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Wpływ ryzobakterii na wzrost rzepaku i rodzime społeczności drobnoustrojów w ryzosferze

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Spis treści

Streszczenie

Ryzobakterie promujące wzrost roślin (PGPR) stanowią przyjazną dla środowiska alternatywę ograniczającą stosowanie chemicznych nawozów i fungicydów. PGPR poprawiają wzrost roślin poprzez wydzielanie fitohormonów, rozpuszczanie fosforanów, wiązanie azotu, zwiększanie wchłaniania składników odżywczych i działanie jako środki biokontroli patogenów. Skuteczność inokulacji PGPR jest powiązana z ich zdolnością kolonizacji korzenia, a także przetrwania w obecności rodzimych zbiorowisk bakterii i grzybów w ryzosferze.

Celem niniejszej rozprawy doktorskiej była charakterystyka i identyfikacja bakterii wyizolowanych z ryzosfery korzeni rzepaku uprawianego na dwóch gospodarstwach wiejskich położonych w Górsku w województwie kujawsko-pomorskim i w Ostródzie w województwie warmińsko-mazurskim, ocena wpływu ryzobakterii na poprawę wzrostu i rozwoju rzepaku, poszukiwanie genów przyczyniających się do promowania wzrostu roślin i biokontroli patogenów roślinnych, a także określenie wpływu inokulacji ryzosfery PGPR na liczebność mikrooganizmów związanych z obiegiem azotu oraz bioróżnorodność rodzimych zbiorowisk bakteryjnych i grzybowych.

W badaniach wstępnych, przeprowadzono analizę określenia liczebności ryzobakterii hodowlanych z różnych faz wzrostu rzepaku (wegetatywnej, kwitnienia i dojrzewania), uprawianego w Górsku i w Ostródzie. Badania wykazały złożoną dynamikę zbiorowisk drobnoustrojów, ujawniając różnice w liczebności hodowlanych ryzobakterii na różnych etapach wzrostu rzepaku. W przypadku ryzosfery rzepaku pobranego z Górska, populacja hodowlanych ryzobakterii była najwyższa w fazie wegetatywnej, zmniejszała się w fazie kwitnienia, a następnie zwiększała się w fazie dojrzałości rośliny. Natomiast w ryzosferze rzepaku pobranego z Ostródy, liczebność hodowlanych bakterii ryzosferowych była najwyższa w fazie kwitnienia, po czym zmniejszyła się kolejno w fazie dojrzałości i wegetatywnej rośliny.

W ramach niniejszej pracy doktorskiej, wyizolowano 300 izolatów bakteryjnych: po 150 izolatów z ryzosfery korzeni rzepaku uprawianego w Górsku i w Ostródzie, w tym po 50 izolatów z każdej fazy wzrostu rośliny: wegetatywnej, kwitnienia i dojrzewania. Następnie, przeprowadzono charakterystykę ich właściwości promujących wzrost roślin (PGP), w tym produkcję kwasu indolilo-3-octowego (IAA), fosforanów, deaminazy ACC, sideroforów, chitynaz, cyjanowodoru (HCN) i amoniaku. Badanie miało na celu sprawdzenie czy istnieje związek między liczebnością ryzobakterii izolowanych z różnych faz wzrostu rośliny, a ich właściwościami PGP. W ryzosferze rzepaku uprawianego w Górsku, największą populację

hodowlanych ryzobakterii stwierdzono w fazie wegetatywnej rzepaku, a cztery z siedmiu badanych cech PGP, takich jak: produkcja deaminazy ACC, sideroforów, HCN i chitynaz były wytwarzane przez najwyższy procent izolatów również w fazie wegetatywnej rośliny. W przypadku ryzosfery rzepaku uprawianego w Ostródzie, najwyższą liczebność mikroorganizmów ryzosferowych odnotowano w próbach pobranych w fazie kwitnienia rzepaku i również z tej fazy wzrostu rośliny wyizolowano najwięcej szczepów zdolnych do produkcji IAA, ACC deaminazy, fosforanów, sideroforów oraz amoniaku.

W kolejnym etapie badań, ryzobakterie, które wykazywały wysokie aktywności co najmniej czterech z siedmiu badanych cech PGP zidentyfikowano i wytypowano do doświadczenia doniczkowego, w celu zbadania ich zdolności promujących wzrost rzepaku w warunkach sterylnych. Badania wazonowe w warunkach sterylnych pozwoliły wytypować szczepy najlepiej promujące wzrost roślin. Należały do nich: *Bacillus paralicheniformis* 2R5, wyizolowany z ryzosfery rzepaku w Górsku oraz *Peribacillus frigoritolerans* 2RO30 i *Pseudomonas sivasensis* 2RO45 pochodzące z ryzosfery rzepaku w Ostródzie. W oparciu o doświadczenia w warunkach sterylnych, *B. paralicheniformis* 2R5, *P. frigoritolerans* 2RO30 i *P. sivasensis* 2RO45 wybrano do badań doniczkowych w niesterylnej glebie. Badania te miały na celu sprawdzenie czy izolaty będą promowały wzrost rzepaku również w obecności rodzimych mikroorganizmów glebowych. Dodatkowo w doświadczeniach doniczkowych z użyciem gleby niesterylnej, wykorzystano konsorcjum składające się z dwóch izolatów *P. frigoritolerans* 2RO30 i *P. sivasensis* 2RO45, aby sprawdzić czy konsorcjum drobnoustrojów będzie wykazywało lepsze działanie promujące niż pojedyncze szczepy. Badania wazonowe w niesterylnej glebie wykazały, że tylko pojedyncze szczepy *B. paralicheniformis* 2R5 i *P. sivasensis* 2RO45 promowały wzrost rzepaku.

Ryzobakterie promujące wzrost rzepaku: *B. paralicheniformis* 2R5 i *P. sivasensis* 2RO45 poddano analizie sekwencjonowania genomu w celu znalezienia genów odpowiedzialnych za promowanie wzrostu roślin i biologiczną kontrolę fitopatogenów. Analiza genomu *B. paralicheniformis* 2R5 wykazała obecność genów odpowiedzialnych za biosyntezę IAA, solubilizację fosforanów, sekwestrację sideroforów i produkcję chitynaz. Natomiast, w genomie *P. sivasensis* 2RO45 znaleziono geny odpowiedzialne za biosyntezę IAA, solubilizację fosforanów, produkcję ACC deaminazy i sekwestrację sideroforów. W genomach tych szczepów zidentyfikowano również wiele biosyntetycznych klastrów genów, które kodują biologicznie czynne metabolity wtórne wykazujące działanie przeciwgrzybowe.

Ponadto, w genomie *B. paralicheniformis* 2R5 znaleziono geny związane z obiegiem azotu, takie jak *narG* oraz *nosZ*. Z tego względu, sprawdzono czy inokulacja *B. paralicheniformis* 2R5 może mieć wpływ na liczbę kopii tych genów w ryzosferze rzepaku oraz oznaczono liczbę kopii innych równie ważnych genów związanych z obiegiem azotu, takich jak *nifH, nirS* i *amoA*. Badania wykazały, że obecność genów *narG* i *nosZ* w genomie *B. paralicheniformis* 2R5, była zbieżna z istotnymi zmianami w liczbie kopii genów związanych z cyklem azotu w ryzosferze. Inokulacja *B. paralicheniformis* 2R5 spowodowała wzrost liczby kopii genów zarówno *narG* i *nosZ*, które występowały w genomie szczepu 2R5, jak również wzrost liczby kopii innych genów, takich jak *nifH* i *nirS*.

Kolejnym etapem badań było określenie wpływu inokulacji *B. paralicheniformis* 2R5 oraz *P. sivasensis* 2RO45 na skład i różnorodność zbiorowisk bakteryjnych i grzybowych w ryzosferze rzepaku. W badaniach zaobserwowano, iż inokulacja *P. sivasensis* 2RO45 nie wpłynęła istotnie na wskaźniki alfa-bioróżnorodności. Jednakże, liniowa analiza dyskryminacyjna wykazała, że inokulacja *P. sivasensis* 2RO45 zmieniła skład taksonomiczny zbiorowisk drobnoustrojów ryzosfery rzepaku, istotnie zwiększając liczbę odczytów korzystnych dla roślin mikroorganizmów, takich jak bakterie z rodziny *Comamonadaceae* i *Vicinamibacteraceae*, rodzaju *Streptomyces* oraz grzyby z rodziny *Nectriaceae*, *Didymellaceae*, rodzaju *Exophiala* i gatunków *Cyphellophora vermispora* i *Mortierella minutissima*. Ponadto, zaobserwowano, że inokulacja szczepem *B. paralicheniformis* 2R5 początkowo zmniejszyła bogactwo gatunkowe społeczności bakteryjnych, podczas gdy po 44 dniach inokulacji wskaźnik alfa-bioróżnorodności wzrósł. Liniowa analiza dyskryminacyjna wykazała, że inokulacja *B. paralicheniformis* 2R5 zmodyfikowała skład taksonomiczny zbiorowisk bakterii i grzybów ryzosfery rzepaku, zwiększając liczbę odczytów pożytecznych dla roślin mikroorganizmów, takich jak *Nitrospira*, *Ramlibacter*, *Sphingomonas*, *Massilia*, *Terrimonas* oraz *Solicoccozyma*, *Schizothecium*, *Cyphellophora*, *Fusicolla*, *Humicola*.

W niniejszej pracy doktorskiej, przeanalizowano również wpływ inokulacji *P. sivasensis* 2RO45 na aktywność metaboliczną i bioróżnorodność funkcjonalną mikroorganizmów ryzosferowych rzepaku. Badania wykazały, że inokulacja szczepem *P. sivasensis* 2RO45 przyczyniła się do wzrostu ogólnej aktywności metabolicznej drobnoustrojów ryzosferowych. Ponadto, cztery źródła węgla, w tym fenole, polimery, kwasy karboksylowe i aminokwasy, były lepiej metabolizowane przez zespół mikroorganizmów ryzosfery rzepaku inokulowanej *P. sivasensis* 2RO45 niż w próbach kontrolnych. W oparciu o uzyskane profile rozkładu substratów węglowych obliczono wskaźniki różnorodności

funkcjonalnej. Badania wykazały, że inokulacja *P. sivasensis* 2RO45 spowodowała wzrost różnorodności funkcjonalnej mikrobiomu ryzosfery rzepaku mierzonej wskaźnikiem bioróżnorodności Shannona-Wienera (*H'*) oraz indeksem równocenności Shannona-Wienera (*E*).

Podsumowując, wyniki przedstawione w niniejszej rozprawie doktorskiej po raz pierwszy opisują gatunki *Bacillus paralicheniformis* i *Pseudomonas sivasensis* jako ryzobakterie promujące wzrost rzepaku. *B. paralicheniformis* 2R5 i *P. sivasensis* 2RO45 promując wzrost rzepaku mogą być obiecującą alternatywą dla chemicznych fungicydów czy nawozów mineralnych. Ich potencjalna zdolność zmiany mikrobiomu poprzez zwiększenie liczby korzystnych dla roślin grup mikroorganizmów wydaje się być ważna z punktu widzenia poprawy wydajności upraw rzepaku. Dalsze badania w kierunku opracowania formulacji preparatu na bazie tych bakterii mogą przyczynić się do lepszej jakości uprawy rzepaku.

Abstract

Plant growth-promoting rhizobacteria (PGPR) represent an environmentally friendly alternative to reducing the use of chemical fertilizers and fungicides. PGPR enhance plant growth by secreting phytohormones, dissolving phosphates, fixing nitrogen, increasing nutrient uptake, and acting as biocontrol agents against pathogens. The effectiveness of PGPR inoculation is linked to their ability to colonize roots, as well as their survival in the presence of native bacterial and fungal communities in the rhizosphere.

The aim of this doctoral thesis was to characterize and identify bacteria isolated from the rhizosphere of canola roots cultivated on two farms located in Górsk in the Kuyavian-Pomeranian Voivodeship and in Ostróda in the Warmian-Masurian Voivodeship, assess the impact of rhizobacteria on improving the growth and development of canola, search for genes contributing to a plant growth promotion and a biocontrol of plant pathogens, and determine the impact of PGPR rhizosphere inoculation on the number of microorganisms associated with the nitrogen cycle and the biodiversity of native bacterial and fungal communities.

In the preliminary studies, an analysis was conducted to determine the abundance of cultivable rhizobacteria from different growth stages of canola (vegetative, flowering, and maturity), cultivated in Górsk and in Ostróda. The studies revealed a complex dynamics of microbial communities, showing differences in the abundance of cultivable rhizobacteria at different growth stages of canola. In the case of the rhizosphere of canola taken from Górsk, the population of cultivable rhizobacteria was highest in the vegetative stage, decreased in the flowering stage, and then increased in the plant maturity stage. Meanwhile, in the rhizosphere of canola taken from Ostróda, the abundance of cultivable rhizosphere bacteria was highest in the flowering stage, after which it decreased successively in the maturity and vegetative stages of the plant.

In this doctoral thesis, 300 bacterial stains were isolated: 150 isolates from the rhizosphere of canola roots cultivated in Górsk and in Ostróda, including 50 isolates from each plant growth stage: vegetative, flowering, and maturity. Subsequently, a characterization of their plant growth-promoting (PGP) properties was conducted, including the production of indole-3-acetic acid (IAA), phosphates, ACC deaminase, siderophores, chitinases, hydrogen cyanide (HCN), and ammonia. The study aimed to check whether there is a relationship between the abundance of rhizobacteria isolated from different plant growth stages and their PGP properties. In the canola rhizosphere cultivated in Górsk, the largest population of

cultivable rhizobacteria was observed in the vegetative stage of canola, and four out of the seven tested PGP traits, such as the production of ACC deaminase, siderophores, HCN, and chitinases were produced by the highest percentage of isolates also in the vegetative stage of the plant. In the case of the canola rhizosphere cultivated in Ostróda, the highest abundance of rhizosphere microorganisms was recorded in samples taken in the flowering stage of canola, and also from this growth stage of the plant, the most strains capable of producing IAA, ACC deaminase, phosphates, siderophores, and ammonia were isolated.

