiales
						Filobasidiales
						Geastrales
						Geminibasidiales
						Gigasporales
						Glomerales
						Glomerellales
						GS04
						GS05
						GS07
						GS10
						GS11
						GS37
						Helotiales
						Holtermanniales
						Hymenochaetales
						Hypocreales
						Jahnulales
						Kickxellales
						Lecanorales
						Leucosporidiales
						Lulworthiales
						Magnaporthales
						Malasseziales
						Melanosporales
						Microascales
						Microbotryales
						Microbotryomycetes_ord_Incertae_sedis
						Monoblepharidales
						Mortierellales
						Mucorales
						Mycocaliciales
						Myrmecridiales
						Olpidiales
						Onygenales
						Ophiostomatales
						Orbiliales
						Ostropales
						Paraglomerales
						Peltigerales
						Pezizales
						Phallales
						Platygloeales
						Pleosporales
						Polyporales
						Pucciniales
						Rhizophlyctidales
						Rhizophydiales
						Russulales
						Saccharomycetales
						Sebacinales
						Sordariales
						Spizellomycetales
						Sporidiobolales
						Taphrinales
						Thelebolales
						Thelephorales
						Trechisporales
						Tremellales
						Tremellodendropsidales
						Trichosphaeriales
						Trichosporonales
						Tubeufiales
						Umbelopsidales
						unidentified
						Ustilaginales
						Venturiales
						Verrucariales
						Xylariales
						Zoopagales
						Unidentified

9. Tekst manuskryptu publikacji P.4

New insight into the soil bacterial and fungal microbiome after phosphorus biofertilizer application as important driver of regenerative agriculture including biodiversity loss reversing and soil health restoration

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Abstract

Nowadays, the exploitation of biofertilizers unfolds vast potential for the advancement of sustainable and organic agriculture and improvement of the arable soils quality. Hereunder, we revealed the capacity of an innovative phosphorus biofertilizer to enhance the activity and diversity of soil microbiome and mycobiome inhabiting chemically degraded soil (type Abruptic Luvisol). Two-year field experiment comprised of the control treatment (FC) devoid of microbial enrichment, optimal dose of fertilizer containing beneficial microorganisms (FA100) and 40% reduced of fertilizer containing microorganisms (FA60). Phosphorus biofertilizer increased soil enzymatic activity immediately after application and between corresponding treatments in subsequent sampling times, alleviated the effects of metabolic stress, improved phytoavailable phosphorus content and increased maize yield. Identification based on the terminal restriction fragments size revealed the presence of microorganisms important for soil health such as phosphorus solubilizers, nitrogen fixers, biological control agents, entomopathogens, mycorrhizal fungi, bioremediators and plant growth promoters. Next Generation Sequencing (NGS) showed that the application of phosphorus biofertilizer changed the relative abundance of different microbial phyla, classes and orders, increased the diversity of soil microorganisms and indicated that the composition of soil microbiome and mycobiome was also dependent on the sampling time. Prediction of bacterial community function using PICRUSt demonstrated that biofertilizer raised the number of operational taxonomic units associated with metabolism and cell processes, including phosphorus compound pathways. FUNGuild showed that saprotrophic and symbiotrophic fungi were more abundant in microbiologically enriched treatments. Our results proved that the phosphorus biofertilizer offers a sustainable and promising solution to lessen traditional mineral fertilizers inputs while ensuring soil microorganisms welfare and enhancing land productivity, and, what is more, can be effectively exploited in the regenerative agriculture and as a factor enhancing resilience to climate change.

Keywords: biofertilizer, soil microorganisms, climate change, sustainable agriculture, soil restoration, regenerative agriculture

41 **1. Introduction**

42 It is beyond doubt that modern agriculture has undergone strong chemization over the last few
43 years to intensify crop production and meet the food requirements of an ever-growing world
44 population which is predicted to hit more than 9 billion people by 2050 (Fasusi et al., 2021;
45 Pirttilä et al., 2021). Mineral fertilizers assist in restoring the chemical balance in nutrient-
46 deficient soils and increase crop biomass, however their unreasonable and excessive
47 application may lead to environmental concerns associated with the soil quality and
48 productivity and also with climate change. Moreover, plants use only a limited amount (30-
49 40%) of nutrients provided with the fertilizers and the rest remains unavailable and
50 contributes to the environmental pollution (Kumar et al., 2022). The deterioration of farmland
51 soils quality due to relentless anthropopression turned to a global concern posing threats to the
52 further progress of agricultural economics (Kopittke et al., 2019).

53 Adverse environmental impacts (e.g. accumulation of heavy metals, water eutrophication,
54 nutrient leaching) arising from the over-use of mineral fertilizers and synthetic pesticides and
55 a policy regulating the quantities of applied agrochemicals led to the development of non-
56 hazardous alternative approaches (Mącik et al., 2020a). The implementation of beneficial
57 microorganisms strains, including bacteria and fungi, in the biofertilizers form is currently
58 one of the "hot topics" in the field of sustainable and organic agriculture and is increasingly
59 gaining attention (Canfora et al., 2021). Moreover, the biological fertilizers important for
60 restoring soil health and reversing biodiversity loss are one of the major components of
61 regenerative agriculture (EASAC, 2022), relevant to achieve the main goals and to meet
62 targets of the Biodiversity and Farm to Fork Strategies. The complex communities of soil
63 bacteria, archaea and fungi shape the soil environment and regulate ecosystem sustainability
64 by directing processes encompassing decomposition of organic matter and chemical elements
65 cycling (Feng et al., 2021; Frąc et al., 2018; Jurys and Feizienė, 2021). Soil microorganisms
66 are also well known as plant growth promoters (PGP) due to traits including synthesis of
67 phytohormones and antibiotics, alleviation of biotic/abiotic stresses and protection against
68 phytopathogens (Fan and Smith, 2021). The exploitation of microbial strains is justified also
69 in the regulation of phosphorus bioavailability, a macronutrient determining the proper cell
70 functioning and indispensable throughout the entire plant life (Meng et al., 2021).
71 Regrettably, most of the soil phosphorus remains unassimilable for plants; this challenge can
72 be overcome with phosphorus solubilizing microorganisms (PSM) (Alori et al., 2017;

Hallama et al., 2021). Representatives of *Bacillus* spp., *Pseudomonas* spp., *Micrococcus* spp., *Xanthomonas* spp., *Serratia* spp., *Rhizobium* spp., *Burkholderia* spp., *Penicillium* spp., *Aspergillus* spp., *Mortierella* spp., *Saccharomyces* spp., *Paecilomyces* spp. and *Cephalosporium* spp. were found to dissolve phosphorus compounds into highly assimilable orthophosphates (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$) due to traits including organic acids synthesis, excretion of phytases and phosphatases and reduction of soil pH (Kumar et al., 2022; Mitra et al., 2020; Mitter et al., 2021; Tian et al., 2021). The utilization of PSM in place of chemical fertilizers is considered as an attractive and compatible with the principles of sustainable agriculture approach, also in view of the fact that some portion of phosphorus provided with mineral fertilizers becomes unavailable through the binding with metal ions occurring in the soil environment (Nacoon et al., 2020).

Soil microorganisms are considered as a reliable bioindicator of ecosystem functioning due to the high vulnerability to external factors and environmental perturbations (Schloter et al., 2018; Tahat et al., 2020). High biodiversity within soil microbial communities is undeniably related to the preservation of stability and continuity of soil exploitation (Bastida et al., 2021). Current soil management practices more frequently integrate microbiome and mycobiome status with overall soil condition and involve the adoption of microbial-based formulations to enhance biodiversity and, consequently, soil health (Ray et al., 2020). Beneficial features carried by microorganisms create them as a powerful and effective tool to counteract the harmful outcomes of human agricultural activity (Mohanty et al., 2021). Biofertilizers improve heavy metals tolerance level of plants (Ahemad, 2019) and increase soil organic matter (SOM) content (Dębska et al., 2016). The adoption of biofertilizers is also gaining momentum in the face of incessant climate change and the threats associated to agroecosystems including droughts, salinity, high temperature stress and spread of phytopatogens. It goes without saying that such occurrences impair the soil's productive potential and diminish the quality and quantity of crop yields (Fadiji et al., 2022; Fiodor et al., 2021; Velásquez et al., 2018). Agriculture remains a sector highly sensitive to climate change, but at the same time the intensification of agricultural practices significantly contributes to global warming, taking into consideration fact that the world food system generates ~21-37% of annual greenhouse gas (GHG) emissions (Lynch et al., 2021; Nelson et al., 2014). Abiotic stresses triggered by climate change can be mitigated with the help of beneficial microbial strains (Sarker et al., 2021). Microorganisms work by several mechanisms to safegaurd plants

against stressful situations including exopolysaccharide synthesis (enhancing water holding capacity and protection from dessication), emission of volatile organic compounds (protection from thermal stress and phytopathogens), ACC deaminase activity (alleviation of ethylene synthesis), excretion of phytohormones, synthesis of osmoprotectants, induced systemic resistance and production of enzymes (superoxidase dismutase, catalase, ascorbate peroxidase and guaiacol peroxidase) removing reactive oxygen species (Fadiji et al., 2022; Fiodor et al., 2021). The improvement of soil microbiological parameters provided by the application of biofertilizers also seems an appealing solution, given that a greater diversity of soil microbiome and mycobiome may strengthen the general soil resilience to environmental stresses (Bertola et al., 2021). Furthermore, it was documented that the application of microbial inoulants decreased CH_4 and N_2O emissions in field studies (Akiyama et al., 2016; Kantachote et al., 2016). The GHG emissions may be also reduced by exploiting the phenomenon of biological nitrogen fixation by representatives of *Rhizobium* spp., *Azotobacter* spp. and *Azospirillum* spp., which would simultaneously limit the use of nitrogen mineral fertilizers (Aasfar et al., 2021; Wen et al., 2021). Biofertilization can also stimulate soil carbon sequestration, potentially not only reducing the CO_2 amount in the atmosphere, but also increasing the soil organic carbon content in the arable lands (Gayathri et al., 2021). The members of *Pseudomonas* spp., *Bacillus* spp., *Paraburkholderia* spp., *Paenibacillus* spp., *Streptomyces* spp. and *Penicillium* spp. were found to demonstrate properties enhancing plant tolerance to environmental stresses arising from the climate change (Fadiji et al., 2022).

Soil organic fertilization is a longstanding and clearly advantageous agronomic procedure (Geng et al., 2019). Numerous reports describe the influence of organic amendments such as compost, chicken manure and spent mushroom substrate on the status of soil microbiome (Frąc et al., 2021; Gryta et al., 2020; Tan et al., 2022). In addition, studies detailing shifts in soil microbial communities triggered by the introduction of beneficial bacterial and fungal strains also appear with increasing frequency (Schütz et al., 2018). On the other hand, research on refining microbial-based practices and enhancing their efficiency are still underway to fill the knowledge gaps and explore the entire spectrum of possibilities provided by microorganisms (Sudheer et al., 2020). In this work we propose the application of phosphorus mineral fertilizer enriched with strains of beneficial bacteria which represents a cutting edge approach in the field of biofertilizers and broadens the fragmentary understanding on this topic. Phosphorus fertilizer was enriched with three bacterial strains

(*Paenibacillus polymyxa* CHT114AB, *Bacillus amyloliquefaciens* AF75BB and *Bacillus* sp. CZP4/4) chosen on the basis of their beneficial properties (Mącik et al., 2020b; Mącik et al., 2022), encompassing synthesis of plant hormones (auxin, cytokinin), increasing the nutrient acquisition (P, N, Fe), excretion of antifungal/antimicrobial compounds, formation of biofilm and alleviation of biotic and abiotic stresses (drought, soil salinity, phytopathogens) (Langendries and Goormachtig, 2021; Radhakrishnan et al., 2017; Sheteiwy et al., 2021).

In this study, we investigated whether application of phosphorus biofertilizer containing beneficial bacterial strains could improve microbiological parameters of degraded soil, including soil enzymatic activity and genetic and functional diversity of soil microbiome and mycobiome. In accordance with the regulations concerning the limitation of mineral fertilizers inputs in agroecosystems, we also suggested a 40% reduced dose of fertilizer containing microbial enrichment in the expectation of its effectiveness in soil health restoration and reversal of microbial biodiversity loss. We hypothesize that phosphorus biofertilizer has legs to ameliorate degraded soil quality and obtained results will provide a scientific guidelines for the development of ecofriendly agricultural practices promoting the soil microorganisms welfare.

2. Materials and methods

2.1. Soil characteristics and field study site

The field experiment was carried out in Poland in 2018-2019 on Abruptic Luvisol (AL) degraded soil located in Basznia, South-East Poland (50°39′ N, 22°65′ E), under maize cultivation (variety of P9241, FAO: K280, Z270, PIONEER). Soil chemical degradation (characterized by acidification (pH_{KCl} of 4.9) and decrease in nutrients content) occurred as a result of the past activity of former sulphur mine situated nearby the study site. The AL presented a low content of Mg (3.6 mg 100 g^{-1} of soil) and a very low content of P (4.8 mg 100 g^{-1} of soil) and K (5.3 mg 100 g^{-1} of soil). The study site was located at an altitude of 230 m above sea level.

The AL soil was fertilized with the following mineral fertilizers: phosphorus mineral fertilizer SUPER FOS DAR 40 (Grupa Azoty, Puławy, Poland), nitrogen fertilizer PULREA PUŁAWSKI MOCZNIK 46 N (Grupa Azoty, Puławy Poland) and potassium salt (BIALCHEM, Poland). The microbial consortium for the phosphorus fertilizer contained the beneficial bacterial strains including *Paenibacillus polymyxa* (CHT114AB), *Bacillus*

168 *amyloliquefaciens* (AF75BB) and *Bacillus* sp. (CZP4/4), mixed in the proportion of 1:1:1 and
169 coated on fertilizer granules (Borowik et al., 2019). The bacterial strains were selected from
170 SYMBIOBANK Collection in the Research Institute of Horticulture (Skierniewice, Poland).
171 The biofertilizers were provided by the Łukasiewicz Research Network - New Chemical
172 Syntheses Institute (Puławy, Poland). The doses of applied (bio)fertilizers were calculated
173 according to maize nutritional requirements and soil mineral content and were described in
174 details by Mącik et al. (2020b).

175 The field experiment comprised of the following treatments: FC (optimal dose of phosphorus
176 mineral fertilizer without microbial enrichment), FA100 (optimal dose of fertilizer enriched
177 with microorganisms) and FA60 (dose reduced by 40% of fertilizer enriched with
178 microorganisms). Reducing the fertilizer dose in FA60 treatment remains in agreement with
179 the principles of sustainable and organic agriculture. Each treatment included three
180 experimental plots with the 15 m x 10 m dimensions with a 6-m interval between particular
181 fertilization method. Soil samples (0-25cm) were collected on the following terms: before
182 bio(fertilizers) application in April 2018 and April 2019 (A18/A19), one week after
183 (bio)fertilization in June 2018 and June 2019 (J18/J19) and after the maize harvest in October
184 2018 and October 2019 (O18/O19). The results obtained from J18 were thoroughly analyzed
185 and discussed in a previous study concerning the immediate effect of phosphorus biofertilizer
186 on the status of the soil microbiome and mycobiome (Mącik et al., 2020b), however for this
187 study the analysis of Next Generation Sequencing results was conducted comprehensively for
188 the whole experimental period, excluding sampling times before (bio)fertilizers application.

189 **2.2. Soil enzyme assays**

190 β-glucosidase activity was determined by the method Eivazi and Tabatabai (1988), modified
191 by Alef and Nannipieri (1995), with measurement of the concentration of p-nitrophenol (PNP)
192 released by soil after 1 h incubation at 37°C with p-nitrophenol glucoside (PNG). The PNP
193 concentration was measured at 400 nm. Acid phosphomonoesterase activity was determined
194 according to Tabatabai and Bremner (1969) with p-nitrophenyl phosphate as a substrate. This
195 activity was determined by the PNP released after 1 h incubation at 37°C. The concentration
196 of PNP was measured at 400 nm. For protease analysis, Tris-HCl (pH 8.1) sodium caseinate
197 was used and after 1 h incubation at 50°C, the concentration of tyrosine was measured at 578
198 nm (Ladd and Butler, 1972 with modification of Alef and Nannipieri, 1995). Urease activity

was assessed using urea as a substrate and determining the release of ammonia after incubation for 18 h at 37°C. The concentration of ammonia was measured colorimetrically at a wavelength of 410 nm (Zantua and Bremner, 1977). The enzymes activities were calculated based on the dry (105°C) weight of the soil.

2.3. Phytoavailable phosphorus content and maize yield

The content of available phosphorus (P_2O_5) was determined by the Egner-Riehm method (according to Polish Standard PN-R-04023: 1996) using a Sherwood flame photometer and Genesys 6 spectrophotometer in District Agricultural Chemical Station (Lublin, Poland). At full maturity, maize was harvested and its yield was measured as a weight of grains and straw from each plot in a particular fertilization method.

2.4. Catabolic fingerprinting of microbial communities

The metabolic abilities of soil microbial communities were investigated using Biolog™ ECO plates for the bacterial community and FF plates in case of fungal community (Biolog Inc., Hayward, CA, USA). One gram portion of fresh soil was shaken in 99 ml of saline peptone water for 20 min at 20°C and followed by incubation for 30 min at 4°C (Gryta et al., 2014). Then, the soil suspension was transferred into ECO and FF plates wells (120 µl and 100 µl, respectively). After inoculation, plates were incubated at 23°C for 216 h. Plates were read every 24 h of incubation period at 590/750 nm (ECO) and 490/750 nm (FF) with a Biolog MicroStation™.

2.5. Total genomic DNA extraction from soil samples

Total genomic DNA extraction of 0.5 g soil was performed using FastDNA SPIN Kit for Feces (MP Biomedicals, Solon, OH, USA) according to the protocol provided by manufacturer. DNA was quantified spectrophotometrically at 260 nm (NanoDrop 2000/2000c Thermo Scientific, West Palm Beach, FL, USA). The extracted DNA was then stored at -20°C for the further analyses including multiplex terminal restriction fragment length polymorphism and Next Generation Sequencing.

