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OPRACOWANIE BAKTERYJNEGO KOMPLEKSU DO NATURALIZACJI RYZOSFERY MALIN

DEVELOPING A BACTERIAL COMPLEX FOR THE NATURALIZATION OF RASPBERRY RHIZOSPHERE

Rozprawa doktorska

Doctoral thesis

Rozprawa doktorska przygotowana pod kierunkiem

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oraz

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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.....

OŚWIADCZENIE PROMOTORA POMOCNICZEGO 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.

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

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

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Streszczenie

Ekologiczna produkcja żywości to nie tylko praktyka pozwalająca na otrzymanie wysokiej jakości produktu, ale również na zmniejszenie negatywnego wpływu rolnictwa na środowisko naturalne. Szacuje się, że około 30% powierzchni ekologicznych gruntów uprawnych znajduje się w Europie, a około 6,4% tych gruntów znajduje się w Polsce. Ponadto produkcja ekologicznych owoców miękkich, w tym malin, w Polsce pomiędzy rokiem 2012 a 2020 wzrosła prawie dwukrotnie z 21 635 ton do 41 870 ton. Intensyfikacja produkcji rolniczej, która nastąpiła w ostatnich latach spowodowała uzależnienie rolników od chemicznych metod ochrony roślin, jako tych najłatwiejszych w zastosowaniu oraz niezawodnych. Nie można jednak pominąć faktu, iż konwencjonalne środki ochrony roślin mogą prowadzić do nabycia odporności przez mikroorganizmy patogeniczne na ich składniki aktywne oraz zmniejszenia bioróżnorodności mikroorganizmów glebowych. Zwiększone zainteresowanie rolnictwem ekologicznym, a w szczególności ekologiczną produkcją owoców miękkich jako tych, często spożywanych bez uprzedniej obróbki, to kolejny czynnik zachęcający producentów do stosowania nowych rozwiązań ekologicznej ochrony i biostymulacji roślin, opartych na pożytecznych mikroorganizmach.

Zmiana strategii ochrony roślin na bardziej przyjazną dla środowiska jest także zgodna z polityką Unii Europejskiej, która od wielu lat stara się promować wśród rolników rolnictwo zrównoważone, ekologiczne i regeneracyjne. Rozporządzenie Rady (WE) nr 834/2007 z dnia 28 czerwca 2007 roku stanowi, że stosowanie konwencjonalnych, chemicznych środków ochrony roślin musi być ograniczone do minimum, a rolnicy powinni stosować ekologiczne środki ochrony roślin, pochodzenia naturalnego. Wprowadzona 20 maja 2020 roku Europejska Strategia na Rzecz Bioróżnorodności do 2030 roku, będąca istotnym elementem Europejskiego Zielonego Ładu, podkreśla znaczenie bioróżnorodności wszystkich środowisk naturalnych, a także potrzebę zwiększania bioróżnorodności obszarów użytkowych rolniczo. Strategia ta ma na celu nie tylko ograniczenie stosowania pestycydów o 50%, ale także zwiększenie powierzchni upraw ekologicznych do ilości równej lub większej niż 25% powierzchni wszystkich gruntów uprawnych. Rolnicy stosujący ekologiczne praktyki uprawy roślin nie mogą stosować wielu konwencjonalnych nawozów i środków ochrony roślin, co może prowadzić do zmniejszenia plonów oraz zwiększenia kosztów produkcji. Wspominanie akty prawne określają również rolę i potrzebę stosowania biologicznych metod ochrony roślin, wykorzystujących ekstrakty roślinne czy biopreparaty zawierające inokulum mikrobiologiczne.

Badania zawarte w niniejszej rozprawie podejmują temat opracowania innowacyjnego biopreparatu mikrobiologicznego zawierającego konsorcjum bakterii pożytecznych wyizolowanych z korzeni malin dzikorosnących, posiadających właściwości hamujące wzrost najbardziej powszechnych patogenów grzybowych oraz grzybopodobnych atakujących uprawy malin. Na proces opracowania biopreparatu składały się pobór próbek gleby, wyprowadzenie czystych kultur bakterii, ich identyfikację oraz wybór bakterii pożytecznych, ocena ich zdolności do hamowania wzrostu wybranych fitopatogenów, wybór izolatów o większym potencjalne antagonistycznym, charakterystyka funkcjonalna wybranych bakterii. Dla wybranych, najbardziej aktywnych 4 szczepów bakterii należących do 3 rodzajów – *Arthorbacter, Pseudomonas* oraz *Rhodococcus,* został zoptymalizowany skład podłoża hodowlanego (źródła oraz zawartość węgla i azotu), warunki hodowli (wartość pH podłoża oraz temperatura hodowli), metoda prezerwacji oraz opracowana została prebiotyczna mieszanka suplementacyjna wspomagająca wzrost tych mikroorganizmów. Zostało również przeprowadzone doświadczenie wazonowe mające na celu sprawdzenie wpływu inokulum bakteryjnego na wzrost roślin malin poddanych działaniu wybranych fitopatogenów – *Botrytis*

cinerea, Colletotrichum acutatum, Phytophthora sp. i *Verticillium* sp., oraz na zbiorowiska mikroorganizmów zasiedlających ryzosferę i fyllosferę malin.

Ocena zdolności do hamowania wzrostu patogenów przez wyizolowane bakterie pożyteczne została przeprowadzona na podstawie pomiaru stref zahamowania wzrostu w testach płytkowych. Charakterystykę funkcjonalną i określenie zdolności katabolicznych pojedynczych izolatów wykonano przy użyciu mikromacierzy fenotypowych na płytkach GEN III z wykorzystaniem systemu BIOLOG™. W doświadczeniu wazonowym zastosowano 3 sposoby aplikacji inokulum mikrobiologicznego oraz 5 patosystemów, co pozwoliło na określenie wpływu inokulum pożytecznych bakterii, jak i obecności fitopatogenów na wzrost roślin. Wpływ zastosowanej naturalizacji oraz obecności fitopatogenów na zbiorowiska mikroorganizmów zasiedlających ryzosferę i fyllosferę malin zbadano z wykorzystaniem płytek ECO i systemu Biolog™, oraz sekwencjonowania następnej generacji (ang. *Next Generation Sequencing*, NGS).

Przeprowadzone badania wykazały, że ryzosfera malin leśnych może być wartościowym i bogatym źródłem pożytecznych mikroorganizmów, a odpowiednio opracowane warunki hodowli i prezerwacji pozwalają na skuteczne namnożenie oraz zachowanie bakterii. Dodatek inokulum bakteryjnego pozytywnie wpłynął na aktywność dehydrogenaz glebowych w obiektach bez obecności patogenów. Aplikacja inokulum mikrobiologicznego wraz z podlewaniem 4 tygodnie po posadzeniu roślin skutkowała największym pozytywnym wpływem na suchą masę malin. Wartość wskaźnika stresu substratowego (SST) dla zbiorowisk mikroorganizmów zasiedlających fyllosferę ulegała obniżeniu w obiektach, gdzie zaaplikowano inokulum mikrobiologiczne. Aplikacja inokulum bakteryjnego wpłynęła na zmniejszenie indeksu bioróżnorodności Shannona dla zbiorowisk grzybów. Wyniki uzyskane na podstawie przeprowadzonych badań wskazują na szerokie i kompleksowe działanie opracowanego biopreparatu mikrobiologicznego. Wyniki wskazują na jego skuteczność w biostymulacji wzrostu malin, wspieraniu zbiorowisk mikroorganizmów zasiedlających otoczenie rośliny oraz hamowaniu wzrostu organizmów fitopatogenicznych.

Słowa kluczowe: biopreparaty, zrównoważone rolnictwo, inokulum mikrobiologiczne, mikroorganizmy glebowe, rolnictwo regeneracyjne, mikromacierze fenotypowe, fitopatogeny owoców miękkich, sekwencjonowanie następnej generacji

Abstract

Organic production of food is not only a farming strategy that allows to achieve a high-quality product but also decrease the negative effect of farming on the natural environment. It is estimated that about 30% of ecological arable land is located in Europe, and about 6.4% of this land is located in Poland. In addition, the production of organic soft fruit, including raspberries, in Poland between 2012 and 2020 almost doubled from 21 635 tons to 41 870 tons. The intensification of agricultural production that has taken place in recent years has made farmers dependent on chemical methods of plant protection as easy to use and reliable. However, it cannot be ignored that conventional plant protection products can lead to the acquisition of resistance by pathogenic microorganisms and a reduction of the soil microorganisms' biodiversity. Increased interest in the field of organic farming, and in particular the organic production of soft fruits as those, often consumed directly, is another factor that encourages producers to use new solutions for ecological protection and biostimulation of plants, based on inter alia beneficial microorganisms.

Changing the plant protection strategy to a more environmentally friendly one is also in line with the policy of the European Union, which for many years has been trying to promote organic, sustainable and regenerative agriculture among farmers. Council Regulation (EC) No 834/2007 of 28 June 2007 stipulates that the use of conventional, chemical plant protection products must be kept to a minimum, and farmers should use organic plant protection products of natural origin. The European Strategy for Biodiversity 2030, introduced on May 20, 2020, being an important element of the European Green Deal, emphasizes the importance of biodiversity of all-natural environments, as well as the need to increase the biodiversity of agricultural areas. This strategy not only aims to reduce the use of pesticides by 50%, but also to increase the area of organic farming to an amount equal to or greater than 25% of all cultivated land. Farmers using ecological plant cultivation practices are obliged not to use many conventional fertilizers and plant protection products, which may lead to a reduction in yields and an increase in production and final product costs. Mentioned legal acts also define the role and the need to use biological methods of plant protection, such as plant extracts or biopreparations containing microbiological inoculum.

This research concerns the development of an innovative microbiological biopreparation containing a consortium of beneficial bacteria isolated from wild raspberry rhizosphere. Beneficial bacteria used in this research have the ability to inhibit the growth of the chosen, most common fungal and fungal like pathogens that infect raspberry plants. The process of biopreparation development consisted of taking soil samples, isolation of bacterial strains, their identification and selection of beneficial bacteria, assessment of their ability to inhibit the growth of selected phytopathogens, selection of the most effective isolates and functional characteristics of selected bacteria. For selected, 4 strains of bacteria belonging to 3 genera - *Arthorbacter, Pseudomonas* and *Rhodococcus*, the composition of the culture medium (carbon and nitrogen sources), the culture conditions (pH value of the medium and culture temperature), the preservation method were optimized and the prebiotic supplementary blend additive was developed. A pot experiment was also carried out to evaluate the effect of the bacterial inoculum on the growth of raspberry plants treated with selected phytopathogens - *Botrytis cinerea*, *Colletotrichum acutatum, Phytophthora* sp. and *Verticillium* sp., and on the communities of microorganisms inhabiting the rhizosphere and phyllosphere of raspberries.

The assessment of the ability to inhibit the growth of pathogens by the isolated beneficial bacteria was carried out on the basis of measuring the growth inhibition zones on the culturing plates. Functional characterization and determination of catabolic capacity of individual isolates

were performed using phenotype microarrays by Biolog^{TM}, with the use of GEN III plates. In the pot experiment, 3 methods of application of the microbial inoculum and 5 pathosystems were used, which allowed to study the relationship between the inoculum of beneficial bacteria and phytopathogens, as well as plant growth. The effect of the applied naturalization and the presence of phytopathogens on the microbial communities inhabiting raspberry rhizosphere and phyllosphere were investigated using the Biolog^{TM} system with ECO microplates, and the Next Generation Sequencing (NGS).

The conducted research has shown that the rhizosphere of wild growing raspberries may be a valuable source of beneficial microorganisms, and that properly developed conditions for culturing and drying allow for effective growth and preservation of bacteria. The application of bacterial inoculum to raspberry plants in the pot experiment positively affected the activity of soil dehydrogenases in objects without the presence of pathogens. The application of the microbial inoculum along with watering 4 weeks after planting resulted in the greatest positive effect on the dry mass of the plant. The value of the substrate stress index (SST) for the microbial communities inhabiting the phyllosphere decreased in the experiments where the microbial inoculum was applied. The application of the bacterial inoculum resulted in the reduction of the Shannon's biodiversity index for the fungal communities inhabiting the raspberry phyllosphere. The results obtained on the basis of the conducted research indicate a broad and comprehensive effect of the developed microbiological biopreparation. The results indicate its effectiveness in biostimulating the growth of raspberries, supporting the communities of microorganisms inhabiting the plant's rhizosphere and phyllosphere and inhibiting the growth of phytopathogenic organisms.

Keywords: biopreparations, sustainable agriculture, microbial inoculum, soil microorganisms, regenerative agriculture, phenotypic microarrays, soft fruit phytopathogens, next generation sequencing

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Lista publikacji stanowiących podstawę rozprawy doktorskiej

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

"Opracowanie bakteryjnego kompleksu do naturalizacji ryzosfery malin":

P.1: **Pylak, M.**, Oszust, K., Frąc M., 2019. Review report on the role of bioproducts, biopreparations, biostimulants and microbial inoculants in organic production of fruit. *Reviews in Environmental Science and Bio/Technology* 18, 597-616. Wskaźnik Impact Factor: 5,261; punktacja MNiSW: 140 punktów.

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

P.2: **Pylak, M.**, Oszust, K., Frąc M., 2020. Searching for New Beneficial Bacterial Isolates of Wild Raspberries for Biocontrol of Phytopathogens-Antagonistic Properties and Functional Characterization. *International Journal of Molecular Sciences,* 21, 9361. Wskaźnik Impact Factor: 5,924; punktacja MNiSW: 140 punktów.

Indywidualny wkład: istotny udział w przeprowadzeniu izolacji środowiskowych szczepów bakterii oraz ich identyfikacji, zbadaniu ich właściwości antagonistycznych oraz katalitycznych, analiza uzyskanych wyników, statystyczne i graficzne opracowanie wyników, przygotowanie manuskryptu.

P.3: **Pylak, M.**, Oszust, K., Frąc M., 2021. Optimization of Growing Medium and Preservation Methods for Plant Beneficial Bacteria, and Formulating a Microbial Biopreparation for Raspberry Naturalization. *Agronomy,* 11, 2521. Wskaźnik Impact Factor: 3,949; punktacja MNiSW: 100 punktów.

Indywidualny wkład: istotny udział w przeprowadzeniu optymalizacji warunków hodowli i prezerwacji szczepów bakterii, zaprojektowaniu i przeprowadzeniu doświadczenia wazonowego, analiza uzyskanych wyników, statystyczne i graficzne opracowanie wyników, przygotowanie manuskryptu.

Uzupełnieniem wyników badań przedstawionych w wyżej wymienionych publikacjach jest analiza zmian strukturalnych i funkcjonalnych w zbiorowiskach mikroorganizmów zasiedlających ryzosferę i fyllosferę malin w wyniku obecności fitopatogenów, a także aplikacji inokulum pożytecznych bakterii. Wyniki zostały przygotowane do publikacji i przedstawione

jako badania uzupełniające niniejszej rozprawy doktorskiej, obejmujące manuskrypt publikacji zatytułowanej:

P.4: **Michał Pylak**, Karolina Oszust, Jacek Panek, Magdalena Frąc, Structural and functional shift of soil rhizosphere and raspberry shoots microbiomes underlying changes caused by phytopahogens contamination and naturalization strategies implementation.

Indywidualny wkład: analiza uzyskanych wyników, statystyczne i graficzne opracowanie wyników, przygotowanie manuskryptu.

1. Wprowadzenie

1.1. Problematyka

Jednym z największych problemów, z którymi musi zmierzyć się nowoczesne rolnictwo jest potrzeba ciągłego zwiększania plonów oraz radzenie sobie z degradacją gleb powodującą zmniejszenie dostępnego, uprawnego areału, a także poszukiwanie alternatywnych rozwiązań dla stale rosnącej chemizacji rolnictwa. Nie mniej istotne jest kontrolowanie obecności fitopatogenów, które mogą powodować nawet 60% strat w plonach [1]. Degradacja gleb może być powodowana przez wiele czynników, które mają negatywny wpływ na właściwości fizyczne, chemiczne i biologiczne gleb [2–4]. Pogorszenie właściwości fizycznych gleby skutkuje zmianą składu granulometrycznego, jej większą gęstością oraz zagęszczeniem [5]. Negatywna zmiana właściwości chemicznych to najczęściej zakwaszenie, obecność soli, spadek zawartości materii organicznej, zmniejszona biodostępność składników odżywczych lub ich brak, a także obecność metali ciężkich [3,6,7]. Degradacja biologiczna to przede wszystkim obniżenie bioróżnorodności edafonu, w tym mikroorganizmów glebowych [8–10]. Nie ulega wątpliwości, że stosowanie chemicznych środków ochrony roślin przyczynia się do opisanego spadku bioróżnorodności środowiska glebowego [11]. Stosowanie nawozów mineralnych pozwala na intensyfikację rolnictwa i zwiększanie plonów, jednakże niewłaściwe i zbyt intensywne ich wykorzystanie może powodować degradację gleb, tak chemiczną jak i biologiczną [12–14]. Zmniejszanie zużycia konwencjonalnych środków ochrony roślin to działanie, które jest niezbędne do ograniczenia postępującej w zatrważającym tempie degradacji gleb uprawnych.

Potrzeba zmiany niektórych praktyk rolnictwa konwencjonalnego na bardziej przyjazne środowisku została również zauważona przez Unię Europejską, która stara się promować gospodarowanie w sposób bardziej ekologiczny i zrównoważony. Odpowiednia polityka uwzględniająca ograniczanie dostępu do niektórych pestycydów, czy zaprzestanie ich stosowania to tylko niektóre z działań pozwalających na ograniczenie najbardziej niekorzystnych skutków intensyfikacji rolnictwa [1,15]. Rozporządzenie Rady (WE) nr 834/2007 z dnia 28 czerwca 2007 roku stanowi, że stosowanie konwencjonalnych, chemicznych środków ochrony roślin powinno być ograniczone do minimum, a rolnicy powinni stosować więcej środków pochodzenia naturalnego takich jak ekstrakty roślinne, produkty zwierzęce, nawozy naturalne czy preparaty mikrobiologiczne [16,17]. Jednym z najbardziej istotnych działań Unii Europejskiej jest przyjęcie Europejskiego Zielonego Ładu,

czyli zbioru inicjatyw politycznych, których celem jest osiągnięcie neutralności dla klimatu w Europie do 2050 roku. Spośród 10 priorytetów określonych przez Komisję Europejską wyróżniają się te, o nazwie „Ekosystemy i bioróżnorodność" oraz „Zdrowa żywność i zrównoważone rolnictwo". W obrębie tych dwóch priorytetów znajdziemy zbiór praktyk, który pomaga zadbać m.in. o stan gleb uprawnych i zdegradowanych, a także o przywrócenie i zachowanie bioróżnorodności tak na obszarach rolniczych jak i dzikich. Jednym z elementów Europejskiego Zielonego Ładu jest wprowadzona 20 maja 2020 roku Europejska Strategia na rzecz Bioróżnorodności do 2030 roku, która podkreśla niezwykłą wagę bioróżnorodności ekosystemów. Strategia ta zakłada między innymi zmniejszenie wykorzystania pestycydów o 50% oraz zwiększenie powierzchni upraw ekologicznych do ilości równej lub większej niż 25% powierzchni wszystkich gruntów uprawnych [18,19].

1.2. Ryzosfera jako źródło pożytecznych mikroorganizmów

Ryzosfera to warstwa gleby otaczająca korzenie roślin i będąca pod wpływem związków przez nie wydzielanych. Jest to tak zwany gorący punkt (ang. *hotspot*) oddziaływań mikrobiologicznych, gdyż to właśnie w ryzosferze zachodzi najwięcej procesów mikrobiologicznych, a interakcje między organizmami są najbardziej intensywne [20,21]. Zbiorowiska mikroorganizmów charakterystycznych dla ryzosfery składają się najczęściej z bakterii, grzybów, alg i archeonów. Ze względu na obecność łatwo przyswajalnych źródeł węgla, ryzosfera zapewnia stabilne środowisko, w którym mogą zachodzić najbardziej istotne procesy związane z interakcjami między mikroorganizmami, rośliną i mikrofauną [22,23]. Rośliny mogą „rekrutować" unikalne mikroorganizmy, które zasiedlając ryzosferę wchodzą w symbiotyczną reakcję hamując wzrost fitopatogenów i stymulując systemiczną odporność roślin [24]. Przez ostatnie lata naukowcy prowadzą coraz więcej badań dotyczących pozyskiwania szczepów mikroorganizmów pożytecznych przydatnych w zwalczaniu chorób roślin, szczególnie powodowanych przez patogeny zasiedlające środowisko glebowe. Mikroorganizmy pożyteczne zasiedlające ryzosferę, które charakteryzują się zdolnością do tworzenia symbiozy z roślinami i mają właściwości antagonistyczne względem fitopatogenów lub mają działanie biostymulujące względem roślin coraz częściej stają się obiektem zainteresowania badaczy [25,26]. Wiele gatunków bakterii zasiedlających ryzosferę roślin często posiada właściwości do biokontroli patogenów roślin na skutek zdolności do produkcji antybiotyków, bakteriocyn, sideroforów, enzymów hydrolitycznych oraz innych metabolitów wtórych [27–30]. Antagonizm przeciwko fitopatogenom jako efekt wydzielania

biopestycydów przez mikroorganizmy pożyteczne to jedna z najważniejszych cech, przyczyniająca się do poprawy zdrowia roślin [31].

1.3. Wpływ pożytecznych mikroorganizmów na rośliny uprawne i fitopatogeny

Wśród mikroorganizmów zasiedlających ryzosferę roślin często można zidentyfikować bakterie pożyteczne, które pozytywnie wpływają na organizmy roślinne wchodząc z nimi w symbiozę [27,32]. Bakterie promujące wzrost roślin i posiadające właściwości antagonistyczne najczęściej należą do rodzajów *Alcaligenes*, *Azospirillum*, *Arthrobacter*, *Acinetobacter*, *Bradyrhizobium*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Allorhizobium*, *Sinorhizobium*, *Frankia*, *Mesorhizob*, *Azoarcus*, *Exiguobacterium*, *Methylobacterium*, *Paenibacillus* i *Pantoea* [23,25,33–38]. Liczne eksudaty korzeniowe powodują, że ryzosfera staje się atrakcyjną niszą ekologiczną dla mikroorganizmów, które są zdolne do przemieszczania w jej kierunku z wykorzystaniem mechanizmu chemotaksji. Efektem tego procesu jest symbiotyczna reakcja pozytywnie wpływająca na zdrowie roślin, oraz plon poprzez zwiększenie biodostępności składników odżywczych, wydzielanie hormonopodobnych związków stymulujących wzrost, zmniejszanie intensywności infekcji fitopatogenicznych oraz reakcji na stresy środowiskowe [27]. Związki takie jak antybiotyki, endotoksyny, bakteriocyny, siderofory, enzymy hydrolityczne, cyjanowodór (HCN), kwas fenazyno-1-karboksylowy (PCA), 2,4-DAPG oraz inne metabolity wtórne produkowane przez bakterie ryzosferowe ograniczają wzrost organizmów fitopatogenicznych, zapobiegając rozwojowi chorób [23,27,38–45].

Na działanie mikroorganizmów pożytecznych składa się wiele mechanizmów, które można podzielić na bezpośrednie i pośrednie. Bezpośrednie działanie mikroorganizmów pożytecznych jest skupione przede wszystkim na hamowaniu wzrostu organizmów patogenicznych przez antybiozę, pasożytnictwo, zmniejszanie rozprzestrzeniania się patogenów oraz poprzez konkurencję o składniki odżywcze [46,47]. Antybioza polega na wydzielaniu przez mikroorganizmy pożyteczne związków alleopatycznych które mogą dyfundować w glebie, do których należą m.in. lipopeptydy, bakteriocyny, antybiotyki, biosurfaktanty czy enzymy zdolne do degradowania ścian komórkowych fitopatogenów, oraz lotnych związków, które wpływają na metabolizm fitopatogenów hamując ich rozwój [46,48,49]. Pasożytnictwo wykorzystywane przez organizmy pożyteczne względem fitopatogenów można również podzielić na dwa rodzaje nekrotroficzne i biotroficzne.

Pasożytnictwo nekrotroficzne polega na zabiciu komórek fitopatogenicznych i wykorzystaniu składników uwolnionych z ich komórek. Pasożyt biotroficzny natomiast wykorzystuje żywe komórki fitopatogenów i żeruje na nich przez dłuższy czas uniemożliwiając ich namnażanie i infekcję roślin [50,51]. Mikroorganizmy pożyteczne mogą ograniczać wirulencję patogenów poprzez wydzielanie enzymów chitynolitycznych i pektynolitycznych, które mogą negatywnie oddziaływać na komórki fitopatogenów oraz ich zarodniki [49,52]. Ponadto mikroorganizmy pożyteczne utrudniają zasiedlenie stref korzeniowych przez fitopatogeny, poprzez konkurencję o składniki odżywcze i przestrzeń do wzrostu. Organizmy pożyteczne mogą także szybko kolonizować strefy korzeniowe oraz produkować biofilm utrudniający kolonizację korzeni przez organizmy patogeniczne [53,54]. Biofilm składający się z komórek organizmów pożytecznych, egzopolisacharydów, białek i kwasów nukleinowych pełniących rolę cząsteczek sygnalizacyjnych ogranicza również wpływ stresów środowiskowych na mikroorganizmy i rośliny poprzez pokrywanie korzeni warstwą zabezpieczającą między innymi przed suszą. Mikroorganizmy, w tym bakterie, sinice i grzyby oddziaływują z tkankami roślinnymi i sobą nawzajem z wykorzystaniem zjawiska *quorum sensing*, polegającego na zdolności do komunikowania się poszczególnych bakterii między sobą oraz wyczuwania liczebności okolicznych mikroorganizmów, a wytwarzany biofilm jest jednym z najważniejszych elementów tego procesu [55–59].

Pośrednie mechanizmy działania mikroorganizmów pożytecznych to indukcja odporności systemicznej i stymulacja wzrostu, a także zwiększanie żyzności gleby [47,60]. Indukcja odporności to zbiór działań, które podejmuje roślina w reakcji na infekcję patogenów lub stymulację poprzez mikroorganizmy pożyteczne. Indukcja odporności powoduje wzmocnienie ścian komórkowych roślin oraz wzbudzenie szlaków metabolicznych odpowiedzialnych za produkcję fitohormonów, związków fenolowych, enzymów chitynolitycznych i glukanolitycznych, kwasu salicylowego oraz roślinnych białek PR (ang. pathogenesis-related protein) [47,61]. Lokalna produkcja kwasu salicylowego w tkankach roślin prowadząca do ich obumierania powoduje zahamowanie rozprzestrzeniania się patogenów w tkankach. Bakterie pożyteczne mogą również stymulować produkcję deaminazy kwasu 1-aminocyklopropano-1-karboksylowego (ACC), który jest prekursorem etylenu. Wpływa to na zmniejszenie produkcji etylenu, który negatywnie oddziałuje na rośliny będące pod wpływem stresu biotycznego i abiotycznego. Bakterie pożyteczne mogą zwiększać odporność roślin na stres abiotyczny, taki jak m.in. susze, powodzie, wysoka temperatura, poprzez stymulację produkcji lub produkcję hormonów regulujących wzrost roślin - kwasu

giberelinowego (GA3), kwasu indolilooctowego (IAA), cytokin czy kwasu abscysynowego (ABA) [61–66].

1.4. Rola preparatów mikrobiologicznych w rolnictwie regeneracyjnym

Zastosowanie preparatów mikrobiologicznych nie musi ograniczać się wyłącznie do bezpośredniego wspierania wzrostu roślin oraz biokontroli fitopatogenów. Biopreparaty mogą również być wartościowym narzędziem służącym regeneracji gleb zdegradowanych oraz przyczyniać się do umożliwienia prowadzenia upraw lub zwiększenia plonów na takich terenach. Wszystkie niekorzystne warunki środowiskowe, które mogą wpływać na bioróżnorodność funkcjonalną mikroorganizmów oraz na właściwości gleby mogą przyczyniać się do powstawania niekorzystnych warunków – stresu abiotycznego. Nadmierna akumulacja metali ciężkich w glebach, powszechna szczególnie na obszarach, na których prowadzona jest działalność wydobywcza przyczynia się do nagromadzenia w glebach nadmiernych stężeń metali ciężkich [67]. Ich obecność w glebie powoduje zmianę wartości pH oraz struktury gleby co skutkuje występowaniem na takich terenach warunków stresowych zarówno dla roślin jak i zbiorowisk mikroorganizmów glebowych [68]. Niektóre bakterie pożyteczne zdolne są do pozakomórkowego wydzielania peptydów wiążących jony metali, co prowadzi do ich bioakumulacji, biosorpcji, precypitacji, oksydacji lub/i zredukowania. Procesy te skutkują zmniejszeniem toksyczności nadmiernego stężenia jonów metali znajdujących się w glebach zdegradowanych [69]. Wydzielanie sideroforów, czyli metabolitów mikrobiologicznych tworzących kompleksy z metalami śladowymi, pozwala na łatwiejsze przyswajanie tych związków przez rośliny, prowadząc do poprawy wzrostu roślin, ale również usunięcia tych związków z gleby [70]. Podobnie produkowane przez mikroorganizmy biosurfaktanty o właściwościach amfifilowych mogą wiązać jony metali, a co za tym idzie zmniejszać ich stężenie w środowisku [71,72]. Do usuwania z gleby jonów metali ciężkich przyczyniają się również wspomniane wcześniej egzopolisacharydy, które zwiększają tempo agregacji i biosorpcji oraz wydzielane zewnątrzkomórkowo, przez bakterie pożyteczne, kwasy organiczne tworzące kompleksy z jonami metali [73–76].

Wieloletnie stosowanie nawozów mineralnych, a także podlewanie wodą zawierającą sól powoduje wzrost zasolenia gleb. Nagromadzenie soli w glebie powoduje zaburzenie homeostazy roślin prowadząc do pogorszenia ich stanu zdrowia i zmniejszenia plonu lub uniemożliwienia wykorzystania gleb zasolonych do uprawy roślin [77,78]. Zasolenie gleby powoduje wytwarzanie reaktywnych form tlenu w korzeniach roślin zarówno uprawnych jak i dzikich. To zjawisko powoduje uszkadzanie komórek i nasilenie stresu oksydacyjnego.

Zarówno mikroorganizmy jak i rośliny aktywują w takiej sytuacji szlaki metaboliczne pozwalające na neutralizację tych niekorzystnych warunków poprzez sekrecję katalazy i peroksydazy askorbinianowej [79]. Produkcja związków o właściwościach antyoksydacyjnych oraz stymulacja roślin do ich produkcji, a także zmniejszenie stężenia etylenu w tkankach roślinnych przez produkcje ACC to kolejne z mechanizmów wpływających na lepszą odporność na nadmierne zasolenie [80–83].

Gleby zdegradowane często charakteryzują się gorszą retencją wody w porównaniu do gleb zdrowych. Mikroorganizmy pożyteczne mogą przyczynić się do poprawy odporności roślin na suszę, zarówno wpływając na rozwój systemów korzeniowych roślin jak i zmieniając zawartość materii organicznej w glebie, która przyczynia się do zwiększenia retencji wody. Zastosowanie pożytecznych bakterii może wpłynąć na wytwarzanie większej ilości i biomasy korzeni o mniejszej średnicy, przy jednocześnie większej długości. Takie parametry korzeni pozwalają roślinom dosięgnąć głębszych warstw gleby, o większej zawartości wody oraz zwiększyć powierzchnię jej czerpania. Zwiększona w ten sposób powierzchnia korzeni wpływa pozytywie na absorbcję wody i składników mineralnych [84–87]. Wykorzystanie biopreparatów zawierających inokulum mikrobiologiczne na glebach podatnych na suszę skutkowało zwiększeniem turgoru o 24% w porównaniu do kontroli [88]. Zmiana właściwości osmotycznych błon i ścian komórkowych roślin, cytoplazmy oraz poszczególnych organelli komórkowych na skutek nagromadzenia odpowiednich białek oraz enzymów, a także innych związków organicznych i nieorganicznych takich jak sacharoza, prolina, betaina, glicyna, mannitol, jony wapnia czy kwas jabłkowy, pozwala zachować turgor i zmniejszyć zużycie wody przez rośliny przy jednoczesnym zachowaniu niezbędnej zawartości wody w roślinach [89–91]. Prolina będąca podstawowym związkiem wpływającym na właściwości osmotyczne tkanek roślinnych podlegających działaniu stresów abiotycznych pomaga stabilizować struktury komórkowe takie jak białka i błony komórkowe, a także bierze udział w łagodzeniu stresu oksydacyjnego [90,92,93]. Obecne w glebie mikroorganizmy pożyteczne mogą również rozkładać dostępną dla nich materię organiczną, co znacząco przyczynia się do zwiększania retencji wody oraz zawartości węgla organicznego w glebie.

Rola biopreparatów zawierających inokulum nie kończy się tylko na biokontroli fitopatogenów, ale jest dużo szersza. Ich wykorzystanie obejmuje zarówno rolnictwo, gdzie mogą wspierać lub nawet całkowicie zastąpić konwencjonalne metody ochrony roślin, ale także mogą być używane w rolnictwie regeneracyjnym czy pełnić istotną rolę w procesach bioremediacji. Opracowanie nowych biopreparatów, zawierających lokalne szczepy

mikroorganizmów pożytecznych pozwoli na stworzenie szerokiego spektrum dostępnych produktów, co przyczyni się do lepszej biokontroli lokalnie występujących fitopatogenów oraz naturalnego radzenia sobie z problemami takimi jak susza, obecność metali ciężkich w glebie czy zasolenie. Badania dotyczące opracowywania nowych biopreparatów to wstęp do nowoczesnych, zrównoważonych, proekologicznych praktyk rolniczych, które pozwolą ograniczyć negatywny wpływ rolnictwa na ekosystem.

Dotychczasowe badania dotyczące poznania mechanizmów działania pożytecznych mikroorganizmów, a także opracowania nowych formulacji biopreparatów skupiają się przede wszystkim na antybiozie i pasożytnictwie. Przegląd literatury wskazuje, że równie istotnym mechanizmem działania biopreparatów jest konkurencja w zasiedlaniu nisz ekologicznych i wykorzystania składników znajdujących się w glebie. Charakterystyka uzdolnień metabolicznych mikroorganizmów, a także poznanie wrażliwych punktów metabolizmu fitopatogenów pozwoli na lepsze dopasowanie konkretnych szczepów mikroorganizmów do nisz ekologicznych i obecnych w środowisku szczepów patogenów. Wykorzystanie nowatorskiego wskaźnika stresu substratowego (SST) pozwala na wykrycie związków wpływających negatywnie lub pozytywnie na mikroorganizmy pożyteczne i patogeniczne, przyczyniając się do określenia na tej podstawie składu prebiotycznej mieszanki suplementacyjnej wspierającej wzrost pożytecznych mikroorganizmów oraz hamującej wzrost fitopatogenów [94]. Również wykorzystanie metod sekwencjonowania następnej generacji przyczynia się do lepszego zrozumienia działania pożytecznych mikroorganizmów poprzez porównanie składu całych zbiorowisk mikroorganizmów i wpływu stosowanych biopreparatów na ich funkcjonowanie i skład taksonomiczny [9,95].

Naturalizacja to nowoczesne biotechnologiczne podejście do uprawy roślin. Polega na inokulacji roślin pożytecznymi mikroorganizmami, takimi jak grzyby czy bakterie, w celu zwiększenia ich wzrostu, a także tolerancji na stres środowiskowy zarówno biotyczny jak i abiotyczny. Oprócz wpływu na rośliny naturalizacja wpływa również pozytywnie na bioróżnorodność środowisk glebowych poprzez poprawę warunków glebowych i poszerzanie nisz środowiskowych, które stają się łatwiejsze do zasiedlenia przez inne mikroorganizmy pożyteczne [96,97]. Badania przedstawione w poniższej rozprawie skupiają się na izolacji i wyborze szczepów bakterii pożytecznych pochodzących z ryzosfery malin dzikorosnących, które jako składnik aktywny biopreparatu naturalizacyjnego będą zdolne do hamowania wzrostu wybranych najbardziej szkodliwych i powszechnych fitopatogenów grzybowych i grzybopodobnych wpływających niekorzystnie na maliny uprawne. Ponadto zastosowanie

wskaźnika stresu substratowego pozwoli na określenie składu mieszanki suplementacyjnej wpływającego pozytywnie na wzrost mikroorganizmów pożytecznych. Sprawdzenie wpływu inokulum bakterii pożytecznych na rośliny malin, skład taksonomiczny i metabolizm zbiorowisk mikroorganizmów glebowych w doświadczeniu wazonowym pozwoliło zweryfikować założone hipotezy, a zastosowane różne rodzaje aplikacji inokulum pomogą w określeniu sposobu naturalizacji zapewniającego największe korzyści.

2. Hipoteza badawcza i cele rozprawy doktorskiej

Hipotezy badawcze

Na podstawie przeglądu literatury dotyczącego obecnie stosowanych biopreparatów w uprawach owoców miękkich, aktualny problem z występowaniem i biokontrolą fitopatogenów grzybowych i grzybopodobnych w rolnictwie ekologicznym i regeneracyjnym, a także na wynikającą z tego potrzebę opracowywania nowych biopreparatów zawierających lokalnie występujące mikroorganizmy pożyteczne, postawiono następujące hipotezy badawcze:

- szczepy bakterii wyizolowane z ryzosfery malin dzikorosnących posiadają właściwości antagonistyczne skierowane przeciwko wybranym fitopatogenom grzybowym i grzybopodobnym owoców miękkich,
- szczepy bakterii wyizolowane z ryzosfery malin dzikorosnących posiadają bogate uzdolnienia metaboliczne oraz enzymatyczne,
- zoptymalizowane parametry podłoża hodowlanego oraz warunków hodowli, a także odpowiednio dobrana metoda prezerwacji pozwala na namnożenie oraz zachowanie aktywności komórek bakteryjnych,
- aplikacja inokulum wybranych izolatów bakterii pożytecznych wpływa pozytywnie na poprawę wzrostu roślin malin oraz aktywność mikroorganizmów glebowych w obecności fitopatogenów grzybowych i grzybopodobnych,
- aplikacja inokulum wybranych izolatów bakterii pożytecznych wpływa pozytywnie na bioróżnorodność mikroorganizmów i aktywność zbiorowisk mikroorganizmów glebowych.

Cel rozprawy doktorskiej

Celem rozprawy doktorskiej było opracowanie biopreparatu mikrobiologicznego do naturalizacji ryzosfery malin oraz określenie wpływu izolatów środowiskowych bakterii pożytecznych wchodzących w jego skład na rośliny malin, a także zbiorowiska oraz aktywność mikroorganizmów glebowych. W związku z tym postawiono następujące cele szczegółowe:

- określenie zdolności izolatów środowiskowych bakterii pożytecznych do hamowania wzrostu wybranych fitopatogenów grzybowych i grzybopodobnych,
- określenie uzdolnień metabolicznych wybranych bakterii pożytecznych,
- optymalizacja składu podłoża hodowlanego, warunków hodowli oraz metody prezerwacji dla wybranych izolatów bakterii pożytecznych, a także określenie składu prebiotycznej mieszanki suplementacyjnej dla wybranych izolatów bakterii pożytecznych oraz fitopatogenów grzybowych i grzybopodobnych,
- określenie wpływu aplikacji inokulum wybranych izolatów bakterii pożytecznych na suchą masę części nadziemnych roślin malin, mokrą masę korzeni oraz aktywność dehydrogenaz,
- określenie składu taksonomicznego zbiorowisk mikroorganizmów zasiedlających ryzosferę i fyllosferę roślin malin oraz wpływu inokulum bakterii pożytecznych na te zbiorowiska w warunkach kontaminacji przez wybrane fitopatogeny.

3. Omówienie wyników przedstawionych w publikacjach i badaniach uzupełniających

W publikacjach P.1, P.2, P.3 oraz w badaniach uzupełniających przedstawionych w manuskrypcie publikacji P.4 podjęto zagadnienie opracowania biopreparatu naturalizacyjnego zawierającego konsorcjum bakterii pożytecznych wyizolowanych z ryzosfery malin dzikorosnących, określenia jego wpływu na wzrost roślin malin, aktywność dehydrogenaz w glebie, uzdolnienia funkcjonalne oraz skład taksonomiczny zbiorowisk mikroorganizmów zasiedlających ryzosferę i fyllosferę malin w doświadczeniu wazonowym.

3.1. Publikacja P.1 (Review report on the role of bioproducts, biopreparations, biostimulants and microbial inoculants in organic production of fruit)

Bibliografia: Pylak, M., Oszust, K. & Frąc, M. (2019). Review report on the role of bioproducts, biopreparations, biostimulants and microbial inoculants in organic production of fruit. *Rev Environ Sci Biotechnol* 18, 597–616 https://doi.org/10.1007/s11157-019-09500-5

W publikacji przeglądowej P.1 przedstawiono charakterystykę dostępnych komercyjnie biopreparatów zawierających pożyteczne mikroorganizmy, ekstrakty roślinne oraz z glonów, a także składniki pochodzenia zwierzęcego. Omówiono znaczenie i skalę rolnictwa ekologicznego w Europie i Polsce, a także problemy z jakimi zmagają się rolnicy decydujący się na produkcję owoców w sposób ekologiczny takie jak wysokie straty w plonach wynikające z obecności fitopatogenów, wyższy koszt i mniejszy wybór środków ochrony roślin, wzrost nakładów pracy potrzebnych do uzyskania produktu, co przekłada się na wyższy koszt produktu końcowego. Podkreślono zgodność ekologicznych praktyk rolniczych z obecnymi trendami promowanymi przez Unię Europejską, która zachęca rolników do znacznego ograniczania konwencjonalnych, chemicznych środków ochrony roślin na korzyść stosowania biopreparatów i środków pochodzenia naturalnego.

W publikacji scharakteryzowano dostępne preparaty o działaniu biostymulującym wzrost roślin. Podkreślono szerokie działanie stymulujące biopreparatów polegające na zwiększaniu biodostępności substancji odżywczych obecnych w glebie, wspieraniu pobierania oraz przyswajania substancji odżywczych przez rośliny. Omówiono najczęstsze czynniki powodujące ograniczenie wzrostu roślin w rolnictwie ekologicznym, takie jak większa niż w rolnictwie konwencjonalnym obecność fitopatogenów, mniejsza zawartość pierwiastków takich jak azot czy fosfor w glebach uprawianych ekologicznie na skutek stosowania uboższych

w te składniki nawozów ekologicznych, mniejsza biodostępność pierwiastków, takich jak żelazo, cynk, miedź i mangan na skutek występowania nieodpowiedniego odczynu gleby. Ponadto, w publikacji P.1 scharakteryzowano komponenty wykorzystywane jako nośniki dla biopreparatów mikrobiologicznych oraz mechanizmy działania biopreparatów, obejmujące stymulację wzrostu korzeni przekładającą się na zwiększenie ich powierzchni, zmianę wartości pH gleby pozwalającą na sprawną wymianę substancji odżywczych, tworzenie kompleksów z nierozpuszczalnymi pierwiastkami, takimi jak żelazo, zwiększającymi ich rozpuszczalność, wiązanie toksycznych jonów metali, stymulację wzrostu mikroorganizmów glebowych zwiększającą ich bioróżnorodność, a także wiązanie azotu atmosferycznego.

W publikacji P.1 przedstawiono najczęściej wykorzystywane w biopreparatach inokulum mikrobiologiczne o złożonym działaniu, polegającym na zwiększaniu asymilacji składników odżywczych przez rośliny, hamowaniu wzrostu fitopatogenów, indukowaniu odporności systemicznej roślin oraz wydzielaniu związków hormonopodobnych. Ponadto, omówiono najczęściej stosowane w biopreparatach grupy mikroorganizmów, wśród których szczególną uwagę poświęcono charakterystyce bakterii i mikrogrzybów należące do rodzajów *Trichoderma, Pythium, Talaromyces, Aureobasidium, Bacillus, Pseudomonas* i *Lactobacillus.* Na przykładzie grzybów z rodzaju *Trichoderma* scharakteryzowano także mechanizmy działania pożytecznych mikroorganizmów skierowane przeciwko fitopatogenom, takie jak konkurencja o składniki odżywcze, antybioza, czyli wydzielanie toksycznych dla innych mikroorganizmów związków chemicznych, mykopasożytnictwo polegające na pasożytowaniu grzyba pożytecznego na innym, w tym przypadku fitopatogenicznym, inaktywacja enzymów grzybów fitopatogenicznych przez organizmy pożyteczne, indukowanie odporności systemicznej roślin.

W końcowej części pracy przedstawiono wyzwania związane z koniecznością opracowania nowych rozwiązań biotechnologicznych, w tym biopreparatów dla rolnictwa oraz przytoczono znaczenie i założenia dokumentów strategicznych Unii Europejskiej.

3.2. Publikacja P.2 (Searching for New Beneficial Bacterial Isolates of Wild Raspberries for Biocontrol of Phytopathogens-Antagonistic Properties and Functional Characterization)

Bibliografia: Pylak, M., Oszust, K., & Frąc, M. (2020). Searching for New Beneficial Bacterial Isolates of Wild Raspberries for Biocontrol of Phytopathogens-Antagonistic Properties and Functional Characterization. *International journal of molecular sciences*, *21*(24), 9361. https://doi.org/10.3390/ijms21249361

W publikacji P.2 scharakteryzowano najczęściej występujące na plantacjach owoców miękkich fitopatogeny grzybowe i grzybopodobne należące do rodzajów *Botrytis*, *Colletotrichum*, *Phytophthora* oraz *Verticillium*. Przedstawiono strategie Unii Europejskiej takie jak Europejski Zielony Ład oraz jego ważny element, którym jest Strategia na Rzecz Bioróżnorodności do 2030 roku z uwzględnieniem wymagań nakładanych przez te działania na rolników ekologicznych. W publikacji omówiono znaczenie izolowania lokalnie występujących szczepów bakterii pożytecznych jako tych, które wykazują cechy/właściwości dopasowane do zwalczania lokalnych fitopatogenów, oraz mają większą szansę na przetrwanie po aplikacji. Celem badań, omówionych w publikacji P.2, było wyizolowanie z gleby ryzosferowej oraz korzeni malin dzikorosnących izolatów bakterii posiadających zdolność do hamowania wzrostu wybranych fitopatogenów owoców miękkich, dokonanie charakterystyki uzdolnień metabolicznych i enzymatycznych wybranych szczepów bakterii pożytecznych, a także określenie składu mieszanki suplementacyjnej stymulującej wzrost bakterii i wywołującej stres substratowy dla organizmów fitopatogenicznych, co zostało ustalone na podstawie wyników analizy uzdolnień metabolicznych mikroorganizmów.

W publikacji zaprezentowano wyniki identyfikacji genetycznej, dokonanej na podstawie sekwencjonowania fragmentu 16S rDNA, 65 szczepów bakterii, należących do 21 rodzajów. Szczepy zostały wyizolowane z gleby ryzosferowej i korzeni malin dzikorosnących. Na podstawie utworzonego drzewa filogenetycznego można zauważyć, że poszczególne rodzaje bakterii tworzą odrębne klastry. Po wykonaniu przeglądu literatury 42 szczepy określono jako potencjalnie pożyteczne i poddano badaniom obejmującym oznaczenie uzdolnień antagonistycznych wobec kluczowych patogenów owoców miękkich. Spośród wybranych 42 izolatów 8 charakteryzowało się wyjątkowo wysokimi właściwościami antagonistycznymi. Były to szczepy *Arthrobacter* sp. B58/18 i B49/18, *Pseudomonas* sp.

B25/18 i B37/18, *Bacillus* sp. B39/18 i B40/18, oraz *Rhodococcus* sp. B12/18 i B73/18. Zdolność do hamowania wzrostu fitopatogenów różniła się pomiędzy poszczególnymi izolatami,, a *Rhodococcus* sp. B12/18 wykazywał zdolność do hamowania wzrostu 11 izolatów fitopatogenów należących do wszystkich 4 rodzajów (*Botrytis*, *Colletotrichum*, *Phytophthora*, *Verticillium*).

Osiem wybranych izolatów poddano analizie uzdolnień metabolicznych z wykorzystaniem systemu Biolog^{TM} i płytek GEN III, które pozwalają na zbadanie zdolności do utylizacji 71 źródeł węgla należących do 6 grup: aminokwasów, peptydów i polipeptydów; kwasów karboksylowych i estrów; węglowodanów, polioli i innych; kwasów cukrowych i ich związków; pochodnych cukrów. Ponadto, badania z wykorzystaniem płytek GEN III pozwoliły na ocenę wpływu 23 czynników stresowych m.in. takich jak zasolenie, skrajne wartości pH czy obecność antybiotyków na przyrost biomasy mikroorganizmów. Na podstawie wyników analizy uzdolnień metabolicznych wykazano, że niektóre badane izolaty bakterii takie jak *Bacillus* sp. B39/18 i B40/18 wykorzystywały poszczególne grupy związków chemicznych w bardzo podobny sposób. Jednakże badane izolaty B58/18 i B49/18 pomimo tego, że należące do tego samego rodzaju *Arthrobacter* znacznie różniły się od siebie zdolnościami metabolicznymi. Podobną zależność zaobserwowano dla bakterii B12/18 i B73/18 należących do rodzaju *Rhodococcus*. Wyniki analizy skupień w formie dendrogramu przedstawione na rysunku 5 w publikacji P.2 prezentują podobieństwo w utylizacji grup związków chemicznych przez badane szczepy bakterii. Wyniki analizy wskazują na utworzenie 3 klastrów o podobnym profilu metabolicznym wśród badanych szczepów bakterii. Wykazano, że badane szczepy bakterii różnią się zdolnością do wzrostu w obecności czynników stresowych. Warto podkreślić, że trzy spośród badanych izolatów: *Rhodococcus* sp. B12/18, *Arthrobacter* sp. B49/18 oraz *Arthrobacter* sp. B58/18, charakteryzowały się bardzo niską odpornością na większość czynników ograniczających wzrost. Analiza uzdolnień enzymatycznych z wykorzystaniem testów API ZYM i metabolicznych z użyciem testów płytkowych wykazała zróżnicowanie w aktywności enzymatycznej i metabolicznej pomiędzy badanymi szczepami bakterii. Izolat *Pseudomonas* sp. B37/18 charakteryzował się najszerszymi zdolnościami metabolicznymi, szczepy *Arthrobacter* sp. B58/18 i *Rhodococcus* sp. B12/18 charakteryzowały się najwyższą aktywnością enzymatyczną.

W oparciu o wyniki analizy uzdolnień metabolicznych z wykorzystaniem systemu Biolog^{TM} i płytek GEN III dla bakterii pożytecznych oraz płytek FF dla fitopatogenów grzybowych i grzybopodobnych wyliczono wskaźnik stresu substratowego (SST).

Na podstawie wartości SST dla poszczególnych związków chemicznych wybrano 4 związki chemiczne, które wykorzystano do stworzenia prebiotycznej mieszanki suplementacyjnej wspierającej wzrost pożytecznych bakterii, ale powodującej stres substratowy dla fitopatogenów. Wybrane związki chemiczne to kwas α-ketoglutarowy, kwas jabłkowy, N-acetylo-D-glukozamina oraz kwas glutarowy.

Na podstawie uzyskanych wyników do opracowania biopreparatu do naturalizacji ryzosfery malin wytypowano cztery szczepy bakterii, dla których przeprowadzono proces optymalizacji wzrostu oraz opracowano formulację i sposób wytwarzania biopreparatu, co zostało przedstawione w kolejnej publikacji P.3.

3.3. Publikacja P.3 (Optimization of Growing Medium and Preservation Methods for Plant Beneficial Bacteria, and Formulating a Microbial Biopreparation for Raspberry Naturalization)

Bibliografia: Pylak, M., Oszust, K., & Frąc, M. (2021). Optimization of Growing Medium and Preservation Methods for Plant Beneficial Bacteria, and Formulating a Microbial Biopreparation for Raspberry Naturalization. *Agronomy, 11*(12), 2521. https://doi.org/10.3390/agronomy11122521

W publikacji P.3 przedstawiono wyniki procesu optymalizacji składu podłoża hodowlanego, metody prezerwacji oraz dodatku prebiotycznej mieszanki suplementacyjnej dla 4 wybranych szczepów bakterii pożytecznych, posiadających potencjał w ograniczaniu wzrostu fitopatogenów ze względu na zróżnicowane właściwości antagonistyczne skierowane przeciwko wybranym grzybowym i grzybopodobnym patogenom roślinnym. Wybrane izolaty należały do rodzajów *Arthrobacter, Psuedomonas* i *Rhodococcus* oraz charakteryzowały się ponadto szerokimi uzdolnieniami metabolicznymi i enzymatycznymi.

W wyniku procesu optymalizacji składu podłoża ustalono, że dodatek sacharozy w stężeniu 3% jako źródła węgla zapewnił najlepszy wzrost bakterii, porównywalny do kontroli wykonanej z wykorzystaniem podłoża minimalnego M9. Dodatek peptonu w stężeniu 0,2% nie wpływał na ilość bakterii po hodowli w obiektach z dodatkiem sacharozy. Spośród przetestowanych źródeł azotu, azotan amonu najbardziej istotnie zwiększył przyrost biomasy badanych bakterii. Temperatura hodowli i pH również wykazały duży wpływ na zdolność bakterii do namnażania. *Rhodococcus* sp. B12/18 wykazywał najwyższe tempo wzrostu w podłożu o wartości pH ok. 6, a pozostałe izolaty preferowały wartość pH podłoża hodowlanego powyżej 7. Optymalną temperaturą dla wzrostu wszystkich szczepów bakterii

była temperatura wyższa niż 24°C i niższa niż 30°C. Dodatek prebiotycznej mieszanki suplementacyjnej składającej się z równych ilości kwasu α-ketoglutarowego, kwasu jabłkowego, N-acetylo-D-glukozaminy oraz kwasu glutarowego, dodanych w ilości 10 g L^{-1} spowodował istotnie statystyczny wzrost gęstości optycznej hodowli bakterii *Rhodococcus* sp. B12/18 i *Pseudomonas* sp. B37/18 zmierzonej po 144 godzinach hodowli w porównaniu do kontroli bez dodatku suplementów.

Podczas optymalizacji metod prezerwacji mikroorganizmów zbadano 3 metody – suszenie konwencjonalne, suszenie próżniowe oraz liofilizację. Ponadto zbadano wpływ 3 metod przygotowania próbki poprzez suszenie peletu bakteryjnego po odwirowaniu i usunięciu supernatantu, suszeniu całego podłoża hodowlanego wraz z mikroorganizmami oraz suszenie podłoża hodowlanego wraz z mikroorganizmami, wysyconego ziemią okrzemkową. Chociaż wielkość populacji żywych komórek bakteryjnych po suszeniu była mniejsza o co najmniej 2 rzędy wielkości w porównaniu do świeżej hodowli, to biopreparat posiadał wystarczającą liczebność mikroorganizmów do zastosowania w rolnictwie. Suszenie konwencjonalne charakteryzowało się długim czasem suszenia oraz uzyskiwaniem bardzo twardego i zbitego produktu. Suszenie próżniowe trwało 72h do czasu pełnego wysuszenia oraz w przypadku suszenia medium wysyconego na ziemi okrzemkowej pozwalało na osiągnięcie produktu w postaci puszystego proszku. Jako najlepszą metodę suszenia zostało wytypowane suszenie próżniowe z 0,1 M dodatkiem trehalozy do podłoża hodowlanego, który wpłynął pozytywnie na przeżywalność bakterii w trakcie procesu suszenia, w szczególności w odniesieniu do szczepu *Rhodococcus* sp. B75/18.

W publikacji P.3 prezentowane są również wyniki badań dotyczące wczesnego efektu aplikacji konsorcjum bakteryjnego na rośliny malin i parametry gleby w doświadczeniu wazowym. Wpływ inokulum bakterii na suchą masę fyllosfery różnił się w zależności od zastosowanej strategii naturalizacji oraz obecności patogenów. W obiektach bez obecności patogenów zastosowanie inokulum bakteryjnego powodowało obniżenie suchej masy części nadziemnych roślin, jednak w obiektach zakażonych patogenami zastosowanie konsorcjum mikrobiologicznego powodowało istotne statystycznie zwiększenie suchej masy w porównaniu do obiektów, w których nie zastosowano inokulum. Aplikacja inokulum spowodowała zmniejszenie masy korzeni w obiektach bez obecności patogenów. W obiektach zakażonych patogenami *Botrytis cinerea* i *Verticillium* sp. oraz w obiektach, w których obecne były wszystkie patogeny aplikacja inokulum spowodowała zwiększenie masy korzeni w porównaniu do kontroli, co wskazuje na ochronne działanie testowanych bakterii na rośliny w warunkach

stresu biotycznego spowodowanego przez fitopatogeny. W obiektach bez obecności patogenów aplikacja inokulum powodowała zwiększenie aktywność dehydrogenaz glebowych w porównaniu do kontroli, podobny efekt uzyskany był w trzech spośród pięciu badanych patosystemów, tj. dla *Colletotrichum acutatum, Phytophthora* sp. oraz dla obiektów zakażonych wszystkimi czterema patogenami. W pozostałych obiektach (*Botrytis cinerea* i *Verticillium* sp.) dodatek inokulum bakteryjnego powodował obniżenie aktywności dehydrogenaz. Zawartość makroskładników w glebie w doświadczeniu wazonowym różniła się w zależności od zastosowanej strategii naturalizacji oraz obecności patogenów. W przypadku obiektów bez obecności patogenów największa zawartość makroskładników w częściach nadziemnych roślin została stwierdzona w obiektach, w których została zastosowana aplikacja inokulum na korzenie w czasie sadzenia. Strategia naturalizacji łącząca aplikację na korzenie podczas sadzenia i podlewanie naturalizacyjne, oraz samo podlewanie naturalizacyjne były najbardziej skuteczne w zwiększaniu zawartości biodostępnych składników pokarmowych w glebie w warunkach stresu biotycznego występującego w patosystemach. Zawartość mineralnych i organicznych form azotu w glebie nie była zależna od zastosowanej naturalizacji, ale w obiektach zawierających wszystkie testowane patogeny powodowała zwiększenie zawartości azotu.

3.4. Badania uzupełniające (Analiza zmian strukturalnych i funkcjonalnych w zbiorowiskach mikroorganizmów zasiedlających ryzosferę i fyllosferę malin w wyniku obecności fitopatogenów, a także aplikacji inokulum pożytecznych bakterii – manuskrypt publikacji P.4 – Structural and functional shift of soil rhizosphere and raspberry shoots microbiomes underlying changes caused by phytopahogens contamination and naturalization strategies implementation)

3.4.1. Wstęp

W nowoczesnym, szybko zmieniającym się świecie, w którym zapotrzebowanie na żywność ciągle rośnie, często zapominamy, że zasoby potrzebne do jej produkcji nie są nieskończone. Konwencjonalne praktyki rolnicze dążą do maksymalizacji plonu oraz zmniejszenia zużycia zasobów takich jak woda, powierzchnia uprawna i energia. Niestety, konwencjonalne praktyki rolnicze, a w szczególności wykorzystanie chemicznych środków ochrony roślin przyczyniło się między innymi do zauważalnego zmniejszenia bioróżnorodności w środowisku glebowym [98]. Ze względu na to jednym z głównych celów rolnictwa ekologicznego, zrównoważonego i regeneracyjnego jest zwiększanie bioróżnorodności, a w szczególności przywracanie i zachowanie bioróżnorodności środowiska glebowego,

a co za tym idzie poprawa funkcjonowania całych ekosystemów [99]. Jedną z najważniejszych praktyk prowadzących do osiągnięcia tych celów jest ograniczenie wykorzystania chemicznych środków ochrony roślin i zastąpienie ich biopreparatami ekologicznymi. Biopreparaty zawierające mikroorganizmy pożyteczne nie tylko pomagają chronić rośliny przed działaniem fitopatogenów oraz wzbogacają zbiorowiska mikroorganizmów glebowych o mikroorganizmy pożyteczne, ale również poprawiają jakość gleby i stymulują wzrost roślin [16,96]. Obecnie na rynku niewiele jest biopreparatów przeznaczonych do stosowania w uprawach owoców miękkich takich jak maliny, a wykazano, że pożyteczne mikroorganizmy wyizolowane z takich samych roślin jak te na których będą stosowane, oraz wyizolowane w tej samej strefie klimatycznej są zacznie bardziej skuteczne w hamowaniu wzrostu lokalnie występujących fitopatogenów [100].