In the next stage of the research, rhizobacteria that showed high activities of at least four of the seven tested PGP traits were identified and selected for a pot experiment to investigate their ability to promote canola growth under sterile conditions. Pot experiments under sterile conditions allowed to choose strains that best promoted plant growth. These included: *Bacillus paralicheniformis* 2R5, isolated from the rhizosphere of canola in Górsk, and *Peribacillus frigoritolerans* 2RO30 and *Pseudomonas sivasensis* 2RO45 from the rhizosphere of canola in Ostróda. Based on sterile condition experiments, *B. paralicheniformis* 2R5, *P. frigoritolerans* 2RO30, and *P. sivasensis* 2RO45 were selected for pot experiments in non-sterile soil to check if the isolates would also promote canola growth in the presence of native soil microorganisms. Additionally, in non-sterile pot experiments, a consortium consisting of two isolates, *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45, was used to check if the microbial consortium would show better promoting effect than individual strains. Pot experiments in non-sterile soil showed that only the individual strains *B. paralicheniformis* 2R5 and *P. sivasensis* 2RO45 promoted canola growth.

Canola growth-promoting rhizobacteria: *B. paralicheniformis* 2R5 and *P. sivasensis* 2RO45 were subjected to genome sequencing analysis to find genes responsible for promoting plant growth and biological control of phytopathogens. Genome analysis of *B. paralicheniformis* 2R5 revealed the presence of genes responsible for the biosynthesis of IAA, phosphate solubilization, siderophore sequestration, and chitinase production. Meanwhile, in the genome of *P. sivasensis* 2RO45, genes responsible for the IAA biosynthesis, phosphate solubilization, ACC deaminase production, and siderophore sequestration were found. In these strains' genomes, many biosynthetic gene clusters coding for biologically active secondary metabolites with antifungal activity were also identified.

Moreover, in the genome of *B. paralicheniformis* 2R5, genes related to the nitrogen cycle, such as *narG* and *nosZ*, were found. Therefore, it was examined whether the inoculation

of *B. paralicheniformis* 2R5 could influence the number of copies of these genes in the canola rhizosphere, and the number of copies of other equally important nitrogen cycle-related genes, such as *nifH*, *nirS*, and *amoA*, were determined. Results showed that the presence of *narG* and *nosZ* genes in the genome of *B. paralicheniformis* 2R5 was not correlated with significant changes in the number of gene copies related to the nitrogen cycle in the rhizosphere. Inoculation with *B. paralicheniformis* 2R5 led to an increase in the number of copies of both *narG* and *nosZ* genes present in the 2R5 genome, as well as an increase in the number of copies of other genes, such as *nifH* and *nirS*.

The next step of the research was to determine the impact of inoculation with *B. paralicheniformis* 2R5 and *P. sivasensis* 2RO45 on the composition and diversity of bacterial and fungal communities in the canola rhizosphere. Results showed that inoculation with *P. sivasensis* 2RO45 did not significantly affect alpha-diversity indices. However, linear discriminant analysis showed that inoculation with *P. sivasensis* 2RO45 changed the taxonomic composition of the canola rhizosphere microbial communities, significantly increasing the number of reads of beneficial microorganisms, such as bacteria from the *Comamonadaceae* and *Vicinamibacteraceae* families, *Streptomyces* genus, and fungi from the *Nectriaceae*, *Didymellaceae* families, *Exophiala* genus, and *Cyphellophora vermispora* and *Mortierella minutissima* species. Additionally, it was observed that inoculation with *B. paralicheniformis* 2R5 initially reduced OTU richness of bacterial communities, while after 44 days of inoculation, the alpha-diversity index increased. Linear discriminant analysis showed that inoculation with *B. paralicheniformis* 2R5 modified the taxonomic composition of the bacterial and fungal communities in the canola rhizosphere, increasing the number of reads of beneficial microorganisms, such as *Nitrospira*, *Ramlibacter*, *Sphingomonas*, *Massilia*, *Terrimonas*, and *Solicoccozyma*, *Schizothecium*, *Cyphellophora*, *Fusicolla*, *Humicola*.

In this doctoral thesis, the impact of inoculation with *P. sivasensis* 2RO45 on the metabolic activity and functional diversity of canola rhizospheric microorganisms was also analyzed. Results showed that inoculation with *P. sivasensis* 2RO45 contributed to an increase in the overall metabolic activity of rhizospheric microorganisms. Additionally, four carbon sources, including phenols, polymers, carboxylic acids, and amino acids, were better metabolized by the microbial community of canola rhizosphere inoculated with *P. sivasensis* 2RO45 than in control samples. Based on the obtained carbon substrate degradation profiles, functional diversity indices were calculated. Results showed that inoculation with *P. sivasensis* 2RO45 caused an increase in the functional diversity of the canola rhizosphere microbiome

measured by the Shannon-Wiener biodiversity index (H') and the Shannon-Wiener evenness index (E).

In summary, the results presented in this doctoral thesis describe for the first time *Bacillus paralicheniformis* and *Pseudomonas sivasensis* as rhizobacteria promoting canola growth. *B. paralicheniformis* 2R5 and *P. sivasensis 2RO45*, by promoting canola growth, may be a promising alternative to chemical fungicides or mineral fertilizers. Their potential ability to change the microbiome by increasing the number of beneficial microorganism groups seems to be important for improving the efficiency of canola cultivation. Further research towards developing a formulation based on these bacteria may contribute to better canola cultivation quality.

I Wstęp

1. Rzepak jako przemysłowa roślina uprawna

Rzepak (*Brassica napus* L.) zajmuje trzecie miejsce na świecie pod względem wielkości upraw roślin oleistych i jest głównym źródłem wysokiej jakości oleju roślinnego i białek wykorzystywanych w przemyśle paszowym (Li i in., 2022). Ze względu na możliwość uprawy w różnych warunkach klimatycznych, a także wysoką zawartość oleju w nasionach osiągającą 40-45%, rzepak uważa się za obiecującą roślinę energetyczną do produkcji ropy naftowej (Saghafi i in., 2018). Jego znaczenie wzrasta także w produkcji biodiesla, gdzie niska zawartość tłuszczów nasyconych i obecność 10% tlenu przyczyniają się do wyższej wydajności spalania, co jest szczególnie korzystne w niskich temperaturach (Blackshaw i in., 2011). Z uwagi na zbliżające się regulacje dotyczące czystych paliw (CFS), prognozuje się wzrost popytu na rzepak. Obecnie olej napędowy zawiera co najmniej 2% biopaliwa, a do 2030 roku spodziewany jest wzrost tej wartości do 8-11%, co ma związek z dążeniami do redukcji emisji gazów cieplarnianych (Ma i in., 2023). Jednakże, uprawa rzepaku wiąże się z wyzwaniami ekologicznymi. Duże zapotrzebowanie na nawozy azotowe prowadzi do zwiększenia emisji gazów cieplarnianych, zanieczyszczenia zbiorników wodnych, zakwaszenia gleby i zmniejszenia różnorodności biologicznej (Ma i Herath, 2016; Martínez-Dalmau i in., 2021). W tym kontekście, kluczowe staje się opracowanie i wdrożenie zrównoważonych praktyk zarządzania uprawami rzepaku, które umożliwią zwiększenie wydajności upraw, jednocześnie minimalizując negatywny wpływ nawozów mineralnych na środowisko. Takie podejście pozwoli na zrównoważone wykorzystanie rzepaku jako źródła biopaliw i innych produktów, jednocześnie chroniąc ekosystemy i promując bardziej zrównoważone rolnictwo.

2. Ryzobakterie promujące wzrost roślin

Liczne badania wykazały, że stosowanie naturalnych i organicznych metod odgrywa ważną rolę w zwiększaniu plonów roślin (Mącik i in., 2020; Kavusi i in., 2022). W szczególności, bakterie zamieszkujące ryzosferę, które wykazują pozytywny wpływ na wzrost i rozwój roślin, czyli tzw. ryzobakterie promujące wzrost roślin (PGPR) uznano za ważną strategię na rzecz zrównoważonego rozwoju rolnictwa, która pozwala na skuteczne ograniczenie lub nawet wyeliminowanie stosowania pestycydów i nawozów mineralnych bez

utraty plonów (Ambrosini i in., 2016). PGPR można wykorzystać w rolnictwie na różne sposoby, w tym jako: (i) bionawozy, które poprawiają dostępność składników odżywczych dla roślin, (ii) fitostymulatory, które pobudzają wzrost roślin, głównie poprzez syntezę fitohormonów, (iii) ryzoremediatory, rozkładające zanieczyszczenia organiczne oraz (iv) biopestycydy, zwalczające choroby roślin (Somers i in., 2004). Ponadto, istnieją dwa rodzaje mechanizmów związane z korzystnym wpływem aktywności PGPR w ryzosferze; jest to typ bezpośredni i pośredni. PGPR mogą bezpośrednio wpływać na rozwój roślin poprzez zwiększenie dostępności składników odżywczych (azot, fosfor i żelazo) lub zwiększając poziom fitohormonów takich jak: kwas indolo-3-octowy, etylen, cytokininy i gibereliny. A także, PGPR mogą pośrednio stymulować wzrost roślin poprzez hamowanie patogenów roślinnych dzięki wytwarzaniu metabolitów przeciwdrobnoustrojowych lub enzymów zewnątrzkomórkowych (Kong i Liu, 2022; Kelbessa i in., 2023).

2.1. Bezpośrednie mechanizmy promujące wzrost roślin

Azot (N) jest uznawany za jeden z najważniejszych składników odżywczych, kluczowych dla wzrostu i rozwoju roślin. Rośliny wykorzystują azot do tworzenia aminokwasów, nukleotydów, fosfolipidów oraz chlorofilu, niezbędnych w procesach takich jak fotosynteza i rozwój komórek (Oleńska i in., 2020). Jednakże, główne źródło azotu w środowisku naturalnym, czyli azot atmosferyczny (N_2) nie jest łatwo dostępny dla roślin (Rilling i in., 2018). Bakterie PGPR za pomocą kompleksu enzymów azotazowych są w stanie przekształcić azot atmosferyczny (N_2) w amoniak (NH_3). Proces ten może przyczynić się do złagodzenia negatywnych skutków stresu abiotycznego, poprzez zwiększenie dostępności azotu dla roślin (Aloo i in., 2020).

Fosfor (P) jest niezbędnym makroelementem, który odgrywa kluczową rolę w procesach wzrostu i metabolizmu roślin. W glebie fosfor występuje zazwyczaj w formie nierozpuszczalnej, zarówno jako fosforany organiczne, jak i nieorganiczne. Bakterie posiadają zdolność do rozpuszczania tych fosforanów do formy rozpuszczalnej i łatwiej przyswajalnej przez rośliny, co ma znaczący wpływ na ich zdrowie i rozwój (Kalayu, 2019). Fosfor jest kluczowym składnikiem wielu ważnych procesów fizjologicznych i biochemicznych w roślinach, w tym fotosyntezy, rozwoju korzeni i łodyg, a także w procesie tworzenia kwiatów i nasion oraz dojrzewania roślin (de Andrade i in., 2023).

Żelazo (Fe) jest kolejnym kluczowym składnikiem odżywczym niezbędnym dla wzrostu roślin. W glebie żelazo występuje głównie w formie trudno rozpuszczalnych jonów żelaza (Fe^{3+}), które są niedostępne zarówno dla roślin, jak i bakterii ryzosferycznych (Silva i in., 2021). Natomiast jony żelaza (Fe^{2+}) są znacznie bardziej rozpuszczalne i dostępne dla roślin, lecz łatwo ulegają utlenieniu do mniej dostępnej formy Fe^{3+} (McLaren i in., 2020). Większość mikroorganizmów, w tym bakterie i grzyby, posiada zdolność wydzielania sideroforów, które są związkami o niskiej masie cząsteczkowej działającymi jako chelatory jonów żelaza Fe^{3+}. Siderofory chelatują jony Fe^{3+}, a następnie redukują je do formy Fe^{2+}, która jest łatwiej przyswajalna przez rośliny, umożliwiając im wykorzystanie tego istotnego składnika odżywczego (Richardson i Simpson, 2011).

Kwas indolo-3-octowy (IAA) jest fitohormonem, uznawanym za jedną z najważniejszych auksyn, czyli organicznych związków niskocząsteczkowych. IAA odgrywa istotną rolę w regulacji różnorodnych procesów wzrostu i rozwoju roślin, w tym w podziale i wydłużaniu komórek, różnicowaniu, reakcjach na bodźce zewnętrzne, procesach starzenia się, opadania liści oraz kwitnienia (Jeyanthi i Kanimozhi, 2018). IAA produkowana przez bakterie ma pozytywny wpływ na rozwój systemu korzeniowego roślin, przede wszystkim na włośniki i korzenie boczne, co zwiększa zdolność rośliny do pobierania składników odżywczych. Tym samym, IAA przyczynia się do poprawy zasobów odżywczych dostępnych dla rośliny, co z kolei wspiera jej wzrost i rozwój (Goswami i in., 2016).

Cytokininy są hormonem roślinnym, który odgrywa kluczową rolę w regulacji wielu procesów wzrostu i rozwoju roślin. Hormony te biorą udział w takich procesach jak podział i różnicowanie komórek, formowanie się włośników korzeniowych i korzeni bocznych, rozwój układu naczyniowego, produkcja chlorofilu, stymulowanie kiełkowania nasion oraz regulacja procesów starzenia się (Heydarian i in., 2021; Mekureyaw i in., 2022). Ponadto, cytokininy pełnią ważną funkcję w transmisji sygnałów od korzeni do pędów roślin, zwłaszcza w warunkach stresu środowiskowego (Boukhatem i in., 2022).

Gibereliny stanowią istotną grupę fitohormonów, które są aktywnie zaangażowane w szereg kluczowych procesów życiowych roślin. Obejmują one regulację wzrostu i spoczynku roślin, kiełkowanie nasion, rozwój systemu korzeniowego, proces kwitnienia oraz dojrzewanie owoców (Binenbaum i in., 2018). Produkcja giberelin ma zasadnicze znaczenie w zwiększaniu odporności roślin na różnorodne stresy abiotyczne, co przekłada się na lepszą adaptację do trudnych warunków środowiskowych (Vanissa i in., 2020).

Kwas 1-aminocyklopropano-1-karboksylowy (ACC) jest prekursorem etylenu, który jest gazowym hormonem roślinnym (Goswami i in., 2016). Etylen, chociaż w niskich stężeniach odgrywa kluczową rolę w regulacji różnych etapów życia roślin, takich jak kiełkowanie nasion, formowanie korzeni przybyszowych, kwitnienie, procesy starzenia i odporność na stres (Batool i in., 2020; Oleńska i in., 2020), to jego nadprodukcja wywołana przez różne stresy abiotyczne może mieć niekorzystny wpływ na wzrost roślin, ograniczając rozwój pędów i korzeni oraz przyspieszając proces starzenia się rośliny (Fiodor i in., 2021). Bakterie PGPR odgrywają istotną rolę w utrzymaniu równowagi etylenu w roślinach, szczególnie w warunkach stresowych, poprzez produkcję enzymu ACC deaminazy. Enzym ten przekształca ACC w amoniak i α-ketomaślan, co skutkuje zmniejszeniem dostępności substratów potrzebnych do syntezy etylenu (Glick, 2014; Jha i Mohamed, 2022).