227 **2.6. Analysis of the soil microbial communities using a multiplex terminal restriction**
228 **fragment length polymorphism (M-tRFLP) approach**

229 M-tRFLP DNA profiling consisted of the following steps: multiplex PCR, purification of
230 PRC products, restriction enzyme digestion and detection of terminal restriction fragments (T-
231 RFs). Multiplex PCR was conducted using literature primer sets for bacteria (63f/1087r HEX)
232 (Hauben et al., 1997; Marchesi et al., 1998), archaea (Ar3f/Ar9r ROX) (Jurgens and Saano,
233 1999; Jurgens et al., 1997) and fungi (ITS1F 6-FAM/ITS4R) (Gardes and Bruns, 1993; White
234 et al., 1990) on Veriti Fast Thermalcycler (Applied Biosystems, Foster City, CA, USA) under
235 the following program: 95°C for 5 min, 30× (95°C for 30 s, 55°C for 30 s, 72°C for 1 min),
236 72°C for 10 min. The obtained PCR products were purified using Exo-BAP-Mix (EURx,
237 Gdańsk, Poland) and then Performa® DTR (Dye Terminator Removal) Gel Filtration
238 Cartridges (EdgeBio, San Jose, CA, USA) according to the protocol supplied by the
239 manufacturer. The purified DNA was quantified by a spectrophotometer at 260 nm
240 (NanoDrop 2000/2000c Thermo Scientific, West Palm Beach, FL, USA). The purified PCR
241 products were digested using restriction enzyme HaeIII (EURx, Gdańsk, Poland). After
242 digestion, 1 µl of the digest were mixed with 9 µl Hi-Di™ formamide and 0.5 µl of DNA size
243 standard GS-600LIZ (Applied Biosystems, Foster City, CA, USA). Prior to fragment analysis,
244 samples were denatured and chilled on ice. Sizes of the fluorescently labelled terminal
245 restriction fragments (T-RFs) were determined on an automated ABI 3130 DNA genetic
246 analyzer through capillary electrophoresis. A detailed specification of M-tRFLP was provided
247 by Gryta and Frąc (2020) and Mącik et al. (2020b).

248 **2.7. Analysis of soil microbial communities using Next Generation Sequencing (NGS)**

249 The Next Generation Sequencing was conducted at Genomed S.A. (Warsaw, Poland) on the
250 MiSeq instrument (Illumina Inc., San Diego, CA, USA). Amplification of bacterial V3-V4
251 16S rDNA and fungal ITS1 rDNA hypervariable regions was performed with primer sets
252 341F/785R (Klindworth et al., 2013) and ITS1FI2/5.8S (Schmidt et al., 2013; Vilgalys
253 Mycology Lab, 1992), respectively, and Q5 Hot Start High-Fidelity 2× Master Mix, according
254 to the manufacturer (NEB Inc., Ipswich, MA, United States). Sequencing was performed in
255 paired-end mode 2 x 250 bp using the v2 Illumina kit.

256

257

2.8. Statistical and bioinformatics analyses

Metabolic efficiency of soil bacterial and fungal communities was calculated as a ratio between mean values of OD 590 nm/OD 750 nm and OD 490 nm/OD 750 nm (Pinzari et al., 2016) from 0-216 h incubation period, respectively, and visualized as heat maps.

Matrix2png web interface (Pavlidis and Noble, 2003) was used to generate heat maps representing the relative abundance of T-RFs. Identification of microorganisms based on the size of T-RFs was conducted using the TRiFLe tool (Junier et al., 2008). Jaccard and Sorensen coefficients were calculated based on the number of T-RFs between particular treatments. Jaccard coefficient (J) was calculated according to the formula: $J=N_{AB}/(N_A+N_B-N_{AB})$, where N_{AB}-number of common T-RFs in both profile A and B, N_A-number of T-RFs in profile A, N_B-number of T-RFs in profile B (Gryta and Frąc, 2020). Sorensen (S) coefficient was calculated with the following formula: S=2C/(A+B), where C-number of common T-RFs in both profile 1 and 2, A-number of T-RFs in profile 1, B-number of T-RFs in profile 2 (Johnston-Monje et al., 2014). Venn diagrams were constructed via the online tool http://bioinformatics.psb.ugent.be/webtools/Venn/. Dendrograms of cluster analysis were prepared based on the relative abundance of T-RFs with a scaled similarity (%) on the axis using Ward's algorithm and Euclidean distance with marked Sneath's criteria (33% and 66%).

The automatic preliminary step of the NGS data analysis was conducted on the MiSeq platform using the MiSeQ Reporter (MSR) v. 2.6 software (Illumina Inc., San Diego, CA) and included demultiplexing and generation of the fastq files. The taxonomical classification of the OTUs was performed with Quantitative Insights into Microbial Ecology (QIIME) (Caporaso et al., 2010) software using Basic Local Alignment Search (BLAST) tool (Altschul et al., 1990) and UNITE v. 8 database (ITS1 region) (Kõljalg et al., 2013; Nilsson et al., 2019) and uCLUST algorithm (Edgar, 2010) and the GreenGenes v. 13.8 database (16S V3-V4 region) (DeSantis et al., 2006).

Variations in beta-diversity within soil microbial communities were visualized with unweighted pair group mean (UPGMA) clustering algorithm and principle coordinate analysis (PCoA) on Bray-Curtis distance matrices of bacterial and fungal operational taxonomic units (OTUs). Bacterial functional profile was predicted from 16S rDNA data using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) algorithm (Douglas et al., 2020) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database

289 (Kanehisa and Goto, 2000). FUNGuild tool was used to parse fungal OTUs with trophic mode
290 and ecological guild (Nguyen et al., 2016).

291 Statistical tests were used to determine differences in soil enzymes activities, number of
292 functional OTUs, maize yield and available phosphorus content between treatments. First step
293 referred to verification of analysis of variances (ANOVA) assumptions including dataset
294 normality and homoscedasticity of the variance with Shapiro–Wilk and Levene tests,
295 respectively. Afterwards, data were analyzed with ANOVA and post hoc Tukey test when
296 ANOVA assumptions were met (maize yield) and with F-Welch and RIR Tukey test for
297 unequal numbers when dataset normality was maintained, but the homogenity of the variance
298 was violated (enzymatic activities, PICRUSt/KEGG pathways, phosphorus content).
299 Statistical analysis was performed with Statistica Software (version 13, StatSoft Inc., Tulsa,
300 OK, USA, 2011) and R software (version 1.0.5.999, R Core Team, 2018, Vienna, Austria).

301 **3. Results**

302 **3.1. Soil enzymatic activity**

303 Application of biofertilizers in J19 increased the activity of protease, urease and acid
304 phosphomonoesterase in both FA100 and FA60, whereas an enhancement of β-glucosidase
305 activity was reported only in FA100, as compared to control treatment. The similar trend,
306 namely increased soil enzymatic activity in FA100 and FA60 was observed in A19 for every
307 studied enzyme, except of urease, for which improvement of activity was noted only in FA60
308 (Table 1).

309 Protease activity throughout the two-year experiment remained higher in FA100 and FA60 on
310 every sampling time apart from FA60(O19). The highest protease activity was reported in
311 FA100(J19), directly after biofertilizers application and, what is more, and upward trend
312 between the equivalent FA100 and FA60 treatments was observed from O18 to J19 sampling
313 times. Relatively elevated protease activities were also observed in A18 treatments.

314 As for urease, samples collected in A19 demonstrated the highest activity. Considering
315 variations within individual sampling times, urease activity remained at similar levels in FC
316 and FA100 treatments at A18, A19 and O19.

317 Analyzing variations in β-glucosidase levels across two year experimental period it was found
318 that samples collected in O19 exhibit the highest activity, with no statistically significant

changes between particular treatments. Interestingly, FA60 experienced a stepwise growth (by 112.03%) of aforementioned enzyme activity, from the A18 to O19. A comparable tendency was also seen for the FA100, in which *β*-glucosidase activity increased by 158.95% between O18 and O19. Concerning the inner-annual shifts, 2019 witnessed an increase in *β*-glucosidase activity between corresponding fertilization treatments with the successive soil sampling times.

The highest acid phosphomonoesterase activities were recorded within J19 (FA100 and FA60) and O19 (FC and FA100) sampling times. An identical trend was observed between the consecutive O18, A19 and J19 sampling times, namely, acid phosphomonoesterase activity increased along with the reduction of the mineral fertilizer dose within particular sampling time.

3.2. Phytoavailable phosphorus content and maize yield

The phytoavailable phosphorus (P_2O_5) content remained at higher level in all FA100 treatments throughout the experimental period with statistically significant differences in comparison with FC and FA60. The highest P_2O_5 content was recorded in FA100(J19), directly after biofertilizers application (an increase of 49.89% with respect to the FC(J19)). Furthermore, at the end of the experiment in O19, the soil phosphorus level increased in FA100 and FA60 as compared to the corresponding treatments from A18. As regards temporal fluctuations of soil P content, FA100 and FA60 experienced an upward trends from A18 to J19 and from O18 to O19, respectively. In 2018 and 2019 application of biofertilizer increased average maize yield in FA60 and FA100 treatments, respectively, with no statistically significant differences between particular treatments (Table 1).

3.3. Metabolic potential of soil microbial communities

3.3.1. Metabolic efficiency of soil bacterial community

The ratio between substrate utilization (OD 590 nm) and bacterial growth (OD 750 nm) calculated for the individual substrates located on BIOLOG™ ECO plates revealed the diversification of metabolic efficiency of soil bacterial communities and indicated C-compounds caused metabolic stress. Application of biofertilizers in J19 lowered the OD590/OD750 ratio for D-xylose, D,L-α-glycerol phosphate, γ-hydroxybutyric acid, L-phenylalanine, tween 40, α-cyclodextrin and phenylethylamine in both FA100 and FA60 as

compared to the control soil. The decline in OD590/OD750 ratio was also reported for α-ketobutyric acid, D-malic acid, i-erythritol and D-glucose-1-phosphate in FA100(J19) and for itaconic acid, D-glucosaminic acid, tween 80, L-asparagine, D-mannitol and L-threonine in FA60(J19). At the beginning of the field experiment in April 2018 2-hydroxy benzoic acid, 4-hydroxy benzoic acid, D-glucosaminic acid, glycyl-L-glutamic acid, L-serine and γ-hydroxybutyric acid were found stressful to soil bacterial community, however the OD590/OD750 ratio for aforementioned substrates decreased at the end of the experiment in October 2019. No stressful situation occurred for β-methyl-D-glucoside, D-mannitol, tween 40, D-xylose, i-erythritol and phenylethylamine across all the analysed samples (Figure 1).

3.3.2. Metabolic efficiency of soil fungal community

Metabolic efficiency of soil fungal community was expressed as a ratio between values of substrates utilization (OD 490 nm) and biomass production (OD 750 nm) for each substrate placed on BIOLOG^{TM} FF plates. Directly after biofertilizers application in June 2019, the OD490/OD750 ratio values decreased for α-D-Lactose, maltose, D-mannitol, α-keto-glutaric acid, L-fucose, D-fructose and D-galactose in both FA100 and FA60. Metabolic stress was also reduced for acetamido-acetic acid and for arbutin, D-glucaric acid and D-sorbitol in FA100(J19) and FA60(J19), respectively. No stressful metabolic situation across the experimental period was observed for e.g. L-arabinose, D-ribose, α-D-glucose, D-arabitol, N-acetyl-D-galactosamine, 2-amino ethanol, D-mannose, xylitol, D-melezitose, stachyose, maltitol, D-raffinose, N-acetyl-D-glucosamine, D-cellobiose, turanose, L-proline, N-acetyl-L-glutamic acid, D-mannose, adonitol and adenosine. Relatively low values of OD490/OD750 ratio values, as compared to other sampling times, were noted in case of dextrin, D-gluconic acid, L-asparagine, D-melibiose, putrescine, L-threonine, D-trehalose and sucrose in J19 (Figure 2).

3.4. Multiplex terminal restriction fragment length polymorphism

PCA analysis regarding the relative abundance of terminal restriction fragments was used to arrange the fertilization treatments in response to the soil sampling time. PCA clustered treatments from particular soil sampling times separately for all three investigated microorganisms groups. Samples collected in A18 and O18 were grouped together, whereas A19, J19 and O19 formed three distinctly separated groups. PC1 explained 23.82%, 35.05% and 22.90% of the variance, while PC2 accounted for 13.70%, 16.28% and 15.07% for

bacteria, archaea and fungi, respectively (Figure 3). A cluster analysis also pointed to a clearly defined grouping of fertilization treatments according to soil sampling time. At both 33% and 66% of Sneath's criterion, samples collected in June 2019 were clearly separated from remaining sampling times in bacterial and fungal communities (Figure S1, S3).

The distribution of terminal restriction fragments during two-year field experiment was expressed through the Venn diagrams which demonstrate the number of T-RFs common to the three fertilization treatments, constituting the "core microbiome" and T-RFs which appeared only in selected treatments, called "satellite microbiome". Archaea was the most abundant group in terms of "core" T-RFs and, simultaneously, the least diversified among the specific T-RFs (Figure 4B). Bacterial community was characterized by the lowest number of T-RFs (Figure 4A), while fungi exhibited the highest number of fragments characteristic to a particular fertilization method (Figure 4C). On the other hand, only archaea experienced an increase in the total number of T-RFs in FA100 and FA60 as compared to control soil (FC-51, FA100-57, FA60-52). The number of bacterial and fungal T-RFs remained at higher level in FA100 than in FA60 (bacteria: FC-29, FA100-24, FA60-20; fungi: FC-54, FA100-47, FA60-41). The identification of microorganisms based on the size of selected T-RFs revealed the presence of various microbial genera. Fungal community was characterized by the highest number of identified microorganisms. In the restriction profile of each microbial group some genera were affiliated to a T-RFs with different sizes, e.g. *Paracoccus* (60 bp, 70 bp, 71 bp), *Cenarchaeum* (150 bp, 160 bp, 170 bp) and *Lobulomyces* (170 bp, 240 bp). Furthermore, TRiFLe tool assigned several different microbial genera to a fragment of a defined size (e.g. 117 bp and 214 bp among bacteria, 273 bp, 345 bp and 373 bp within archaea and 54 bp, 88 bp, 157 bp and 452 bp inside fungi). In bacterial and archaeal restriction profiles there were some T-RFs common for all treatments and sampling times (e.g. archaea: 60 bp, 100 bp, 120 bp, 200 bp, 320 bp and 360 bp; bacteria: 168 bp and 174 bp), whereas for fungi no fragment appeared simultaneously in all treatments throughout the experimental period. However, the occurrence of fungal T-RFs 100 bp and 114 bp was not observed only in the June 2019, but the aforementioned fragments were the most abundant in October 2019. Microorganisms identified among fragments classified as a "core microbiome" were also shown in heatmaps (Figure S1-S3), representing variations in the relative abundance and number of individual T-RFs. After biofertilizers application in J19, the number of T-RFs decreased within bacterial and fungal communities, and then increased in O19. Sorensen and Jaccard coefficients, based

412 on the number of T-RFs were calculated to indicate the similarities between fertilization
413 treatments. The highest values of aforementioned indices were reached between FA100 and
414 FA60 within all investigated microbial groups.

415 **3.5. Next Generation Sequencing**

416 **3.5.1. Bacterial and archaeal communities composition**

417 The structure of bacterial and archaeal communities in AL soil was analyzed at phylum, class
418 and order level. NGS revealed the presence of total 46 phyla with 41 common to all
419 fertilization treatments ("core microbiome"), 4 shared between FA100 and FA60
420 (Deferrisomatota, Nanoarchaeota, TX1A-33 and WS4) and 1 unique to FA60 (Dadabacteria)
421 (Figure S4A). At phylum level soil was dominated by Actinobacteriota (25.43%-36.21%),
422 Proteobacteria (20.56%-26.59%), Acidobacteriota (12.38%-16.55%) and Chloroflexi (5.81%-
423 8.37%). Acidobacteriota were more abundant in FA100 and FA60 throughout the whole
424 experiment, while Chloroflexi decreased only in FA100-FA60(O18), as compared to control
425 treatments (Figure 5A).

426 123 classes were shared between FC, FA100 and FA60. Classes common to FA100 and FA60
427 included Coriobacteriia, D8A-2, Defferrisomatia, Nanoarchaeia, Syntrophobacteria,
428 Thermodesulfovibrionia, TX1A-33 and WS4, whereas Dadabacteriia and Sulfobacillia were
429 unique to FA60 (Figure S4B). The dominant soil bacterial classes were Alphaproteobacteria
430 (12.74%-15.52%), Actinobacteria (10.58%-16.33%), Gammaproteobacteria (6.92%-11.83%)
431 and Thermoleophilia (9.37%-12.38%). Gammaproteobacteria and Vicinamibacteria were
432 present in higher abundance in FA100 and FA60 across all sampling times in comparison
433 with controls. The relative abundance of Blastocatellia was enhanced (>3%) in FA100(O18,
434 J18, O19) and FA60(J18, J19) (Figure 5B).

435 300 OTUs at order level were common to all fertilization treatments. FA100 and FA60
436 included 5 (B55-F-B-G02 (Firmicutes), Candidatus_Buchananbacteria, Ga0077536
437 (Proteobacteria), Leptolyngbyales, Piscirickettsiales) and 2 (Dadabacteriales, Sulfobacillales)
438 unique orders, respectively, while FC was characterized by the presence of 1 individual order
439 (JTB23 (Proteobacteria)). 20 orders shared between FA100 and FA60 included Arenicellales,
440 Defferrisomatales, Dehalococcoidales, Euzebyales, Puniceispirillales, Syntrophobacterales
441 and Woesearchaeales while Ammonifexales and Gloeobacterales were reported in both FC
442 and FA60 (Figure S4C). At the order level, Rhizobiales predominated bacterial community

443 (7.64%-9.58%), followed by Gaiellales (5.85%-7.94%), Burkholderiales (5.09%-7.54%),
444 Vicinamibacteriales (3.16%-6.31%), Gemmatimonadales (3.25%-4.48%) and
445 Solirubrobacterales (3.00%-4.16%). Burkholderiales and Vicinamibacterales were more
446 abundant in FA100 and FA60 across all sampling times as compared to FC. The relative
447 abundance of Rhizobiales was higher only in FA60(J18) and FA100(O18) in comparison with
448 corresponding controls (Figure 5C).

449 Principle coordinate analysis, based on the Bray-Curtis distances, pointed to a well-defined
450 clustering of samples with respect to the fertilization treatment and soil sampling time.
451 Samples collected directly after biofertilizers application (J18, J19) were clearly separated
452 from the autumn sampling times (O18, O19), and, furthermore, PCoA highlighted the
453 distinction of controls from FA100 and FA60 not only in J18 and J19, but also in O19.
454 Regarding the sample arrangement in PCoA space, it was found that FC(O18) and
455 FA60(O18) formed one cluster, while the second one comprised of FA100(O18) and FA100-
456 FA60(O19) (Figure 5D). On the other hand, the distribution of samples in the circular
457 UPGMA dendrogram revealed that the main clustering factor was the fertilization regime.
458 FA100 and FA60 from all sampling times (except FA60(O18)) formed a single group within
459 which FA100 were separated from FA60 (Figure 5F). The increased Shannon diversity index
460 (H) were reported in FA60(J18), FA100-FA60(O18), FA100-FA60(J19) and FA100(O19) in
461 comparison with corresponding FC. H index reached the highest values in FA60(J19), while
462 FC(O18) and FA60(O19) were characterized by the greatest dispersion of H values (Figure
463 5E).