Sekwencjonowanie następnej generacji to metoda pozwalająca między innymi na zbadanie relacji pomiędzy mikroorganizmami, a także między roślinami i mikroorganizmami. Metoda ta pozwala na zróżnicowanie i porównanie zbiorowisk mikroorganizmów grzybowych i bakteryjnych, a także zbadanie wpływu aplikowanego biopreparatu na zbiorowiska mikroorganizmów zasiedlających ryzosferę i fyllosferę roślin naturalizowanych. Ze względu na to, że mikroorganizmy glebowe biorą udział w wielu procesach zachodzących w glebie takich jak obieg węgla i azotu, zwiększanie biodostępności mikro- i makro-składników czy rozkład materii organicznej, różnice w składzie zbiorowisk mogą wpływać na reakcję roślin zarówno na abiotyczne stresy środowiskowe, jak i na stres biotyczny powodowany przez fitopatogeny [101–103].

W związku z powyższym, uzupełnieniem badań przedstawionych w opublikowanych dotychczas artykułach naukowych, wchodzących w skład niniejszej rozprawy doktorskiej, było określenie wpływu inokulum bakterii pożytecznych na właściwości funkcjonalne oraz skład taksonomiczny zbiorowisk mikroorganizmów zasiedlających ryzosferę oraz fyllosferę malin w doświadczeniu wazonowym. Do przeprowadzenia analiz wykorzystano płytki ECOPlate systemu BIOLOG^{TM} oraz sekwencjonowanie następnej generacji z wykorzystaniem platformy MiSeq Ilumina^{TM}.

3.4.2. Materiały i metody

3.4.2.1. Doświadczenie wazonowe

Doświadczenie wazonowe zostało przeprowadzono w kontrolowanych warunkach temperatury i wilgotności w pokoju fitotronowym. Sadzonki malin zostały posadzone

w doniczkach zawierających 3 kg przesianej, niesterylnej gleby pochodzącej z nieuprawianej działki. Zastosowano 5 wariantów zakażenia fitopatogenami: *Botrytis cinerea* G277/18 (B), *Verticillium* sp. G296/18 (V), *Colletotrichum acutatum* G172/18 (C) oraz *Phytophthora* sp. G408/18 (P), jako kontrolę zastosowano obiekt z dodatkiem wody zamiast inokulum fitopatogenów. Dla każdego wariantu zakażenia zastosowano 4 metody naturalizacji zawierającej inokulum pożytecznych bakterii wyizolowanych z ryzosfery malin dzikorosnących: *Rhodococcus* sp. B12/18 (GenBank: MW255650), *Pseudomonas* sp. B37/18 (GenBank: MW255651), *Arthrobacter* sp. B58/18 (GenBank: MW255652), *Rhodococcus* sp. B75/18 (GenBank: MW255653) [104]. 4 testowane strategie naturalizacji obejmowały inokulację korzeni sadzonek malin podczas sadzenia (R), naturalizację poprzez podlewanie zawiesiną bakterii 4 tygodnie po posadzeniu (W), naturalizację łączoną obejmującą zarówno naturalizację korzeni podczas sadzenia jak i podlewanie naturalizacyjne 4 tygodnie po posadzeniu (RW) oraz kontrolę bez naturalizacji (N). Takie podejście miało na celu odwzorowanie stanu plantacji - nowo zakładanych (naturalizacja korzeni - R) oraz już istniejących (podlewanie naturalizacyjne - W). Pochodzenie oraz dokładna charakterystyka szczepów pożytecznych bakterii oraz fitopatogenów grzybowych i grzybopodobnych, a także szczegółowy opis eksperymentu wazonowego zostały przedstawione w publikacjach P.2 i P.3.

3.4.2.2. Płytki ECO systemu Biolog™ – różnorodność funkcjonalna

Badania przeprowadzone na płytkach ECO przeprowadzono poprzez odważenie 1 g gleby ryzosferowej lub fyllosfery roślin i zawieszenie materiału w 99 ml sterylnej soli fizjologicznej z peptonem (ang. *saline peptone water*), wytrząsanie przez 20 minut w temperaturze pokojowej oraz inkubację w temperaturze 4°C przez 30 minut [12]. Następnie do każdej studzienki na płytce ECO Biolog™ dodano 120 µl przygotowanej zawiesiny i inkubowano w temperaturze 25°C przez 6 dni. Każdy eksperyment został wykonany w trzech powtórzeniach (n=3). W czasie inkubacji płytek, co 24 godziny, mierzono absorbancję każdej studzienki dla 2 długości fali światła – 590 nm i 750 nm. Pomiar absorbancji dla dwóch długości fal światła pozwala na ocenienie zarówno przyrostu biomasy – 750 nm, jak również stopnia wykorzystania substratu – 590 nm. Ponadto na podstawie tych dwóch pomiarów wyliczony został współczynnik stresu substratowego. Wyniki zostały pogrupowane w zależności od patosystemów oraz zastosowanych strategii naturalizacji, a analiza statystyczna wyników została przeprowadzona z wykorzystaniem oprogramowania *Statistica* 13.1.

3.4.2.3. Izolacja DNA i sekwencjonowanie następnej generacji

DNA środowiskowe (e-DNA) z gleby ryzosferowej oraz fyllosfery malin zostało wyizolowane przy użyciu zestawu do izolacji FAST DNA Spin Kit for Feces (MP Biomedicals, Santa Ana, USA) zgodnie ze zmodyfikowanym protokołem producenta opisanym przez Oszust i in. [105]. Następnie regiony ITS1 oraz 16S V3-V4 zostały poddane amplifikacji z użyciem starterów specyficznych zgodnie z Toju i in. [106]: ITS1f-KYO1 i ITS2_KYO2 dla ITS1, oraz zgodnie z Klindworth i in. [107] dla 16S. Reakcje prowadzone były w objętości 10 µl i składały się z 5 µl mieszaniny reakcyjnej KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA), 0,2 µM starterów ITS1 Forward lub 16S Forward, 1,6 µl wody wolnej od nukleaz oraz 3 µl wyizolowanego DNA. Reakcja została przeprowadzona zgodnie z następującym protokołem: 180 sekund w 95°C; 30 cykli po 30 sekund w 95°C, 30 sekund w 55°C, 60 sekund w 72°C; oraz końcowe 300 sekund w 72°C. Następnie próbki zostały oczyszczone z przy użyciu urządzenia do magnetycznego oczyszczania próbek MagMAX™ (Applied Biosystems, USA) z wykorzystaniem komercyjnego zestawu MagMAX™ Express Magnetic Particle Processor (Applied Biosystems, Waltham, MA, USA) z dodatkiem 18 µl zawiesiny kuleczek magnetycznych CleanPCR (CleanNA, Waddinxveen, Niderlandy) oraz 50 µl 10 mM Tris-HCl pH 8.0 (EURx, Gdańsk, Polska). Następnym krokiem było indeksowanie produktów PCR podwójnymi indeksami oraz sekwencjami adaptorowymi Ilumina™ z wykorzystaniem zestawu Nextera XT Index Kit v2 (Illumina™, San Diego, CA, USA). Reakcje prowadzone były w objętości 10 µl i składały się z 5 µl KAPA HiFi HotStart ReadyMix, po 1 µl indeksów i5 oraz i7, 1 µl wody wolnej od nukleaz oraz 2 µl oczyszczonego amplikonu. Reakcja została przeprowadzona zgodnie z następującym protokołem: 180 sekund w 95°C; 10 cykli po 30 sekund w 95°C, 30 sekund w 55°C, 60 sekund w 72°C; oraz końcowe 300 sekund w 72°C. Po indeksowaniu próbki zostały ponownie oczyszczone z przy użyciu urządzenia do magnetycznego oczyszczania próbek MagMAX™ Express Magnetic Particle Processor (Applied Biosystems, Waltham, MA, USA) z dodatkiem 18 µl zawiesiny kuleczek magnetycznych CleanPCR (CleanNA, Waddinxveen, Niderlandy) oraz 50 µl 10 mM Tris-HCl pH 8.0 (EURx, Gdańsk, Polska). Oczyszczone i zindeksowane amplikony zostały znormalizowane, spulowane oraz rozcieńczone do stężenia 4nM. Następnie 5 µl przygotowanej w ten sposób biblioteki zdenaturowano poprzez dodanie 5 µl 0,2 N NaOH i rozcieńczono do stężenia 12 pM przy użyciu buforu HT1 (Illumina, San Diego, CA, USA). Kolejny krok stanowiło połączenie 540 µl przygotowanej biblioteki z 60 µl 10 pM zdenaturowanej w powyższy sposób biblioteki kontrolnej PhiX (Illumina, San Diego, CA, USA). Następnie biblioteka została ponownie zdenaturowana termicznie poprzez 2 minuty inkubacji

w temperaturze 96°C oraz natychmiastowe umieszczenie jej w lodzie na 5 minut. W kolejnym kroku biblioteka została załadowana do wkładu z odczynnikiem MiSeq v3 2x300 (Illumina, San Diego, CA, USA), oraz poddana sekwencjonowaniu w aparacie MiSeq (Illumina, San Diego, CA, USA).

Uzyskane wyniki zostały przeanalizowane z wykorzystaniem środowiska QIIME2 i PICRUSt oraz narzędzia FUNGuild z wykorzystaniem baz danych SILVA i UNITE odpowiednio dla bakterii i grzybów [107–111].

Tabela 1. Sekwencje starterów do amplifikacji i sekwencjonowania regionów ITS1 oraz 16S V3-V4

Nazwa startera	Sekwencja 5'→ 3'	Źródło
ITS1f-KYO1	CTHGGTCATTTAGAGGAASTAA	[106]
ITS2-KYO2	TTYRCTRCGTTCTTCATC	[106]
16S_V3V4_F	CCTACGGGNGGCWGCAG	[107]
16S_V3V4_R	GACTACHVGGGTATCTAATCC	[107]
Overhang F	TCGTCGGCAGCGTCAGA TGTGTATAAGAGACAG	Illumina
Overhang R	GTCTCGTGGGCTCGGAG ATGTGTATAAGAGACAG	Illumina

3.4.2.4. Analiza statystyczna

W celu analizy wpływu inokulum bakteryjnego na różnorodność funkcjonalną zbiorowisk mikroorganizmów wygenerowano wykresy w oparciu o średnią wartość obliczoną z trzech powtórzeń. Aby określić wpływ strategii naturalizacji i patosystemów na zróżnicowanie zbiorowisk drobnoustrojów obliczono wskaźnik zróżnicowania Shannon'a, wskaźnik równości Pielou i wskaźnik zróżnicowania filogenetycznego Faith'a. Uzyskane wyniki poddano analizie statystycznej z wykorzystaniem jednokierunkowej analizy wariancji ANOVA ($p < 0,05$) oraz testu Tukey'a w celu ujawnienia statystycznie istotnych różnic między strategiami naturalizacji oraz patosystemami.

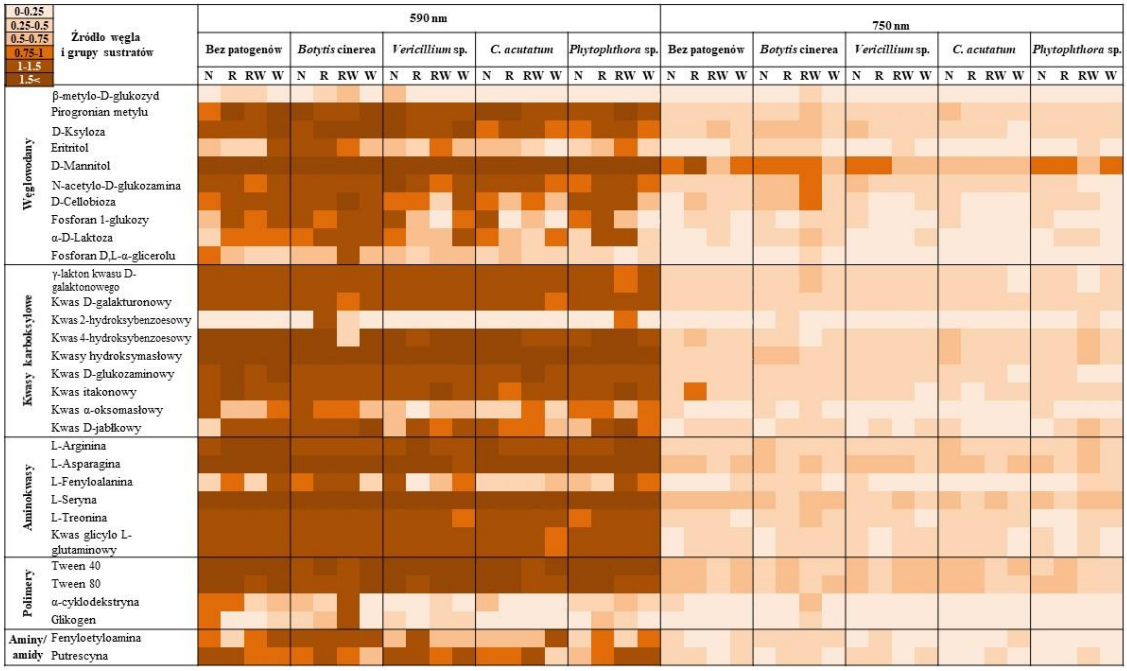
3.4.3. Wyniki i dyskusja

3.4.3.1. Różnorodność funkcjonalna zbiorowisk mikroorganizmów

Analiza uzdolnień funkcjonalnych zbiorowisk mikroorganizmów może przyczynić się do lepszego zrozumienia wpływu inokulum bakterii pożytecznych na ekosystem w miejscu aplikacji. W tabeli 2 zostały przedstawione wartości absorbancji zmierzonej dla długości fali 590 nm i 750 nm dla zbiorowisk mikroorganizmów zasiedlających ryzosferę malin. W wariantach bez patogenów zastosowanie inokulum bakteryjnego skutkowało zmniejszeniem

utylizacji polioli, kwasu α-ketomasłowego i glikogenu, jednak skutkowało wyższym wykorzystaniem kwasu D-jabłkowego, węglowodanów takich jak α-D-laktoza, glukozo-1-fosforan oraz aminokwasu L-fenyloalaniny. Zwiększenie poziomu wykorzystania kwasu D-jabłkowego może być powiązane z przystosowaniem zbiorowisk do zasiedlania strefy korzeniowej roślin, które są zdolne do wydzielania kwasu jabłkowego w reakcji na obecność fitopatogenów. Bakterie zdolne do utylizacji tego związku mogą być zdolne do wytwarzania silniejszej symbiozy z roślinami [112]. W wariantach obejmujących infekcję patogenami największe zmiany zaobserwowano w patosystemach kontaminowanych *Verticillium* sp., *Phytophthora* sp. i *C. acutatum*. W tych obiektach dodatek inokulum bakteryjnego powodował mniejsze wykorzystanie węglowodanów takich jak D-celobioza, D-glukozo-1-fosforan i α-D-laktoza. Infekcja patogenami *B. cinerea, Verticillium* sp. i *C. acutatum* skutkowała zwiększeniem wykorzystania α-D-laktozy w doświadczeniach bez zastosowanej naturalizacji w porównaniu z kontrolą bez patogenu i którymkolwiek z zabiegów naturalizacyjnych. Zarówno obecność fitopatogenów jak i aplikacja inokulum pożytecznych bakterii wpływały na metabolizm węglowodanów przez mikroorganizmy glebowe. Zwiększenie utylizacji węglowodanów może świadczyć o przystosowaniu do zasiedlania nisz ekologicznych związanych ze strefami korzeniowymi ze względu na to, że 20% węglowodanów obecnych w glebie jest pochodzenia roślinnego [113]. Jednakże w doświadczeniach bez kontaminacji patogenem, inokulum bakteryjne zmniejszyło zdolność do wykorzystania α-cyklodekstryny i glikogenu, jednak, gdy rośliny malin zostały zakażone *B. cinerea*, aplikacja inokulum bakterii pożytecznych zarówno jako naturalizacja korzeni, jak i podlewanie naturalizacyjne, zwiększyły zdolność zbiorowisk mikroorganizmów do wykorzystania tych związków. Wartości absorbancji dla długości fali 750 nm nie różniły się tak bardzo, jak dla długości fali 590 nm. Zastosowanie inokulum bakteryjnego spowodowało zwiększenie intensywności wzrostu mikroorganizmów glebowych w obiektach zakażonych *B. cinerea*. Intensywność wzrostu mikroorganizmów glebowych, wyrażona produkcją biomasy, przy wykorzystaniu różnych źródeł węgla była podobna we wszystkich zastosowanych patosystemach i strategiach naturalizacji. Zmniejszenie utylizacji badanych substratów przez zbiorowiska mikroorganizmów może świadczyć o negatywnej interakcji tych patogenów i mikroorganizmów zasiedlających ryzosferę malin. Negatywny efekt obecności fitopatogenów może być zniesiony poprzez poprawny dobór strategii naturalizacji [114,115].

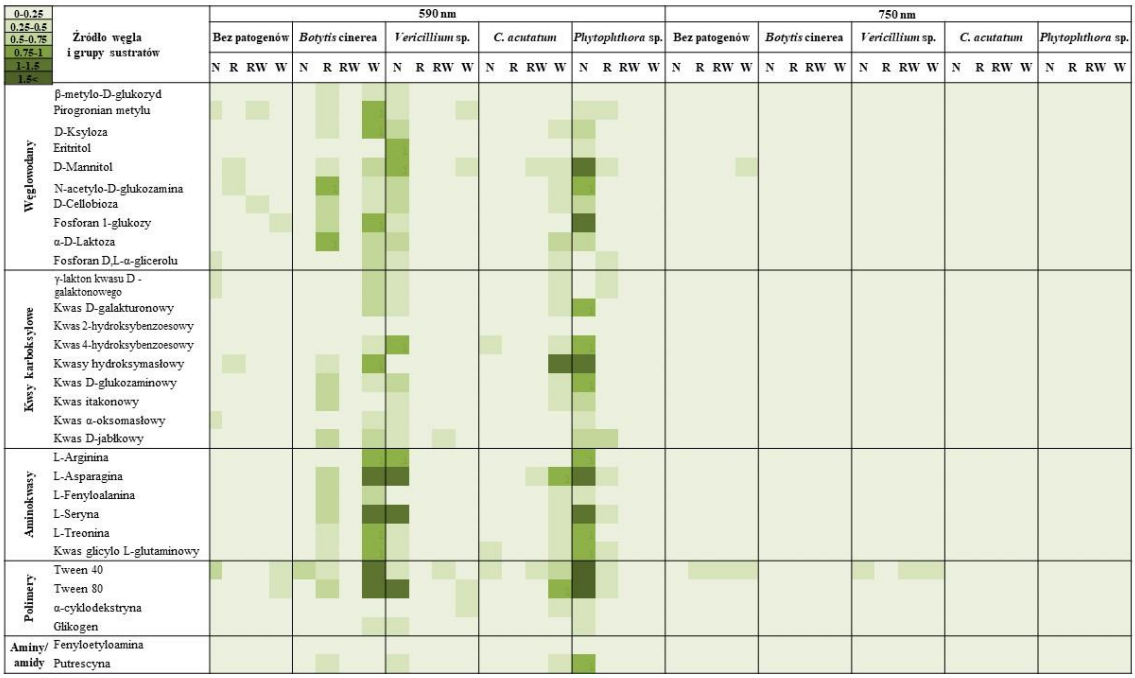
Tabela 2. Wartości absorbancji dla długości fali 590 nm i 750 nm dla różnych źródeł węgla. Próbki do analizy pobrano z ryzosfery malin. N – bez naturalizacji, R – naturalizacja korzeni podczas sadzenia, W – podlewanie naturalizacyjne 4 tygodnie po posadzeniu, RW – naturalizacja łączona obejmująca naturalizację korzeni podczas sadzenia i podlewanie naturalizacyjne 4 tygodnie po posadzeniu.



Wartości absorbancji zmierzone dla długości fali 590 nm i 750 nm, dla zbiorowisk mikroorganizmów zasiedlających części nadziemne malin przedstawiono w tabeli 3. W doświadczeniach bez zanieczyszczenia patogenami zastosowanie inokulum bakteryjnego zwiększyło wykorzystanie węglowodanów. D-Mannitol, N-acetylo-D-glukozamina, D-celobioza i D-glukozo-1-fosforan były wykorzystywane w większym stopniu w obiektach poddanych działaniu inokulum bakteryjnego. W wariantach kontaminowanych *B. cinerea*, w których nie zastosowano żadnej strategii naturalizacji, stwierdzono obniżenie różnorodności funkcjonalnej mikroorganizmów glebowych, wyrażone zmniejszonym wykorzystaniem testowanych substratów węglowych. Jednakże, gdy zastosowano strategię naturalizacji poprzez podlewanie, zaobserwowano zwiększenie wykorzystania źródeł węgla w porównaniu z kontrolą bez naturalizacji. Naturalizacja poprzez podlewanie spowodowała intensyfikację wykorzystania węglowodanów, kwasów karboksylowych, polimerów, a zwłaszcza aminokwasów. Naturalizacja korzeni była również skuteczna, jednak efekt ten był mniejszy niż podlewanie naturalizacyjne. Zakażenie malin *Verticillium* sp. skutkowało zwiększonym

wykorzystaniem źródeł węgla w obiektach bez żadnej z badanych strategii naturalizacji w porównaniu do obiektów kontrolnych. W wariantach kontaminowanych *Colletotrichum acutatum* podlewanie z dodatkiem pożytecznych bakterii okazało się najbardziej skuteczne w zwiększaniu zdolności do wykorzystania zróżnicowanych źródeł węgla przez społeczności mikroorganizmów, zwłaszcza aminokwasów i kwasów karboksylowych. Zbiorowiska mikroorganizmów w obiektach z zakażeniem *Phytophthora* sp. wykazały zwiększone wykorzystanie źródeł węgla w obiektach bez zastosowania naturalizacji. W obiektach zainfekowanych *Phytophthora* sp., w których zastosowano naturalizację korzeni, wykorzystanie fosforanu DL-α-glicerolu i laktonu kwasu D-galaktonowego było zwiększone w porównaniu z obiektami doświadczalnymi bez żadnej strategii naturalizacji. Na wartości absorbancji dla długości fali 750 nm nie miała wpływu ani obecność patogenów, ani dodanie inokulum bakterii pożytecznych. W obiektach bez obecności patogenów zastosowanie pożytecznych bakterii skutkowało zwiększonym wykorzystaniem D-mannitolu i kwasu glicylo-L-glutaminowego w porównaniu z kontrolą.

Tabela 3. Wartości absorbancji dla długości fali 590 nm i 750 nm dla różnych źródeł węgla. Próbki do analizy pobrano z fyllosfery malin. N – bez naturalizacji, R – naturalizacja korzeni podczas sadzenia, W – podlewanie naturalizacyjne 4 tygodnie po posadzeniu, RW – naturalizacja łączona obejmująca naturalizację korzeni podczas sadzenia i podlewanie naturalizacyjne 4 tygodnie po posadzeniu.

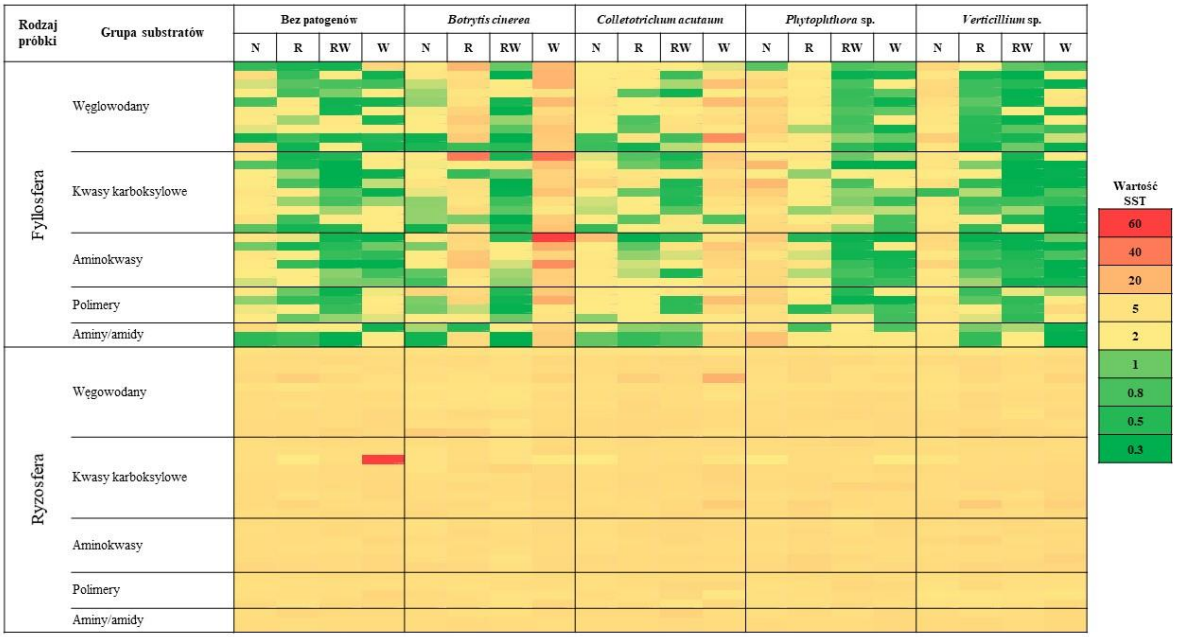


W tabeli 4 przedstawiono wartości wskaźnika stresu substratowego (SST) obliczone na podstawie wartości absorbancji dla obu długości fali 590 nm i 750 nm. Im niższa wartość SST, tym wyższa produkcja biomasy przy niższym wykorzystaniu substratu, co odpowiada lepszym warunkom wzrostu dla zbiorowisk mikroorganizmów. Na wartość wskaźnika stresu

substratowego dla zbiorowisk mikroorganizmów zasiedlających ryzosferę nie miała wpływu ani obecność patogenu, ani też zastosowana strategia naturalizacji. Jednak duże zróżnicowanie zaobserwowano w wartościach SST obliczonych dla zbiorowisk mikroorganizmów zasiedlających fyllosferę malin. W obiektach niekontaminowanych patogenami wprowadzenie inokulum bakteryjnego skutkowało obniżeniem SST dla wszystkich zastosowanych strategii naturalizacji. Naturalizacja łączona, obejmująca zarówno naturalizację korzeni podczas sadzenia, jak i podlewanie z dodatkiem inokulum pożytecznych bakterii 4 tygodnie po posadzeniu, skutkowała największą redukcją wskaźnika stresu substratowego w porównaniu z kontrolą, dla wszystkich grup substratów. W patosystemie kontaminowanym *B. cinerea*, wartość wskaźnika SST dla wariantów bez naturalizacji była wyższa niż w kontroli. Aplikacja inokulum bakteryjnego w obiektach z tym patogenem skutkowała wzrostem wartości wskaźnika stresu substratowego w wariantach z zastosowaniem naturalizacji łączonej. W obiektach, w których obecnych był fitopatogen *C. acutatum*, bez zastosowanej naturalizacji, wartość SST była niższa w porównaniu z kontrolą bez patogenów. Podlewanie naturalizacyjne spowodowało wzrost wartości SST, natomiast naturalizacja korzeni i naturalizacja łączona spowodowała obniżenie wartości wskaźnika stresu substratowego. Zakażenie roślin przez *Phytophthora* sp. powodowało zwiększenie stresu substratowego. Każda strategia naturalizacji skutkowała obniżeniem wartości wskaźnika SST, jednak podlewanie naturalizacyjne i naturalizacja łączona skutkowały największym obniżeniem wartości SST. Widoczne zróżnicowanie stresu substratowego dla zbiorowisk mikroorganizmów zasiedlających fyllosferę w porównaniu do braku zróżnicowania mikroorganizmów zasiedlających ryzosferę może być skutkiem naturalnej większej stabilności zbiorowisk mikroorganizmów występujących w glebie przykorzeniowej. Mikroorganizmy glebowe są mniej narażone na zmiany temperatur, wiatr, promieniowanie UV, a ich nisze ekologiczne charakteryzują się większą wilgotnością. Ze względu na wymienione czynniki środowiskowe zbiorowiska mikroorganizmów są bardziej stabilne i mniej podatne na zmiany [116,117]. Zastosowanie naturalizacji spowodowało obniżenie wartości stresu substratowego przede wszystkim w obiektach, w których zastosowana została naturalizacja łączona. Wielokrotna aplikacja mikroorganizmów charakteryzowała się lepszym efektem naturalizacyjnym, co często jest cechą biopreparatów mikrobiologicznych [16,118]. Niższe wartości wskaźnika stresu substratowego w obiektach, w których obecny był fitopatogen *B. cinerea*, a nie została zastosowana żadna z badanych strategii naturalizacji, może być efektem oddziaływania patogenów na zbiorowiska mikroorganizmów [95,103,119]. Wskaźnik stresu substratowego

jest wartościowym narzędziem pozwalającym na pełniejsze zrozumienie interakcji pomiędzy zbiorowiskami mikroorganizmów.

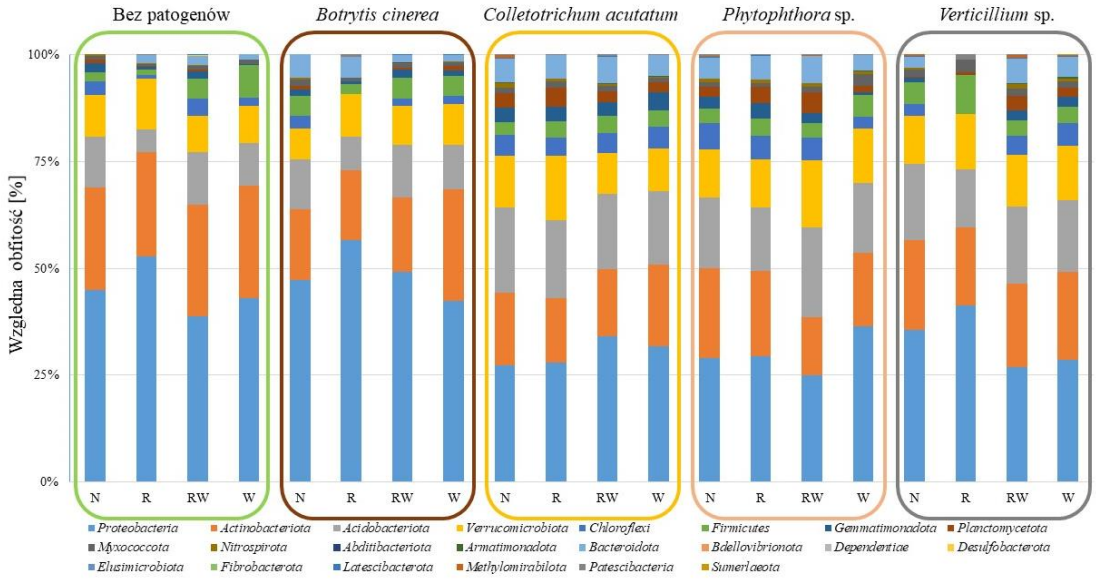
Tabela 4. Wartości SST obliczone na podstawie wartości absorbancji zmierzonych dla długości fali 590 nm i 750 nm dla różnych źródeł węgla, zarówno dla próbek gleby ryzosferowej jak i fyllosfery malin. N – bez naturalizacji, R – naturalizacja korzeni podczas sadzenia, W – podlewanie naturalizacyjne 4 tygodnie po posadzeniu, RW – naturalizacja łączona obejmująca naturalizację korzeni podczas sadzenia i podlewanie naturalizacyjne 4 tygodnie po posadzeniu.



3.4.3.2. Względna obfitość typów bakterii i grzybów w ryzosferze malin

Wykorzystanie sekwencjonowania następnej generacji pozwoliło na określenie struktury zbiorowisk mikroorganizmów glebowych. Wykazano różnice w składzie taksonomicznym mikrobiomów bakteryjnych i grzybowych w zależności od poszczególnych strategii naturalizacji i patosystemów. Na rysunkach 1 i 2 zostały przedstawione różnice w składzie taksonomicznym zbiorowisk mikroorganizmów grzybowych i bakteryjnych zasiedlających ryzosferę malin w zależności od zastosowanej strategii naturalizacji oraz patosystemu kontaminowanego fitopatogenami. W próbkach gleby ryzosferowej zidentyfikowano 22 typy bakterii, spośród których najbardziej powszechne były *Proteobacteria* (27-55%) i *Actinobacteriota* (16-22%). Trzeci i czwarty pod względem obfitości typ stanowiły odpowiednio *Acidobacteriota* (8-21%) i *Verrucomicbiota* (9-15%). Względne obfitości w próbkach gleby ryzosferowej odpowiadają tym obserwowanym przez

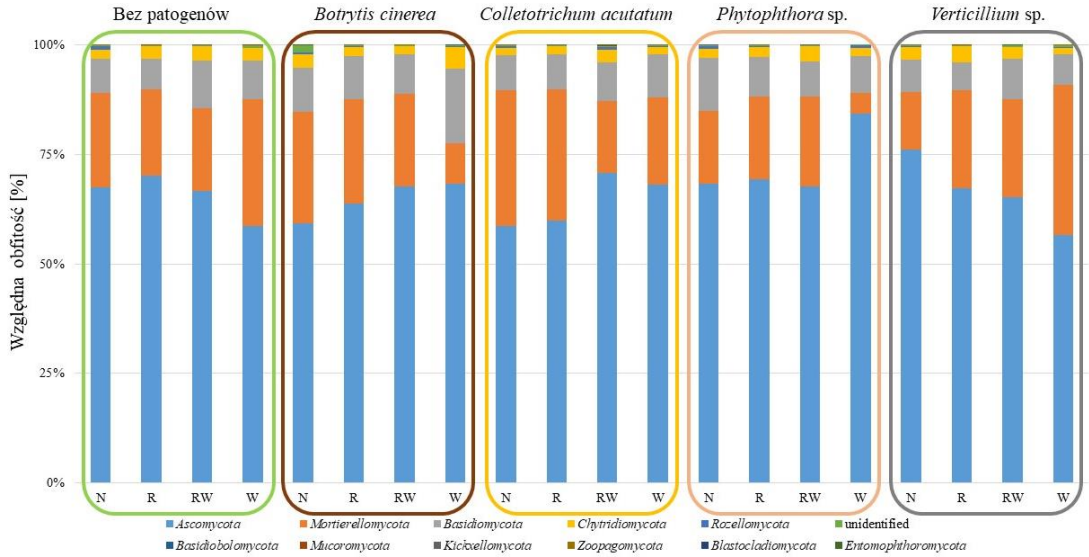
innych badaczy [120,121]. Bez względu na zastosowany patosystem aplikacja inokulum pożytecznych bakterii zwiększyła względną obfitość *Proteobacteria*, jednak infekcja fitopatogenami skutkowała zmniejszeniem względnej obfitości tego typu bakterii (rys. 1.).



Rysunek 1. Skład taksonomiczny zbiorowisk bakterii glebowych zasiedlających ryzosferę malin w doświadczeniu wazonowym. N – bez naturalizacji, R – naturalizacja korzeni podczas sadzenia, W – podlewanie naturalizacyjne 4 tygodnie po posadzeniu, RW – naturalizacja łączona obejmująca naturalizację korzeni podczas sadzenia i podlewanie naturalizacyjne 4 tygodnie po posadzeniu.

Pomimo tego, że w próbkach ryzosfery malin zidentyfikowano 11 typów grzybów, 3 z nich odpowiadają za ponad 95% względnej obfitości: *Ascomycota* (55-85%), *Mortierellomycota* (4-32%) i *Basidiomycota* (6-20%). Podobnie jak dla zbiorowisk bakterii tak i w tym przypadku zaobserwowane względne obfitości grzybów w próbkach ryzosfery są zgodne z obserwacjami innych zespołów badających skład taksonomiczny mykobiomu glebowego [7,120–122]. Aplikacja inokulum pożytecznych bakterii spowodowała zwiększenie względnej obfitości typu *Ascomycota*, a największy wzrost zaobserwowano w obiektach kontaminowanych fitopatogenem *Phytophthora* sp. z zastosowaniem podlewania naturalizacyjnego. W tych samych obiektach odnotowano największy spadek obfitości *Mortierellomycota*. Zastosowanie podlewania naturalizacyjnego w obiektach zakażonych *Verticillium* sp. spowodowało wzrost względnej obfitości *Basidiomycota* przy zmniejszeniu względnej obfitości *Mortierellomycota*. Na podstawie uzyskanych wyników należy wyciągnąć wniosek, że zastosowana strategia naturalizacji powinna być dostosowana do obecnych w miejscu aplikacji patogenów, gdyż może to zagwarantować większą skuteczność zabiegu [123,124]. Ponadto wcześniejsze badania wskazywały, że przedstawiciele *Ascomycota* należący do taksonów *Hypocreales* i *Maasoglossum* są najczęstszymi saprotrofami

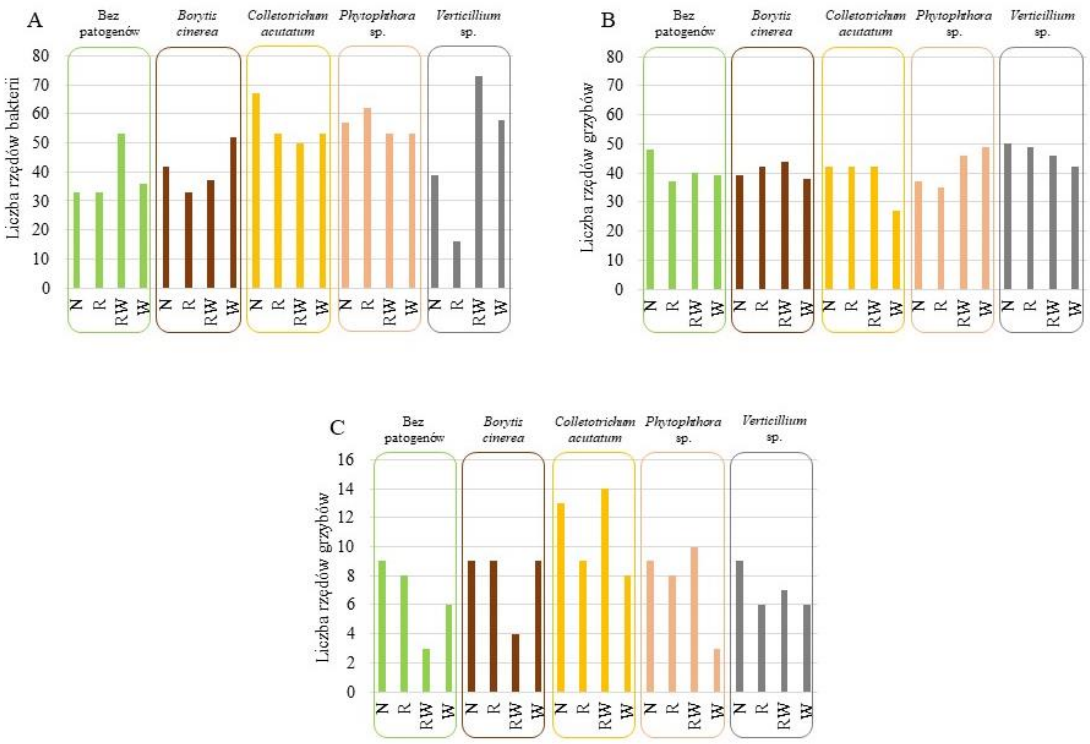
w ryzosferze maliny sadzonej i dzikorosnącej [101]. Wykazano również, że zastosowanie nawozu mineralnego wzbogaconego o szczepy pożytecznych bakterii wiązało się z występowaniem przedstawicieli *Ascomycota* i *Basidiomycota*, takich jak: *Falcocladiales*, *Myrangiales* czy *Tilletiales* [125].



Rysunek 2. Skład taksonomiczny zbiorowisk grzybów zasiedlających ryzosferę malin w doświadczeniu wazonowym. N – bez naturalizacji, R – naturalizacja korzeni podczas sadzenia, W – podlewanie naturalizacyjne 4 tygodnie po posadzeniu, RW – naturalizacja łączona obejmująca naturalizację korzeni podczas sadzenia i podlewanie naturalizacyjne 4 tygodnie po posadzeniu.

3.4.3.3. Liczba rzędów bakterii i grzybów w ryzosferze malin

Liczba rzędów mikroorganizmów zidentyfikowanych w próbkach gleby to wartościowa informacja, która może przyczynić się do poszerzenia wiedzy na temat wpływu zastosowania konkretnej naturalizacji lub patosystemu na skład zbiorowisk mikroorganizmów glebowych. Uzyskane wyniki liczby rzędów bakterii przedstawiono na rysunku 3.

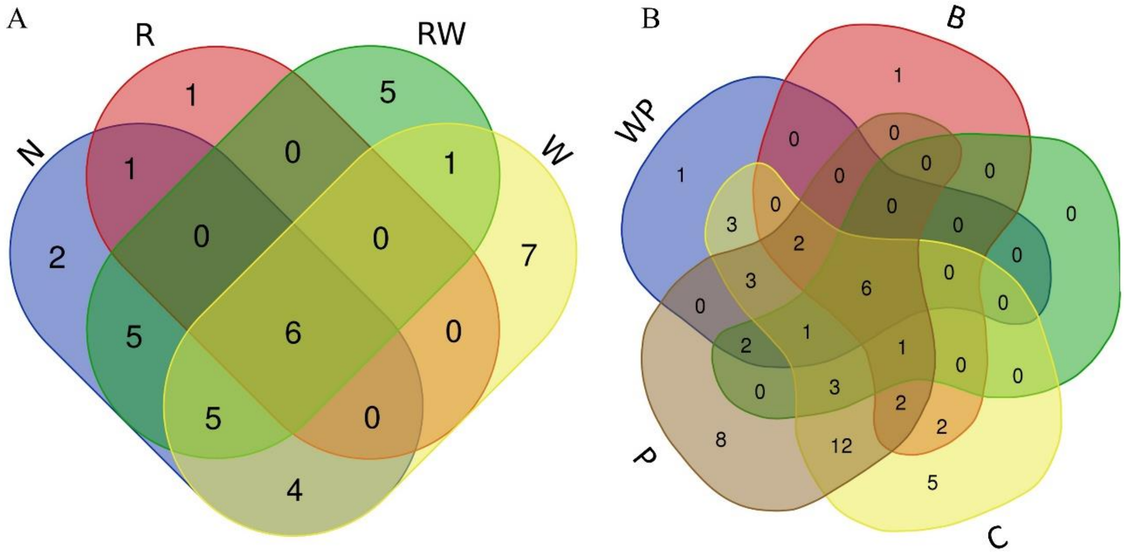


Rysunek 3. Liczba rzędów bakterii (A) i grzybów (B) obecnych w ryzosferze malin oraz liczba rzędów grzybów w próbkach fyllosfery (C) w zależności od zastosowanej strategii naturalizacji i patosystemu. N – bez naturalizacji, R – naturalizacja korzeni podczas sadzenia, W – podlewanie naturalizacyjne 4 tygodnie po posadzeniu, RW – naturalizacja łączona obejmująca naturalizację korzeni podczas sadzenia i podlewanie naturalizacyjne 4 tygodnie po posadzeniu.

Liczba zidentyfikowanych rzędów bakterii i grzybów w próbkach gleby ryzosferowej podlega zarówno wpływom zastosowanych strategii naturalizacji jak i obecności fitopatogenów [126]. We wszystkich obiektach z wyjątkiem wariantu z zakażeniem *C. acutatum* aplikacja inokulum powodowała zwiększenie liczby zidentyfikowanych rzędów bakterii. Aplikacja inokulum w postaci naturalizacji korzeni podczas sadzenia spowodowała zwiększenie liczby rzędów w obiektach z zakażeniem *Phytophthora* sp., aplikacja łączona była najbardziej skuteczna w obiektach z zakażeniem *Verticillium* sp. oraz w obiektach bez zakażenia patogenami, a podlewanie naturalizacyjne najlepiej sprawdziło się w obiektach zakażonych *B. cinerea*. Liczba rzędów grzybów znajdujących się w ryzosferze była bardziej stabilna i nie ulegała tak dużym zmianom jak liczba organizmów bakteryjnych. Można zaobserwować, że w obiektach bez infekcji patogenów, tych zakażonych *C. acutatum* oraz *Verticillium* sp. naturalizacja powodowała zmniejszenie liczby rzędów grzybów, a w obiektach zakażonych *B. cinerea* oraz *Phytophthora* sp., spowodowała zwiększenie ich liczby. Wyniki przedstawione na rysunku 6C przedstawiają liczbę rzędów grzybów zasiedlających fyllosferę malin. Zastosowanie naturalizacji inokulum bakterii pożytecznych powodowało zmniejszenie liczby mikroorganizmów grzybowych zasiedlających fyllosferę malin.

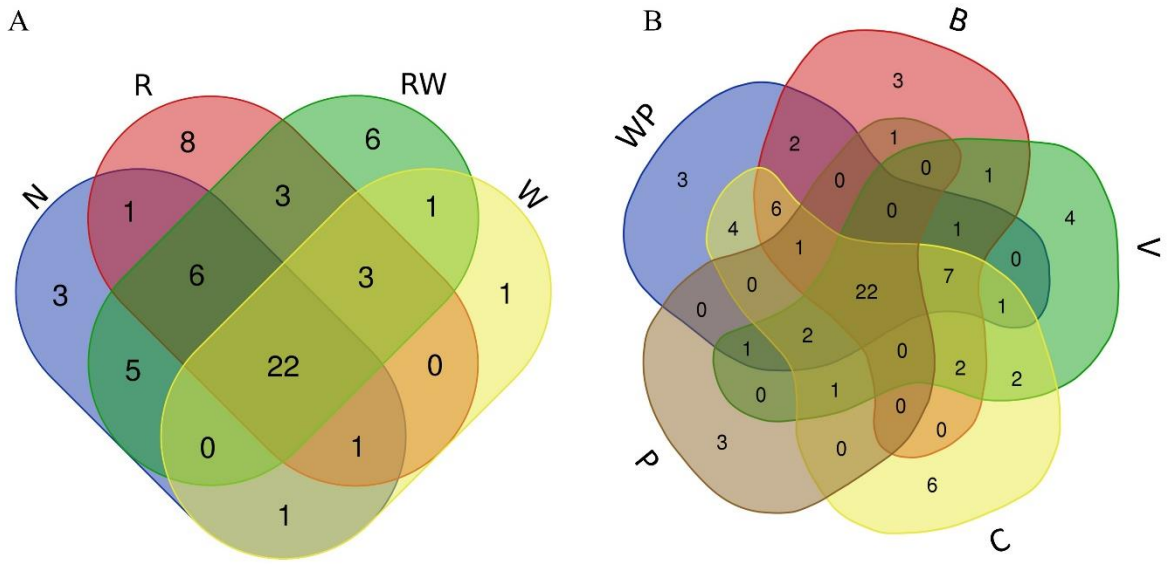
3.4.3.4. Skład rdzeniowego mikrobiomu ryzosfery i fyllosfery malin

Skład taksonomiczny mikrobiomu rdzeniowego (ang. core microbiome) ryzosfery malin może mieć kluczowe znaczenie dla prawidłowego funkcjonowania zbiorowisk mikroorganizmów. Jest to grupa organizmów obecnych w większości próbek. Liczbę mikroorganizmów rdzeniowych zasiedlających ryzosferę malin przedstawiono na rysunku 4.



Rysunek 4. Diagramy Venn’a przestawiające liczbę bakterii rdzeniowych w próbkach ryzosfery w zależności od zastosowanej strategii naturalizacji (A) i patosystemu (B). B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P – *Phytophthora* sp., N – bez naturalizacji, R – naturalizacja korzeni podczas sadzenia, W – podlewanie naturalizacyjne 4 tygodnie po posadzeniu, RW – naturalizacja łączona obejmująca naturalizację korzeni podczas sadzenia i podlewanie naturalizacyjne 4 tygodnie po posadzeniu.

Jak przedstawiono na rysunku 4A w obiektach z zastosowaniem podlewania naturalizacyjnego liczba poszczególnych bakterii była najwyższa, a w naturalizacji korzeni najniższa. Dla wszystkich strategii naturalizacji zaobserwowano 6 wspólnych wariantów sekwencji amplikonu ASV (Amplicon Sequence Variant) dla bakterii. Rycina 4B przedstawia liczbę bakterii wchodzących w skład mikrobiomu rdzeniowego charakterystycznych dla poszczególnych patosystemów. Patosystem *Phytophthora* sp. charakteryzował się 8 ASV dla bakterii, które występowały tylko w tym patosystemie. Przeprowadzone badania wykazały 6 bakteryjnych wariantów sekwencji amplikonu wspólnych dla wszystkich patosystemów.



Rysunek 5. Diagramy Venn'a przestawiające liczbę grzybów rdzeniowych w próbkach ryzosfery w zależności od zastosowanej strategii naturalizacji i patosystemu. B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P – *Phytophthora* sp., N – bez naturalizacji, R – naturalizacja korzeni podczas sadzenia, W – podlewanie naturalizacyjne 4 tygodnie po posadzeniu, RW – naturalizacja łączona obejmująca naturalizację korzeni podczas sadzenia i podlewanie naturalizacyjne 4 tygodnie po posadzeniu.

Liczbę rdzeniowych mikroorganizmów grzybowych dla poszczególnych strategii naturalizacji i patosystemów przedstawiono na rysunku 5. Najwięcej charakterystycznych mikroorganizmów grzybowych stwierdzono w wariantach z zastosowaniem naturalizacji korzeni i naturalizacji łączonej. Najwięcej grzybów związanych z określonym patosystemem odnotowano dla obiektów zakażonych *Colletotrichum acutatum*. Ponadto 22 zidentyfikowane mikroorganizmy grzybowe występowały we wszystkich próbkach bez względu na zastosowany patosystem czy strategię naturalizacji. Mikroorganizmy rdzeniowe charakterystyczne dla poszczególnych strategii naturalizacji i patosystemów występujących w doświadczeniu przedstawiono w tabeli 5. Większy udział grzybów niż bakterii w mikrobiomie rdzeniowym jest spowodowany zmianą stosunku ilości bakterii do grzybów na korzyść grzybów w składzie taksonomicznym zbiorowisk mikroorganizmów zasiedlających gleby uprawne [127,128].

Tabela 5. Skład mikrobiomu rdzeniowego w zależności od zastosowanych strategii naturalizacji i patosystemów. B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P – *Phytophthora* sp., N – bez naturalizacji, R – naturalizacja korzeni podczas sadzenia, W – podlewanie naturalizacyjne 4 tygodnie po posadzeniu, RW – naturalizacja łączona obejmująca naturalizację korzeni podczas sadzenia i podlewanie naturalizacyjne 4 tygodnie po posadzeniu.

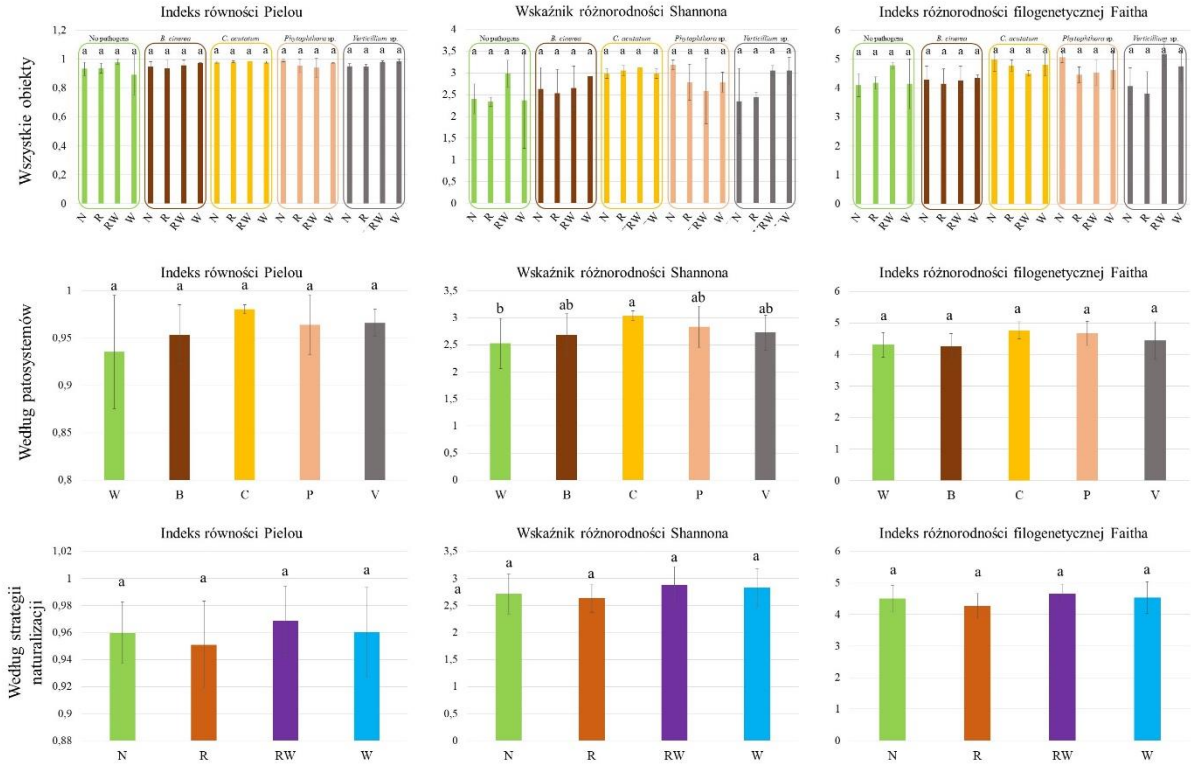
	Strategie naturalizacji			
	N	**R**	**RW**	**W**
Bakterie	Burkholderiales	Xanthobacteraceae	Micrococcaceae	Actinobacteriota MB-A2-108
	Bradyrhizobium		Chitinophagaceae	Mycobacterium
			Acidimicrobiia IMCC26256	Solirubrobacterales 67-14
			Gemmatimonadaceae	Methyloligellaceae
			Pedosphaeraceae ADurb.Bin063-1	Xanthobacteraceae
				Gaiellales
				Acidobacteriales
Grzyby	Hypocreales	Hypocreales	Microascales	Pleosporales
	Helotiales	Agaricales	Sordariales	
	Trechisporales	Agaricales	Helotiales	
		Chaetothyriales	Agaricales	
		Pleosporales	Capnodiales	
		Saccharomycetales	Pyxidiophorales	
		Ascomycota		

	Patosystemy				
	WP	**B**	**V**	**C**	**P**
Bakterie	Xanthobacteraceae	Pseudolabrys		Ktedonobacteria C0119	Acetobacteraceae
				Acidimicrobiia IMCC26256	Solirubrobacterales 67-14
				Parafilimonas	Blastocatellaceae
				Flavisolibacter	Reyranella
				Pedosphaeraceae ADurb.Bin063-1	Rhodanobacter
					Pedosphaeraceae Ellin517
					Rhizobium
Grzyby	Microascales	Pleosporales	Chaetothyriales	Chaetothyriales	Hypocreales
	Sordariales	Pleosporales	Pezizales	Helotiales	Eurotiales
	Cantharellales	Ascomycota	Agaricales	Chaetothyriales	
			Pyxidiophorales	Pleosporales	
				Hypocreales	
				Helotiales	

3.4.3.5. Bioróżnorodność mikroorganizmów bakteryjnych i grzybowych w ryzosferze i fyllosferze malin

Bioróżnorodność drobnoustrojów w zebranych próbkach została oceniona za pomocą 3 powszechnie wykorzystywanych wskaźników bioróżnorodności: wskaźnika różnorodności Shannona, równości Pielou i różnorodności filogenetycznej Faitha. Wskaźnik równości Pielou opisuje jak różni się liczba wariantów sekwencji amplikonu (ang. *Amplicon Sequence Variant*, ASV)/operacyjnych jednostek taksonomicznych (ang. *Operational Taxonomic Unit*, OTU)

w badanych próbkach. Indeks różnorodności Shannona pozwala na porównanie liczby ASV/OTU zasiedlających daną niszę ekologiczną i ich względną obfitość. Indeks różnorodności filogenetycznej Faitha bierze pod uwagę pokrewieństwo filogenetyczne poszczególnych mikroorganizmów, nie uwzględniając ich obfitości [129–131]. Analizę przeprowadzono dla zbiorowisk bakteryjnych zasiedlających ryzosferę malin oraz zbiorowisk grzybowych zasiedlających ryzosferę i części nadziemne malin, a wartości wskaźników przedstawiono na rysunku 6.

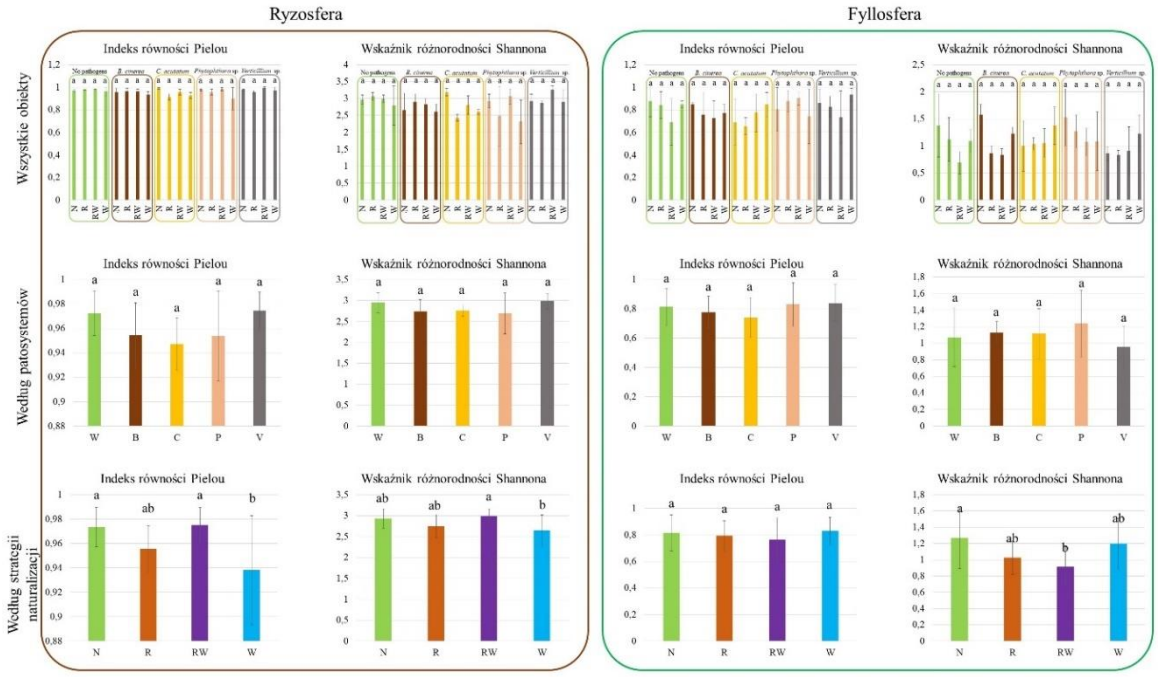


Rysunek 6. Wartości wskaźników bioróżnorodności dla zbiorowisk bakteryjnych zasiedlających ryzosferę malin. B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P – *Phytophthora* sp., N – bez naturalizacji, R – naturalizacja korzeni podczas sadzenia, W – podlewanie naturalizacyjne 4 tygodnie po posadzeniu, RW – naturalizacja łączona obejmująca naturalizację korzeni podczas sadzenia i podlewanie naturalizacyjne 4 tygodnie po posadzeniu.

Inokulacja roślin patogenami powodowała wzrost wartości wskaźników bioróżnorodności dla zbiorowisk bakterii w porównaniu z kontrolą. Spośród wszystkich przetestowanych strategii naturalizacji naturalizacja łączona spowodowała najwyższy wzrost wartości wskaźników bioróżnorodności dla zbiorowisk bakterii. Obecność fitopatogenów spowodowała wzrost różnorodności w obiektach bez zastosowanej żadnej strategii naturalizacji w porównaniu z kontrolą. W obiektach bez patogenów zarówno wskaźniki Shannona, jak i Faitha wskazywały na naturalizację łączoną jako najbardziej skuteczną w zwiększaniu bioróżnorodności zbiorowisk bakteryjnych. Obecność fitopatogenów w badanych obiektach

wpłynęła na zbiorowiska mikroorganizmów podwyższając wartość wskaźnika różnorodności Shannona [132].