2.2. Pośrednie mechanizmy promujące wzrost roślin

Jednym z głównych mechanizmów stosowanych przez PGPR w celu zapobiegania namnażaniu się fitopatogenów jest synteza antybiotyków. Antybiotyki obejmują heterogenną grupę organicznych związków o niskiej masie cząsteczkowej, które są szkodliwe dla wzrostu i aktywności metabolicznej innych mikroorganizmów (Upadhyay i in., 2022). Mechanizm działania polega głównie na hamowaniu syntezy ścian komórkowych patogenu, zaburzaniu funkcjonowania błony komórkowej oraz hamowaniu syntezy kwasów nukleinowych i/lub białek (Baran i in., 2023).

Wzrost i aktywność patogenów, PGPR mogą również zahamować poprzez wydzielanie enzymów hydrolitycznych (Jeyanthi i Kanimozhi, 2018). Enzymy degradujące ścianę komórkową, takie jak β-1,3-glukanaza i chitynaza wydzielane przez szczepy PGPR, wywierają hamujący wpływ na wzrost strzępek patogenów grzybowych poprzez degradację ich ściany komórkowej. Chitynaza rozkłada chitynę, nierozpuszczalny liniowy polimer β-1,4-N-acetylo-glukozaminy, który jest głównym składnikiem ściany komórkowej grzybów (Singh i in., 2017). Natomiast β-1,3-glukanazy odpowiadają za hydrolizę β-1,3- i β-1,3-1,6-glukanów znajdujących się w ścianach komórkowych grzybów (Perrot i in., 2022).

Ponadto, PGPR mogą wywołać u roślin zjawisko znane jako systemiczna indukowana oporność (ISR), która fenotypowo podobna jest do systemicznej oporności nabytej (SAR) występującej, gdy rośliny aktywują swoje mechanizmy obronne w odpowiedzi na infekcję z czynnikiem chorobotwórczym (Pieterse i in., 2009). ISR obejmuje sygnalizację jasmonianową

i etylenową w roślinie, a hormony te stymulują reakcje obronne rośliny żywicielskiej na szereg patogenów (Verhagen i in., 2004). ISR nie wymaga bezpośredniej interakcji między PGPR wywołującym oporność a patogenem (Bakker i in., 2007).

Cyjanowodór (HCN) jest lotnym metabolitem wtórnym syntetyzowanym przez wiele ryzobakterii, który może wpływać na zakorzenienie się roślin lub hamować rozwój chorób roślin (Lanteigne i in., 2012). Ponadto, HCN bierze udział w procesach geochemicznych, na przykład chelatacji metali, prowadząc do pośredniego wzrostu dostępności składników odżywczych dla roślin (Rijavec i Lapanje, 2016).

3. Mikrobiom ryzosfery a inokulacja PGPR

Ryzosfera to ściśle związana z korzeniami warstwa gleby, w której zachodzą liczne interakcje między rośliną a mikroorganizmami (Glick, 2012). Rośliny za pośrednictwem korzeni aktywnie uwalniają do gleby różnorodne związki organiczne i nieorganiczne, co nosi nazwę wydzielania korzeniowego lub ryzodepozycji (Uren, 2000). Wydzieliny korzeniowe zawierają głównie organiczne związki o niskiej masie cząsteczkowej, takie jak aminokwasy, kwasy organiczne, cukry i związki fenolowe, a także związki o większej masie cząsteczkowej, w tym polisacharydy i białka (Badri i Vivanco, 2009; Zhalnina i in., 2018). Są one istotnym źródłem składników odżywczych dla mikroorganizmów ryzosfery, dlatego wszelkie zmiany w ich stężeniu lub składzie mogą istotnie wpływać na kształtowanie się mikrobiomu ryzosfery, wywierając pozytywny, negatywny lub neutralny wpływ na wzrost drobnoustrojów (Canarini i in., 2019).

Wprowadzenie specyficznych PGPR do środowiska napotyka na szereg wyzwań, w tym konkurencję i antagonizm ze strony rodzimych mikroorganizmów glebowych (Clinton i Rumbaugh, 2016). W środowisku glebowym konkurencja o ograniczone składniki odżywcze, które dostarczają zaledwie około 5% wymaganych zasobów (Ashman i Puri, 2013), jest intensywna. Tylko te mikroorganizmy, które są wyjątkowo konkurencyjne, są w stanie przetrwać podczas ciągłej rywalizacji o składniki odżywcze, takie jak na przykład węgiel i azot (Stengel i Gelin, 1998). W większości badań utrzymuje się pogląd, że aby PGPR był kompetentny ekologicznie, musi być w stanie kolonizować środowisko roślinne, jednocześnie pozytywnie oddziałując z rodzimymi drobnoustrojami, aby poprawić wzrost roślin (Trivedi i in., 2012). Efektywność wprowadzonego PGPR jest zatem silnie zależna od różnorodności

rodzimej społeczności mikroorganizmów. PGPR mogą napotkać na antagonistyczne działanie ze strony rodzimych drobnoustrojów, co jest powszechnym mechanizmem obronnym przeciwko inwazyjnym mikroorganizmom; na przykład, stwierdzono, że 90% *Actinomycete* sp. wyizolowanych z ryzosfery wykazują takie zachowanie w stosunku do *Bradyrhizobium japonicum* (Pugashetti i in., 1982). Ponadto, drobnoustroje glebowe mogą zmieniać charakterystykę wydzielin korzeniowych, ograniczając tym samym przyciąganie i kolonizację inokulowanego PGPR (Gupta Sood, 2003). Z drugiej strony, PGPR stosowane jako środki biokontroli mogą eliminować patogeny zamieszkujące korzenie (Glick i in., 2007) lub inne nieszkodliwe drobnoustroje, co prowadzi do zmian w mikrobiomie ryzosfery rośliny (Trabelsi i Mhamdi, 2013).

Ze względu na złożoną rolę inokulantów mikrobiologicznych, ich oddziaływanie na rodzime zbiorowiska drobnoustrojów w ryzosferze nadal pozostaje przedmiotem dyskusji. Na przykład, Marwa i in. (2020) wykazali, że szczepy *Paracoccus versutus* NM01 i *Aeromonas caviae* NM04 sprzyjały rozwojowi paproci, jednocześnie zwiększając różnorodność bakteryjną w ryzosferze. Natomiast, po wprowadzeniu *Bacillus amyloliquefaciens* FH-1, który wspomagał wzrost ogórka, odnotowano obniżenie różnorodności bakterii w ryzosferze (Wang i in., 2021). Z drugiej strony, Piromyou i in. (2011) stwierdzili, że inokulacja PGPR, *Pseudomonas* sp. SUT 19 i *Brevibacillus* sp. SUT 47, nie miała wpływu na strukturę społeczności drobnoustrojów. Biorąc pod uwagę te doniesienia, niezbędne staje się przeprowadzenie dogłębnych badań dotyczących wpływu inokulacji PGPR na strukturę i funkcjonowanie mikrobiomu ryzosfery, zanim PGPR zostaną wykorzystane na skalę komercyjną (Martínez-Hidalgo i in., 2019). Te badania pozwolą na stworzenie efektywnego współdziałania pomiędzy roślinami a drobnoustrojami, co będzie sprzyjać wzrostowi i ogólnej kondycji roślin (Kong i Liu, 2022).

4. Metody oznaczenia bioróżnorodności drobnoustrojów

Wśród technik oznaczenia różnorodności populacji drobnoustrojów w glebie można wyróżnić metody hodowlane i niehodowlane. Metody hodowlane obejmują hodowlę płytkową i mikropłytkową. W przypadku hodowli płytkowej, wykonuje się posiew na podłoża agarowe i określa ogólną liczbę bakterii, wyrażoną w jednostkach tworzących kolonię na gram suchej masy gleby (Kirk i in., 2004; Alain i Querellou, 2009). Metoda ta jest prosta, szybka, niedroga i pozwala na izolację mikroorganizmów o określonych właściwościach. Jednak potrafi wykryć

jedynie najbardziej aktywne i szybko rosnące mikroorganizmy heterotroficzne (Kirk i in., 2004). Ponadto, ze względu na brak możliwości zapewnienia optymalnych warunków wzrostowych dla wszystkich mikroorganizmów, frakcja mikroorganizmów hodowlanych wynosi zaledwie od 1 do 10% całej mikrobioty obecnej w glebie (Nannipieri i in., 2003; Alain i Querellou, 2009).

W metodach mikropłytkowych, zawiesinę gleby inokuluje się na specjalistyczne podłoża znajdujące się w 96-dołkach, a odczyt wykonuje się spektrofotometrycznie i poddaje analizie statystycznej (Kirk i in., 2004). Analizę tę można wykonać przy użyciu mikropłytek Biolog EcoPlates zawierających 31 różnych substratów węglowych, które są powszechnie występującymi związkami w wydzielinach korzeniowych roślin (Campbell i in., 1997). Metoda ta wykorzystując całkowitą hodowlaną frakcję bakterii glebowych, pozwala na uzyskanie profili metabolizowania różnych substratów (CLPP – Community Level Physiological Profiles), co umożliwia określenie funkcjonalnej bioróżnorodności badanej społeczności mikroorganizmów. Jednak, należy pamiętać, że analiza ta nie oddaje całkowitego potencjału funkcjonalnego całej społeczności drobnoustrojów, ponieważ tylko mikroorganizmy hodowlane, zdolne do wzrostu w warunkach bogatych w składniki odżywcze, przyczyniają się do konsumpcji substratów węglowych (Nannipieri i in., 2003; Stefanowicz, 2006).

Wśród niehodowlanych metod umożliwiających analizę metagenomu możemy wyróżnić sekwencjonowanie nowej generacji (NGS). Metody sekwencjonowania o dużej przepustowości umożliwiają analizę genomów wszystkich mikroorganizmów w próbce, nie tylko tych, które nadają się do hodowli. Jedną z takich metod jest metagenomika typu shotgun, czyli nieselektywne („shotgun") sekwencjonowanie wszystkich („meta-") genomów mikrobiologicznych („genomics") obecnych w próbce (Hamady i Knight, 2009). Badanie metagenomiczne typu shotgun składa się z pięciu etapów (i) pobranie próbek, izolacja DNA i sekwencjonowanie; (ii) wstępne przetwarzanie odczytów sekwencjonowania, czyli tzw. obróbka wstępna surowych danych; (iii) analiza sekwencji w celu sprofilowania taksonomicznych, funkcjonalnych i genomowych cech mikrobiomu; (iv) obróbka końcowa przetworzonych danych oraz (v) walidacja (Quince i in., 2017). W celu określenia bioróżnorodności strukturalnej społeczności bakteryjnych najczęściej bada się gen kodujący 16S rRNA, natomiast w przypadku grzybów badane są fragmenty ITS (ang. Internal Transcribed Spacer) (Satam i in., 2023). Ze względu na to, iż proces jest ukierunkowany, często konieczna jest wstępna amplifikacja genów, po której następuje sekwencjonowanie i analiza bioinformatyczna (Oulas i in., 2015). Jest to szczególnie ważne, gdy próbka zawiera niewielką

liczbę genów przeznaczonych do analizy. Według niektórych ekspertów, takie metody nie powinny być klasyfikowane jako badania metagenomiczne, ponieważ zakres analizy genów jest ograniczony i nie obejmuje całej puli genetycznej obecnej w próbce (Quince i in., 2017).

II Cel pracy, hipotezy badawcze i zakres badań

Cel pracy

Głównym celem naukowym rozprawy doktorskiej była charakterystyka i identyfikacja bakterii wyizolowanych z ryzosfery korzeni rzepaku uprawianego na dwóch gospodarstwach wiejskich położonych w Górsku w województwie kujawsko-pomorskim i w Ostródzie w województwie warmińsko-mazurskim, ocena wpływu ryzobakterii na poprawę wzrostu i rozwoju rzepaku, poszukiwanie genów przyczyniających się do promowania wzrostu roślin i biokontroli patogenów roślinnych, a także określenie wpływu inokulacji ryzosfery PGPR na liczebność mikroorganizmów związanych z obiegiem azotu oraz bioróżnorodność rodzimych zbiorowisk bakteryjnych i grzybowych.

Hipotezy badawcze

1. Ryzosfera korzeni rzepaku uprawianego w gospodarstwach położonych w różnych obszarach Polski w Górsku i w Ostródzie stanowi źródło gatunków bakterii promujących wzrost rzepaku.
2. Genomy ryzobakterii promujących wzrost rzepaku zawierają geny odpowiedzialne za stymulację wzrostu roślin i biokontrolę fitopatogenów.
3. Ryzobakterie promujące wzrost rzepaku mają wpływ na bioróżnorodność mikroorganizmów rodzimych w ryzosferze.

Zakres i metodyka badań

1. Oznaczenie liczebności i izolacja hodowlanych ryzobakterii (publikacja nr 1 i 3).

Liczebność hodowlanych ryzobakterii oznaczono dla trzech różnych faz wzrostu rzepaku (wegetatywnej, kwitnienia i dojrzewania) uprawianego w Górsku w województwie kujawsko-pomorskim (publikacja nr 1) i w Ostródzie w województwie warmińsko-mazurskim (publikacja nr 3).

Miejsce izolacji ryzobakterii wytypowano ze względu na różne położenie geograficzne, warunki atmosferyczne oraz stosowanie różnych zabiegów agrotechnicznych, takich jak

nawożenie gleby czy stosowanie środków ochrony roślin. Liczebność hodowlanych ryzobakterii oznaczono metodą płytkową Kocha na podłożu standardowym.

2. Charakterystyka ryzobakterii potencjalnie promujących wzrost roślin (publikacja nr 1 i 3).

Łącznie 300 izolatów ryzosferowych scharakteryzowano pod kątem ich właściwości promujących wzrost roślin (PGP), w tym produkcję kwasu indolilo-3-octowego, fosforanów, deaminazy ACC, sideroforów, chitynaz, cyjanowodoru i amoniaku. Badania wykonano według standardowych technik mikrobiologicznych i biochemicznych.