464 **3.5.2. Predictive functions of bacterial community**

465 Predictive functional profile of bacterial community was dominated by sequences connected
466 to "Metabolism" (~81.09%), "Genetic Information Processing" (~12.08%), "Cellular
467 Processes" (~4.12%) and "Environmental Information Processing" (~2.04%). "Organismal
468 Systems" and "Human Diseases" pathways represented the least abundant main KEGG
469 classes, accounted for ~0.38% and ~0.30%, respectively (Figure 6A). The application of
470 biofertilizers increased the number of sequences in FA100(J18), while FA100(J19) and
471 FA60(J19) experienced a decline in the number of functional OTUs in comparison with
472 control treatments. However, directly after biofertilizers application in J19, the number of
473 OTUs increased as compared to J18 and O18. FA100 and FA60 in O18 and O19 recorded an

upward trend in the number of sequences associated with main KEGG classes as compared to control treatments. Furthermore, FA100(O18) and FA100(O19) displayed approximately 150% and 250% higher number of OTUs, respectively, than the corresponding FC(O18) and FC(O19). The number of functional OTUs remained at similar level between FA100(J18)-FA100(O18) and between FA100(J19)-FA100(O19). In general, total number of functional sequences was higher in 2019 than in 2018 (Figure 6B). "Metabolism" was the most diverse main KEGG class and included 11 sub-classes with the highest number of functional OTUs attributed to amino acids and carbohydrates metabolism, followed by "Metabolism of cofactors and vitamins" and "Metabolism of terpenoids and polyketides". Relatively high number of sequences was also assigned to "Xenobiotics biodegradation and metabolism". In contrast, pathways associated with nucleotide and glycan biotransformations were characterized by the lowest number of OTUs (Figure 6C). Considering the remaining main KEGG classes, "Cellular Processes" were dominated by "Cell growth and death" and "Cell motility", "Environmental Information Processing" was mainly composed of sequences related to "Membrane transport", while OTUs linked to processes including "Folding, sorting and degradation", "Replication and repair" and "Translation" were the most abundant within "Genetic Information Processing" (Figure S5). For each sub-class the number of functional sequences increased in FA100 and FA60 at O18 and O19 as compared to corresponding controls. In depth analysis of bacterial functional profile revealed that the biofertilization influenced phosphorus compounds biotransformations. Six different P-related biochemical pathways were distinguished: "Phosphotransferase system (PTS)", "Pentose phosphate pathway", "Inositol phosphate metabolism", "Oxidative phosphorylation", "Glycerophospholipid metabolism" and "Phosphonate and phosphinate metabolism". Similarly to the main classes and sub-classes, the number of functional OTUs associated with P-compounds processes remained at higher level in FA100 and FA60 at autumn dates (O18 and O19) (Figure 6D).

3.5.3. Fungal community composition

The composition of fungal community was analyzed, similarly to bacteria and archaea, at phylum, class and order level. The "core mycobiome" consisted of 13 phyla, with 1 phylum unique to FC (Calcarisporiellomycota) and 1 phylum shared between FA100 and FA60 (Blastocladiomycota) (Figure S6A). The fungal assemblages were dominated by Ascomycota (46.63%-66.86%), Mortierellomycota (8.16%-21.95%) and Basidiomycota (>3.00%-22.92%).

506 The relative abundance of Ascomycota increased in a selected treatments where biofertilizers
507 were applied (FA100(J18), FA100-FA60(O18), FA60(J19) and FA100-FA60(O19)), likewise
508 for Basidiomycota (FA100-FA60(J18), FA100-FA60(O18) and FA100(J19)) (Figure 7A).

509 36 OTUs at class level were shared among FC, FA100 and FA60. Both FC and FA60
510 included 3 characteristic classes encompassing Calcarisporiellomycetes, GS37 (Ascomycota),
511 Wallemiomycetes and Chytridiomycetes, Endogonomycetes, Tritirachiomycetes, respectively.
512 Simultaneously, Laboulbeniomycetes and Olpidiomycetes were common to FA100 and FA60
513 (Figure S6B). Sordariomycetes was the most abundant class (20.48%-39.26%), followed by
514 Mortierellomycetes (8.16%-21.95%), Dothideomycetes (3.63%-16.91%), Agaricomycetes
515 (>3.00%-21.45%) and Eurotiomycetes (3.32%-11.43%). The application of biofertilizers
516 stimulated the occurrence of Agaricomycetes (FA100-FA60(J18), FA100(O18, J19)),
517 Eurotiomycetes (FA100-FA60(O19)), Leotiomycetes (FA100-FA60(J18, O18)),
518 Pezizomycetes (FA100(J19), FA60(O19)) and Sordariomycetes (FA100-FA60(J18, J19,
519 O19). Conversely, the decline in the relative abundance of Archaeorhizomycetes and
520 Mortierellomycetes was observed in FA100 and FA60 (Figure 7B).

521 88 orders from a total 108 constituted the "core mycobiome", 4 orders were specific to both
522 FC (Calcarisporiellales, GS37, Microstromatales, Wallemiales) and FA60 (Chytridiales,
523 Corticiales, GS20 (Mucoromycota), Tritirachiales); at the same time Botryosphaeriales,
524 Gigasporales and Phacidiales were unique to FA100 (Figure S6C). The dominant orders
525 included Hypocreales (11.31%-23.75%), Mortierellales (8.16%-21.95%), Sordariales (3.64%-
526 14.30%) and Agaricales (>3.00%-20.91%). Selected FA100 and FA60 had greater abundance
527 of Agaricales (FA100-FA60(J18), FA100(J19)), Coniochaetales (FA100(O18), FA60(O19)),
528 Helotiales (FA100(O18)), Hypocreales (FA100-FA60(J18), FA60(O18), FA100-FA60(J19,
529 O19)), Pezizales (FA100(J19), FA60(O19)), Pleosporales (FA100(O19)) and Sordariales
530 (FA100-FA60(J18), FA60(J19)) (Figure 7C).

531 PCoA analysis showed that samples were grouped according to soil sampling time, with vivid
532 distinction of J18 from the O18, J19 and O19. Samples collected in J19 and O19 were
533 relatively in a close proximity to each other in the PCoA space (Figure 7D). Grouping of
534 treatments according to sampling time was also evidenced by UPGMA circular dendrogram;
535 samples collected in J18, O18 and J19 formed three clearly separated clusters. Furthermore,
536 most of the samples collected in O18, J19 and O19 formed one cluster (Figure 7F). Within

fungal community, Shannon diversity index increased in FA100-FA60(J18), FA100(O18), FA60(J19) and FA100-FA60(O19) as compared to controls. The highest values of H index were reported in FA100(J18) and FA60(J18) (Figure 7E).

3.5.4. Fungal functional profile

FUNGuild revealed that AL soil was dominated by the representatives of the following trophic modes: "Pathotroph-Saprotroph-Symbiotroph" (15.79%-44.58%), "Saprotroph" (22.25%-38.91%) and "Saprotroph-Symbiotroph" (18.72%-33.52%). As compared to corresponding control treatments, the relative abundance of "Saprotroph" increased in FA100(J18), FA60(O18) and FA100(O19), "Saprotroph-Symbiotroph" was more abundant in FA100-FA60(O19) and "Symbiotroph" recorded an upward trend in FA100-FA60(J18), FA100-FA60(O18), FA60(J19) and FA60(O19). Meanwhile, "Pathotroph" noted a decline in the relative abundance in FA100(J19) and FA60(O19) (Figure 8A). Deeper insight into fungal functional profile showed that the application of biofertilizer increased the relative abundance of "dung saprotroph" (FA60(J18), FA60(O18), FA100-FA60(J19), FA100(O19)), "dung saprotroph-plant saprotroph" (FA100(J19), FA100-FA60(O19)), "plant saprotroph-wood saprotroph" (FA60(J18), FA60(O18), FA100(O19)), "undefined saprotroph" (FA100(J18), FA60(O18), FA100(O19)), "arbuscular mycorrhizal" (FA60(O18), FA60(J19), FA60(O19)), "ectomycorrhizal" (FA100(J18), FA60(J18), FA60(J19)), "endophyte" (FA100(J18)), "ectomyccorhizal-undefined saprotroph" (FA100(O19)), "endophyte-litter saprotroph-soil saprotroph-undefined saprotroph" (FA100(O19), FA60(O19)) and "animal pathogen" (FA100(O18)). Plant pathogens were more abundant in almost all FA100 and FA60 treatments except for FA100(J19) and FA60(O19), however the abundance of phytopathogens decreased between corresponding FA100-FA60 treatments in 2018 and between FA60 treatments in 2019 (Figure 8C). PCA analysis of fungal functional OTUs showed the clear clustering of treatments with respect to the soil sampling time (Figure 8B).

4. Discussion

The enzymatic response of soil microbial communities, due to sensitivity to management practices, is commonly used bioindicator of soil quality, reflecting variations in the soil environment under the influence of external factors (Lee et al., 2020). During field experiment we observed variations in the activity of acid phosphomonoesterase, urease, protease and β-glucosidase triggered by introduction of phosphorus biofertilizer and, as Dick

568 and Kandeler (2005) stated, enzymatic activity may change rapidly within two years after
569 implementation of new fertilization regime. The improvement of enzymatic activity in
570 FA100(J19) and FA60(J19) remains in agreement with other studies concerning the influence
571 of biofertilizers on soil microbial properties. Acid phosphomonoesterase activity increased in
572 soil inoculated with *Azotobacter chroococcum, Acetobacter diazotrophicus and Aspergillus*
573 *awamori* (Srivastava and Singh, 2022), enhanced urease activity was observed in soil
574 amended with microbial inoculant containing *Frankia casuarinae CcI3* (Qi et al., 2022),
575 application of *Bradyrhizobium* sp. significantly improved protease activity during growing
576 seasons in 2018 and 2019 (El-Sawah et al., 2021), while upward trend in *β*-glucosidase
577 activity was reported in case of seed-applied biofertilizers TripleN®, Rhizosum N® and
578 Rhizosum PK® in common wheat cultivation (Dal Cortivo et al., 2020). The higher level of
579 soil enzymatic activity may be associated with the elevated action of soil microorganisms and,
580 concomitantly, with a remobilization and increased availability of nutrients (Chaudhary et al.,
581 2022). In addition, enhanced acid phosphomonoesterase activity may facilitate the P uptake
582 from organic sources (El-Sawah et al., 2021). A study conducted by Ramesh et al. (2011)
583 revealed the improvement of acid and alkaline phosphatases activities in the rhizosphere of
584 soybean treated with *Bacillus* sp. strains, *Bacillus amyloliquefaciens* and *Bacillus cereus*.
585 Improved soil enzymatic activity and higher availability of essential nutrients is of particular
586 importance in the light of climate change, as environmental stresses and increment in
587 greenhouse gases concentration in the atmosphere may contribute to reduced mineral content
588 in plants (Elbasiouny et al. 2022;Sangiorgio et al., 2020). Relatively high enzymatic activity
589 in A18 and A19, however, may refer to the acceleration of chemical transformations
590 occurring in the soil at the beginning of the growing season. The increase in soil enzymatic
591 activity within the corresponding treatments between particular sampling times may suggest
592 that the application of phosphorus biofertilizer not only improves this parameter but also
593 maintains such an outcome over time.

594 Phosphorus biofertilizer improved the level of available P in FA100 treatment. The increased
595 P content in the soil was also observed after inoculation with P-solubilizing bacterium
596 *Paenibacillus* sp. B1 (Li et al., 2020), *B. licheniformis* and *B. amyloliquefaciens* (Wang et al.,
597 2021a) and *Bacillus* sp. strain SD01N-014 (Liu et al., 2018). The enhanced level of
598 phytoavailable phosphorus content may result from the P-solubilizing activity of
599 microorganisms provided with the biofertilizer. Beneficial microorganisms could also

stimulate the activity of native microbiome towards biotransformations of P compounds. Furthermore, the gradual increase in P content in the FA100 and FA60 across field experiment may suggest that biofertilizer favours the accumulation of this element in the soil. Climate change may limit the P availability in the soil which, in turn, impairs the drought tolerance of plants. An increase in the mineral component content in the soil through the biofertilization may not only increase its uptake but also promote resistance to environmental disturbances (Elbasiouny et al., 2022; Tariq et al., 2018). Improved maize yield may be associated with the higher soil enzymatic activity and, concomitantly, enhanced nutrients availability. According to Schmidt and Gaudin (2018) maize yield increased by an average 15.3% after biofertilization in field experiments conditions. Higher yields is thought to be reflected by increased number of functional OTUs associated with amino acids and carbohydrates metabolism in the soil, compounds that can be assimilated and utilized by plants (Moe, 2013). It is worth to mention that, although the maize yield increase after phosphorus biofertilizer was not statistically significant, the 40% reduction of mineral fertilizer application is more beneficial from the environmental protection point of view and without any loss of the yield. This observation is very valuable for farmers and also in the context of assumptions of modern, sustainable and regenerative agriculture and in the view of the deteriorating state of crop yields as a result of climate change .

Metabolic abilities of soil bacterial and fungal communities were evaluated as a ratio between substrate utilization and growth pattern. According to Pinzari et al. (2016) the lower values of abovementioned ratio indicate the more dynamic microbial growth accompanied by a little substrate consumption. In contrast, higher ratio points on a stressful metabolic situation when small microbial biomass is developed with high utilization of carbon compounds. Our results showed that the phosphorus biofertilizer has a potential to alleviate metabolic stress of some C-substrates belonging to different compound groups (carbohydrates, carboxylic acids, polymers, amino acids) thus enhancing metabolic efficiency of soil microorganisms. Such a change may result from the different metabolic activity of beneficial microorganisms provided with the biofertilizer as compared to indigenous microbiota. Application of biofertilizer exert not only the immediate effect on metabolic stress, but also the prolonged impact especially on carboxylic acids in ECO plates. No stressful situation across the experimental period for some substrates (e.g. carbohydrates) indicate that certain compound can be effectively metabolized regardless of soil sampling time and constitute the preferred

carbon source. However, variations in the metabolic efficiency of soil microbial communities throughout the field experiment may refer to seasonal temperature fluctuations and maize photosynthetic activity and roots exudates. Broadening the catabolic potential of soil microorganisms may also entail greater resistance to various environmental stresses, including those induced by climate change.

Apart from enzymatic activity and metabolic potential, analysis of genetic diversity of bacterial, archaeal and fungal communities using the M-tRFLP approach was also carried out. Consequently, restriction profiles, called "genetic fingerprinting", unique to each of the microbial group were obtained. Throughout the experimental period we observed variations in both number of T-RFs and their relative abundance, which may reflect the susceptibility of microorganisms to changes induced by the introduction of beneficial microorganisms to the soil. Reduction in the number of T-RFs in bacterial community in J19 remains in agreement with results of Zhang et al. (2010) presented decline in the richness within bacteria inhabiting soil inoculated with *Rhizobium leguminosarum* strain. In case of bacteria and fungi, we observed increased number of T-RFs in O19 in comparison with J19, which may indicate that the favourable impact of biofertilizer becomes apparent after a certain time. Such a trend was also reported by Mącik et al. (2022) where different soil type (Brunic Arenosol) was treated with the same phosphorus biofertilizer. Archaeal "core microbiome" consisted of relatively high number of T-RFs accompanied by low diversity among specific fragments, possibly indicating archaea as the most stable microbial group when confronted with a novel fertilization technique. Furthermore, increased total number of archaeal T-RFs in FA100 and FA60 may point that biofertilizer stimulates a selected microbial group. Higher number of T-RFs in FA100 than in FA60 among bacteria and fungi may suggest, instead, a preferable solution is application of phosphorus fertilizer at an optimal dose, but microbiologically enriched. The higher values of Sorensen and Jaccard indices between FA100 and FA60 treatments indicate greater similarity of restriction profiles with respect to the number of T-RFs (Gryta and Frąc, 2020; Johnston-Monje et al., 2014). PCA analysis showed a clear clustering of treatments with respect to the soil sampling time. The arrangement of samples in PCA space indicated that changes in t-RFLP patterns were more pronounced in the second year of the field experiment. Grouping of different sampling times within a particular year mainly resulted from the dissimilarities in the relative abundance and number of individual T-RFs in the restriction profile. The common clustering of the A18 and O18 sampling times

may suggest that the microbial communities quickly achieved their pre-biofertilization status. Nevertheless, the subsequent application of biofertilizer in 2019 resulted in a greater diversity within restriction profiles as evidenced by the vivid distinction of samples according to sampling time.

TRiFLe tool, based on the size of selected T-RFs, allowed identification of different microorganisms providing essential services for the preservation of soil productive potential and soil health. Ecologically important archaea may be represented by the members of *Nitrososphaera* spp., ammonia oxidizing archaea, with wide metabolic abilities, involved in nitrification process (Miranda et al., 2019; Mukhtar et al., 2019). Bacterial genera demonstrating beneficial traits included *Rhizobium* and *Bradyrhizobium* (biological nitrogen fixation) (Hara et al., 2019; Wang et al., 2018c), *Lysobacter* (biological control agent against fungal phytopathogens) (Nian et al., 2021), *Pseudomonas* (phytohormones synthesis) (Hassen et al., 2018), *Bacillus* (mitigation of abiotic and biotic stresses factors) (Saxena et al., 2020), *Anabaenopsis* (phytohormones synthesis) (Kollmen and Strieth, 2022), *Aeromonas* (siderophore synthesis) (Seenivasagan and Babalola, 2021), *Sodalis* (decomposition of deadwood and plant biopolymers) (Tláskal et al., 2021) and *Burkholderia (*bioremediation) (Min et al., 2017). Furthermore, some representatives of *Citrobacter* spp., *Lysobacter* spp., *Pseudomonas* spp., *Bacillus* spp., *Burkholderia* spp. and *Klebsiella* spp. were found to exhibit phosphate solubilizing properties (Castagno et al., 2021; Chen et al., 2019; Liu et al., 2020; Mažylytė et al., 2022; Nacoon et al., 2020; Suleman et al., 2018). Among fungal community, selected T-RFs were associated with saprotrophic fungi (*Psathyrella*, *Conocybe*) (Padamsee et al., 2008; Ogura-Tsujita et al., 2009), entomopathogens (*Cordyceps*, *Metarhizium*) (Hussain et al., 2021; Brunner-Mendoza et al., 2019), nematophagous fungi (*Dactylellina*) (Degenkolb and Vilcinskas, 2016), lichenized fungi (*Caloplaca*, *Biatora*, *Cladonia*) (Castillo and Beck, 2012; Printzen, 2014; Tuovinen et al., 2015), mycorrhizal fungi (*Suillus*, *Sarcodon*, *Tomentella*, *Paraglomus*) (Li et al., 2021; Grupe et al., 2015, Suvi et al., 2010; Błaszkowski et al., 2012), plant growth promoting microorganisms (*Penicillium*, *Trichoderma*) (Zhang et al., 2018; Radhakrishnan et al., 2014) and fungi exerting bioremediation properties (*Solicoccozyma*) (Du et al., 2022). What is more, TRiFLe revealed the presence of fungi which may be potentially involved in phosphate solubilization and mobilization in the soil (*Oidiodendron*, *Penicillium*, *Trichoderma*, *Pichia*, *Aspergillus* and *Saccharomyces*) (Alori et al., 2017). Some microorganisms identified with TRiFLe may also participate in the

mitigation of climate change induced stresses in crops including cold and freezing (*Pantoea* spp., *Pseudomonas* spp.), high temperature (*Bacillus* spp., *Pseudomonas* spp.), salinity (*Pseudomonas* spp., *Burkholderia* spp.) and drought (*Klebsiella* spp., *Citrobacter* spp., *Serratia* spp., *Penicillium* spp.) (Fadiji et al., 2022).