Na rysunku 7 przedstawiono wartości wskaźników bioróżnorodności dla zbiorowisk mikroorganizmów grzybowych zasiedlających ryzosferę i fyllosfery malin. W przeciwieństwie do rosnących wartości wskaźników bioróżnorodności dla zbiorowisk bakteryjnych, wartości dla zbiorowisk grzybowych ulegały obniżeniu w patosystemach oraz po zastosowaniu strategii naturalizacji.



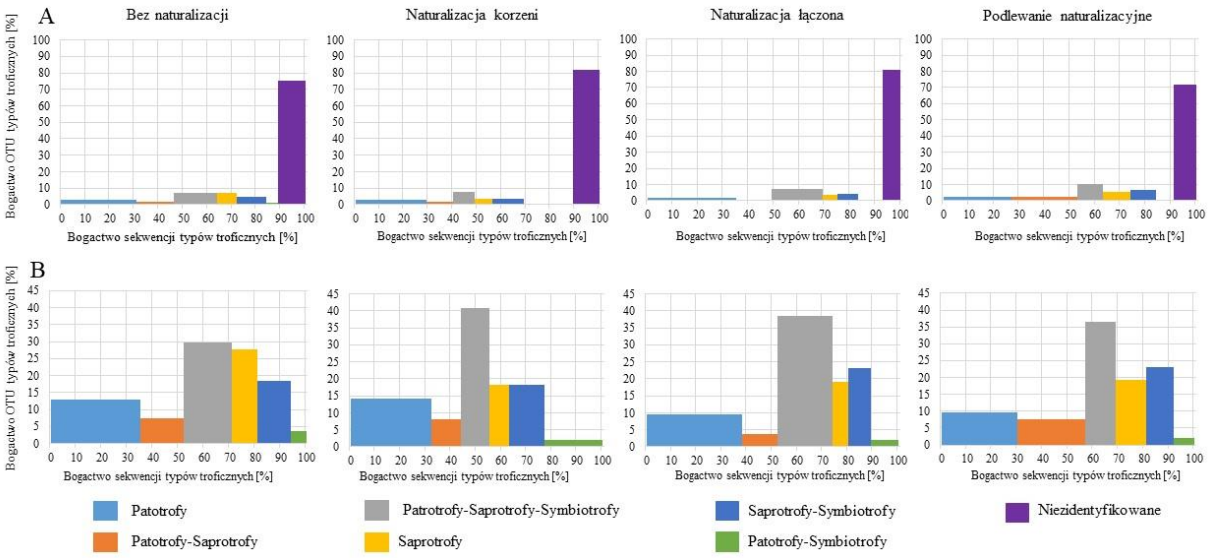
Rysunek 7. Wartości wskaźników bioróżnorodności dla zbiorowisk grzybowych zasiedlających ryzosferę malin. B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P – *Phytophthora* sp., N – bez naturalizacji, R – naturalizacja korzeni podczas sadzenia, W – podlewanie naturalizacyjne 4 tygodnie po posadzeniu, RW – naturalizacja łączona obejmująca naturalizację korzeni podczas sadzenia i podlewanie naturalizacyjne 4 tygodnie po posadzeniu.

Naturalizacja łączona nie spowodowała istotnej statystycznie różnicy w wartościach wskaźnika równości Pielou, w porównaniu do kontroli. Jednakże zastosowanie zarówno wyłącznie naturalizacji korzeni lub podlewania naturalizacyjnego spowodowało obniżenie wartości wskaźnika równości Pielou oraz bioróżnorodności Shannona dla zbiorowisk zasiedlających ryzosferę. Bioróżnorodność zbiorowisk mikroorganizmów zasiedlających fyllosferę malin podlegała zmianom wywołanym zarówno przez obecność patogenów (choć te zmiany nie były istotne statystycznie) jak również zastosowane strategie naturalizacji. Szczególną uwagę warto zwrócić na obniżenie wartości wskaźnika różnorodności Shannona, którego wartość była istotnie niższa pod wpływem każdego z badanych sposobów naturalizacji,

a najniższa była dla strategii naturalizacji łączonej. Zbiorowiska grzybów zasiedlające fyllosferę charakteryzowały się niższymi wartościami równości Pielou, oraz większą zmiennością wartości wskaźnika różnorodności Shannona, spowodowanymi większym narażeniem na warunki środowiskowe [133]. Statystycznie istotne obniżenie wskaźnika różnorodności Shannona dla zbiorowisk grzybowych zasiedlających fyllosferę może być spowodowane przeciwgrzybiczymi właściwościami zastosowanego inokulum pożytecznych bakterii.

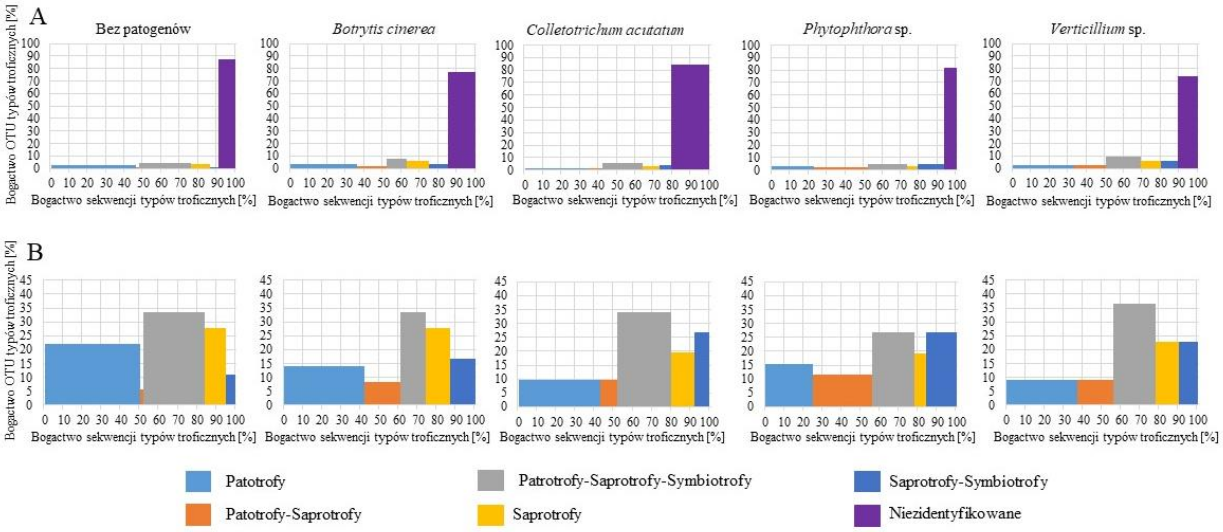
3.4.3.6. Porównanie typów troficznych mikroorganizmów grzybowych zasiedlających ryzosferę malin

Typy troficzne mikroorganizmów grzybowych obecnych w próbkach ryzosfery zidentyfikowano za pomocą narzędzia FUNGuild, a wyniki przedstawiono na rysunkach 8 i 9. Pozwoliło to na dalszą analizę zbiorowisk mikroorganizmów i ich interakcji. Zidentyfikowane i niezidentyfikowane mikroorganizmy podzielono na typy proficzne obejmujące patotrofy, saprotrofy, symbiotrofy i ich kombinacje, biorąc pod uwagę strategie naturalizacji oraz zastosowane patosystemy. Niezidentyfikowane i niehodowlane mikroorganizmy stanowią zdecydowaną większość obecnych w glebie OTU [134]. We wszystkich próbkach przeważały mikroorganizmy niezidentyfikowane, a ich względne bogactwo OTU stanowiło 70-80% wszystkich obecnych OTU. Jednak bogactwo ich sekwencji wynoszące od 7% do 10% było porównywalne z innymi grupami troficznymi mikroorganizmów, oprócz patrotrofów.



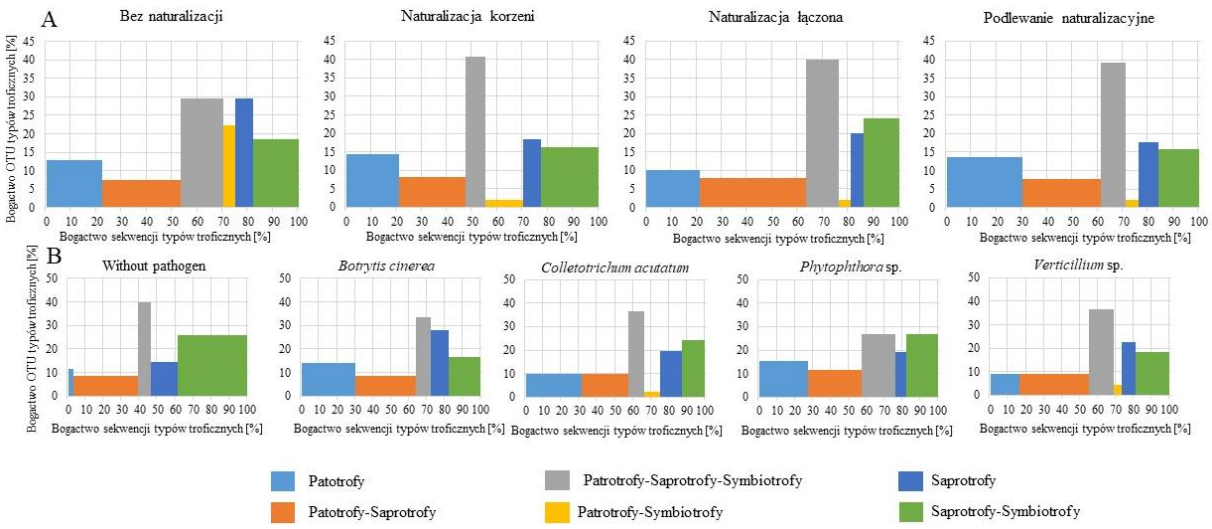
Rysunek 8. Względne bogactwo OTU i sekwencji dla grzybów zidentyfikowanych w ryzosferze pogrupowane ze względu na zastosowaną strategię naturalizacji. Część A przedstawia wyniki z uwzględnieniem niezidentyfikowanych grzybów, część B przedstawia wyniki z wyłączeniem grzybów niezidentyfikowanych. Wysokość kolumny reprezentuje obfitość OTU, a szerokość kolumny reprezentuje obfitość sekwencji.

Zmniejszenie się bogactwa OTU organizmów patotroficznych było obserwowane w obiektach, w których zastosowano naturalizację łączoną lub podlewanie naturalizacyjne. Zastosowanie naturalizacji było jednak również związane ze zwiększeniem bogactwa OTU organizmów multitroficznych oraz obniżeniem bogactwa OTU organizmów saprotroficznych, co może być efektem stymulacji bioróżnorodności zbiorowisk mikroorganizmów glebowych przez inokulum pożytecznych bakterii [95].



Rysunek 9. Względne bogactwo OTU i sekwencji dla grzybów zidentyfikowanych w ryzosferze pogrupowane ze względu na zastosowany patosystem. Część A przedstawia wyniki z uwzględnieniem niezidentyfikowanych grzybów, część B przedstawia wyniki z wyłączeniem grzybów niezidentyfikowanych. Wysokość kolumny reprezentuje obfitość OTU, a szerokość kolumny reprezentuje obfitość sekwencji.

Porównując wyniki dla poszczególnych patosystemów, przedstawione na rysunku 9, można zauważyć, że obecność fitopatogenów grzybowych spowodowała zmniejszenie bogactwa OTU organizmów patotroficznych w zbiorowiskach, jednocześnie zwiększając bogactwo OTU organizmów sapro-symbiotroficznych. Obecność inokulum fitpoatogenów znacznie zwiększyła również względne bogactwo sekwencji wykrywanych OTU należących do mieszanego trybu troficznego: pato-saprotroficznych.



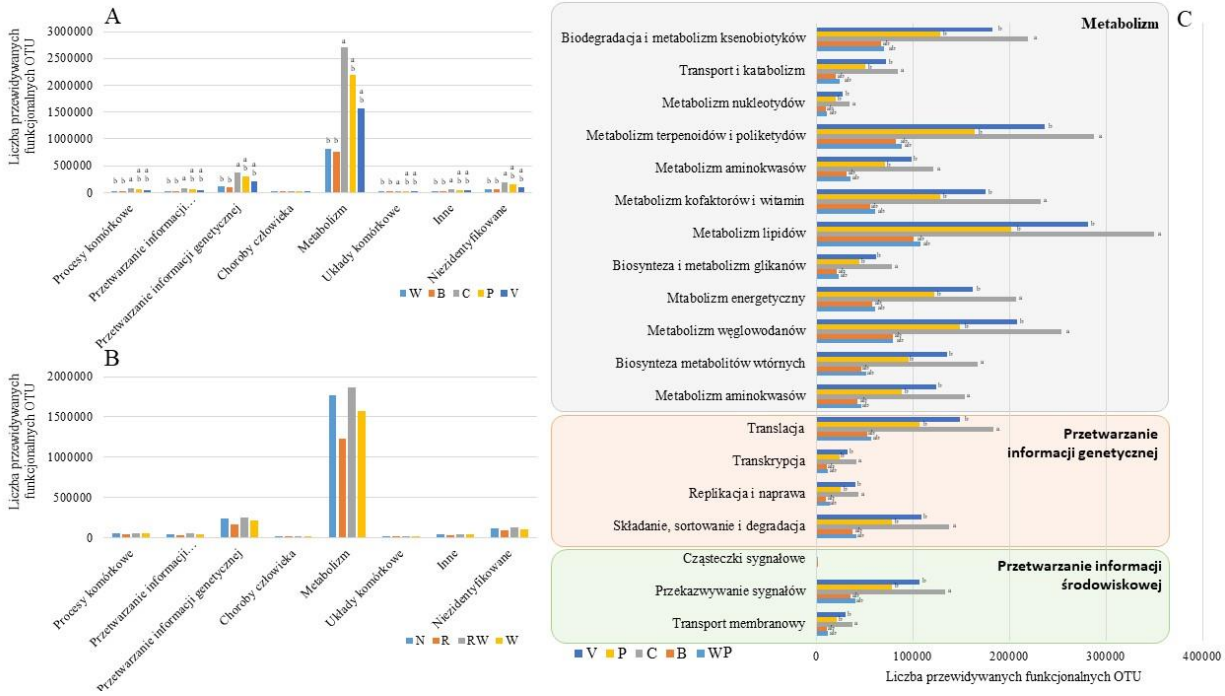
Rysunek 10. Procentowa częstotliwość występowania OTU i sekwencji dla grzybów zidentyfikowanych w fyllosferze pogrupowana ze względu na zastosowany patosystem. Część A przedstawia wyniki z uwzględnieniem niezidentyfikowanych grzybów, część B przedstawia wyniki z wyłączeniem grzybów niezidentyfikowanych. Wysokość kolumny reprezentuje procentowy udział OTU, a szerokość kolumny reprezentuje udział sekwencji.

Analizując wyniki analizy bogactwa typów troficznych dla OTU zidentyfikowanych w próbkach fyllosfery, zaprezentowane na rysunku 10, można zauważyć znaczne obniżenie bogactwa OTU organizmów pato-symbiotroficznych w obiektach, w których zastosowano dowolną ze strategii naturalizacji przy jednoczesnym wzroście bogactwa OTU mikroorganizmów multitroficznych. Występowanie OTU organizmów patotroficznych było najmniejsze w obiektach, w których zastosowano naturalizację łączoną. W przypadku obiektów, w których obecne było inokulum badanych fitopatogenów zaobserwowano zwiększone bogactwo sekwencji organizmów patotroficznych w próbkach w porównaniu do kontroli bez obecności fitopatogenów. Obecność fitopatogenów wpłynęła również na zmniejszenie bogactwa OTU organizmów multitroficznych. Interakcje zachodzące pomiędzy natywnymi mikroorganizmami związanymi z roślinami, obecność fitopatogenów a także dodatek inokulum bakterii pożytecznych mogą zmieniać proporcje typów troficznych mikroorganizmów glebowych [135,136].

3.4.3.7. Analiza funkcjonalna zbiorowisk mikroorganizmów bakteryjnych zasiedlających ryzosferę malin

Zidentyfikowane ASV bakteryjne poddane zostały analizie z wykorzystaniem środowiska PICRUSt i bazy KEGG, która pozwoliła na podział ASV na odpowiedzialne za odpowiednie grupy procesów metabolicznych. Wyniki tej analizy przedstawione są na rysunku 11. Większość zidentyfikowanych ASV odpowiedzialna była za procesy związane z metabolizmem komórkowym, co jest związane z faktem, że stanowią

one największą grupę szlaków metabolicznych obecnych w procesach metabolicznych bakterii [137]. Obecność patogenów za wyjątkiem *B. cinerea* powodowała zwiększenie obfitości przewidywanych funkcjonalnych ASV, a największe zróżnicowanie metabolizmu zbiorowisk bakteryjnych widoczne było dla obiektów zakażonych *C. acutatum*. 3 klasy procesów komórkowych, które wykazywały najwyższą aktywność zostały podzielone na podklasy, aby lepiej zobrazować poszczególne zależności pomiędzy obecnością fitopatogenów a aktywnością komórek. Zwiększenie aktywności szklaków metabolicznych zostało także zaobserwowane w glebach, do których wprowadzono nawozy mineralne wzbogacone pożytecznymi szczepami bakterii [7,125].



Rysunek 11. Przewidywany profil funkcjonalny zbiorowisk bakterii na podstawie zidentyfikowanych ASV, pod względem patosystemów (A i C) oraz naturalizacji (B). B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P – *Phytophthora* sp., N – bez naturalizacji, R – naturalizacja korzeni podczas sadzenia, W – podlewanie naturalizacyjne 4 tygodnie po posadzeniu, RW – naturalizacja łączona obejmująca naturalizację korzeni podczas sadzenia i podlewanie naturalizacyjne 4 tygodnie po posadzeniu.

Najsilniej reprezentowanymi podklasami w metabolizmie zbiorowisk bakteryjnych były szlaki związane z metabolizmem lipidów, węglowodanów, terpenoidów, poliketydów, ksenobiotyków, a także metabolizmem kofaktorów i witamin. Podobne wyniki zaobserwowano dla podklas związanych z przetwarzaniem informacji genetycznych i środowiskowych, jednak najbardziej reprezentowanymi podklasami były odpowiednio translacja i transdukcja sygnałów. Gdy wyniki pogrupowano według strategii naturalizacji, obiekty, w których zastosowano naturalizację łączoną wykazały najsilniejszy wzrost liczby genów odpowiedzialnych za metabolizm w porównaniu z kontrolą bez zastosowanej naturalizacji,

jednak różnice nie były istotne statycznie. Wzrost obserwowany w tej strategii naturalizacji może być spowodowany podwójną aplikacją inokulum, gdyż wielokrotna aplikacja preparatów mikrobiologicznych wzmacnia ich działanie, a często jest niezbędna do zaobserwowania efektów ich działania [95,138].

4. Podsumowanie i wnioski

Niezmiennie narastająca intensyfikacja rolnictwa charakteryzująca się zwiększonym zużyciem chemicznych środków ochrony roślin, niezbędnych do produkcji odpowiedniej ilości plonów, może prowadzić do znacznej degradacji środowiska glebowego. Ograniczenie tego procesu oraz przywracanie utraconej bioróżnorodności to kluczowy element rolnictwa ekologicznego, zrównoważonego i regeneracyjnego. Wykorzystanie biopreparatów nie ogranicza się do zastosowań wyłącznie rolniczych, gdyż tego typu preparaty mogą być również stosowane do przywracania do stanu pierwotnego gleb zdegradowanych. Zastosowanie środowiskowych izolatów szczepów bakteryjnych jako składnika aktywnego biopreparatów pozwala wykorzystać ich właściwości i uzdolnienia do hamowania wzrostu fitopatogenów, biostymulacji wzrostu roślin, zwiększania biodostępności składników odżywczych oraz kontroli transformacji materii organicznej w środowisku glebowym. Pomimo ciągłych badań nad mikroorganizmami i nowymi formulacjami preparatów mikrobiologicznych ważne jest opracowywanie nowych preparatów, zawierających lokalnie występujące szczepy mikroorganizmów pożytecznych. Zapewni to większą kompatybilność produktu z roślinami i zbiorowiskami mikroorganizmów glebowych, a co za tym idzie lepsze efekty stosowanej naturalizacji. Ze względu na zmieniające się dynamicznie warunki klimatyczne, zasięgi występowania fitopatogenów, a także zmieniające się praktyki rolnicze tematyka związana z opracowywaniem nowych biopreparatów i badaniem ich wpływu na rośliny, fitopatogeny i zbiorowiska mikroorganizmów pozostaje tematem aktualnym i wymagającym dalszych, ukierunkowanych badań, które pozwolą nie tylko na opracowanie nowych formulacji, ale także na lepsze wykorzystanie zasobów zarówno w rolnictwie ekologicznym, jak i konwencjonalnym.

Na podstawie wyników przeprowadzonych badań można sformułować następujące wnioski:

- W zbiorowiskach mikroorganizmów zasiedlających ryzosferę malin leśnych znajdują się bakterie o działaniu hamującym wzrost fitopatogenów, spośród których wytypowano 4 komplementarne szczepy o zróżnicowanej aktywności i właściwościach uzupełniających swoje działanie:
    - *Rhodococcus* sp. B12/18 (GenBank: MW255650),
    - *Pseudomonas* sp. B37/18 (GenBank: MW255651),
    - *Arthrobacter* sp. B58/18 (GenBank: MW255652),
    - *Rhodococcus* sp. B75/18 (GenBank: MW255653).

- Przeprowadzone badania dotyczące uzdolnień metabolicznych wybranych szczepów bakterii pozwoliły na określenie składu prebiotycznej mieszanki suplementacyjnej, której 1% dodatek pozytywnie wpływał na wzrost pożytecznych bakterii. W jej skład wchodzą:
    - kwas α-ketoglutarowy,
    - kwas jabłkowy,
    - N-acetylo-D-glukozamina,
    - kwas glutarowy.
- Zaproponowany skład podłoża hodowlanego, warunki hodowli oraz suszenia pozwalają na otrzymanie funkcjonalnego biopreparatu mikrobiologicznego. Opracowana formulacja obejmuje w szczególności:
    - źródło węgla – sacharoza 3%
    - źródło azotu – azotan amonu 6%
    - pH podłoża hodowlanego – 7-8
    - temperaturę hodowli – 24-30℃
- Aplikacja inokulum bakteryjnego pozytywnie wpłynęła na aktywność dehydrogenaz w obiektach, w których nie zastosowano dodatku inokulum wybranych fitopatogenów.
- Aplikacja inokulum pożytecznych bakterii poprzez podlewanie naturalizacyjne 4 tygodnie po posadzeniu, wywołała efekt biostymulujący wzrost roślin, wpływając pozytywnie na suchą masę fyllosfery w obiektach, w których obecne były fitopatogeny. Wybór strategii naturalizacji powinien w dużej mierze zależeć od rodzaju fitopatogenów występujących w miejscu aplikacji.
- Aplikacja inokulum pożytecznych bakterii spowodowała obniżenie wskaźnika stresu substratowego dla zbiorowisk zasiedlających fyllosferę malin.
- Zbiorowiska mikroorganizmów zasiedlających ryzosferę malin są bardziej odporne na zmiany różnorodności funkcjonalnej niż zbiorowiska zasiedlające fyllosferę.
- Aplikacja inokulum pożytecznych szczepów bakterii spowodowała zmniejszenie wskaźnika różnorodności Shannona dla zbiorowisk mikroorganizmów grzybowych zasiedlających ryzosferę malin.
- Aplikacja inokulum pożytecznych szczepów bakterii spowodowała statystycznie istotne zmiany w metabolizmie zbiorowisk drobnoustrojów glebowych, zwiększając liczbę wariantów sekwencji amplikonów związanych z metabolizmem bakterii i procesami komórkowymi.

- Inokulum bakteryjne będące składnikiem aktywnym biopreparatu jest efektywnym środkiem hamującym wzrost wybranych patogenów grzybowych i grzybopodobnych, wpływającym biostymulująco na rośliny malin, oraz stymulująco na zbiorowiska mikroorganizmów zasiedlających ryzosferę i fyllosferę malin uprawnych.

5. Tekst publikacji P.1

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REVIEW PAPER

Review report on the role of bioproducts, biopreparations, biostimulants and microbial inoculants in organic production of fruit

Michał Pylak · Karolina Oszust · Magdalena Frąc

Published online: 15 July 2019

Abstract The demand for ecologically cultivated fruits is growing each year, but the yields in organic farming are still lower than the yields in conventional farming. Moreover plant pathogens are a serious threat in organic fruit production and the assortment of conventional pesticides is limited in organic farming. The European Commission has established regulations that state which types of bioproducts can be used in organic farming. Appropriately chosen biopreparations might be a solution to this problem. Biopreparations are products used to inhibit the growth of pathogenic fungi or bacteria, stimulate plants growth and enhance plant nutrient uptake. They can be composed of plant growth promoting bacteria and fungi, plant extracts or animals-derived compounds. The second category of bioproducts useful for enhancing yield and nutrient uptake are biostimulants. They can be composed of microorganisms, protein hydrolysates, seaweed extracts and other substances. Bacteria, fungi and yeasts are used in biocontrol of plant pathogens and in enhancing plants growth by producing hormone-like substances and reducing symptoms of environmental stress caused by weather or soil factors such as drought or low nutrient availability.

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Keywords Strawberries · Horticulture · Plant pathogens · Nutrients availability · Plant growth promoting fungi · Plant growth promoting rhizobacteria

1 Introduction

It is hard to deny that organic farming is one of the most important practices in fruits production, in the context of modern agriculture (Barłowska et al. 2017). Approximately a third part of the world's organic farming is located in Europe, and 6.4% of European organic farming is currently in Poland. The area of organic cultivation has increased over recent years significantly. The fastest growth of organic farming was observed between 1999 and 2013. Throughout this period, the number of organic food producers in Poland rose from 27 to 26 499, and the cultivated area increased from 300 to 674 694 ha (Kiełbasa 2015). Increased demand for organic fruits has been observed in the past years. The total area under organic cultivation in the EU-28 has risen from just over 4 million ha in 2002, to 12 million ha in 2016 (McEldowney 2018). It is important to know that when farmers decide to cultivate an area by means of the organic method, they are obliged not to use most of the commercially available pesticides. Because of this fact, the costs of organic food production are higher,

and the yield is usually lower comparing to the conventional farming (Kiełbasa 2015). Organic agriculture very often is considered as a system oriented towards revitalization of soil as a self-regulatory mechanism in case of respecting the main agronomic and ecological regularities. In such a system application of external inputs, even of biological origin, should be limited and before application each biopreparation or solution should be tested comprehensively. Therefore, this restriction also stimulated the scientific research in terms of biological methods of plant protection and biostimulation. European Commission regulations state that bioproducts such as Azadirachtin extracted from *Azadirachta indica* (Neem tree), lecithin with fungicidal properties, plant essential oils or microorganism-based biopreparations are allowed in organic production of fruits (appendix to WE No. 889/2008). WE No. 834/2007 states that using chemical pesticides must be limited to the absolute minimum and farmers are encouraged to use substances of natural origin. During past years of research many different types of products were invented including: bioproducts, biopreparations, biostimulants and microbial inoculants, to enhance plants health, vigor, growth and yield or/and protect them against abiotic and biotic stress factors including plant pathogens.

Biopreparations is any product derived from a living organism or its metabolites. Bioproducts or bio-based products are materials, chemicals, and energy from renewable biological resources (Singh et al. 2003). Biostimulants are materials, other than fertilizers, that promote plant growth when applied in low quantities (du Jardin 2015). Microbial inoculants are amendments containing beneficial microorganisms, able to promote plants health.

Biopreparations are products used to inhibit the growth of pathogenic fungi or bacteria. They can be made from a variety of bioproducts obtained from natural sources. This includes plant extracts, humic substances, polysaccharides, e.g. chitosan. Biopreparations can also contain a great variety of beneficial microorganisms, bacteria or fungi. It has been found that in many cases they may be at least as efficient in biocontrol of fruit pathogens as conventional, commercially available products (Wagner and Hetman 2016). It is important to remember, that the efficacy of biopreparations varies and is highly dependent on many factors such as soil and air humidity or rainfalls (Pačuta et al. 2018). Storage conditions can also influence the germination rates of conidia in biopreparations based on fungi like *Trichoderma harzianum* (Leal et al. 2016). Also, it should be borne in mind that different species of the pathogen genus may not be sensitive to identical biopreparation to the same extent (Hussein et al. 2014).

The aim of this review is to characterize various commercially available biopreparations and bioproducts based on plant growth promoting bacteria and fungi, plant and algae extracts, and animals-derived bioproducts such as chitosan. Furthermore, as the review characterize biostimulants and their performance, as well as overviews most common microbial inoculants used for plant pathogens biocontrol and the enhancement plants growth. The scheme summarizing the content of this reviews is given at Fig. 1.

2 Biostimulants

Organic horticulture aims to reduce impact of fruit production on ecosystems. However, it generates lower yields and more land is needed compering to conventional one (Dorais and Alsanius 2015). Substances other than fertilizers, that have the ability to promote plant growth even when applied in low quantities e.g. Kelpak Sl are defined as biostimulants (du Jardin 2015). Application of biostimulants is intended to increase crop productivity in organic farming more widely (Trewavas 2001). Yield in organic farming may be lower by 5–32% in comparison to conventional farming practices, depending on the plants tested (Ponisio et al. 2015). Many factors contribute to the situation and previous research suggests it might be associated with fungal or bacterial pathogens and nutrient limitations (De Ponti et al. 2012). Some of the studies have shown that the nutrient availability (mostly N and P) is the main cause of lower yield in organic horticulture (Berry et al. 2002). Commonly used organic fertilizers are mostly: pelleted chicken manure, fish and meat meal, seabird and bat guano and abattoir waste. Due to their characteristics they fail to supply plants with the most needed nutrients during the intense growing period (Tuomisto et al. 2012; Zhao et al. 2009). Another factor that needs to be taken under consideration is bioavailability of other nutrients e.g. Fe, P, Zn, Cu, Mn. The uptake of these elements is often reduced in

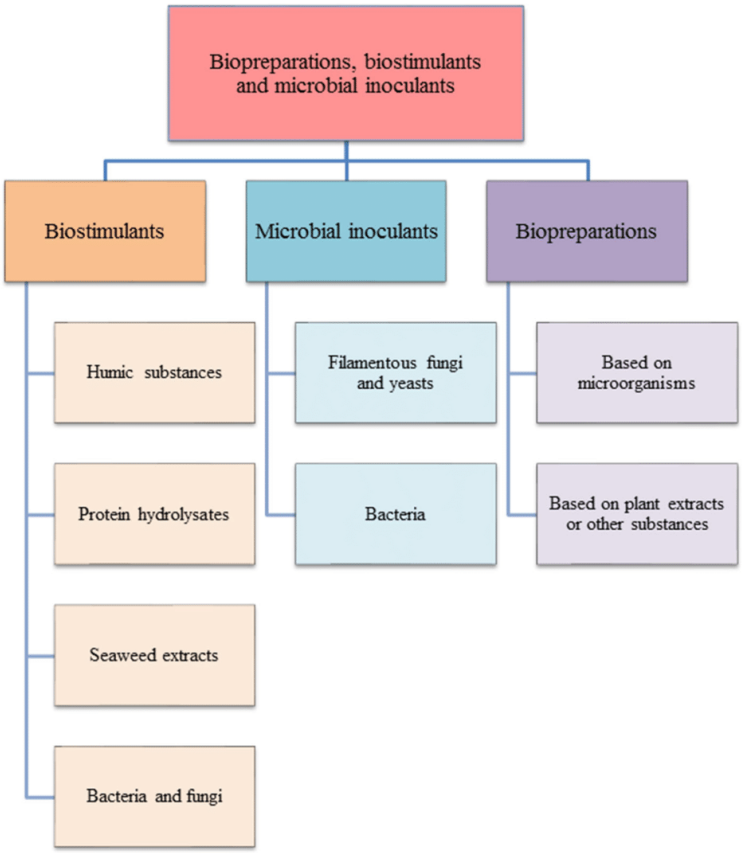

Fig. 1 Presentation of biopreparations, biostimulants and microbial inoculants

unfavourable soil pH, which means these elements form insoluble compounds in alkaline or acidic soils (Barbieri et al. 2015). Du Jardin (2015) defined plant biostimulants (PBs) as any substance or microorganisms supplied to plants primarily with the aim of enhancing nutrition uptake efficiency, but also increasing abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content. The most promising PBs are: seaweed extracts, protein hydrolysates, humic and fulvic acids, silicon, chitosan, inorganic compounds and beneficial fungi and bacteria (Ruzzi and Aroca 2015).

The enhancement of the nutrient uptake and assimilation is one of the benefits of using biostimulants. It is often attributed to at least one of the following factors: biostimulants can increase the activity of soil both microbiologically and enzymatically, they are able to affect the root structure, and change the solubility and transportability of micronutrients (Colla and Rouphael 2015; Ertani et al. 2009; Lucini et al. 2015). In the past few decades not only the use of biostimulants in organic horticulture was under research, but also the use of microbial inoculum. Plant growth promoting bacteria and fungi might be essential for maintaining proper growth, despite nutrient limitation commonly occurring in organic farming (Rouphael et al. 2017). They can be combined with Si to enhance their ability to alleviate biotic stress (Etesami 2018). Nutrient uptake facilitated by microorganisms resulting in better plant growth, is a result of diverse mechanisms such as: supplying nitrogen to soil by biological fixation of N_2; enhancing

bioavailability of soil nutrients by secreting enzymes (i.e. phosphatases), the presence of siderophores or organic acids that have the ability of mineral phosphates solubilisation and other nutrients; increasing the surface contact between roots and soil and consequently increasing plant's access to nutrients (Calvo et al. 2014; Colla et al. 2015a; Rouphael et al. 2015).

2.1 Nutrient availability in soil

It is common for plants in organic farming to experience nutrient deficiencies due to the low amounts of soil nutrients or their low solubility. One of the roles of plant biostimulants is increasing the amount of nutrients available for plants by increasing soil cation exchange via providing nitrogen and enhancing solubility of soil nutrients (De Pascale et al. 2017).

Some of the most important plant biostimulants commonly used for many years are humic substances. They are formed as the result of the chemical and biological decomposition of organic matter, including microbiological processes (du Jardin 2015). Humic substances are recognized as an indispensable part of physico-chemical soil properties. They stimulate root growth and thus increase soil nutrient availability due to an increase in the area contact between soil and roots (Canellas et al. 2015). Humic compounds are capable of increasing soil cation exchange capacity and neutralize soil pH. They also create complexes with insoluble elements like Fe and make them available for plants. This is an important property, because it allows to supply plants in micronutrients, which are not easily available (García-Mina et al. 2004). Fulvic acids, which are a part of humus substances can be absorbed by a plant as a complex with cations, because of their small molecular mass. It is also confirmed that humic substances have the ability to inctease plasma membrane H^+-ATPase activity, enhancing H^+ secretion which lowers the pH of soil and root surface. Lower pH assists with nutrient availability and uptake (Canellas et al. 2015; Nardi et al. 2002). Humic substances have also the ability to impact stress reduction and the production of secondary metabolites. Humic and fulvic acids can bind heavy metals, therefore there are less likely to be assimilated by the plant during nutrient uptake (Yang et al. 2013). The Pb^{2+} is a toxic lead ion often found in lead polluted soils. It can be complexed by humic compounds, so that it becomes less soluble and its uptake is reduced. Depending on soil pH the efficiency of this process might vary and it was observed, that humic substances are not particularly effective in reducing the solubility of heavy metals in acidic soils ($pH < 5$) (Park et al. 2013; Yang et al. 2013).

Protein hydrolysates (PH) are another principal group of plant biostimulants. There are a mixture of oligopeptides, polypeptides and amino acids that are being made out of protein used partial hydrolysis (Schaafsma 2009). They can be applied as foliar sprays or dosed into the soil near the plant's roots (Colla et al. 2015b). They not only enhance soil properties like respiration but also act as growth stimulants for soil microbiota due to their ability to use the PH are an easy carbon and nitrogen sources readily available sources for microorganisms. Protein hydrolysates can also complex and chelate soil micro- and macronutrients so that these become more accessible to plants (du Jardin 2015; Farrell et al. 2014).

Another important category of plant biostimulants are bacteria and fungi that have the ability to promote plants growth. There are many species that are symbiotic to plants e.g. *Azorhizobium, Allorhizobium, Bradyrhizobium, Mezorhizobium, Rhizobium*, and *Sinorhizobium* and non-symbiotic nitrogen-fixing bacteria like *Azospirillum, Azotobacter, Bacillus* and *Klebsiella* (Bhardwaj et al. 2014; Calvo et al. 2014; Hayat et al. 2010; Miransari 2011). They are used to enhance plants growth by increasing the amount of nitrogen, phosphorus and other micronutrients in soil. The usefulness of those bacteria goes beyond fixing the N_2, they also have the ability to recycle organic matter. The biostimulants can mineralize organic nitrogen through nitrite to nitrate that is easily absorbed by plants (Miransari 2011). One of the most important and well-studied nitrogen binding bacteria species is *Azospirillum* spp. (Calvo et al. 2014). An increased soil nitrogen content was observed after inoculating sugarcanes plantation with *A. dizaotrophicus*, with an increase of up to 60–80% compared to conventional agriculture (Boddey et al. 1991). Furthermore, some *Bacillus* species i.e. *B. megaterium, B. circulans, B. subtilis, B. polymyxa, B. sircalmous* have the ability to solubilise phosphates. The mechanisms of plant growth promoting by bacteria and fungi are complex, and still not fully understood (Satyaprakash et al. 2017). Some of them are capable of producing

plant hormones for instance auxins, cykokinins, gibberellins, ethylene and abscisic acid (Hayat et al. 2010). Auxins are well known for their capacity to stimulate root growth and in consequence nutrient and water uptake. Cykokinins are responsible for intensifying mitotic cell division in shoots and roots. Gibberellins affect flower and fruit formation, as well as seed germination. Finally, abscisic acid plays an important role in responses to draught, high salinity, and other environmental stresses (Sah et al. 2016). Some strains of bacteria are also able to produce phosphatases and organic acids which are essential to solubilise inorganic phosphate. This leads to increasing concentration of phosphate in soil so that it is more readily available for plants. Mycorrhizal fungi are also responsible for enhancing plants growth in organic farming. They have the ability to live in a symbiosis with plants—hyphae growing into plants roots increase their surface area resulting in increased capacity for absorbing nutrients and water. Fungi also produce siderophores, which chelates iron ions, and secrete phosphatase, and other organic compounds which are essential to enhancing P availability (Rouphael et al. 2015).

2.2 Uptake of nutrients by plants

The ability to uptake nutrients depends on many factors like e.g. environmental conditions, plant species, development of root system, and microorganisms living in symbiosis with roots. The development of roots plays a fundamental role in plants ability to effectively absorb nutrients specially in organic horticulture, where nutrients may appear in soil in low concentrations (De Pascale et al. 2017). It was proved that plant biostimulants enhance the root growth leading to larger root surface area and soil penetration, but recent studies suggest, that the auxins concentration in many biostimulants is too low to efficiently stimulate root grow (Wally et al. 2013). It was observed, that organic compounds, like amino acids, aromatic carboxylic acids and linear carboxylic acids found in humic acids can act as auxins to the plant stimulating the root growth. Moreover, humic substances are capable of stimulate the expression of genes instrumental in responding to presence of auxins, and enhancing the synthesis of plasma membrane H^+-ATPase, which stimulates root growth.

Protein hydrolysates also play an important role in stimulating plant's roots growth and have been proven to do so not only in crops like tomatoes or lettuce but also in fruits like strawberries (Lucini et al. 2015; Marfà et al. 2009). This property is thought to be the effect of peptides and amino acids capable of acting as signalling molecules like hormones (Matsubayashi and Sakagami 2006). Many studies have been conducted over the years showing that, for example tryptophan can be linked to inducing auxins production in plants fertilized with biostimulants, glutamate might be a key to the change roots architecture. It has the ability to stimulate the root branching, which advances plants ability to exploit the soil (Colla et al. 2014; Forde and Lea 2007). Moreover, biostimulants also intensify the growth of bacteria and fungi, which might have the ability to produce auxins-like substances (Luziatelli et al. 2016).

Some research reported enhanced growth of the plant roots cultivated with the addition of seaweed extract (SWE). It is a complex mixture composed of polysaccharide, fatty acids, vitamins, phytohormones and mineral nutrients (Battacharyya et al. 2015). It has been found, that addition of SWE can increase the root biomass in hydroponically grown plants even with low nutrient concentration in the medium (Vernieri et al. 2006). The authors of a publication from 2014 have proved that the foliar application of seaweed extract three times in concentration of 2 ml l^{-1} have induced positive effects on the plant growth, fruit yield, and quality of Sweet Charlie strawberry plants produced from cold stored transplants (Youssef and Metwally 2014). Other researchers have found out that fertilising strawberry plants with Ascophyllum extract in form of solution weekly added to the soil, increased total root length, root surface area, root volume per plant, leaf area and shoot fresh/dry weights of strawberry. The soil microbial colony counts and total microbial physiological activity in soil were also increased (Alam et al. 2013).

Biostimulants based on microorganisms are also capable of enhancing plants root growth, for instance *Trichoderma* spp. can promote plants root growth, by secreting auxin-like compounds in its hyphae, or enhancing auxin production in mycorrized plant roots (Colla et al. 2015a; Frąc et al. 2018). A meta-analysis conducted by Rubin et al. (2017) showed, that bacteria promoting root growth in plants may increase root mass up to 35% in well-watered conditions and up to

43% in drought conditions. Endophytic fungi and plant growth promoting bacteria enhances root growth, in combination with external mycorrhizal mycelium enlarge the volume of soil available for plant nutrient uptake, and it enhances resilience with respect to low nutrient concentration in soil.

The usefulness of biostimulants in enhancing the nutrient uptake in plants consists not only in enhancing root growth, but also on increasing the nutrient uptake itself. Some studies suggest, that biostimulators such as humic substances, protein hydrolysates or seaweed extracts are up-regulating genes involved in nutrients transport. For example it has been proven, that humic acids can up-regulate genes (BnNRT1.1 and BnNRT2.1) responsible for nitrogen transport in *Brassica napus* (Jannin et al. 2012). A similar property was observed by Cerdán et al. (2013). The authors reported, that root application of protein hydrolysates, extracted from plants, can enhance plants Fe(III)-chelate reductase activity. This results in increased the capacity to uptake iron ions from the soil. It has also been found that inoculating soil with arbuscular mycorrhizal fungi (AMF) (e.g. *Scutellospora calospora*, *Acaulospora laevis*, *Gigaspora margarita*, *Glomus aggregatum*, *Rhizophagus irregulare* (syn *G. intraradices*), *Funneliformis mosseae* (syn *G. mosseae*), *G. fasciculatum*, *G. etunicatum,* and *G. Deserticola*) and/or plant growth-promoting rhizobacteria (PGPR) (e.g. *Bacillus amyloliquefaciens, B. brevis, B. circulans*, *B. coagulans*, *B. firmus*, *B. halodenitrificans*, *B. laterosporus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, *B. pasteurii*, *B. polymyxa*, and *B. subtilis*) up-regulate the genes responsible for nitrate transport (NRT1.1, NRT2, and NAR2.2), which resulted in enhancing nitrogen uptake and increasing the overall nitrogen in durum wheat biomass (Saia et al. 2015). Studies also show, that root inoculation of potted pear plants with *Staphylococcus* and *Pantoea* lead to increased Fe absorption from soil. It is suspected to be the effect of lowering pH value of rhizosphere and the resulting increase in the activity of root Fe(III)-chelate reductase.

2.3 Assimilation of nutrient by plants

The least studied property of plant biostimulants is their capacity to stimulate expression of genes responsible for enzymes important in plants' metabolism and in assimilation of absorbed nutrients. From over 30,000 genes analyzed 300 genes were expressed in a different way in plants treated with humic substances 3 days before the analysis, compare to non-treated plants. A month later still over a 100 genes were being expressed differently. 50% of upregulated genes under investigation were taking part in nitrogen assimilation or in photosynthesis (Jannin et al. 2012). Similar research conducted on protein hydrolysates has shown, that this type of biostimulants can enhance enzymes activity, in particular enzymes responsible for carbon metabolism, and nitrate assimilation (Schiavon et al. 2008). Related studies were conducted on seaweed extracts, and the results proved that the seaweed extract applied by a foliar spray also has the ability to stimulate nitrogen assimilation enzymes (Zhang et al. 2010). Such experiments were prepared using microorganisms. Plants inoculation with arbuscular mycorrhizal fungi *Glomus intraradices* or plant growth-promoting rhizobacteria *Pseudomonas mendocina* enhanced the activity of nitrate reductase in leaves (Kohler et al. 2008).

3 Microbial inoculants

While discussing bioproducts and biopreparations it is important to take a closer look at microorganisms included the products. Organisms used in commercially available bioproducts are mostly plant growth promoting fungi and bacteria. They are able to enhance nutrient uptake by plants, to parasite on plant pathogens, to induce resistance of plants, or to secrete hormone-like compounds.

3.1 Fungi and yeasts

Fungi and yeasts presented in this section are some of the most commonly used species in the current research, and currently available biopreparations.

3.1.1 Trichoderma spp.

One of the most effective antagonists of plant pathogens are the fungi belonging to *Trichoderma* genus. *Trichoderma harzianum*, as a compound of some biopreparations is one of the most commonly used plant pathogen antagonist. It not only has the capacity to function as a mycoparasite, but it also produces antibiotic substances and due to high grow

rate it is an excellent nutrient competitor. Morover, it can also stimulate plant defense mechanisms (Benítez et al. 2004). Biopreparations with *Trichoderma harzianum* are used in many countries e.g. in Poland, and marketed as a Trianum-P with the T-22 strain. Many scientist have studied the production of antimicrobial compounds by *Trichoderma* sp. and they have found out that the fungi are capable of producing, among other things, viriden, peptaboils, gliotoxines, sesquiterpenes and isonitryles. Those compounds are toxic enzymes that are being used by the fungi to inhibit the growth of other competitors in the ecological niche (Berg et al. 2004). Due to the capacity to produce chitinase the fungi belonging to *Trichoderma* can act as a mycoparasite through the degradation of pathogen cell walls (Benítez et al. 2004; El-Katatny et al. 2000; Kubicek et al. 2001; Larkin 2016; Ozbay and Newman 2004). The spectrum of pathogens affected by *Trichoderma* sp. is very broad, and includes the following genera: *Armillaria, Botrytis, Chondrostereum, Colletotrichum, Dematophora, Diapor- the, Endothia, Fulvia, Fusarium, Fusicladium, Helmin- thosporium, Macrophomina, Monilia, Nectria, Phoma, Phytophthora, Plasmopara, Pseudoperonospora, Pythium, Rhizoctonia, Rhizopus, Sclerotinia, Sclerotium, Venturia, Verticillium* (Datnoff et al. 1995; De Melo and Faull 2000; Monte 2001; Tronsmo and Dennis 1977). It is important to notice, that some fungi belonging to *Trichoderma* genus such as *Trichoderma atroviride* G79/11 are known to produce cellulases, but also can produce other enzymes, which makes them suitable for antifungal biopreparations (Oszust et al. 2017a, b).

Trichoderma sp. fungi illustrate, the most common mechanisms of antagonism against pathogenic fungi (Fig. 2). There are five main mechanisms involved in attacking other fungi and promoting plant growth: competition for nutrients and space, production of inhibitory compounds, mycoparasitism, inactivation of the pathogen enzymes and induced resistance (Elad et al. 1999; Haran et al. 1996; Lorito et al. 1996; Roco and Pérez 2001; Yedidia et al. 1999, 2000).

Competition is one of the most common biological control activities. Fungi belonging to *Trichoderma* sp. are known for very fast growth and are treated as aggressive competitor. They quickly colonizes substrates excluding slower growing pathogens such as *Fusarium* sp. (Papavizas 1985). This property may be very useful for protecting plants by seed treatments, because the protection against pathogens is critical during the germination. *Trichoderma* sp. added to the soil during seeding or applied beforehand during seed growth along the expanding root system (Harman 2000; Harman et al. 1998; Sivan 1989).

Antibiosis is the antagonisms mechanism of numerous *Trichoderma* spp. isolates discovered to be capable of producing antibiotic substances. These compounds are often inhibiting the growth of pathogens. For example Howell and Stipanovic (1983) discovered an antibiotic—glovirin, isolated from *Trichoderma virens,* that was found as a growth inhibitor of *Phytophthora* species (Howell and Stipanovic 1983). Other studies shown that *T. harzianum* T12, and *T. koningii* T8 are useful to control of roots rot on peas (Lifshitz and Baker 1986).

Mycoparasitism is another activity taking part in the antipathogenic repertoire of *Trichoderma* genus members. This mechanism makes *Trichoderma* spp. a potential biocontrol agents. *Trichoderma* sp. typically grow towards other fungi hyphae and coil around them. They are able to secrete lytic enzymes that degrade cell walls of pathogenic fungi. This very process is called a mycoparasitism. Special adaptations like hooks, hyphae coiling and appressorium like bodies make it possible to attach to other fungi (Ozbay and Newman 2004). Most fungi belonging to *Trichoderma* genus are known to produce high amounts of cell-degrading enzymes like α-1,3-glucanases and other chitinolytic enzymes. One of the most efficient enzyme producers is *Trichoderma harzianum*, therefore it is often used in biopreparations. Scientist have also proved that some enzymes produced by antipathogenic fungi inhibit the spore germination and growth of pathogenic fungi hyphae (Szekeres et al. 2004). Studies on the influence of fungi from the genus *Trichoderma* and *Gliocladium* on fungal plant pathogen—*Botrytis cinerea*, growing on strawberries *Gliocladium virens* (G2 and G8) and *T. koningii* (T21)—revealed maximum inhibition of the pathogen growth. *Trichoderma* sp. and *G. virens* managed to colonize and sporulate on sclerotia and caused their lysis within 7-21 days (Alizadeh et al. 2007).

Inhibiting or inactivation pathogen enzymes is another mechanism that enables fungi to control the growth of pathogens. There are specific strains of *Trichoderma* like *T. harzianum* T39 that are able to produce proteases. Those enzymes inactivate pathogen enzymes that are supposed to hydrolyse plant

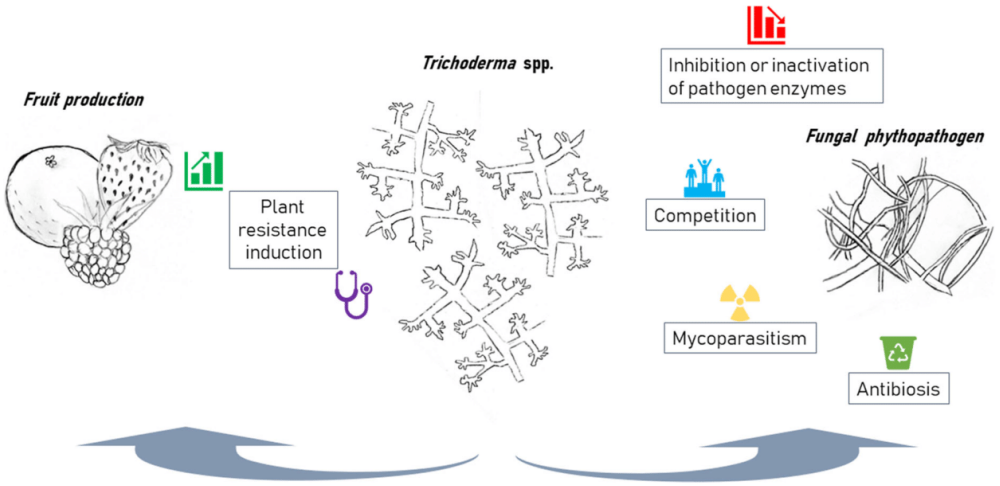

Fig. 2 Presentation of mechanisms involved in attacking other fungi and promoting plant growth

tissues. This strain inhibited growth of *Alternaria alternata* by over 50% by degrading endopolygalacturonase (endo-PG) and pectate lysate (Roco and Pérez 2001). *Botrytis cinerea* is another example of a strawberry pathogen whose's growth is inhibited by fungi of *Trichoderma* genus. It is producing pectinases, glucanase, cutinase and chitinase, and all those enzymes are suppressed by the protease secreted by *Trichoderma* sp. (Elad 2000).

Induced resistance is a process of enhancing plant's resistance to pathogens by other organisms. Specific *Trichoderma* strains are able to colonize plant's root tissues and star a series of biochemical and morphological changes. It induces plant defense response resulting in the activation of induced systemic resistance. It was shown that *Trichoderma harzianum* T39 applied to the soil can induce systemic resistance in strawberries attacked by powdery mildew, caused by *Podosphaera aphanis* and consequently inhibit the growth of the pathogen (Harel et al. 2011).

3.1.2 Pythium oligandrum

It is a fungus used in biocontrol of strawberry gray mould. Apart from this, it can reduce the growth of leaf spot (*Mycosphaerella fragariae*) and powdery mildew (*Sphaerotheca macularis*) of strawberries. *Botrytis cinerea* is one of the most important strawberry diseases is capable of decreasing the yield by up to 80%. It grows quickly and spreads easily by spores transferred by wind, rain drops or animals, and it can be successfully managed by *Pythium oligandrum*. *P. oligandrum* has also the cpacity to parasite other fungi e.g. *Fusarium oxysporum* or *Verticillium albo-atrum,* by producing enzymes (cellulases or chitinases) that degrade pathogens cell walls (Benhamou et al. 1999). *P. oligandrum* also secretes other extracellular enzymes i.e. lipases, proteases and β-1,3-glucanases, which affect pathogenic fungi (Picard et al. 2000).

3.1.3 Talaromyces flavus

Talaromyces flavus is a fungus widely spread around the world. *Talaromyces* genus belongs to heat-resistant fungi (HRF) group, and *T. flavus* is one of the most common fungi belonging to this group. The HRF are known for their ability to withstand high temperature treatment, such as pasteurization process. It is able to survive heating to 90 °C for 6 min and to 95 °C for 1 min in glucose tartrate heating medium of pH 5.0 and 16°Brix (Frac 2015; Panek and Frąc 2018). It is also known for its ability to produce bioactive compounds such as actofunicone, deoxy- funicone and vermistatin (Proksa 2010). Because of these

compounds and their ability to grow fast and compete for nutrients it is a promising material for further research in the field of pathogen biocontrol. Dethoup et al. (2007) has shown the ability to control the growth of *Phytophthora palmivora*, *P. parasitica*, *Colletotrichum capsici, C. gloeosporioides, Fusarium oxysporum* by *Talaromyces flavus* in in vitro conditions (Dethoup et al. 2007).

3.1.4 Aureobasidium pullulans

Another microorganism commonly used around the world in strawberry farming is *Aureobasidium pullulans*, because its high efficacy in the strawberry protection against *Botrytis cinerea* and *Rhizopus stolonifer*. It is a fungus similar to yeast colonizing plants in their natural habitat (Mounir et al. 2007). The development of *Aureobasidium pullulans* depends on many factors such as temperature, pH value, nutrient availability in the substrate. Its efficacy in strawberry protection has been proved by many scientists (Lima et al. 1997; Prokkola and Kivijärvi 2007; Sylla et al. 2013; Wagner and Hetman 2016). These fungi compete with other fungi for space and nutrients, they might be a direct parasite of pathogenic fungi and they produce antimicrobial enzymes and antibiotics (Chi et al. 2009). The anipathogenic effect and reduced severity of the disease is not yet fully understood and it is suspected to be a reaction on a many different levels. Fungal competition for nutrients and space weakens pathogen's cells and makes them more receptive to host enzymes and potential antibiotic compounds produced by the plant or the antagonist (Adikaram et al. 2002). *Aureobasidium pullulans* L47 was proven to be the most effective against both *B. cinerea* and *R. stolonifer* (Lima et al. 1997).

3.1.5 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi are organisms similar in their effects to plant growth promoting rhizobacteria (PGPR), they are obligate symbionts, belonging to the phylum Glomeromycota (Berruti et al. 2016). They have the capacity to develop a symbiotic association with plants. This relationship provides benefits for both fungi and plants e.g. fungi enhances the growth of roots and increases theirs surface area, which improves provision of water and nutrients. Even though the symbiosis is not specific it has been found, that some combinations of fungi and plants are more effective in different conditions (Miransari 2011). They can be used as biofertilisers and are useful in organic farming of fruits increasing their yields and decreasing effects of environmental stresses (Berruti et al. 2016; Stewart et al. 2005; Zardak et al. 2018).

3.2 Bacteria

3.2.1 Bacillus spp.

As it was mentioned earlier the gray mould of strawberries is one of the most economically important diseases in strawberry farming. It has been reported, that not only fungi, but also some bacteria can help with controlling this disease. *Bacillus lentimorbus, B. megaterium, B. pumilis*, *B. subtilis* are species capable of inhibiting the growth of *B. cinerea* during in vitro studies. They were not only inhibiting the growth itself but also reducing the conidia germination on strawberry fruits by up to 80% (Donmez et al. 2011).

3.2.2 Pseudomonas fluorescens

Pseudomonas sp. is a commonly occurring fungi in almost all cultivated areas. This genus and especially the species *Pseudomonas fluorescens* are widely studied with respect to biocontrol activities. One of the strains that is particularly important for organic farming is *Pseudomonas fluorescens* Pf-5. It grows quickly, produces siderophores, which might act as a growth factors and phenazines. Phenazines are a large group of compounds that act as a growth stimulator in plants. They also have the capacity to elicit induced systemic resistance (Pierson and Pierson 2010). Their ability to inhibit the growth of *B. cinerea* on strawberries has been demonstrated both in vitro and in vivo. The bacterial inoculum was added to 0.01% glycerol oil and used through foliar application on strawberry plants. The research has proven that due to this treatment severity of the disease were decreased in comparison with fungicide (Haggag and Abo El Soud 2012).

3.2.3 LAB: lactic acid bacteria

It is a wide group of bacteria, consisting of different species capable of producing lactic acid during

fermentation. Some of them have been categorized by the U.S. Food and Drug Administration as Generally Regarded as Safe (GRAS) and by the European Food Safety Authority as having Qualified Presumption of Safety. Some LAB bacteria produce biologically active compounds, for instance bacteriocins or organic acids (Reis et al. 2012). Current research has shown, that two strains of *Lactobacillus plantarum* PM411 and TC92 prevented *Xanthomonas fragariae* in strawberry. *X. fragariae* is a bacterial plant pathogen causing the angular leaf spot of strawberry. It spreads mostly via water drops splashing or via mechanical means during the farming or harvesting. Strawberries were sprayed with the inoculum at 10^8 CFU ml^{-1} concentration. The bacteria were able to live on the leaves at the concentration of about 10^4 CFU per leaf. The test strains reduced disease incidence from 40% to 10–12% (Daranas et al. 2018).

4 Examples of commercially available biopreparations and bioproducts

Biopreparations are substances obtained from a living organism or even its metabolites. However materials, chemicals and energy derived from renewable biological resources are called bioproducts (Singh et al. 2003). There are many of biopreparations and bioproducts used in organic fruit production e.g. Micosat F, Biosept 33 SL. They are based on different active ingredient such as microbial inoculum (e.g. *Pythium oligandrum),* plant extracts (e.g. *Allium sativum*—garlic), or substances derived from animals (e.g. chitosan). Table 1 presents examples of plant derived bioproducts that have been found as effective in biocontrol of some fungal pathogens. Those biopreparations are valued by farmers due to their effectiveness and safety not only for plants themselves but also for animals (Bala et al. 2009; Marjanska-Cichon and Sapieha-Waszkiewicz 2011; Reddy et al. 2000).

4.1 Bioproducts and biopreparations based on microbial components

Polyversum WP is a biopreparation consisting a mixture of *Pythium oligantrum* spores. *Pythium oligandrum* is a fungus that is capable of mycoparasiting common plant pathogenic fungi. Intensive research has found that they are capable of inducing plant's resistance, enhance plant's growth, and produce hydrolytic enzymes, e.g. cellulases or chitinases. It has been found, that lyophilized filtrate after *P. oligantrum* culture, diluted to proper concentration (1:10, 1:100) is capable of inhibiting the growth of *Botrytis cinerea* causing grey mould disease of strawberries, one of the most dangerous strawberry pathogens in contemporary agriculture (Bala et al. 2009).