3. Identyfikacja taksonomiczna wytypowanych ryzobakterii (publikacja nr 1 i 3).

Identyfikację taksonomiczną wykonano na podstawie analizy sekwencji nukleotydowej genu 16S rRNA. Genomowe DNA ryzobakterii izolowano przy użyciu zestawu GeneMATRIX Bacterial and Yeast Genomic DNA Purification Kit. Amplifikację fragmentu genu kodującego 16S rRNA prowadzono metodą PCR (ang. Polymerase Chain Reaction) z wykorzystaniem starterów 27F i 1492R. Produkty PCR sekwencjonowano przy użyciu BigDye Terminator v 3.1 Cycle Sequencing Kit. Przynależność taksonomiczną określono z wykorzystaniem serwera EzBioCloud.

4. Ocena wpływu wytypowanych ryzobakterii na poprawę wzrostu rzepaku w warunkach sterylnych i niesterylnych (publikacja nr 1 i 3).

Do badań wazonowych w warunkach sterylnych wykorzystano sterylny wermikulit i piasek w stosunku 1:1, natomiast warunki niesterylne stanowiła niesterylna gleba pobrana z Górska (publikacja nr 1) i z Ostródy (publikacja nr 3). Nasiona rzepaku inokulowano metodą otoczkowania. Po zbiorach, oznaczono następujące parametry roślin: zawartość chlorofilu, długość korzeni, pędów i epikotylu, powierzchnię fotosyntetyczną liści oraz masę korzeni, pędów, epikotylu, ogonków i liści, a także obliczono dwa wskaźniki wzrostu roślin: powierzchnię właściwą liści (SLA – ang. Specific Leaf Area) oraz wskaźnik wagowy liści (LWR – ang. Leaf Weight Ratio).

5. Analiza genomów szczepów promujących wzrost rzepaku (publikacja nr 1 i 3).

Analizę genomów wykonano w oparciu o metodę sekwencjonowania genomu (NGS – ang. Next Generation Sequencing). Biblioteki genomowego DNA przygotowano na Microlab STAR Liquid Handler przy użyciu zestawu Nextera XT Library Preparation kit. Pomiar stężenia bibliotek wykonywano z zastosowaniem urządzenia Roche Light Cycler 96 qPCR oraz zestawu odczynników Kapa Biosystems Library Quantification Kit. Biblioteki sekwencjonowano z

wykorzystaniem sekwenatora HiSeq (Illumina). Sekwencjonowanie przebiegało w trybie sparowanych końców (250 pz).

Analizę identyfikacji genów odpowiedzialnych za promowanie wzrostu roślin wykonano z wykorzystaniem bazy danych KEGG (ang. Kyoto Encyclopedia of Genes and Genomes), a analizę biosyntetycznych klastrów genów (BGCs) przeprowadzono za pomocą oprogramowania anti-SMASH.

6. Ocena wpływu inokulacji na liczebność różnych grup fizjologicznych mikroorganizmów związanych z obiegiem azotu (publikacja nr 1).

Analizę ilościową mikroorganizmów związanych z obiegiem azotu w ryzosferze wykonano z wykorzystaniem metody ilościowej reakcji łańcuchowej polimerazy (qPCR – ang. quantitaive polymerase chain reaction). W badaniach oznaczono liczbę kopii genów zaangażowanych w nitryfikację (*nifH*), denitryfikację (*narG*, *nirS* i *nosZ*) oraz amonifikację (*amoA*). DNA izolowano z ryzosfery z wykorzystaniem zestawu DNeasy Power Soil Kit. Amplifikację prowadzono w urządzeniu CFX Opus 96 Real-Time PCR System. Oznaczenia ilości kopii genów dokonano na podstawie krzywych standardowych (produkt reakcji PCR wklonowany w wektor pCR4 TOPO). Uzyskane liczebności kopii genów przeliczono na gram suchej masy gleby.

7. Ocena wpływu inokulacji na bioróżnorodność strukturalną populacji bakterii i grzybów ryzosfery rzepaku (publikacja nr 2 i 4).

Analizę bioróżnorodności strukturalnej wykonano w oparciu o metodę sekwencjonowania nowej generacji (NGS – ang. Next Generation Sequencing). Genomowe DNA wyizolowano przy użyciu DNeasy Power Soil Kit. Amplifikację fragmentu genu kodującego 16S rRNA wykonano z użyciem primerów S-D-Bact-0341-b-S-17 i S-D-Bact-0785-a-A-21, a amplifikację transkrybowanego drugiego odstępnika wewnętrznego (ITS2 - ang. Internal Transcribed Spacer) przy użyciu ITS3 i ITS4. Biblioteki NGS przygotowano zgodnie z protokołem ISC (ang. Illumina Support Center). Sekwencjonowanie wykonano z wykorzystaniem platformy MiSeq (Illumina) w technologii sparowanych końców (2 x 250 pz), z zastosowaniem MiSeq Reagent Kit v2 (500 cykli). Za pomocą programu Mothur v1.44.3, uzyskane sekwencje połączono i poddano jakościowej filtracji, a następnie klastrowaniu w operacyjne jednostki taksonomiczne (OTU - ang. Operational Taxonomic. Units) na zasadzie podobieństwa do innych sekwencji w próbce (próg podobieństwa 97%). Przypisanie OTU do odpowiednich poziomów taksonomicznych zostało przeprowadzone zgodnie z SOP MiSeq mothur przy

użyciu bazy danych ARB-SILVA SSU oraz UNITE v8.3, odpowiednio dla społeczności bakteryjnych i grzybowych.

W badaniach obliczono następujące wskaźniki alfa-bioróżnorodności: bogactwo gatunkowe (OTU richness), wskaźniki bioróżnorodności (Shannon-Wienner, H'; Invert Simpson, 1/D). Natomiast, w celu wskazania grup taksonomicznych, których liczebność względna zmieniała się istotnie po inokulacji, zastosowano liniową analizę dyskryminacyjną LEfSe (ang. Linear discriminant analysis Effect Size).

8. Ocena wpływu inokulacji na bioróżnorodność funkcjonalną mikrobiomu ryzosfery rzepaku (publikacja nr 4).

Analizę wykonano przy użyciu mikropłytek Biolog EcoPlates, które umożliwiają profilowanie hodowlanej frakcji zespołów drobnoustrojów pod kątem aktywności metabolicznej względem szeregu substratów węglowych (CLPP – ang. Community Level Physiological Profiles), co pozwala na wyznaczenie bioróżnorodności funkcjonalnej. W badaniach oznaczono następujące wskaźniki różnorodności funkcjonalnej: indeks bioróżnorodności Shannona-Wienera (*H'*), indeks równocenności Shannona-Wienera (*E*) oraz indeks bioróżnorodności Simpsona (*D*).

III Wykaz publikacji wchodzących w skład rozprawy doktorskiej

1.	**Świątczak, J.,** Kalwasińska, A., Felföldi, T.,Swiontek Brzezinska, M., 2023. *Bacillus paralicheniformis* 2R5 and its impact on canola growth and N-cycle genes in the rhizosphere. FEMS Microbiology Ecology, 99(9), p.fiad093.	IF – 4,519 MNiSW – 100
2.	**Świątczak, J.,** Kalwasińska, A., Szabó, A.,Swiontek Brzezinska, M., 2023. The effect of seed bacterization with *Bacillus paralicheniformis* 2R5 on bacterial and fungal communities in the canola rhizosphere. Microbiological Research, 275, p.127448.	IF – 6,7 MNiSW –100
3.	**Świątczak, J.,** Kalwasińska, A., Swiontek Brzezinska, M., 2024. Plant growth–promoting rhizobacteria: *Peribacillus frigoritolerans* 2RO30 and *Pseudomonas sivasensis* 2RO45 for their effect on canola growth under controlled as well as natural conditions. Frontiers in Plant Science, 14, p.1233237.	IF – 6,627 MNiSW –100
4.	**Świątczak, J.,** Kalwasińska, A., Szabó, A.,Swiontek Brzezinska, M., 2023. *Pseudomonas sivasensis* 2RO45 inoculation alters the taxonomic structure and functioning of the canola rhizosphere microbial community. Frontiers in Microbiology, 14, p.1168907.	IF – 6,064 MNiSW – 100
		Sumaryczny IF – 23,91 **Suma punktów MNiSW – 400**

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Bacillus paralicheniformis 2R5 and its impact on canola growth and N-cycle genes in the rhizosphere

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Research Article

Bacillus paralicheniformis 2R5 and its impact on canola growth and N-cycle genes in the rhizosphere

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Abstract

Chemical fertilization has a negative impact on the natural environment. Plant growth-promoting (PGP) rhizobacterial biofertilizers can be a safer alternative to synthetic agrochemicals. In this research, a culture-based method was used to assess the population size of rhizobacteria at the vegetative, flowering, and maturity stages of canola. Rhizobacteria were then isolated from each of the canola growth stages, and their seven PGP traits were determined. The highest abundance of culturable bacteria was found at the vegetative stage of the plants. Furthermore, four out of seven PGP traits were produced by the highest % of isolates at the vegetative stage. In the greenhouse experiment that included six rhizobacterial strains with best PGP traits, the greatest canola growth promotion ability under sterile conditions was observed after the introduction of *Bacillus paralicheniformis* 2R5. Moreover, under nonsterile conditions, 2R5 significantly increased canola growth. The presence of the *trpA*, *B*, *C*, *D*, *E*, *F* and *pstA*, and *S* genes in the 2R5 genome could be associated with canola growth promotion abilities. The *chiA* and *mbtH* genes could contribute to 2R5 antifungal activity against fungal pathogens. Moreover, the introduction of 2R5 significantly increased the abundance of the *narG*, *nosZ*, *nifH*, and *nirS* genes, which can prove that the 2R5 strain may be an important member of the soil bacterial community.

Keywords: *Bacillus paralicheniformis* 2R5, PGPR, biofertilizers, canola, N-cycle genes

Introduction

Canola (*Brassica napus* L.) is one of the most significant oilseed crops and it is widely used as a source of oil for food and industrial applications (Raboanatahiry et al. 2021). *Brassica napus* L. is susceptible to many fungal diseases and insect pests, which results in its yield loss (Obermeier et al. 2022). It is well known that nitrogen (N) availability is a limiting factor in canola production (Riar et al. 2020). Low N use efficiency promotes excessive N chemical fertilization, which enhances crop production costs and has a negative effect on the environment (Calvo et al. 2019). Therefore, plant growth-promoting rhizobacteria (PGPR) improving plant development have been studied as biofertilizers to enhance crop productivity. The biofertilizers can promote plant growth through various mechanisms, e.g. phytohormone production, phosphate solubilization, siderophore sequestration, and atmospheric N fixation (Du et al. 2019, Chandran et al. 2021).

The genus *Bacillus* is one of the best-studied rhizobacteria for plant growth promotion (Hashmi et al. 2019). *Bacillus* sp. PG-8 was found to be an *Arachis hypogea* growth promoter (Gohil et al. 2022), while in another study, *Bacillus subtilis* L2 promoted the growth of *Zingiber officinale* Roscoe (Jabborova et al. 2021). There were reports where the application of *Bacillus* spp. inoculants increased plant growth through N fixation. For example, N_2-fixing *B. pumilus* significantly increased maize dry biomass (Kuan et al. 2016). Furthermore, a consortium of *Bacillus* isolates increased plant growth and it was associated with the expression of nitrate (NO_3^-) and ammonium (NH_4^+) uptake genes in *Arabidopsis thaliana* (Calvo et al. 2019).

The aim of this study was to isolate and characterize the plant growth-promoting (PGP) properties of several rhizobacterial strains from three different stages of canola growth. Based on their biochemical traits, the strains were selected for a greenhouse experiment to assess their impact on canola growth. The strain showing the best performance, *Bacillus paralicheniformis* 2R5, was further subjected to whole-genome sequencing (WGS) to identify genes associated with its ability to promote canola growth and biologically control plant pathogens. Additionally, the effect of *B. paralicheniformis* 2R5 on the abundance of N-cycling genes in the rhizosphere of inoculated plants was investigated.

Materials and methods

Experimental site

Plant samples of canola (*Brassica napus* L. var. *napus*) were collected from the arable soil in Górsk (Poland 53°01′46.1″N, 18°26′59.4″E). Plants were sampled at the vegetative (R), flowering (2R), and maturity (3R) growth stages.

Isolation of canola rhizospheric bacteria

Canola roots were washed with sterile water to remove the adhering soil particles. The roots were cut into 2 cm pieces, macerated in sterile phosphate buffer (PBS), and subjected to a 10-fold serial dilution process. Each dilution was spread on nutrient agar (NA, BioMaxima, Warsaw, Poland) with 40 μg/ml antifungal agent amphotericin B for enumerating the culturable rhizospheric bacteria

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population. Plates were incubated for 3 days at 28°C. From each canola growth stage, 50 bacterial colonies were selected based on their different morphological parameters: shape, size, color, elevation, and capacity (Table S1), and were preserved in glycerol stocks at −80°C for further use.

PGP characteristics *in vitro*

Qualitative estimations

All the analyses were made in triplicate.

Phosphorus solubilization

Phosphorus solubilization ability was assessed using Pikovskaya's agar plates (Pikovskaya 1948). Pikovskaya's agar plates were incubated at 26 °C for 7 days for the observation of halo zones around the colonies. The solubilization index was evaluated as the ratio of total diameter (colony + halo zone) to colony diameter (Luziatelli et al. 2019).

Siderophore screening

Siderophore sequestration was estimated on chrome azurol sulphonate (CAS) medium (Alexander and Zuberer 1991). The CAS plates were incubated for 4 days at 26 °C for the observation of an orange halo zone around the colonies. The siderophore index (SI) was calculated as the ratio of the halo zone diameter to colony diameter.

Chitynolytic activity

To test the chitynolytic activity, bacteria were inoculated on a medium containing (g/l) peptone 1.0; $FeSO_4 \cdot 7H_2O$ 0.1; iron gluconate 0.1; yeast extract 0.1; colloidal chitin 7.0 g dry mass; and agar 15.0 (Swiontek Brzezinska et al. 2013). Plates were incubated for 14 days at 22°C to observe the clearing zone around the colonies. The colloidal chitin was prepared using the Lingappa and Lockwood method (1962).

Hydrogen cyanide test

Hydrogen cyanide (HCN) production was assessed using NA plates supplemented with glycine (0.44%) according to Lorck (1948). A sterile filter paper (Whatman No. 1) was soaked in a solution of picric acid (0.5%) and sodium carbonate (2%) and placed on the plate lid. After 4 days of incubation at 28°C, a color change of the filter paper from yellow to brown was considered a positive indicator for HCN production.

Ammonia production

Ammonia production was assessed by inoculating bacteria in nutrient broth (BioMaxima, Poland) for 3 days at 26°C. After adding the Nessler reagent (0.5 ml), the development of an orange color was considered as a positive result.