Next Generation Sequencing was employed to gain deeper understanding of microbial community structure and functionality in soil amended with phosphorus biofertilizer. Bacterial and archaeal communities composition was analyzed at phylum, class and order level and each taxonomic level showed a higher number of OTUs in FA100 and FA60 treatments, indicating a beneficial impact of the biofertilizer on the diversity of soil microorganisms. Probably, the phosphorus biofertilizer stimulated the occurrence of specific microorganisms and boosted their proliferation, thereby broadening the range of services provided by the microbiome. The higher number of OTUs at phylum, class and order level within bacterial community was also observed by Wang et al. (2021b) in soil inoculated with biocontrol strains *Paenibacillus jamilae* HS-26 and *Bacillus amyloliquefaciens* subsp. *plantarum* XH-9. Specific orders for the FA100 and FA60 included Dehalococcoidales, which representatives are able to remediate halogenated ethenes and chloroorganic aromatic compounds (Löffler et al., 2013) and Syntrophobacterales, a sulfate-reducing bacteria (Plugge et al., 2011). The higher biodiversity within bacterial community may be also supported by the increased Shannon index values. The higher values of Shannon index within soil bacterial community were also observed after inoculation with *B. subtilis* (Wang et al., 2021a) and *Trichoderma* biofertilizer (Zhang et al., 2022). According to Pang et al. (2017) higher diversity within microbial communities ensures a well-balanced microbiome with greater resistance to environmental stresses and pathogens activity which is essential for fostering adaptation to climate change.

The application of biofertilizer modified the relative abundance of bacterial groups from phylum to order level. It is not easy to pinpoint the driver of such a change, however shifts in the community structure may result from the competition for an ecological niche, a certain imbalance arising from the introduction of active, beneficial microorganisms and interactions between indigenous microbiome and bacteria provided with the biofertilizer. Proteobacteria and Actinobacteriota are ubiquitous in soils, and, Actinobacteriota were found to synthesize hydrolytic enzymes degrading plant polymers (Gao et al., 2019; Zhang et al., 2019). Members of Acidobacteriota colonize acidic and nutrient deficient environments (Wang et al., 2018c),

728 and, as Kalam et al. (2020) described, Acidobacteriota are involved in soil organic matter
729 decomposition, biogeochemical cycles and also affect plant growth. The increased relative
730 abundance of Chloroflexi and Acidobacteriota was also observed by Chen et al. (2021) after
731 soil inoculation with *B. subtilis* and *P. polymyxa*. In selected FA100 and FA60 treatments
732 biofertilizer stimulated the occurrence of Blastocatellia, which members can breakdown
733 complex proteins (Ivanova et al., 2020). Similar trend was reported by Pongsilp and Nimnoi
734 (2020) where distribution of Blastocatellia increased in soil amended with liquid medium
735 containing *Ensifer fredii*. Members of Rhizobiales participate in N_2 fixation and are
736 commonly used as a ecofriendly biofertilizers, however certain species are plant pathogens
737 (Wang et al., 2018b), therefore a decline in their relative abundance may be considered as a
738 favourable biofertilizer impact.

739 Shifts in the fungal community, similarly to bacteria and archaea, were analyzed at the
740 phylum, class and order level. Fungi belonged to Ascomycota are widely distributed soil
741 organic matter decomposers (Muneer et al., 2021), Basidiomycota can breakdown
742 lignocellulose and form symbiotic relationships with plants (Wang et al., 2022) while
743 Mortierellomycota exert plant growth promoting properties including phosphorus
744 solubilization (Ozimek and Hanaka, 2021; Wolińska et al., 2022). Application of biofertilizer
745 promoted the abundance of Ascomycota, especially in O18 and O19, however such a trend
746 might derive from the accumulation of organic matter in the soil and the enhanced activity of
747 microorganisms involved in its breakdown. Cao et al. (2022) also observed that the
748 Ascomycota was more abundant in soil treated with microbial inoculant containing *Bacillus*
749 spp. strains. Biofertilizer used in this study stimulated the occurrence of some fungal orders
750 involving microorganisms important for soil microbial health such as Helotiales (saprotrophs
751 and ericoid mycorrhizal fungi) (Wang et al., 2006), Sordariales (saprotrophs growing on dung
752 or decaying plants and exerting antifungal activity) (Luo et al., 2022) and Agaricales
753 (saprotrophs synthesizing hydrolytic and oxidative enzymes involved in lignocellulose
754 breakdown) (Ruiz-Dueñas et al., 2020). Variations in the composition of soil mycobiome
755 between particular treatments may be attributed to the fact that the expansion of certain fungal
756 groups inhibits the development of other fungi due to nutrient consumption (Yin et al., 2022).
757 The increased Shannon diversity index within fungal community remains in line with results
758 obtained by Zhao et al. (2022) where soybean seeds were inoculated with *Bradyrhizobium*

japonicum 5038 and the combination of *Bradyrhizobium japonicum* 5038 and *Bacillus aryabhattai* MB35-5.

PICRUSt-based functional inference revealed variations in the bacterial biological functions after fertilization with phosphorus biofertilizer. In general, increased number of functional sequences associated with main KEGG classes and sub-classes in both FA100 and FA60 in O18 and O19 may suggest that the positive effect of the biofertilizer emerges gradually. Furthermore, similar number of sequences between FA100 treatments within particular year may indicate that the action of optimal dose of fertilizer in combination with beneficial bacteria is maintained over time. We speculated that an upward trend in the number of functional OTUs in J19 in comparison with J18 and O18 resulted from soil supplementation with nutrients contained in fertilizers and with the acceleration of processes involved in their breakdown. In other studies, it was observed that biofertilizers increased the abundance of genes involved in energy production and conversion, lipid transport and metabolism, cell motility, secondary metabolites biosynthesis, transport, and catabolism (Qi et al., 2022), xenobiotics biodegradation and metabolism (Wang et al., 2021a), signal transduction mechanisms and nucleotide and coenzyme transport and metabolism (Tian et al., 2022). Higher number of functional sequences in FA100 and FA60 in autumn sampling times may indicate that microorganisms gradually adapted their metabolic pathways to altered environmental conditions.

The majority of obtained functional OTUs was assigned to metabolic-related pathways, pointing that metabolism of various compounds may be a determinant of proper functioning of soil microbiome and indicator of soil health (Qi et al., 2022). Relatively high number of sequences was also assigned to "Genetic Information Processing" suggest that processes associated with DNA replication and gene expression are also fundamental for the soil microorganisms prosperity. Different sub-classes within "Metabolism" main KEGG class indicate the broad spectrum of metabolic capabilities of bacteria inhabiting AL soil. The highest number of functional OTUs was connected to "Amino acids metabolism" and "Carbohydrates metabolism" suggesting that the biotransformations of these compounds occurs with the greatest intensity. For the improvement of degraded soils quality important metabolic pathways included degradation of xenobiotics, compounds responsible for soil contamination, and synthesis of secondary metabolites such as antibiotics (Mishra et al., 2021; Sharrar et al., 2020). As Singh et al. (2014) and Crits-Christoph et al. (2018) stated enzymes

involved in xenobiotics degradation and metabolism of terpenoids and polyketides are harbored by a small group of soil microorganisms and, according to Xun et al. (2019), specialized functions play a crucial role in mitigation of environmental stresses and ensuring a high-quality microbiome. On the other hand, synthesis of antibiotics may broaden the antagonistic capacity of microorganisms towards phytopathogens. A more profound insight into the predictive functional profile revealed that biofetilizer also affected pathways associated with P compounds. In long-term study conducted by Wang et al. (2021c) inoculation with *Burkholderia cepacia* ISOP5 and *Rhodopseudomonas palustris* ISP-1 decreased the abundance of genes associated with P uptake and transport, but increased genes responsible for organic P mineralization and inorganic P solubilization such as *phoN* (acid phosphatase), *phnA* (phosphonoacetate hydrolase) and *phnFGHIJKLMNOP* (C-P lyase subunit). In our study, phosphorus biofertilizer increased the number of functional OTUs associated with processes referred to formation of cell wall membranes ("Glycerophospholipid metabolism") (Kondakova et al., 2015), decomposition of compounds constituting the P source ("Phosphonate and phosphinate metabolism") (Tapia-Torres et al., 2016), synthesis of NADPH and ATP ("Pentose phosphate pathway" and "Oxidative phosphorylation") (Borisov et al., 2011; Spaans et al., 2015) and transport of carbohydrates and sugar derivatives ("Phosphotransferase system") (Somavanshi et al., 2016). The increased number of functional sequences associated with "Oxidative phosphorylation" in soil amended with biofertilizers was also observed by Cao et al. (2022). In general, results obtained from PICRUSt indicated that phosphorus biofertilizer may be beneficial to soil bacterial community by promoting the abundance of functional OTUs associated with basic and specialized biochemical processes determining resilience and adaptability of soil bacteria. Enhanced metabolism of P compounds may strengthen bacterial cells and support the establishment of a high quality soil microbiome.

Assignment of trophic modes and ecological functional groups (guilds) with FUNGuild database revealed that the phosphorus biofertilizer may stimulate the occurrence of fungi important for protection and preservation of soil health, which is one of the most relevant challenges of regenerative agriculture. Saprotrophic fungal communities are involved in the decomposition of organic matter of various origin while mycorrhizal species improve nutrient uptake in plants and display antagonism towards phytopathogens (Fang et al., 2020; Frąc et al., 2018). Mycorrhizal fungi can also increase drought tolerance in plants by improving water

retention, root biomass and synthesis of substances (e.g. glomalin) exerting aggregating properties on soil structure (Fadiji et al., 2022). Moreover, mutualistic interactions between arbuscular mycorrhizal fungi and plant roots can increase drought resilient by regulating glucose exudation and rhizosphere expansion (Hoang et al., 2022). The increase in the relative abundance of saprotrophs and mycorrhizal fungi was also observed by Mącik et al. (2022) in soil amended with the same phosphorus biofertilizer as used in this study. Biofertilization also promoted the increase in plant pathogens abundance, however we assume that such an outcome can be controlled due to higher genetic and improved functional diversity as well as the presence of microbial biocontrol agents.

Both PCoA and the UPGMA clustering revealed that not only fertilization treatment, but also soil sampling time affected the composition of soil microbiome and mycobiome. The distinction of controls from microbiologically amended treatments within bacterial community in PCoA space was also observed by Wang et al. (2018a, 2021b). Yang et al. (2020) observed that the soil samples inoculated with microbial fertilizer were grouped according to wheat growing periods. The samples arrangement within bacterial community in both PCoA and UPGMA tree may point that bacteria are more sensitive to seasonal environmental changes and new fertilization regime as compared to fungi. The distribution of samples in PCoA space may also indicate that fungal community underwent shifts after the first application of biofertilizer and afterwards remained more resilience to the subsequent fertilization showing simultaneously greater stability under fluctuating environmental conditions. Close proximity of fungal samples collected in O18, J19 and O19 and their common grouping may point that variations after biofertilizers application maintained over time.

5. Conclusions

The development of sustainable soil management practices by involving microbial-based bioformulations is highly required in time of agricultural intensification, climate change and to meet targets of regenerative agriculture including soil health restoration and biodiversity loss reversing. Owing to the invaluable contribution of soil microorganisms to the maintenance of the ecosystem ecological balance, we suggested the application of an innovative phosphorus biofertilizer as a tool to improve the biodiversity of microbiome and mycobiome inhabiting degraded soil. The obtained results confirmed the favourable influence

of the biofertilizer on the soil microbiological properties including enzymatic activity, presence of microorganisms exhibiting plant growth promoting properties, composition of functional profiles and metabolic efficiency. What is more, we showed that phosphorus biofertilizer has a potential to increase the phytoavailable phosphorus content and enhance maize yield. These results revealed the comprehensive action of biofertilizer on the soil environment. In summary, this work showed that the microbiologically enriched phosphorus mineral fertilizer can be adopted in sustainable and regenerative agriculture combining the status of soil microbial communities with the general soil health and beneficial properties provided by microorganisms open up entirely new doors for the improvement of the arable soils quality and productivity. Finally, enhanced soil ecosystem biodiversity and the replacement of agrochemicals with microbial inoculants can support the formation of a healthier and stronger microbiome and mycobiome more resistant to damaging consequences of climate change.

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FIGURES CAPTIONS

Figure 1. Ratio between values of substrate utilization (OD 590) and biomass production (OD 750) determining theoretical metabolic efficiency of soil bacterial community on different carbon sources located on Biolog™ ECO plates. A ratio of >4 indicates a stressful metabolic situation for soil bacterial community functioning. A ratio of <4 points on a balance between substrate utilization and biomass production. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), A18-April 2018, O18-October 2018, A19-April 2019, J19-June 2019, O19-October 2019.

Figure 2. Ratio between values of substrate utilization (OD 490) and biomass production (OD 750) determining theoretical metabolic efficiency of soil fungal community on different carbon sources located on Biolog™ FF plates. A ratio of >2 indicates a stressful metabolic situation for soil fungal community functioning. A ratio of <2 points on a balance between substrate utilization and biomass production. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), A18-April 2018, O18-October 2018, A19-April 2019, J19-June 2019, O19-October 2019.

Figure 3. The principal components plots of terminal restriction fragment patterns within bacterial (A), archaeal (B) and fungal (C) communities. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), A18-April 2018, O18-October 2018, A19-April 2019, J19-June 2019, O19-October 2019.

Figure 4. Venn diagrams showing the distribution and number of common and unique terminal restriction fragments (T-RFs) within bacterial (A), archaeal (B) and fungal (C) communities and identification of microorganisms based on the size of selected T-RFs. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), A18-April 2018, O18-October 2018, A19-April 2019, J19-June 2019, O19-October 2019, S-Sorensen coefficient, J-Jaccard coefficient.

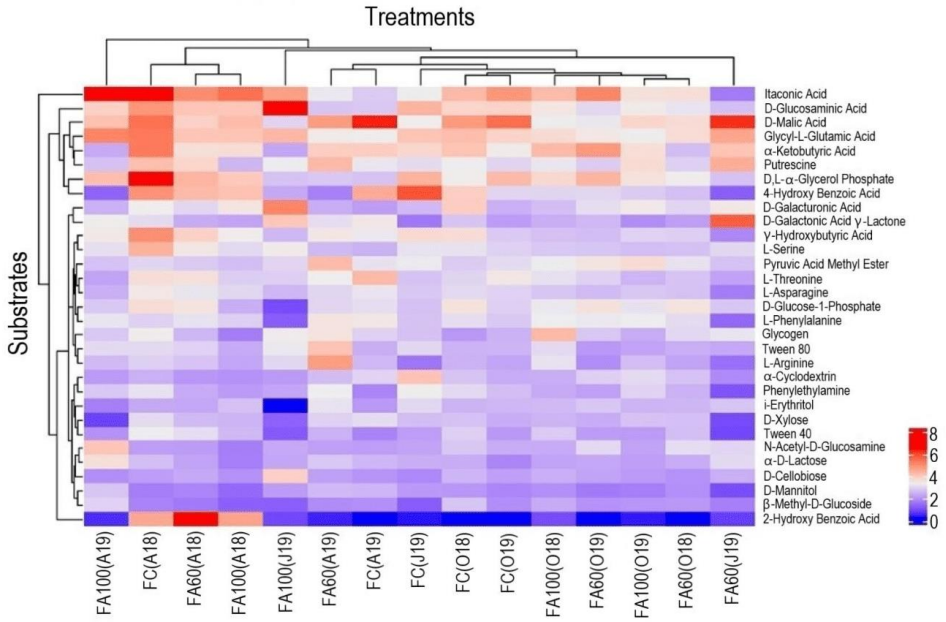
Figure 5. Effects of the phosphorus biofertilizer on the composition of bacterial and archaeal communities. (A) - the relative abundance of dominant bacterial phyla, (B)- the relative abundance of dominant bacterial classes, (C) - the relative abundance of dominant bacterial orders, (D) - principle coordinate analysis (PCoA) of bacterial and archaeal OTUs based on Bray-Curtis distances, (E) - Shannon diversity index, (F) - UPGMA dendrogram constructed from a Bray-Curtis distances of the bacterial and archaeal OTUs. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced

by 40%), J18-June 2018, O18-October 2018, J19-June 2019, O19-October 2019.

Figure 6. PICRUSt prediction of the bacterial functional profile. (A) - the relative abundance of main KEGG classes, (B) - number of functional operational taxonomic units (OTUs) assigned to main KEGG classes, (C) - number of functional OTUs associated with metabolism of various compounds, (D) - number of functional OTUs associated with phosphorus processes. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), J18 - June 2018, O18- October 2018, J19-June 2019, O19-October 2019. Different letters indicate statistically significant differences ($p < 0.05$) calculated for each sampling time separately.

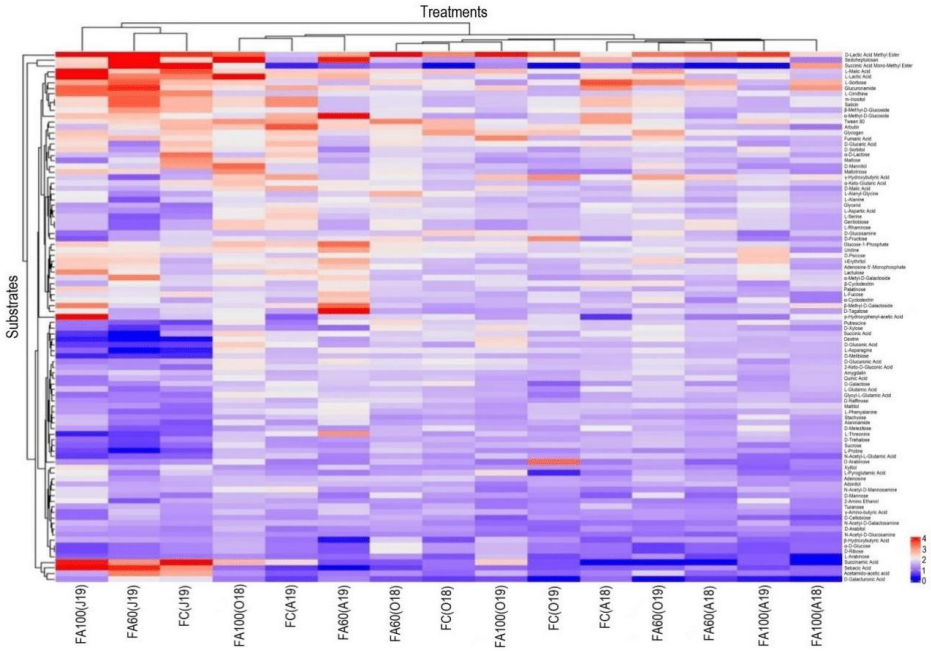
Figure 7. Effects of the phosphorus biofertilizer on the composition of fungal community. (A) - the relative abundance of dominant fungal phyla, (B)- the relative abundance of dominant fungal classes, (C) - the relative abundance of dominant fungal orders, (D) - principle coordinate analysis (PCoA) of fungal OTUs based on Bray-Curtis distances, (E) - Shannon diversity index, (F) - UPGMA dendrogram constructed from a Bray-Curtis distances of the fungal OTUs. Explanation: FC-optimal dose of fertilizer, FA100- optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), J18- June 2018, O18-October 2018, J19-June 2019, O19-October 2019.