Micosat F is a biopreparation produced by an Italian company CCS Aosta. It is a composition of arbuscular mycorrhizal fungi: *Glomus species*, *Trichoderma viride*, and rhizosphere bacterial species (*Bacillus subtilis, Pseudomonas fluorescens* and *Streptomyces* spp.). This biopreparation contains 40% C, 0.15% N, 431 mg kg^{-1} P and 9.558 mg kg^{-1} K and comes in the form of granules. Such microorganisms live in symbiosis with a wide variety of cultivated crops and enhance plant's nutrient uptake. Furthermore they reduce the influence of environmental stress, such as drought, on plants. The research into the influence of this biopreparation onto the growth of fruits in organic farming was conducted and revealed that the biopreparation enhances the growth of apple trees ('Topaz') and sour cherry ('Debreceni Bötermö') (Grzyb et al. 2015).

Trianum-P (Koppert BV, Netherlands) is a biopreparation containing *Trichoderma harzianum Rifai* strain T-22. It come in the form of granules to be dissolved in water. This biopreparation contains 10^9 CFU spores of *T. harzianum* g^{-1} of bioproduct that are capable of germinating and growing in a variety of soils, and different pH values (4–8.5). It decreases the infection rate of *Fusarium* sp., *Rhizoctonia* sp., *Pythium* sp. on different plants including strawberries. This species of *Trichoderma* is known for its capacities to produce antibiotic substances, enhance root growth, and stimulate plants defence systems (Benítez et al. 2004). Similar biopreparations basing on *T. harzianum* are commercially available as fungicides in the following countries the Czech Republic (Supresivit), USA (T-Gro), Australia (Rootshield WP), New Zeland (Trichodex) (Woo et al. 2014).

Worth mentioning is the broad spectrum of humus bioproducts. They consist of many beneficial organisms like bacteria or fungi, and organic matter created during the humification processes. Due to their properties (active microorganisms and complex chemical compounds) it is often sold as a liquid prepared to

Table 1 Biological control of fungal pathogens

Plant	Bioproduct	Pathogen	References
Garlic (*Allium sativum*)	Extract	*Alternaria* spp. *Phytophtora infestans*	Slusarenko et al. (2008)
Lemon grass (*Cymbopogon citratus*) Basil (*Ocimum basilicum*)	Essential oil	*Phytophthora capsicum* *Phytophthora drechsleri* *Phytophthora melonis*	Amini et al. (2016)
Neem (*Azadirachta indica*)	Seed kernel extract	*Monilinia fructicola,* *Penicillium expansum* *Trichothecium roseum* *Alternaria alternata*	Wang et al. (2010)
Oleander (*Nerium oleander*) Monkeypod (*Pithecolobium dulce*)	Oil cake	*Bipolaris oryzae*	Harish et al. (2008)
Thyme (*Thymus vulgaris*)	Essential oil	*Aspergillus flavus* *Fusarium moniliforme*	Massoud et al. (2012)
Warionia saharae	Essential oil	*Alternaria* spp. *Penicillium expansum* *Rhizopus stolonifer*	Znini et al. (2013)
Wild betel (*Piper sarmentosum*)	Extract	*Colletotrichum gloeosporioides*	Bussaman et al. (2012)

dilution. They are predominantly applied in a diluted form directly to the soil near the plants. Biopreparations used this way: Humus UP (Ekodarpol, Poland), Wspomag (BIOHUMUSECO, Poland), HUMVIT-EKO UNIWERSALNY (BIOHUMUSECO, Poland), TOTALHUMUS (THE, Poland), BIO-HUMUS EXTRACT "RASKILA" (LLC EKO ZEME, Latvia). Some of the bioproducts like WORM HUMUS (Humus Versol, Spain), or BIO–HUMUS (LLC EKO ZEME, Latvia) are soil-like formulations, and should be mixed with soil before planting in 1:5 ratio (Derkowska et al. 2015; Piotrowski et al. 2015).

Seaweed biopreparations such as Alga 600 (Agrocoast, USA) are another type of commercially available and useful biopreparations. Most of the seaweed extracts are derived from *Ascophyllum nodosum*, *Fucus* spp., *Laminaria* spp., *Sargassum* spp., and *Turbinaria* spp. (Hong et al. 2007; Ugarte et al. 2006). They stimulate the growth of roots and upper parts of plants and enhance the yield in strawberry plants (Alam et al. 2013).

4.2 Plant extracts, and other type of bioproducts

Plants are known for the production of aromatic secondary metabolites such as phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins. Some of them like carvacrol, eugenol or thymol may inhibit the growth of the pathogens due to their phenolic structures. The compounds have antimicrobial properties and they are a part of plants defence mechanisms (Das et al. 2010; Gurjar et al. 2012). Plant extracts, and other type of bioproducts described in this section are depicted at Fig. 3.

Plant extracts (PE) are eco-friendly and biodegradable, so they can be used in organic farming. Moreover, they are often cheaper than conventional fungicides. One of the most important steps in preparing a plant extract is choosing the proper plant material and the method of extraction. Some researchers recommend preparing PE from fresh plant materials, because the presence of some active compounds prone to degradation. Many plants are used in a dry form, due to different water content in plants. They are air dried before extraction to maintain the same amount of plant tissue in a sample (Salie et al. 1996; Tiwari et al. 2012). Choosing a right solvent is important for the extracting maximum of active substance. Even though water is the most common solvent for preparing plant extracts, it has been found that substances extracted with organic solvent display more consistent antimicrobial activity. For this reason

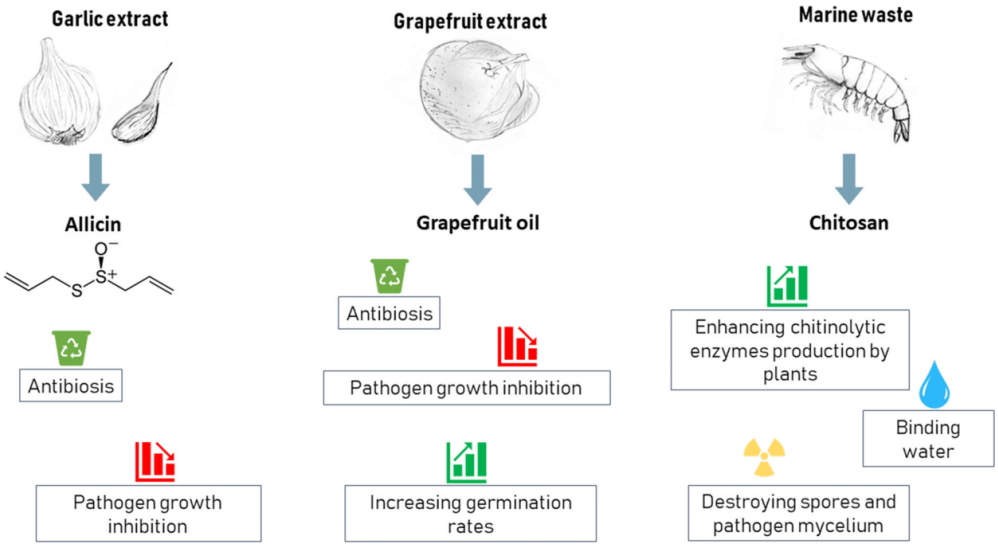

Fig. 3 Presentation of different sources of bioproducts and properties of such bioproducts

most of identified antimicrobial compounds are aromatic or saturated organic compounds, and they are mostly extracted using organic solvents like methanol or ethanol (Härmälä et al. 1992; Parekh et al. 2004). It is important to maintain a standardised extraction and solvents to decrease the variation in PE's antimicrobial activity. Plant material should be ground into small particles. Research has shown that 5-min extraction of 10 µm plant particles resulted in higher yield than a 24 h extraction of larger particles (Eloff 1998). Various plants can be used as sources of bioproducts useful in plant disease management for example turmeric (*Curcuma longa Linn.*), ginger (*Zingiber officinale Rosc.*)—*Phytopthora infestans,* neem/margosa (*Azadirachta indica* A. Juss.)—*Alternaria alternata*, holy basil (*Oscimum sanctum Linn.*), peach (*Prunus persica Linn.*)—*Botrytis cinerea*, oregano (*Origanum hercleoticum*)—*Fusarium oxysporum* (Gurjar et al. 2012).

Garlic extract is one of the most common used in biocontrol is. Slicing or crushed garlic cloves result in mixing of vacuolar enzyme alliin lyase and its substrate—alliin. The product of this reaction is thiosulphenic acid, which immediately and spontaneously dimerises to diallylthiosulphinate—allicin. This is the very compound that gives garlic its characteristic smell (Slusarenko et al. 2008). Allicin has been known to be a major antimicrobial substance in garlic since 1944 and it has been reported to be effective against a wide range of plant pathogens (Curtis et al. 2004). It has been proven that garlic extract is capable of inhibiting the growth of *Phytophthora infestans* both in vitro and in field studies. Researchers have studied the application of garlic extract or allicin both as foliar spray (100 µg ml^{-1}) and as alginate capsules applied to the soil around *Phytophtora*-inoculated plants. They have found that both ways of application were effective. It also has been proven that they are effective in seed priming reducing the *Alternaria* spp. infections after germination in carrot plants (Slusarenko et al. 2008). Garlic extract acquired by ethanol and water extraction from dried garlic cloves has been found capable of inhibiting the growth of *B. cinerea*. 40% aqueous garlic extract reduced the mycelial growth by 92%, and 60% and 80% extracts reduced the growth by 100%. 40% $\leq$ concentration of water extract inhibited all conidial germination of *Botrytis cinerea* (Daniel et al. 2015). One of the biopreproducts that contains garlic extract is Bioczos Płynny (Himal, Poland). It has been found to reduce the severity of grey mould disease in strawberries, and its efficacy was comparable to

commercially available fungicide—Switch 62.5 WG (cyprodynil + fludioxonil) (Marjanska-Cichon and Sapieha-Waszkiewicz 2011).

Another plant extract used in biopreparations, e.g. in Biosept 33 SL is 33% grapefruit extract (GE). It is derived from both the pulp and the seeds of grapefruit (*Citrus paradise*) (Xu et al. 2007). It contains a broad variety of antibiotic substances such as endogenous flavonoids, and it does not have any observed side effects. Studies upon the grapefruit extract showed, that it act as a strong growth inhibition in many species of bacterial and fungal plant pathogens (Jamiołkowska 2009). It has been proved that dressage of seeds with 0.2% Biosept 33 SL causes better germination and fewer diseases in plants like peas or bean. It also decreased an amount of plant pathogenic fungi such as *F. oxysporum, A. alternata, B. cinerea* isolated from those plants in later studies (Patkowska 2006). Grapefruit extract has also been proven to inhibit the spore germination of *B. cinerea* in vitro and in vivo—on grape berries. A 0.5% grapefruit extract showed better efficacy that commonly used fungicide (0.1% of thiabendazole) during in vitro studies—only 14% of spores germinated in the sample treated with GE, and 29% in the sample treated with thiabendazole. The immersion of grapes in 0.5% GE was significantly more effective than the immersion in 0.1% thiabendazole resulting in 17.2 infected berries per kilogram of fruits and 23.2 infected berries per kilogram of fruit respectively (Xu et al. 2007).

Chitosan is one of the modern bioproducts and is found *inter alia* in biopreparations such as BIOCHIKOL (earlier Biochikol 020 PC) produced by Poli-Farm. It is a polymer produced from the chitin elements of arthropod's exoskeleton and marine waste (Reddy et al. 2000). Many researchers have proved that it can be effective in the plant protection against pathogenic fungi (El Ghaouth et al. 1991a, b). Apart from inhibiting growth of pathogens chitosan also induces an increase in the activity of chitinase and phytoalexines production in plants which enables the treated plants to destroy pathogen's cell walls (El Hadrami et al. 2010). Phytoalexines are antimicrobial substances that are capable of accumulating rapidly in the areas of pathogen infection (Urban et al. 2004). Chitosan can also bind water to make a moisture barrier and delay the aging process in plants which lowers the rate of fungi infections (Reddy et al. 2000). Moreover, the compound can directly damage pathogenic fungi by destroying spores and mycelium (Urban et al. 2004). An experiment with 10 g l^{-1} chitosan applied as a foliar spray to strawberry plants resulted in effective controlling of the infection of *B. cinerea* in strawberries. Furthermore, the studies show that to maintain the efficacy of this treatment chitosan should be applied regularly in 10-days intervals (Reddy et al. 2000). Researchers have proved, that foliar application of 500 and 1000 ppm chitosan solution previously dissolved in 0.1 N HCl and diluted with distilled water with pH adjusted at 6.5 by 0.1 NaOH resulted in significant enhancement of plants height, root length, total fruit weight, total anthocyanins, carotenoids, total flavonoids, phenolics contents and antioxidant activity of fresh strawberry fruits (Rahman et al. 2018).

It is instructive to compare conventional chemical fungicides to commercially available biopreparations as far as phytopathogen controlling effectiveness. Polyversum WP (Target, Poland), a biopreparation containing *Pythium oligandrum* showed similar results in reducing the severity of grey mold, leaf spot and powdery mildew on strawberries in field tests. The efficiency of this product was the same or only a little lower than fungicides (Signum 33 WG (boscalid + piraclostrobin), Folpan 80 WG (folpet), Teldor 500 SC (fenhexamid) and against leaf spot and powdery mildew: Domark 100 EC (tetraconazole), Zato 50 WG (trifloxystrobin), Topsin M 500 SC (thiophanate methyl) (Meszka and Bielenin 2010). Biosept 33 SL (Target, Poland) and Biochikol 020 PC (Poli-Farm. Poland) are biopreparations made out of bioproducts—Biosept 33 SL contains grapefruit oil, and Biochikol 020 PC contains chitosan. Both of them were tested to determine their ability to inhibit development of *Topospora myrtilli* (Feltg.) Boerema on stems of highbush blueberry. Both biopreparations tested inhibited the growth of pathogen, but their protective effects were weaker compared to Dithane M45 80WP containing mancozeb (Szmagara 2008). Another research shows that combining conventional fungicides with biopreparations might lead to the reduction of the amount of fungicides required. The application of Serenade biopreparation (Bayer, Germany) containing *Bacillus subtilis* QST 713 with Fracture fungicide (CEV, Portugal) containing BLAD polypeptide results in controlling Botrytis blossom blight affecting wild blueberries. The research has

shown that fungicide usage can be reduced without loses in disease control (De Curtis et al. 2019).

It should be borne in mind that management systems might affect soil microorganisms. One of the purposes of using biopreparations is to increase soil biodiversity. Applying organic fertilizers, soil tillage or cover crops may change the expected output of the use of biopreparations. Cover crops produce root exudates, which are C-rich compounds such as amino acids, organic acids, phenolics and secondary metabolites. All these substances are attractive for microbes including arbuscular mycorrhizal fungi and nitrogen fixing bacteria (Vukicevich et al. 2016) and also cover crops may enhance the soil microbial community by providing a legacy of increased microbial biomass P, and phosphatase activity (Hallama et al. 2018). Due to this, it can be expected that the addition of biopreparations based on microorganisms will enhance soil biodiversity even more and cover crops increasing soil moisture by cover crops might enhance their survivability (De Vries et al. 2012). However, it should be borne in mind that unfortunately increasing soil diversity by cover crops might also lead to the increase in the amount of host specific plant pathogens as a result of pathogens being attracted to root exudates (Hofmann et al. 2009). Soil tillage is another agricultural management practice that can directly affect soil microorganisms, and thus needs to be taken into consideration when using biopreparations because it negatively affects most soil microbes (López-Piñeiro et al. 2013). Tillage, in turn leads to the reduction of both AM fungi and plant growth promoting bacteria (Brito et al. 2012; Lupwayi et al. 1998). Nevertheless, tillage might be mandatory in some specific circumstances, e.g., replanting, but the loss of soil microbial diversity caused by tillage might be counterproductive when applying microbial based biopreparations. Moreover, tillage in some cases have a positive effect on soil suppressiveness to phytopathogens (Bongiornoa et al. 2019). Organic fertilizers are known to increase organic matter content in soil which lead to increased amount of fungi and bacteria in soil. Furthermore, the soil treated with organic fertilizers is characterized by better water retention and higher amount of organic compounds. Using organic fertilizers in known to be beneficial for both plants and microorganisms. The use of both organic fertilizers and biopreparations can have positive effects on the soil and the plants (Escobar and Solarte 2015), however on the basis of long term experiments the effect of organic matter addition on soil suppressiveness to *Pythium ultimum* was not significant (Bongiornoa et al. 2019).

5 Summary and future targets

There are many ways to help plants grown in organic horticulture. The ban on the use of conventional plant protectants has led to increased demand for now, biologically based products. Properly prepared and tested preparations may be the future of agriculture. The use of biopreparations can contribute to maintaining a proper soil structure, high content of organic matter, increased water retention, or an increase in the number of beneficial soil microorganisms. Reducing the amount of mineral fertilizers and chemical fungicides can contribute to increase of biodiversity in arable areas. The EU laws and especially Council Directive of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources (91/676/EEC) should be taken into account in the context mineral fertilizers; the laws state, that the amount of nitrogen containing fertilizers used in agriculture and horticulture should be reduced. Taking everything into consideration, future research should focus on developing new bioproducts, new biopreparations, and formulations, as well as testing their effect in practice.

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6. Tekst publikacji P.2

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Searching for New Beneficial Bacterial Isolates of Wild Raspberries for Biocontrol of Phytopathogens-Antagonistic Properties and Functional Characterization

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Abstract: The threat caused by plants fungal and fungal-like pathogens is a serious problem in the organic farming of soft fruits. The European Commission regulations prohibit some commercially available chemical plant protection products, and instead recommend the use of natural methods for improving the microbial soil status and thus increasing resistance to biotic stresses caused by phytopathogens. The solution to this problem may be biopreparations based on, e.g., bacteria, especially those isolated from native local environments. To select proper bacterial candidates for biopreparation, research was provided to preliminarily ensure that those isolates are able not only to inhibit the growth of pathogens, but also to be metabolically effective. In the presented research sixty-five isolates were acquired and identified. Potentially pathogenic isolates were excluded from further research, and beneficial bacterial isolates were tested against the following plant pathogens: *Botrytis* spp., *Colletotrichum* spp., *Phytophthora* spp., and *Verticillium* spp. The eight most effective antagonists belonging to *Arthrobacter*, *Bacillus*, *Pseudomonas*, and *Rhodococcus* genera were subjected to metabolic and enzymatic analyses and a resistance to chemical stress survey, indicating to their potential as components of biopreparations for agroecology.

Keywords: beneficial bacteria; rhizosphere; metabolic analysis; phenotypic microarray

1. Introduction

Pathogenic microorganisms are a serious threat to crops, especially under the conditions in which the observed soil biodiversity is lowered. *Botrytis* spp., *Colletotrichum* spp., *Phytophthora* spp., and *Verticillium* spp. are just examples of the most symptomatic microorganisms that can cause plant diseases in the organic production of soft fruits including raspberries [1]. *Phytophthora* genus belongs to a common pathogens of raspberry and is one of the most crucial dangers in organic farming [2]. Research shows that representatives of the *Colletotrichum* genus can survive in soil and in any remaining plant residues for up to 30 months which might lead to re-infection even after defeating the disease in a particular growing season [3]. *Botrytis* genus is considered to be one of the most important plant fungal pathogens due to its potential to cause great losses in fruit yield [4].

The intensification of agricultural production, which has occurred in recent years, has led to farmers becoming dependent on chemical methods of plant protection as these methods are reliable and easy to use. In contrast to these advantages, chemical methods of plants protection may cause negative effects, i.e., the development of resistance by the targeted pathogen and a decrease in soil biodiversity [5].

Another disadvantage of conventional intensive cropping systems is the constantly growing cost of pesticides and the growing number of pathogens resistant to chemicals. The increase interest in organic farming and in particular, in the organic production of fruits could be considered as another factor encouraging farmers to develop new solutions in plant biostimulation and protection with the use of natural microbe-based products. European Commission regulation No. 834/2007 states that using chemical pesticides must be limited to the absolute minimum and farmers are encouraged to use substances of natural origin.

Furthermore, the EU Biodiversity Strategy for 2030, which was brought to life on 20 May 2020, sets forth the importance of biodiversity in all environments, and this includes increasing the biodiversity of agricultural land. This strategy for the coming years aims not only to reduce the use of pesticides by at least 50% but also to increase the area of organic farming until it reaches up to 25% of the total agricultural area. Organic farmers are obliged not to use many commercially available chemical pesticides and fertilizers. This can lead to lowering yields and an increase in the costs of this farming method. The aforementioned regulations also state that bioproducts, i.e., plant-based essential oils or biopreparations based on microorganisms are allowed and should be used in organic food production [6].

It was previously proven that deliberately selected native root-associated bacterial isolates, de novo introduced to cultivation, e.g., as biopreparations, can enhance plant growth [7]. In addition, they can rescue crops from diseases acting as biocontrol agents against a wide range of pathogens [8]. For this purpose, bacteria belonging to different genera, e.g., *Arthrobacter*, *Bacillus*, *Pseudomonas*, and *Rhodococcus* are frequently used [9–11].

Current research shows that locally isolated bacteria might be more effective against local pathogens than bacteria from different regions of the world [12]. Moreover, each plant species likely benefits from recruiting a specialized consortium of bacteria, which is specific for each plant system. Therefore, evidence of phytoprotective roles of microbes isolated from native environment should be evaluated under in vitro experiments and then in phytotron and agricultural conditions [8]. This indicates that it is important to collect new organisms from local sources and form new biopreparations based on them. Another factor is that bacteria isolated from wild raspberries growing in forests are less likely to have contact with pesticides which might decrease their antagonistic potential [13]. It also increases the survival chances of those organisms since they are still living in a similar ecological niche in terms of humidity or temperature.

Surely, the most expected outcome is that microorganisms introduced into the rhizosphere would present abilities to decompose organic matter, solubilize phosphorus, change soil structure creating small soil aggregates, and thus improve water retention. Beyond a doubt, the rhizosphere is a medium sufficient for bacterial growth., It contains organic acids, inorganic phosphorus, and organic carbon from rhizodeposition [14–16].

Nevertheless, the level of isolates survival and activity properties may be raised and influenced by different soil pH values or salinity levels in different ecological niches which they inhabit [10–16]. The very first and preliminary research suggesting prebiotic' additives for the future application into microbial-based biopreparations was published [17].

As a consequence, these capabilities need to be taken into consideration during biopreparation construction and application. In novel probiotechnology actions, serving precision agriculture and targeted solutions, it seems to be reasonable to provide a wide range of descriptions of skills determinants. Among others, there is a potential to survive in different ecological niches, which we aimed to achieve the presented study. The objectives of the study were to acquire and widely characterize bacterial isolates from a native niche of raspberry plants in order to indicate its potential as components of future biotization and naturalization biopreparations for agroecology.

Referring to the above touchstones, the hypotheses of our study include (i) wild raspberry rhizosphere and the root are a valuable sources of new beneficial bacterial isolates, (ii) some of the isolates show a great but varied potential to inhibit the growth of *Botrytis*, *Colletotrichum*, *Phytophthora*, and/or *Verticillium* genera representatives, and (iii) based on metabolic abilities, enzymatic properties and resistance to chemical stresses of beneficial bacterial isolates and phytopathogens, it is possible to select the most probable additives for future biopreparations.

2. Results

2.1. Identification of Bacterial Isolates

In the very first step, 65 different bacterial isolates belonging to 21 genera were isolated from wild raspberries and identified based on 16S rDNA fragment. The share of identified species is presented in Figure 1. Nine of the isolated bacteria were described as plant pathogens, and 42 isolates were recognized as potentially beneficial for plants. Among others, these were representatives belonging to the following genera *Arthrobacter* (15%), *Bacillus* (14%), *Pseudomonas* (28%), and *Rhodococcus* (3%).

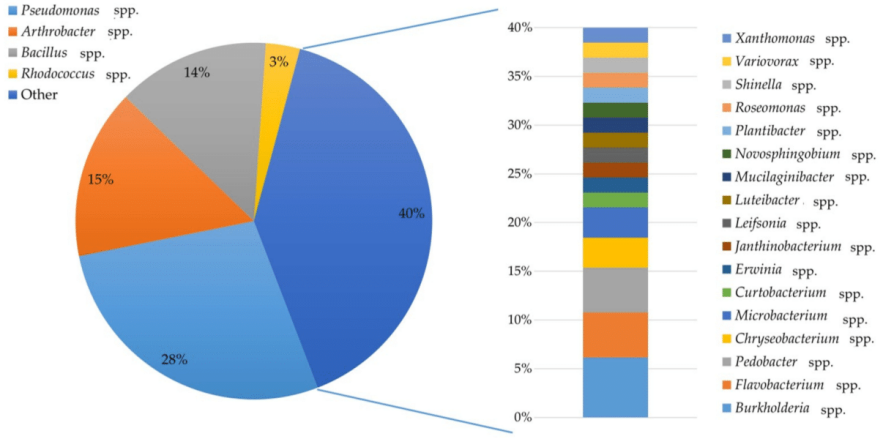

Figure 1. The percentage of bacteria belonging to individual genera among the isolates acquired from wild raspberry following identification based on 16S rDNA gene.

Based on sequences obtained via the sequencing of the DNA fragment of 16S ribosomal DNA (rDNA) a phylogenetic tree was created including all identified isolates (Figure 2). All genera were easily distinguishable from each other and create their separate clusters. Genetic analysis revealed that there are two clusters among isolates belonging to the *Pseudomonas* genus. It is worth noting that both *Pseudomonas* sp. that were chosen based on their antagonistic properties belonged to only one small group. This suggests that they are closely related isolates.

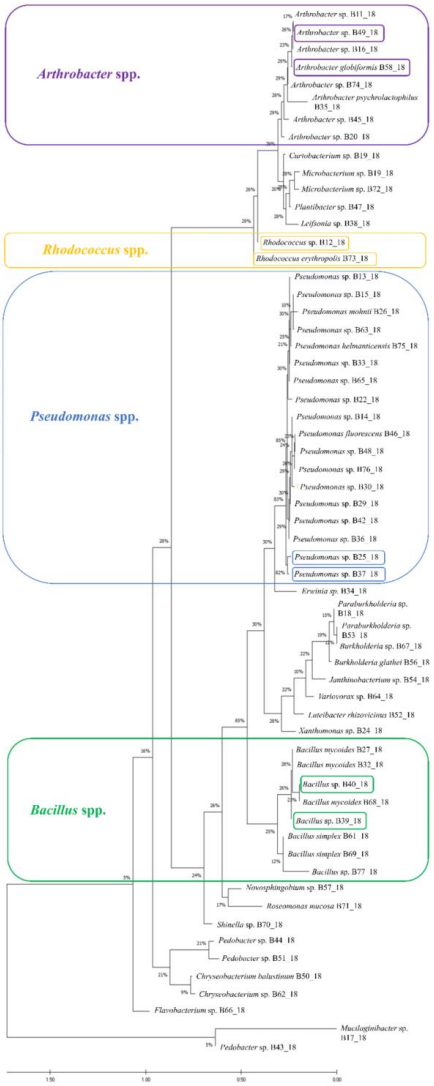

Figure 2. The evolutionary history of bacterial isolates of wild raspberry inferred by using the Maximum Likelihood method and Tamura-Nei model and bootstrap method based on 500 replicates. The tree with the highest log likelihood (−11,216.66) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 60 nucleotide sequences. There were a total of 1629 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Colored brackets represent the main genera used in this research, and small brackets point to particular isolates tested in presented experiments.

2.2. Antagonistic Properties

The second step of our study covered isolates screening for their antagonistic abilities against the fungal and fungal-like pathogens. The results of these analyses are presented in Appendix A Table A1 and most promising isolates are summarized in Table 1. The list of tested isolates was narrowed in this step to the potentially beneficial ones. Among this, there were eight isolates of bacteria found to be characterized by relatively high degree of efficiency in inhibiting pathogens growth, which had no antagonistic properties to each other (data are not shown), this ensures that they will not interact in any negative way. These were the following isolates: *Arthrobacter* sp. B58/18 and B49/18, *Pseudomonas* sp. B25/18 and B37/18, *Bacillus* B39/18 and B40/18, and *Rhodococcus* sp. B12/18 and B73/18. The three bacterial isolates were the most effective in inhibiting the growth of pathogens. *Arthrobacter* sp. B58/18, *Pseudomonas* sp. B25/18 and *Rhodococcus* sp. B12/18 presented strong antagonistic properties against tested isolates from both *Colletotrichum* spp. Almost all of the tested bacteria were successful against *Phytophthora* spp. and *Verticillium* spp. representatives. Five bacterial isolates were effective in inhibiting the growth of *Botrytis* spp. and they were *Rhodococcus* sp. B12/18, *Bacillus* sp. B39/18, *Arthrobacter* sp. B 49/18 and B58/18, as well as *Rhodococcus* sp. B73/18.

The antagonistic properties varied among the examined bacterial isolates and different pathogens, nevertheless all bacteria except *Bacillus* sp. B40/18 were effective in inhibiting the growth of at least 7 strains of plant pathogens belonging to at least 2 genera. *Rhodococcus* sp. B12/18 influenced the growth of 11 different strains of pathogens and isolate *Bacillus* sp. B40/18 affected the growth of only 6. It is noteworthy that the bacteria examined did not cause a strong inhibition of fungal pathogens growth on plates but sometimes caused the inhibition of sporulation, e.g., *Arthrobacter* sp. B58/18 against *Botrytis* spp. G277/18. Appendix A Table A1 presents extended table of antagonisms against plants fungal pathogens of all isolated and potentially beneficial bacterial isolates.

Table 1. Antagonistic properties of bacteria against 4 chosen fungal and fungal-like plants pathogenic isolates. Paper circles inoculated with bacterial inoculum were placed on a PDA plates inoculated with plants pathogen. Pathogen growth inhibition zones were evaluated after 96 h. For each pathogenic fungus, the size of the inhibition zone was determined individually as mean values. For *Colletotrichum* spp. it was <10 mm, 10–15 mm, >15 mm,, for +, ++, +++ respectively, for *Verticillium* spp. it was <10 mm, 10–20 mm, >20 mm, for +, ++, +++ respectively, for *Phytophthora* spp. it was <20 mm, 20–30 mm, >30 mm for +, ++, +++ respectively, for *Botrytis* spp. it was <10 mm, 10–20 mm, >20 mm, for +, ++, +++ respectively. – - means phytopathogen growth without inhibition.

Examined Isolates of Bacteria		**Phytopathogens**											
		***Colletotrichum* spp.**			***Verticillium* spp.**			***Phytophthora* spp.**			***Botrytis* spp.**		
		G172/18	**G371/18**	**G166/18**	**G293/18**	**G296/18**	**G297/18**	**G368/18**	**G373/18**	**G369/18**	**G275/16**	**G277/18**	**G276/18**
Rhodococcus sp.	B12/18	+	+	++	+	++	++	+++	+	++	–	++	+++
Pseudomonas sp.	B25/18	++	++	+++	+++	++	++	++	+	+++	–	–	–
Pseudomonas sp.	B37/18	–	–	++	+++	+++	++	+++	+	++	–	–	–
Bacillus sp.	B39/18	–	++	++	++	++	++	++	+	+++	++	–	++
Bacillus sp.	B40/18	–	–	–	++	+	++	+	+	+	–	–	–
Arthrobacter sp.	B49/18	–	–	++	++	++	++	+++	+	++	–	+++	–
Arthrobacter sp.	B58/18	++	++	++	++	+	+++	+	+	–	–	++	–
Rhodococcus sp.	B73/18	–	–	–	++	++	++	++	+	++	–	++	–

2.3. Analysis of Metabolic Abilities

Following great antagonistic abilities of isolates *Arthrobacter* sp. B58/18 and B49/18, *Pseudomonas* sp. B25/18 and B37/18, *Bacillus* B39/18 and B40/18, and *Rhodococcus* sp. B12/18 and B73/18, they were next surveyed with metabolic abilities, namely carbon substrates—sugar, sugar derivatives, sugar acids (Figure 3a–c respectively).

Sugars are known to be the most commonly used carbon sources in growing substrates for bacteria. Even though sugars are the broadest group in this study only a few of them were attainable for the examined bacteria. In this study the most utilized carbon sources were α-D-glucose and D-trehalose followed by D-mannose and D-fructose. D-glucose was also the only compound utilized by every bacterium in this study, and *Arthrobacter* sp. B58/18 was able to make use of the broadest range of substances as a carbon source.

It has been established that some sugar derivatives can be used as a carbon source in microbial growth or in biotransformation [18]. In this case, four compounds were utilized the most, and the result of the cluster analysis is presented in Figure 3 and these are as follows: D-fructose 6-phosphate and D-glucose 6-phosphate, inosine and N-acetyl. Only four bacteria were able to fully exploit these four substrates *Pseudomonas* sp. (B37/18), *Rhodococcus* sp. (B73/18), and *Bacillus* spp. (B40/18 and B39/18). Other substrates were not utilized in a significant way and it is noteworthy that *Arthrobacter* sp. (B58/18) was able to utilize only three of the sugar derivatives presented. Among many chemical compounds belonging to the group of sugar acids and their compounds, quinic acid was the one that was utilized by most of the isolates (Figure 4). D-gluconic acid was the second most utilized compound in this group. No other compounds were utilized at all.

As presented in Figure 4, every substrate except gelatin, D-serine, and D-aspartic acid was utilized. The only substrate that was utilized by every examined bacterium was L-serine. L-arginine, L-pyroglutamic acid, L-aspartic acid, L-glutamic acid, and L-histidine were utilized most profoundly by five isolates *Pseudomonas* spp. B25/18 and B37/18, *Bacillus* spp. B40/18 and B39/18, and *Rhodococcus* sp. B73/18. Similar results were obtained when checking the OD using the wavelength of 750 nm. The ratio between both absorbances for almost every isolate was above 1, which suggests a stressful metabolic situation (Table 2).

Table 2. The OD590/OD750 ratio of the categorized substrates located on Biolog® GEN III microplates. A ratio of >1 indicates a stressful metabolic situation for bacterial isolate functioning. A ratio value of <1 suggests good utilization of substrate (balance between respiration and biomass formation). A ratio value over 1 suggests a stressful metabolic situation, $n = 3$.

Isolate Names	Isolate Number	Amino Acids, Peptides and Polypeptides	Carboxylic Acids and Esters	Polyols	Sugar Acids and Their Compounds	Sugar Derivatives	Sugars
Arthrobacter sp.	B49/18	0.54	0.84	0.76	0.00	1.43	0.54
Arthrobacter sp.	B58/18	2.15	4.76	1.44	3.19	1.47	2.31
Bacillus sp.	B39/18	2.45	2.31	2.18	0.77	1.66	0.85
Bacillus sp.	B40/18	2.26	2.01	2.19	0.76	1.14	0.56
Pseudomonas sp.	B25/18	1.81	1.49	1.79	0.58	1.26	0.51
Pseudomonas sp.	B37/18	2.19	2.17	2.41	0.80	1.39	0.62
Rhodococcus sp.	B73/18	2.44	2.20	1.91	0.77	1.32	0.61
Rhodococcus sp.	B12/18	1.28	1.41	0.89	0.25	1.75	0.81

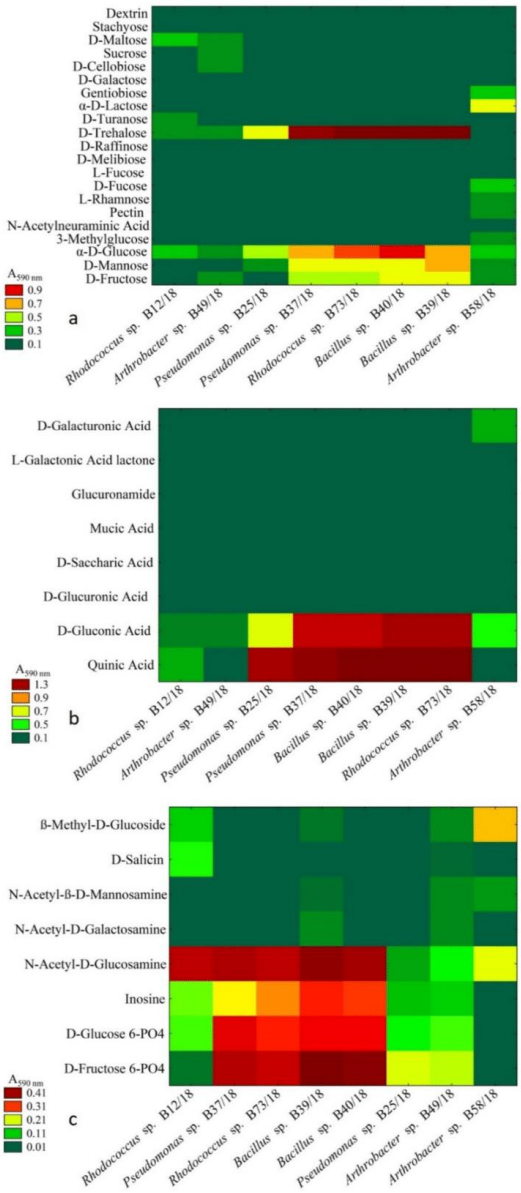

Figure 3. Cluster analysis of the examined bacterial isolates which depended on the utilization of carbon substrates–sugars (**a**), sugar acids (**b**), sugar derivatives (**c**). Measurements were taken for the wavelength of 590 nm, $n = 3$.

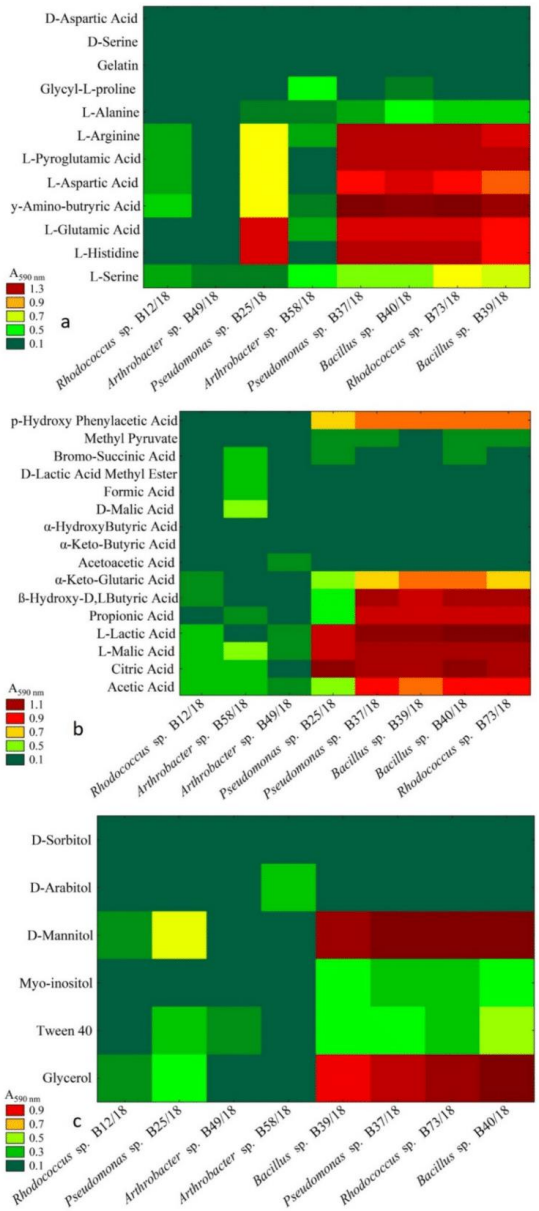

Figure 4. Cluster analysis of the examined bacterial isolates depended on the utilization of carbon substrates–amino acids, peptides and polypeptides (**a**), carboxylic acids and esters (**b**), polyols and others (**c**). Measurements were taken at a wavelength of 590 nm, $n = 3$.

The carboxylic acids shown in the presented graph (Figure 4) are also known for being a potential carbon source for microorganisms [19]. In this case, the ones that were the most utilized by most

isolates were acetic acid, citric acid, L-malic acid, L-lactic acid, propionic acid, β-hydroxy-D, L-butyric acid. It is worth noting that L-malic acid was the only compound that was utilized by every examined isolate. Isolates such as the following: *Rhodococcus* sp. B12/18, *Arthrobacter* spp. B58/18, and B49/18, were able to utilize the smallest number of compounds. The highest absorbance ratio values were presented for *Arthrobacter* sp. B58/18 (4.76), the values for the other isolates varied from 0.84 to 2.31.

It has been proven that for some microorganisms polyols can serve an important role as carbon sources for their metabolism [20]. The polyols used in this study were glycerol and D-mannitol (Figure 4). *Arthrobacter* sp. B58/18 was able to utilize only one substrate D-arabitol, while D-sorbitol was not utilized by any isolate at all. Only two isolates were not able to grow on Tween 40 as the main carbon source and these were *Rhodococcus* sp. B12/18 and *Arthrobacter* sp. B58/18. Similar to the results of carboxylic acids and esters group utilization, the highest absorbance ratio values were demonstrated by *Arthrobacter* sp. B58/18 for sugars group as well as sugar acids and their compounds indicating the stressful metabolic situation, whereas the value for the other isolates varied from 0.00 to 0.85, which indicates a favourable utilization of substrate and a balance between respiration and biomass production.

As the graph in Figure 5 shows, all bacteria except *Arthrobacter* sp. B49/18 were similar in their abilities to utilize particular groups of carbon sources. It barely utilized any compounds belonging to the following groups: amino acids, peptides and polypeptides, sugar acids and their compounds, and polyols and others. Instead, it was much better at utilizing sugars and sugar derivatives. Results for the other examined isolates were comparable. When considering Sneath's stringent criterion (33%) there are three similar groups which may be distinguished (A–C). Regarding a less restrictive criterion (66%), the number of similar groups is only two.

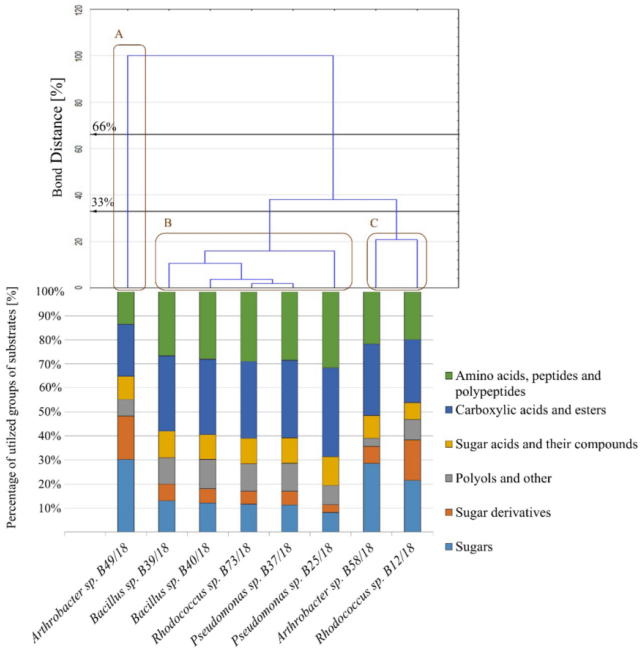

Figure 5. Bar graph presenting the ability to utilize different groups of substrates by the examined bacteria. The dendrogram presents a similarity in carbon sources utilization, clustering according to Sneath's stringent criterion (33%) and the less restrictive criterion (66%). Utilization is calculated based on absorbance values at 590 nm, $n = 3$.

2.4. Resistance to Chemical Stresses

Resistance to various factors, that may be encountered in a variety of environments is one of the most important features that characterize microorganisms intended to be used in biopreparations. Many factors influence the growth of microorganisms, and as it is widely known that a lack of carbon is one of the most significant [21]. As another factor, soil pH may be considered. Most European soils can be considered acidic as they have a pH value between 4 and 6. Over 21% of agricultural land in Poland is acidic (pH below 5.5) and this is a common problem for farmers [22,23].

Also, soil salinity may be a limiting factor in microbial growth [24]. As previously mentioned, during this study, absorbances were measured at two different wavelengths. The reason for this was that the absorbances for those wavelengths depend on different factors: 750 nm represents optical density, and 590 nm represents substrate usage. While presenting the results for different carbon source utilization we focused on the absorbances measured for 590 nm. In presenting the results of chemical resistance, we focused on the absorbance measured at 750 nm which represents optical density (OD) directly and is directly correlated with the number of bacterial cells in the growing medium.

As the data presented in Figure 6 show, the majority of the examined bacteria (*Pseudomonas* sp. B25/18, *Pseudomonas* sp. B37/18, *Rhodococcus* sp. B73/18, *Bacillus* sp. B39/18, *Bacillus* sp. B40/18) were resistant to most of the limiting factors with the exception of 8% NaCl, D-serine, and sodium bromate. They were able to grow almost in each given condition including the acidic pH (pH = 5), different salinity levels (1% and 4% NaCl). Some of tested isolates were also able to survive in the presence of selected antibiotics such as vancomycin, lincomycin, rifamycin, minocycline, fusidic acid, aztreonam, nalidixic acid and troleandomycin. Three of the examined isolates, i.e., *Rhodococcus* sp. (B12/18) and *Arthrobacter* spp. (B49/18 and B58/18), were very vulnerable to the conditions presented and barely able to grow at all. Nalixidic acid was also able to inhibit the growth of *Pseudomonas* sp. B25/18. 5 compounds were also effective in limiting the growth of all examined bacteria and these were 4% NaCl, sodium butyrate, guanidine hydrochloride, and lithium.

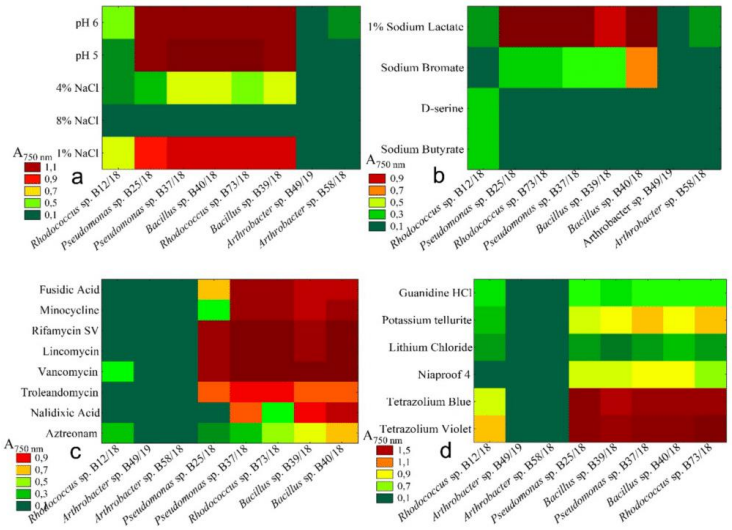

Figure 6. Charts presenting the medium absorbance values measured at 750 nm wavelength for different additives representing chemical stresses. The 4 charts represent the 4 groups of substrates: (**a**) chemical soil properties; (**b**) organic compounds, (**c**) antibiotics, (**d**) toxic substances.

2.5. Enzymatic Activity of Selected Bacterial Isolates

The enzymatic activity of the three selected isolates were evaluated using both API ZYM tests (bioMérieux SA, Marcy l'Etoile, France) and Petri plates with media with appropriate substrates. The API tests allowed for the evaluation of the ability of bacteria to secrete 19 different enzymes and Petri tests allowed for eight more enzymatic abilities to be checked. The results are presented in Table 3.

Table 3. Enzymatic abilities of selected beneficial bacterial isolates. '-' corresponds to a negative reaction, '+++' to a reaction of maximum intensity and values '+' or '++' are intermediate reactions depending on the level of intensity, $n = 3$.

Metabolic Abilities	Substrate Additive	*Rhodococcus* sp. B12/18		*Pseudomonas* sp. B37/18		*Arthrobacter* sp. B58/18	
		24 h	168 h	24 h	168 h	24 h	168 h
Proteolytic	4% skim milk	–	–	+	+++	–	–
	4% gelatin	–	–	+++	+++	–	+
Amylolytic	1% starch	–	–	++	+++	+	++
Amonification	4% skim milk	+	+	+++	+++	++	++
	4% urea	++	++	–	–	+	+
Denitryfication	0.1% KNO_3	+	+	–	–	+	++
Nitrogen fixation	Medium without nitrogen	–	–	–	–	–	–
Cellulolytic	Shredded straw	–	+	+	+++	++	+++
Nitryfication	Nitryfication medium	–	–	–	–	–	–
Phosphate solubilization	Pikovska medium	–	–	–	–	–	–

Enzymatic activity	Substrate	*Rhodococcus* sp. B12/18	*Pseudomonas* sp. B37/18	*Arthrobacter* sp. B58/18
Alkaline phosphatase	2-naphthyl phosphate	++	–	–
Esterase (C 4)	2-naphthyl butyrate	++	++	+
Lipase esterase (C 8)	2-naphthyl caprylate	+++	+	+
Lipase (C 14)	2-naphthyl myristate	–	–	+
Leucine arrylamidase	L-leucyl-2-naphthyllamide	+++	++	+++
Valine arrylamidase	L-valyl-2-naphthlamide	++	–	++
Cystine arrylamidase	L-cystyl-2-naphthlamide	+	–	++
Trypsin	N-benzoyl-DL-arginine-2-naphthyllamide	–	–	–
α-chymotrypsin	N-glutaryl-phenylalanine-2-naphthyllamide	+	–	–
Acid phosphatase	2-naphthyl phosphate	++	+	+
Naphthyl-AS-BI phosphohydrolase	Naphthyl AS-BI-phosphate	+++	++	++
α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	–	–	++
ß-galactosidase	2-naphthyl-ßD-galactopyranoside	–	–	–
ß-glucuronidase	Naphthyl-AS-BI-ßD-glucuronide	–	–	–
α-glucosidase	2-naphthyl-αD- glucopyranoside	+++	–	++
ß-glucosidase	6-Br-2-naphthyl-ßD-glucopyranoside	+++	–	–
N-acetyl-ß-glucosaminidase	1-naphthyl-N-acetyl-ßD-glucosaminide	–	–	–
α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	–	–	+
α-fucosidase	2-naphthyl-αL-fucopyranoside	–	–	–

The bacteria were different in terms of their metabolic and enzymatic abilities. Some enzymatic and metabolic abilities were greatly enhanced after a longer period of time such as the proteolytic abilities of *Pseudomonas* sp. B37/18. Although isolate B37/18 presented mediocre enzymatic abilities using API ZYM tests it was much more effective than the others when considering metabolic abilities. *Rhodococcus* sp. B12/18 presented limited metabolic potential with the capacity to conduct ammonification, denitrification and it only has slight cellulolytic abilities. However, B12/18 presented a broad spectrum of enzymatic abilities, being able to produce 11 different enzymes. *Arthrobacter* sp. B58/18 also secreted 11 enzymes used to evaluate enzymatic ability through API ZYM tests, but the overall activity was lower than that for *Rhodococcus* sp. B12/18.

2.6. Prebiotic Supplement Mixture against Fungal and Fungal-Like Pathogens

Based on the calculated ratio for both beneficial bacterial isolates and plant fungal pathogens, it was possible to note that some chemical compounds might be favourable as an addition to bacterial biopreparations, enhancing beneficial bacteria and influencing against plant fungal and fungal-like pathogens. The ratio results for all of the tested compounds are presented in Table A2. Those compounds are D-malic acid, D-saccharic acid, N-acetylo-D-galactosamine, and α-keto-glutaric acid. The results of our study indicated that they not only stimulate the growth of beneficial bacteria, but also, at the same time, cause a stressful metabolic situation for plant pathogens.

3. Discussion

The antagonistic properties of bacteria against fungal and fungal-like plant pathogens have been described previously, and efforts have been made to utilize these organisms in biological plant protection [25–27]. However, to the best of our knowledge, the metabolic characteristics of the presented organisms complied to a limited extent with their antagonistic properties against certain plant fungal pathogens, as proposed and determined in this research.

3.1. The Properties of Bacteria Belonging to Arthrobacter Genus

Arthrobacter sp. the isolate B58/18 was able to inhibit growth of selected phytopathogens and in this study was characterized by the ability to utilize a few carbon sources. Generally, the *Arthrobacter* genus representatives are known to produce amylase, lipase and protease [28]. Furthermore, *Arthrobacter* spp. can inhibit the growth of *Fusarium roseum* by releasing chitinase [29]. The ability to produce amylase may be useful in the utilization of compounds such as gentiobiose, pectin or other polysaccharides. In contrast, the production of protease can influence the utilization of glycyl-L-proline.

This particular isolate was also effective in the utilization of D-malic acid which may lead to the formation of acetic acid [30]. The previously mentioned abilities to produce chitinolytic enzymes may be a contributing factor to its noteworthy antagonistic properties. *Arthrobacter* spp. were successful in inhibiting the growth of the almost every isolate of chosen pathogens, especially isolates belonging to *Colletotrichum* spp. and *Phytophthora* spp.

It was established that *Arthrobacter* sp. B58/18 was susceptible to environmental stresses such as the presence of NaCl or antibiotics. However, this isolate does not pose a risk of introducing antibiotic-resistant organisms into the environment, which could cause unforeseen consequences for the environment and human. This result is important for further field application goals, when the formulated biopreparation based on this bacterial isolate is used for specific soil conditions. Thus, *Arthrobacter* sp. B58/18 is expected to be an effective antagonist, and the conditions for its proper development are predefined.

By contrast, *Arthrobacter* sp. B49/18 also is characterized by the lack of the ability to utilize a broad variety of carbon sources. However, compared to *Arthrobacter* sp. B58/18 which utilizes simple sugars such as D-fructose, D-mannose, and α-D-glucose, it is also similar in that it is vulnerable to environmental stresses. *Arthrobacter* sp. B49/18 proved itself to be less effective in inhibiting the growth of selected plant fungal and fungal-like pathogens than *Arthrobacter* sp. B58/18. These findings indicate that *Arthrobacter* sp. B49/18 is a rather weak candidate to be a constituent of a microbial biopreparation.

3.2. The Properties of Bacteria Belonging to Rhodococcus Genus

Furthermore, bacteria belonging to the *Rhodococcus* genus were also previously characterized in the literature as potentially beneficial microorganisms antagonistic to some plant pathogens. They were described to produce antimicrobial compounds such as the antifungals rhodopeptins, antimycobacterial lariatins or antibacterial aurachin RE, rhodostreptomycins A and B, saframycin A. These substances were proven to be successful in inhibiting the growth of many both bacterial and fungal plant pathogens which are common in agriculture [31–34].

In our study, we examined two isolates belonging to *Rhodococcus* genus that were isolated from wild raspberry roots and the rhizosphere. These were *Rhodococcus* sp. B73/18 and B12/18. Both isolates have been proven to be different from each other in their antagonistic properties against pathogens and their metabolic abilities and resistances to chemical stresses. The first one was successful in the growth inhibition of *Verticillium* spp. isolates. In turn, the second one inhibited the growth of *Phytophthora* spp. and two isolates of the *Botrytis* genera. Isolate B12/18 was overall better in its antagonistic properties inhibiting the growth of 11 isolates of phytopathogens. In terms of metabolic abilities and carbon source utilization, there is an easily visible contrast between those two isolates. Isolate B73/18 is much more effective in utilizing many different carbon sources than isolate B12/18. However, B73/18 isolate was resistant to many antibiotics. Therefore, due to the emergence of bacterial resistance to antibiotics, which is considered a worldwide public health problem, this isolate is a weaker candidate than B12/18 isolate as a biopreparation component.

However, the OD590/OD750 ratios were much lower for isolate B12/18 which might suggest that even though isolate B73/18 can utilize a broader spectrum of substrates it also triggers some stress-related mechanisms. OD590 increases during culturing but, OD750 does not increase accordingly. Although *Rhodococcus* sp. B12/18 was more vulnerable to different stress factors in comparison to isolate B73/18, it is the only bacteria in this study that was able to grow in the presence of D-serine and sodium bromate. Since developing a formulation of microorganisms for a biopreparation requires the step for satisfactory biomass production, this study includes a preliminary selection of media ingredients that may trigger biomass production with simultaneous relatively low level of metabolic reactivity. The metabolic response should not be very significant since this means that a rather stressful situation is in progress, as was explained in detail in recent years [17].

3.3. The Properties of Bacteria Belonging to Bacillus Genus

The bacteria belonging to the *Bacillus* genera are commonly used in many biopreparations [6]. They produce a wide array of antimicrobial compounds and have the ability to induce plant systemic resistance against pathogens such as *Botrytis cinerea* [35,36]. One of the most important substances produced by this genus of bacteria is lipopeptides which are highly antimicrobial [37]. Both of the tested *Bacillus* spp. isolates presented antagonistic properties against isolates of pathogens belonging to the *Verticillium* and *Phytophthora* genera. Even though *Bacillus* sp. isolate B39/18 was not producing a very strong antagonistic reaction to *Verticillium* spp., this isolate was not easily overgrown by these fungi and was able to compete for nutrients that might be vital to colonize soil and promote plants growth [38].

Furthermore, *Bacillus* sp. isolate B39/18 inhibited the growth of *Colletotrichum* sp. G166/18. Although some researchers have described the antagonistic properties of *Bacillus* sp. against *Colletotrichum* spp. results may vary depending on the particular isolates [39,40]. Isolate B39/18 was showing antagonistic properties against *Botrytis* spp. In terms of carbon sources utilization, both isolates were incredibly similar. It was only possible to observe small differences such as the broader spectrum of compounds utilized by *Bacillus* isolate B39/18, especially sugar derivatives. Although they are similar, the OD590/OD750 ratio suggests that *Bacillus* sp. isolate B40/18 handles stressful conditions better (ratios are almost the same or lower for this isolate).

3.4. The properties of Bacteria Belonging to Pseudomonas Genus

Last but not least, there are bacteria representatives belonging to the *Pseudomonas* genus used in commercially available bioproducts [6]. *Pseudomonas* strains were found to be effective in decreasing severity of red raspberry cane spur blight [41]. It has been proven that *Pseudomonas* spp. isolated from the rhizosphere of trees are able to inhibit the growth of some soil-borne plant pathogens e.g., *Verticillium* spp. [42]. *Phytophthora* spp. is also a pathogen that is inhibited by *Pseudomonas* spp. representatives. Research suggest that a treatment composed of *Pseudomonas fluorescens* and olive oil is

more effective in reducing the severity of Phytophthora Blight of Pepper than acibenzolar-S-methyl (ASM) which is a newly introduced fungicide and systemic resistance stimulant [43,44].

In our research there were two isolates belonging to the *Pseudomonas* genus included. In terms of their antagonistic properties against fungal and fungal-like plant pathogens *Pseudomonas* sp. isolate B25/18 presented a very strong antagonistic mode against *Colletotrichum* spp., and the two isolates of *Phytophthora* genus. Isolate B25/18 was also effective in inhibiting the growth of *Verticillium* spp. In contrast, *Pseudomonas* sp. isolate B37/18 presented very weak antagonism against *Colletotrichum* spp. and similarly to B25/18, isolate B37/18 was found to inhibit the growth of *Verticillium* spp. and *Phytophthora* spp. (Table 1). Both isolates presented no antagonistic properties against *Botrytis* spp. Both isolates presented different carbon utilization patterns. Isolate B37/18 was more effective in terms of carbon utilization from different carbon sources. Isolate B25/18 grew less intensively on media containing amino acids, peptides, and polypeptides such as L-aspartic acid, L-pyroglutamic acid, L-arginine, and L-serine compared to isolate B37/18.

It has been proven that some bacteria belonging to the *Pseudomonas* genera can utilize the aforementioned amino acids as their sole source of carbon and nitrogen [45] which was also confirmed in the presented research. Both tested isolates, *Pseudomonas* sp. B25/18 and B37/18 were able to use carboxylic acids as a carbon source, although isolate B25/18 was less effective in utilizing acetic acid, propionic acid, β-hydroxy-D-butyric acid and α-keto-glutaric acid in comparison with the to isolate B37/18. The ability to utilize carboxylic acids by *Pseudomonas* spp. has previously been noted and corresponds with our results [46]. Myo-inositol was not utilized by *Pseudomonas* sp. isolate B25/18, as opposed to isolate B37/18, which was able to use it as a carbon source. Glycerol and D-mannitol which were not available for *Pseudomonas* sp. isolate B25/19, were utilized at a high rate by isolate B37/18. Glycerol has proved to be a sufficient carbon source for *Pseudomonas* spp. representatives. Bacteria cultured on a medium containing glycerol as the only carbon source tend to produce additional substances such as glycolipids [47]. Both isolates utilized the same 4 sugar derivatives: D-fructose 6-phosphate, D-fructose 6-phosphate, inosine, and N-acetyl-D-glucosamine, but the ability to utilize them was different. Isolate B37/18 was more effective in the utilization of those compounds. *Pseudomonas* sp. isolate B37/18 along with isolate B25/18 were able to grow on a medium containing one of three sugars: D-mannose, D-glucose, and D-trehalose. Moreover, isolate B37/18 grows on a medium containing D-fructose as the sole carbon source.