Quantitative estimations

All the analyses were made in triplicate.

Indole acetic acid production

Indole acetic acid (IAA) production was quantified using medium (g/l) peptone 5.0; yeast extract 3.0; and L-tryptophan 1.0 following the modified method of Ehmann (1977). After 4 days of incubation at 28°C, culture suspension was centrifuged at 10 080 *g* for 10 min. The supernatant was mixed with Salkowski reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$) and incubated for 30 min in the dark. Intensity of the color was measured at 530 nm using a Hitachi U-2500 spectrophotometer.

ACC deaminase activity

Quantitative estimation of ACC (1-aminocyclopropane-1-carboxylate) deaminase was carried out according to the modified method described by Honma and Shimomura (1978). Bacterial cultures were inoculated in a nutrient broth medium (BioMaxima, Warsaw, Poland) followed by incubation at 30°C for 24 h in a rotary shaker. Culture suspension (30 ml) was centrifuged at 6000 *g* for 10 min (4°C) and Dworkin and Foster (DF) salt minimal medium (5 ml) was added to the pellet (Dworkin and Foster 1958). Culture suspension was centrifuged again at 6000 *g* for 10 min (4°C) and DF salt minimal medium (5 ml) with 0.5 M ACC (30 µl) was added to the pellet. After incubation at 30°C for 24 h, culture suspension was centrifuged at 4032 *g* for 10 min (4°C) and bacterial cells were washed with 0.1 M Tris–HCl (5 ml; pH 7.6). After centrifugation at 10 000 *g* for 5 min, 0.1 M Tris–HCl (600 µl; pH 8.5), toluene (30 µl), and 0.5 M ACC substrate (20 μl) were added to the pellet followed by incubation at 30°C for 30 min. HCl at a concentration of 0.56 M (1 ml) was added and culture suspension was centrifuged at 10 000 *g* for 5 min. Centrifuged culture suspension supernatant (1 ml) was mixed with 0.56 M HCl (800 µl) and 0.2% 2,4-dinitrophenylhydrazine (300 µl). After a 30-min-long incubation at 30°C, 2 M NaCl (2 ml) was added and the absorbance was determined at 540 nm using a Hitachi U-2500 spectrophotometer. The ACC deaminase was expressed in terms of nanomoles of α-ketobutyrate produced per milligram protein per hour. Protein content was estimated according to the Bradford (1976) method.

16S rRNA gene sequencing

Genomic DNA of the six best-performing strains in the PGP tests was extracted using the GeneMATRIX Bacterial and Yeast Genomic DNA Purification Kit (EURx, Gdańsk, Poland) following the prescribed protocol. The 16S rRNA gene was amplified by PCR in a reaction mixture consisting of 1 U *Taq* DNA polymerase, 0.2 mM deoxynucleoside triphosphate mixture, Polbuffer B with 1.5 mM $MgCl_2$, 0.25 µM of universal primers, and 1 µl of genomic DNA (total reaction volume of 20 µl). The following primers were used: 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-TACGGTTACCTTGTTACGACTT-3; Kalwasińska et al. 2020). The thermocycling conditions were initial denaturation at 95°C for 3 min, 30 cycles of amplification (denaturation at 95°C for 30 s, annealing at 52°C for 20 s, and extension at 72°C for 1 min 40 s), and final extension at 72°C for 5 min. To check the PCR amplicons, 1% (w/v) agarose gel stained with Midori Green DNA Stain (Nippon Genetics Europe GmbH, Düren, Germany) was used. PCR products were sequenced using the BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with universal primers 27F and 1492R. This procedure was performed in accordance with the manufacturer's procedure. Capillary electrophoresis was performed by the Sequencing and Oligonucleotides Synthesis Laboratory (IBB, Warsaw, Poland). The taxonomic affiliation of bacterial isolates was determined using the EzBioCloud online tool (Yoon et al. 2017).

In vivo pot experiment

Canola seeds (variety of Arazzo) were sterilized with 1% NaOCl for 30 min and were rinsed three times with sterile distilled water. The bacterial inocula were prepared in 10 ml of nutrient broth supplemented with 0.05 g carboxymethyl cellulose (CMC) to make a suspension of 10^8 CFU/ml. The disinfected seeds were added to the cell suspension and agitated for 30 min. Control seeds were

treated with 10 ml of nutrient broth supplemented with 0.5% CMC without bacterial inocula (Rudolph et al. 2015).

The seeds were sowed in pots (four seeds per pot) in eight replicates for each treatment. The pots contained (i) sterile sand and vermiculite (1:1) or (ii) nonsterile soil taken from the field of Górsk (Poland 53°01′46.1″N 18°26′59.4″E). The physicochemical soil parameters such as pH, the content of P_2O_5, K_2O, Mg, N–NO_3, N–NH_4, and C_{org} were determined by the District Chemical and Agricultural Station in Bydgoszcz (Poland) (PN-ISO 10390: 1997, PN-R-04022: 1996, PN-R-04022: 1996/Az1: 2002, PN-R-04020: 1994/Az1: 2004, PN-R-04028: 1997, PN-ISO 14235: 2003). The plants were grown at 22°C with a photoperiod of 16/8 h (light/dark; 100 μmol/m^2/s). The pots were moistened with an equal amount of water. The canola plants were harvested 44 days after planting.

After harvesting, the CCI of canola leaves was measured by a handheld meter (CCM-200 plus, Opti-Sciences, Inc, Hudson, NH, USA). Soil adhering to plant roots was washed with distilled water and canola morphological parameters, including roots, shoots, and epicotyl length, were measured. In the case of plants under nonsterile soil conditions, only length of roots and epicotyl were measured. Photosynthetic area of leaves was determined with the DigiShape 1.3 software (Moraczewski 2005). The plant roots, shoots, epicotyl, petioles, and leaves were dried at 85°C for 48 h to determine their dry weight. For nonsterile conditions, only the dry weight of plant roots, epicotyl, petioles, and leaves was determined. Two following plant growth indexes were calculated as follows: SLA (specific leaf area) = assimilation area (cm^2) / dry biomass of leaves (g) and LWR (leaf weight ratio) = dry biomass of leaves (g) / total plant dry biomass (g) (Piernik et al. 2017).

The WGS of *B. paralicheniformis* 2R5

The whole genome of *B. paralicheniformis* 2R5 was sequenced at the University of Birmingham (UK) on Illumina HiSeq (Illumina Inc, San Diego, CA, USA) following the 250 bp paired-end protocol. Library preparation was carried out using the Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the prescribed protocol with a slight modification (1 min PCR elongation time instead of 30 s long; 2 ng of genomic DNA instead of 1 ng). A microlab STAR automated liquid handling system (Hamilton, Switzerland) was used for DNA quantification and library preparation. Pooled libraries were quantified on a Roche LightCycler 96 qPCR machine (Roche, Basel, Switzerland) using the Kapa Biosystems Library Quantification Kit for Illumina platforms (Kapa Biosystems, Wilmington, MA, USA). Reads were adapter trimmed using the Trimmomatic 0.30 with a sliding window quality cutoff of Q15 (Bolger et al. 2014). De novo assembly was performed on samples using SPAdes version 3.7 (Bankevich et al. 2012), and contigs were annotated using the Prokka 1.11 (Seemann 2014). Further annotation was performed using the NCBI's Prokaryotic Genome Annotation Pipeline (PGAP). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis (https://www.kegg.jp/kegg/mapper/reconstruct.html) was used to predict the PGP genes. Biosynthesis gene clusters (BGCs) were detected using the antiSMASH (version 6.0; https://antismash.secondarymetabolites.org).

Biocontrol activities of *B. paralicheniformis* 2R5 against plant pathogens

Biocontrol activity of *B. paralicheniformis* 2R5 was assayed against seven plant pathogens, *Alternaria alternata* 783, *Botrytis cinerea* 873, *Fusarium culmorum* 2333, *F. oxysporum* 872, *F. solani* 25, *Phytophthora cactorum* 1925, and *P. megasperma* 404 all obtained from the Plant Pathogenic Bank of the Institute of Plant Protection in Poznan (Poland). *Bacillus paralicheniformis* 2R5 was grown on potato dextrose agar (PDA) for 24 h at 26°C. Fungi were grown on PDA for 5 days at 26°C and their 5 mm discs were placed in the center of PDA plates with a spread bacterial isolate. Diameter of fungal pathogens was estimated after incubating inoculated plates for 7 days at 26°C. Plates inoculated only with a fungi agar disc were used as control. Each experiment was performed in triplicate. The plant pathogen growth inhibition zone was calculated from the following equation: inhibition % = (diameter of fungi control − diameter of fungi that grew with bacteria) / diameter of fungi control × 100 (Wonglom et al. 2019).

Quantitative PCR

To quantify gene copies involved in N-cycle, including nitrogen fixing (based on *nif*H gene), nitrate reduction (*narG*), denitrification (*nir*S and *nos*Z), and ammonia oxidizing (*amo*A), quantitative PCR (qPCR) was performed. The qPCR reaction was carried out according to the method described in Kalwasińska et al. (2022) using 5.0 μl of SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (2×), 2.8 μl of nuclease-free water, 0.6 μl of each primer (10 μM), and 1.0 μl of template DNA on a CFX Opus 96 Real-Time PCR System (Bio-Rad, Hemel Hempstead, UK).

DNA was extracted from samples of rhizosphere treated with *B. paralicheniformis* 2R5 and from samples of untreated rhizosphere from 0 to 44 days referred to as T0, T22, and T44 for days 0, 22, and 44, respectively (four replicates; 0.25 g of each) using a DNeasy Power Soil Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The concentration of DNA was determined using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The soil DNA was further cloned into pCR4 TOPO vector (Invitrogen, Waltham, MA, USA) and was used as a control DNA for standard curve preparation. Target copy numbers were calculated from the standard curves, and the number of copies per gram of dry soil was determined. Standards, samples, and the negative control were amplified in triplicate. The efficiency of the qPCR assays was between 94% and 114%. The used primers and thermocycling conditions are present in the supplementary data (Table S2).

Statistical analysis

Differences between treatments in sterile conditions were tested with analysis of variance following the Tukey post-hoc test in Past v 3.25 (Hammer et al. 2001). The Shapiro–Wilk test was used to check the assumptions of normality, while Levene's test was used to assess the homogeneity of variances. For non-normally distributed data, a test for equal medians (Kruskal–Wallis) was performed. To determine differences between noninoculated seedlings and seedlings inoculated with *B. paralicheniformis* 2R5 under nonsterile conditions, a *t*-test for equal means was applied.

Differences in copy numbers of genes involved in the N-cycle between plants treated with *B. paralicheniformis* 2R5 and nontreated plants at T0, T22, and T44 after bacterization were tested with a *t*-test for equal means in Past v 3.08. For non-normally distributed data, a test for equal medians (Mann–Whitney) was performed.

Results

Enumerating culturable rhizobacteria and characterization of PGP traits

The culturable rhizobacteria were enumerated at the vegetative, flowering, and maturity canola growth stages (Fig. S1). The highest

population of culturable bacteria ($P < .05$) was found in vegetative stage (17.2×10^3 CFU/ml) samples followed by samples at the maturity (15.9×10^3 CFU/ml) and flowering (12.6×10^3 CFU/ml) stages.

PGP traits of the rhizobacteria isolated from three different canola growth stages were determined (Table S3). At the vegetative stage, ACC deaminase, siderophores, HCN, and chitinases were produced by the highest % of isolates (96%, 72%, 9%, and 22%, respectively), whereas at the maturity stage, IAA (> 8 μg/ml), phosphates and ammonia were produced by the highest % of isolates (31%, 18%, and 47%, respectively; Fig. S2).

High values were observed in four out of seven tested PGP properties of the six strains: R27, R38, R44, 2R5, 3R27, and 3R43. Strain R27 produced a high concentration of IAA (24 μg/ml), solubilized phosphates (SI = 6), sequestrated siderophores (SI = 4.2), and was an HCN producer. Strain R38 showed high ACC deaminase activity (2174.2 nmol α-ketobutyrate/mg protein/h), IAA production (21.66 μg/ml), siderophore production (SI = 4.3), and HCN production. Strain R44 demonstrated a high concentration of ACC deaminase (1007.8 nmol α-ketobutyrate/mg protein/h) and IAA (12.01 μg/ml) and displayed high phosphate (SI = 7) and siderophore (SI = 4.3) production. Strain 2R5 had high ACC deaminase activity (2226.1 nmol α-ketobutyrate/mg protein/h), IAA (8.49 μg/ml), and siderophore (SI = 3.3) and chitinase (1 mm) production, whereas the 3R27 and 3R43 strains produced a high concentration of ACC deaminase (1314.4 and 1117.3 nmol α-ketobutyrate/mg protein/h), IAA (15.97 and 8.55 μg/ml), solubilized phosphates (SI = 4 and 4), and sequestrated siderophores (SI = 4.7 and 3.1), respectively.

Plant growth promotion in canola

Six rhizobacterial isolates were selected for molecular identification and a pot experiment under sterile conditions based on their PGP characteristics. These strains were identified as *Pseudomonas* sp. R27, *P. atacamensis* R38, *Pseudomonas* sp. R44, *B. paralicheniformis* 2R5, *P. piscium* 3R27, and *P. brenneri* 3R43. Under sterile conditions, canola plants inoculated with *B. paralicheniformis* 2R5 showed the highest shoot weight, leaf weight, and CCI with significant increases ($P < .05$) over the other inoculant treatments and uninoculated control (Fig. 1). *Bacillus paralicheniformis* 2R5 had a significant increase ($P < .05$) in leaf weight ratio compared to uninoculated control and other inoculant treatments, but not in the case of *P. brenneri* 3R43 (Fig. 1). No significant differences were observed for the following canola growth promotion parameters: weight (roots, epicotyl, petioles), length (roots, shoots), and specific leaf area (data not shown).

Bacillus paralicheniformis 2R5 turned out to be the best-performing strain under sterile conditions. Therefore, 2R5 was chosen for a greenhouse experiment in nonsterile soil (Table 1) to check if it is still able to promote canola growth in the presence of native soil microorganisms. Under nonsterile conditions, seedlings inoculated with *B. paralicheniformis* 2R5 showed a significantly higher weight and length of roots, weight of leaves, and chlorophyll content index (CCI) compared to noninoculated seedlings. The physicochemical parameters of the nonsterile soil are shown in Table S4.

Ultimately, *B. paralicheniformis* 2R5 increased canola leaf weight and chlorophyll content as compared to uninoculated control both under sterile and nonsterile conditions. In addition, under sterile conditions, *B. paralicheniformis* 2R5 increased shoot weight and leaf weight ratio, while under nonsterile conditions, it increased the weight and length of roots. *Bacillus paralicheniformis* 2R5 effects on canola growth under sterile and nonsterile conditions are shown in Fig. S3.