Figure 8. Fungal functional profile inferred by FUNGuild. (A) - the relative abundance of fungal trophic modes, (B) - principle component analysis (PCA) of number of operational taxonomic units (OTUs) associated with fungal trophic modes, (C) - the relative abundance of fungal ecological guilds; OTUs with relative abundance equal or greater than 0.5% were analyzed. Explanation: FC-optimal dose of fertilizer, FA100- optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), J18- June 2018, O18-October 2018, J19-June 2019, O19-October 2019.



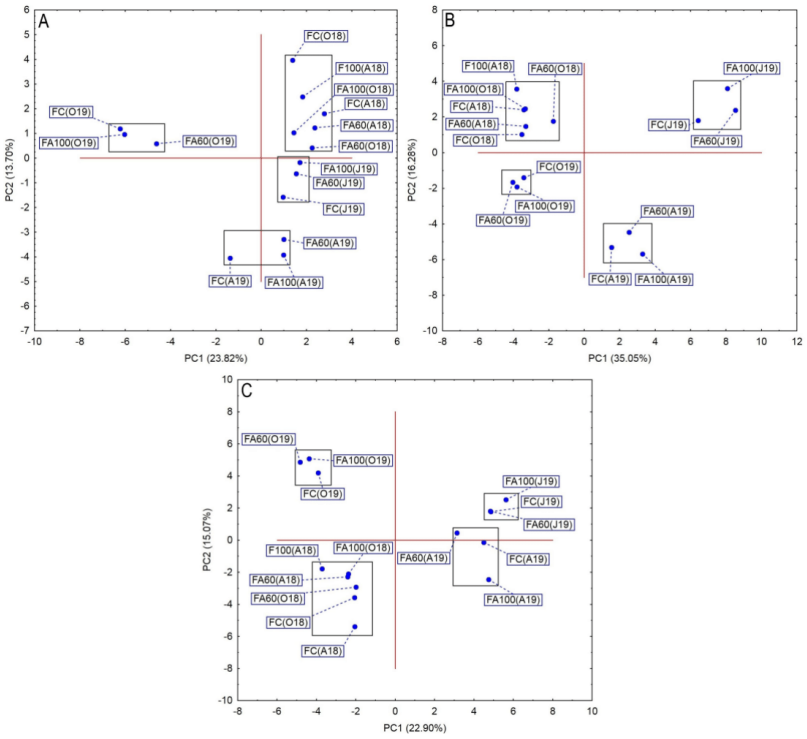
1514

1515 **FIGURE 1**



1516

1517 **FIGURE 2**



1518

1519 **FIGURE 3**

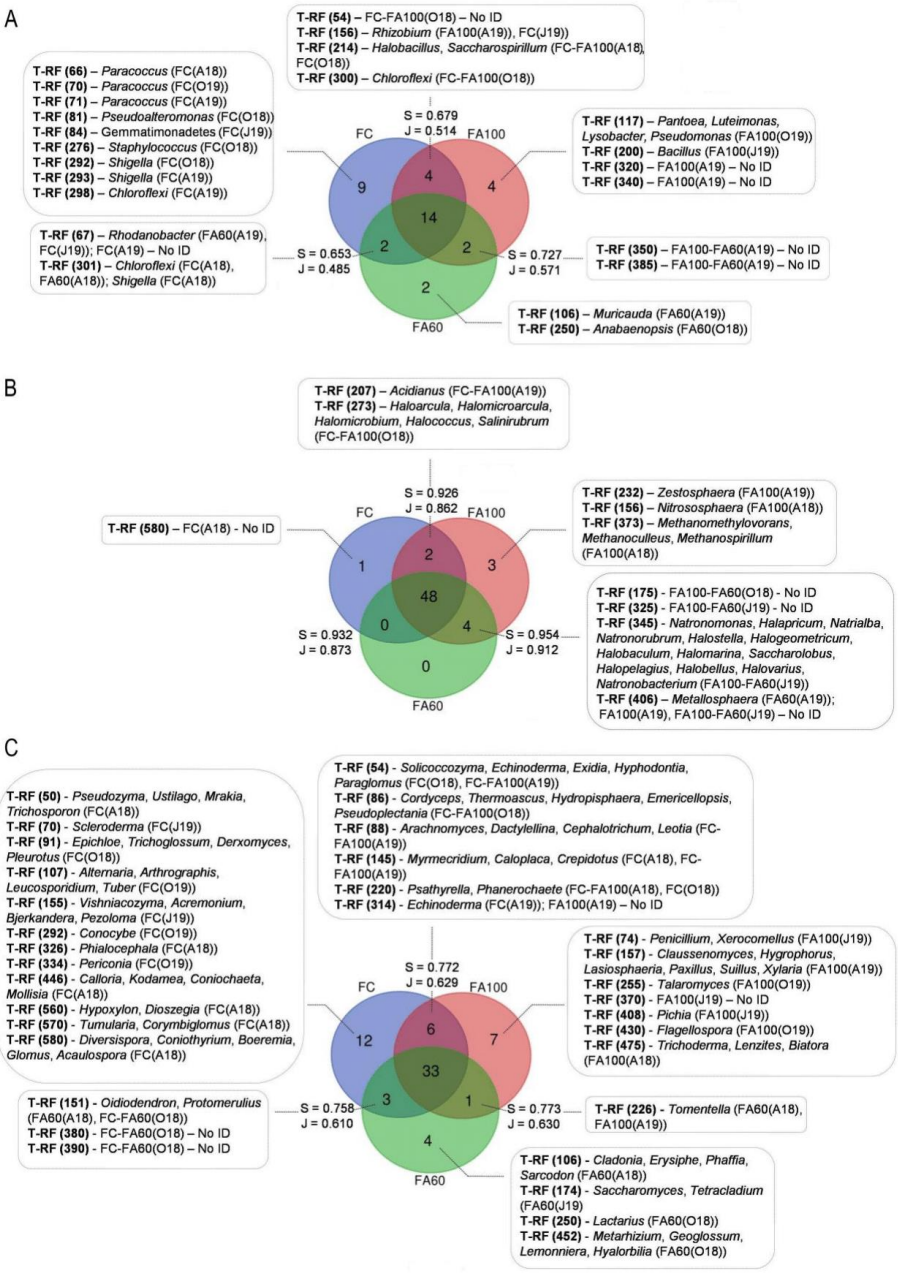
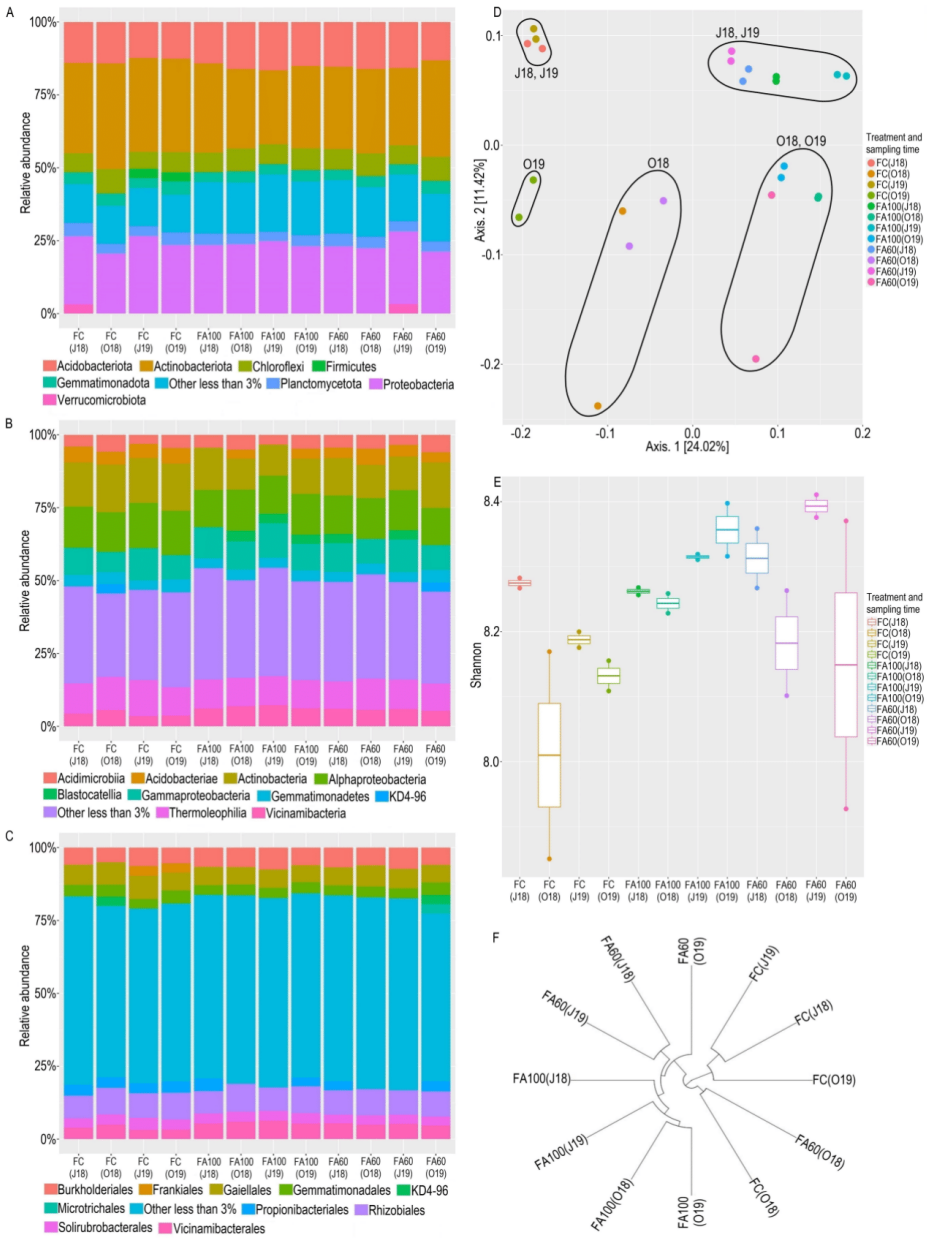

A
T-RF (54) – FC-FA100(O18) – No ID
T-RF (156) – Rhizobium (FA100(A19)), FC(J19))
T-RF (214) – Halobacillus, Saccharospirillum (FC-FA100(A18), FC(O18))
T-RF (300) – Chloroflexi (FC-FA100(O18))
T-RF (66) – Paracoccus (FC(A18))
T-RF (70) – Paracoccus (FC(O19))
T-RF (71) – Paracoccus (FC(A19))
T-RF (81) – Pseudoalteromonas (FC(O18))
T-RF (84) – Gemmatimonadetes (FC(J19))
T-RF (276) – Staphylococcus (FC(O18))
T-RF (292) – Shigella (FC(O18))
T-RF (293) – Shigella (FC(A19))
T-RF (298) – Chloroflexi (FC(A19))
S = 0.679
J = 0.514
FC
FA100
9
4
4
14
2
2
2
FA60
T-RF (117) – Pantoea, Luteimonas, Lysobacter, Pseudomonas (FA100(O19))
T-RF (200) – Bacillus (FA100(J19))
T-RF (320) – FA100(A19) – No ID
T-RF (340) – FA100(A19) – No ID
T-RF (67) – Rhodanobacter (FA60(A19), FC(J19)); FC(A19) – No ID
T-RF (301) – Chloroflexi (FC(A18), FA60(A18)); Shigella (FC(A18))
S = 0.653
J = 0.485
S = 0.727
J = 0.571
T-RF (350) – FA100-FA60(A19) – No ID
T-RF (385) – FA100-FA60(A19) – No ID
T-RF (106) – Muricauda (FA60(A19))
T-RF (250) – Anabaenopsis (FA60(O18))
B
T-RF (207) – Acidianus (FC-FA100(A19))
T-RF (273) – Haloarcula, Halomicroarcula, Halomicrobium, Halococcus, Salinirubrum (FC-FA100(O18))
S = 0.926
J = 0.862
FC
FA100
T-RF (580) – FC(A18) - No ID
1
2
3
48
0
4
0
FA60
T-RF (232) – Zestosphaera (FA100(A19))
T-RF (156) – Nitrososphaera (FA100(A18))
T-RF (373) – Methanomethylovorans, Methanoculleus, Methanospirillum (FA100(A18))
S = 0.932
J = 0.873
S = 0.954
J = 0.912
T-RF (175) - FA100-FA60(O18) - No ID
T-RF (325) - FA100-FA60(J19) - No ID
T-RF (345) - Natronomonas, Halapricum, Natrialba, Natronorubrum, Halostella, Halogeometricum, Halobaculum, Halomarina, Saccharolobus, Halopelagius, Halobellus, Halovarius, Natronobacterium (FA100-FA60(J19))
T-RF (406) – Metallosphaera (FA60(A19)); FA100(A19), FA100-FA60(J19) – No ID
C
T-RF (50) - Pseudozyma, Ustilago, Mrakia, Trichosporon (FC(A18))
T-RF (70) - Scleroderma (FC(J19))
T-RF (91) - Epichloe, Trichoglossum, Derxomyces, Pleurotus (FC(O18))
T-RF (107) - Alternaria, Arthrographis, Leucosporidium, Tuber (FC(O19))
T-RF (155) - Vishniacozyma, Acremonium, Bjerkandera, Pezoloma (FC(J19))
T-RF (292) - Conocybe (FC(O19))
T-RF (326) - Phialocephala (FC(A18))
T-RF (334) - Periconia (FC(O19))
T-RF (446) - Calloria, Kodamea, Coniochaeta, Mollisia (FC(A18))
T-RF (560) - Hypoxylon, Dioszegia (FC(A18))
T-RF (570) - Tumularia, Corymbiglomus (FC(A18))
T-RF (580) - Diversispora, Coniothyrium, Boeremia, Glomus, Acaulospora (FC(A18))
T-RF (54) - Solicoccozyma, Echinoderma, Exidia, Hyphodontia, Paraglomus (FC(O18), FC-FA100(A19))
T-RF (86) - Cordyceps, Thermoascus, Hydropisphaera, Emericellopsis, Pseudoplectania (FC-FA100(O18))
T-RF (88) - Arachnomyces, Dactylellina, Cephalotrichum, Leotia (FC-FA100(A19))
T-RF (145) - Myrmecridium, Caloplaca, Crepidotus (FC(A18), FC-FA100(A19))
T-RF (220) - Psathyrella, Phanerochaete (FC-FA100(A18), FC(O18))
T-RF (314) - Echinoderma (FC(A19)); FA100(A19) – No ID
S = 0.772
J = 0.629
FC
FA100
12
6
7
33
3
1
4
FA60
T-RF (74) - Penicillium, Xerocomellus (FA100(J19))
T-RF (157) - Claussenomyces, Hygrophorus, Lasiosphaeria, Paxillus, Suillus, Xylaria (FA100(A19))
T-RF (255) - Talaromyces (FA100(O19))
T-RF (370) - FA100(J19) – No ID
T-RF (408) - Pichia (FA100(J19))
T-RF (430) - Flagellospora (FA100(O19))
T-RF (475) - Trichoderma, Lenzites, Biatora (FA100(A18))
T-RF (151) - Oidiodendron, Protomerulius (FA60(A18), FC-FA60(O18))
T-RF (380) - FC-FA60(O18) – No ID
T-RF (390) - FC-FA60(O18) – No ID
S = 0.758
J = 0.610
S = 0.773
J = 0.630
T-RF (226) - Tomentella (FA60(A18), FA100(A19))
T-RF (106) - Cladonia, Erysiphe, Phaffia, Sarcodon (FA60(A18))
T-RF (174) - Saccharomyces, Tetracladium (FA60(J19)
T-RF (250) - Lactarius (FA60(O18))
T-RF (452) - Metarhizium, Geoglossum, Lemonniera, Hyalorbilia (FA60(O18))