The chemical resistances of both isolates were similar but isolate B25/18 presented no resistance to nalidixic acid, and weaker resistance to potassium tellurite, 1% NaCl, fusidic acid, and minocycline compared to isolate B37/18. It has been proven that concentrations of NaCl higher than 1.75% reduce the amount of *Pseudomonas* bacteria growing in the medium [48]. Upon the initial review isolate B37/18 seemed to be much more effective in utilizing the presented compounds as carbon sources than isolate B25/18, but comparing these results to the OD590/OD750 ratio, it is visible that for isolate B37/18 the conditions presented were more stressful than for *Pseudomonas* sp. isolate B25/18. Therefore, while constructing biopreparations it should be taken into consideration that there are discrepancies between the two candidate isolates concerning antagonistic and substrate utilization properties, suggesting better potential of B37/18 isolate as a candidate for biotization and naturalization biopreparations.

3.5. Summary of Bacterial Properties as Potential Candidates to Biopreparations for Agroecology

However, it is worth noting that D-trehalose is utilized by the majority of the bacteria examined. Trehalose is a compound that can be used by bacteria as a carbon source but it is also produced by microorganisms while they are subjected to stressful conditions, especially on an osmotic basis [49]. It is also commonly used to increase the survivability rate when bacteria become too dry [50,51], which is an element of microorganism preservation. Those properties of trehalose make it an ingredient worth considering as an additive to bacterial biopreparations. It might not only increase the survivability

of the bacterial formulation during storage, but it may also can enhance their growth in the natural environment after application.

Surprisingly, isolates such as *Pseudomonas* sp. B25/18 and B37/18, *Bacillus* sp. B39/18 and B40/18, *Rhodococcus* sp. B73/18 grew very effectively indeed in the presence of antibiotics. The release of antibiotics into soils creates a potential threat to microorganisms in this environment and may impact on the functional, genetic, and structural diversity of microbial communities, therefore probably some isolates acquired from soil environment pose such resistance. These compounds, if they occur in soil, are proven to affect its microbiota negatively, thereby decreasing soil microbial activity [52]. When choosing microorganisms to be used in biopreparation, especially for agroecology and organic production it might be important to select isolates that are not resistant to antibiotics. This will ensure a slower spread of antimicrobial properties against native soil inhabitants [53]. Since introducing antibiotic-resistant isolates to new ecological niches might have unforeseen consequences, it is needed to limit this type of organisms to a minimum and use them only when needed. Isolates B12/18 and B58/18 presented not only high metabolic activity, but also almost no resistance to antibiotics, making them good candidates for future biopreparation formulations.

The enzymatic and metabolic abilities may be the key to properly understanding the possible functions of those bacteria in the environment. The broader the spectrum of secreted enzymes the more environmental niches that bacteria can grow in and the more competitive they can be against other possibly pathogenic microorganisms [54]. The cellulolytic abilities presented by all of the examined isolates might contribute to an increase in the amount of organic matter in soil [55]. It is also worth noting that the addition to the soil of microorganisms that have a high degree of metabolic and enzymatic activity might stimulate different processes in the soil environment [56] and drive soil ecosystem services that are important in the soil quality evaluation [57]. Therefore, one of the strategies for agroecology could be the application of microbes used as a drivers of soil services [58] by participation in crucial processes in the soil environment through enzymatic activity of selected isolates and their consortia.

It is widely known that plants have their own probiotic bacteria similarly to animals. It is worth noting that when considering new formulations of biopreparations it is possible to enhance the further growth of microorganisms in particular environment by using certain additives [59]. It is also important for the additives not to enhance the growth of pathogens such as pathogenic fungi, especially if a biopreparation is to be used not only to stimulate the growth of plants, but mainly as an agent of plant fungal and fungal-like pathogen biocontrol.

The choice of the appropriate additives requires very specific research in order to evaluate their influence not only on probiotic microorganisms, but also on pathogens [60]. Table A2 presents different compounds whose influence on both beneficial bacterial isolates as well as fungal pathogens was tested in presented study. This approach allows one to perform a rapid and cost-effective evaluation over 40 different compounds. D-malic acid, D-saccharic acid, N-acetylo-D-galactosamine, and α-keto-glutaric acid that were indicated to be the best choice as prebiotic supplementary blend were not only easy to obtain, but also economical. Based on the OD590/OD750 and OD490/OD750 results, they seem to be the best out of all compounds tested.

This research is essential for future work focused on making a biopreparation composed of microorganisms to be used in the organic farming of soft fruits. An advanced knowledge base concerning the abilities of various bacteria to utilize different carbon sources might be important in deciding the future composition of a bacterial growing media or carrier for microorganisms. A deeper knowledge concerning how bacteria deal with stress factors, such as different pH or salinity levels, may help in predicting if they will be able to survive in the biopreparation or in the environment.

4. Materials and Methods

4.1. Bacterial Isolates Acquisition and Identification

The bacterial isolates used in this study were derived from the wild raspberries rhizosphere and root samples derived from Janów Lubelski, Kraśnik, Łuków, Puławy, Siedlce, and Świdnik Forest Distincts. First raspberry plants with adhering soil were collected and initially prepared as described by Oszust and Frąc [61] and followed by bacterial isolation procedures (from rhizosphere and roots). For this one gram of rhizosphere soil or root, the sample was placed in 9 mL sterile water (10^{-1}) and shaken for 10 min at room temperature. One (1.0) mL of this suspension was transferred into a 9 mL blank (10^{-2}) and serially diluted up to 10^{-6}. Then, 100 µL of each dilution from 10^{-4} to 10^{-6} series was added to Petri dishes with Plate Count Agar (PCA, BioMaxima, Lublin, Poland) or medium based on soil extract [62] and incubated at 25 °C for 48–72 h. The morphologically distinct bacterial colonies were isolated and subcultured on Potato Dextrose Agar medium (PDA, A&A Biotechnology, Gdynia, Poland). At this stage of research 65 different bacterial isolates were distinguished, 63 from wild raspberry rhizosphere and 2 isolates from its roots therein.

Then, bacterial isolates were identified based on genetic identification with using commercially available Applied Biosystems™ Kits based on universal primers included (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA) as described below. All analyses were performed according to the producer's manual in the Veriti™ 96-Well Thermal Cycler (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA) as presented below.

In the first step, the genomic DNA was extracted from a single colony for each bacterial isolate using PrepMan Ultra reagent (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA). Then there were PCR reactions conducted to amplify 16S rDNA gene with FAST MicroSeq™ 500 16S rDNA PCR Kit (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA). Thermal cycling conditions for this step were as follows: initial denaturation at 96 °C for 1 min, then 30 cycles of 15 s at 64 °C of annealing, and a final extension at 72 °C for 1 min.

Next, the quality of obtained DNA amplicons was assessed in 2% agarose gel in electrophoresis and followed by purification using ExoSAP-IT™ PCR Product Cleanup Reagent (Affymetrix Inc., Santa Clara, CA, USA) with the procedure that included incubation at 37 °C for 15 min and at 80 °C for 15 min was performed. Then, forward and reversed-sequencing reactions were prepared, and the cycle sequencing run was performed using MicroSEQ™ 500 16S rDNA Sequencing Kit (Life Technologies Ltd., Warrington, United Kingdom by Thermo Fisher Scientific, Waltham, MA, USA). The thermal cycling conditions for this step were as follows–initial denaturation at 96 for 1 min, 10 s melting at 96 °C, 50 °C, 5 s annealing in 25 cycles, and followed by 75 s extending at 60 °C. Subsequently, Performa Spin Columns (EdgeBio, San Jose, CA, USA) were applied to rid reagent residues. After that, the material was subjected to identification via Sanger sequencing [63] with Applied Biosystems 3130 sequencer (Applied Biosystems, Foster City, CA, USA).

In the next step, all obtained sequences were aligned and compared with the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). Subsequently, phylogenetic tree was prepared using the maximum likelihood method employing MEGA (University Park, PA, USA) software [64]. For this purpose, the Tamura–Nei model [65] and bootstrap method phylogeny testing were applied.

The detailed information about each bacterial isolate covering identification, accession number of sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank/), the sample from which it was isolated, the medium used for isolation, and the forest where samples were obtained with GPS-coordinates, are summarized in Table A3.

4.2. Biocontrol Efficacy and Antagonistic Abilities of Bacterial Isolates

Eight selected isolates, namely *Arthrobacter* spp. B49/18 and B58/18, *Bacillus* spp. B39/18 and B40/18, *Pseudomonas* spp. B25/18, B37/18, and *Rhodococcus* spp. B12/18 and B73/18, were tested for their antagonistic properties against 12 isolates of 4 selected plant fungal and fungal-like pathogens (three

isolates of each pathogen): *Botrytis* spp., *Colletotrichum* spp., *Phytophthora* spp. and *Verticillium* spp. Phytopathogens characteristics including a way and source of isolation, as well as identification within GenBank accession numbers list were described previously by Malarczyk et al. [66]. The first step was to suspend centrifuged bacteria in 1 mL of water. After that, 90-mm diameter Petri dishes with 20 cm^3 of PDA medium (A&A Biotechnology, Gdynia, Poland) were inoculated with 100 µL of homogenized pathogen mycelium by spreading pathogen inoculum (70% T) over the solid medium using sterile disposable spreaders. After inoculation, three 4 mm sterile cellulose paper circles were placed on the medium, at the same distance from each other. Then, 15 µL of bacterial suspension (90% T) was pipetted onto every circle and the plates were incubated at 25 °C for 96 h. The pathogens growth inhibition zones surrounding the circles with bacterial inoculum were measured after incubation.

The next step was to evaluate how the examined bacteria react to each other. This step ensures that it is possible to combine certain bacteria into one biopreparation. Any mutual antagonism would prevent those bacteria from being in one formulation. To do this, 90-mm Petri dishes, containing 20 cm^3 of PDA medium (A&A Biotechnology, Gdynia, Poland) were inoculated with 100 µL of bacterial suspension (90% T). The bacterial inoculum was spread using sterile spreaders. After that 16 paper circles with a diameter of 4 mm were placed on the plates, in a pattern that guarantees the same distance from one paper circle to another. Next, 15 µL of bacterial suspension was pipetted onto every circle (90% T). The growth inhibition zone was measured after 96 h of incubation at 25 °C.

4.3. Metabolic Abilites of Isolated Beneficial Bacterial Isolates

The eight selected bacteria were tested on GEN III plates (Biolog Inc., Hayward, CA, USA) due to their very diverse and noteworthy substrate composition. One plate allows the researcher to check bacterial growth both on many carbon sources and in different stress conditions. There were 71 different carbon sources in a separate well of the plate, and we divided them, for further analysis, into 6 different groups based on their chemical structure: amino acids, peptides, and polypeptides; carboxylic acids and esters; polyols and other; sugar acids and their compounds; sugar derivatives; sugars.

Furthermore, carrying out tests considering two wavelengths of light allows us to draw more specific conclusions. This is because absorbances for those wavelengths depend on different factors: 750 nm stands for optical density, and 590 nm for substrate usage [67]. Based on this knowledge it is possible to calculate the ratio between them [68]. The authors suggested that different ratios might inform varied metabolism responses of examined organisms. According to this research, the lower the ratio the more efficient the metabolism is, and the higher the ratio, the more stressful situation is for the microorganisms [68,69]. Even though the mentioned research was carried out for fungi, this method seems so interesting and universal that we decided to use this approach for research on bacteria.

Table 4 presents the division of chemical compounds present in the GEN III plate (Biolog Inc., Hayward, CA, USA) into the different groups. Moreover, there are 23 chemical resistance assays composed of different pH and salinity levels, antibiotics, and chemical compounds (Table 5). For the most part, a single colony was chosen and mixed in with the Biolog® inoculating fluid IF-A (Biolog Inc., Hayward, CA, USA) to make a solution of the desired turbidity 90–98%. After that, each bacterial suspension was poured into the multichannel pipette reservoir and all wells were filled with 100 µL of prepared suspension. For each isolate there were 3 biological replicates as separate microplates to ensure adequate statistical evidence. The plates were incubated at 28 °C for 72 h in aerobic conditions. The microplates were read manually using the MicroStation (Biolog Inc., Hayward, CA, USA) semiautomated reader at 0, 4, 8, 20, 24, 28, 44, 48, 52, 68, and 72 h for two different wavelengths at 590 nm and 750 nm. The average absorbance for all wells within each category was calculated.

Table 4. The assignment of individual carbon sources located in the GEN III plates (Biolog Inc., Hayward, CA, USA) into categorized groups.

Amino Acids, Peptideds and Polypeptides	Polyols and Others	Sugars	Sugar Acids and Their Compounds	Carboxylic Acids and Esters	Sugar Derivatives
D-Aspartic Acid	D-Sorbitol	Dextrin	D-Galacturonic Acid	p-Hydroxyphenylacetic Acid	β-Methyl-D-glucoside
D-Serine	D-Mannitol	D-Maltose	L-Galactonic Acid lactone	L-Lactic Acid	D-Salicin
Glycyl-L-proline	D-Arabitol	D-Trehalose	D-Gluconic Acid	Citric Acid	N-Acetyl-D-glucosamine
L-Alanine	Myo-inositol	D-Cellobiose	D-Glucuronic Acid	α-Ketoglutaric Acid	N-Acetyl-β-D-mannosamine
L-Arginine	Glycerol	Gentiobiose	Glucuronamide	D-Malic Acid	N-Acetyl-D-galactosamine
L-Aspartic Acid	Tween 40	Sucrose	Mucic Acid	L-Malic Acid	Inosine
L-Glutamic Acid		D-Turanose	Quinic Acid	Bromosuccinic Acid	D-Glucose-6-PO_4
L-Histidine		Stachyose	D-Saccharic Acid	α-Hydroxybutyric Acid	D-Fructose-6-PO_4
L-Pyroglutamic Acid		D-Raffinose		β-Hydroxy-D	
L-Serine		α-D-Lactose		α-Ketobutyric Acid	
γ-Amino-butyric Acid		D-Melibiose		Acetoacetic Acid	
Gelatin		N-Acetylneuraminic Acid		Propionic Acid	
		α-D-Glucose		Acetic Acid	
		D-Mannose		Formic Acid	
		D-Fructose		Methyl Pyruvate	
		D-Galactose		D-Lactic Acid Methyl Ester	
		3-Methylglucose		L-Butyric Acid	
		D-Fucose			
		L-Fucose			
		L-Rhamnose			
		Pectin			

Table 5. The assignment of individual chemical compounds located in the GEN III plates (Biolog Inc., Hayward, CA, USA) into categorized groups.

Antibiotics	Organic Compounds	Chemical Soil Properties		Toxic Substances
		pH	Salinity	
Fusidic Acid	1% Sodium Lactate	pH 6	1% NaCl	Guanidine HCl
Troleandomycin	D-serine	pH 5	4% NaCl	Niaproof 4
Rifamycin SV	Sodium Butyrate		8% NaCl	Tetrazolium Violet
Minocycline	Sodium Bromate			Tetrazolium Blue
Lincomycin				Lithium Chloride
Vancomycin				Potassium tellurite
Nalidixic Acid				
Aztreonam				

Afterward, the results were exported and subjected to further analysis such as cluster analysis, using Statistica 13.1 software (StatSoft Inc., Tulsa, OK, USA). This analysis was essential for identifying the differences and similarities between the microorganisms used in this study. Furthermore, Sneath's criteria were applied to hierarchical clustering to find similar groups. Based on the analysis conducted using the metabolic capacity and chemical sensitivity, as well as on antagonistic potential of selected bacteria presented in the first part of the research, there were 3 isolates selected as the best potential candidates to be used in at future biopreparation. Those isolates were *Rhodococcus* sp. B12/18, *Pseudomonas* sp. B37/18, *Arthrobacter* sp. B58/18 and were subjected to additional metabolic and enzymatic analyses using API tests (bioMérieux SA, Marcy l'Etoile, France) and microbiology test plates.

4.4. Enzymatic Activity of Selected Bacterial Isolates

Enzymatic activities were evaluated using API ® ZYM strips (bioMérieux SA, Marcy l'Etoile, France). This analysis allows the researcher to detect the presence of 19 different enzymes secreted by the tested microorganisms. Organic farming uses numerous ways of adding exogenous organic matter to soil including organic waste application. Selecting isolates presenting numerous enzymatic activities increases their chance to not only survive after application but also to enhance soil quality utilizing compounds from organic fertilizer making them more accessible to plants. In order to prepare this test, a bacterial inoculum was prepared to achieve the desired opacity equivalent to that of a McFarland No. 5–6 standard. Then, 5 mL of distilled water was added to the incubation chamber to ensure adequate humidity.

The 65 µL volume of the prepared bacterial solution was added to each measuring cell. Trays with measuring cells were placed inside plastic incubation trays, these in turn were placed in an incubator at 30 °C for 4 h. After this time 5 µL of ZYM A reagent and 5 µL of ZYM B reagents were added to each measuring cell. The strips were set aside for 5 min for the colour to develop. Next, values ranging from '−' to '+++' were assigned, corresponding to the colours developed: '−' corresponded to a negative reaction, '+++' corresponds to a reaction of maximum intensity and the values '+' or '++' are intermediate reactions depending on the level of intensity. All procedures were performed accordingly to the manufacturer's manual (bioMérieux SA, Marcy l'Etoile, France).

Other enzymatic activities, including denitrification and nitrification, ammonification, amylolytic, proteolytic, and cellulolytic abilities, nitrogen fixation, and solubilizing phosphate, were determined using common microbiological procedures on Petri dishes with properly selected media. For solid agar media on Petri dish, the plates were inoculated by a loop of bacteria placed at the middle of sterile Petri dish with an appropriate medium. Liquid media were inoculated with a 10% addition of 70% T bacterial solution. Degradation zones for solid media and absorbances for liquid media were measured after 24 and 168 h of incubation.

The proteolytic activity was evaluated using two different media containing nutrient agar and one with the 4% addition of skimmed milk and a second with a 4% gelatin. For the medium that contained gelatin, the degradation zones were measured after covering the plates with Frazier's reagent.

The amylolytic abilities were evaluated using nutrient agar medium with the addition of 1% starch and the degradation zones were measured after covering the plates with Lugol solution.

The ammonification abilities were evaluated using two different nutrient broths, one containing a 4% addition of skimmed milk and the second one containing 4% addition of urea. A filter-sterilized urea suspension was added to the medium after sterilization to ensure that it has not degraded during sterilization. The absorbance values were determined after the addition of Nessler's reagent at a wavelength of 410 nm.

Denitrification abilities were determined using a nutrient broth medium with 0.1% addition of KNO_3. Durham tubes were placed in the test tubes with the medium and bacterial inoculum, and the production of gas was evaluated during a 1-week incubation. Nitrogen fixation abilities were evaluated using a liquid medium without nitrogen source according to the handbook of microbiological media [70]. The absorbance for the wavelength of 600 nm was measured after 24 h and the full incubation period.

The ability of the bacteria to degrade cellulose was evaluated by growing bacteria in 50 mL flasks containing 0.5 g of sterile shredded straw and 2 mL of water. The flasks were inoculated with 1.5 mL of 70% transmittance bacterial solution. After 24 and 168 incubation hours the liquid from the bottom of the flask was transferred to clean test tubes and a reaction with 3,5-dinitrosalicylic acid (DNS) was performed. The absorbance was measured for a wavelength of 550 nm.

In order to evaluate ability to solubilize phosphate, bacteria were inoculated to Pikovskaya agar medium according to the Handbook of Microbiological Media [70]. After 24 and 168 incubation hours, the clean medium degradation zones were measured.

The evaluation of nitrification abilities was performed using the universal mineral medium containing: $(NH_4)_2SO_4$ 2 g/L, K_2HPO_4 1 g/L, $MgSO_4$ 0.5 g/L, NaCl 0.2 g/L, $FeSO_4$ 5 mg/L, $MnSO_4$ 5 mg/L, $CaCO_3$ 5 g/L. Then, 100 mL flasks with 30 mL of sterile medium were inoculated with 1 mL of 70% transmittance bacterial suspension. In order to confirm the presence of NO_2^- ions 1 mL of bacterial culture was added to a well of a 24-well plate. Next, 2 drops of sulphanilic acid and 2 drops of 1-naphthylamine were added to the well. The presence of a pink or brown colour suggests a positive result for the first phase of nitrification. In order to confirm the presence of NO_3^- ions 1 mL of bacterial culture was added to a well of a 24-well plate. Next, 2 drops of diphenylamine were added to the well. The presence of blue or dark blue colour suggests the positive result for the second phase of nitrification. Next, a value ranging from '−' to '+++' was assigned, corresponding to the colours developed: '−' corresponded to a negative reaction, '+++' to a reaction of maximum intensity and values of '+' or '++' are intermediate reactions depending on the level of intensity.

4.5. Metabolic Abilities of Selected Fungal and Fungal-Like Plant Pathogens

Metabolic abilities of selected phytopathogens were performed on the FF MicroPlate (Biolog®, Hayward, CA, USA) containing 95 different carbon sources in the wells. Inoculation was performed according to the manufacturer's protocol with modifications described by Oszust et al. [17]. After the homogenization of the mycelium suspension in inoculating fluid (FF-IF, Biolog®, Hayward, CA, USA), the transmittance was adjusted to 75% using a turbidimeter (Biolog®, Hayward, CA, USA). A volume of 100 µL of the mycelium suspension was added to each well. The inoculated microplates were incubated in darkness at 25 °C for 10 days. Absorbances were measured daily at the wavelengths of 490 nm and 750 nm, then the ratio of those values was calculated.

4.6. Prebiotic Supplement Blend as Possible Component of Future Biopreparation

After GEN III and FF microplate analysis, compounds common for both types of the microplates were chosen. For those substrates, a ratio of absorbance values was calculated, the OD590/OD750 for GEN III and the OD490/OD750 for FF microplates. The ratio was calculated based on the average value

from all days of the incubation. Based on the ratio it was possible to indicate some compounds that were causing a stressful metabolic situation to the fungal and fungal-like plant pathogens but were not inhibiting bacterial growth. Those substrates might be considered as a valuable source of additional nutrients for bacterial isolates in biopreparation, thereby further inhibiting growth of pathogenic fungi and/or enhancing beneficial bacterial consortia.

5. Conclusions

- Bacteria isolated from raspberry rhizosphere have antagonistic properties against common fungal and fungal-like plant pathogens such as *Botrytis* spp., *Colletotrichum* spp., *Phytophthora* spp., and *Verticillium* spp.
- Describing the utilization abilities of different substrates by microorganisms such as bacteria is a study that might benefit from the application of a calculation method of the 590–750 nm ratio of absorbances. This may not only give the researchers a broader spectrum of results, but also emphasize differences between the tested isolates as far as functional response is concerned, namely the metabolic activity in juxtaposition with biomass production.
- Bacteria to be used in biopreparation need to present some desired features. They should be antagonistic against pathogens, have a broad spectrum of utilized compounds to be used as a carbon source, survive in many different conditions such as different pH values. Due to the fact, that since resistance to antibiotics becomes more common in soil microorganisms it is worth considering that bacteria used in biopreparations are not resistant to antibiotic compounds. This practice reduces spreading this undesirable feature among other microorganisms. From tested bacteria, isolates B12/18 and B58/18 presented needed features and at the same time, they were not resistant to most tested antibiotics. Those two isolates might be worth to be used in future testing and formulations. It is worth considering using other isolates in some special cases and under restrictions due to their good antagonistic and metabolic properties.
- A carbon substrate, such as D-trehalose was utilized by the tested bacteria in a balanced way, namely without causing a stressful metabolic situation that, might be a beneficial addition to a bacterial formulation providing enhanced growth and survivability.
- Metabolic and enzymatic abilities analysis provides important information about particular environmental isolates which might be essential to achieving a complete understanding of their functioning in biopreparations or in future environmental niches. The more enzymatic abilities are demonstrated by the isolates, the easier it might be for those isolates to acclimatize to new environments or stress conditions.
- Carefully selected chemical compounds are a valuable additive to biopreparations. Additives such as D-malic acid, D-saccharic acid, N-acetylo-D-galactosamine, and α-keto-glutaric acid may not only enhance or stimulate bacterial growth but also inhibit the growth of plant fungal and fungal-like pathogens.
- Future research will focus on in planta testing bacterial formulations on raspberry plants combined with introducing fungal and fungal-like pathogens to the experimental treatments. Due to the fact that microbial inoculation may cause tremendous changes in the dynamics of soil microbial communities, subsequent research should include the soil microbiome and mycobiome status and its functional shifts after selected bacterial isolates application. Moreover, regarding the suggested blend of prebiotic supplements, it should be tested for its effect on the growth of beneficial microorganisms and pathogens. Moreover, the study should focus on the development of a biopreparation with carriers and technology conditions appropriate for the selected bacterial isolates, considering future needs arising from the Biodiversity Strategy for 2030.

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Abbreviations

16S rDNA	16S ribosomal DNA gene
ASM	Acibenzolar-S-methyl
MEGA	Molecular Evolutionary Genetics Analysis
OD590/OD750	The ratio of absorbances value measured at 590 nm and 750 nm
OD490/OD750	The ratio of absorbances value measured at 490 nm and 750 nm
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
T	Transmittance

Appendix A

Table A1. Antagonistic properties of all bacteria isolated from soil and potentially beneficial bacteria against 4 selected plant fungal pathogens. Paper circles inoculated with bacterial inoculum were placed on a PDA plate inoculated with plant fungal pathogen. Pathogen growth inhibition zones were evaluated after 96 incubation hours. Pluses and minuses represent the size of an inhibition zone. For each pathogens the size of the inhibition zone was determined individually as mean values. For *Colletotrichum* spp. it was 18 mm ± 2.6, 22 mm ± 10.2, 30.6 mm ± 6.1, for +, ++, +++ respectively, for *Verticillium* spp. it was 13.0 mm ± 5.0, 14.9 mm ± 2.7, 18.2 mm ± 2.5, for +, ++, +++ respectively, for *Phytophthora* spp. it was 18.0 mm ± 7.6, 23.3 mm ± 2.5, 45.0 mm ± 6.5 for +, ++, +++ respectively, for *Botrytis* spp. it was 14.4 mm ± 1.9, 17.6 mm ± 2.7, 26.1 mm ± 3.1 for +, ++, +++ respectively. – means good growth of phytopathogens without inhibition effect.

Bacteria		**Phytopathogenic Fungi and Fungal-Like Pathogens**											
		***Colletotrichum* spp.**			***Verticillium* spp.**			***Phytophthora* spp.**			***Botrytis* spp.**		
		G172/18	**G371/18**	**G166/18**	**G293/18**	**G296/18**	**G297/18**	**G368/18**	**G373/18**	**G369/18**	**G275/16**	**G277/18**	**G276/18**
Flavobacterium sp.	B10/18	–	–	–	–	–	–	–	–	–	-	–	–
Rhodococcus sp.	B12/18	+	+	++	+	++	++	+++	+	++	-	++	+++
Pseudomonas sp.	B13/18	–	–	–	–	–	–	–	–	–	–	–	–
Pseudomonas sp.	B14/18	–	–	–	–	–	–	–	–	+	–	++	–
Burkholderia sp.	B18/18	–	–	–	–	–	–	–	–	–	–	–	–
Curtobacterium sp.	B19/18	–	–	–	–	–	–	–	–	–	–	–	–
Arthrobacter sp.	B20/18	–	–	–	–	–	–	–	–	–	–	+	–
Pseudomonas sp.	B21/18	–	–	–	–	–	–	–	+	–	–	–	+
Pseudomonas sp.	B22/18	–	–	–	–	–	–	–	–	–	–	–	–
Microbacterium sp.	B23/18	–	–	–	–	–	–	–	++	-	-	-	–-
Pseudomonas sp.	B25/18	++	++	+++	+++	++	++	++	+	+++	–	–	–
Pseudomonas sp.	B26/18	–	–	–	–	–	–	–	–	–	–	–	–
Bacillus sp.	B27/18	–	–	–	–	+	+	–	–	–	–	–	–
not identified	B28/18	–	–	–	–	–	–	–	+	–	–	–	–
Pseudomonas sp.	B29/18	–	–	–	++	+	–	–	–	–	–	–	–
Pseudomonas sp.	B30/18	–	–	+	+	+	–	–	+	–	–	–	–
Bacillus sp.	B31/18	–	–	–	–	–	–	–	–	–	–	–	–
Bacillus sp.	B32/18	–	–	–	–	–	+	–	–	–	–	–	–
Pseudomonas sp.	B33/18	+	–	–	+	+	–	–	++	–	–	–	–
Arthrobacter sp.	B35/18	–	–	–	–	–	–	–	++	–	–	–	–
Pseudomonas sp.	B37/18	–	–	++	+++	+++	++	+++	+	++		–	
Bacillus sp.	B39/18	–	++	++	++	++	++	++	+	+++	++	–	++
Bacillus sp.	B40/18	–	–	–	++	+	++	+	+	+	–	–	–
Flavobacterium sp.	B41/18	–	–	–	–	–	–	+	–	–	–	–	–
Pseudomonas sp.	B42/18	–	–	–	–	–	–	–	++	+	–	–	–
Pedobacter sp.	B44/18	–	–	–	–	–	+	++	++	+	–	–	–

Table A1. *Cont.*

Bacteria		Phytopathogenic Fungi and Fungal-Like Pathogens											
		Colletotrichum spp.			*Verticillium* spp.			*Phytophthora* spp.			*Botrytis* spp.		
		G172/18	G371/18	G166/18	G293/18	G296/18	G297/18	G368/18	G373/18	G369/18	G275/16	G277/18	G276/18
Arthrobacter sp.	B45/18	–	–	–	–	–	–	–	++	+	+	–	–
Pseudomonas sp.	B46/18	-	-	-	-	+	+	-	+	-	-	–	–
Plantibacter sp.	B47/18	–	–	–	–	–	–	–	–	–	–	–	–
Pseudomonas sp.	B48/18	–	–	–	–	–	–	–	–	++	–	–	–
Arthrobacter sp.	B49/18	-	-	++	++	++	++	+++	+	++	-	+++	-
Pedobacter sp.	B51/18	–	–	–	–	–	–	–	–	–	–	–	–
Janthinobacterium sp.	B54/18	–	–	–	–	–	–	–	–	–	–	–	–
Novosphingobium sp.	B57/18	–	–	–	–	++	++	–	+++	–	–	–	–
Arthrobacter sp.	B58/18	++	++	++	++	+	+++	+	+	–	–	++	–
Arthrobacter sp.	B59/18	–	–	–	–	–	–	–	–	++	–	–	–
not identidied	B60/18	–	–	–	–	+	+	++	–	–	–	–	–
Bacillus sp.	B61/18	–	–	–	–	–	–	–	–	–	–	–	–
Flavobacterium sp.	B66/18	–	–	–	++	+	++	–	+++	–	–	–	–
Bacillus sp.	B68/18	–	–	–	–	+	–	–	-	–	–	–	–
Bacillus sp.	B69/18	–	–	–	++	-	–	–	+++	–	–	–	–
Shinella sp.	B70/18	–	–	–	–	–	–	–	+++	–	–	–	–
Microbacterium sp.	B72/18	–	–	–	–	–	–	++	+	+	–	–	–
Rhodococcus sp.	B73/18	-	-	-	++	++	++	++	+	++	–	++	–
Arthrobacter sp.	B74/18	+	–	++	+	–	–	–	++	–	–	–	–
Pseudomonas sp.	B75/18	–	+++	–	+	–	–	–	+++	+	–	++	–
Bacillus sp.	B77/18	–	–	–	–	+	+	++	+	–	–	+	–

Table A2. The table presents values of the ratio calculated based on absorbance values the OD590/OD750 for beneficial bacterial isolates and the OD490/OD750 nm for plant fungal and fungal-like pathogens. Ratios were calculated from average absorbance values from all measurements. Values higher than >1 suggest stressful metabolic situation, values lover than <1 suggest good utilization of substrates. Underlined substrates are those that are neutral for bacteria, but cause stressful metabolic situation for plant fungal and fungal-like pathogens.

Substrate	Beneficial Bacteria			Fungal and Fungal-Like Plant Pathogens									
	Rhodococcus sp.	*Pseudomonas* sp.	*Arthrobacter* sp.	*Botrytis* spp.			*Colletotrichum* spp.			*Phytophthora* spp.	*Verticillium* spp.		
	B12/18	B37/18	B58/18	G275/18	G276/18	G277/18	G166/18	G172/18	G371/18	G408/18	G293/18	G296/18	G297/18
Bromosuccinic Acid	0.00	0.16	8.60	2.05	2.91	3.84	17.67	0.00	11.44	0.00	13.91	8.50	12.38
D-Arabitol	0.00	0.90	8.37	1.23	1.60	1.49	1.19	1.53	1.29	1.86	6.82	3.48	3.42
D-Cellobiose	0.00	0.00	3.17	1.55	1.44	1.20	1.81	1.65	1.16	1.24	2.53	2.05	2.42
Dextrin	9.32	0.00	1.56	0.00	1.85	4.14	2.38	2.98	1.74	1.93	2.27	2.08	2.31
D-Fructose	22.84	0.00	18.46	1.39	1.42	1.24	1.80	1.53	1.73	1.30	1.64	1.61	1.67
D-Galactose	0.00	0.00	0.00	1.38	1.42	1.37	1.35	1.59	1.31	1.39	1.61	1.59	1.65
D-Galacturonic Acid	0.00	0.00	16.97	1.65	1.95	1.43	2.49	2.03	1.78	1.64	0.00	0.00	0.00
D-Glucuronic Acid	0.00	0.00	30.14	1.54	2.16	49.57	1.73	3.40	2.35	7.11	2.18	1.99	2.04
D-Lactic Acid Methyl Ester	0.00	0.00	9.94	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D-Malic Acid	**0.00**	**0.00**	**7.43**	**2.70**	**2.92**	**4.66**	**4.80**	**5.24**	**2.59**	**6.13**	**2.09**	**2.07**	**2.11**
D-Mannitol	2.31	24.86	19.51	1.36	1.42	1.40	1.18	1.42	1.33	1.30	2.44	2.23	3.00
D-Mannose	3.30	3.76	7.48	1.62	1.54	1.34	3.28	1.90	1.55	1.31	1.73	1.66	1.80
D-Melibiose	0.00	0.00	0.00	1.70	1.43	1.43	1.34	1.55	1.21	1.41	1.46	1.38	1.51
D-Raffinose	6.03	0.00	2.70	1.40	1.45	1.34	1.24	1.39	1.33	1.23	1.52	1.51	1.52
D-Saccharic Acid	**0.00**	**0.00**	**54.50**	**1.69**	**2.76**	**7.70**	**2.04**	**8.50**	**2.57**	**0.00**	**3.58**	**3.63**	**2.64**
D-Sorbitol	19.00	0.00	0.00	1.38	1.36	1.20	1.46	1.93	1.41	1.22	6.71	7.47	6.36
D-Trehalose	3.76	0.00	4.15	1.76	1.43	1.54	1.76	1.30	1.35	1.71	1.52	1.56	1.54
Gentiobiose	68.20	0.00	9.61	1.56	1.50	1.46	2.40	1.65	1.41	1.23	1.68	1.57	1.70
Glucuronamide	0.00	0.00	17.05	0.00	0.00	3.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Glycerol	2.50	6.47	0.00	1.39	1.63	1.27	1.66	1.57	1.63	1.33	1.70	1.97	1.96
L-Alanine	3.82	1.17	19.17	2.26	2.19	1.89	1.48	1.77	1.43	1.78	2.10	2.42	2.59
L-Aspartic Acid	3.44	2.93	24.17	3.09	5.22	4.05	2.85	3.28	2.69	0.00	3.35	3.70	3.56
L-Fucose	0.00	0.00	127.57	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.70	2.33	3.91
L-Glutamic Acid	0.00	2.46	9.13	2.27	2.42	2.00	2.94	5.13	1.63	3.34	3.23	2.46	3.97
L-Lactic Acid	3.42	3.41	0.00	0.00	0.00	0.00	0.00	0.00	13.15	0.00	255.50	5.43	14.76
L-Malic Acid	3.01	30.46	8.28	0.00	4.90	0.00	6.63	5.89	2.83	0.00	3.94	3.92	4.49
L-Pyroglutamic Acid	2.34	5.90	0.00	0.00	0.00	0.00	2.19	1.82	1.80	0.00	0.00	0.00	0.00
L-Rhamnose	0.00	0.00	82.45	1.27	1.47	1.33	1.26	1.18	1.25	1.20	1.53	1.51	1.58
L-Serine	3.30	0.52	6.45	1.92	24.50	3.03	2.28	2.96	1.90	6.94	2.58	2.54	2.82
N-Acetyl-D-Galactosamine	**0.00**	**0.00**	**15.67**	**1.50**	**1.73**	**3.20**	**1.16**	**1.40**	**1.23**	**1.49**	**1.56**	**1.78**	**1.70**
N-Acetyl-D-Glucosamine	2.23	0.00	3.99	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
N-Acetyl-D-Mannosamine	0.00	0.00	6.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Quinic Acid	2.26	0.00	11.40	1.57	1.94	1.74	1.41	1.70	1.30	2.57	3.61	2.65	2.87
Stachyose	21.00	0.00	5.45	1.37	1.33	1.43	1.33	1.62	1.26	1.34	1.46	1.46	1.50
Sucrose	9.46	0.00	3.71	1.45	1.40	1.46	1.68	1.53	1.73	1.32	1.54	1.55	1.56
α-D-Glucose	1.51	4.07	8.24	1.56	1.40	1.41	1.70	1.33	1.18	1.46	1.62	1.62	1.68
α-D-Lactose	139.50	0.00	13.03	1.42	1.39	1.33	1.46	1.83	1.41	1.26	0.00	0.00	0.00
α-Keto-glutaric Acid	**5.97**	**0.00**	**0.00**	**78.29**	**5.07**	**0.00**	**3.80**	**5.58**	**8.88**	**21.06**	**8.26**	**5.57**	**6.92**
β-Methyl-D-Glucoside	38.80	0.00	7.34	1.79	1.51	1.73	1.49	1.68	1.44	1.45	1.52	1.44	1.54
γ-Amino-butyric Acid	2.00	0.00	1.74	1.41	7.05	3.19	1.39	1.38	1.43	2.17	2.11	2.30	2.50

Table A3. Data presenting information about all isolates including identification, accession number of sequences in GenBank, the sample from which isolation was made, the medium used for isolation, and forest, where samples were obtained. LMEM – Laboratory of Molecular and Environmental Microbiology of the Institute of Agrophysics, Polish Academy of Sciences.

Isolate Code LMEM	Identification	The Accession Number of Sequences in GenBank	Compartment	Microbiological Medium	Forest Location in Poland	Coordinates	Forest Distinct in Poland
B10/18	*Flavobacterium* sp.	MW255682	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01061 E 022.01705	Kraśnik
B11/18	*Arthrobacter* sp.	MW255683	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01061 E 022.01705	Kraśnik
B12/18	*Rhodococcus* sp.	MW255650	wild raspberry rhizosphere	Agar with soil extract	Chruślanki Józefowskie	N 51.01061 E 022.01705	Kraśnik
B13/18	*Pseudomonas* sp.	MW255684	wild raspberry rhizosphere	Agar with soil extract	Chruślanki Józefowskie	N 51.01061 E 022.01705	Kraśnik
B14/18	*Pseudomonas* sp.	MW255685	wild raspberry rhizosphere	Agar with soil extract	Chruślanki Józefowskie	N 51.01061 E 022.01705	Kraśnik
B15/18	*Pseudomonas* sp.	MW255686	wild raspberry rhizosphere	Plate Count Agar	Pomorze	N 51.17137 E 021.95993	Kraśnik
B16/18	*Arthrobacter* sp.	MW255687	wild raspberry rhizosphere	Agar with soil extract	Wierzchowiska	-	Janów Lubelski
B17/18	*Mucilaginibacter* sp.	MW255688	wild raspberry rhizosphere	Agar with soil extract	Wierzchowiska	-	Janów Lubelski
B18/18	*Burkholderia* sp.	MW255689	wild raspberry rhizosphere	Agar with soil extract	Świdnik	N 51.54462 E 022.28306	Świdnik
B19/18	*Curtobacterium* sp.	MW255690	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01083 E 022.01578	Kraśnik
B20/18	*Arthrobacter* sp.	MW255691	wild raspberry rhizosphere	Plate Count Agar	Pomorze	N 51.17137 E 021.95993	Kraśnik
B21/18	*Pseudomonas* sp.	-	wild raspberry rhizosphere	Plate Count Agar	Pomorze	N 51.17137 E 021.95993	Kraśnik
B22/18	*Pseudomonas* sp.	MW255692	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01061 E 022.01705	Kraśnik
B23/18	*Microbacterium* sp.	MW255693	wild raspberry rhizosphere	Agar with soil extract	Chruślanki Józefowskie	N 51.01051 E 022.01672	Kraśnik
B24/18	*Xanthomonas* sp.	MW255694	wild raspberry rhizosphere	Plate Count Agar	Wierzchowiska	-	Janów Lubelski
B25/18	*Pseudomonas protegens*	MW255695	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.00903 E 022.01139	Kraśnik
B26/18	*Pseudomonas mohnii*	MW255696	wild raspberry rhizosphere	Plate Count Agar	Pomorze	N 51.17247 E 021.95057	Kraśnik
B27/18	*Bacillus mycoides*	MW255697	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.00903 E 022.01139	Kraśnik
B29/18	*Pseudomonas* sp.	MW255698	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01083 E 022.01578	Kraśnik
B30/18	*Pseudomonas* sp.	MW255699	wild raspberry rhizosphere	Plate Count Agar	Wierzchowiska	-	Janów Lubelski
B31/18	*Bacillus* sp.	MW255700	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.00936 E 022.01220	Kraśnik
B32/18	*Bacillus mycoides*	MW255701	wild raspberry rhizosphere	Plate Count Agar	Bobowiska	N 51.41395 E 022.24402	Puławy
B33/18	*Pseudomonas* sp.	MW255702	wild raspberry rhizosphere	Plate Count Agar	Bobowiska	N 51.41395 E 022.24402	Puławy
B34/18	*Erwinia* sp.	MW255703	wild raspberry rhizosphere	Plate Count Agar	Krzywda	-	Łuków
B35/18	*Arthrobacter psychrolactophilus*	MW255704	wild raspberry rhizosphere	Plate Count Agar	Krzywda	-	Łuków
B36/18	*Pseudomonas* sp.	MW255705	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01061 E 022.01705	Kraśnik
B37/18	*Pseudomonas* sp.	MW255651	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01061 E 022.01705	Kraśnik
B38/18	*Leifsonia* sp.	MW255706	wild raspberry rhizosphere	Plate Count Agar	Śmiary	-	Siedlce
B39/18	*Bacillus thuringiensis*	MW255707	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01051 E 022.01672	Kraśnik
B40/18	*Bacillus* sp.	MW255708	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01061 E 022.01705	Kraśnik
B41/18	*Flavobacterium* sp.	-	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.00926 E 022.01120	Kraśnik
B42/18	*Pseudomonas* sp.	MW255709	wild raspberry rhizosphere	Plate Count Agar	Bobowiska	N 51.41395 E 022.24402	Puławy
B43/18	*Pedobacter* sp.	-	wild raspberry rhizosphere	Plate Count Agar	Bobowiska	N 51.41395 E 022.24402	Puławy
B44/18	*Pedobacter* sp.	MW255710	wild raspberry rhizosphere	Plate Count Agar	Bobowiska	N 51.41395 E 022.24402	Puławy
B45/18	*Arthrobacter* sp.	MW255711	wild raspberry rhizosphere	Plate Count Agar	Pomorze	N 51.17100 E 021.95998	Kraśnik
B46/18	*Pseudomonas fluorescens*	MW255712	wild raspberry rhizosphere	Plate Count Agar	Pomorze	N 51.17100 E 021.95998	Kraśnik
B47/18	*Plantibacter* sp.	MW255713	wild raspberry rhizosphere	Plate Count Agar	Pomorze	N 51.17100 E 021.95998	Kraśnik
B48/18	*Pseudomonas* sp.	MW255714	wild raspberry rhizosphere	Plate Count Agar	Pomorze	N 51.17100 E 021.95998	Kraśnik
B49/18	Arthrobacter sp.	MW255715	wild raspberry rhizosphere	Plate Count Agar	Bobowiska	N 51.41395 E 022.24402	Puławy
B50/18	*Chryseobacterium balustinum*	MW255716	wild raspberry rhizosphere	Plate Count Agar	Śmiary	-	Siedlce
B51/18	*Pedobacter* sp.	MW255717	wild raspberry rhizosphere	Plate Count Agar	Śmiary	-	Siedlce

Table A3. *Cont.*

Isolate Code LMEM	Identification	The Accession Number of Sequences in GenBank	Compartment	Microbiological Medium	Forest Location in Poland	Coordinates	Forest Distinct in Poland
B52/18	*Luteibacter rhizovicinus*	MW255718	wild raspberry rhizosphere	Plate Count Agar	Śmiary	-	Siedlce
B53/18	*Burkholderia* sp.	MW255719	wild raspberry rhizosphere	Agar with soil extract	Śmiary	-	Siedlce
B54/18	*Janthinobacterium lividum*	MW255720	wild raspberry rhizosphere	Agar with soil extract	Śmiary	-	Siedlce
B56/18	*Burkholderia* sp.	MW255721	wild raspberry rhizosphere	Agar with soil extract	Chruślanki Józefowskie	N 51.00926 E 022.01120	Kraśnik
B57/18	*Novosphingobium* sp.	MW255722	wild raspberry rhizosphere	Agar with soil extract	Pomorze	N 51.17137 E 021.95993	Kraśnik
B58/18	*Arthrobacter globiformis*	MW255652	wild raspberry rhizosphere	Agar with soil extract	Wierzchowiska	-	Janów Lubelski
B59/18	*Arthrobacter* sp.	-	wild raspberry rhizosphere	Agar with soil extract	Pomorze	N 51.17137 E 021.95993	Kraśnik
B61/18	*Bacillus simplex*	MW255723	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01083 E 022.01578	Kraśnik
B62/18	*Chryseobacterium* sp.	MW255724	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01083 E 022.01578	Kraśnik
B63/18	*Pseudomonas* sp.	MW255725	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01083 E 022.01578	Kraśnik
B64/18	*Variovorax* sp.	MW255726	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01083 E 022.01578	Kraśnik
B65/18	*Pseudomonas* sp.	MW255727	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.00926 E 022.01120	Kraśnik
B66/18	*Flavobacterium* sp.	-	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01083 E 022.01578	Kraśnik
B67/18	*Burkholderia* sp.	MW255728	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.00903 E 022.01139	Kraśnik
B68/18	*Bacillus mycoides*	MW255647	wild raspberry roots	Plate Count Agar	Chruślanki Józefowskie	N 51.00903 E 022.01139	Kraśnik
B69/18	*Bacillus simplex*	MW255648	wild raspberry roots	Plate Count Agar	Chruślanki Józefowskie	N 51.01083 E 022.01578	Kraśnik
B70/18	*Shinella* sp.	MW255729	wild raspberry rhizosphere	Plate Count Agar	Wierzchowiska	-	Janów Lubelski
B71/18	*Roseomonas mucosa*	MW255730	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.00926 E 022.01120	Kraśnik
B72/18	*Microbacterium* sp.	MW255731	wild raspberry rhizosphere	Plate Count Agar	Śmiary	-	Siedlce
B73/18	*Rhodococcus erythropolis*	MW255732	wild raspberry rhizosphere	with soil extract	Bobowiska	N 51.41395 E 022.24402	Puławy
B74/18	*Arthrobacter* sp.	MW255733	wild raspberry rhizosphere	Plate Count Agar	Pomorze	N 51.17137 E 021.95993	Kraśnik
B75/18	*Arthrobacter* sp.	MW255653	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.00926 E 022.01120	Kraśnik
B76/18	*Pseudomonas* sp.	MW255734	wild raspberry rhizosphere	Plate Count Agar	Chruślanki Józefowskie	N 51.01083 E 022.01578	Kraśnik
B77/18	*Bacillus* sp.	MW255735	wild raspberry rhizosphere	Plate Count Agar	Wierzchowiska	-	Janów Lubelski

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7. Tekst publikacji P.3

agronomy

MDPI

Article

Optimization of Growing Medium and Preservation Methods for Plant Beneficial Bacteria, and Formulating a Microbial Biopreparation for Raspberry Naturalization

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Abstract: The current study focuses on the optimization of bacterial growing medium composition, including the carbon and nitrogen source in different concentrations, the pH value of the medium and the temperature. Optimization was performed for four environmental bacterial isolates belonging to the genera *Arthrobacter*, *Pseudomonas* and *Rhodococcus*, which were previously obtained from wild raspberries. These bacteria proved to be potent antagonists against certain fungal and fungal-like plant pathogens. Furthermore, three preservation methods and three sample preparation techniques were evaluated. In addition, a prebiotic supplementary blend based on previous research was tested. The research included a pot experiment to estimate the influence of bacterial cultures on the growth of plant shoots and roots, on the soil enzymatic activity and the content of macronutrients, minerals and nitrogen in the soil depending on the naturalization strategy. The best carbon and nitrogen source were chosen. The addition of a supplementary blend resulted in the increased growth of two bacterial isolates. Bacterial inoculum applied to the roots and watering resulted in increased shoot mass in objects infected with plant pathogens, although in plants without the pathogen infection, bacterial inoculum resulted in the decreased mass of plants. Naturalization strategy should be matched to the pathogens present at plantations.

Keywords: biopreparation; organic farming; fungal plant pathogens; optimization; preservation; pot experiment

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1. Introduction

Microorganisms have been utilized more and more often in recent years for plant growth and resistance stimulation, which includes biocontrol, as well as soil microbial activity and functionality maintenance or improvement. Bacterial biopreparations and bioproducts are now common in organic farming. Current trends in plant cultivation and pest control are shifting towards methods safe for the environment and microbial diversity [1]. The European Union promotes the use of organic methods of crop production, including pest control, but also bans compounds that are damaging to environmental biodiversity and are detrimental to soil microbial communities [2]. The European Union Biodiversity Strategy for 2030 clearly states that ecological biodiversity degradation must be stopped and one of the most important factors for achieving this goal is agricultural and horticultural production systems based on natural solutions, including beneficial microbes, as well as decreasing the application of mineral fertilizers and reducing the application of chemicals used in pest control. Furthermore, WE No. 834/2007 states that using chemical pesticides must be limited to the absolute minimum and that farmers are encouraged to use substances of natural origin [3]. Biopreparations may consist of microorganism inoculum, a carrier and supplements that support the growth of beneficial microorganisms and their survivability in different ecological niches [4,5]. Biopreparations are products composed of living microorganisms or their metabolites. Currently, there are biopreparations available to farmers, i.e., Micosat F

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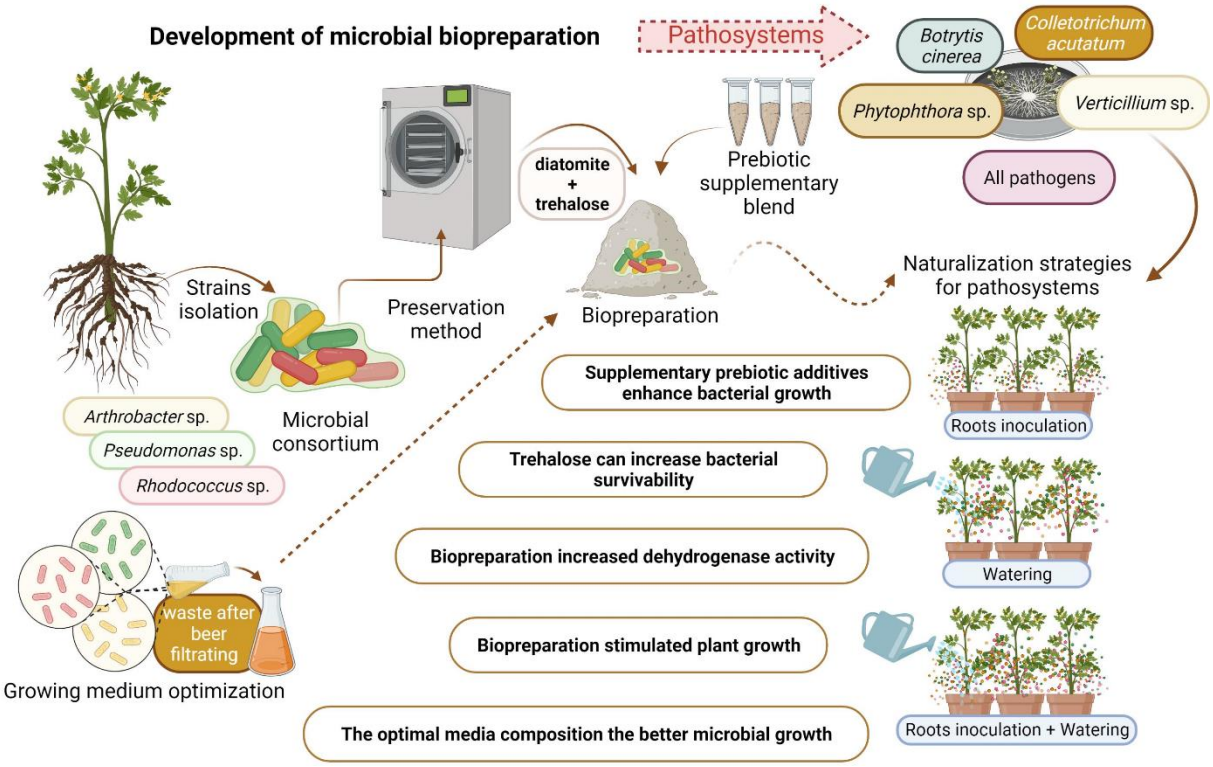

or Polyversum WP based, respectively, on the fungi *Trichoderma* spp. and *Pythium oligandrum*. There are other biopreparations available that inhibit the growth of soft fruit pathogens such as *Phytophthora* spp. (PREV-AM), *Colletotrichum* spp. (chitosan), and *Verticillium* spp. (Bioczos S) [6–8]. However, there is still a niche available for another agroecology product, combining plant growth and resistance stimulation, as well as being appropriate for the maintenance of soil microbial activity and diversity, and thus appropriate for use in the organic production of fruits.

Since the number of plant protection products targeting phytopathogens which are allowed to be used in organic farming is decreasing, because they are being steadily forbidden on an official basis, the demand for new methods of plant protection is rising. The most promising alternatives to these products are biopreparations based on local and highly effective bacterial isolates. The application of bacteria that are locally occurring in wild plants to plants in plantations can be called a naturalization. In our previous research, we isolated and characterized four bacterial isolates belonging to three different genera *Arthrobacter* sp., *Bacillus* sp., and *Rhodococcus* sp., and examined their ability to inhibit the growth of fungal and fungal-like plant pathogens such as *Botrytis* spp., *Colletotrichum* spp., *Phytophthora* spp. and *Verticillium* spp. Moreover, we selected four chemical compounds that have the ability to stimulate bacterial growth and are not metabolized by pathogens and might therefore be used as a supplementary prebiotic blend [5].

In this paper, we focus on the development of biopreparation formulations based on previously isolated and examined bacterial isolates. The experiments conducted concentrated on the optimization of the bacterial growth and drying of bacteria, including testing the prebiotic supplementary blend and pot experiment to examine the influence of selected bacterial isolates on plants and pathogens. The optimization of the growing media can have a high degree of influence over the speed of growth for different microorganisms. Different microorganisms utilize different chemical compounds such as a carbon or nitrogen sources. Environmental isolates may show a preference for different medium pH values or growing temperatures that are often difficult to predict. Optimization can also influence the production of enzymes or other metabolites [9–11]. Preservation methods may differ for different bacterial species. Appropriate preservation techniques ensure that survivability is maintained and that the biopreparation is stable and the shelf life of the future product is sufficiently long. It is also worth noting that an appropriate choice of carrier may shorten the drying time by increasing the surface area of the dried sample.

The aim of this research was to formulate a biopreparation for plant growth and resistance stimulation for raspberry naturalization, defined as the application of previously chosen beneficial bacterial strains, isolated from natural habitats of wild raspberries. The optimization process of the growing medium was focused on the carbon source, the nitrogen source, the pH value of the medium, the growing temperature, carrier choice and supplementary blend additive. The optimization of the drying process focused on finding a drying method and sample preparation that ensured the highest degree of survivability. We also examined the influence of pure cultures of those bacteria on raspberry plants in the pot experiment, evaluating the dry mass of shoots, the wet mass of roots, and soil dehydrogenase activity in experiments treated with different types of naturalization strategies using prepared biopreparations. The hypothesis of the presented research is that environmental isolates of bacteria belonging to *Arthrobacter, Pseudomonas*, and *Rhodococcus* genera are effective in promoting the early growth of raspberry plants and furthermore are suitable to be a part of biopreparation formulation.

2. Materials and Methods

2.1. Isolation and Identification of Bacterial Strains

Bacteria were isolated from raspberry roots and soil collected in the following Forest Districts: Łuków, Świdnik, Janów Lubelski, Kraśnik, and Siedlce. A full description of the methodology of the sampling techniques used is presented in Oszust et al. (2020) [12]. The bacteria were genetically identified based on a 16S rDNA fragment as described in

Pylak et al. (2020) [5]. The sequences obtained were submitted to the National Center for Biotechnology Information (NCBI) database: *Rhodococcus* sp. B12/18 (GenBank: MW255650), *Pseudomonas* sp. B37/18 (GenBank: MW255651), *Arthrobacter* sp. B58/18 (GenBank: MW255652), *Rhodococcus* sp. B75/18 (GenBank: MW255653). The antagonistic and metabolic abilities of the isolated strains, including important features for plant growth promotion, were evaluated and presented in previous research [5]. These strains were isolated from the rhizosphere of wild raspberry, taking into account that plant–microbe interactions in the rhizosphere determine plant health, as well as soil productivity and fertility [13].

2.2. Optimization of the Growing Medium for Bacterial Isolates

Optimization of the growing media was performed for four isolates of bacteria. The basic medium used for optimization consisted of the residues of beer filtering on diatomite (1 L), $NaHPO_4$ (7 g/L), KH_2PO_4 (3 g/L), NH_4Cl (1 g/L), and microelements solution (10 mL/L) containing 100 mg/L $MnCl_2$*$4H_2O$, 50 mg/L $ZnCl_2$, 50 mg/L $CuCl_2$*$2H_2O$, 50 mg/L $CoCl_2$*$6H_2O$, and 50 mg/L Na_2MoO_4*$2H_2O$. The optimization process focused on 3 sugars: glucose, lactose and sucrose were tested as additional carbon sources, including 3 different concentrations: 3%, 6% and 9%, and 4 different nitrogen sources: $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, including 3 different concentrations: 1%, 3% and 6%. The pH value was set to 7.2 using 1 M NaOH. A total of 15 mL of medium subjected to optimization was poured into 50 mL Erlenmeyer flasks; these were plugged with cellulose stoppers and sterilized at a temperature of 121 °C for 20 min. After sterilization, the medium in the flasks was inoculated with a 1.5 mL solution of bacteria in the water, for which the turbidity was set to 90% of transmittance using a turbidimeter (Biolog®, Hayward, CA, USA), which represents the optical density of 0.23–0.28 (0.23 for B37/18, B75/18 and 0.28 for B12/18, B58/18) McFarland measured with the use of a DEN-1B densitometer (Biosan®, Riga, Latwia) or absorbance value of 0.1–0.12 (0.1 for B37/18, B75/18 and 0.12 for B12/18, B58/18) measured at the wavelength of 600 nm with the use of an Infinite M200PRO spectrophotometer (Tecan®, Männedorf, Switzerland). Bacteria used for inoculum preparation were cultured for 48 h on PDA plates at a temperature of 30 °C. After choosing the best carbon and nitrogen sources and their concentrations, the culturing temperature and pH value of the medium were subjected to optimization. The following temperatures, 18 °C, 24 °C, 30 °C, and the set of pH values 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 were reviewed. For each of the aforementioned variants, carbon source, nitrogen source, the pH value of the medium and temperature of cultivation were optimized. After 72 h of cultivation in the tested conditions, 1 mL of medium with bacteria was diluted with water using the serial dilution method. From the chosen dilutions (10^{-6}, 10^{-7}, 10^{-8}) 100 µL was spread on a PDA plate using a sterile cell spreader [14]. The plates were then cultivated for 48 h at 30 °C. Afterwards, the colonies were counted and the composition of the optimized medium was evaluated.