Genome analysis and biocontrol activity

The WGS analysis of *B. paralicheniformis* 2R5 revealed a sequence read count of 491 153, a genome size of 4.55 Mb and GC content of 45.9%. The number of contigs was 78, while the N50 and L50 values were 456 496 and 4, respectively. The WGS analysis revealed 4749 genes and 94 pseudogenes. The genome assemblies and annotations are summarized in Table S5.

Bacillus paralicheniformis 2R5 harbored genes involved in IAA production, which include *trpA*, *trpB*, *trpD*, *trpC*, *trpE*, and *trpF*. Genes involved in phosphate solubilization (*pstA*, *pstS*), siderophore sequestration (*mbtH*), and chitinase production (*chiA*) were identified in the genome of *B. paralicheniformis* 2R5. Nitrogen fixation genes such as *narG* and *nos* were found in the 2R5 genome. The identified genes associated with plant growth promotion are present in Table 2.

The BGCs nonribosomal peptide synthetases (NRPS; bacillibactin, fengycin, lichenysin, bacitracin), thiopeptides and RiPP-like (butirosin A / butirosin B), lanthipeptide-class-ii and cyclic-lactone-autoinducers (haloduracin β / haloduracin α), lassopeptide, CDPS, RiPP-like, terpene, and T3PKS were detected in the *B. paralicheniformis* 2R5 genome. *Bacillus paralicheniformis* 2R5 exhibited 100% similarity to gene type NRPS with the most similar known clusters of lichenysin and bacitracin. *Bacillus paralicheniformis* 2R5 inhibited growth of six plant pathogens with the highest inhibition rate of 24% and 20% against *A. alternata* and *P. cactorum*, respectively. The predicted secondary metabolite cluster genes of *B. paralicheniformis* 2R5 and its biocontrol activity are presented in Table 3.

Quantification of genes involved in the N-cycle

The copy number of N-cycle genes showed statistical differences between samples treated with *B. paralicheniformis* 2R5 and untreated samples (Table 4). Although there were no significant differences in the number of ammonia-oxidizing gene *amoA*, a significant increase in denitrifying nitrous oxide reductase gene *nosZ* at each time-point in the *B. paralicheniformis* 2R5-treated rhizosphere was observed. The copy number of nitrate-reducing gene *narG* and nitrite reductase gene *nirS* significantly increased at T0 and T44 in the treated rhizosphere, while N-fixing gene *nifH* was significantly higher than in untreated control at T22 and T44.

Discussion

A plant rhizosphere, an important ecological niche, teems with a rich tapestry of microorganisms, exerting a profound influence on plant physiology and morphology. These microbial communities hold the potential to stimulate plant growth and provide protection against harmful phytopathogens (Alawiye and Babalola 2019). Within the rhizosphere, microbial life thrives abundantly and exhibits heightened activity (Zhang et al. 2017).

Our research findings further shed light on the intricate dynamics of microbial communities within the rhizosphere, revealing variations in rhizobacteria density across different stages of plant growth. These disparities can be attributed to the differential production of secretions during various plant stages, thereby reshaping the structure of the rhizosphere microbiome. This remodeling process enhances the growth of specific community members while inhibiting others, as elucidated by the studies of Jacoby et al. (2020) and Vives-Peris et al. (2020). In a research con-

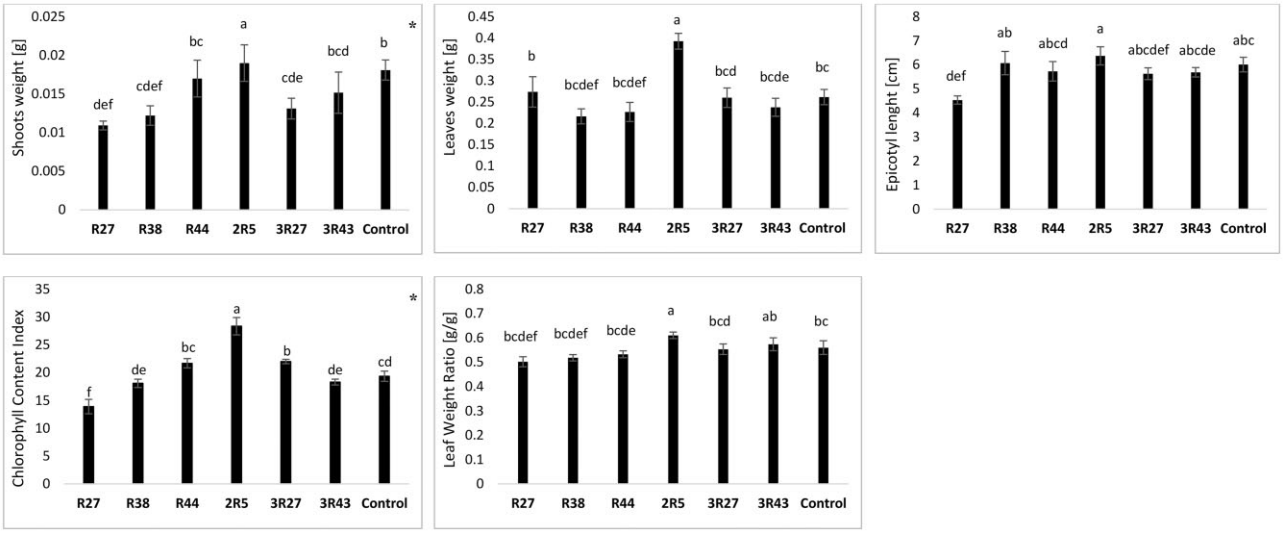

Figure 1. PGP effect of the selected isolates evaluated under sterile conditions. Different letters indicate significant differences ($P < .05$) between the means of the groups, determined using the Tukey HSD test as a post-hoc analysis; ∗ denotes variables with a non-normal distribution (tested for equal medians using Mann–Whitney test). No significant differences were observed for the following canola growth promotion parameters: root weight, epicotyl weight, petiole weight, root length, shoot length, and specific leaf area.

Table 1. PGP effect under nonsterile conditions between seedlings inoculated with *B. paralicheniformis* 2R5 and those noninoculated seedlings.

Parameter	Seedlings inoculated with *B. paralicheniformis* 2R5	Noninoculated seedlings	*t*	*P*-value
Weight of roots [g]	0.005	0.003	5.663	.001**
Weight of epicotyl [g]	0.005	0.004	2.237	ns
Weight of petioles [g]	0.005	0.003	2.413	ns
Weight of leaves [g]	0.011	0.008	2.935	.026*
Length of roots [cm]	14.15	9.15	8.771	1.23E-04***
Length of epicotyl [cm]	8.32	8.50	−0.392	ns
SLA (specific leaf area) [cm^2/g]	244.40	200.24	1.558	ns
LWR (leaf weight ratio) [g/g]	0.407	0.430	−1.199	ns
CCI (chlorophyll content index)	13.80	10.42	6.352	.001**

ns—nonsignificant; significant differences (*$P < .05$, **$P < .01$, ***$P < .001$) according to the *t*-test; values for seedlings are expressed as mean ($n = 8$).

Table 2. *Bacillus paralicheniformis* 2R5 genes involved in plant growth promotion.

Characteristics	2R5	Genes
IAA production	+	*trpA*, *trpB*, *trpD*, *trpC*, *trpE*, *trpF*
	−	*trpG*, *ipdC*
ACC deaminase production	−	*acdS*, *acdA*, *rimM*, *dcyD*
Phosphate solubilization	+	*pstA*, *pstS*
	−	*pstB*, *pstC*, *pqq*
Siderophore production	+	*mbtH*
	−	*pvd*, *fpvA*, *acrA*, *acrB*, *fhu*, *asbF*, *entD*, *fetB*, *fbpA*, *feoB*
HCN production	−	*hcnA*, *hcn B*, *hcnC*
Chitinase production	+	*chiA*
	−	*chiB*, *chiC*, *chiD*
Acetoin and butanediol synthesis		
Nitrogen fixation	−	*poxB*, *budA*, *budB*, *budC*
	+	*narG*, *nosZ*
	−	*nirS*, *amoA*, *nifH*

+, positive result; −, negative result.

ducted by Bashir et al. (2020), the endophytic population in sunflower (*Helianthus annuus*) was compared between its vegetative and reproductive stages. The researchers unveiled a decline in the culturable root endophytic bacteria during the reproductive stage, showcasing the intricate interplay between plant development and microbial inhabitants. Similarly, the work of Muñoz-Rojas and Caballero-Mellado (2003) highlighted a decrease in the population of *Gluconacetobacter diazotrophicus* with the aging of plants, potentially triggered by activation and intensification of plant defence mechanisms. In our research, the canola plants carried the largest population of culturable rhizobacteria at the vegetative stage (17.2×10^3 CFU/ml) followed by the maturity (15.9×10^3 CFU/ml) and flowering (12.6×10^3 CFU/ml) stages. Marag and Suman (2018) enumerated culturable root endophytic bacteria from two different varieties of maize and at three different stages of the plant growth. A similar endophytic population size ($\times 10^3$ CFU/ml) was observed; however, the highest size was observed at the flowering stage followed by the vegetative and then the maturity stage in both maize varieties (Marag and Suman 2018).

The remarkable microbial ecology of the rhizosphere is exemplified by the plant growth promoting abilities of some microbial species like phosphate-solubilization or indole-3-acetic acid (IAA)

Table 3. Estimate of biosynthetic cluster genes and biocontrol activity of the *B. paralicheniformis* 2R5.

Biosynthetic cluster genes						
Region	**From**	**To**	**Most similar known cluster**		**Type**	**Similarity**
Rg 1.1	217 680	240 141	None		Lassopeptide	
Rg 1.2	460 805	507 971	Bacillibactin	NRP	NRPS	53%
Rg 2.1	1	39 645	Fengycin	NRP	NRPS	66%
Rg 4.1	191 123	256 560	Lichenysin	NRP	NRPS	100%
Rg 5.1	237 721	258 470	None		CDPS	
Rg 6.1	112 577	122 921	None		RiPP-like	
Rg 6.2	217 154	300 043	Bacitracin	NRP	NRPS	100%
Rg 7.1	1	21 912	Fengycin	NRP	NRPS	20%
Rg 7.2	117 215	139 104	None		terpene	
Rg 7.3	192 252	233 349	None		T3PKS	
Rg 9.1	86 793	128 035	Butirosin A / butirosin B	Saccharide	thiopeptide, RiPP-like	7%
Rg 10.1	36 241	51 704	None		siderophore	
Rg 12.1	1	25 573	Haloduracin β / haloduracin α	RiPP: lanthipeptide	Lanthipeptide-class-ii, cyclic-lactone-autoinducer	40%
Rg 20.1	1	13 425	Fengycin	NRP	NRPS	20%
Inhibition of plant pathogens						
A. alternata	*B. cinerea*	*F. culmorum*	*F. oxysporum*	*F. solani*	*P. cactorum*	*P. megasperma*
24%	13%	18%	19%	17%	20%	0%

Rg—region, NRPS—nonribosomal peptide synthetases, RiPP—ribosomally synthesized and post-translationally modified peptide, NRP—nonribosomal peptide.

production by *Bacillus* spp. (Ribeiro et al. 2018). Among the diverse strains within the *Bacillus* genus, *B. siamensis*, *B. cereus*, and *B. subtilis* have emerged as notable producers of IAA, which is crucial for plant development, influencing cell elongation, and division (Wagi and Ahmed 2019; Widawati 2020). Intriguingly, the genomic analysis of *B. paralicheniformis* 2R5 unveiled the presence of *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, and *pstA* and *pstS* genes, responsible for IAA biosynthesis and phosphate solubilization, respectively. The *pst* gene, encoding the phosphate transport pathway in bacterial genomes, assumes a pivotal role in enhancing phosphate bioavailability within phosphate-limited environments, thereby promoting plant growth (Nascimento et al. 2020). Świątczak et al. (2023) made a comparative genome analysis of the *Brevibacillus laterosporus* K75 genome and *B. laterosporus* MG64 genome published by Li et al. (2020). The analysis revealed that *B. laterosporus* K75 genome contained additional genes involved in phosphate transport pathways (*pqq*, *pstA*, *B*) and IAA production (*trpA*, *B*, *C*, *D*, *E*, *F*, *S*), suggesting that these genes were responsible for the *B. laterosporus* K75 stimulatory effect on maize plants (Świątczak et al. 2023). Our findings strongly support the notion that *B. paralicheniformis* 2R5 stmulates canola growth under both sterile and nonsterile conditions through its remarkable IAA and phosphate traits. The microbial ecology within the rhizosphere, with its diverse array of microorganisms and their intricate molecular interactions, continues to unveil the fascinating mechanisms underpinning plant–microbe interactions and holds immense potential for agricultural applications.

The microbial ecology aspect of *B. paralicheniformis* species becomes apparent in their remarkable ability to control pathogenic fungi through the production of secondary metabolites, as highlighted by Du et al. (2019). In our study, *B. paralicheniformis* 2R5 exhibited significant antifungal activity against a wide range of tested fungal pathogens, complemented by the identification of several biosynthetic gene clusters within its genome. These findings underscore the potential application of *B. paralicheniformis* 2R5 as a valuable tool for biological control of plant diseases. Our results align with another study where *B. paralicheniformis* MDJK30 emerged as a potential biocontrol agent. This strain displayed antifungal activity against *F. solani*, and similar gene clusters associated with secondary metabolites, such as lichenysin, fengycin, bacitracin, and bacillibactin, were identified in its genome (Du et al. 2019). As a member of PGPR, the 2R5 strain also exhibited the ability to produce chitinases and siderophores, as confirmed by the presence of the *chiA* and *mbtH* genes. These characteristics likely contribute to its heightened antifungal activity. In fact, previous research demonstrated that *Bacillus subtilis* F29-3, expressing the *chiA* gene, exhibited a larger inhibition zone against *B. elliptica* compared to a control strain of *B. subtilis* (Chen et al. 2004) that was lacking chitinase activity. Additionally, the *mbtH* protein is involved in siderophore mycobactin (Mbt) biosynthesis in *Mycobacterium tuberculosis*. This protein plays a crucial role in the functioning of nonribosomal peptide synthetases (NRPS), which are well known for their antimicrobial, immunosuppressant, cytostatic, and antiviral activities (Zwahlen et al. 2019). These discoveries reinforce the notion that *B. paralicheniformis* species possess robust capabilities in combating plant pathogens and providing further support for their application in disease management strategies.