FIGURE 4



1522

1523 **FIGURE 5**

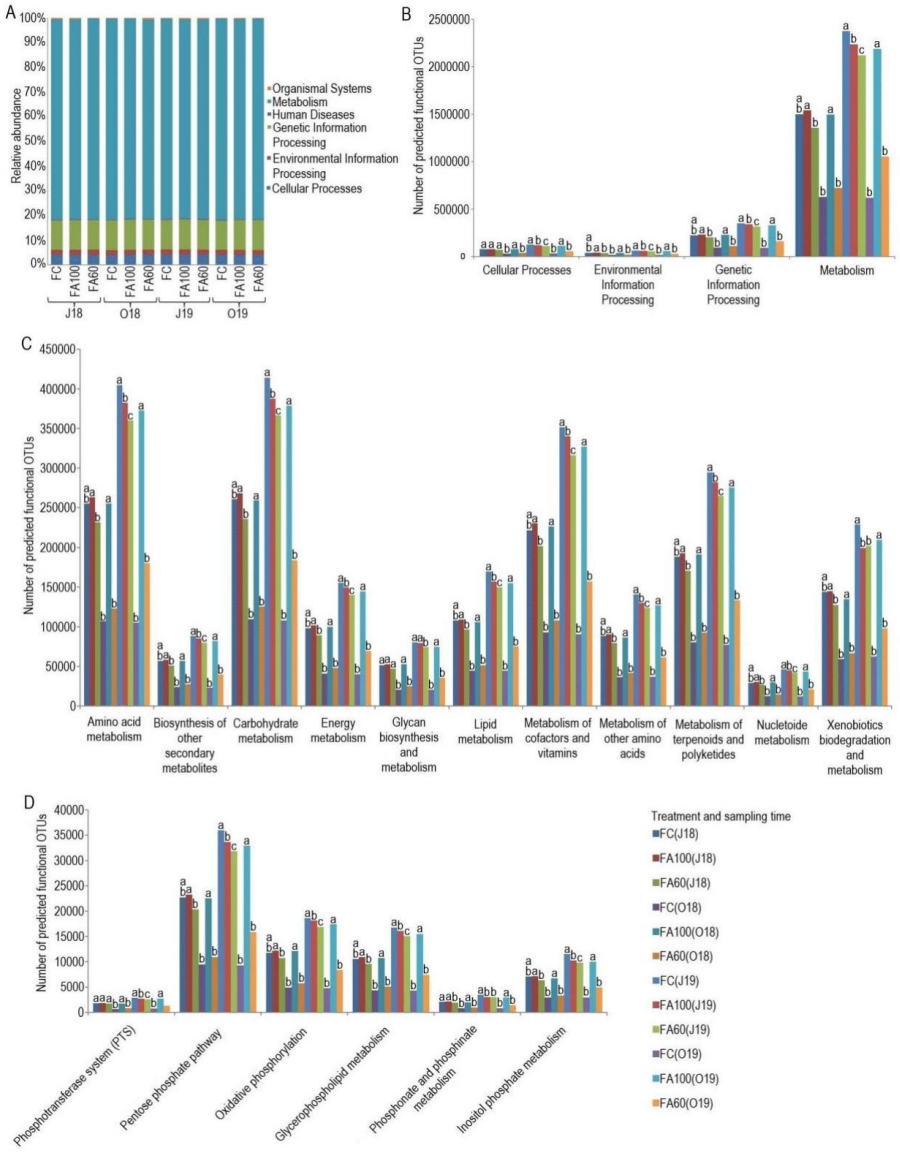
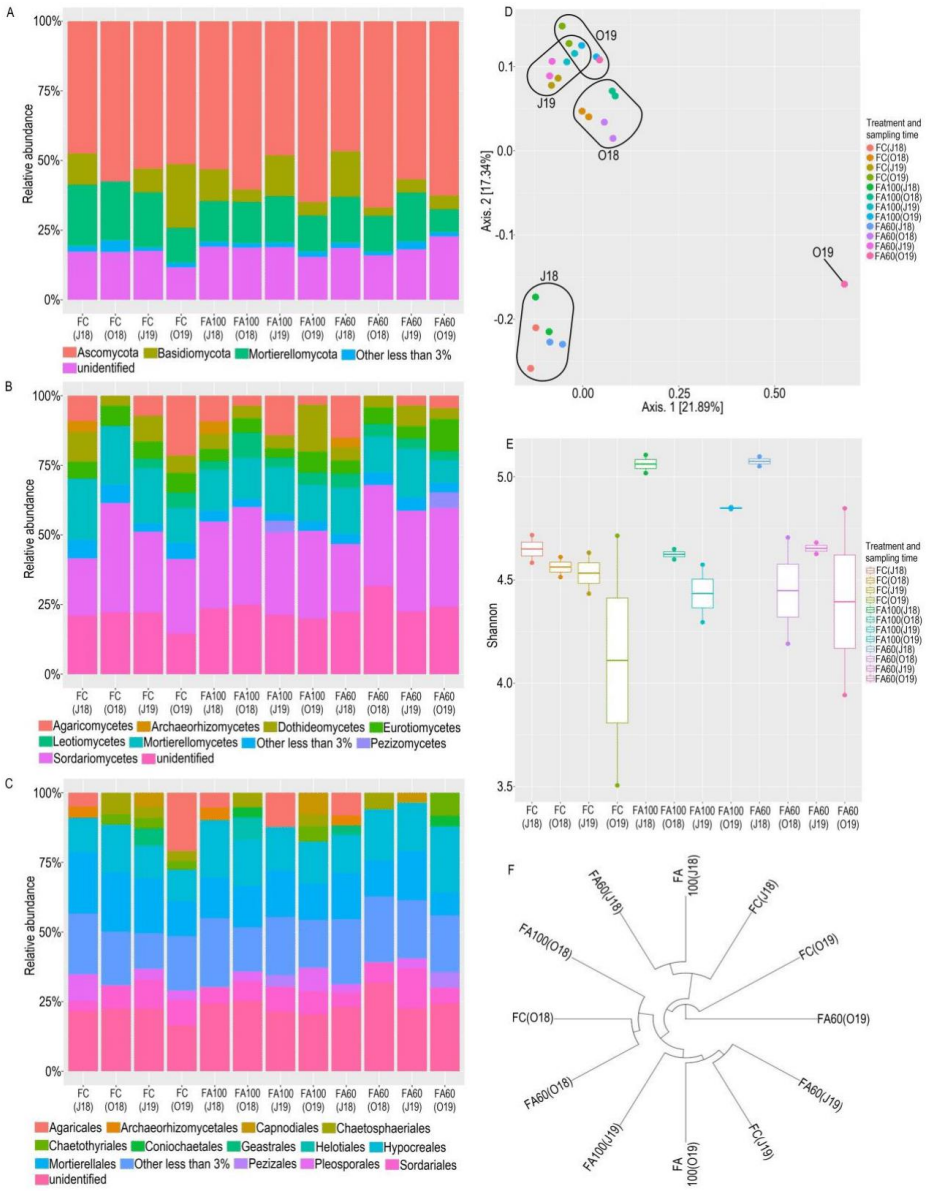

A
Relative abundance
Organismal Systems
Metabolism
Human Diseases
Genetic Information Processing
Environmental Information Processing
Cellular Processes
FC
FA100
FA60
J18
O18
J19
O19
B
Number of predicted functional OTUs
Cellular Processes
Environmental Information Processing
Genetic Information Processing
Metabolism
C
Number of predicted functional OTUs
Amino acid metabolism
Biosynthesis of other secondary metabolites
Carbohydrate metabolism
Energy metabolism
Glycan biosynthesis and metabolism
Lipid metabolism
Metabolism of cofactors and vitamins
Metabolism of other amino acids
Metabolism of terpenoids and polyketides
Nucleotide metabolism
Xenobiotics biodegradation and metabolism
D
Number of predicted functional OTUs
Phosphotransferase system (PTS)
Pentose phosphate pathway
Oxidative phosphorylation
Glycerophospholipid metabolism
Phosphonate and phosphinate metabolism
Inositol phosphate metabolism
Treatment and sampling time
FC(J18)
FA100(J18)
FA60(J18)
FC(O18)
FA100(O18)
FA60(O18)
FC(J19)
FA100(J19)
FA60(J19)
FC(O19)
FA100(O19)
FA60(O19)

FIGURE 6



1527

1528 **FIGURE 7**

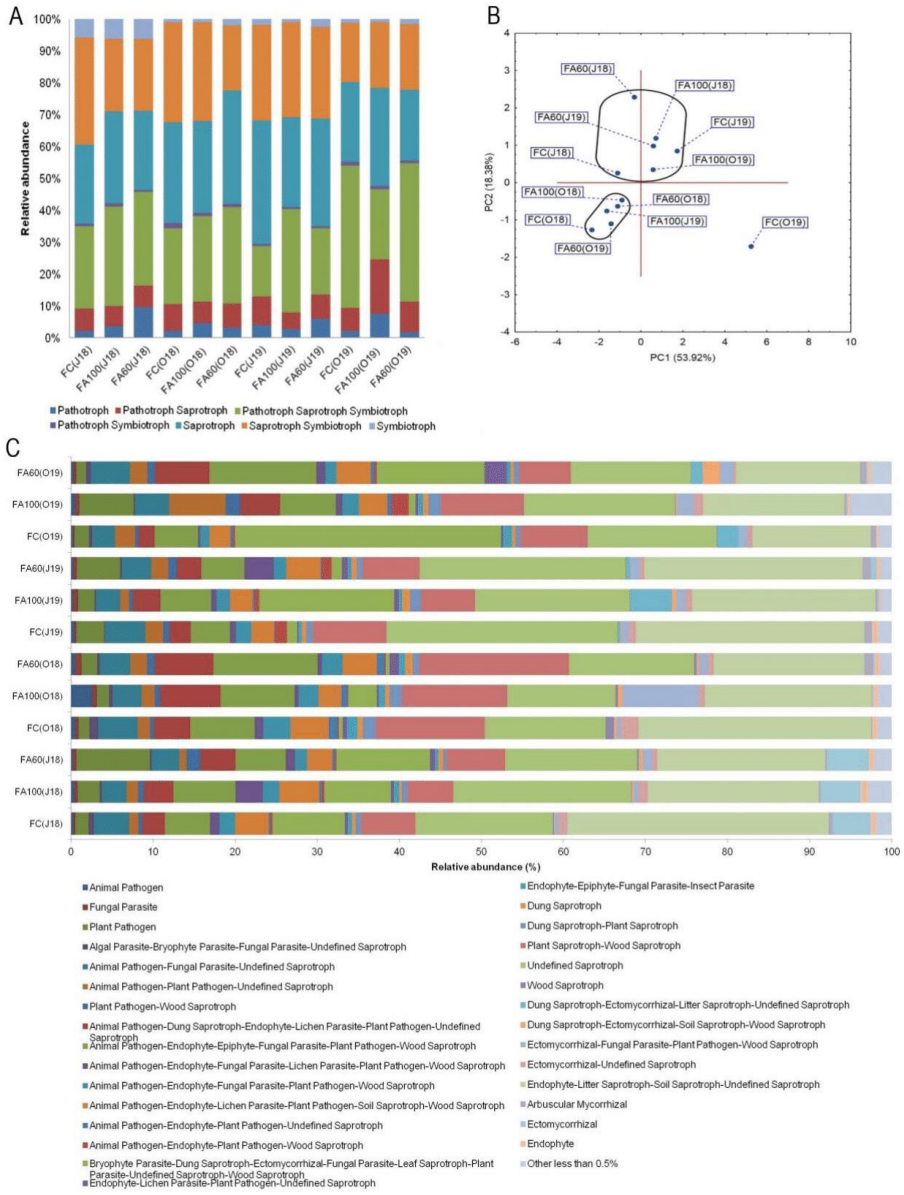

A
Relative abundance
FC(J18)
FA100(J18)
FA60(J18)
FC(O18)
FA100(O18)
FA60(O18)
FC(J19)
FA100(J19)
FA60(J19)
FC(O19)
FA100(O19)
FA60(O19)
Pathotroph
Pathotroph Saprotroph
Pathotroph Saprotroph Symbiotroph
Pathotroph Symbiotroph
Saprotroph
Saprotroph Symbiotroph
Symbiotroph
B
PC2 (18.38%)
PC1 (53.92%)
C
Relative abundance (%)
Animal Pathogen
Fungal Parasite
Plant Pathogen
Algal Parasite-Bryophyte Parasite-Fungal Parasite-Undefined Saprotroph
Animal Pathogen-Fungal Parasite-Undefined Saprotroph
Animal Pathogen-Plant Pathogen-Undefined Saprotroph
Plant Pathogen-Wood Saprotroph
Animal Pathogen-Dung Saprotroph-Endophyte-Lichen Parasite-Plant Pathogen-Undefined Saprotroph
Animal Pathogen-Endophyte-Epiphyte-Fungal Parasite-Plant Pathogen-Wood Saprotroph
Animal Pathogen-Endophyte-Fungal Parasite-Lichen Parasite-Plant Pathogen-Wood Saprotroph
Animal Pathogen-Endophyte-Fungal Parasite-Plant Pathogen-Wood Saprotroph
Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Soil Saprotroph-Wood Saprotroph
Animal Pathogen-Endophyte-Plant Pathogen-Undefined Saprotroph
Animal Pathogen-Endophyte-Plant Pathogen-Wood Saprotroph
Bryophyte Parasite-Dung Saprotroph-Ectomycorrhizal-Fungal Parasite-Leaf Saprotroph-Plant Parasite-Undefined Saprotroph-Wood Saprotroph
Endophyte-Lichen Parasite-Plant Pathogen-Undefined Saprotroph
Endophyte-Epiphyte-Fungal Parasite-Insect Parasite
Dung Saprotroph
Dung Saprotroph-Plant Saprotroph
Plant Saprotroph-Wood Saprotroph
Undefined Saprotroph
Wood Saprotroph
Dung Saprotroph-Ectomycorrhizal-Litter Saprotroph-Undefined Saprotroph
Dung Saprotroph-Ectomycorrhizal-Soil Saprotroph-Wood Saprotroph
Ectomycorrhizal-Fungal Parasite-Plant Pathogen-Wood Saprotroph
Ectomycorrhizal-Undefined Saprotroph
Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph
Arbuscular Mycorrhizal
Ectomycorrhizal
Endophyte
Other less than 0.5%

FIGURE 8

TABLE CAPTIONS

Table 1. Variations in soil enzymatic activity, maize yield and phytoavailable phosphorus content. Different letters indicate statistically significant differences ($p < 0.05$) calculated for each sampling time separately. ± means standard deviation. Explanation: FC-optimal dose of fertilizer, FA100-optimal dose of fertilizer enriched with microorganisms, FA60-fertilizer enriched with microorganisms (dose reduced by 40%), A18-April 2018, O18-October 2018, A19-April 2019, J19-June 2019, O19-October 2019, PNP-p-nitrophenol.

1553 **TABLE 1.**

	Sampling time/Treatment														
	A18			O18			A19			J19			O19		
	FC	FA100	FA60	FC	FA100	FA60	FC	FA100	FA60	FC	FA100	FA60	FC	FA100	FA60
β-glucosidase (mg PNP kg^{-1} hr^{-1})	1.95 ± 0.05 a	1.88 ± 0.19 a	1.33 ± 0.43 a	1.18 ± 0.15 b	0.95 ± 0.06 b	1.61 ± 0.17 a	1.36 ± 0.64 a	1.85 ± 0.63 a	1.84 ± 0.54 a	1.94 ± 0.33 a	2.09 ± 0.22 a	1.89 ± 0.29 a	2.86 ± 0.50 a	2.46 ± 0.90 a	2.82 ± 0.27 a
Urease (µg N-NH_4 kg^{-1} hr^{-1})	52.47 ± 11.02 a	50.31 ± 0.93 a	50.94 ± 6.06 a	61.34 ± 1.73 a	54.56 ± 6.27 a	62.53 ± 0.79 a	86.37 ± 16.51 a	84.28 ± 4.83 a	95.84 ± 9.77 a	48.71 ± 2.13 a	59.26 ± 13.51 a	55.57 ±5.04 a	71.89 ± 7.77 a	72.31 ± 7.38 a	54.53 ± 4.59 b
Protease (mg tyrosine kg^{-1} hr^{-1})	13.44 ± 1.81 b	19.39 ± 2.43 a	17.14 ± 0.37 ab	7.67 ± 0.22 a	8.55 ± 1.30 a	8.93 ± 0.74 a	6.23 ± 1.22 b	11.67 ± 2.39 a	10.97 ± 1.21 a	10.15 ± 0.69 b	20.42 ± 2.97 a	15.12 ± 4.90 ab	8.81 ± 1.11 a	9.86 ± 1.08 a	8.1 ± 0.21 a
Acid phosphomonoesterase (mmol PNP kg^{-1} hr^{-1})	31.14 ± 1.08 a	28.75 ± 2.22 a	32.29 ± 6.14 a	23.82 ± 6.16 a	24.54 ± 2.97 a	27.39 ± 4.93 a	8.73 ± 1.28 b	16.03 ± 2.66 a	17.9 ± 0.96 a	28.96 ± 3.74 a	34.02 ± 4.83 a	39.81 ± 7.25 a	39.86 ± 2.96 a	36.78 ± 1.22 a	14.8 ± 4.06 b
Maize yield (t ha^{-1})	-	-	-	13.53 ± 2.91 a	12.42 ± 2.15 a	13.60 ± 0.87 a	-	-	-	-	-	-	14.18 ± 2.29 a	14.78 ± 1.90 a	13.89 ± 0.88 a
P_2O_5 content (mg 100 g^{-1})	3.93 ± 0.25 b	5.30 ± 0.20 a	3.47 ± 0.21 b	4.37 ± 0.25 b	5.47 ± 0.21 a	2.83 ± 0.15 c	3.83 ± 0.25 b	5.90 ± 0.17 a	3.13 ± 0.12 c	4.67 ± 1.27 b	7.00 ± 0.10 a	3.53 ± 0.06 b	3.97 ± 0.21 b	5.33 ± 0.23 a	4.03 ± 0.23 b

AUTHOR CONTRIBUTIONS

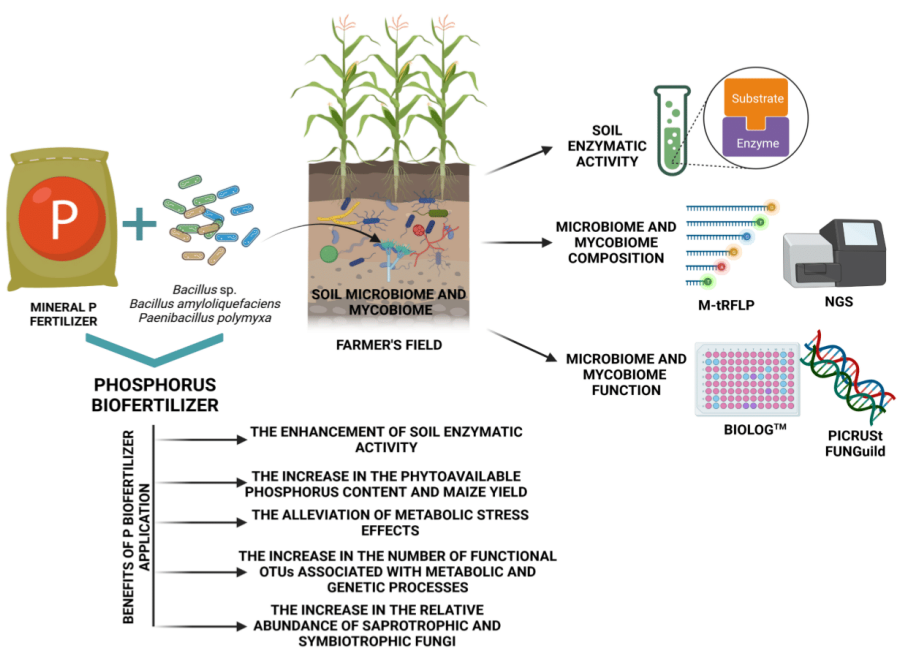
Mateusz Mącik: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft, Writing- review & editing

Agata Gryta: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing- review & editing

Lidia Sas-Paszt: Conceptualization, Funding acquisition, Project administration

Magdalena Frąc: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing- review & editing