2.3. Optimization of the Preservation Method for Bacterial Isolates

The optimization of the preservation method was performed for bacteria growing on an optimized medium. Three techniques were chosen: D—drying in a conventional dryer (Termaks® TS 9053, Bergen, Norway), VD—drying in a vacuum dryer (Memmert® Vacuum Oven VO49, Büchenbach, Germany) and LF—lyophilization (Labconco® FreeZone 2.5, Kansas City, MO, USA) of the bacterial isolates. For the conventional dryer, the temperature was set at a the level of 35 °C for the first 24 h, 42 °C for the second 24 h and 50 °C for the final 24 h and the whole drying process took 72 h. Vacuum drying was performed at 30 °C and a pressure of 50 mbar was applied for 72 h in the presence of $CaCl_2$ as a drying agent. Lyophilization was performed at a pressure of 0.01 mbar and the temperature of the condenser was set at −56 °C. There were three methods used for sample preparation for drying. The first one was to dry the whole growing medium with bacteria, the second was the centrifugation of the growing medium and only drying the bacterial residue after the

removal of the supernatant, and the third involved mixing the whole growing medium and bacteria with the carrier—diatomite. In the centrifugation variant, the bacterial suspension was centrifuged for 10 min at 2880 RCF, then the supernatant was removed and the pellet was subjected to the drying process. In the process of mixing the carrier variant with the medium containing bacteria after culturing, 10% (*w*/*w*) dextrose was added, as Kumar, Mallik and Sarkar (2017) showed that dextrose can increase the survivability of bacteria during the drying process.

Furthermore, the addition of trehalose up to a concentration of 0.1 M in the growing medium and the drying process were evaluated in a similar way to dextrose to increase bacterial survivability [15,16]. After culturing the medium was mixed with diatomite CELATOM FW-14 (EP Minerals, Reno, NV, USA) in a 2:1 (*w*/*w*) ratio. A later preparation was subjected to drying in the presented conditions. After the drying process was complete, 1 g of the preparation with bacteria was suspended in water and shaken for at least 20 min, and then diluted with water using the serial dilution method. From the chosen dilutions (10^{-6}, 10^{-7}, 10^{-8}), 100 µL was spread on the PDA plate using a sterile cell spreader [14]. The plates were then cultivated for 48 h at 30 °C. Afterwards, the colonies were counted and the composition of the optimized medium was evaluated. Each bacterium was dried in a separate container and each dryer was sterilized via heating up to 200 °C for 2 h between batches.

2.4. Prebiotic Supplementary Blend

The enhancement of bacterial growth of the 4 tested isolates by the supplementary blend consisting of D-malic acid, N-acetyl-D-glucosamine, α-keto-glutaric acid, and γ-aminobutyric acid (in an equal weight ratio, in total 10 g/L) was evaluated during 144 h of culturing in liquid culture. The medium used in the experiment was an M9 Minimal Salts medium (Merck, Darmstadt, Germany) with the 10 mL/L addition of micronutrient solution ($MnCl_2$*$4H_2O$ (100 mg/L), $ZnCl_2$ (150 mg/L), $CuCl_2$*$2H_2O$ (50 mg/L), $CoCl_2$*$6H_2O$ (50 mg/L), Na_2MoO_4*$2H_2O$ (50 mg/L)). Culturing took place under aerobic conditions, at pH 7.2, at 28 °C in 50 mL flasks, 15 mL of medium, 105 rpm rotation, and 50 µL of 10^6 bacterial cells per flask of inoculum. Control was provided for each isolate culture, which constituted the medium and culture conditions but without the addition of supplements. The cultures were performed in triplicate (n = 3). Every 24 h, 200 µL from each flask was placed in a 96-well plate and the optical density of the bacterial culture at a wavelength of 600 nm (OD600nm) was measured using a spectrophotometric microplate reader, Infinite M200PRO (Tecan®, Männedorf, Switzerland). If flocculation appeared and its severity might have had an influence on the measurement, the samples were lightly homogenized or mixed via pipetting to destroy the aggregates.

In detail, the supplements were as follows: D-malic acid (Nanga®, Złotów, Poland), N-acetyl-D-glucosamine (Glentham Life Sciences®, Corsham, UK), α-keto-glutaric acid (MyProtein®, Manchester, UK), and γ-aminobutyric acid (Ostrovit®, Zambrów, Polska). These supplements were chosen according to previous research by [5] concerning certain metabolic characteristics and features of selected bacterial isolates using GEN III plates (Biolog®, Hayward, CA, USA).

2.5. Phytotron Raspberry Pot Experiment

Seedlings of raspberry (variety *Polana*) were planted in pots, the diameter of which was 19 cm. Each pot contained 3 kg of soil collected from the top layer at the organic raspberry field, carefully sieved through a 5 mm sieve beforehand to remove stones and roots. According to District Chemical and Agricultural Station in Lublin (Lublin, Poland), the soil was classified as dense. The soil was not sterilized to ensure that the conditions were close to natural. One raspberry seedling was planted in one pot, with its roots and shoots trimmed to the same size as the others, which is a standard procedure used by farmers when establishing new raspberry plantations. After planting the surface of the soil was thoroughly covered with a blend of vermiculite and perlite (mixed 1:1) to prevent water loss from the soil due to evaporation. Soil moisture was measured using a method of

TDR (Time Domain Reflectometry) sensor FOM/mts (ETest, Poland) and kept constant at 18–20% throughout the experiment. Water was poured into the stands (diameter 23 cm) according to the TDR measurement method. The temperature in the phytotron was kept constant at 20–22 °C. The constant photoperiod of the day and night system was set at 16 h for the day and 8 h for the night. The duration of the pot experiment was two months (January and February 2020). The pot experiment was set up in triplicate (three pots per variant).

The following five variants of contamination with pathogens (pathosystems) were used: *Botrytis cinerea* G277/18 (B), *Verticillium* sp. G296/18 (V), *Colletotrichum acutatum* G172/18 (C), and *Phytophthora* sp. G408/18 (P), the variants of all of the above-mentioned tested pathogens, and controls without the addition of pathogen, but with the addition of water instead.

Pathogen inoculation was as follows: 10 mL of a freshly prepared sterile water suspension of the respective pathogens containing 10^8 conidia per ml was applied per each pot in the case of a single pathogen variant (B, V, C, P). For all of the tested pathogen variants, the suspension consisted of 2.5 mL of 10^8/mL conidia of each pathogen applied together. Pathogen inoculum was applied twice during the pot experiment. The first inoculation was performed during raspberry planting (pipetted into the soil near the roots) and the second one month after raspberry planting. In this case, the *Phytophthora* sp. and *Verticillium* sp. suspension were introduced to the soil near the roots, while *Botrytis cinerea* and *Colletotrichum acutatum* were inoculated on the plant using sterile inoculation sticks.

For every variant of pathogen contamination, four methods of naturalization strategy, including the application of beneficial bacterial strains selected from natural habitats of wild raspberries, were tested. These options correspond to situations in which biopreparations might be used in the future. The first (R) was to apply the bacterial consortium on the roots of plants during planting in pots—this method might be used when setting up a new raspberry plantation. The second was to apply the bacteria both during planting and one month after naturalization watering with the bacterial consortium (RW), which reflects newly set up plantations with both methods being used. The third one (W) corresponds to already existing plantations where it is only possible to apply microorganisms to all plants during watering. The last one was planted without any naturalization to serve as a control. Table A1 presents the experimental design of the pot experiment.

As with the pathogen inoculum, the bacterial consortium was applied in a 10 mL total volume of bacterial suspension (2.5 mL of each bacterial strain) with an abundance of 10^8/mL into the soil regardless of the naturalization variant (R, RW, W). Bacterial abundance in the inoculum was determined using the Thoma cell counting chamber and verified using serial dilution, spreading 100 µL on PCA plates and culturing for 48 h at 30 °C. After that time, colonies were counted and based on their number the number of bacteria in the inoculum was confirmed.

To obtain the appropriate number of bacteria for the pot experiment, the tested isolates were cultured on an optimized medium for 4 days. Then, the required concentration was provided through serial dilution, based on Thom's cell counting chamber results (BLAUBRAND®, Wertheim, Germany).

After the experiment, soil, root and plant samples were collected, measured and stored for future analysis. The soil samples to be used for soil analyses were stored at 4 °C. Root and shoot samples were weighed and dried, and then stored at room temperature.

2.6. Plant and Soil Analyses

The physicochemical analyses of plant and soil material from the pot experiment were performed by the District Chemical and Agricultural Station in Lublin (Lublin, Poland), according to standard procedures used in the Station described below.

Plant material (shoots) testing was performed to determine foliar feeding (N, P, K, Ca, Mg). In the soil, there was a determination of the N mineral content, the pH, P_2O_5, K_2O, Mg, the organic carbon and the soil organic matter contents.

The determination of P in plant material after mineralization in H_2SO_4 and H_2O_2 was performed using the vanadomolibdate method (KQ/PB-24) [17]. The method is based on the spectrophotometric measurement of the intensity of the yellow colour of the phosphorus–vanadium–molybdic acid complex, which is formed by orthophosphate and vanadium ions in the presence of molybdate in an acidic environment through the use of a colorimeter.

The determination of N in plant material was performed through the use of the distillation method after mineralization in H_2SO_4 and H_2O_2. Determination of nitrogen content consists of converting the amide form of nitrogen into ammonia through mineralization in concentrated sulfuric acid (VI), distilling ammonia from the alkaline medium, absorption in a known volume of a standard sulfuric acid solution, and the titration of excess acid with a standard sodium hydroxide solution (KQ/PB-70) using the Kjeldahl method with Buchi B-324 distillation apparatus and a digital burette.

The amount of Mg content in raspberry shoots was determined after mineralization in H_2SO_4 and H_2O_2 (KQJPB-26). The principle of the method consists of measuring the absorption of radiation by magnesium atoms released when the test solution is sprayed into an acetylene–air flame using a Perkin ELMER atomic absorption spectrometer.

The determination of K and Ca content in shoots was determined after mineralization in H_2SO_4 and H_2O_2 (KQ/PB-25). It involved the measurement of radiation emitted by a suitably excited sample. Excitation source—burner; flame—propane–butane air. The equipment used was a Jenway flame photometer.

The determination of nitrate and ammonium content in the soil (N-NO_3, N-NH_4) was performed using a colorimetric method which involved the use of the SKALAR SCAN ++ SYSTEM flow autoanalyzer after extraction in 1% K_2SO_4 (KQ/PB-71). Nitrate was determined using the nitrogen-reduction method with the use of cadmium. As for ammoniacal nitrogen, the modified Berthelot reaction was used.

The organic carbon content was determined using Tiurin's method with a heating plate and a Titronic R 300 digital burette being used. The soil organic matter (SOM) was evaluated through the use of a weight method using a laboratory balance, SLW 115 dryer and a Nabarthetm muffle furnace [17].

The pH in KCl was determined (PN-ISO 10390:1997) using pH-meter CP-505; the phosphorus (P_2O_5) and potassium content (K_2O) were evaluated by following the Egner–Riehm method (PN-R-04022:1996 + Az1:2002), using a Sherwood flame photometer and Genesys 6 spectrophotometer; and the magnesium content was detected through the use of the Schachtschabel method (PN-R-04020:1994 + Az1:2004), using an AAS-3 atomic absorption spectrometer.

At the end of the study, the above-ground part of the plants was cut off, and the dry weight (g), as well as height (cm) of the plants and the number of branches, were determined. Soil adhered to the roots was removed prior to weighing, shaking and washing in distilled water. In addition, the fresh weight (g) of the roots of the plants was measured. Both measurements were carried out using a laboratory balance (RADWAG, WLC 0.6/A1/C/2). All samples were stored at 4 °C before analysis for about 1 week.

For the determination of the enzymatic activity of fresh soil samples with natural moisture, samples were taken and stored at 4 °C before analysis. In the soil samples, the activity of dehydrogenase (EC 1.1.1.1) was determined using the Thalmann method [18] and modified by Alef [19] in three repetitions. Formazan equivalents released in the reaction were converted to SI units presented as cm^3 H_2. All samples were analysed on the same day.

2.7. Statistical Analysis

The colony count in the growing medium and the drying method optimization mean value were calculated based on the repetitions. For the analysis of supplementary blend influence, graphs were generated based on the mean value calculated from three replicates. For the early effect of naturalization strategies on plant growth, the mean value and

standard deviation of dehydrogenase activity in the soil were calculated and a two-way ANOVA with $p < 0.05$ was performed to check differences, and Tukey tests were also performed to reveal statistically significant differences between naturalization strategies. For the analysis of the effect of the prebiotic supplement on the growth of bacteria the mean value and standard deviation were calculated and a one-way ANOVA with $p < 0.05$, $p < 0.01$, and $p < 0.001$ was performed to check differences, and Tukey tests were also performed to reveal its statistical significance.

3. Results

3.1. Optimization of the Growing Medium

Optimizing the growing medium for bacterial growth focused on four parameters: carbon source, nitrogen source, culturing temperature and the pH value of the growing medium. As presented in Table 1, sucrose addition was optimal in supporting bacterial growth. Both glucose and lactose were also utilized by the bacteria. Peptone addition was not crucial, however, as it intensified growth when combined with a 3% concentration of lactose. In further experiments, a 3% addition of sucrose without peptone was chosen. The variant with bacteria on the control M9 medium was also considered to compare the growth rate on a classic store-bought medium to the growth achieved on a medium based on supernatant from diatomite used for beer filtration. The number of bacteria growing on the M9 medium was one order of magnitude lower for *Arthrobacter* sp. B58/18, and similar for the other tested microorganisms when compared to the 3% sucrose variant.

Table 1. The effect of different carbon sources (glucose, lactose, sucrose), their concentrations 3%, 6%, 9% and the addition of peptone on the growth of bacteria in liquid culture. The control was prepared using the M9 minimal medium containing KH_2PO_4 15 g/L, NaCl, 2.5 g/L, Na_2HPO_4, 33.9 g/L, NH_4Cl, 5 g/L. *Rhodococcus* sp. B12/18, *Pseudomonas* sp. B37/18, *Arthrobacter* sp. B58/18, and *Rhodococcus* sp. B75/18, pH = 7.2, culturing temperature = 30 °C.

Carbon Source		Peptone Concentration		Colony-Forming Units (CFU/mL)			
Sugar	**Concentration**	**Additive**	**Concentration**	**B12/18**	**B37/18**	**B58/18**	**B75/18**
Glucose	3%	Peptone	0%	2.45×10^{11}	1.50×10^{12}	6.15×10^{10}	5.46×10^{11}
	3%		0.20%	$<10^8$	1.37×10^{12}	1.80×10^{11}	1.50×10^{12}
	6%	Peptone	0%	1.00×10^9	1.50×10^{12}	3.00×10^9	1.25×10^{10}
	6%		0.20%	6.80×10^9	4.45×10^{10}	5.00×10^8	1.66×10^{11}
	9%	Peptone	0%	$<10^8$	$<10^8$	$<10^8$	$<10^8$
	9%		0.20%	$<10^8$	3.00×10^9	$<10^8$	$<10^8$
Lactose	3%	Peptone	0%	9.10×10^{11}	7.50×10^{11}	$<10^8$	7.96×10^{11}
	3%		0.20%	1.50×10^{12}	1.50×10^{12}	1.00×10^{10}	6.25×10^{10}
	6%	Peptone	0%	7.61×10^{11}	7.50×10^{11}	$<10^8$	$<10^8$
	6%		0.20%	4.00×10^{10}	5.20×10^{10}	$<10^8$	3.50×10^9
	9%	Peptone	0%	$<10^8$	$<10^8$	$<10^8$	$<10^8$
	9%		0.20%	$<10^8$	$<10^8$	$<10^8$	$<10^8$
Sucrose	3%	Peptone	0%	1.50×10^{12}	1.22×10^{12}	1.50×10^{12}	1.28×10^{12}
	3%		0.20%	1.50×10^{12}	1.50×10^{12}	1.50×10^{12}	1.50×10^{12}
	6%	Peptone	0%	1.50×10^{12}	1.50×10^{12}	8.66×10^{11}	1.50×10^{12}
	6%		0.20%	8.50×10^9	5.20×10^{10}	7.00×10^9	2.03×10^{11}
	9%	Peptone	0%	2.24×10^{11}	1.00×10^{10}	1.50×10^{10}	3.29×10^{11}
	9%		0.20%	2.15×10^{11}	6.20×10^{10}	1.30×10^{10}	1.80×10^{11}
Control using M9 medium				1.11×10^{12}	1.50×10^{12}	1.42×10^{11}	1.46×10^{12}

The results presented in Table 2 show that out of all of the tested nitrogen compounds, ammonium nitrate provided the best growth conditions for the bacteria *Pseudomonas* sp. B37/18 and *Arthrobacter* sp. B58/18. A 6% addition of this N source caused bacteria to grow to the highest concentration of 5.20×10^9. Isolate *Rhodococcus* sp. B12/18 grew in the narrowest

spectrum of nitrogen compounds and their concentrations, while *Rhodococcus* sp. B75/18 utilized all tested nitrogen compounds in the broadest spectrum of their concentrations. Ammonium nitrate (NH_4NO_3) was utilized by a broad spectrum of bacteria, but only in a higher concentration of 6%. For future experiments upon growth optimization, 6% of NH_4NO_3 was chosen as the best nitrogen source for all tested bacteria.

Table 2. The effect of different nitrogen sources ($NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl) and their concentrations of 1%, 3%, 6%, on the growth of bacteria in a liquid culture. *Rhodococcus* sp. B12/18, *Pseudomonas* sp. B37/18, *Arthrobacter* sp. B58/18, and *Rhodococcus* sp. B75/18, pH = 7.2, culturing temperature = 30 °C.

Nitrogen Source		Colony-Forming Units (CFU/mL)			
Compound	**Concentration**	**B12/18**	**B37/18**	**B58/18**	**B75/18**
$NaNO_3$	1%	2.50×10^9	$<10^8$	6.50×10^9	4.4×10^{11}
	3%	$<10^8$	$<10^8$	$<10^8$	3.1×10^{11}
	6%	$<10^8$	$<10^8$	$<10^8$	$<10^8$
NH_4NO_3	1%	$<10^8$	$<10^8$	1.30×10^{10}	2.05×10^{10}
	3%	$<10^8$	1.10×10^{11}	2.00×10^{10}	2.7×10^{10}
	6%	5.20×10^9	2.23×10^{11}	1.20×10^{10}	2.6×10^{11}
$(NH_4)_2SO_4$	1%	3.00×10^9	1.00×10^9	$<10^8$	$<10^8$
	3%	1.00×10^9	1.50×10^{10}	$<10^8$	$<10^8$
	6%	4.00×10^9	6.00×10^9	1.40×10^{10}	3.5×10^{11}
NH_4Cl	1%	4.26×10^8	4.50×10^9	1.50×10^9	6.47×10^{11}
	3%	$<10^8$	$<10^8$	3.00×10^9	5.26×10^{11}
	6%	6.00×10^9	4.00×10^9	$<10^8$	4.67×10^{11}

As presented in Figure 1, the temperature affected the growth of bacteria. The most optimal temperature for all bacteria was a temperature of 24 °C. The results also indicate that a temperature of 30 °C provided sufficient growth of bacteria, except B58/18 (*Arthrobacter* sp.). From all of the bacteria tested, only isolate B12/18 was able to grow in acidic conditions, when the medium had a pH value of 5.5–6, and isolate B37/18 grew in a medium with a pH value of 6.5. The most optimal pH values of the optimized medium which allowed for the growth of all examined bacteria ranged between 7 and 8.

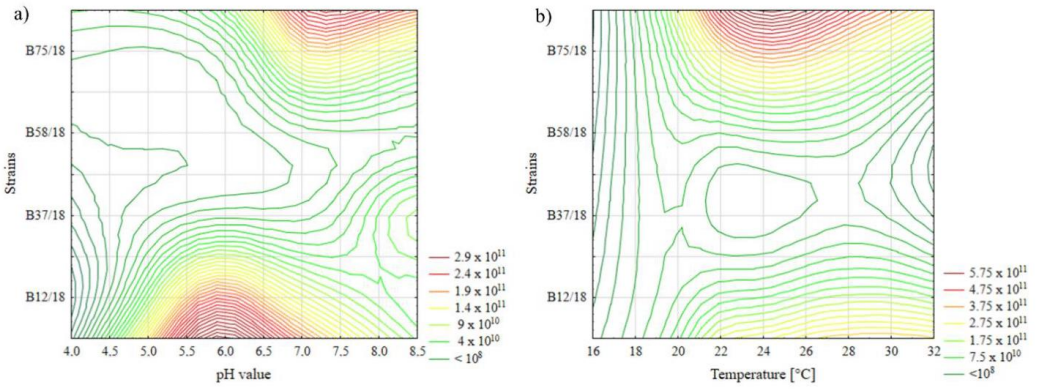

Figure 1. The effect of different medium pH values—(**a**) and temperatures—(**b**) on the growth of bacteria in a liquid culture. *Rhodococcus* sp. B12/18, *Pseudomonas* sp. B37/18, *Arthrobacter* sp. B58/18, *Rhodococcus* sp. B75/18.

3.2. Influence of Bacterial Prebiotic Supplement Addition to the Growing Medium

The enhancement of growth is presented in Figure 2; this was caused by the 1% addition of the supplementary blend and was observed for two out of the four tested bacterial isolates growing in the M9 medium. An increase in optical density measured at 600 nm was observed for *Rhodococcus* sp. B12/18 and *Pseudomonas* sp. B37/18. After 100 h of cultivation, both *Arthrobacter* sp. B58/18 and *Rhodococcus* sp. B75/18 isolates began to flocculate, which resulted in the need to lightly homogenize the cultures to destroy the aggregates and ensure the accuracy of the measured optical density. All cultures were lightly homogenized to ensure a similar testing environment. Isolates B58/18 and B75/18 presented similar growth results with or without the prebiotic supplementary blend.

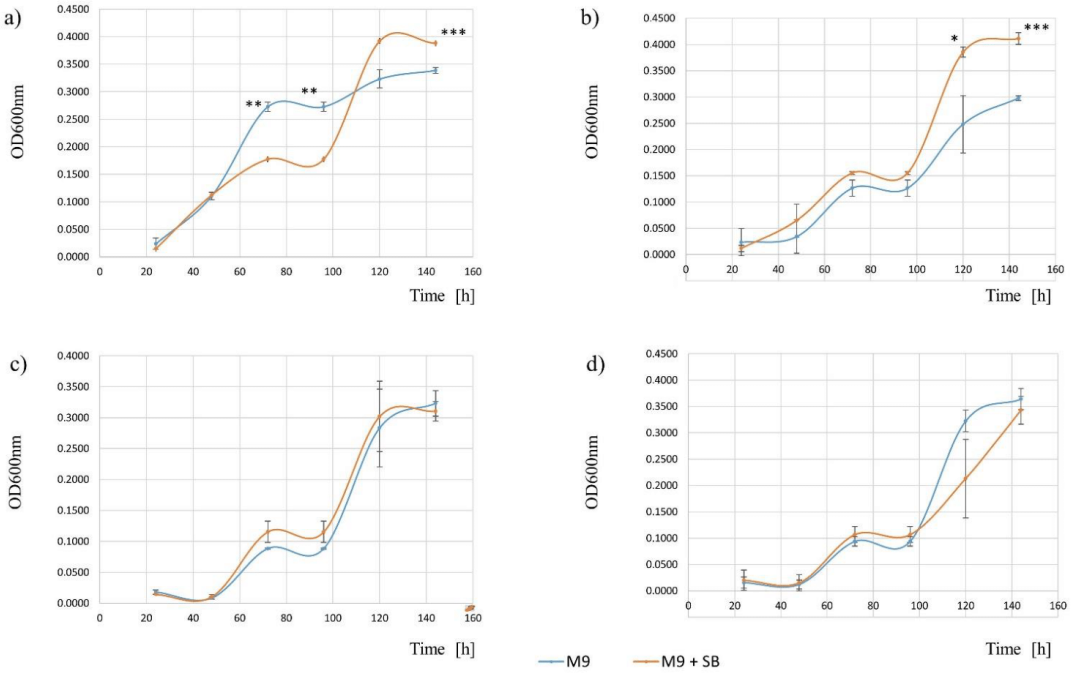

Figure 2. Effect of the 1% supplementary blend addition (D-malic acid, N-acetyl-D-glucosamine, α-keto-glutaric acid, γ-aminobutyric acid) on the growth of bacteria during 144 h of culturing in the M9 medium. Figures represent OD600nm measured during cultivation every 24 h for particular strains of bacteria accordingly: (**a**) *Rhodococcus* sp. B12/18, (**b**) *Pseudomonas* sp. B37/18, (**c**) *Arthrobacter* sp. B58/18, (**d**) *Rhodococcus* sp B75/18; n = 3, error bars represent standard sample deviation. Stars represent the level of significance (*p*-value) of the differences within the particular hour. Three stars—*p*-value < 0.001, two stars—*p*-value < 0.01, one star—*p*-value < 0.05.

3.3. Optimization of Preservation Technique

In conventional drying (D) of the bacterial pellet, for the medium with bacteria and the medium with bacteria absorbed on diatomite the number of bacteria after drying ranged from 10^6–10^8, through to 10^6–10^8 to 10^6–10^{10}, respectively. In vacuum drying (VD) of the bacterial pellet, for the medium with bacteria and the medium with bacteria absorbed on diatomite the number of bacteria after drying ranged from 10^6–10^7, through to 10^6–10^8 to 10^6–10^9, respectively. In the lyophilization (LF) of the bacterial pellet, in the medium with bacteria and in the medium with bacteria absorbed on diatomite the number of bacteria after drying ranged from 10^6 to 10^8 depending on the sample preparation strategy.

All drying techniques, including D—drying, VD—vacuum drying, and LF—lyophilization, and sample preparation had a significant influence on the survivability of the bacteria. The controls included fresh culture and bacterial pellet after centrifugation at the speed of 2800 rcf for 10 min to examine the influence of spinning on several viable bacteria in the sample. As presented in Table 3, the number of viable cells after centrifugation was reduced by up to two orders of magnitude, as in the case of *Rhodococcus* sp. B12/18 or by 5.6 times as for *Rhodococcus* sp. B75/18. Lyophilization resulted in the lowest decrease in bacterial number in the samples without the differentiation in sample type. Lyophilization of the bacterial pellet resulted in the survival of all tested isolates in a number higher than 10^6. The vacuum drying of the medium with bacteria absorbed on diatomite resulted in the survival of most strains in a number higher than 10^8 except for *Rhodococcus* sp. B12/18. This type of drying was chosen as the most effective and least costly in terms of energy compared to the other preservation methods, based on the results achieved. Even though lyophilization of the bacterial pellet resulted in improved survivability compared with vacuum drying, its energy cost and difficulty to adapt in commercial processes, when compared to vacuum drying, resulted in the resignation of this method for future research. Further preparation of biopreparation was achieved with the use of vacuum drying.

Table 3. The effect of different drying methods and sample preparations on the survivability of bacteria used in naturalization biopreparation. *Rhodococcus* sp. B12/18, *Pseudomonas* sp. B37/18, *Arthrobacter* sp. B58/18, *Rhodococcus* sp. B75/18, D—drying, VD—vacuum drying, LF—lyophilization.

Drying Technique and Sample Type		Colony-Forming Units (CFU/g)			
		B12/18	**B37/18**	**B58/18**	**B75/18**
Control	Fresh culture	7.50×10^{10}	6.00×10^{8}	4.10×10^{9}	8.50×10^{9}
	Bacterial pellet	1.00×10^{8}	2.00×10^{7}	2.00×10^{8}	1.50×10^{9}
D	Bacterial pellet	$<10^6$	$<10^6$	1.00×10^{6}	1.00×10^{8}
	Medium with bacteria	$<10^6$	$<10^6$	$<10^6$	1.00×10^{8}
	Medium with bacteria absorbed on diatomite	4.00×10^{10}	1.00×10^{6}	9.90×10^{8}	$<10^6$
VD	Bacterial pellet	$<10^6$	$<10^6$	1.00×10^{7}	2.00×10^{7}
	Medium with bacteria	2.00×10^{8}	$<10^6$	$<10^6$	$<10^6$
	Medium with bacteria absorbed on diatomite	$<10^6$	5.30×10^{9}	7.00×10^{8}	4.70×10^{9}
LF	Bacterial pellet	7.00×10^{6}	1.00×10^{7}	2.00×10^{6}	2.00×10^{8}
	Medium with bacteria	3.00×10^{8}	$<10^6$	1.00×10^{7}	1.00×10^{8}
	Medium with bacteria absorbed on diatomite	7.00×10^{8}	$<10^6$	1.00×10^{6}	1.00×10^{8}

The effect of the addition of trehalose to the culturing of bacteria subject to conventional drying (D) is presented in Table 4. The results show an increase in the units (CFU) of *Arthrobacter* sp. B58/18 after drying by one order of magnitude from 10^8 to 10^9. The addition of trehalose to the culturing of bacteria subjected to vacuum drying (VD) resulted in an increase in CFU for *Rhodococcus* sp. B75/18 after drying by one order of magnitude, from 10^9 to 10^{10}. The addition of trehalose both for the culturing and drying of bacteria subjected to conventional drying (D) resulted in a survival rate that ranged from 10^7 to 10^8 for *Rhodococcus* sp. B12/18 and *Arthrobacter* sp. B58/18, respectively. The addition of trehalose to the culturing and drying of bacteria subjected to vacuum drying (VD) resulted in a survival rate that ranged from 10^8 to 10^9 for *Pseudomonas* sp. B37/18 and *Rhodococcus* sp. B75/18, respectively. Comparing the CFU after the conventional drying (D) of mixed batches combining *Rhodococcus* sp. B12/18 and *Arthrobacter* sp. B58/18 with the trehalose added only during culturing and for both culturing and drying, it may be noted that the results were 1.50×10^9 and 1.67×10^8, respectively. However, from the CFU after the vacuum drying (VD) of mixed batches that combined *Pseudomonas* sp. B37/18 and *Rhodococcus* sp. B75/18 with the trehalose added only

during culturing and during both culturing and drying, it may be noted that the results were 3.97×10^9 and 8.87×10^9, respectively.

Table 4. The effect of trehalose addition and the chosen drying methods on bacterial survivability in drying (D) and vacuum drying (VD) processes used in manufacturing and in the naturalization biopreparation. *Rhodococcus* sp. B12/18, *Pseudomonas* sp. B37/18, *Arthrobacter* sp. B58/18, *Rhodococcus* sp. B75/18, D—drying, VD—vacuum drying.

Bacterial Isolate	Trehalose Addition		Drying Technique	Colony-Forming Units (CFU/g)
	To the Culture	To Drying		
B12/18	0.1 M	-	D	$<10^7$
B58/18	0.1 M	-	D	1.20×10^9
B37/18	0.1 M	-	VD	2.00×10^8
B75/18	0.1 M	-	VD	1.96×10^{10}
B12/18	0.1 M	0.1 M	D	$<10^7$
B58/18	0.1 M	0.1 M	D	1.00×10^8
B37/18	0.1 M	0.1 M	VD	1.00×10^8
B75/18	0.1 M	0.1 M	VD	5.50×10^9
B12/18 + B58/18	0.1 M	-	D	1.50×10^9
B37/18 + B75/18	0.1 M	-	VD	3.97×10^9
B12/18 + B58/18	0.1 M	0.1 M	D	1.67×10^8
B37/18 + B75/18	0.1 M	0.1 M	VD	8.87×10^9

The addition of trehalose during culturing increased the survivability of *Rhodococcus* sp. B75/18 in vacuum drying and *Arthrobacter* sp. B58/18 during conventional drying.

The addition of trehalose during culturing and after culturing did not produce as much of a significant result as the addition which occurred only during culturing. It is worth mentioning that drying bacteria in mixed batches did not result in a reduction in survivability.

3.4. The Early Effect of the Bacterial Consortium on Raspberry Plants and on Soil Properties

The influence of bacterial isolates on raspberry plants was evaluated based on four parameters: dry mass of the shoots which allows for a comparison to be made with regard to plants size, the wet mass of the roots which may correlate with microorganisms influencing the plant rhizosphere, overall soil dehydrogenase activity which is correlated with the influence of the biopreparations on the soil microbial community, and the effect of naturalization strategies on soil micro- and macronutrients content.

3.4.1. The Early Effect of the Bacterial Consortium on Plant Mass

As presented in Figure 3, the early effect of the bacterial consortium used on raspberry plants depended on the mass of shoots and differed depending on the variant used, but the bio-stimulating effect of the developed biopreparation was observed in plants that were inoculated with pathogens (Figure A1). In general, the mass of shoots was higher in raspberry plants treated with biopreparation. In the variant with the *Botrytis cinerea* pathosystem, the highest mass of shoots was measured for watering with the addition of a biopreparation, which suggests that this method of biopreparation application might be the most appropriate choice against *B. cinerea* infection. For the *Verticillium* sp. inoculation variant, the highest mass was observed for the plants that were treated with biopreparation during planting (Figure 3).

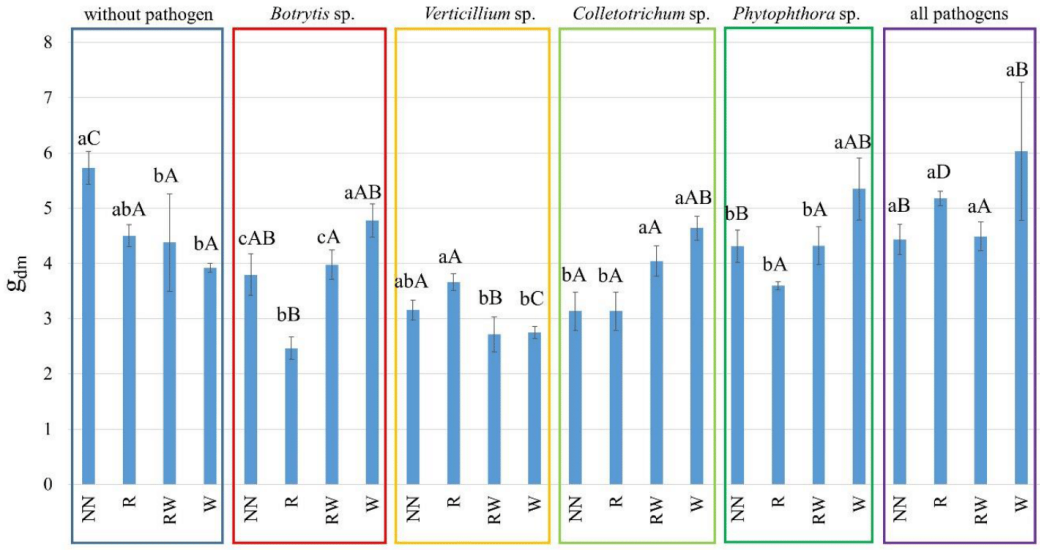

Figure 3. The early effect of the bacterial consortium on *Polana* raspberry plant growth in the pot experiment depends on the pathosystem and naturalization strategy applied. The dry biomass of shoots varies, according to pathosystems *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp., and all pathogens (*Botrytis*, *Verticillium*, *Colletotrichum*, *Phytophthora*) used and also without pathogens, within different biopreparation application strategies: no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W). The error bars indicate a standard deviation, $n = 3$, different lowercase letters above the bars indicate differences within the particular pathosystems, and different capital letters above the bars indicate differences within the particular naturalization strategies (according to ANOVA with $p < 0.05$).

Other tested naturalization strategy variants were less successful in inhibiting the negative effect of pathogens on plants. For inhibiting the influence of *Colletotrichum acutatum* on raspberries, the most effective method was watering with the addition of biopreparation, or this method combined with root naturalization during planting. Only root naturalization had no effect on the plants. Again, for plants inoculated with *Phytophthora* sp., the most effective method was watering the plants with the addition of biopreparation. Root inoculation during planting without additional treatment was not effective enough. However, the plants that were treated with all four pathogens had the highest dry mass of shoots when watered with the biopreparation. Root naturalization during planting was more effective than no biopreparation or watering naturalization combined with root naturalization.

Appendices C and D Figure A3 present the changes in the number of shoots and the average length of plants for different pathosystems.

3.4.2. The Early Effect of the Bacterial Consortium Used on Root Mass

Figure 4 shows that the roots collected from plants that were not inoculated with any pathogens were the heaviest for variants without biopreparation application. Application during planting and application during watering combined with further application with watering was less effective than watering application. Inoculation of the plants with *Botrytis cinerea* caused a decrease in the mass of the roots compared to the variants without the pathogen. The application of bacterial biopreparation resulted in an increasing mass of roots in the plants that were treated during planting as compared to the plants without any bacterial inoculum applied. In the *Verticillium* sp. variants, the application of the tested biopreparation during planting or watering caused an increase in root mass in comparison with the application during planting and after that with water. *Colletotrichum acutatum* increased the mass of

the root in objects without any naturalization treatment when compared to the control, although the difference was not statistically significant. The application of biopreparation was not effective in plant biostimulation in pathosystem with *C. acutatum*. In the plants inoculated with *Phytophthora* sp. root growth was reduced both in the variants without naturalization and when all naturalization strategies were applied. In plants that were inoculated with all 4 pathogens and had no naturalization strategy applied the growth of the roots was decreased in comparison with the objects without any naturalization strategy applied. The most effective method in inhibiting the negative influence of phytopathogens was watering with biopreparation and its application during planting along with subsequent watering with biopreparation.

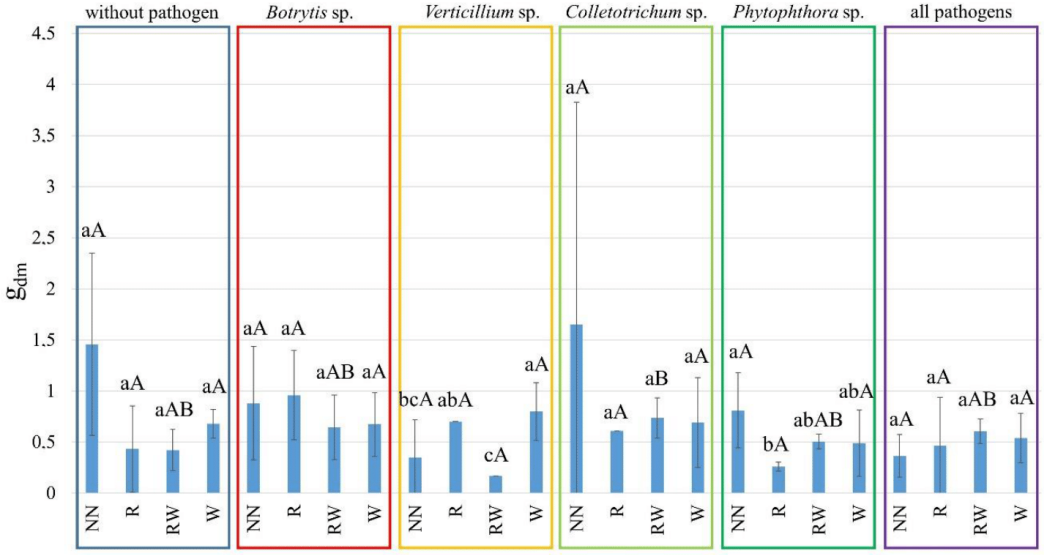

Figure 4. The early effect of the bacterial consortium on *Polana* raspberry plant growth in the pot experiment depended on the pathosystem and naturalization strategy applied. The wet biomass of the roots, according to pathosystems *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp., and all pathogens (*Botrytis*, *Verticillium*, *Colletotrichum*, *Phytophthora*) and without pathogens, within different biopreparation application strategies: no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W). The error bars indicate a standard deviation, $n = 3$, different lowercase letters above the bars indicate differences within the particular pathosystem, and different capital letters above the bars indicate differences within the particular naturalization strategies (according to ANOVA with $p < 0.05$).

3.4.3. The Early Effect of the Bacterial Consortium on Soil Dehydrogenase Activity

The results of the naturalization strategies with regard to dehydrogenase activity are shown in Figure 5. Both the application of the biopreparation and the presence of a pathogen influenced dehydrogenase activity in the soil. For experiments conducted without the presence of pathogens, dehydrogenase activity was highest in the experiments in which both types of naturalization strategies were applied—root naturalization and watering naturalization.

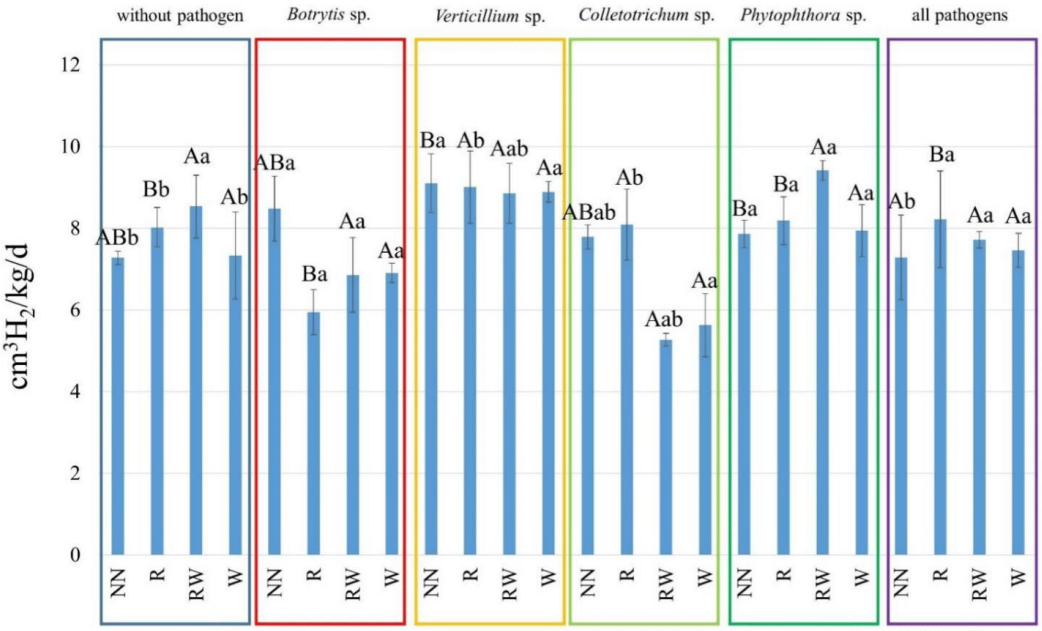

Figure 5. The effect of the bacterial consortium on dehydrogenase activity in the soil samples depending on the pathosystem and naturalization strategy applied. Dehydrogenase activity varied according to the pathosystems used, *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp., and all pathogens (*Botrytis*, *Verticillium*, *Colletotrichum*, *Phytophthora*) and without pathogens, within different biopreparation application strategies: no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W). Error bars indicate the standard deviation, $n = 3$, different lowercase letters above the bars indicate differences within the particular pathosystem, and different capital letters above the bars indicate differences within the particular naturalization strategies (according to ANOVA with $p < 0.05$).

The second highest activity was observed in naturalization strategies with only root inoculation, while watering with biopreparation resulted in no effect on the dehydrogenase level when compared to the strategies without the naturalization applied. For plants inoculated with *Botrytis cinerea*, dehydrogenase activity was higher without any naturalization, and a decrease in the dehydrogenase activity to lower levels than in the control was observed after the application of biopreparation. The highest levels of dehydrogenase activity, despite the overall decrease, were observed when both naturalization strategies were applied including watering naturalization and roots naturalization. In experiments carried out in the presence of *Verticillium* sp., enzymatic activity was comparable to the control regardless of any naturalization strategies, although activities in those objects were the highest among all other trials. In *Colletotrichum acutatum*, the pathosystem activity of the dehydrogenase was the highest while root naturalization was applied; however, it was only a tendency, without significant differences. Furthermore, both watering naturalization and combined naturalization resulted in reducing dehydrogenase activity in this pathosystem. In experiments involving an inoculation with the fungal-like pathogen *Phytophthora* sp., a combined naturalization strategy was most effective in increasing dehydrogenase activity. Other application methods had a similar effect and were comparable with the no-naturalization variant. In experiments that were subjected to inoculation with all four pathogens, dehydrogenase activity was the highest for experiments treated with biopreparation during planting. The second highest result was achieved by the

combined naturalization strategy, and last but not least watering with the addition of the biopreparation.

3.4.4. The Early Effect of the Bacterial Consortium on Soil Macronutrients, Minerals and Nitrogen Content

The amount of macronutrients according to pathosystems *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp. and all pathogens (*Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp.) and without pathogens, within different biopreparation naturalization strategies (no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W)) was evaluated, and the results are presented in Table 5.

Table 5. The effect of the bacterial consortium on the macronutrient amount in the plant, root and in the soil samples depends on the pathosystem and naturalization strategy applied. The amount of macronutrients varied according to the pathosystem used, *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp. and all pathogens (*Botrytis*, *Verticillium*, *Colletotrichum*, *Phytophthora*) and without pathogens, within different biopreparation application strategies: no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W).

Pathogen Contamination	Naturalization	Macronutrients in Shoots					Absorbable Forms of Minerals in the Soil			Nitrogen in Soil		
		N	P	K	Ca	Mg	P_2O_5	K_2O	Mg	$N\text{-}NO_3$	$N\text{-}NH_4$	N_{min}
		% d.m.					mg/100 g Soil			mg/kg d.m.		kg/ha
without pathogen	NN	1.44	0.29	2.47	1.22	0.44	18.3	32.5	14.3	4.41	6.26	45.90
	R	1.44	0.3	2.63	1.38	0.48	17.6	31.8	12.9	4.18	6.34	45.20
	RW	1.4	0.27	2.15	1.2	0.48	20.3	33.9	14.5	1.41	7.87	39.90
	W	1.72	0.29	2.46	1.12	0.48	20.5	34.3	15	3.33	7.10	44.80
Botrytis cinerea	NN	1.41	0.28	2.12	1.13	0.46	19.6	35.6	12.6	3.65	7.71	48.80
	R	1.52	0.29	2.65	1.28	0.48	20.2	35.3	12.7	10.7	3.35	60.40
	RW	1.48	0.3	2.29	1.21	0.44	21.1	36.1	13.9	3.11	7.40	45.20
	W	1.34	0.29	2.21	1.22	0.42	20.5	35.4	14.9	2.83	6.61	40.60
Verticilium sp.	NN	1.51	0.29	2.04	1.2	0.42	16.1	31.8	14	2.23	9.37	49.90
	R	1.66	0.3	2.31	1.16	0.4	15.5	29.8	13.5	2.75	8.02	46.30
	RW	1.65	0.29	2.35	1.15	0.32	16.1	31.7	14.6	2.45	8.94	49.00
	W	1.34	0.27	2.05	1.33	0.4	18.1	33.9	14.8	1.34	8.74	37.60
Colletotrichum acutatum	NN	1.18	0.26	2.01	1.33	0.38	17.5	31.8	12.8	2.78	7.45	44.00
	R	1.6	0.27	2.03	1.43	0.44	18	33.1	12	1.36	8.06	40.50
	RW	1.62	0.29	2.17	1.34	0.4	18	33.6	13.7	9.36	3.72	56.20
	W	1.67	0.29	2.63	1.36	0.42	18.4	33.2	13.4	12.1	1.96	60.70
Phytophthora sp.	NN	1.6	0.28	2.56	1.36	0.4	18.5	32.3	14.4	4.40	6.61	47.30
	R	1.47	0.24	2.14	1.3	0.38	19.7	36.7	15	4.33	5.36	41.70
	RW	1.16	0.24	2.04	1.17	0.32	15.5	32.9	13.9	2.84	7.85	46.00
	W	1.61	0.28	2.81	1.39	0.42	17.4	29.2	13.1	3.38	9.83	56.80
all pathogens	NN	1.54	0.27	2.23	1.24	0.39	16.7	30.4	12.9	4.32	9.15	57.90
	R	1.57	0.27	2.21	1.22	0.38	14.9	28.8	13.1	4.11	9.55	58.70
	RW	1.44	0.3	2.19	1.33	0.44	16.3	32.4	13.4	4.59	7.8	53.30
	W	1.34	0.3	2.26	1.2	0.38	18.4	34.7	13.9	2.65	9.39	51.80

The amount of nitrogen in shoots was the highest in the control experiments without any pathogens, but with the biopreparation applied via watering. For all tested pathosystems, the application on roots during planting caused an increase in the level of nitrogen in the shoots as compared to the experiments without the application of naturalization. Differences in the phosphorus level in the dry mass of the plants were negligible. The amount of potassium in the plants differed between all of the samples studied. The highest level was observed in the *Botrytis cinerea* pathosystem when roots naturalization was applied and in the *Phytophthora* sp. pathosystem when watering naturalization was applied. For *Botrytis cinerea* and *Verticillium* sp. variants, root application and combined naturalization were the most effective methods. For *Colletotrichum acutatum* and *Phytophthora* sp., application via watering was the most effective method, and in both cases levels of this element were higher than in the control.

The calcium levels were the highest in *B. cinerea* and *C. acutatum* pathosystems with roots treated during planting, and in *Verticillium* sp. and *Phytophthora* sp. treated during watering. The amount of magnesium in plants was the highest in the control experiments and *B. cinerea* variants with treated roots. Levels of this element were low in all other pathosystems except for *C. acutatum* experiments treated during planting and experiments with both naturalization strategies applied in pathosystem composed of all four pathogens.

In the control experiments, without the presence of pathogens, the highest amount of absorbable forms of minerals in soil were measured under plants that had been treated using a combined naturalization strategy of watering and the root application of biopreparation, and under plants that were watered with biopreparation.

The number of absorbable forms of minerals in the soil in experiments involving an exposure to pathogens was highest in the *B. cinerea* pathosystem, and in this case, the most effective method was watering with biopreparation. A high amount of minerals in the soil was observed in experiments involving an exposure to *Phytophthora* sp. with roots treated during planting.

The concentration of nitrogen in the soil was evaluated in terms of its three most important forms: nitrate-nitrogen (N-NO_3), ammoniacal nitrogen (N-NH_4) and mineral nitrogen (N_{min}). The highest levels of the nitrate form of nitrogen were found in soil samples collected in experiments involving an exposure to *Botrytis* sp. and naturalized with biopreparation during planting. High levels of this type of nitrogen were found in *Phytophthora* sp. variants treated with both root naturalization and naturalization watering and variants only treated with naturalization watering. Ammoniacal nitrogen was found in the soil at its highest concentration in samples collected from experiments involving an exposure to *Verticillium* sp., *Phytophthora* sp. and all four pathogens treated with both naturalization strategies, watering and roots naturalization, respectively. Mineral nitrogen content was the highest in soil collected from experiments involving an exposure to *Botrytis cinerea* involving the application of root treatment. *Colletotrichum acutatum* and *Phytophthora* sp. pathosystems also contained high levels of soil mineral nitrogen. Experiments involved combined naturalization strategies and watering naturalization for *Colletotrichum acutatum* and a naturalization for *Phytophthora* sp., respectively. In the pathosystem including all tested fungal and fungal-like plant pathogens, mineral nitrogen levels were high for variants with roots naturalization, and lower for variants that included watering with a biopreparation, such as the combined and watering naturalization strategies.

4. Discussion

4.1. Optimization of Growing Medium

The optimization of the growing medium is an essential part of designing any microbial biopreparation to be used in agriculture. This type of process can be applied to reduce the cost of microbial culturing and increase the number of viable cells in the finished biopreparation. This may result in decreasing the costs of the final product and the energy that must be used during the manufacturing processes [20–22]. Additionally, it is important to mention that changes in the growing medium can influence bacterial antagonistic properties against fungal plant pathogens [23]. All of the tested isolates of bacteria were able to utilize all three tested sugars as carbon sources, but sucrose was the only sugar that in the lowest examined concentration of 3% allowed all four isolates to reach numbers as high as 10^{12} in 48 h of culturing. Peptone addition did not provide any notable advantage which might suggest that the supernatant from diatomite used for beer filtering might contain a source of substances similar to those in peptone, such as an organic nitrogen source, peptides and polypeptides, vitamins and amino acids [24]. Comparing the obtained results of bacterial growth on the proposed medium with a 3% sucrose content to the commercially available M9 medium, it may be noted that growth on both media is similar with one order of magnitude higher on the tested medium as compared to the M9 medium for *Arthrobacter* sp. B58/18.

Ammonium nitrate has been previously proven to be efficient as a carbon source in bacterial growth similar, to other tested mineral nitrogen sources [25–27]. It is a cheap and efficient nitrogen source not only for bacteria but also for plants. It may be assumed that residues of ammonium nitrate in biopreparation can be utilized by bacteria and plants after the application of the biopreparation. It might be critical to evaluate the temperature and pH value of the culturing medium while optimizing the growing medium, especially for environmental isolates that have not been cultured and fully understood previously. The optimal soil pH value for most plants and plant-associated bacteria is slightly acidic or neutral. As we have proven for these environmental isolates, the optimal pH value of the growing medium is in the range between 7 and 8.

Carefully chosen medium additives have the potential to influence bacterial growth in a very effective way. In previous research we chose four chemical compounds based on their efficient utilization by bacteria [13]. D-malic acid, N-acetyl-D-glucosamine, α-keto-glutaric acid, and γ-amino-butyric acid were added to the medium in a 1% m/v total concentration. D-malic acid may be a stimulant for soil bacteria since some plants secrete this compound to the soil near their roots to possibly attract plant-growth-promoting bacteria [28]. N-acetyl-D-glucosamine takes part in cell signaling and in the construction of the cell wall [29,30]. The α-ketoglutaric acid molecule is an antioxidant; this organic acid is also a part of the Krebs cycle and its addition may stimulate bacterial growth [31,32]. The γ-amino-butyric acid molecule is an amino acid that can be used as a carbon or nitrogen source by some bacterial isolates, e.g., *Pseudomonas* sp. [33,34]. Although supplement addition was only 1% of the total medium mass, it had a positive impact on the growth of two isolates—*Rhodococcus* sp. B12/18 and *Pseudomonas* sp. B37/18. Two other isolates were not affected by the addition of supplements when compared to the control.

4.2. The Preservation of Bacteria

The preservation of bacterial cells to ensure their viability after long-term storage is not an easy task. What is more important is that the isolates used in this study were non-spore-forming Gram-negative bacteria. This makes their preservation much more difficult, and thus it is important to optimize this process correctly, ensuring a broad spectrum of methods, and at the same time it must be borne in mind that this process needs to be scalable to industrial processes. Chosen methods such as conventional drying, vacuum drying and lyophilization are typically used in the preservation of different strains of bacteria. Survivability depends on the type of bacteria and process parameters [15,35–37]. This research proved that environmental isolates of non-spore-forming bacteria can be preserved using many methods and sample preparation techniques. It has been proven that spinning bacterial cultures affected the number of viable cells in the medium. Another study indicated that the longer the storage time and the higher the RCF, the more bacteria are destroyed [38].

In this research, centrifugation resulted in a decrease in the number of viable cells, from five times up to over two orders of magnitude. Different sample preparation techniques produce many advantages and disadvantages. Preserving only the bacterial pellet results in a decrease in the drying time due to the removal of most of the water with the supernatant, but also the removal of secondary metabolites and substances that might help bacteria deal with the temperature and moisture stress [39]. Drying the whole medium with the bacteria ensures that the bacteria are surrounded by all of its metabolites and any remaining sucrose that might help bacteria to survive the drying process, but the increasing osmotic pressure caused by removing water from the drying medium might negatively affect bacteria during the drying process. The removal of large amounts of liquid takes more time by lengthening the drying process and subjecting bacteria to longer periods of high temperature [40,41]. The sample that consisted of medium absorbed on diatomite dried far more rapidly than the sample without diatomite due to its much larger surface area. The location of the absorbing medium with bacteria into diatomite results in the bacteria being surrounded by its growing medium, but because diatomite is a very porous material it is a great binder

to bacteria. However, diatomite is proven to have a negative ion load that may tightly bind bacteria without their ability to escape after biopreparation application [42]. Another advantage of using diatomite in this type of biopreparation is that it is chemically inert and can store large amounts of water, which, after the application of biopreparation, may help both plants and bacteria by improving soil water retention [43]. A classic sample drying resulted in the Maillard reaction and the process took over 72 h. Even though lyophilization was effective in the preservation of almost every sample type and bacterial isolate, due to its energy cost, sample preparation time (freezing), and the small amounts that can be lyophilized at once, it was decided that this type of drying technique will not be used in future biopreparation production. Vacuum drying was chosen as the best option for those particular isolates and for future application in biopreparation manufacturing due to its easy scalability to industrial processes. Furthermore, drying using vacuum drying resulted in the biopreparation having the form of a light and airy powder with few to no harder granules. This may help with ease of use by farmers and may also result in less of a caking tendency.

Trehalose is known to serve a role as a cryoprotectant during lyophilization and microbiological preparation drying. Its ability to both stabilize biomolecules and decrease the negative effect of drought and high temperatures makes it an ideal additive for the preservation of bacterial cells [44,45]. Research by Crove, Reid and Crowe (1996) suggests that trehalose may be superior to other sugars for preserving biomaterials [15,46]. Research has also shown that the addition of trehalose to the growing medium before culturing may stimulate bacteria to prepare for temperature and drought stress, thereby improving their survivability during preservation and enhancing the production of heat shock proteins [47]. The addition of trehalose to growing medium and preparation before drying had an impact on the survivability of some isolates such as *Rhodococcus* sp. B75/18. Since trehalose may facilitate the drying process, it was included in the final growing medium as one of the components.

4.3. Effect of Biopreparation on the Early Growth of Plants

Scientists agree that the early growth period is a crucial time for plants, which can affect their whole life. It is not only important to ensure that the soil is well prepared but also that it is rich in microorganisms that can enhance the growth of young plants such as raspberry seedlings and protect them from pathogens that are present in the soil [48–50]. Soilborne pathogens can be transported from the nursery to newly set up plantations, and from soil to leaves by soil splash that periodically occurs, e.g., during the rain [51,52]. The early effect of selected strains of bacteria on the growth of raspberry plants varied depending on the phytopathogen contamination variant and naturalization strategy. All experiments involving contamination with pathogens benefitted due to naturalization when compared with the experiments conducted without naturalization, in all pathosystems except *Phytophthora* sp. Additionally, for all pathogens, the highest shoot mass was lower than the control without any pathogens. This leads to the conclusion that the addition of biopreparation to plants infected with pathogens resulted in a higher plant mass compared to experiments without bacterial treatments. Inoculation with pathogens resulted in a decrease in plant mass as compared to the control, which shows that the plants were truly affected by the pathogens. Although the shoot mass suggested that biopreparation may be an important factor in inhibiting the effects of pathogen infection in plants, the mass of the roots decreased in most experiments involving a microbial treatment. All pathogens except *Colletotrichum acutatum*, which along with *Verticillium* sp. was inoculated on shoots, caused a decrease in root mass compared with the control. In plants without pathogen infection, naturalization caused a decrease in plant mass. This is an important observation, and may be connected to the stress of plants due to the colonization of plant tissues by microorganisms. However, the experiment was conducted for 8 weeks, which might be not enough to observe the full naturalization effect. It is known that stress-induced bacterial genes are associated with plant–bacterial interactions and plant-

associated bacteria can coordinate interactions with plants [13]. Therefore, we anticipate that after the first stress, plants will adapt to naturalization conditions and a positive effect will be possible, as was observed in pathosystems combined with naturalization strategies. Moreover, according to the literature [13], in the future there lies great potential in plant growth-promoting microorganisms as inoculants of agricultural soils.

Dehydrogenase activity is often used as an indirect indicator of overall soil microbial activity, because it occurs intracellularly in all living microbial cells and does not accumulate in the soil [53–55]. An increase in enzymatic activity might suggest that the soil microbiota present a high degree of activity and that the soil with the naturalization presents a sufficient medium for microbial life [54,56]. In experiments not involving pathogen infection, dehydrogenase activity was higher than in experiments treated with the naturalization treatment. In all pathosystems except *Botrytis cinerea*, at least one biopreparation application method caused an increase in soil enzymatic activity. Root naturalization and combined naturalization strategy (root treatment and naturalization watering) were the most effective treatment. This suggests that overall biopreparation has a positive effect on soil microbial communities by enhancing the dehydrogenase activity. It is worth noting that even though *Botrytis cinerea* and *Colletotrichum acutatum* were applied to the shoots of raspberries, they negatively affected the soil activity by the highest factor. This might be due to the response of the plant to pathogens and a defensive strategy that might involve secreting chemical compounds affecting microbes in the rhizosphere [57].