The N dynamics in the context of plant growth and development is of paramount importance in microbial ecology (Anas et al. 2020). Studies have suggested that increased levels of N-cycle genes after soil inoculation with PGPR can have positive effects on plant growth and development by enhancing the availability of N (Bhattacharyya et al. 2018). Intriguingly, the introduction of *B. paralicheniformis* 2R5 resulted in a substantial increase in the copy number of genes such as *narG*, *nifH*, *nosZ*, and *nirS*. These findings align with the observations made by Bhattacharyya et al. (2018), who demonstrated that the PGPR *Proteus vulgaris* JBLS202 increased the abundance of N-cycle genes in the rhizosphere.

However, it is crucial to consider the specific actions of N-cycle genes. The *nifH* genes enable bacteria to fix atmospheric nitrogen (N_2) into ammonia (NH_3), which is readily assimilated by plants (Gresshoff et al. 2015, Dasgupta et al. 2021). On the other hand, the genes *narG*, *nirS*, and *nosZ* are involved in the denitrification process, which represents a significant nitrogen loss pathway in agricultural soils (Igiehon and Babalola 2018, Zhu et al. 2018). In our study, the presence of the nitrate-reducing gene *narG* and denitrification gene *nosZ* in the genome of *B. paralicheniformis* 2R5 was

Table 4. qPCR quantifications of genes involved in the N-cycle (copy number/$g_{dry\ soil}$).

Sample	*nosZ*			*narG*			*nifH*			*amoA*			*nirS*		
	Mean	*P*	*t*	Mean	*P*	*t*	Mean	*P*	*t*	Mean/Median	*P*	*t/z*	Mean	*P*	*t*
CT0	3.79E+09	5.23E−05	18.31	1.39E+07	.001	9.76	1.91E+06	ns	0.25	7.21E+05	ns	0.64	1.88E+06	.006	5.42
BpT0	6.88E+09			5.02E+07			2.02E+06			2.85E+06			5.24E+06		
CT22	7.53E+09	.001	8.44	6.05E+07	ns	0.52	2.10E+06	.026	3.45	1.08E+06[nnd]	ns	1.57	1.40E+06	ns	0.06
BpT22	1.25E+10			7.59E+07			4.76E+06			2.42E+06[nnd]			1.42E+07		
CT44	8.23E+09	.002	7.30	7.87E+07	2.26E-06	40.32	2.51E+06	3.43E-04	11.35	1.00E+06[nnd]	ns	1.77	3.95E+06	4.42E-04	10.64
BpT44	1.29E+10			1.04E+08			1.44E+07			2.50E+06[nnd]			1.46E+07		

ns—nonsignificant; nnd—not normal distribution (test for equal medians: Mann–Whitney); C—control, untreated plants; Bp—plants treated with *Bacillus paralicheniformis* 2R5; T0, T22, and T44—time after bacterization in days.

concurrent with notable changes in the abundance of N-cycle genes in the rhizosphere after treatment. It is noteworthy that the untreated samples also exhibited an increase in the abundance of N-cycle genes over time. Previous research has indicated that plants, through root exudates, shape the rhizosphere microbiome, thereby influencing the abundance and activity of N-cycle microbes, including nitrate reducers and denitrifiers (Henry et al. 2008, Weng et al. 2013). Furthermore, the rhizosphere environment can impact the copy number and relative abundance of functional genes. Wang et al. (2015) demonstrated that changes in the microbial community and network interactions within the rhizosphere contributed to alterations in functional genes responsible for nitrification efficiency in maize plants. Therefore, further research is warranted to elucidate the intricate interactions between the introduced *B. paralicheniformis* 2R5 and the native microbial communities. The modulation of N-cycle genes by *B. paralicheniformis* 2R5, along with the influence of the rhizosphere environment, holds significant implications for nutrient availability and plant growth promotion. Exploring these complex microbial interactions will provide valuable insights into optimizing plant–microbe interactions for sustainable agriculture.

Conclusion

This research paves the way for the development and implementation of innovative strategies harnessing the ecology of *B. paralicheniformis* for sustainable agriculture. The commercialization of preparations based on this species holds immense promise in promoting canola growth, boosting resistance to pathogens, and optimizing nutrient cycling dynamics. Further exploration of the intricate interactions between *B. paralicheniformis* 2R5 and the native microbial community will undoubtedly unveil new insights into the microbial ecology of canola-associated rhizobacteria and facilitate the development of effective bioformulations for agricultural applications.

Author contributions

Joanna Świątczak (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing), Agnieszka Kalwasińska (Methodology, Supervision, Validation, Writing – review & editing), Tamás Felföldi (Formal analysis, Investigation), and Maria Swiontek Brzezinska (Conceptualization, Project administration, Supervision, Validation)

Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

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Data availability

The almost complete 16S rRNA gene sequences of R27, R38, R44, 2R5, 3R27, and 3R43 are available under the accession numbers

MW599213, MW599214, MW599217, MW599219, MW599220, and MW599221, respectively. The whole-genome shotgun project of strain 2R5 has been deposited at DDBJ/ENA/GenBank under the accession JAOWAK000000000 (BioProject PRJNA876229, BioSample SAMN31236553).

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Supplementary Materials

Table S1. Morphological parameters of bacterial colonies isolated from three different canola growth stages.

Strains	Shape	Size	Color	Elevation	Opacity
			Vegetative stage		
R1	Circular	Small	Brown	Convex	Translucent
R2	Circular	Small	Brown	Raised	Translucent
R3	Circular	Pinpoint	White	Raised	Translucent
R4	Irregular	Medium	White	Flat	Translucent
R5	Circular	Small	White	Convex	Opaque
R6	Circular	Pinpoint	Brown	Convex	Translucent
R7	Circular	Small	Orange	Flat	Translucent
R8	Irregular	Small	White	Flat	Translucent
R9	Irregular	Medium	Creamy	Raised	Translucent
R10	Irregular	Large	White	Flat	Translucent
R11	Filamentous	Large	White	Flat	Translucent
R12	Rhizoidal	Large	Creamy	Flat	Translucent
R13	Circular	Pinpoint	Brown	Raised	Translucent
R14	Circular	Pinpoint	White	Convex	Opaque
R15	Circular	Pinpoint	White	Raised	Opaque
R16	Filamentous	Medium	White	Flat	Translucent
R17	Circular	Small	Yellow	Convex	Translucent
R18	Circular	Small	Yellow	Raised	Translucent
R19	Circular	Medium	Yellow	Convex	Translucent
R20	Circular	Medium	White	Convex	Opaque
R21	Rhizoidal	Small	Creamy	Flat	Translucent
R22	Circular	Pinpoint	White	Flat	Translucent
R23	Circular	Medium	Orange	Raised	Translucent
R24	Circular	Medium	White	Raised	Opaque
R25	Circular	Pinpoint	Creamy	Raised	Translucent
R26	Filamentous	Small	White	Flat	Translucent
R27	Irregular	Small	White	Flat	Opaque
R28	Circular	Medium	Creamy	Convex	Translucent
R29	Circular	Small	Creamy	Raised	Translucent
R30	Circular	Pinpoint	White	Convex	Translucent
R31	Filamentous	Large	Creamy	Flat	Translucent
R32	Rhizoidal	Medium	Creamy	Flat	Translucent
R33	Circular	Medium	Creamy	Raised	Translucent
R34	Circular	Pinpoint	Yellow	Raised	Translucent
R35	Filamentous	Medium	Creamy	Flat	Translucent
R36	Circular	Pinpoint	Creamy	Flat	Translucent
R37	Circular	Medium	Creamy	Flat	Translucent
R38	Irregular	Medium	Creamy	Raised	Translucent
R39	Irregular	Large	Creamy	Raised	Translucent
R40	Circular	Small	Creamy	Convex	Translucent
R41	Circular	Pinpoint	Creamy	Convex	Translucent
R42	Circular	Large	White	Raised	Opaque
R43	Circular	Pinpoint	Yellow	Convex	Translucent
R44	Irregular	Small	White	Raised	Opaque
R45	Circular	Small	White	Flat	Opaque
R46	Circular	Small	Creamy	Flat	Translucent
R47	Filamentous	Small	Creamy	Flat	Translucent

R48	Rhizoidal	Large	White	Flat	Translucent
R49	Circular	Large	White	Convex	Opaque
R50	Rhizoidal	Medium	White	Flat	Translucent
			Flowering stage		
2R1	Irregular	Small	Creamy	Flat	Translucent
2R2	Irregular	Medium	Creamy	Flat	Translucent
2R3	Filamentous	Small	Creamy	Flat	Translucent
2R4	Filamentous	Medium	Creamy	Flat	Translucent
2R5	Filamentous	Large	Creamy	Flat	Translucent
2R6	Rhizoidal	Small	White	Flat	Translucent
2R7	Rhizoidal	Medium	Creamy	Flat	Translucent
2R8	Rhizoidal	Large	Creamy	Flat	Translucent
2R9	Circular	Small	Creamy	Flat	Translucent
2R10	Circular	Large	White	Convex	Translucent
2R11	Circular	Small	Creamy	Raised	Translucent
2R12	Circular	Medium	White	Convex	Translucent
2R13	Circular	Small	Brown	Convex	Translucent
2R14	Circular	Pinpoint	Brown	Raised	Translucent
2R15	Circular	Small	White	Convex	Translucent
2R16	Circular	Large	White	Raised	Translucent
2R17	Circular	Small	White	Flat	Translucent
2R18	Circular	Small	White	Raised	Translucent
2R19	Circular	Medium	White	Raised	Translucent
2R20	Circular	Small	Orange	Convex	Translucent
2R21	Circular	Pinpoint	Orange	Convex	Translucent
2R22	Circular	Pinpoint	Orange	Flat	Translucent
2R23	Irregular	Medium	White	Flat	Translucent
2R24	Circular	Large	White	Flat	Translucent
2R25	Circular	Medium	White	Flat	Translucent
2R26	Circular	Large	Creamy	Raised	Translucent
2R27	Irregular	Small	Creamy	Flat	Opaque
2R28	Circular	Pinpoint	Creamy	Raised	Translucent
2R29	Irregular	Medium	Creamy	Flat	Opaque
2R30	Circular	Medium	Creamy	Flat	Translucent
2R31	Rhizoidal	Medium	White	Flat	Translucent
2R32	Rhizoidal	Small	Creamy	Flat	Translucent
2R33	Irregular	Medium	Creamy	Raised	Translucent
2R34	Irregular	Large	Creamy	Flat	Translucent
2R35	Irregular	Medium	Orange	Raised	Translucent
2R36	Filamentous	Medium	Creamy	Raised	Opaque
2R37	Circular	Small	Yellow	Convex	Translucent
2R38	Circular	Medium	Yellow	Convex	Translucent
2R39	Circular	Small	Creamy	Convex	Translucent
2R40	Circular	Large	Yellow	Convex	Translucent
2R41	Irregular	Small	White	Flat	Translucent
2R42	Circular	Medium	Orange	Convex	Translucent
2R43	Circular	Medium	Orange	Raised	Translucent
2R44	Irregular	Large	White	Flat	Translucent
2R45	Rhizoidal	Large	White	Flat	Translucent
2R46	Irregular	Large	Creamy	Raised	Translucent
2R47	Irregular	Small	Yellow	Flat	Translucent
2R48	Circular	Medium	Creamy	Flat	Opaque
2R49	Irregular	Medium	Yellow	Raised	Translucent
2R50	Circular	Large	Creamy	Flat	Opaque
			Maturity stage		
3R1	Circular	Medium	Yellow	Raised	Translucent
3R2	Filamentous	Small	Creamy	Flat	Translucent
3R3	Circular	Small	Creamy	Convex	Translucent

3R4	Filamentous	Large	Creamy	Flat	Translucent
3R5	Filamentous	Large	White	Flat	Translucent
3R6	Irregular	Medium	Creamy	Flat	Translucent
3R7	Circular	Medium	Creamy	Convex	Translucent
3R8	Circular	Small	Creamy	Raised	Translucent
3R9	Circular	Small	Creamy	Flat	Translucent
3R10	Circular	Pinpoint	Creamy	Flat	Translucent
3R11	Circular	Medium	Creamy	Flat	Translucent
3R12	Circular	Large	White	Raised	Translucent
3R13	Circular	Large	Creamy	Flat	Translucent
3R14	Circular	Medium	Creamy	Raised	Translucent
3R15	Rhizoidal	Large	Creamy	Flat	Translucent
3R16	Rhizoidal	Medium	Creamy	Flat	Translucent
3R17	Rhizoidal	Small	Creamy	Flat	Translucent
3R18	Filamentous	Small	White	Flat	Translucent
3R19	Circular	Small	Yellow	Raised	Translucent
3R20	Irregular	Large	White	Flat	Translucent
3R21	Circular	Medium	Yellow	Convex	Translucent
3R22	Circular	Medium	Yellow	Flat	Translucent
3R23	Irregular	Medium	Yellow	Flat	Translucent
3R24	Irregular	Large	Creamy	Raised	Opaque
3R25	Irregular	Large	Creamy	Flat	Opaque
3R26	Circular	Medium	Yellow	Raised	Opaque
3R27	Circular	Small	White	Convex	Translucent
3R28	Filamentous	Large	Creamy	Flat	Opaque
3R29	Irregular	Medium	Creamy	Flat	Opaque
3R30	Circular	Medium	Orange	Convex	Translucent
3R31	Irregular	Small	Creamy	Flat	Translucent
3R32	Irregular	Large	Creamy	Flat	Translucent
3R33	Irregular	Small	Creamy	Flat	Opaque
3R34	Irregular	Small	Creamy	Raised	Opaque
3R35	Circular	Small	Orange	Raised	Translucent
3R36	Irregular	Medium	White	Flat	Translucent
3R37	Circular	Small	Yellow	Raised	Opaque
3R38	Circular	Small	White	Flat	Translucent
3R39	Circular	Medium	Yellow	Convex	Opaque
3R40	Irregular	Medium	Creamy	Raised	Opaque
3R41	Circular	Medium	White	Flat	Translucent
3R42	Circular	Small	Orange	Convex	Translucent
3R43	Circular	Small	White	Convex	Opaque
3R44	Circular	Medium	White	Raised	Translucent
3R45	Circular	Large	White	Flat	Translucent
3R46	Filamentous	Medium	White	Flat	Translucent
3R47	Irregular	Small	Yellow	Flat	Opaque
3R48	Irregular	Medium	Yellow	Flat	Opaque
3R49	Filamentous	Medium	Creamy	Flat	Translucent
3R50	Irregular	Small	White	Flat	Translucent

Size: large (>1mm) • medium (approximately =1mm) • small (<1mm) • pinpoint (<0.5mm)

Table S2. qPCR conditions and primers characteristics of genes involved in N-cycle (Kalwasińska et al. 2022).