1576 **GRAPHICAL ABSTRACT**



1577

1578 The investigation of soil microbial diversity after phosphorus biofertilizer application

9.1. Materiały uzupełniające manuskryptu publikacji P.4

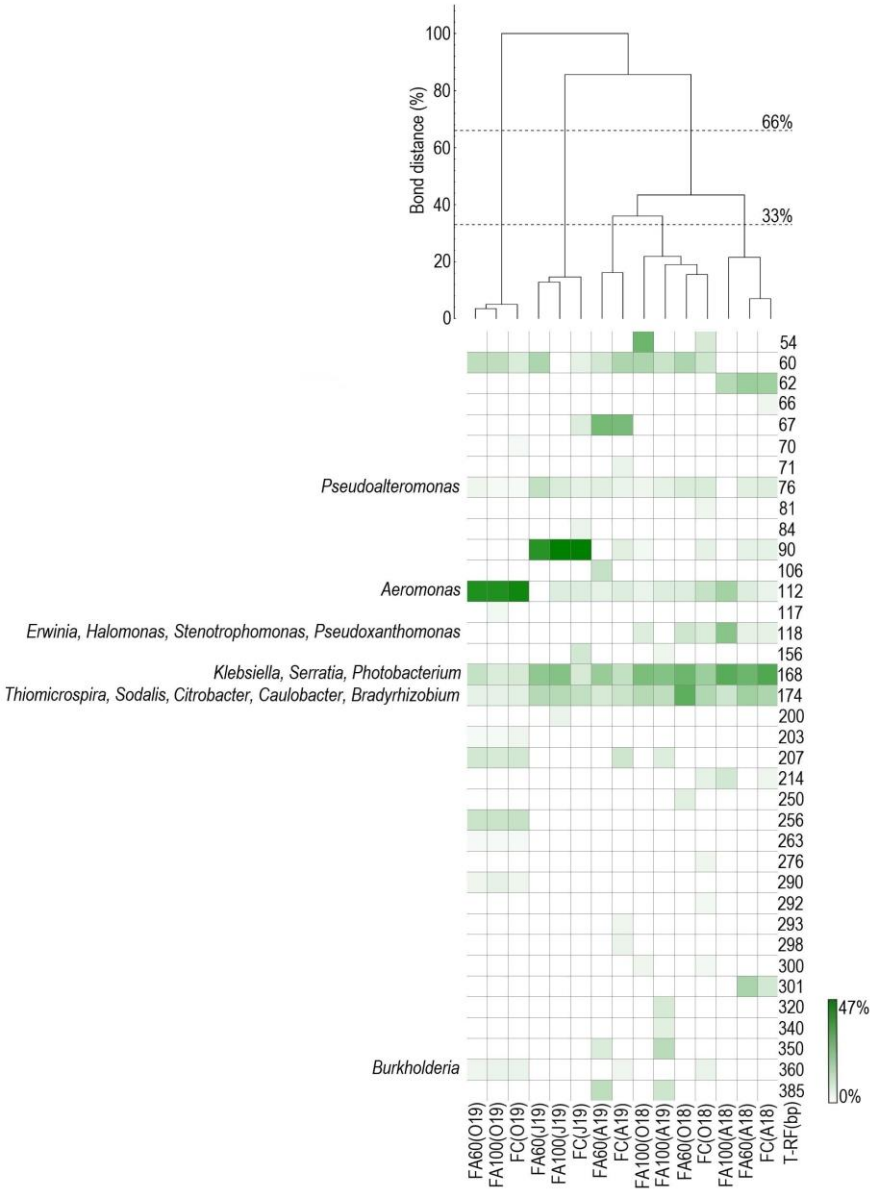


Figure S1. Dendrogram based on the t-RFLP profile of soil bacterial community, heat map representing the relative abundance of terminal restriction fragments (T-RFs) and identification of microorganisms based on the size of selected T-RFs. Explanation: FC - optimal dose of fertilizer, FA100 - optimal dose of fertilizer enriched with microorganisms, FA60 - fertilizer enriched with microorganisms (dose reduced by 40%), A18 - April 2018, O18 - October 2018, A19 - April 2019, J19 - June 2019, O19 - October 2019.

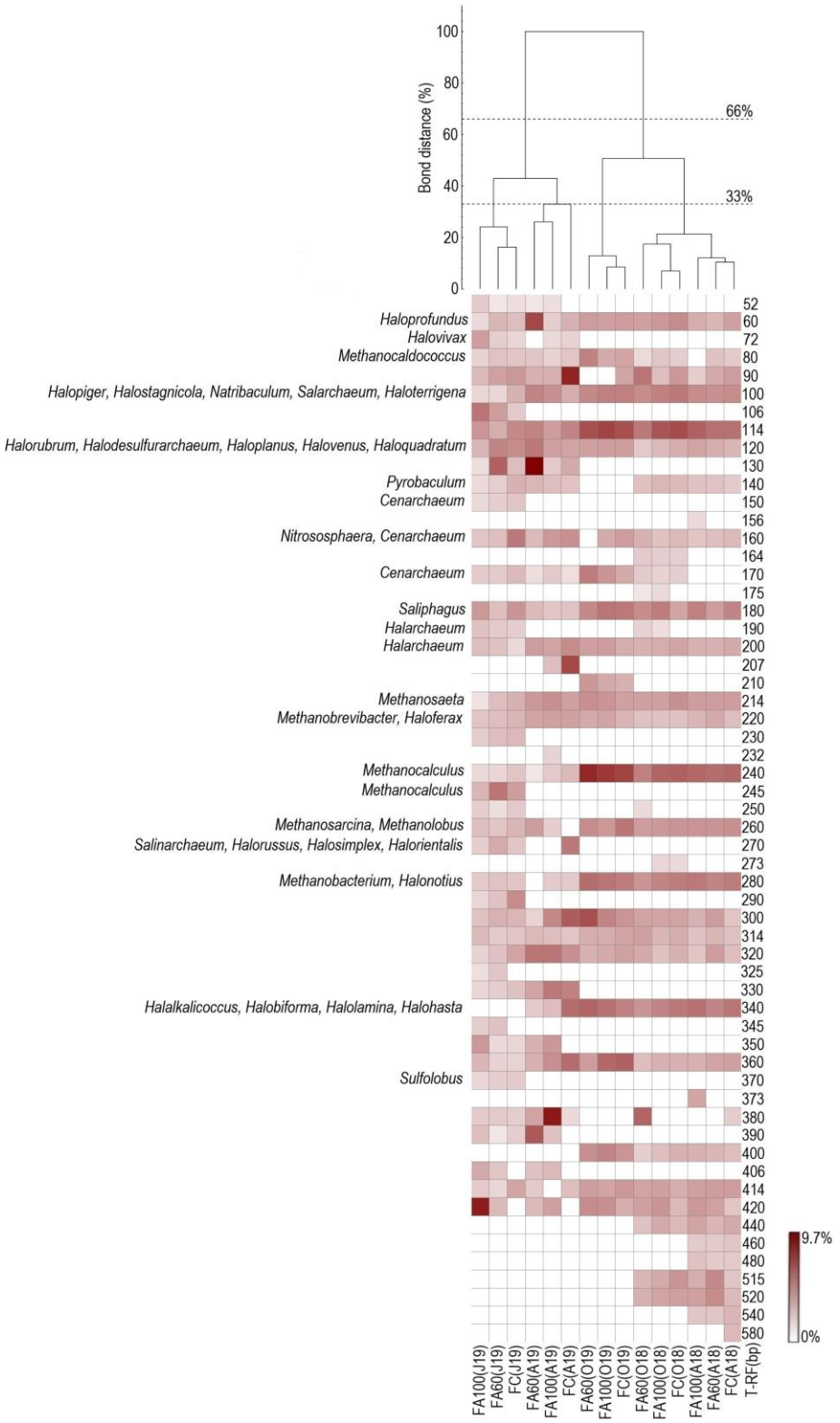


Figure S2. Dendrogram based on the t-RFLP profile of soil archaeal community, heat map representing the relative abundance of terminal restriction fragments (T-RFs) and identification of microorganisms based on the size of selected T-RFs. Explanation as in the Figure S1.

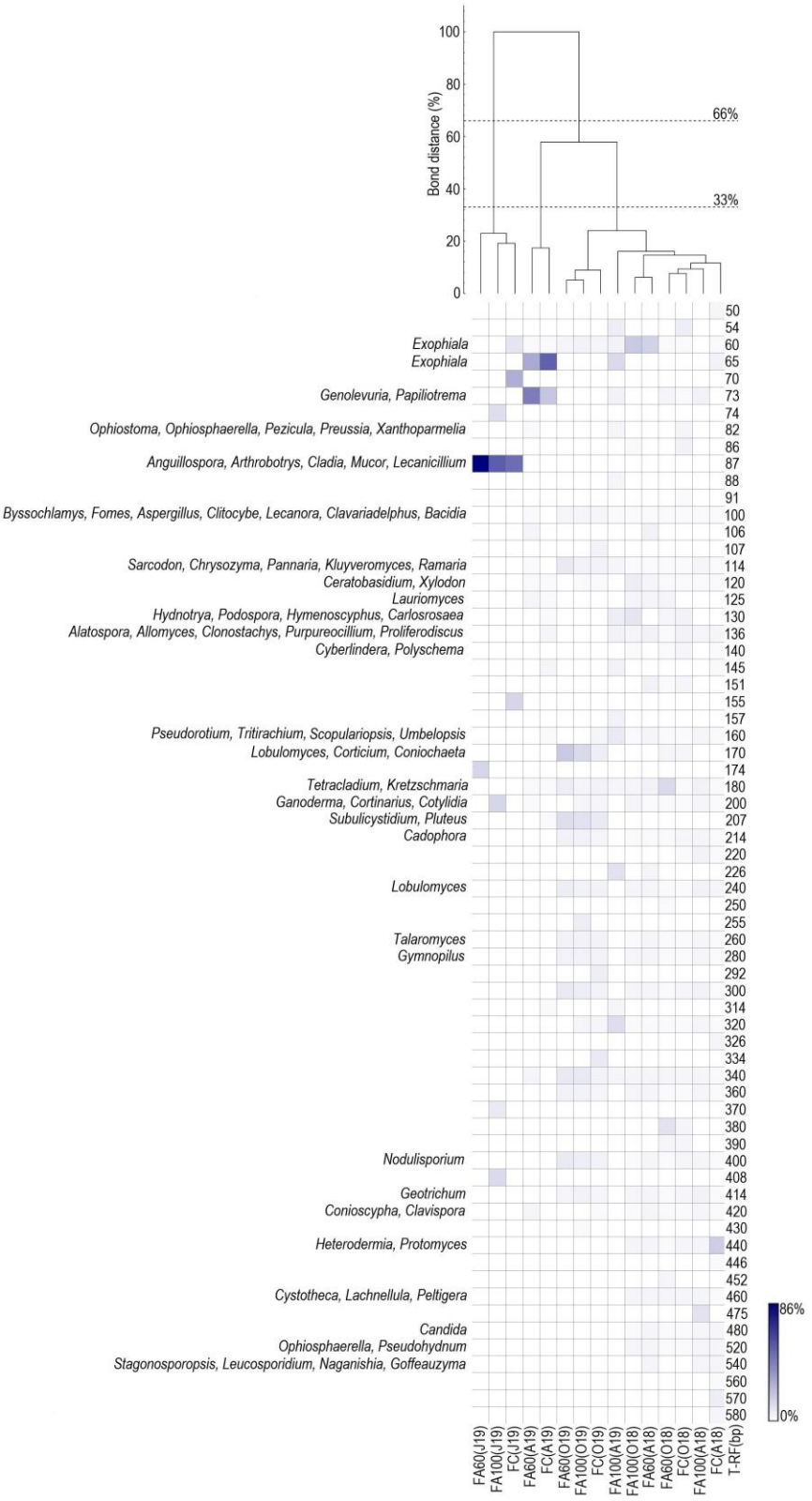


Figure S3. Dendrogram based on the t-RFLP profile of soil fungal community, heat map representing the relative abundance of terminal restriction fragments (T-RFs) and identification of microorganisms based on the size of selected T-RFs. Explanation as in the Figure S1.

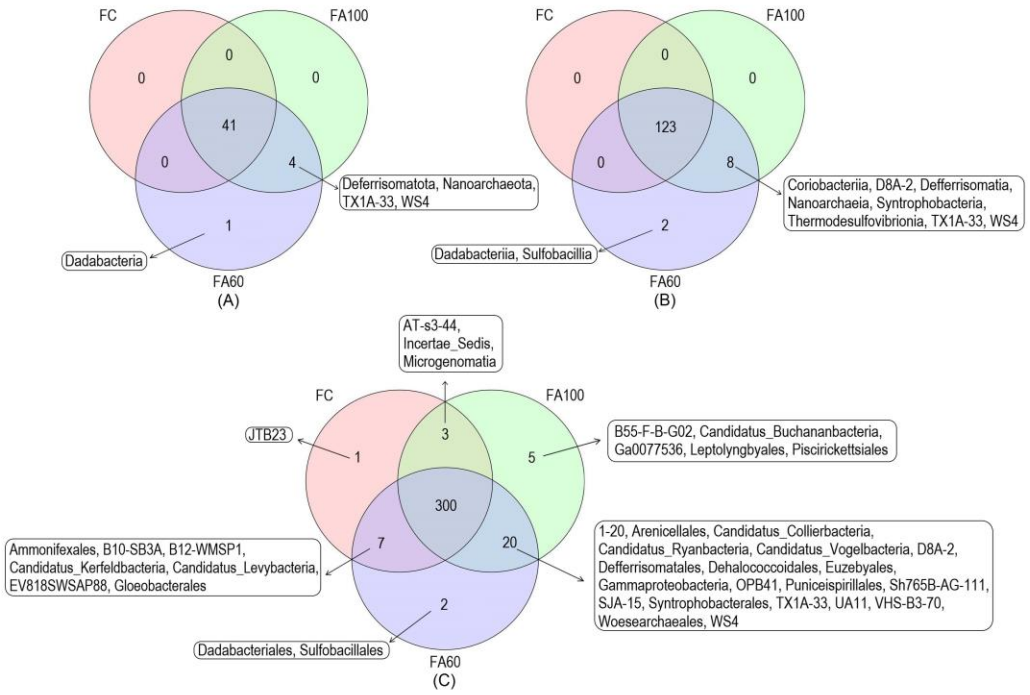


Figure S4. The distribution of bacterial and archaeal operational taxonomic units (OTUs) identified at phylum (A), class (B) and order (C) level. Explanation as in the Figure S1.

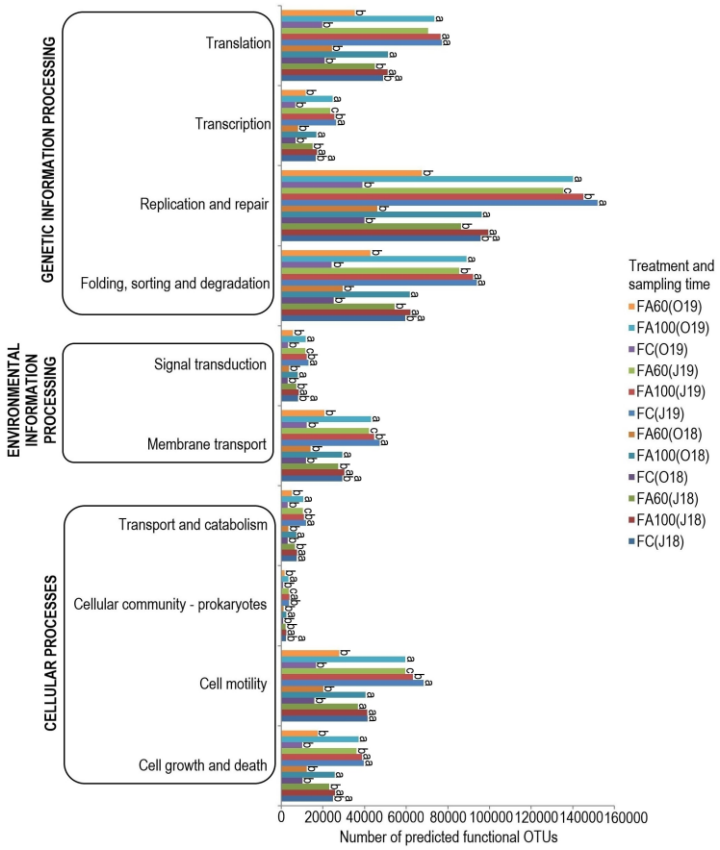


Figure S5. The number of functional operational taxonomic units (OTUs) assigned to pathways associated with Genetic Information Processing, Environmental Information Processing and Cellular Processes. Explanation as in the Figure S1. Different letters indicate statistically significant differences ($p < 0.05$) calculated for each sampling time separately.

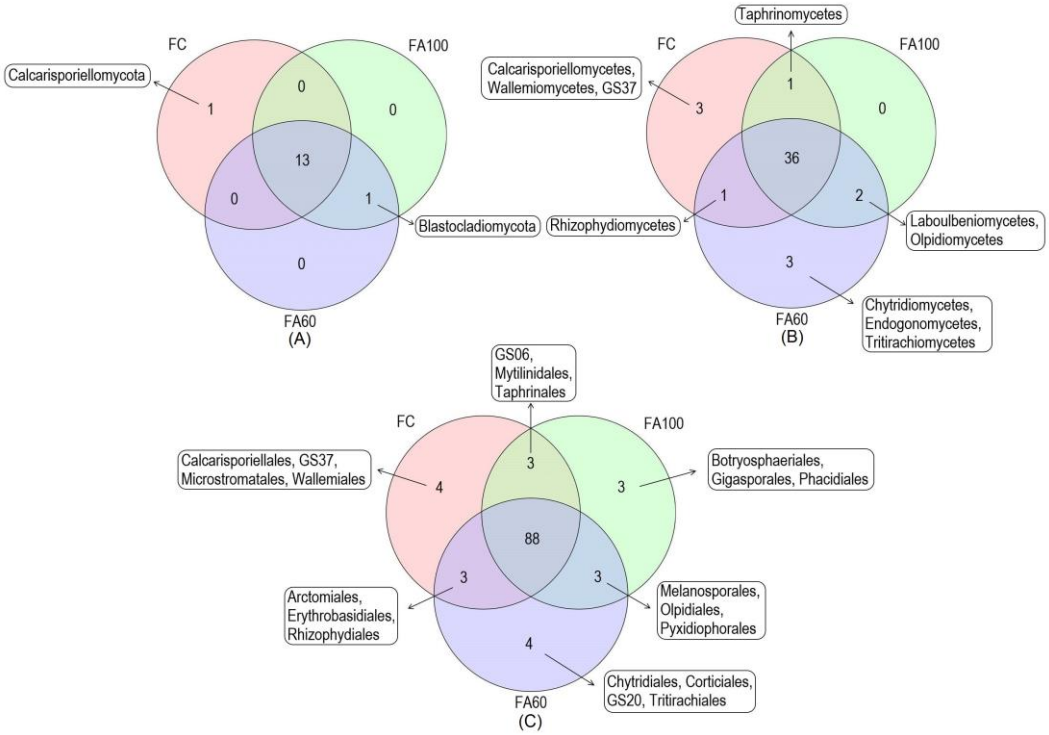


Figure S6. The distribution of fungal operational taxonomic units (OTUs) identified at phylum (A), class (B) and order (C) level. Explanation as in the Figure S1.