The macronutrients content in plant shoots can vary depending on many different factors, such as the stage of growth, the soil microbial community, environmental conditions, and the presence of pests [58–63]. The experiments without pathogen root naturalization resulted in the highest increase in macronutrients in plant shoots. This may be due to an increase in the solubility of compounds near the roots which leads to easier absorption. Root naturalization also proved its effectiveness in the presence of pathogens. It is worth noting that inoculation with *Botrytis cinerea* caused an increase in the absorbable forms of minerals in soil when compared with the control. Combined naturalization strategies and naturalization watering were the most effective for most of the pathosystems. Microorganisms in the soil may contribute to enhancing the solubility of some compounds that might be beneficial for plants [64]. The amount of nitrogen in the soil was not highly dependent on the tested naturalization strategy, although we found that some particular pathogens increased the amount of nitrogen in soil when compared with the control, especially *Verticillium* sp. and all pathogens combined. Some research shows that fungal and fungal-like plant pathogens such as *Phytophthora* sp. may benefit from soil nitrogen imbalance and a higher amount of nitrogen in soil may promote the growth of phytopathogens [65].

5. Conclusions

- Optimization of bacterial media composition and growing conditions leads to improved growth.
- Supplementary prebiotic additives enhance the growth of bacteria even in low concentrations, such as 1%.
- Vacuum drying growing medium absorbed on diatomite is effective in supporting bacterial survivability. The trehalose additive may be an important factor increasing survivability.
- Soil dehydrogenase activity was positively affected by the biopreparation used in control experiments, and in the presence of pathogens dehydrogenase activity varied.
- Different naturalization strategies affected the amounts of nutrients in the soil and shoots differently.
- Naturalization via watering with bacterial inoculum caused the biostimulation of plant growth through an increase in the mass of shoots in pathosystems with pathogen contamination. Therefore, naturalization strategy should be matched to the pathogens occuring at the plantations.

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Data Availability Statement: The data are presented within the manuscript, Appendices A–D, as well as the obtained nucleotide sequences are deposited in the GenBank repository (https://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers: MW255650 (*Rhodococcus* sp. B12/18), MW255651 (*Pseudomonas* sp. B37/18), MW255652 (*Arthrobacter* sp. B58/18), MW255653 (*Rhodococcus* sp. B75/18).

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Abbreviations

PCA	Plate count agar
PDA	Potato dextrose agar
IF-A	Biolog® GEN III Inoculating Fluid
D	Drying
VD	Vacuum drying
LF	Lyophilization
CFU	Colony-forming units
NN	No naturalization
R	Roots naturalization
RW	Roots + watering naturalization
W	Watering naturalization
NCBI	National Centre for Biotechnology Information
RCF	Relative centrifugal force
RPM	Rotations per minute
TDR	Time Domain Reflectometry

Appendix A

Table A1. Design of plant pot experiment including pathosystems, naturalization strategies and repetitions.

	Pathosystem	Naturalization Strategy	Repetitions		
1	without pathogens	no naturalization	a	b	c
2	without pathogens	bacteria roots	a	b	c
5	without pathogens	bacteria roots plus watering	a	b	c
8	without pathogens	bacteria watering	a	b	c
11	Infection *Botrytis cinerea* G277/18	no naturalization	a	b	c
12	Infection *Botrytis cinerea* G277/18	bacteria roots	a	b	c
15	Infection *Botrytis cinerea* G277/18	bacteria roots plus watering	a	b	c
18	Infection *Botrytis cinerea* G277/18	bacteria watering	a	b	c
21	Infection *Verticillium* sp. G296/18	no naturalization	a	b	c
22	Infection *Verticillium* sp. G296/18	bacteria roots	a	b	c
25	Infection *Verticillium* sp. G296/18	bacteria roots plus watering	a	b	c
28	Infection *Verticillium* sp. G296/18	bacteria watering	a	b	c

Table A1. *Cont.*

	Pathosystem	Naturalization Strategy	Repetitions		
31	Infection *Colletotrichum* sp. G172/18	no naturalization	a	b	c
32	Infection *Colletotrichum* sp. G172/18	bacteria roots	a	b	c
35	Infection *Colletotrichum* sp. G172/18	bacteria roots plus watering	a	b	c
38	Infection *Colletotrichum* sp. G172/18	bacteria watering	a	b	c
41	Infection *Phytophthora* sp. G402/18	no naturalization	a	b	c
42	Infection *Phytophthora* sp. G402/18	bacteria roots	a	b	c
45	Infection *Phytophthora* sp. G402/18	bacteria roots plus watering	a	b	c
48	Infection *Phytophthora* sp. G402/18	bacteria watering	a	b	c
51	Infection with all 4 pathogens	no naturalization	a	b	c
52	Infection with all 4 pathogens	bacteria roots	a	b	c
55	Infection with all 4 pathogens	bacteria roots plus watering	a	b	c
58	Infection with all 4 pathogens	bacteria watering	a	b	c

Appendix B

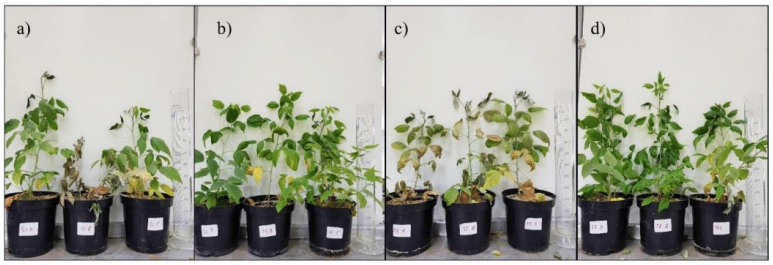

Figure A1. Effect of plant growth stimulation caused by the different naturalization strategies on plants infected with fungal and fungal-like plant pathogens *Botrytis cinerea* (G277/18), *Colletotrichum* sp. (G172/18), *Phytophthora* sp. (G408/18), and *Verticillium* sp. (G296/18): (**a**) no naturalization strategy, (**b**) naturalization of roots, (**c**) roots naturalization and naturalization watering, (**d**) naturalization watering.

Appendix C

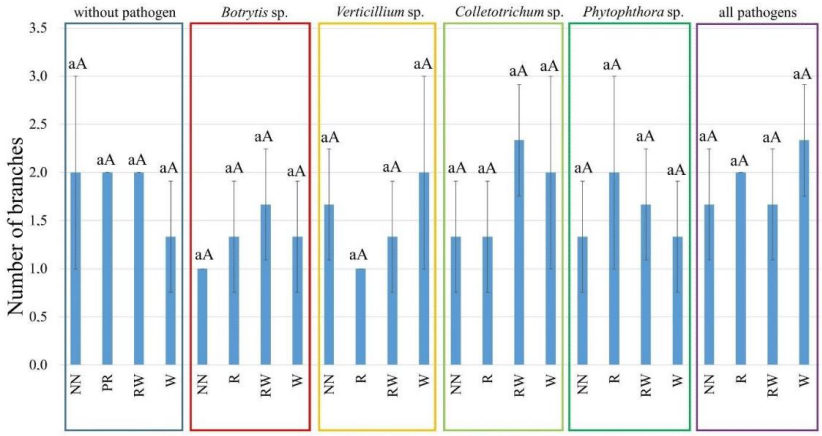

Figure A2. The early effect of the bacterial consortium on *Polana* raspberry plant growth in the pot experiment depended on

the pathosystem and naturalization strategy applied. The average number of stems, according to pathosystems *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp., and all pathogens (*Botrytis*, *Verticillium*, *Colletotrichum*, *Phytophthora*) and without pathogens, within different biopreparation application strategies: no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W). Error bars indicate a standard deviation, $n = 3$, different lowercase letters above the bars indicate differences among the particular pathosystem, and different capital letters above the bars indicate differences among the particular naturalization strategies (according to ANOVA with $p < 0.05$).

Appendix D

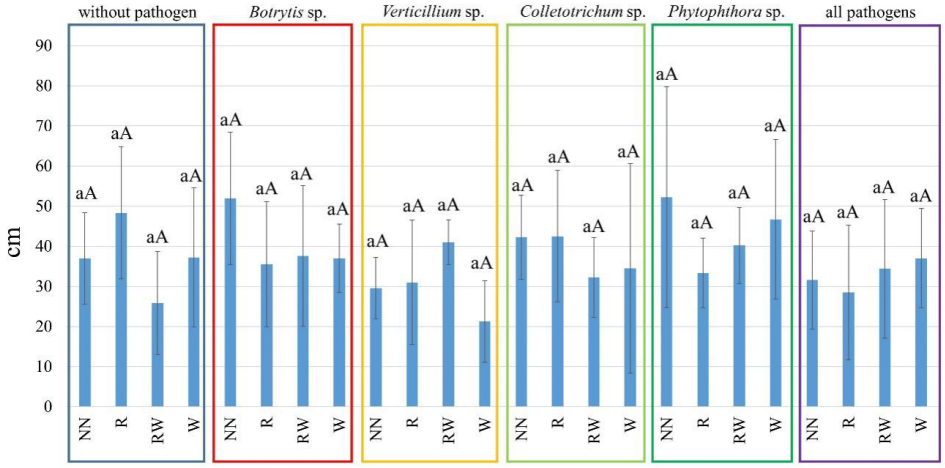

Figure A3. The early effect of the bacterial consortium on *Polana* raspberry plant growth in the pot experiment depended on the pathosystem and naturalization strategy applied. The average length of the stems, according to pathosystems *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp., and all pathogens (*Botrytis*, *Verticillium*, *Colletotrichum*, *Phytophthora*) and without pathogens, within different biopreparation application strategies: no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W). Error bars indicate a standard deviation, $n = 3$, different lowercase letters above the bars indicate differences among the particular pathosystem, and different capital letters above the bars indicate differences among the particular naturalization strategies (according to ANOVA with $p < 0.05$).

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8. Tekst manuskryptu publikacji P.4

Structural and functional shift of soil rhizosphere and raspberry shoots microbiomes underlying changes caused by phytopahogens contamination and naturalization strategies implementation

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Abstract

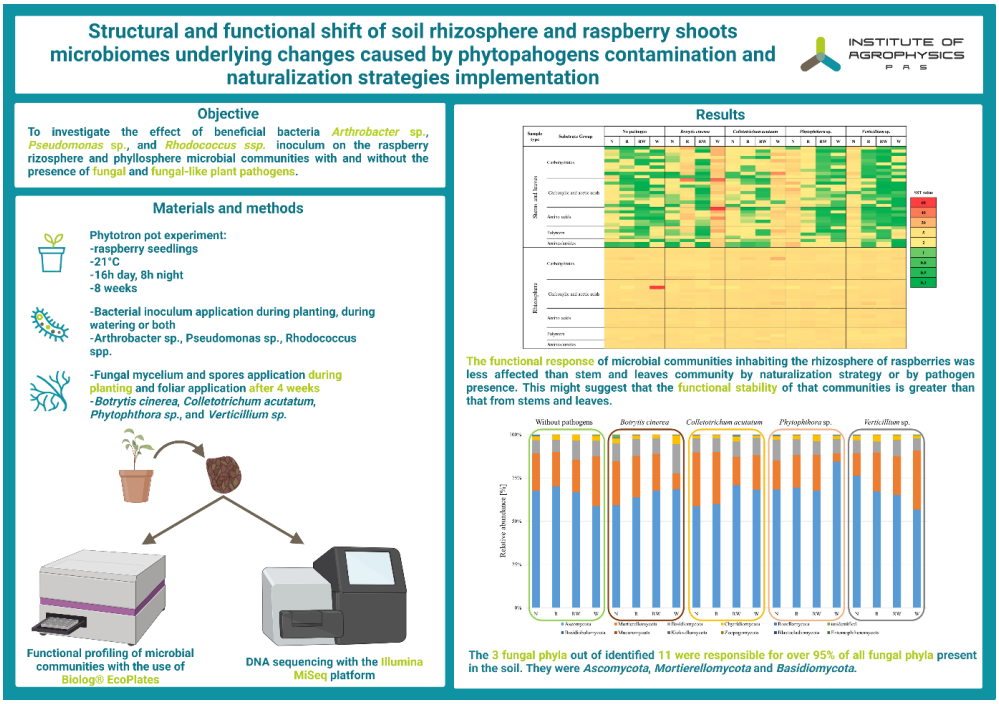
Biopreparations based on the inoculum of beneficial bacteria may become an essential tool in the prevention of fungal and fungal-like phytopathogens causing a decrease in the yield of raspberries, especially in organic farming, in which the use of conventional plant protection products is prohibited. Research is based on the pot experiment including 4 different naturalization strategies and 5 different pathosystems. The application of environmental isolates of plant beneficial bacteria belonging to genera *Arthrobacter*, *Pseudomonas*, and *Rhodococcus* to the raspberry plant was performed to characterize their effect on the microbial communities surrounding the raspberry plant. The application of pathosystems consisting of fungal and fungal-like pathogens belonging to *Botrytis*, *Colletotrichum*, *Phytophthora*, and *Verticillium* genera allowed for analyzing the naturalization treatments effect on microbial communities of raspberry plants infected with phytopathogens. Rhizosphere and shoots samples were subjected to functional profiling analysis of their microbial communities with the use of Biolog® EcoPlates. Futehrmore the DNA was isolated from the rhizosphere and shoots of raspberries and sequenced with the Illumina MiSeq platform. The application of bacterial inoculum

resulted in the decrease of substrate stress index of raspberry shoots microbiota. Over 95% of fungal phyla found in rhizosphere consisted of 3 phyla: *Ascomycota*, *Mortierellomycota*, and *Basidiomycota*. The obtained results indicated, that the application of plant beneficial bacteria to raspberry plants roots during planting and afterward during the watering, may be an important way of inhibiting the negative effect of plant pathogens and enhancing microbial communities' functional abilities.

Keywords: beneficial bacteria, fungal plant pathogens, naturalization, NGS, microbial communities, plants microbiome

Highlights:

- Inoculum application decreased the stress substrate index in shoots microbial communities
- Root microorganisms are resilient to changes in the presence of biopreparations or pathogens
- Majority of soil microbiota are composed of 4 or less phyla
- Trophic mode assessment allows for more comprehensive functional analysis



1. Introduction

In the modern, changing world when the food demand is still growing we often forget that the resources for its production are not limitless. Modern agriculture improves the efficiency of agricultural processes while minimizing the use of resources such as water, space, and energy. Unfortunately, conventional agricultural habits in particular the use of chemical plant protection products that was practiced for over 50 years resulted in a notable decrease in soil biodiversity (Tal, 2018). Therefore, one of the main aim of organic, sustainable and regenerative agriculture is biodiversity increase, especially restore and maintain soil biodiversity, enhancing in this way ecosystem services (EASAC, 2022). To achieve this purpose also organic farming practices are factors that may contribute to the restoration of soil biodiversity. Novel biopreparations in organic farming of soft fruits are a necessity in a world where conventional fungal plant pathogens control strategies are limited. Numerous EU Commission regulations underline how important are proper farming practices in maintaining good soil quality and health. One of the most important parts of those practices is limiting the amount of agrochemicals by farmers and using more natural practices, including biopreparations application. Especially biopreparations based on microorganisms are recommended due to their ability to protect plants against plant pathogens but also enhance the soil microbial community in beneficial strains of bacteria. Properly formulated biopreparations may not only decrease the severity of pest infection in plants but also enhance the soil health, soil microbial quality and stimulate the plants' growth (Pylak et al., 2019; Ważny et al., 2022). Although there are plenty of biopreparations for farmers, there are no biopreparations formulated especially for soft fruits such as raspberries. Moreover, research done by Fikri et. al. (2018) show that beneficial strains of microorganisms isolated locally are much more effective in inhibiting the growth of locally occurring plant pathogens (Fikri et al., 2018). This implies that research on new biopreparations formulations should focus on isolating new, local

beneficial strains of bacteria and formulating new biopreparations targeted against locally occurring fungal plant pathogens. This research strategy ensures that farmers will have a broad spectrum of biopreparations to choose from and will be able to increase their chance of successfully controlling fungal infections in their plantations. Fungal plant diseases are a serious threat to the farming of fruits. Currently when not treated properly fungal plant pathogens belonging to genera *Botrytis*, *Colletotrichum*, *Phytophthora*, or *Verticillium* can cause up to 50% yield loss in soft fruits farming. That yield loss contributes to the higher price of a final product and decreases the availability of organic products for people. Without the use of conventional plant protection products, it is harder for farmers to achieve and maintain proper final product quality (Pylak et al., 2019).

Next-generation sequencing is a way of evaluating relations between microorganisms, as well as plant-microbes interactions. It allows us to distinguish and compare the abundance of soil bacteria and fungi. This type of research tool may be a useful part of the research focused on formulating new biopreparations. It may allow not only help in evaluating the effect of biopreparation on plants but also their effect on soil microbial communities. Microorganisms and their communities take part in various ecological interactions that happen in soil including nutrient mobilization, organic matter decomposition, and gas fluxes (Oszust and Frąc, 2020; Soliman et al., 2017). Differences in the soil microbial communities may contribute to plants' reactions to both different environmental stresses and plant fungal pathogens. Furthermore, introducing new strains of bacteria into the soil can influence the soil's native microbiota, thus it is important to fully understand the possible results of this action (Soliman et al., 2017; Topalović et al., 2020).

In our previous research, we isolated and examined beneficial bacterial strains. The 4 strains belonging to the genera *Arthrobacter, Pseudomonas,* and *Rhodococcus* were chosen as the best antagonists against fungal plant pathogens. Moreover, we examined their ability to

utilize different carbon sources, withstand chemical as well as physical stresses, and produce a spectrum of extracellular enzymes. We have optimized a medium for their growth and drying method for their preservation. Furthermore, we evaluated their effect on the growth of raspberry plants and soil enzymatic activity (Pylak et al., 2021, 2020).

This research aimed to analyze the effect of bacterial inoculum on the functional properties and taxonomic composition of soil microbial communities with the use of Biolog® ECOPlate system and Ilumina® next-generation sequencing of the rhizosphere soil and shoots samples collected from the pot experiment. The presented research hypothesizes that environmental isolates of bacteria belonging to *Arthrobacter*, *Pseudomonas* and *Rhodococcus* genera applied to raspberry plants are affecting soil microbial communities, associated with raspberries, both changing their taxonomic composition and shifting their metabolism.

2. Materials and methods

2.1. Pot experiment

The pot experiment including raspberry plants was conducted in control conditions of temperature and humidity in phytotron room. Raspberry seedlings were planted in pots containing 3 kg of soil. The following five variants of contamination with pathogens (pathosystems) were used: *Botrytis cinerea* G277/18 (B), *Verticillium* sp. G296/18 (V), *Colletotrichum acutatum* G172/18 (C), and *Phytophthora* sp. G408/18 (P), and control experiments without the addition of pathogen, but with the addition of water instead. For every variant of pathogen contamination, four methods of naturalization strategy, including the application of beneficial bacterial strains selected from natural habitats of wild raspberries, were tested. 4 previously isolated and characterized bacterial strains were used: *Rhodococcus* sp. B12/18 (GenBank: MW255650), *Pseudomonas* sp. B37/18 (GenBank: MW255651), *Arthrobacter* sp. B58/18 (GenBank: MW255652), *Rhodococcus* sp. B75/18 (GenBank: MW255653) (Pylak et al., 2020). Naturalization strategies included the application of bacterial

inoculum to raspberry roots during planting, application of bacterial inoculum during watering 4 weeks after planting and applying the bacteria both during planting and one month after naturalization watering with the bacterial consortium. This approach ensures that during research different types of plantations are taken into consideration, newly set up plantations (root naturalization) and already existing ones (watering naturalization). After the experiment, soil and plant samples were collected and stored for future analysis. The soil samples to be used for soil analyses were stored at -24°C.

The source and characterization of beneficial bacteria strains and plant-fungal and fungal-like pathogens, design, and the detailed description of the raspberry plant pot experiment are presented in our previous research papers (Pylak et al., 2021, 2020).

2.2. Biolog EcoPlates

1 g of rhizosphere soil or shoots was suspended in 99-ml sterile saline peptone water and shaken for 20 minutes at 20°C and then was incubated at 4°C for 30 minutes (Gryta et al., 2020). Next, each well of the Biolog® EcoPlate was inoculated with 120 µl of the prepared suspension and incubated at 25°C. Each experiment was performed in triplicates (n=3). Absorbance at 590 nm and 750 nm were measured on Biolog® Microstation after 24, 48, 72, 96, 120, and 144 incubation hours. Substrates were divided into five groups of carbon sources including carbohydrates, carboxylic and ketonic acids, amines and amides, amino acids, and polymers, according to Weber and Legge (Weber and Legge, 2009). Carrying out tests considering two wavelengths of light allows us to draw more specific conclusions. This is because absorbances for those wavelengths depend on different factors: 750 nm stands for optical density, and 590 nm for substrate usage (Sofo and Ricciuti, 2019). The absorbance values and stress substrate factor was calculated, and the results were grouped according to particular pathosystems and naturalization strategies, afterwards, the statistical analysis was performed with the use of *Statistica* 13.1.

2.3. Isolation of the DNA and NGS sequencing

The environmental DNA (e-DNA) from raspberry rhizosphere soil and raspberry shoots was isolated using a FAST DNA Spin Kit for Feces (MP Biomedicals, Santa Ana, USA) according to the protocol, as described previously by Oszust et al. (Oszust et al., 2014). Then ITS1 and 16S V3-V4 regions were amplified with locus-specific primers based on Toju et al. (2012) (Toju et al., 2012): ITS1f-KYO1 and ITS2_KYO2 for ITS1 amplification and Klindworth et al. (2013) for 16S amplification containing Illumina Overhang sequences presented in Table 1 (Klindworth et al., 2013; Toju et al., 2012). Reactions were performed in 10 µl and consisted of 5 µl of KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, US), 0.2 µM of pooled ITS1 forward primers or 16S forward primer, 0.2 µM of pooled ITS1 reverse primers or 16S reverse primer, 1.6 µl of nuclease-free water and 3 µl of the tested sample. Reactions were performed in the following conditions: 180 seconds at 95°C; 30 cycles of 30 seconds, 95°C; 30 seconds, 55°C; 60 seconds, 72°C; and finally 300 seconds at 72°C. After reactions were performed, samples were purified with MagMAX™ Express Magnetic Particle Processor (Applied Biosystems, Waltham, MA, US) using 18 µl of CleanPCR (CleanNA, Waddinxveen, The Netherlands) paramagnetic beads, and eluted in 50 µl of 10 mM Tris-HCl pH 8.0 (EURx, Gdańsk, Poland). After purification, the Index PCR step was performed by attaching dual indexes and Illumina sequencing adapters using Nextera XT Index Kit v2 (Illumina, San Diego, CA, US). Reactions consisted of 5 µl of KAPA HiFi HotStart ReadyMix, 1 µl of i5 and i7 index each, 1 µl of nuclease-free water, and 2 µl of the purified amplicon. Conditions of Index PCR were as follows: 180 seconds at 95°C; 10 cycles of: 30 seconds at 95°C, 30 seconds at 55°C, 60 seconds at 72°C; and 300 seconds at 72°C. Next step was purifying indexed amplicons with MagMAX™ Express Magnetic Particle Processor using 18 µl of CleanNGS (CleanNA, Waddinxveen, The Netherlands) paramagnetic beads, and eluted in 50 µl of 10 mM Tris-HCl pH 8.0. After that samples were normalized, pooled together

and diluted with 10mM Tris-HCl pH8.0 to achieve 4nM concentration. Then 5 µl of 4 nM library was denatured with 5 µl of 0.2 N NaOH and diluted to achieve 12 pM concentration with Illumina HT1 buffer (Illumina, San Diego, CA, US). 540 µl of prepared library was combined with 60 µl of 10pM denatured as above PhiX control library (Illumina, San Diego, CA, US). Then library was heat denatured by incubating in 96°C for 2 minutes and immediately placing it on the ice for 5 minutes. After that library was loaded on MiSeq v3 2x300 reagent cartridge (Illumina, San Diego, CA, US) and sequenced with Illumina MiSeq platform (Illumina, San Diego, CA, US). Results were then analyzed with QIIME2 environment and with FUNGuild tool using UNITE or SILVA databases (Bolyen et al., 2019; Kessy et al., 2020; Klindworth et al., 2013; Nguyen et al., 2016; Quast et al., 2013). Furthermore Shannon's diversity index, Pielou evennes index and Faith's phylogenetic diversity index were calculated to assess insights for ecological differences in microbial community. Results were grouped according to particular pathosystems and naturalization strategies, afterwards, the statistical analysis was performed with the use of *Statistica* 13.1.

2.4. Statistical analysis

For the analysis of the effect of bacterial inoculum on the functional diversity, graphs were generated based on the mean value calculated from three replicates. For the effect of naturalization strategies and pathosystems on the microbial communities diversity Shannon's diversity index, Pielou evennes index and Faith's phylogenetic diversity index were calculated and a one-way ANOVA with $p < 0.05$ was performed to check differences, and Tukey tests were also performed to reveal statistically significant differences between naturalization strategies and pathosystems.

3. Results

3.1. The functional diversity of microbial communities

The analysis of the functional abilities of microbial communities may contribute to an overall better understanding of the effect of bacterial inoculum on the ecosystem at the application site. Absorbance values for the wavelength of 590 nm and 750 nm are presented in Table 2. Absorbance values for the wavelength of 590 nm correspond to the ability of microorganisms to utilize certain compounds. In experiments without pathogens application of bacterial inoculum resulted in the decrease of polyols, α-Ketobutyric acid, and Glycogen utilization, however, resulted in higher utilization of D-Malic acid, carbohydrates such as α-D-Lactose, D-Glucose-1-Phosphate and amino acid L-Phenylalanine. In experiments including the pathogen inoculation the most profound changes were observed in variants contaminated with *Verticillium* sp., *C. acutatum*, and *Phytophthora* sp. in those objects the addition of bacterial inoculum resulted in lower utilization of carbohydrates such as D-Cellobiose, D-Glucose-1-Phosphate and α-D-Lactose. The presence of *B. cinerea, Verticillium* sp. and *C. acutatum* resulted in the enhancement of utilization of α-D-Lactose in experiments without the naturalization strategy applied when compared to the control without the pathogen and any of the naturalization treatments. However, in experiments without the pathogen inoculation, bacterial inoculum reduced the ability to utilize α-Cyclodextrin and Glycogen, however when raspberry plants were infected with *B. cinerea* the naturalization strategy of both root naturalization and watering enhanced the ability of the microbial community to utilize those compounds. Absorbance values for the wavelength of 750 nm correspond to the ability of microorganisms to produce biomass when utilizing certain compounds. The absorbance values for the wavelengths of 750 nm were not as different as for the wavelength of 590 nm. However, the addition of bacterial inoculum resulted in enhancement of biomass production in objects infected with *B. cinerea* and with root and watering naturalization treatments applied. Biomass

production while utilizing different carbon sources was similar among all pathosystems and naturalization strategies applied however.

The absorbance values for the wavelength of 590 nm and 750 nm for the microbial communities isolated from shoots of raspberry plants are presented in Table 3. In experiments without the pathogen contamination, the application of bacteria inoculum increased carbohydrates utilization. D-Mannitol, N-Acetyl-D-Glucosamine, D-Cellobiose and D-Glucose-1-Phosphate were utilized at higher level in objects treated with bacterial inoculum. In experiments with *B. cinerea* presence objects without the naturalization applied were negatively affected. However, when the watering naturalization strategy was applied the utilization of carbon sources was enhanced when compared to the control without the naturalization. The watering naturalization strategy resulted in the intensification of carbohydrates, carboxylic acids, polymers, and especially amino acids utilization. Root naturalization strategy was also effective but not as profoundly as the watering naturalization. Raspberry inoculation with *Veriticillium* sp. resulted in increased utilization of carbon sources in objects without any of the naturalization strategies applied when compared to the control experiments. In experiments with the presence of *Colletotrichum acutatum* watering with the addition of beneficial bacteria proved to be the most effective in enhancing microbial communities' carbon utilization abilities, especially amino acids and carboxylic acids. Microbial communities in experiments including *Phytophthora* sp. inoculation showed enhanced utilization of carbon sources in objects without any naturalization treatment. In experiments with *Phytophthora* sp. pathosystem when root naturalization strategy was applied utilization of DL-α-Glycerol Phosphate and D-Galactonic Acid Lactone was enhanced when compared to experimental objects without any naturalization strategy. Absorbance values for the wavelength of 750 nm were not as affected by both presence of pathogens and the addition of beneficial bacterial inoculum. In objects without the pathogen

presence application of beneficial bacteria resulted in increased utilization of D-Mannitol and Glycyl-L-Glutamic Acid when compared to the control.

Table 4 presents values of stress substrate index (SST) calculated based on values of absorbance for both wavelengths of 590 nm and 750 nm. The lower the SST value the higher the biomass production at lower substrate utilization which corresponds to better growing conditions for the microbial community in given conditions. As presented in Table 4 the stress substrate index value for microbial communities inhabiting rhizosphere was not affected by both pathogen presence and the naturalization strategy applied. However high diversity can be observed in the SST values calculated for samples from shoots. In experimental treatments without the pathogen inoculation, the addition of bacterial inoculum resulted in lowered SST for all types of naturalization strategies. The combined naturalization strategy including both root treatment during planting and watering with the addition of beneficial bacteria inoculum 4 weeks after planting resulted in the greatest reduction of stress substrate index when compared to the control for all substrate groups. When raspberry plants were inoculated with *B. cinerea* the SST value for experiments without the naturalization was higher than in the control. The addition of bacterial inoculum to objects with this pathogen resulted in the increase of substrate stress index value for root and watering naturalization strategy, however it caused a lowering of stress substrate index value in objects with combined naturalization strategy. In objects with the presence of *C. acutatum,* but without the bacterial inoculum applied the SST value was lower when compared to the control. The watering naturalization strategy caused an increase in SST value, however, root naturalization and combined naturalization caused a lowering of stress substrate index value. Infection of plants by *Phytophthora* sp. increased SST value. Each naturalization strategy resulted in the lowering of stress substrate index value, however, naturalization watering and combined naturalization strategy resulted in the highest reduction of SST value. Similarly *Phytophthora* sp. infection with *Verticillium* sp. causes increased SST

value for objects without any naturalization strategy applied. Nonetheless, each naturalization strategy resulted in a lowering of the SST value.

3.2. The relative abundance of bacterial and fungal phyla in rhizosphere samples

The structure of the soil microorganisms community was analyzed with the use of next-generation sequencing. The results indicated differences in the taxonomic composition of both bacterial and fungal microbiomes according to particular naturalization strategies and pathosystems are presented in Figure 1 and Figure 2.

The 22 bacterial phyla were identified in raspberry rhizosphere samples. The most abundant bacterial phyla in the raspberry rhizosphere were *Proteobacteria* (27-55%) and *Actinobacteriota* (16-22%). The third and fourth most numerous phyla constituted *Acidobacteriota* (8-21%) and *Verrucomicrobiota* (9-15%), respectively. In every pathosystem, the application of beneficial bacteria inoculum increased the relative abundance of *Proteobacteria*, however, the addition of pathogen inoculum to the experiments resulted in a decrease in the relative abundance of *Proteobacteria* (Figure 1).

Although in samples of raspberry rhizosphere 11 fungal phyla were identified 3 of them are responsible for over 95% of all relative abundance. Ascomycota (55-85%), Mortierellomycota (4-32%) and Basidiomycota (6-20). The application of beneficial bacteria resulted in the increased relative abundance of Ascomycota and the highest increase was observed in *Phytophthora* sp. pathosystem experiments in which the watering naturalization strategy was applied. In the same objects, the highest decrease of Mortierellomycota was noted. Application of watering naturalization strategy in *Verticillium* sp. resulted in the increase in the relative abundance of Basidiomycota with the reduction of Mortierellomycota relative abundance presented in Figure 2.

3.3. The core microbiome of raspberry rhizosphere

The core microbiome of the raspberry rhizosphere may be crucial for the proper functioning of microbial communities. It is a group of organisms that are present in most samples. The number of rhizosphere bacterial core microorganisms is presented in Figure 3.

As presented in Figure 3A, each naturalization strategy was associated with the characteristics of the bacteria. In objects with the watering naturalization, the strategy applied the number of individual bacteria was the highest, and in the root naturalization strategy was the lowest. All naturalization strategies shared the 6 bacteria that were present in samples treated with all naturalization strategies. Figure 3B presents the number of core bacteria characteristics for all pathosystems. The most numerous individual bacteria were present in experiments involving *Phytophthora* sp. infection. All pathosystems shared the 6 core bacteria that were present in samples coming from every pathosystem.

The number of fungal organisms specific for naturalization strategies and pathosystems is presented in Figure 4. The most specific fungi among all naturalization strategies were associated with root treatment and combined naturalization including root treatment and watering with beneficial bacteria. Most fungi associated with a particular pathosystem were noted for *Colletotrichum acutatum* pathosystem. The fungal core microbiome consisted of 22 identified fungi that were present in all samples of the rhizosphere.

Core microorganisms characteristic for particular naturalization strategies and pathosystems present in the experiments are presented in the table 5.

3.4. The number of microbial orders in the raspberry rhizosphere

The number of bacterial orders identified in soil samples may be a valuable resource that expands our understanding of particular treatment or pathosystem applied to the soil microbial communities composition, results of the number of bacterial orders are presented in Figure 5.

315 The effect of bacterial inoculum application on the number of bacterial orders present
316 in rhizosphere samples is presented in Fugure 5A. Experiments without any naturalization
317 treatment applied and no pathosystems present were characterized by the lowest number of
318 bacterial orders identified except for *Verticillium* sp. pathosystem with the root naturalization
319 applied. In objects without any pathosystems combined naturalization strategy (RW) resulted
320 in the highest increase of bacterial orders identified from 33 orders in the object without any
321 naturalization to 53 in the object with a combined naturalization strategy applied. Infection with
322 *Botrytis cinerea* increased the number of bacterial orders when compared to the control from
323 33 to 42. The watering naturalization strategy caused the increase from 42 to 52 in experiments
324 including the *B. cinerea* pathosystem. In experiments infested with *Verticillium* sp. the infection
325 caused the increase of bacterial orders in objects without any naturalization strategy applied. In
326 experiments with the root and watering naturalization strategy the highest increase in the
327 number of bacterial orders was noted, from 39 to 73. However only root naturalization without
328 any other treatment resulted in the decrease of bacterial orders from 39 to 16. In experiments
329 with *Colletotrichum acutatum,* all naturalization strategies resulted in the decrease of the
330 bacterial orders identified when compared to *C. acutatum* objects without naturalization. In
331 experiments infected with *Phytophthora* sp. root naturalization during planting increased
332 bacterial orders, the contrary to other methods which resulted in a slight decrease when
333 compared to objects without treatment applied. The pathogen presence increased bacterial
334 orders present in the rhizosphere of raspberry plants when compared to the control.
335 The effect of the naturalization strategies and pathogen infection on the number of
336 fungal orders identified in raspberry shoots samples presented in Figure 5B was less diverse
337 than in the rhizosphere samples. In experiments without the pathogen and with *C. acutatum*
338 contamination all naturalization strategies resulted in the decrease of fungal orders identified.
339 The presence of *Botrytis cinerea, Verticillium* sp., and *Phytophthora* sp. caused a decrease in

the number of other fungal orders in plant shoots when compared to experiments without pathogen contamination. In objects inoculated with *B. cinerea* naturalization strategy including both root naturalization and naturalization watering applied were the most efficient in enhancing the number of fungal diversity. In experiments with *Verticillium* inoculation naturalization, watering caused the biggest increase in the number of fungal orders identified. Experiments with *Phytophthora* sp. presence were not affected by the naturalization strategy applied except for watering treatment which resulted in the decrease of bacterial orders to 27. The numbers of fungal orders identified in shoots samples are presented in Figure 5C. The diversity in the number of fungal orders was more prefund than in the rhizosphere samples. Similar to results obtained for the rhizosphere samples, the application of bacterial inoculum resulted in the decrease of fungal orders identified in almost all experiments except those infected with the *C. acutatum* and in objects inoculated with *Phytophthora* sp. naturalized during both planting and watering. In experiments without the pathogen presence and with the *B. cinerea* infection combined naturalization strategy resulted in the biggest decrease in the number of fungal orders identified. However this naturalization strategy proved itself to be the most effective in increasing the number of fungal orders in all remaining experiments – contamination with *Verticillium* sp., *Phytophthora* sp. and *C. acutatum*.

3.5. Bacterial and fungal biodiversity in the rhizosphere and shoots samples

Microbial biodiversity in collected samples was evaluated via 3 common alpha diversity indicators: Shannon's diversity index, Pielou evenness, and Faith's phylogenetic diversity. Analysis was performed for bacterial communities inhabiting raspberry roots and fungal communities inhabiting raspberry roots and shoots. Figure 6 presents values of mentioned biodiversity indicators for bacterial communities.

On average plant inoculation with pathogens resulted in increasing the values of the biodiversity indicators for bacterial communities when compared to the control. Experiments

with the *Colletotrichum acutatum* inoculation were characterized by the highest increase in the values of the biodiversity indicators, however, except for the Shannon diversity index differences were not statistically significant. From all tested naturalization strategies the combined naturalization including root naturalization during planting and naturalization watering 4 weeks after planting resulted in the highest increase of the biodiversity indicators values for bacterial communities. When evaluating results from all samples it can be noted that the evenness of microbial communities remains similar, however, values of Shannon's diversity and Faith phylogenetic diversity indexes vary. Pathogens infection resulted in the increase in diversity in all pathosystems in objects without the naturalization strategy applied when compared to the control. In objects without pathogens both Shannon's and Faith indexes pointed towards combined treatment as the most profound in increasing the diversity of bacterial communities. In objects with *Botrytis cinerea* infection watering naturalization was the most successful in increasing the microbial Shannon's diversity, although phylogenetic diversity was not affected as much. The application of naturalization strategies did not result in a sufficient increase in microbial diversity, however, resulted in a decrease in phylogenetic diversity when compared to experiments without the naturalization applied. Similar results were achieved for *Phytophthora* sp. infection. The application of combined treatment and watering treatment alone resulted in the highest increase both in microbial diversity and phylogenetic diversity.

Figure 7 presents biodiversity indicators values for fungal communities inhabiting raspberry roots and shoots. Contrary to the bacterial communities root fungal microbial communities biodiversity is reduced when the pathogen inoculum is added when compared to the control. Biodiversity indicators values were less affected in experiments including *Verticillium* sp. inoculation. Combined naturalization including root naturalization during planting and naturalization watering 4 weeks after planting resulted in a slight increase in biodiversity Pielou evenness index, however, both roots and especially watering naturalization

resulted in a statistically significant decrease in biodiversity Pielou evenness index. The biodiversity of shoots microbiota was also affected by pathosystems present and naturalization strategies applied. Pielou's evenness index values were less affected both according to pathosystems and naturalization strategies, however, Shannon's diversity index was affected more. Although the evenness of the microbial communities is similar the diversity according to Shannon's diversity changes. Inoculation with *Phytophthora* sp. resulted in the highest increase in biodiversity when compared to the control. Different naturalization strategies resulted in the maintenance of similar microbial evenness, however, the diversity was reduced in objects with combined treatment applied.

3.6. The trophic mode assignments of fungal OTUs (operational taxonomic unit) found in rhizosphere samples

The trophic mode of fungal microorganisms identified in rhizosphere samples was evaluated with the use of the FUNGuild tool and the results are presented in Figures 8 and 9. This enabled to further analyze microbial communities and their interactions. Identified and unidentified microorganisms were divided according to the naturalization strategy, pathosystems, and their trophic modes to pathotrophs, saprotrophs, symbiotrophs, and their combinations. In all samples, unassigned microorganisms were a majority and their OTU richness reached the percentage of 70-80% of all OTUs present. However, their sequence richness was comparable to the other microorganisms ranging between 7% and 10%. In objects with roots naturalization strategy applied OTU and sequence richness of pathotrophs remained similar, however, sequence richness of pathotrophs-saprotrophs decreased. OTU richness of multitrophic pathotrophs-saprotrophs-symbiotrophs increased from 29% to over 40% when compared to the control, and their sequence richness slightly decreased. OTU richness of saprotrophs decreased and OTU richness of sapro-symbiotrophs remained the same. Furthermore, although the OTU richness of patho-symbiotrophs decreased from 4% to 2% their

sequence richness increased from 8% to 22%. In objects with the combined naturalization strategy applied OTU richness of pathotrophs decreased from 13% to 10%, however their sequence richness increased. OTU richness of patho-saprotrophs decreased from 7% to 4%. Both OTU and sequence richness of patho-sapro-symbiotrophs increased from 29% to 37% and from 18% to 21%, respectively. OTU richness of saprotrophs decreased to 19% and OTU richness of sapro-symbiotrophs increased from 18% to 23%. OTU richness of patho-symbiotrophs decreased but their sequence richness increased to 10%. The application of the watering naturalization strategy alone resulted in the decrease of pathotrophs OTU and sequence richness, however, it increased the patho-saprotrophs OTU and sequence richness. Patho-sapro-symbiotrophs OTU richness remained elevated similarly to other naturalized experiments, however, the sequence richness was not as high as in control objects or combined naturalization objects. Saprotrophs and sapro-symbiotrophs OTU richness were similar to those in combined naturalization strategy experiments, however their sequence richness was higher. OTU richness of patho-symbiotrophs remained the same when compared to combined naturalization strategy experiments, but sequence richness decreased.

Figure 9 presents the results of FUNGuild trophic mode assignments to fungal samples according to the pathosystem applied in the experiment. OTU richness of all samples that were not identified was between 70% and 80% of all identified and assigned samples. Sequence richness of unassigned samples was highest in experiments involving *C. acutatum* infection and lowest in *Phytophthora* sp. infested samples. After removing unassigned microorganisms from the analysis the results can be further analyzed. In experiments without any pathogen contamination, pathotrophs OTU richness was at 22% and their sequence richness was at 50%. Patho-saprotrophs OTU richness and sequence richness were at 3% and 5%, respectively. Patho-sapro-symbiotrophs OTU and sequence richness were at 29% and 30%, respectively. Saprotrophs OTU richness was at 27% and sequence richness was at 12%. Sapio-symbiotrophs

OTU and sequences richness were at 18% and 14%, respectively and patho-symbiotrophs OTU and sequence richness were at 3% and 6%, respectively.

The presence of *Botrytis cinerea* in the experiments resulted in the reduction of OTU and sequence richness for pathotrophic fungi from 22% to 14% and from 50% to 42%, respectively. Patho-saprotrophs OTU richness increased from 5% to 7% and their sequence richness increased from 2% to 17%. Saprotrophs OTU and sequence richness remained similar to the control, however OTU richness of sapro-symbiotrophs increased from 11% to 15% and their sequence richness increased to 16% when compared to the control experiments. In experiments including *C. acutatum* contamination pathotrophs OTU richness decreased even more than in *B. cinerea* experiments from 22% to 10%. Patho-saprotrophs OTU richness increased to 10%, but sequence richness decreased to 9%. Multitrophic patho-sapro-symbiotrophs OTU richness remained similar to the control, however sequence richness slightly decreased to 28%. Saprotrophs OTU richness decreased to 19% and sapro-symbiotroph OTU richness increased to 26%, while their sequence richness remained similar. In experiments including *Phytophthora* sp. contamination, pathotrophs OTU and sequence richness decreased to 15% and 25% respectively, however, patho-saprotrophs OTU richness increased from 5% to 12% and their sequence richness increased from 2% to 32%. Both patho-sapro-symbiotrophs and saprotrophs OTU richness decreased by around 8% when compared to the control and their sequence richness decreased by 7%. Sapro-symbiotrophs OTU and sequence richness increased from 11% to 26% and from 11% to 14%, respectively. The presence of *Verticillium* sp. in soil resulted in the highest reduction of pathotrophs OTU richness of all pathosystems applied from 22% to 9% and sequence richness from 50% to 36%. Patho-saprotrophs OTU and sequence richness increased to 9% and 20%, respectively. In this pathosystem the highest increase in OTU richness of multitrophic patho-sapro-symbiotrophs was observed from 33% to 36%, however the sequence richness decreased from 29% to 21%. Saprotrophs OTU richness

decreased to 23%, and sapro-symbiotrophs OTU richness increased to 23%. Their sequence richness was placed at 12% and 9%, respectively.

3.7. The trophic mode assignments of fungal OTUs in the shoots

Results shown in Figure 10A present trophic mode OTU and sequence richness of fungal communities isolated in raspberry shoots according to naturalization strategies. In the analysis of shoots samples, all microorganisms were assigned to proper trophic modes. In experiments with roots naturalization strategy during planting OTU richness of multitrophic patho-sapro-symbiotrophs increased by 12% when compared to the control, however their sequence richness decreased. Fungi belonging to the trophic mode of patho-symbiotrophs were less present in those samples (decrease from 22% to 2% however their sequence richness increased by 10% when compared to the control). Saprothrops OTU richness decreased by 12% and the sequence richness remained unchanged. In experiments involving combined naturalization strategy pathotrophs OTU richness decreased by 3% when compared to the control. Patho-saprotrophs OTU richness slightly increased and sequence richness decreased. OTU richness of patho-sapro-symbiotrophs was similar to other experiments with naturalization applied at 40%, however their sequence richness decreased to 11%. OTU and sequence richness of patho-symbiotrophs both were at 5%. OTU and sequence richness of saprotrophs decreased by 10% and 3%, respectively, when compared to the control. Sapro-symbiotrophs OTU reaches increased by 8% however their sequence richness decreased by 9% when compared to objects without any naturalization strategy applied. The application of the watering naturalization strategy resulted in changes similar to the root naturalization strategy, however sequence richness of multitrophic fungi was higher by 2%, and OTU richness of patho-symbiotrophs was lower by 8%.

Figure 10B presents trophic mode OTU and sequence richness for fungal microorganisms grouped according to applied pathosystems. The presence of each pathosystem

resulted in the increase of sequence richness for pathotrophic organisms, however the OTU richness varied from 9% to 15%. OTU richness of patho-saprotrophs remained on a similar level of 9-11%, although the sequence richness was the highest in *Verticillium* sp. pathosystem reaching up to 39%. Patho-sapro-symbiotrophs OTU richness was the highest in experiments without the pathogen inoculation and the lowest in the *Phytophthora* sp. pathosystem. Sequence richness of multitrophic microorganisms was the highest in experiments including *Phytophthora* sp. pathosystem. *C. acutatum* and *Verticillium* sp. were the only pathosystems where path-symbiotrophs were present, however their OTU and sequence richness was lower than 5%. Saprotrophs OTU richness was the highest in the *B. cinerea* pathosystem, however sequence richness was the highest in experiments without the pathogens. In experiments without the additional pathogen inoculation, sapro-symbiotrophs OTU richness was the highest – 26% in comparison to *B. cinerea*, *C. acutatum* and *Verticillium* sp. contamination, and its sequence richness reached 39% which was the highest of all tested pathosystems.

3.8. The functional analysis of bacterial communities in the rhizosphere

Identified bacterial ASVs (amplicone sequence variants) were subjected to PICRUSt analysis which evaluated genes present in samples. Furthermore, genes responsible for more than 6000 enzymes were divided into groups with the use of the KEGG database. Results from this analysis are presented in Figure 11.

ASVs responsible for taking part in metabolism processes were the majority of all identified ASV. Proportions of all predicted functional ASVs were similar and their statistical significance was the same. It can be noted, that in objects without the pathogen presence were lower than in objects with the pathogen contamination except for the *Botrytis cinerea* infection. In treatment infested with *Colletotrichum acutatum* the highest statistically significant increase in the number of genes responsible for metabolic processes was observed when compared to the control. Experiments with the *Phytophthora* sp. and *Verticillium* sp. contamination

increased genes when compared to the control, but the number was not as high as in the *C. acutatum* experiments. In general, the number of sequences of particular KEGG classes was the highest for *C. acutatum* experiments and the lowest for experiments without any pathogens or with *B. cinerea* contamination. Furthermore, 3 classes that were the most represented in examined bacterial communities were divided into subclasses to fully evaluate the composition and shares of bacterial functions. The most profound subclasses in bacterial communities metabolism were the metabolism of lipids, carbohydrates, terpenoids, polyketides, xenobiotics, as well as the metabolism of cofactors and vitamins. Similar results were observed for genetic and environmental information processing subclasses, however the most profound subclasses were translation and signal transduction, accordingly. A similar tendency in terms of statistically significant differences was observed both in main classes and in subclasses. When results were grouped according to the naturalization strategies, experiments with the combined naturalization treatment applied proved to increase the number of genes responsible for metabolism, when compared to the control, however the differences were not statically significant.

4. Discussion

4.1. The analysis of functional diversity of microbial communities

Analysis of the functional abilities of microbial communities is a crucial part of understanding their interactions and effect on soil and plants. Interactions between soil microbial communities and newly naturalization treatments and pathogens presence may answer important questions about the nature of those interactions and their future implications. Microbial communities inhabiting the raspberry rhizosphere were affected both by pathogen inoculation and by the naturalization strategy applied. The enhancement of D-Malic acid utilization by microbial communities may correspond to bacterial adaptation to living next to plant roots, due to the fact, that plants can secrete Malic acid as a response to pathogen infection.

Bacteria having the ability to utilize this compound can form a tighter symbiotic bond between plants and themselves (Rudrappa et al., 2008). Both pathogen presence and naturalization strategies applied resulted in the change of sugars metabolism by rhizosphere microbial communities. Better utilization of carbohydrates present in soil may contribute to bacterial growth enhancement near the plant roots as 20% of soil-present carbohydrates are the effect of plant secretion or decomposition (Gunina and Kuzyakov, 2015).

Microbial communities inhabiting the raspberry shoots were not highly affected by the application of microbial inoculum when not subjected to the pathogen presence. However, the presence of plant pathogens changed the microbial substrate utilization. The presence of both *B. cinerea* sp. and *C. acutatum* resulted in the decrease of substrate utilization by shoots microbial communities in objects without the naturalization applied which leads to the conclusion that those fungal pathogens are causing a negative interaction with shoots microbial communities, which can be compensated or even overpowered by proper naturalization strategy applied (Chaudhry et al., 2021; Hirano and Upper, 2000). On the contrary, the presence of *Verticillium* sp. and *Phytophthora* sp. leads to enhancement of substrate utilization by foliar microbiota in objects without the naturalization strategy applied. This may be beneficial if this utilization is caused by pathogenic fungus or fungal-like organisms or disadvantageous if the higher microbial substrate utilization rate is an effect of microbial reaction to pathogens presence and thus decreasing the utilization is also a decrease of community resistance ability.

Although the rhizosphere and shoots microbial biomass production was similarly alike, the substrate stress index (SST), presented in Table 4, allows observing a diverse effect of both pathogen and naturalization interaction with plant microbial communities. Rhizosphere microbial communities were less affected by both naturalization strategy and plant pathogens presence. This may be due to the natural stability of soil microbial communities, which are more stable than shoots communities. Soil microbial communities are less affected by changing

temperatures, wind, and UV radiation, and are predominantly livening in a humid environment. Due to these environmental factors, communities may be more balanced and less prone to change, and even though some metabolic response is changing the core microbiota remains well established (Huang et al., 2021; Jiao et al., 2022). On the contrary the stress substrate index for microbial communities inhabiting the shoots diverse and changes according to the applied naturalization strategy and pathosystem present. The application of bacterial inoculum resulted in a decrease in stress index, especially in experiments where both root and watering naturalization treatments were applied. The combined activity of both root inoculation during planting and future naturalization watering resulted in the highest decrease in stress substrate index. The presence of all pathogens except for *B. cinerea* increased the stress substrate index when compared to the control which leads to the conclusion that microbial communities are reacting to pathogen infection. The application of naturalization treatment resulted in lowering the stress of microbial communities and especially combined root and watering naturalization treatment proved to be the most suitable for most applications, and that multiple applications resulted in better effect which is common to ecological plant protection products (Grabas et al., 2016; Pylak et al., 2019). It can be noted that *B. cinerea* presence resulted in the reduction of the SST index in experiments without the naturalization treatment applied, which agrees with current knowledge that some pathogens organisms can have a stimulating activity on microbial communities (Stefanowicz, 2006; Topalović et al., 2020; Trabelsi and Mhamdi, 2013). The stress substrate index even may be a valuable tool allowing to perceive microbial interactions beyond the basic absorbance values.

590 **4.2. The analysis of taxonomical diversity of microbial communities**

591 The conducted identification of the taxonomical composition of soil microbial
592 communities resulted in the identification of the 22 most common bacterial phyla in the
593 raspberry rhizosphere. Achieved relative abundances of bacterial taxa are consistent with
594 existing research on this topic (Janssen, 2006; Jiménez-Bueno et al., 2016). Results showed,
595 that application of bacterial inoculum to roots or via watering can influence the taxonomic
596 composition of rhizosphere bacteria in the soil. The presence of all pathogens except *Botrytis*
597 *cinerea* resulted in the decrease of the most abundant *Proteobacteria* in the objects without the
598 naturalization applied, when compared to the control. It can be concluded, that the presence of
599 *B. cinerea* does not affect the taxonomic composition of rhizosphere bacteria as much as the
600 other pathogens in the experiment.

601 The relative abundance of identified 11 fungal phyla allows to determine the most
602 common fungal phyla in the raspberry rhizosphere. Achieved results correspond to the data
603 concerning main fungal phyla determined in soils (Frąc et al., 2021, 2020; Janssen, 2006;
604 Jiménez-Bueno et al., 2016; Mącik et al., 2020). The relative abundances of Ascomycota might
605 be the most important phylum in the fungal raspberry plant pathogens research since the most
606 important raspberry fungal and fungal-like pathogens belong to the Ascomycota phylum. It can
607 be noted, that the relative abundance of Ascomycota phylum decreased as the effect of
608 naturalization strategies only in the experiments without the pathogens or in the *Verticillium*
609 pathosystem. This also corresponds to our previous research pointing out that naturalization
610 strategies should be carefully suited to the pathogens present at the plantation where the
611 biopreparations are applied (Oszust et al., 2021; Pylak et al., 2021). Moreover, previous
612 research of Oszust and Frąc (2021) indicated that representatives of Ascomycota belonging to
613 the taxa of Hypocreales and Maasoglossum were the most common saprotrophs in the
614 rhizosphere of planted and wild raspberry (Oszust and Frąc, 2020). It was also proeven that

615 incorporation of mineral fertilizer enriched with beneficial bacterial strains were connected with
616 occurrence of Ascomycota and Basidiomycota representatives such as: Falcocladiales,
617 Myrangiales or Tilletiales (Mącik et al., 2022).

618 The core microbiome includes microorganisms that are present in soil samples and may
619 change according to applied naturalization strategy or the presence of pathogens (Risely, 2020).
620 Venn diagrams in Figures 1 and 2 present the distribution of core bacterial and fungal
621 microorganisms identified in analyzed samples. Core microbiota is responsible for the
622 functional stability of the soil microbiome. Application of bacterial inoculum resulted in
623 changes in specific core bacterial and fungal microorganisms depending on both applied
624 naturalization strategies and presence of pathogens. It can be noted that the bacterial core
625 microbiome consisted of fewer microorganisms than the fungal core microbiome which
626 corresponds to the current knowledge that agricultural lands have a core fungal to bacterial ratio
627 shifted towards fungal microorganisms (Anan'eva et al., 2010; Bailey et al., 2002). The number
628 of bacterial orders in the rhizosphere was overall positively affected by at least one
629 naturalization strategy except for the *C. acutatum.* Combined, multiple naturalization
630 treatments of root naturalization during planting and naturalization watering 4 weeks after
631 planting resulted in the increase of bacterial orders number in 2 out of 5 tested pathosystems.
632 The presence of fungal and fungal-like pathogens resulted in the increased number of fungal
633 orders present in samples when compared to the control. The number of fungal orders in the
634 raspberry rhizosphere was less diverse than bacterial orders. In experiments without the
635 pathogen infection application of naturalization strategies resulted in the decrease of fungal
636 orders present in samples which agrees with our previous research stating that the naturalization
637 strategy needs to be matched to the pathogens occurring at the plantations (Pylak et al., 2021).

638 Application of bacterial inoculum to objects without the pathogen contamination
639 resulted in the decrease of fungal orders found in samples of raspberry shoots when compared

to the control. Similar effects were observed for experiments involving *B. cinerea* and *Verticillium* sp. inoculation for the combined treatment and involving *C. acutatum* and *Phytophthora* sp. for the watering treatment. This may be a beneficial effect of naturalization treatments inhibiting the growth of fungal organisms, which are often pathogenic, on the shoots of the plant (Tao et al., 2021).

4.3. The analysis of microbial communities biodiversity

Biodiversity indicators such as the Shannon diversity index, Faith's phylogenetic diversity, or Pielou evenness are valuable metrics that allow calculating how microbial communities vary, and what affects them the most. Pielou evenness index describes how even is the number of different ASV/OTU in tested samples. Shannon's diversity index takes into account the number of species living in a habitat (richness) and their relative abundance (evenness). Faith's phylogenetic diversity presents the total branch length on a phylogenetic tree that is spanned by a community, however, the abundance of each species in the community is not considered (Faith and Baker, 2006; Help et al., 1998; Morris et al., 2014). Pielou evenness of rhizosphere bacterial communities of samples grouped according to the pathosystem applied, presented in Figure 6 was the highest in samples infested by *C. acutatum*, which leads to the conclusion that in that particular pathosystem the number of different ASVs was the most similar. Values calculated for Shannon's diversity index and Faith's phylogenetic diversity pointed that the *C. acutatum* experiments were characterized by the highest amount of different bacterial ASV. Combined naturalization strategy including root naturalization during planting and naturalization watering 4 weeks after planting proved to be the most successful in equalizing the proportions between particular ASV in samples and increasing the biodiversity. The effect achieved by this naturalization strategy is profound due to double inoculum application compared to only a single application in other naturalization strategies.

The presence of pathogens in the experiments resulted in the decrease of the Pielou's evenness of fungal communities inhabiting the rhizosphere. The presence of pathogens inoculum caused an imbalance in microbial communities that are responding to stressful conditions, however the diversity of the communities remained similar (Masenya et al., 2021). Fungal communities inhabiting the shoots were less diverse and less even than those inhabiting soil, probably due to higher environmental stresses such as mentioned before UV light, wind, changing temperatures, and lower humidity caused by climate conditions and changes (Leveau, 2018). Statistically significant changes in Shannon's diversity of fungal communities inhabiting the shoots, particularly the decrease in the diversity of fungal communities may be the effect of the antifungal working of beneficial bacteria inoculum used in this experiment.

FUNGuild is an important tool in the functional analysis of fungal microorganisms and their response to tested conditions. The majority of unassigned fungi remains consistent with the assumption that the majority of fungi present in soil are still unidentified and uncultivable (Tedersoo et al., 2020). The application of bacterial inoculum via watering resulted in the decrease of OTU richness of pathotrophic fungi, which may be associated with antifungal properties of beneficial strains of bacteria used in this research. The decrease was also observed in the combined treatment naturalization strategy, however it was not noticed in only root naturalization strategy experiments. Due to this fact it can be presumed that the decrease of pathotrophs was the effect of only the watering naturalization strategy. On the contrary, the decrease of patho-saprotrophs is a correlated effect of both root and watering naturalization strategies because a similar decrease was not observed in single naturalization strategies. The increase of multitrophic fungal OTU richness may also be a response to bacterial diversity stimulation as the effect of bacterial inoculum application (Trabelsi and Mhamdi, 2013). The presence of pathogens in the experimental setup resulted in the change of fungal trophic mode composition. The interaction between groups of microorganisms can shift the proportions of

690 different trophic modes (Geisen et al., 2022; Sweeney et al., 2020). The inoculation with
691 pathogens increased patho-saprotrophic fungi, however the number of pathotrophs decreased
692 and the number of saprotrophs remained similar. Not much research can be found regarding the
693 effect of pathogen presence on soil microbial communities, therefore new research concerning
694 this topic is needed to fully understand the relationships between bacterial biopreparations,
695 pathogen presence, and soil microbial diversity.

696 PICRUSt analysis is a method of phylogenetic profiling that allows predicting
697 metagenome functional content based on the marker genes, such as the 16S rRNA gene for
698 bacterial organisms. The changes in metabolic functioning of microorganisms caused by the
699 addition of bacterial inoculum were observed, however they were not statistically significant.
700 The shifts in metabolic fingerprints, especially increase in metabolic pathways sequences
701 number, were observed after application of mineral phosphorus fertilizer enriched with
702 beneficial bacterial strains (Mącik et al., 2020; Mącik et al., 2022). The increase in the number
703 of predicted ASV caused by the combined naturalization treatment, including root
704 naturalization during planting and watering naturalization after 4 weeks, was the highest. This
705 observation agrees with the assumption, that the repeated use of biopreparation leads to better
706 results than a single application (Trabelsi and Mhamdi, 2013; von Bennewitz and Hlusek,
707 2006). Statically significantly different results were observed when their were grouped
708 according to particular pathosystems. Every pathogen except for *B. cinerea* significantly shifted
709 the bacterial metabolism. The most affected metabolic pathway group were metabolic pathways
710 which are also the most abundant group in bacterial metabolism (Gupte, 2006). Again, similarly
711 to the biodiversity indexes and the number of microbial orders, the *Colletotrichum acutatum*
712 proved itself to be the most potent to change the microbial community metabolism among all
713 tested pathosystems. The possible cause of this effect is, that *Colletotrichum* sp. produces an
714 extremely wide spectrum of secondary metabolites including nitrogen-containing metabolites,

sterols, terpenes, pyrones, phenolics, and fatty acids (Kim and Shim, 2019). Pathogens used in this study are the most important fungal and fungal-like pathogens for raspberries. Each produces a variety of secondary metabolites that affect greatly the soil microbial communities. Secreted metabolites can affect the microbial composition of the rhizosphere – and change its taxonomic composition and metabolic abilities. It can be observed that the introduction of pathogens caused an extensive reaction of rhizosphere bacterial communities.

The results presented in this study suggest that the application of beneficial bacteria to raspberry plants cultivation can greatly affect the microbiome of the rhizosphere and shoots of raspberry plants. Examined types of naturalization strategies resulted in different effects. Furthermore, the naturalization strategy should be carefully suited for the particular raspberry plantations, depending on the fungal and fungal-like plant pathogens present in the area. Those results correspond to our previous research that proved that the application of bacterial inoculum can enhance raspberry plant growth in the presence of fungal and fungal-like phytopathogens (Pylak et al., 2021).

5. Conclusions

- The application of bacterial inoculum decreased the value of the stress substrate index (SST) in microbial communities inhabiting the raspberry shoots.
- Microbial communities inhabiting plants rhizosphere are more resilient to changes in functional diversity.
- The 4 bacterial phyla out of the 22 identified were responsible for over 75% of all bacterial phyla present in the soil. They were Proteobacteria, Actinobacteriota, Acidobacteriota and Verrucomicrobiota.
- The 3 fungal phyla out of identified 11 were responsible for over 95% of all fungal phyla present in the soil. They were Ascomycota, Mortierellomycota and Basidiomycota.

- The fungal core microbiome consisted of 22 core fungal species.
- The decrease of the Shannon's diversity in fungal communities of raspberry shoots may be the effect of the antifungal effect of beneficial bacterial inoculum application.
- The assignment of trophic modes to identify fungal taxa is a valuable tool in evaluating the functional diversity of soil microbial communities.
- The application of bacterial inoculum results in statistically significant changes in the metabolism of soil microbial communities.
- More research is needed on this topic to fully understand the effect of the application of microbial biopreparations on rhizosphere and shoots microbial communities of raspberries.

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FIGURE CAPTIONS

Fig. 1. The distribution of bacterial phyla in rhizosphere samples followed by the next-generation sequencing of DNA extracted from rhizosphere samples. B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P – *Phytophthora* sp., N – no naturalization strategy applied, R – root naturalization during planting, W – naturalization during watering 4 weeks after planting, RW – combined naturalization including root naturalization during planting and naturalization watering 4 weeks after planting.

Fig. 2. The distribution of fungal phyla in rhizosphere samples followed by the next-generation sequencing of DNA extracted from rhizosphere samples. B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P – *Phytophthora* sp., N – no naturalization strategy applied, R – root naturalization during planting, W – naturalization during watering 4 weeks after planting, RW – combined naturalization including root naturalization during planting and naturalization watering 4 weeks after planting.

Fig. 3. Venn diagrams of core bacteria in rhizosphere samples depending on: A - naturalization strategy, B - pathosystem. WP- without pathogen, B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P – *Phytophthora* sp., N – no naturalization strategy applied, R – root naturalization during planting, W – naturalization during watering 4 weeks after planting, RW – combined naturalization including root naturalization during planting and naturalization watering 4 weeks after planting.

Fig. 4. The Venn diagrams of core fungi in rhizosphere samples depend on: A - naturalization strategy, B - pathosystem. WP- without pathogen, B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P – *Phytophthora* sp., N – no naturalization strategy applied, R – root naturalization during planting, W – naturalization during watering 4 weeks after planting, RW – combined naturalization including root naturalization during planting and naturalization watering 4 weeks after planting.

Fig. 5. The number of bacterial (A) and fungal (B) orders present in the rhizosphere and fungal orders in the shoots (C) samples depending on naturalization strategy and pathosystem. N – no naturalization strategy applied, R – root naturalization during planting, W – naturalization

963 during watering 4 weeks after planting, RW – combined naturalization including root
964 naturalization during planting and naturalization watering 4 weeks after planting.
965
966 **Fig. 6.** The biodiversity indicators values for bacterial communities inhabiting raspberry roots.
967 W – without pathogen, B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*,
968 P – *Phytophthora* sp., N – no naturalization strategy applied, R – root naturalization during
969 planting, W – naturalization during watering 4 weeks after planting, RW – combined
970 naturalization including root naturalization during planting and naturalization watering 4 weeks
971 after planting. Different lowercase letters represent statistically significant differences.
972
973 **Fig. 7.** The biodiversity indicators values for fungal communities inhabiting raspberry roots. W
974 – without pathogen, B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P
975 – *Phytophthora* sp., N – no naturalization strategy applied, R – root naturalization during
976 planting, W – naturalization during watering 4 weeks after planting, RW – combined
977 naturalization including root naturalization during planting and naturalization watering 4 weeks
978 after planting. Different lowercase letters represent statistically significant differences.
979
980 **Fig. 8.** The trophic mode OTU richness and sequence richness for fungi identified in soil
981 rhizosphere samples, according to naturalization strategy. Part A presents results with
982 unassigned fungal microorganisms included, part B presents results with unassigned fungi
983 excluded. Column height represents OTU richness and column width represents sequence
984 richness.
985
986 **Fig. 9.** Trophic mode OTU richness and sequence richness for fungal microorganisms identified
987 in soil rhizosphere samples, according to applied pathosystem. Part A presents results with

unassigned fungi included, part B presents results with unassigned fungal microorganisms excluded. Column height represents OTU richness and column width represents sequence richness.

Fig. 10. The trophic mode OTU richness and sequence richness for fungal microorganisms identified in soil shoots samples, according to the applied pathosystem. Part A presents results grouped according to the naturalization strategy applied, part B presents results grouped according to applied pathosystems. Column height represents OTU richness and column width represents sequence richness.

Fig. 11. Functional OTU assignments for bacterial OTUs identified in soil rhizosphere samples, according to pathosystem (A, C) and naturalization strategy (B). W – without pathogen, B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P – *Phytophthora* sp., N – no naturalization strategy applied, R – root naturalization during planting, W – naturalization during watering 4 weeks after planting, RW – combined naturalization including root naturalization during planting and naturalization watering 4 weeks after planting.

TABLE CAPTIONS

Table 1. The sequence of primers used in ITS1 and 16S V3-V4 regions amplification and sequencing.

Table 2. The absorbance values for the wavelength of 590 nm and 750 nm for different carbon sources. Samples for the analysis were taken from the rhizosphere of raspberry plants. N – no naturalization strategy applied, R – root naturalization during planting, W – naturalization

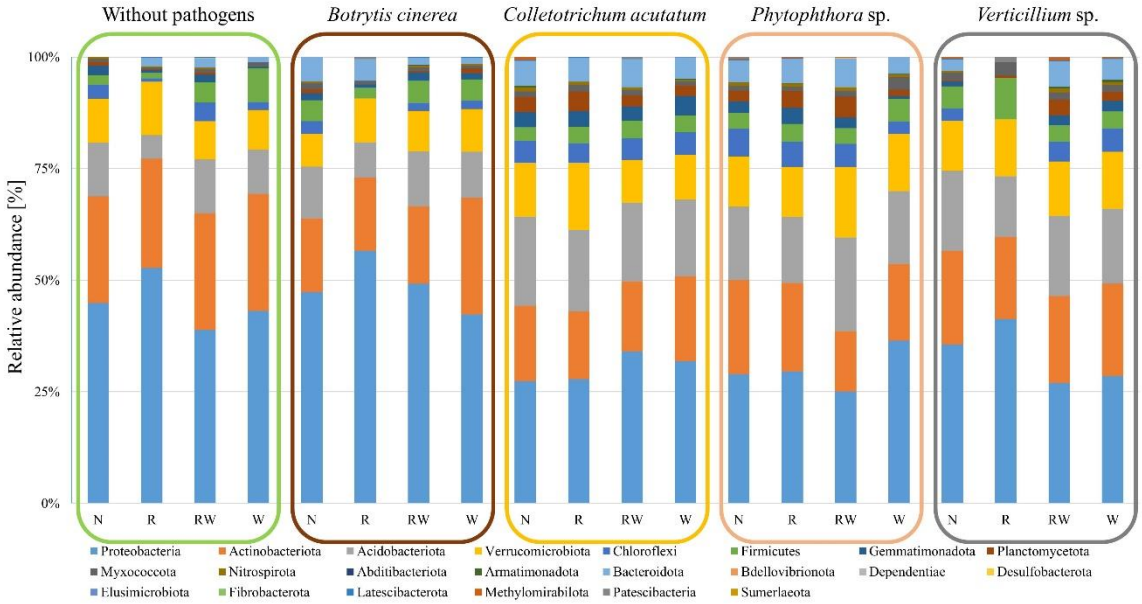
during watering 4 weeks after planting, RW – combined naturalization including root naturalization during planting and naturalization watering 4 weeks after planting.

Table 3. The absorbance values for the wavelength of 590 nm and 750 nm for different carbon sources. Samples for the analysis were taken from the shoots of raspberry plants. N – no naturalization strategy applied, R – root naturalization during planting, W – naturalization during watering 4 weeks after planting, RW – combined naturalization including root naturalization during planting and naturalization watering 4 weeks after planting.

Table 4. The SST values calculated for the wavelengths of 590 nm and 750 nm for different carbon sources, both for rhizosphere and shoots samples. N – no naturalization strategy applied, R – root naturalization during planting, W – naturalization during watering 4 weeks after planting, RW – combined naturalization including root naturalization during planting and naturalization watering 4 weeks after planting.

Table 5. Core bacterial and fungal microorganisms characteristic for naturalization strategies and pathosystems accordingly. N – no naturalization strategy applied, R – root naturalization during planting, W – naturalization during watering 4 weeks after planting, RW – combined naturalization including root naturalization during planting and naturalization watering 4 weeks after planting, WP- without pathogen, B – *Botrytis cinerea*, V – *Verticillium* sp., C – *Colletotrichum acutatum*, P – *Phytophthora* sp.

1038 FIGURE 1



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1040 FIGURE 2

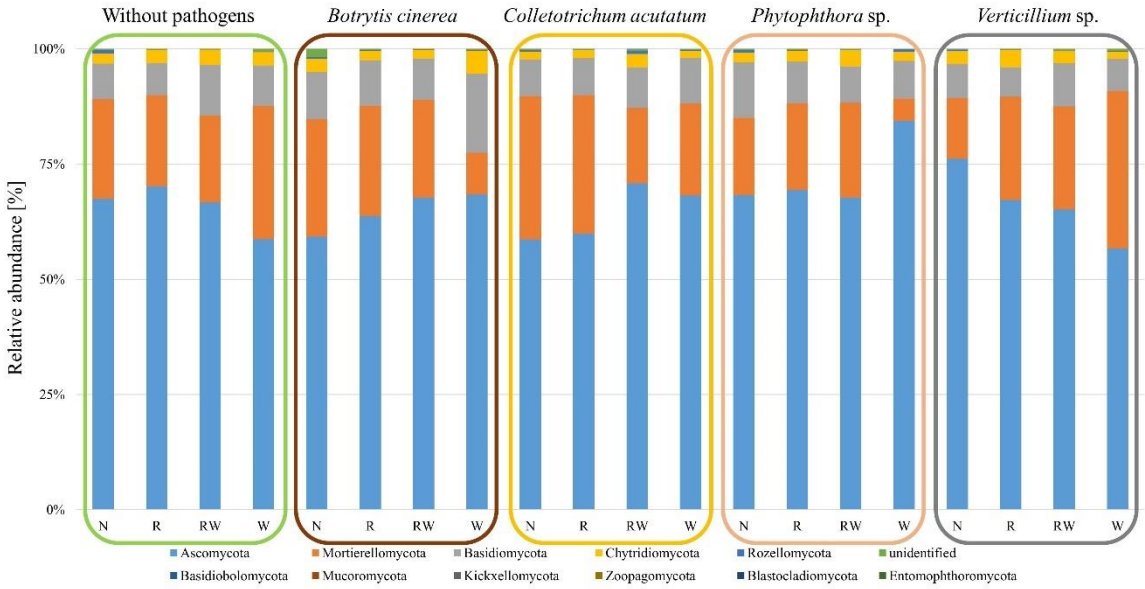

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FIGURE 3

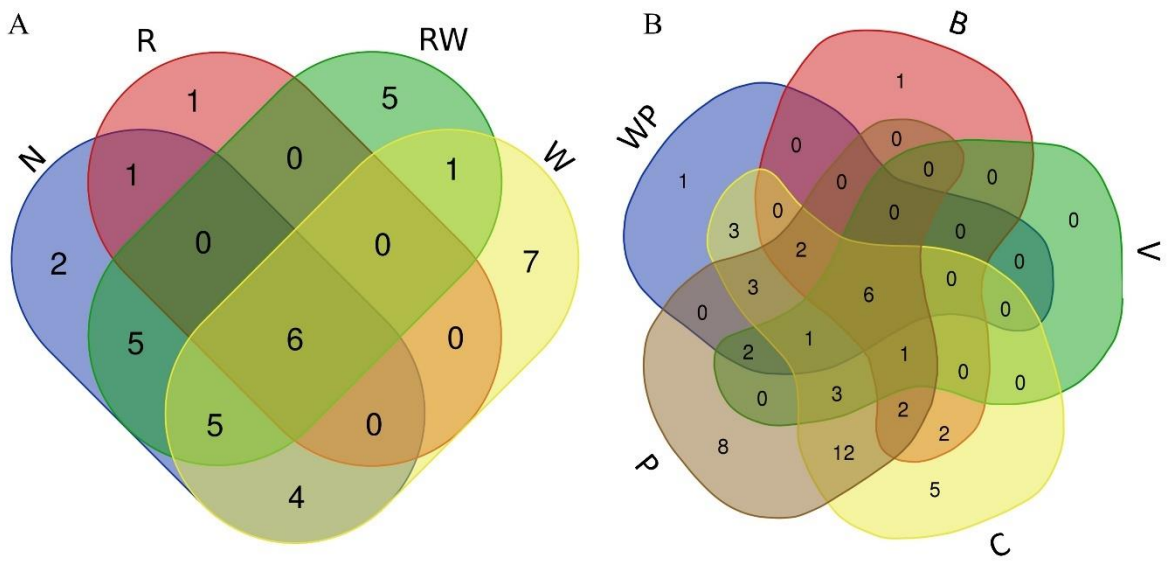
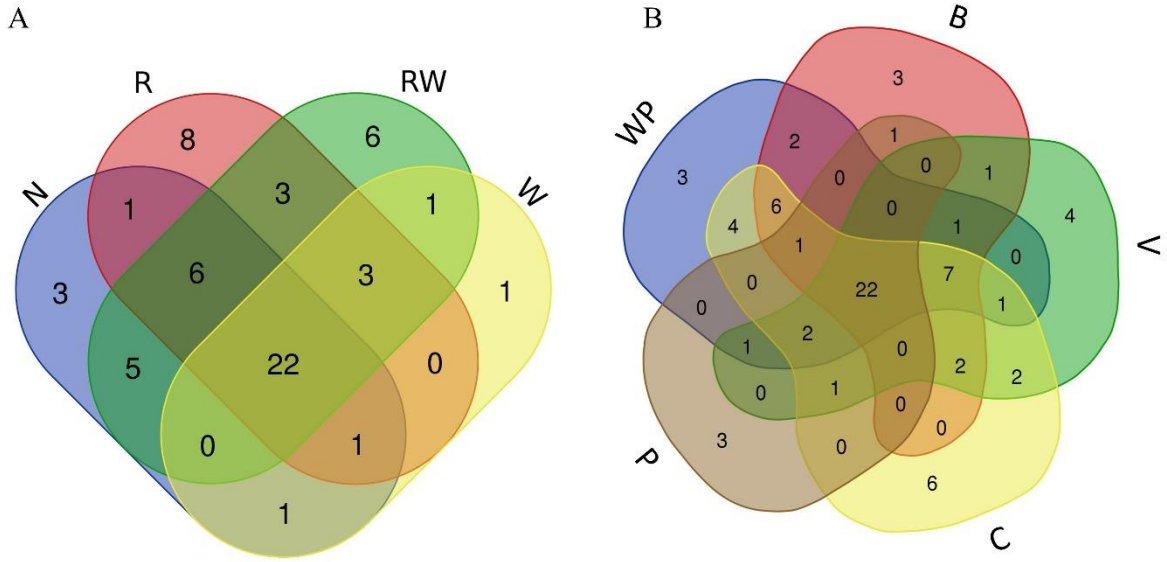
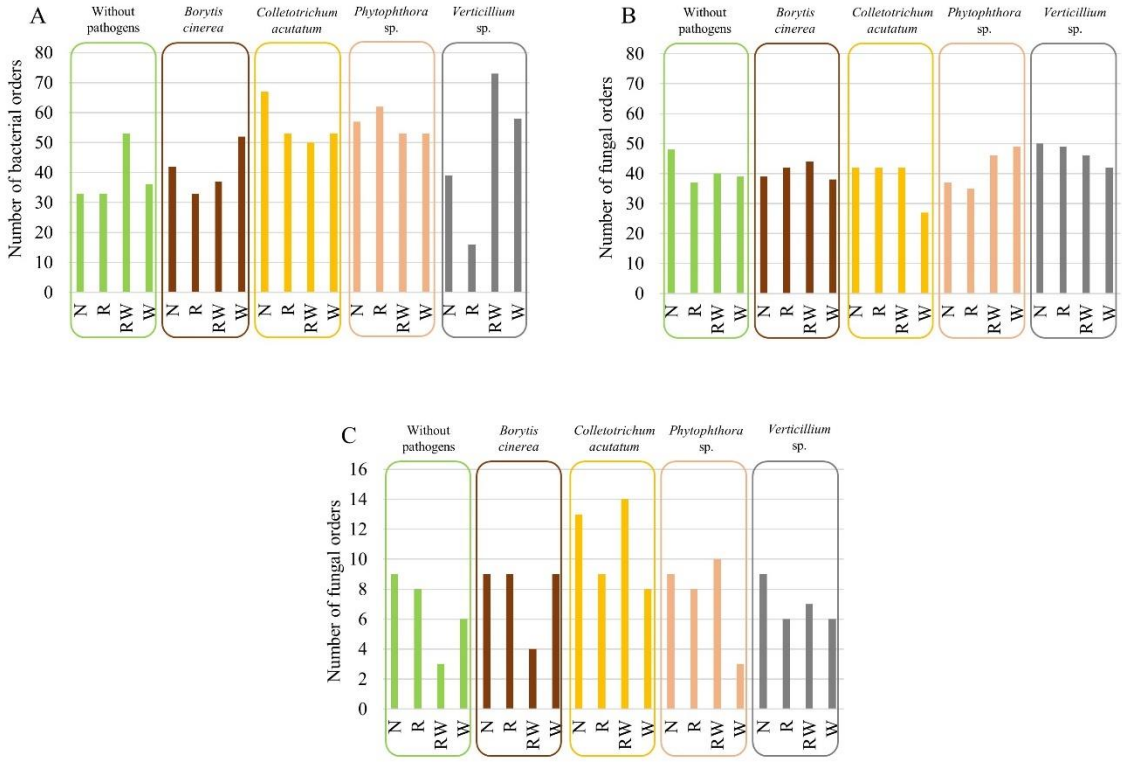


FIGURE 4



1054 FIGURE 5



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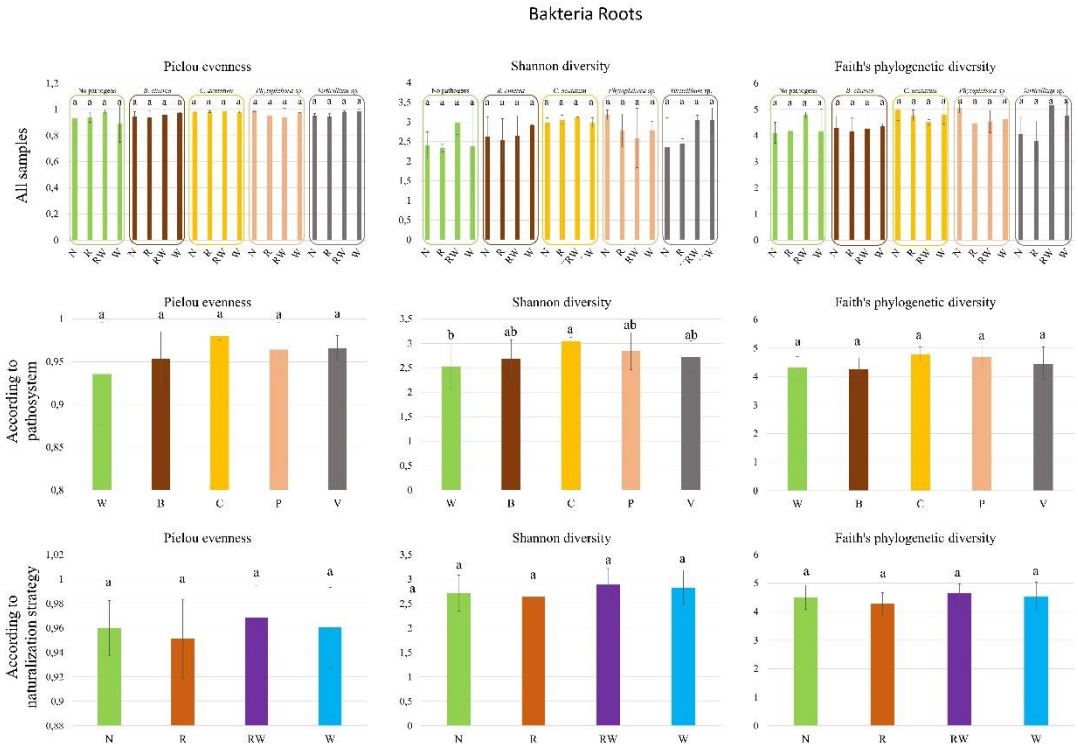
1057 FIGURE 6



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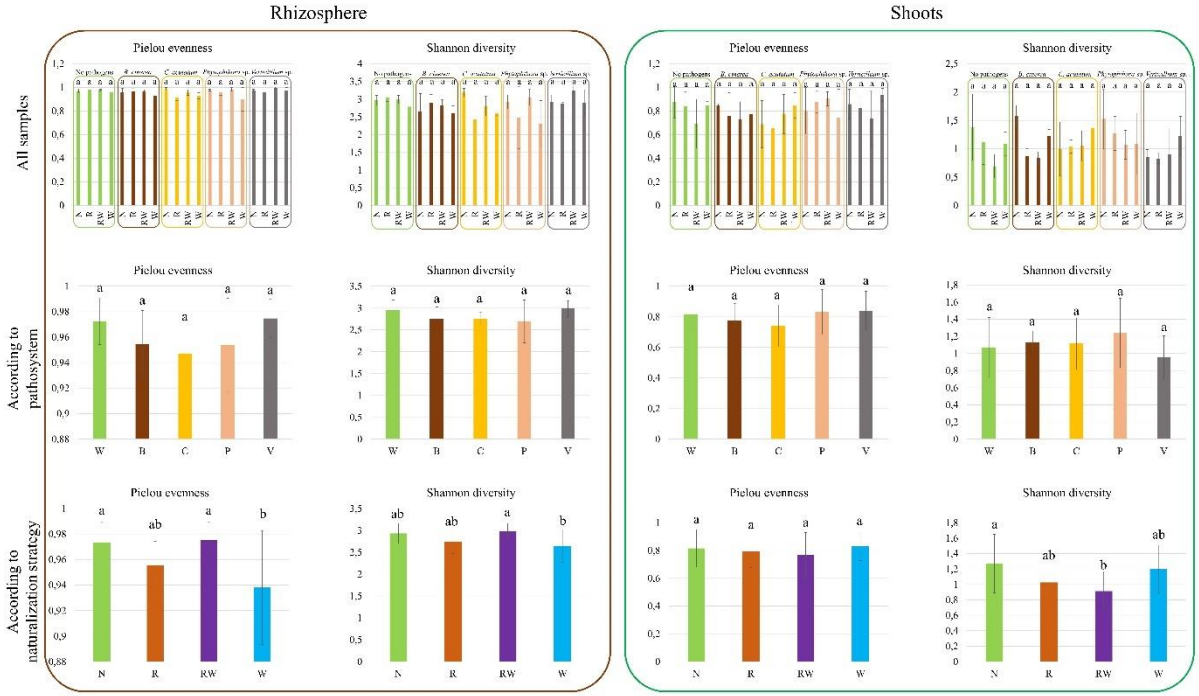
1059 FIGURE 7



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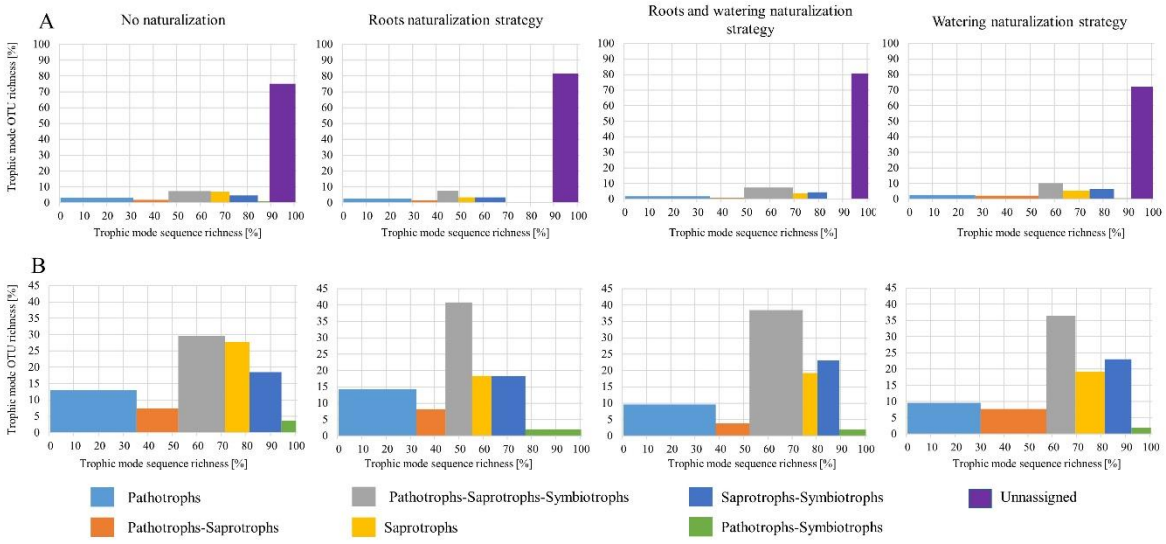
1061 FIGURE 8



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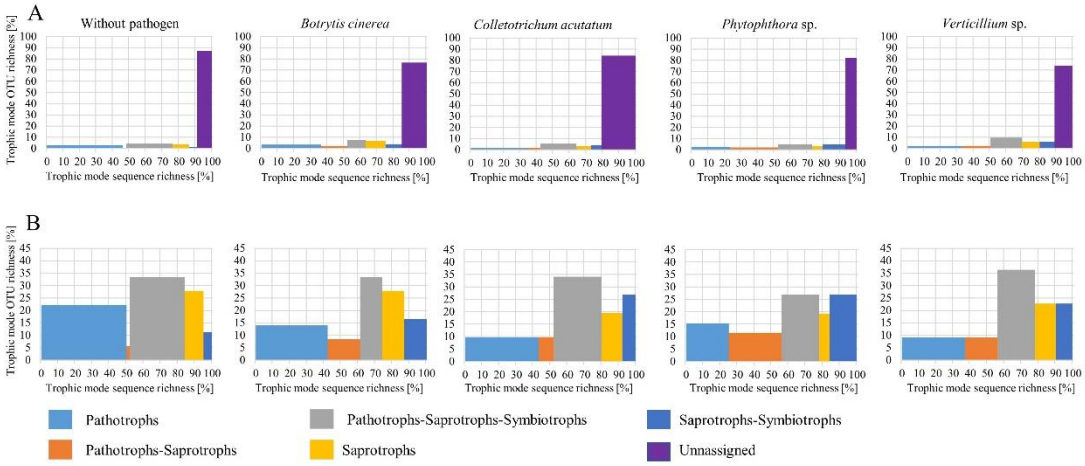
1063 FIGURE 9



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1065 FIGURE 10

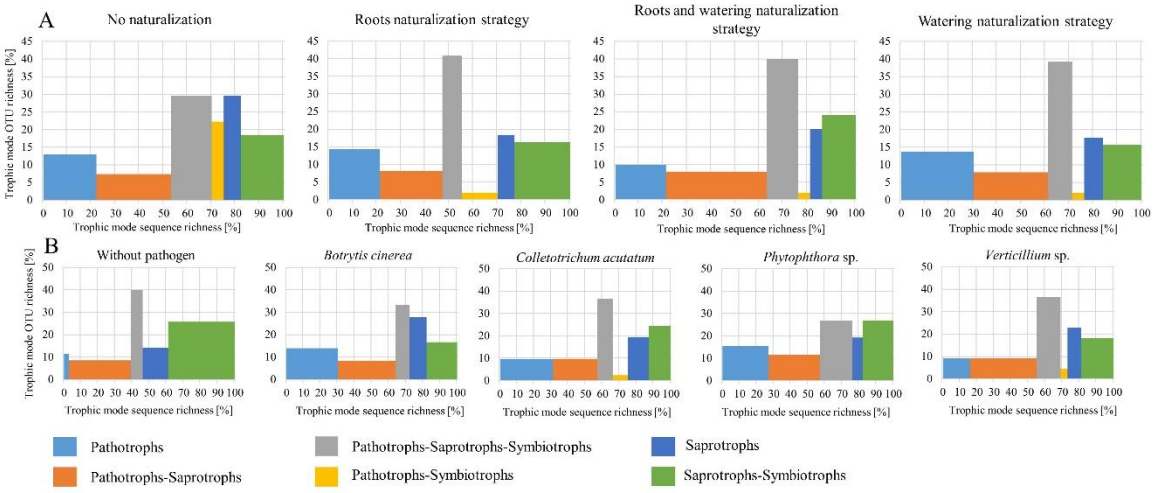

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FIGURE 11

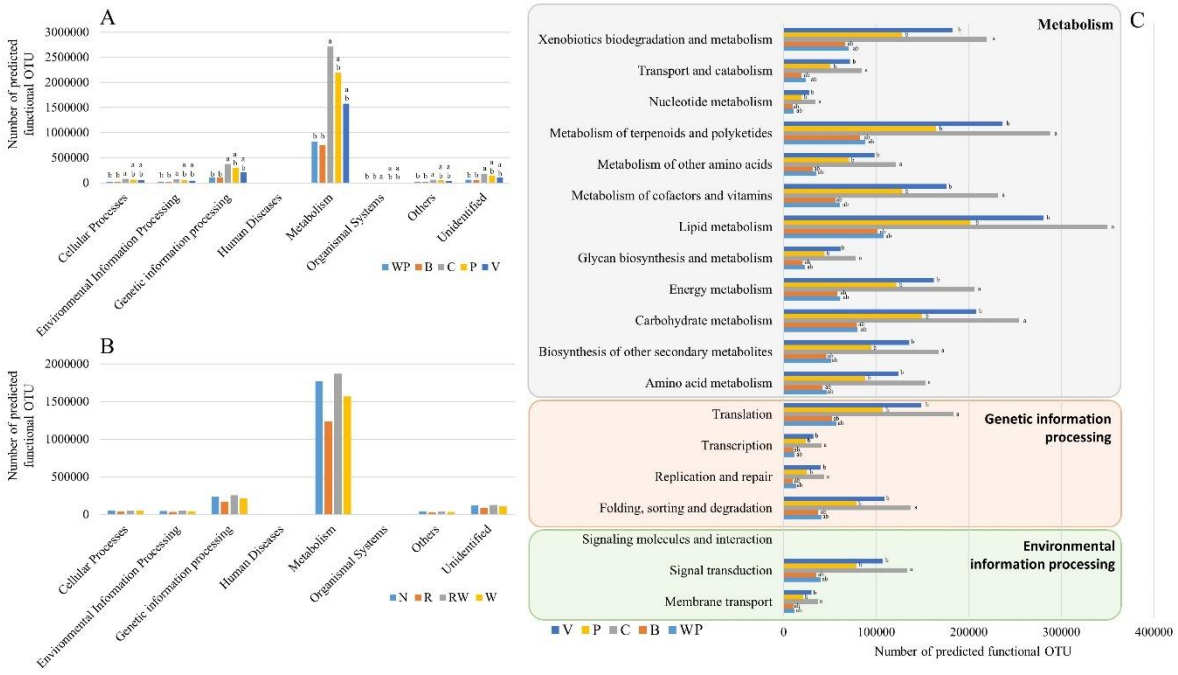
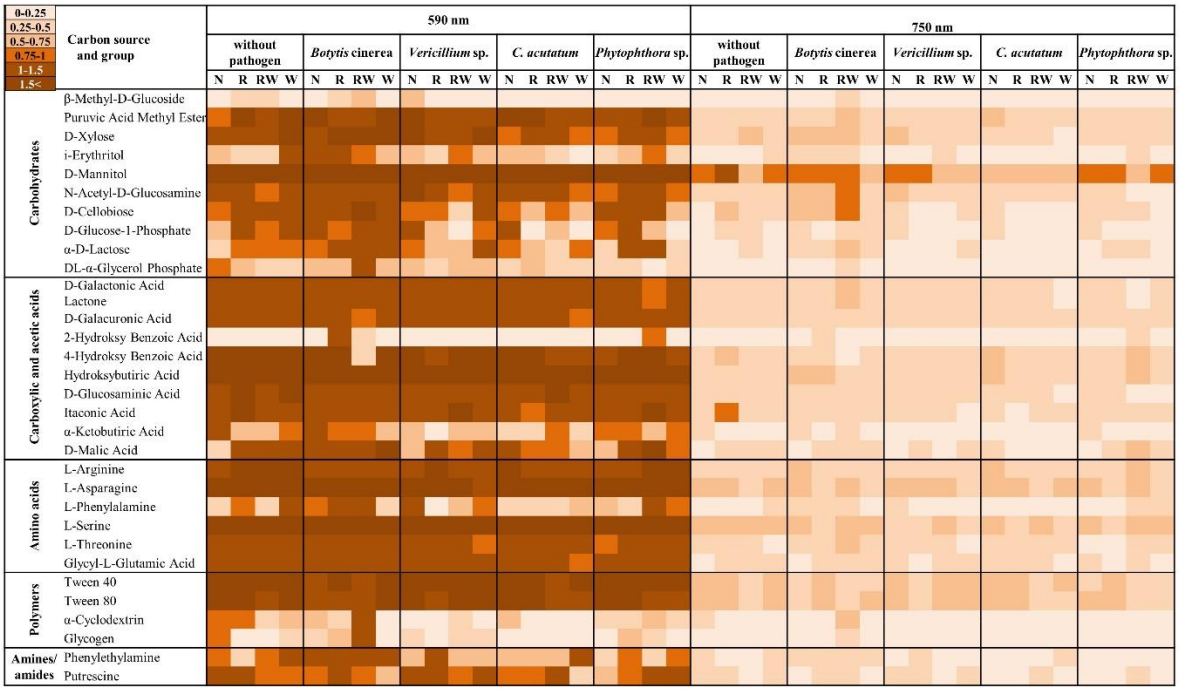


TABLE 1

Starter name	Sequence 5'→ 3'	Reference
ITS1f-KYO1	CTHGGTCATTTAGAGGAASTAA	(Toju et al., 2012)
ITS2-KYO2	TTYRCTRCGTTCTTCATC	(Toju et al., 2012)
16S_V3V4_F	CCTACGGGNGGCWGCAG	(Klindworth et al., 2013)
16S_V3V4_R	GACTACHVGGGTATCTAATCC	(Klindworth et al., 2013)
Overhang F	TCGTCGGCAGCGTCAGA TGTGTATAAGAGACAG	Illumina
Overhang R	GTCTCGTGGGCTCGGAG ATGTGTATAAGAGACAG	Illumina

1076 TABLE 2



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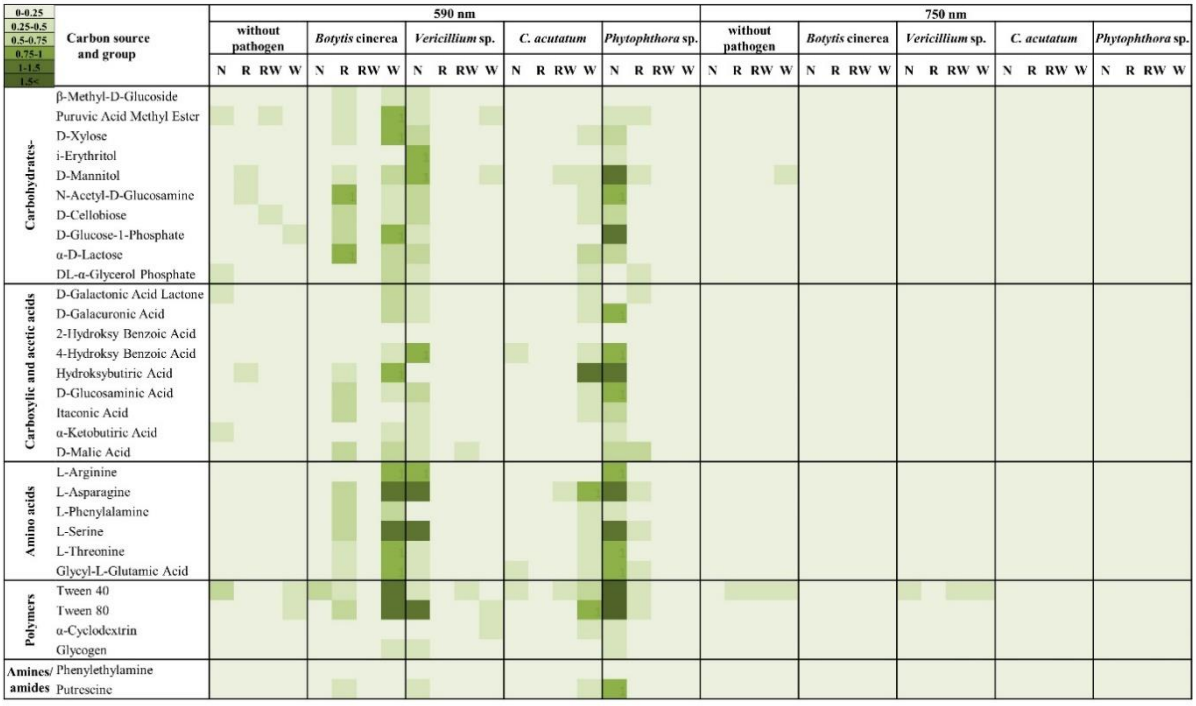
1078 TABLE 3



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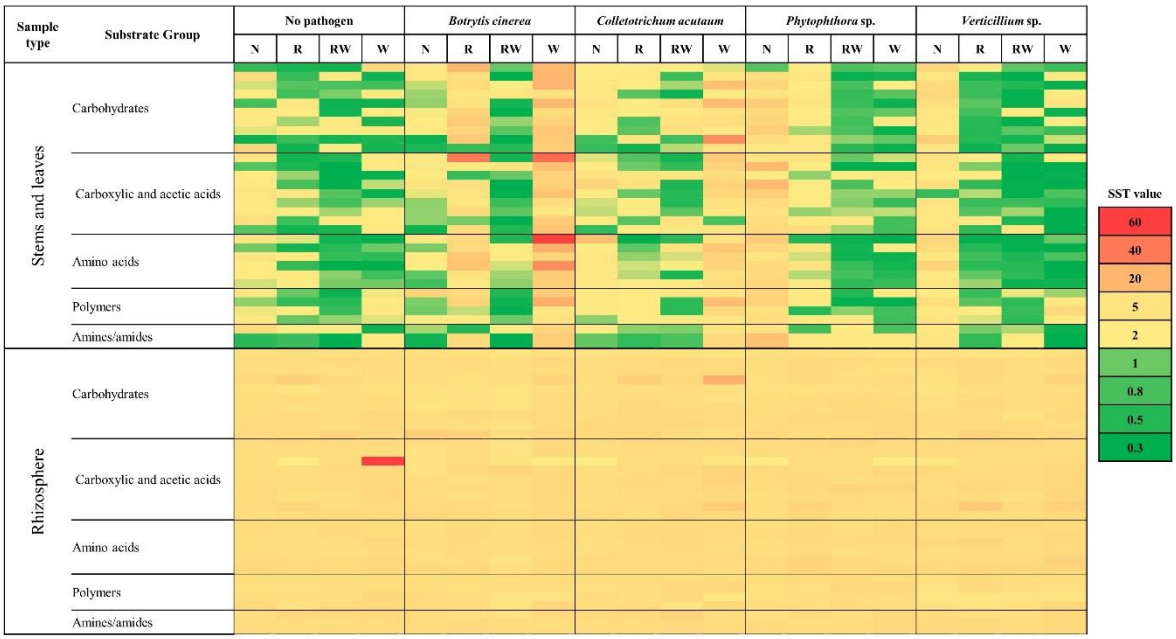
1081 TABLE 4



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1084 TABLE 5

	Naturalization strategies			
	N	**R**	**RW**	**W**
Bacteria	Burkholderiales	Xanthobacteraceae	Micrococcaceae	Actinobacteriota MB-A2-108
	Bradyrhizobium		Chitinophagaceae	Mycobacterium
			Acidimicrobiia IMCC26256	Solirubrobacterales 67-14
			Gemmatimonadaceae	Methyloligellaceae
			Pedosphaeraceae ADurb.Bin063-1	Xanthobacteraceae
				Gaiellales
				Acidobacteriales
Fungi	Hypocreales	Hypocreales	Microascales	Pleosporales
	Helotiales	Agaricales	Sordariales	
	Trechisporales	Agaricales	Helotiales	
		Chaetothyriales	Agaricales	
		Pleosporales	Capnodiales	
		Saccharomycetales	Pyxidiophorales	
		Ascomycota		

	Pathosystems				
	WP	**B**	**V**	**C**	**P**
Bacteria	Xanthobacteraceae	Pseudolabrys		Ktedonobacteria C0119	Acetobacteraceae
				Acidimicrobiia IMCC26256	Solirubrobacterales 67-14
				Parafilimonas	Blastocatellaceae
				Flavisolibacter	Reyranella
				Pedosphaeraceae ADurb.Bin063-1	Rhodanobacter
					Pedosphaeraceae Ellin517
					Rhizobium
Fungi	Microascales	Pleosporales	Chaetothyriales	Chaetothyriales	Hypocreales
	Sordariales	Pleosporales	Pezizales	Helotiales	Eurotiales
	Cantharellales	Ascomycota	Agaricales	Chaetothyriales	
			Pyxidiophorales	Pleosporales	
				Hypocreales	
				Helotiales	

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10. Oświadczenia

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

Mgr inż. Michał Pylak
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

Oświadczenie

Niniejszym oświadczam, że w poniższej pracy inicjatywa podjętych badań jest moim wkładem intelektualnym.

P1: Pylak, M., Oszust, K. & Frąc, M. (2019). Review report on the role of bioproducts, biopreparations, biostimulants and microbial inoculants in organic production of fruit. *Rev Environ Sci Biotechnol* 18, 597–616 https://doi.org/10.1007/s11157-019-09500-5

Mój wkład w powyższą pracę polegał na:

- wykonaniu przeglądu literatury dotyczącego biopreparatów stosowanych w rolnictwie i mechanizmów ich działania,
- przygotowaniu manuskryptu.

Michał Pylak

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

Mgr inż. Michał Pylak
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

Oświadczenie

Niniejszym oświadczam, że w poniższej pracy inicjatywa podjętych badań jest moim wkładem intelektualnym.

P2: Pylak, M., Oszust, K., & Frąc, M. (2020). Searching for New Beneficial Bacterial Isolates of Wild Raspberries for Biocontrol of Phytopathogens-Antagonistic Properties and Functional Characterization. *International journal of molecular sciences*, *21*(24), 9361. https://doi.org/10.3390/ijms21249361

Mój wkład w powyższą pracę polegał na:

- przeprowadzeniu izolacji środowiskowych szczepów bakterii oraz ich identyfikacji,
- zbadaniu właściwości antagonistycznych oraz katalitycznych wyizolowanych szczepów,
- analizie uzyskanych wyników,
- statystycznym i graficznym opracowaniu wyników,
- przygotowaniu manuskryptu.

Michał Pylak

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

Mgr inż. Michał Pylak
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

Oświadczenie

Niniejszym oświadczam, że w poniższej pracy inicjatywa podjętych badań jest moim wkładem intelektualnym.

P3: Pylak, M., Oszust, K., & Frąc, M. (2021). Optimization of Growing Medium and Preservation Methods for Plant Beneficial Bacteria, and Formulating a Microbial Biopreparation for Raspberry Naturalization. *Agronomy*, *11*(12), 2521. https://doi.org/10.3390/agronomy11122521

Mój wkład w powyższą pracę polegał na:

- przeprowadzeniu optymalizacji warunków hodowli i prezerwacji szczepów bakterii,
- zaprojektowaniu i przeprowadzeniu doświadczenia wazonowego,
- analizie uzyskanych wyników,
- statystycznym i graficznym opracowaniu wyników,
- przygotowaniu manuskryptu.

Michał Pylak

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

Mgr inż. Michał Pylak
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

Oświadczenie

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

P4: Pylak M., Oszust K., Panek J., Frąc M.: Structural and functional shift of soil rhizosphere and raspberry shoots microbiomes underlying changes caused by phytopahogens contamination and naturalization strategies implementation). Praca w recenzji.

Mój wkład w powyższą pracę polegał na:

- analizie uzyskanych wyników,
- statystycznym i graficznym opracowaniu wyników,
- przygotowaniu manuskryptu.

Michał Pylak

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

prof. dr hab. Magdalena Frąc
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

Oświadczenie

Niniejszym oświadczam, że w poniższej pracy inicjatywa podjętych badań jest wkładem intelektualnym mgra inż. Michała Pylaka.

P1: Pylak, M., Oszust, K. & Frąc, M. (2019). Review report on the role of bioproducts, biopreparations, biostimulants and microbial inoculants in organic production of fruit. *Rev Environ Sci Biotechnol* 18, 597–616 https://doi.org/10.1007/s11157-019-09500-5

Mój wkład w powyższą pracę polegał na:

- określeniu koncepcji, problematyki i zakresu pracy,
- edycji i recenzji manuskryptu.

Jednocześnie wyrażam zgodę, aby praca została wykorzystana w rozprawie doktorskiej mgra inż. Michała Pylaka.

Magdalena Frąc

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

prof. dr hab. Magdalena Frąc
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

Oświadczenie

Niniejszym oświadczam, że w poniższej pracy inicjatywa podjętych badań jest wkładem intelektualnym mgra inż. Michała Pylaka.

P2: Pylak, M., Oszust, K., & Frąc, M. (2020). Searching for New Beneficial Bacterial Isolates of Wild Raspberries for Biocontrol of Phytopathogens-Antagonistic Properties and Functional Characterization. *International journal of molecular sciences*, *21*(24), 9361. https://doi.org/10.3390/ijms21249361

Mój wkład w powyższą pracę polegał na:

- określeniu koncepcji, problematyki i zakresu badań,
- edycji i recenzji manuskryptu,
- nadzorowaniu kolejnych etapów badań oraz procesu przygotowania manuskryptu,
- kierowaniu przebiegiem badań zgodnie z założeniami projektu,
- pozyskiwaniu środków finansowych umożliwiających realizację badań.

Jednocześnie wyrażam zgodę, aby praca została wykorzystana w rozprawie doktorskiej mgra inż. Michała Pylaka.

Magdalena Frąc

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

prof. dr hab. Magdalena Frąc
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

Oświadczenie

Niniejszym oświadczam, że w poniższej pracy inicjatywa podjętych badań jest wkładem intelektualnym mgra inż. Michała Pylaka.

P3: Pylak, M., Oszust, K., & Frąc, M. (2021). Optimization of Growing Medium and Preservation Methods for Plant Beneficial Bacteria, and Formulating a Microbial Biopreparation for Raspberry Naturalization. *Agronomy*, *11*(12), 2521. https://doi.org/10.3390/agronomy11122521

Mój wkład w powyższą pracę polegał na:

- określeniu koncepcji, problematyki i zakresu badań,
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Jednocześnie wyrażam zgodę, aby praca została wykorzystana w rozprawie doktorskiej mgra inż. Michała Pylaka.

Magdalena Frąc

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

prof. dr hab. Magdalena Frąc
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

Oświadczenie

Niniejszym oświadczam, że w niżej wymienionym manuskrypcie artykułu naukowego inicjatywa podjętych badań jest wkładem intelektualnym mgra inż. Michała Pylaka.

P4: Pylak M., Oszust K., Panek J., Frąc M.,: Structural and functional shift of soil rhizosphere and raspberry shoots microbiomes underlying changes caused by phytopahogens contamination and naturalization strategies implementation). Praca w recenzji.

Mój wkład w powyższy manuskrypt polegał na:

- określeniu koncepcji, problematyki i zakresu badań,
- edycji manuskryptu,
- nadzorowaniu kolejnych etapów badań oraz procesu przygotowania manuskryptu,
- kierowaniu przebiegiem badań zgodnie z założeniami projektu,
- pozyskiwaniu środków finansowych umożliwiających realizację badań.

Jednocześnie wyrażam zgodę, aby powyższy manuskrypt artykułu naukowego został wykorzystany w rozprawie doktorskiej mgra inż. Michała Pylaka.

Magdalena Frąc

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

dr Karolina Oszust
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

Oświadczenie

Niniejszym oświadczam, że w poniższej pracy inicjatywa podjętych badań jest wkładem intelektualnym mgra inż. Michała Pylaka.

P1: Pylak, M., Oszust, K. & Frąc, M. (2019). Review report on the role of bioproducts, biopreparations, biostimulants and microbial inoculants in organic production of fruit. *Rev Environ Sci Biotechnol* 18, 597–616 https://doi.org/10.1007/s11157-019-09500-5

Mój wkład w powyższą pracę polegał na:

- określeniu problematyki i zakresu pracy,
- edycji i recenzji manuskryptu.

Jednocześnie wyrażam zgodę, aby praca została wykorzystana w rozprawie doktorskiej mgra inż. Michała Pylaka.

K. Oszust

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

dr Karolina Oszust
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

Oświadczenie

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Mój wkład w powyższą pracę polegał na:

- określeniu problematyki i zakresu badań,
- analizie i interpretacji wyników badań,
- edycji i recenzji manuskryptu,
- nadzorowaniu kolejnych etapów badań oraz procesu przygotowania manuskryptu.

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K. Oszust

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

dr Karolina Oszust
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

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Jednocześnie wyrażam zgodę, aby praca została wykorzystana w rozprawie doktorskiej mgra inż. Michała Pylaka.

K. Osz

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

dr Karolina Oszust
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

Oświadczenie

Niniejszym oświadczam, że w niżej wymienionym manuskrypcie artykułu naukowego inicjatywa podjętych badań jest wkładem intelektualnym mgra inż. Michała Pylaka.

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- edycji manuskryptu,
- określeniu problematyki i zakresu badań,
- analizie i interpretacji wyników badań,
- nadzorowaniu kolejnych etapów badań oraz procesu przygotowania manuskryptu.

Jednocześnie wyrażam zgodę, aby powyższy manuskrypt artykułu naukowego został wykorzystany w rozprawie doktorskiej mgra inż. Michała Pylaka.

K. Oszust

INSTYTUT AGROFIZYKI PAN

Lublin, 31.08.2022

dr inż. Jacek Panek
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

Oświadczenie

Niniejszym oświadczam, że w niżej wymienionym manuskrypcie artykułu naukowego inicjatywa podjętych badań jest wkładem intelektualnym mgra inż. Michała Pylaka.

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Mój wkład w powyższą pracę polegał na:

- przeprowadzeniu sekwencjonowania NGS,
- analizie wyników sekwencjonowania NGS z wykorzystaniem środowiska QIIME2 i PICRUSt.

Jednocześnie wyrażam zgodę, aby powyższy manuskrypt artykułu naukowego został wykorzystany w rozprawie doktorskiej mgra inż. Michała Pylaka.

11. Aneks – Życiorys naukowy

Dane personalne:

Imię i nazwisko: Michał Pylak

Wykształcenie:

2010 - 2013

Prywatne Liceum Ogólnokształcące im. Królowej Jadwigi w Lublinie, profil biologiczno-chemiczny.

2013 - 2017

Uniwersytet Przyrodniczy w Lublinie, Wydział Nauk o Żywności i Biotechnologii, studia stacjonarne I°, kierunek: biotechnologia

2017 – uzyskanie tytułu **inżynier**, praca pt.: Biotechnologiczna produkcja mannitolu, wykonana w Katedrze Biotechnologii, Mikrobiologii i Żywienia Człowieka pod opieką promotorską dr Kennetha Udeha.

2017 - 2018

Uniwersytet Przyrodniczy w Lublinie, Wydział Nauk o Żywności i Biotechnologii, studia stacjonarne II°, kierunek: biotechnologia, specjalność: nowoczesne techniki analityczne, 2018 – uzyskanie tytułu **magister**, praca pt.: Molekularne metody identyfikacji oraz różnicowania szczepów bakterii należących do gatunku *Lactobacillus casei*, wykonana w Katedrze Biotechnologii, Mikrobiologii i Żywienia Człowieka pod opieką promotorską dr Piotra Jarockiego.

2017-2018 - wykonawca projektu badawczego nr 2016/23/D/NZ9/02661 pt. „Analiza obecności, bioróżnorodności oraz fizjologicznego znaczenia sekwencji bakteriofagowych występujących w genomach bakterii jelitowych z grupy *Lactobacillus casei*" finansowanego przez Narodowe Centrum Nauki i realizowanego przez Uniwersytet Przyrodniczy w Lublinie, Wydział Nauk o Żywności i Biotechnologii pod kierownictwem dr inż. Piotra Jarockiego.

2018 – obecnie

Instytut Agrofizyki im. Bohdana Dobrzańskiego Polskiej Akademii Nauk, Zakład Badań Systemu Gleba-Roślina, studia stacjonarne III°.

Tytuł pracy doktorskiej: **Opracowanie bakteryjnego kompleksu do naturalizacji ryzosfery malin**, wykonywanej w Zakładzie Badań Systemu Gleba-Roślina pod opieką promotora prof. dr hab. Magdaleny Frąc oraz promotora pomocniczego dr Karoliny Oszust.

2019 – obecnie

Instytut Agrofizyki im. Bohdana Dobrzańskiego Polskiej Akademii Nauk,
Zakład Badań Systemu Gleba-Roślina.

Praca na stanowisku **specjalista inżynieryjno-badawczy** przy projekcie „Nowe rozwiązania biotechnologiczne w diagnostyce, zwalczaniu i monitoringu kluczowych patogenów grzybowych w ekologicznej uprawie owoców miękkich", finansowanego przez Narodowe Centrum Badań i Rozwoju w ramach programu BIOSTRATEG, numer umowy BIOSTRATEG3/344433/16/NCBR/2018, realizowanego przez Instytut Agrofizyki im. Bohdana Dobrzańskiego Polskiej Akademii Nauk pod kierownictwem prof. dr hab. Magdaleny Frąc.

Publikacje:

1. **Pylak, M.**, Oszust, K., & Frąc, M. (2021). Optimization of Growing Medium and Preservation Methods for Plant Beneficial Bacteria, and Formulating a Microbial Biopreparation for Raspberry Naturalization. *Agronomy*, *11*(12), 2521. https://doi.org/10.3390/agronomy11122521. Wskaźnik Impact Factor: 3,949; punktacja MNiSW: 100 punktów.
2. Oszust K, **Pylak M**, Frąc M. *Trichoderma*-Based Biopreparation with Prebiotics Supplementation for the Naturalization of Raspberry Plant Rhizosphere. *International Journal of Molecular Sciences*. 2021; 22(12):6356. https://doi.org/10.3390/ijms22126356. Wskaźnik Impact Factor: 6,010; punktacja MNiSW: 140 punktów.
3. **Pylak, M.**, Oszust, K., & Frąc, M. (2020). Searching for New Beneficial Bacterial Isolates of Wild Raspberries for Biocontrol of Phytopathogens-Antagonistic Properties and Functional Characterization. *International journal of molecular sciences*, *21*(24), 9361. https://doi.org/10.3390/ijms21249361. Wskaźnik Impact Factor: 5,924; punktacja MNiSW: 140 punktów.
4. **Pylak, M.**, Oszust, K. & Frąc, M. (2019). Review report on the role of bioproducts, biopreparations, biostimulants and microbial inoculants in organic production of fruit. *Rev Environ Sci Biotechnol* 18, 597–616 https://doi.org/10.1007/s11157-019-09500-5. Wskaźnik Impact Factor: 5,261; punktacja MNiSW: 140 punktów.

Doniesienia zjazdowe:

2018 – 2022

Konferencje ogólnopolskie: **11**

Konferencje międzynarodowe: **5**

w tym wyróżnienie w konkursie na najlepszy poster:

1. Wpływ szczepów bakterii użytych do naturalizacji ryzosfery malin, wyizolowanych z ryzosfery roślin dzikorosnących, na społeczność bakterii glebowych w doświadczeniu wazonowym na V Ogólnopolskim Sympozjum Mikrobiologicznym 2021 **„Metagenomy Różnych Środowisk”**, Warszawa 2021.

Zgłoszenia patentowe:

1. **Zgłoszenie patentowe** P.434148 z dnia 2020-06-01, Oszust K., **Pylak M.**, Frąc M. Sposób otrzymywania biopreparatu do naturalizacji ryzosfery roślin malin o właściwościach antagonistycznych w stosunku do fitopatogenów grzybowych należących do rodzajów *Botrytis, Verticillium, Colletotrichum* i *Phytophthora* z zastosowaniem szczepów grzyba z rodzaju *Trichoderma*, biopreparat do naturalizacji ryzosfery roślin malin, sposób prowadzenia hodowli szczepów grzybów z rodzaju *Trichoderma* do zastosowania w biopreparacie oraz kompozycja podłoża namnażającego dla grzybów z rodzaju *Trichoderma*.
2. **Zgłoszenie patentowe** P.435777 z dnia 2020-10-26, Frąc M., Oszust K., **Pylak M.** Sposób otrzymywania bakteryjnego biopreparatu do naturalizacji ryzosfery roślin malin o działaniu biostymulacyjnym dla roślin, poprawiającym jakość gleby, w tym transformacje materii organicznej, przy jednoczesnych właściwościach antagonistycznych w stosunku do fitopatogenów grzybowych należących do rodzajów *Botrytis, Verticillium, Colletotrichum* i *Phytophthora* oraz biopreparat do naturalizacji ryzosfery roślin malin.

Podnoszenie kwalifikacji poprzez udział w szkoleniach:

8-12.08.2022 – Szkolenie bioinformatyczne dotyczące analizy danych metagenomicznych z wykorzystaniem środowisk QIIME2 oraz PICRUSt, oraz FUNGuild, baz danych SILVA i UNITE odpowiednio dla bakterii i grzybów przeprowadzonym przez holenderską firmę GreenFinch Research.

03.2022 – Szkolenie „Data Analytics With R” dotyczące analizy danych statystycznych z wykorzystaniem środowiska R i programu RStudio, przeprowadzone w formie zdalnej przez firmę NobleProg Polska.

30.01.2020 – Szkolenie „Podstawy i bezpieczeństwo sterylizacji parowej” dotyczące obsługi sterylizatorów parowych, przeprowadzone przez firmę Cheminst Polska.

01.2019 – Szkolenie aplikacyjne obejmujące szlak wykonywania bibliotek, metagenomikę i sekwencjonowanie małych genomów, przeprowadzone przez firmę ANALITYK.