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11. Oświadczenia

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Oświadczenie

Niniejszym oświadczam, że w niżej wymienionych pracach inicjatywa podjętych badań jest moim wkładem intelektualnym, a mój udział obejmował:

P1: **Mącik, M.**, Gryta, A., Frąc M., 2020. **Biofertilizers in agriculture: An overview of concepts, strategies and effects on soil microorganisms**. *Advances in Agronomy* 162, 31-87.

- współudział w opracowaniu koncepcji manuskryptu,
- szczegółową analizę danych literaturowych,
- przygotowanie rysunków oraz tabel do manuskryptu,
- przygotowanie manuskryptu,
- uczestnictwo w odpowiedziach do recenzentów oraz edycji manuskryptu.

P2: **Mącik, M.**, Gryta, A., Sas-Paszt, L., Frąc, M., 2020. **The Status of Soil Microbiome as Affected by the Application of Phosphorus Biofertilizer: Fertilizer Enriched with Beneficial Bacterial Strains**. *International Journal of Molecular Sciences* 21, *8003*

- współudział w opracowaniu koncepcji badań,
- współudział w założeniu doświadczeń polowych,
- współudział w zebraniu próbek gleby,
- współudział w badaniach różnorodności funkcjonalnej i genetycznej zbiorowisk mikroorganizmów glebowych oraz aktywności enzymatycznej gleby,
- analizę i interpretację wyników badań,
- szczegółową analizę danych literaturowych,
- przygotowanie rysunków oraz tabel,
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INSTYTUT AGROFIZYKI PAN

P3: **Mącik, M.**, Gryta, A., Sas-Paszt, L., Frąc, M., 2022. **Composition, activity and diversity of bacterial and fungal communities responses to inputs of phosphorus fertilizer enriched with beneficial microbes in degraded Brunic Arenosol**. *Land Degradation & Development* 33(6), 844-865

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Mateusz Mącik

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Oświadczenie

Niniejszym oświadczam, że w niżej wymienionym manuskrypcie artykułu naukowego inicjatywa podjętych badań jest moim wkładem intelektualnym, a mój udział obejmował:

P4: **Mącik, M.**, Gryta, A., Sas-Paszt, L., Frąc, M., **New insight into the soil bacterial and fungal microbiome after phosphorus biofertilizer application as important driver of regenerative agriculture including biodiversity loss reversing and soil health restoration.**

- współudział w opracowaniu koncepcji badań,
- współudział w założeniu doświadczeń polowych,
- współudział w zebraniu próbek gleby,
- współudział w badaniach różnorodności funkcjonalnej i genetycznej zbiorowisk mikroorganizmów glebowych oraz aktywności enzymatycznej gleby,
- analizę i interpretację wyników badań,
- szczegółową analizę danych literaturowych,
- przygotowanie rysunków oraz tabel,
- przygotowanie manuskryptu.

Mateusz Mącik

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Oświadczenie

Niniejszym oświadczam, że w poniższych pracach inicjatywa podjętych badań jest wkładem intelektualnym mgra Mateusz Mącika.

P1: Mącik, M., Gryta, A., Frąc M., 2020. Biofertilizers in agriculture: An overview of concepts, strategies and effects on soil microorganisms. *Advances in Agronomy* 162, 31-87.

P2: Mącik, M., Gryta, A., Sas-Paszt, L., Frąc, M., 2020. The Status of Soil Microbiome as Affected by the Application of Phosphorus Biofertilizer: Fertilizer Enriched with Beneficial Bacterial Strains. *International Journal of Molecular Sciences* 21, *8003*.

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Mój wkład w powyższe prace polegał na:

- określeniu problematyki i zakresu prac,
- współudziale w opracowaniu koncepcji badań,
- zapewnieniu materiałów i aparatury umożliwiających realizację badań,
- kierowaniu przebiegiem badań zgodnie z założeniami projektu,
- nadzorowaniu procesu przygotowania manuskryptu,
- udziale w analizie i interpretacji wyników badań,
- edycji i korekcie manuskryptu,
- uczestnictwie w odpowiedziach na recenzje.

Jednocześnie wyrażam zgodę, aby prace zostały wykorzystane w rozprawie doktorskiej mgra Mateusza Mącika.

Magdalena Frąc

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Oświadczenie

Niniejszym oświadczam, że w niżej wymienionym manuskrypcie artykułu naukowego inicjatywa podjętych badań jest wkładem intelektualnym mgra Mateusza Mącika.

P4: Mącik, M., Gryta, A., Sas-Paszt, L., Frąc, M., New insight into the soil bacterial and fungal microbiome after phosphorus biofertilizer application as important driver of regenerative agriculture including biodiversity loss reversing and soil health restoration.

Mój wkład w powyższy manuskrypt polegał na:

- określeniu problematyki i zakresu prac,
- współudziale w opracowaniu koncepcji badań,
- zapewnieniu materiałów i aparatury umożliwiających realizację badań,
- kierowaniu przebiegiem badań zgodnie z założeniami projektu,
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- udziale w analizie i interpretacji wyników badań,
- edycji i korekcie manuskryptu.

Jednocześnie wyrażam zgodę, aby powyższy manuskrypt artykułu naukowego został wykorzystany w rozprawie doktorskiej mgra Mateusza Mącika.

Magdalena Frąc

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Lublin, 31.08.2022

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Oświadczenie

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Mój wkład w powyższe prace polegał na:

- współudziale w opracowaniu koncepcji badań,
- współudziale w założeniu doświadczeń polowych, pobieraniu próbek gleby i badaniach różnorodności funkcjonalnej i genetycznej zbiorowisk mikroorganizmów glebowych i aktywności enzymatycznej gleby
- kierowaniu przebiegiem badań laboratoryjnych zgodnie z założeniami projektu,
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- edycji i korekcie manuskryptu,
- uczestnictwo w odpowiedziach na recenzje.

Jednocześnie wyrażam zgodę, aby prace zostały wykorzystane w rozprawie doktorskiej mgra Mateusza Mącika.

Gryta Agata

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Oświadczenie

Niniejszym oświadczam, że w niżej wymienionym manuskrypcie artykułu naukowego inicjatywa podjętych badań jest wkładem intelektualnym mgra Mateusza Mącika.

P4: Mącik, M., Gryta, A., Sas-Paszt, L., Frąc, M., New insight into the soil bacterial and fungal microbiome after phosphorus biofertilizer application as important driver of regenerative agriculture including biodiversity loss reversing and soil health restoration.

Mój wkład w powyższą pracę polegał na:

- współudziale w opracowaniu koncepcji badań,
- współudziale w założeniu doświadczeń polowych, pobieraniu próbek gleby i badaniach różnorodności funkcjonalnej i genetycznej zbiorowisk mikroorganizmów glebowych i aktywności enzymatycznej gleby
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- udziale w analizie i interpretacji wyników badań,
- edycji i korekcie manuskryptu.

Jednocześnie wyrażam zgodę, aby powyższy manuskrypt artykułu naukowego został wykorzystany w rozprawie doktorskiej mgra Mateusz Mącika.

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Oświadczenie

Niniejszym oświadczam, że w poniższych pracach inicjatywa podjętych badań jest wkładem intelektualnym mgra Mateusza Mącika.

P2: Mącik, M., Gryta, A., Sas-Paszt, L., Frąc, M., 2020. The Status of Soil Microbiome as Affected by the Application of Phosphorus Biofertilizer: Fertilizer Enriched with Beneficial Bacterial Strains. *International Journal of Molecular Sciences* 21, *8003*.

P3: Mącik, M., Gryta, A., Sas-Paszt, L., Frąc, M., 2022. Composition, activity and diversity of bacterial and fungal communities responses to inputs of phosphorus fertilizer enriched with beneficial microbes in degraded Brunic Arenosol. *Land Degradation & Development* 33(6), 844-865.

Mój wkład w powyższe prace polegał na:

- współudziale w opracowaniu koncepcji badań,
- pozyskaniu środków finansowych umożliwiających realizację badań,
- edycji i korekcie manuskryptu.

Jednocześnie wyrażam zgodę, aby prace zostały wykorzystane w rozprawie doktorskiej mgra Mateusza Mącika.

INSTYTUT OGRODNICTWA
PAŃSTWOWY INSTYTUT BADAWCZY
Zakład Mikrobiologii i Ryzosfery
Kierownik Zakładu Mikrobiologii i Ryzosfery
Prof. dr hab. Lidia Sas-Paszt

Lidia Sas-Paszt

Lublin, 31.08.2022

prof. dr hab. Lidia Sas-Paszt
Zakład Mikrobiologii i Ryzosfery
Instytut Ogrodnictwa – Państwowy Instytut Badawczy
ul. Pomologiczna 18, 96-100 Skierniewice

Oświadczenie

Niniejszym oświadczam, że w niżej wymienionym manuskrypcie artykułu naukowego inicjatywa podjętych badań jest wkładem intelektualnym mgra Mateusza Mącika.

P4: Mącik, M., Gryta, A., Sas-Paszt, L., Frąc, M., New insight into the soil bacterial and fungal microbiome after phosphorus biofertilizer application as important driver of regenerative agriculture including biodiversity loss reversing and soil health restoration.

Mój wkład w powyższe manuskrypt polegał na:

- współudziale w opracowaniu koncepcji badań,
- pozyskaniu środków finansowych umożliwiających realizację badań,
- edycji i korekcie manuskryptu.

Jednocześnie wyrażam zgodę, aby prace zostały wykorzystane w rozprawie doktorskiej mgra Mateusza Mącika.

INSTYTUT OGRODNICTWA
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Kierownik Zakładu Mikrobiologii i Ryzosfery
Prof. dr hab. Lidia Sas-Paszt

12. Aneks – życiorys naukowy

WYKSZTAŁCENIE

- 2018 - obecnie - studia doktoranckie (III°) w Instytucie Agrofizyki im. Bohdana Dobrzańskiego Polskiej Akademii Nauk w Lublinie. Temat rozprawy doktorskiej: "Wpływ fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie na aktywność i bioróżnorodność mikroorganizmów glebowych."

- 2015 - 2017 - studia magisterskie (II°) na kierunku biotechnologia medyczna na Uniwersytecie Marii-Curie Skłodowskiej w Lublinie. Temat pracy magisterskiej: "Charakterystyka molekularna genu *pssA* uczestniczącego w syntezie egzopolisacharydu *Rhizobium leguminosarum* bv. trifolii."

- 2012 - 2015 - studia licencjackie (I°) na kierunku biotechnologia na Uniwersytecie Marii-Curie Skłodowskiej w Lublinie. Temat pracy licencjackiej: "Sygnały molekularne, struktury powierzchniowe i białka zewnątrzkomórkowe rizobiów uczestniczące w symbiozie z roślinami bobowatymi (Fabaceae)."

OSIĄGNIĘCIA ZWIĄZANE Z ROZPRAWĄ DOKTORSKĄ

Referaty wygłoszone na konferencjach międzynarodowych

- Mącik M., Gryta A., Frąc M., 2020, Genetic diversity of microbial communities in soils amended with biofertilizers, 19th International Workshop for Young Scientists "BioPhys Spring 2020", May 19-21, Prague, Czech Republic

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2021, Seasonal variations in the genetic diversity of fungal communities in the degraded soil amended with phosphorus biofertilizer, 20th International Workshop for Young Scientists "BioPhys Spring 2021", May 18, Lublin, Poland

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2022, The investigation of archaeal genetic diversity in the degraded soil amended with phosphorus biofertilizer, 21th International Workshop for Young Scientists "BioPhys Spring 2022", May 30-31, Nitra, Slovakia

Referaty wygłoszone na konferencjach krajowych

- Mącik M., Gryta A., Oszust K., Frąc M., 2019, Wpływ nawozów mineralnych wzbogaconych mikrobiologicznie na różnorodność funkcjonalną zbiorowisk grzybów i aktywność enzymatyczną gleby, II Konferencja Doktorantów pt. "Cztery żywioły - współczesne problemy w naukach o życiu", 24.10.2019, Lublin

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2020, Charakterystyka zbiorowisk grzybów zasiedlających zdegradowane gleby nawożone fosforowym nawozem

mineralnym wzbogaconym mikrobiologicznie, Konferencja naukowa "Bioróżnorodność środowiska glebowego - wskaźniki oceny", 05.11.2020, Puławy

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2020, Wpływ fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie na strukturę zbiorowisk bakterii zasiedlających gleby zdegradowane chemicznie, III Konferencja Doktorantów pt. "Cztery żywioły - współczesne problemy w naukach o życiu", 18.12.2020, Warszawa

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2021, Ocena różnorodności strukturalnej i funkcjonalnej społeczności grzybów w zdegradowanej chemicznie glebie nawożonej fosforowym bionawozem, II Konferencja "MycoRise Up! Młodzi w mykologii", 23-25.04.2021, Warszawa

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2021, Sezonowe zmiany różnorodności genetycznej bakterii w glebie zdegradowanej nawożonej fosforowym bionawozem, V Ogólnopolskie Sympozjum Mikrobiologiczne "Metagenomy różnych środowisk", 17-18.06.2021, Warszawa

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2021, Genetyczny odcisk palca zbiorowisk bakterii w glebie nawożonej fosforowym nawozem mineralnym wzbogaconym mikrobiologicznie, IV Konferencja Doktorantów "Cztery żywioły - współczesne problemy w naukach o życiu", 14.12.2021, Lublin

Postery zaprezentowane na konferencjach międzynarodowych

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2021, Seasonal dynamics of bacterial functional diversity in degraded soil amended with phosphorus biofertilizer, 13th International Conference on Agrophysics: Agriculture in changing climate, 15-16.11.2021, Lublin, Poland

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2022, Catabolic fingerprinting and diversity of fungi in the degraded soil amended with phosphorus biofertilizer, "Myco Rise Up! Youth in mycology, 27-29.05.2022, Warsaw, Poland

Postery zaprezentowane na konferencjach krajowych

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2021, Zróżnicowanie funkcjonalne zbiorowisk grzybów w glebie zdegradowanej nawożonej fosforowym bionawozem, 54 Konferencja Mikrobiologiczna "Mikroorganizmy różnych środowisk", 20-21.09.2021, Lublin

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2022, Ocena różnorodności genetycznej zbiorowisk bakterii w zdegradowanej glebie nawożonej fosforowym bionawozem, VI Ogólnopolskie Sympozjum Mikrobiologiczne "Metagenomy różnych środowisk", 23-24.06.2022, Puławy

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2022, Wpływ fosforowego bionawozu na zróżnicowanie populacji fitopatogenów zasiedlających glebę zdegradowaną chemicznie, Konferencja "Nowoczesne spojrzenie na fitopatologię" Polskiego Towarzystwa Fitopatologicznego, 7-8.09.2022, Poznań

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2022, Zmienność struktury zbiorowisk grzybów w zdegradowanej glebie nawożonej bionawozem fosforowym, XXIX Zjazd Polskiego Towarzystwa Mikrobiologów, 15-17.09.2022, Warszawa

- Mącik M., Gryta A., Sas-Paszt L., Frąc M., 2022, Analiza metagenomiczna zbiorowisk grzybów w zdegradowanej glebie nawożonejfosforowym bionawozem, Konferencja z okazji 10-lecia Polskiego Towarzystwa Mykologicznego, 24-28.09.2022, Poznań

Współautorstwo doniesień konferencyjnych

- Gryta A., Frąc M., Oszust K., Mącik M., Pertile G., 2019, Różnorodność funkcjonalna zbiorowisk grzybów w glebie nawożonej nawozami mineralnymi wzbogaconymi mikrobiologicznie, 53 Ogólnopolska Konferencja Mikrobiologiczna "Mikroorganizmy w zrównoważonym rolnictwie, ochronie środowiska i procesach biotechnologicznych", 8-11.09.2019, Poznań

- Gryta A., Frąc M., Oszust K., Mącik M., Pertile G., 2019, Genetic diversity of fungal community in soil amendment with mineral fertilizers enriched with microorganisms, XVIII Congress of European Mycologists, 16-21.09.2019, Warsaw-Białowieża, Poland

- Pertile G., Gryta A., Mącik M., Frąc M., 2019, Changes of fungal diversity in soils amended with biofertilizers, XVIII Congress of European Mycologists, 16-21.09.2019, Warsaw-Białowieża, Poland

- Gryta A., Frąc M., Oszust K., Mącik M., Pertile G., 2019, Metaboliczny odcisk zbiorowisk mikroorganizmów w glebie nawożonej nawozami mineralnymi wzbogaconymi mikrobiologicznie, Konferencja "Ochrona bioróżnorodności gleby warunkiem zdrowia obecnych i przyszłych pokoleń, 16.10.2019, Skierniewice

- Frąc M., Oszust K., Panek J., Pertile G., Gryta A., Mącik M., Pylak M., Siegieda D., 2022, Soil microbiome and mycobiome as an important driver of plant health and soil quality, 22nd World Congress of Soil Science, 31.07-05.08.2022, Glasgow, Scotland

Wyróżnienia

- Wyróżnienie za referat wygłoszony podczas III Konferencji Doktorantów "Cztery Żywioły - współczesne problemy w naukach o życiu" pod tytułem "Wpływ fosforowego nawozu mineralnego wzbogaconego mikrobiologicznie na zbiorowiska bakterii zasiedlających gleby zdegradowane chemicznie", 18.12.2020, Warszawa

- Wyróżnienie referat wygłoszony podczas IV Konferencji Doktorantów "Cztery Żywioły - współczesne problemy w naukach o życiu" pod tytułem "Genetyczny odcisk palca zbiorowisk bakterii w glebie nawożonej fosforowym nawozem

mineralnym wzbogaconym mikrobiologicznie", 14.12.2021, Lublin

- Wyróżnienie za prezentację posterową przedstawioną na VI Ogólnopolskim Sympozjum Mikrobiologicznym "Metagenomy różnych środowisk" pod tytułem "Ocena różnorodności genetycznej zbiorowisk bakterii w zdegradowanej glebie nawożonej fosforowym bionawozem", 23-24.06.2022, Puławy

UDZIAŁ W PROJEKTACH

- 10.2021 - 09.2022 - udział w projekcie "Nowe rozwiązania biotechnologiczne w diagnostyce, zwalczaniu i monitoringu kluczowych patogenów grzybowych w ekologicznej uprawie owoców miękkich" (Akronim: EcoFruits) w ramach konkursu NCBR BIOSTRATEG III na stanowisku badawczo-technicznym

- 10.2019 - 09.2021 - udział w projekcie "Opracowanie technologii innowacyjnych nawozów mineralnych wzbogaconych mikrobiologicznie" (Akronim: BIO-FERTIL) w ramach konkursu NCBR BIOSTRATEG III na stanowisku inżynieryjnym