Primers	Sequence 5'-3'	qPCR conditions
***nif*H**		
nifHF	AAAGGYGGWATCGGYAARTCCACCAC	initial denaturation at 98°C for 3 min followed by 40 cycles of denaturation at 98°C for 15 s, annealing 62.5°C for 60 s, extension and plate read at 60°C for 5 s, melting curve step included annealing at 65°C for 5 s and continuous temperature rise to 95°C
nifHRb	TGSGCYTTGTCYTCRCGGATBGGCAT	
***amo*A**		
amoA_F	GGHGACTGGGAYTTCTGG	initial denaturation at 98°C for 3 min followed by 40 cycles of denaturation at 98°C for 15 s, annealing 57°C for 30 s, extension and plate read at 60°C for 5 s, melting curve step included annealing at 65°C for 5 s and continuous temperature rise to 95°C
amoA_R	CCTCKGSAAAGCCTTCTTC	
***nar*G**		
NARG F	TCGCCSATYCCGGCSATGTC	initial denaturation at 98°C for 3 min followed by 40 cycles of denaturation at 98°C for 15 s, annealing 63°C for 30 s, extension and plate read at 60°C for 5 s, melting curve step included annealing at 65°C for 5 s and continuous temperature rise to 95°C
NARG R	GAGTTGTACCAGTCRGCSGAYTCSG	
***nir*S**		
NIRS4Q F	GTSAACGYSAAGGARACSGG	initial denaturation at 98°C for 3 min followed by 40 cycles of denaturation at 98°C for 15 s, annealing 63°C for 30 s, extension and plate read at 60 °C for 5 s, melting curve step included annealing at 65°C for 5 s and continuous temperature rise to 95°C
NIRS6Q R	GASTTCGGRTGSGTCTTSAYGAA	
***nos*Z**		
nosZ1840_F	CGCRACGGCAASAAGGTSMSSGT	initial denaturation at 98°C for 3 min followed by 40 cycles of denaturation at 98°C for 15 s, annealing 67°C for 30 s, extension and plate read at 60°C for 5 s, melting curve step included annealing at 65°C for 5 s and continuous temperature rise to 95°C
nosZ2090_R	CAKRTGCAKSGCRTGGCAGAA	

Table S3. Plant growth – promoting characteristics of rhizobacterial strains isolated from vegetative, flowering and maturity growth stages of canola**.**

Strains	IAA production (µg/ml)	P-solubilization (SI)	ACC deaminase activity (nmol α-ketobutyrate/mg protein/h)	Siderophore production (d_{halo}/d_{colony})	Chitinase production (zone in mm)	HCN production	Ammonia production
			Vegetative stage				
R1	4.31±0.47	0.0	30.6	2.3	0.0	-	-
R2	4.75±0.32	0.0	67.9	2.5	0.0	-	-
R3	7.82±0.66	0.0	29.9	0.0	0.0	-	+
R4	5.31±0.41	0.0	35.5	3.5	0.0	-	-
R5	6.54±0.86	0.0	60.2	3.4	0.0	-	-
R6	5.42±0.59	0.0	152.9	0.0	1.0	-	-
R7	25.40±1.23	0.0	3975.7	5.2	0.0	-	-
R8	4.03±0.19	0.0	168.2	0.9	1.0	-	-
R9	4.53±0.33	0.0	33.5	0.0	0.0	-	-
R10	5.03±0.21	0.0	47.1	2.3	0.0	-	-
R11	8.10±0.27	0.0	96.8	5.0	0.0	-	-
R12	4.98±0.22	0.0	183.4	1.9	0.0	-	-
R13	2.86±0.11	0.0	230.3	0.9	1.0	-	-
R14	3.02±0.61	0.0	89.2	0.0	0.0	-	-
R15	3.64±0.55	0.0	10.5	2.1	0.0	-	-
R16	5.31±0.45	0.0	128.8	0.8	1.0	-	-
R17	6.43±0.75	0.0	49.3	1.5	0.0	-	-
R18	4.30±0.28	0.0	57.1	0.0	0.0	-	-
R19	4.59±0.39	0.0	573.1	0.9	1.0	-	-
R20	4.47±0.19	0.0	190.4	0.8	1.0	-	-
R21	5.76±0.21	0.0	16.7	0.0	0.0	-	-
R22	4.53±0.63	0.0	203.4	2.3	0.0	-	-
R23	5.53±0.54	0.0	162.2	0.0	1.0	-	-
R24	4.98±0.46	0.0	130.6	3.3	0.0	-	-
R25	5.53±0.26	0.0	206.5	0.0	0.0	-	-
R26	2.15±0.16	0.0	25.0	2.2	3.0	-	-
R27	**24.00±0.11**	**6.0**	**403.7**	**4.2**	**0.0**	**+**	**-**
R28	7.04±0.89	0.0	12.4	2.5	0.0	-	-
R29	4.36±0.28	0.0	0.0	2.5	0.0	-	-
R30	5.53±0.33	0.0	76.8	3.0	0.0	+	-
R31	4.20±0.48	0.0	188.2	0.0	3.0	-	-
R32	4.81±0.72	0.0	12.3	2.5	3.0	-	-
R33	4.81±0.41	0.0	10.2	0.0	0.0	-	-
R34	29.02±1.81	2.3	354.7	4.7	0.0	-	-
R35	ng	ng	ng	ng	ng	ng	ng
R36	4.81±0.25	0.0	38.8	4.6	0.0	-	-
R37	3.08±0.18	0.0	321	5.0	0.0	-	-
R38	**21.66±1.01**	**0.0**	**2174.2**	**4.3**	**0.0**	**+**	**-**
R39	4.20±0.77	0.0	14.4	2.3	0.0	-	-
R40	4.47±0.56	0.0	67.0	0.0	0.0	-	-
R41	5.26±0.11	0.0	288.6	3.0	0.0	+	-
R42	28.80±1.11	2.3	896	4.0	0.0	-	-
R43	28.19±1.23	3.7	513.9	3.6	0.0	-	-
R44	**12.01±0.87**	**7.0**	**1007.8**	**4.3**	**0.0**	**-**	**-**
R45	3.47±0.19	0.0	352.2	0.0	0.0	-	-
R46	4.08±0.23	0.0	0.0	2.3	0.0	-	-
R47	ng	ng	ng	ng	ng	ng	ng

R48	ng	ng	ng	ng	ng	ng	ng
R49	3.36±0.11	0.0	75.3	0.0	0.0	-	-
R50	ng	ng	ng	ng	ng	ng	ng
				Flowering stage			
2R1	4.25±0.21	0.0	52.2	3.0	1.0	-	-
2R2	5.09±0.26	0.0	165.2	3.3	1.0	-	-
2R3	4.75±0.37	0.0	91.0	0.0	0.0	-	-
2R4	8.04±0.68	0.0	828.3	3.0	0.0	-	-
2R5	**8.49±0.74**	**0.0**	**2226.1**	**3.3**	**1.0**	**-**	**-**
2R6	3.69±0.17	0.0	17.1	0.0	0.0	-	-
2R7	6.76±0.29	0.0	144.1	0.0	0.0	-	+
2R8	4.03±0.19	0.0	185.6	0.0	0.0	-	-
2R9	4.08±0.13	0.0	82.0	0.0	0.0	-	-
2R10	6.15±0.56	0.0	0.0	0.0	1.5	-	-
2R11	4.59±0.11	0.0	112.0	0.0	0.0	-	-
2R12	4.92±0.22	0.0	100.2	0.0	0.0	-	-
2R13	4.75±0.27	0.0	120.2	0.0	0.0	-	-
2R14	5.42±0.31	0.0	0.0	2.2	1.0	-	-
2R15	10.28±0.94	0.0	48.1	0.0	0.0	-	-
2R16	8.38±0.88	0.0	76.4	0.0	0.0	-	-
2R17	5.09±0.53	0.0	58.5	0.0	0.0	-	-
2R18	6.76±0.47	0.0	67.4	0.0	0.0	-	-
2R19	5.26±0.39	0.0	17.3	2.5	1.0	-	-
2R20	4.86±0.33	0.0	30.4	0.0	0.0	-	-
2R21	6.59±0.41	0.0	50.5	0.0	0.0	-	-
2R22	6.32±0.57	0.0	156.1	1.0	0.0	-	-
2R23	4.70±0.32	0.0	75.9	0.0	0.0	-	-
2R24	5.87±0.45	0.0	14.7	0.0	1.0	-	-
2R25	5.48±0.64	0.0	0.0	1.9	0.0	-	-
2R26	9.55±0.98	0.0	0.0	2.0	1.5	-	-
2R27	6.15±0.54	0.0	0.0	0.0	0.0	-	-
2R28	4.92±0.17	0.0	130.2	0.0	0.0	-	-
2R29	6.93±0.12	0.0	76.1	0.0	0.0	-	-
2R30	4.64±0.52	1.3	0.0	0.0	0.0	-	-
2R31	4.08±0.34	0.0	77.1	2.0	0.0	-	-
2R32	7.82±0.77	1.0	0.0	2.0	0.0	-	-
2R33	3.75±0.11	0.0	0.0	1.5	0.0	-	-
2R34	4.59±0.25	0.0	29.3	0.6	0.0	-	-
2R35	4.64±0.12	0.0	68.7	1.3	0.0	-	+
2R36	4.64±0.17	0.0	63.9	0.0	0.0	-	-
2R37	3.86±0.23	0.0	0.0	1.3	0.0	-	+
2R38	8.94±0.49	0.0	17.2	3.0	0.0	-	-
2R39	4.47±0.47	0.0	69.1	1.8	0.0	-	-
2R40	8.55±0.69	8.7	183.1	3.0	0.0	-	-
2R41	4.64±0.32	0.0	0.0	1.7	0.0	-	-
2R42	5.53±0.43	0.0	0.0	0.0	0.0	-	-
2R43	6.76±0.44	0.0	81.6	0.0	0.0	-	-
2R44	6.04±0.53	0.0	0.0	0.0	0.0	-	-
2R45	ng	ng	ng	ng	ng	ng	ng
2R46	4.53±0.32	0.0	0.0	1.5	0.0	-	-
2R47	4.92±0.52	0.0	65.1	0.0	0.0	-	-
2R48	4.47±0.44	0.0	0.0	0.0	0.0	-	-
2R49	6.48±0.94	0.0	0.0	0.0	0.0	-	-
2R50	ng	ng	ng	ng	ng	ng	ng
				Maturity stage			
3R1	5.70±0.57	0.0	0.0	0.0	0.0	-	+
3R2	6.59±0.41	0.0	0.0	1.2	0.0	-	+
3R3	4.47±0.33	0.0	0.0	1.5	0.0	-	-

3R4	5.53±0.27	0.0	0.0	0.0	0.0	+	-
3R5	4.14±0.19	0.0	0.0	0.0	0.0	-	-
3R6	32.54±1.53	0.0	0.0	2.8	0.0	-	+
3R7	4.36±0.42	0.0	64.4	0.0	0.0	-	+
3R8	4.86±0.51	0.0	0.0	1.2	0.0	-	-
3R9	8.04±0.98	0.0	18.6	4.0	0.0	-	-
3R10	5.03±0.32	0.0	35.7	0.0	1.0	-	-
3R11	6.65±0.43	0.0	0.0	0.0	0.0	-	+
3R12	8.60±0.88	0.0	0.0	0.0	0.0	-	+
3R13	ng	ng	ng	ng	ng	ng	ng
3R14	3.86±0.12	0.0	16.7	3.9	0.0	-	-
3R15	4.98±0.31	0.0	0.0	0.0	0.0	+	-
3R16	9.33±0.71	0.0	0.0	3.2	0.0	-	-
3R17	7.93±0.67	0.0	12.3	1.4	0.0	-	+
3R18	5.42±0.35	0.0	69.2	0.0	0.0	-	+
3R19	5.87±0.31	0.0	50.0	0.0	0.0	-	-
3R20	6.98±0.47	4.3	56.0	0.0	0.0	-	+
3R21	5.65±0.38	0.0	0.0	0.0	0.0	-	-
3R22	8.27±0.74	0.0	0.0	0.0	0.0	-	-
3R23	5.87±0.33	0.0	0.0	0.0	0.0	-	-
3R24	6.59±0.45	0.0	22.8	0.0	0.0	-	-
3R25	10.56±0.92	0.0	152.1	0.0	0.0	-	-
3R26	5.81±0.41	0.0	8.4	1.1	2.0	-	+
3R27	**15.97±1.11**	**4.0**	**1314.4**	**4.7**	**0.0**	**-**	**-**
3R28	6.98±0.47	0.0	78.1	1.2	0.0	-	+
3R29	6.32±0.18	0.0	0.0	1.8	0.0	-	-
3R30	22.94±1.27	0.0	17.1	2.1	0.0	-	-
3R31	19.76±1.11	0.0	92.0	1.2	0.0	-	+
3R32	14.41±0.98	0.0	0.0	1.5	0.0	-	+
3R33	7.46±0.55	0.0	9.4	1.2	2.0	-	+
3R34	6.98±0.76	1.7	56.9	1.2	0.0	-	+
3R35	18.70±1.34	4.7	15.6	3.0	0.0	-	-
3R36	11.67±1.02	3.0	37.0	4.0	0.0	-	-
3R37	5.26±0.31	0.0	495.5	0.0	0.0	-	+
3R38	19.04±1.32	5.7	384.5	3.5	0.0	-	-
3R39	25.73±1.89	4.7	0.0	1.9	0.0	-	-
3R40	4.59±0.22	0.0	0.0	1.2	0.0	-	+
3R41	4.64±0.31	0.0	74.9	1.3	0.0	-	+
3R42	5.76±0.54	4.7	178.1	3.7	0.0	-	-
3R43	**8.55±1.01**	**4.0**	**1117.3**	**3.1**	**0.0**	**-**	**-**
3R44	7.26±0.86	0.0	111.1	1.1	0.0	-	-
3R45	7.26±0.63	0.0	0.0	1.1	0.0	-	-
3R46	5.26±0.54	0.0	0.0	1.3	0.0	-	+
3R47	4.59±0.22	0.0	0.0	1.0	1.0	-	+
3R48	5.31±0.41	0.0	0.0	1.5	2.0	-	+
3R49	4.98±0.18	0.0	88.3	1.2	0.0	-	+
3R50	5.03±0.28	0.0	123.8	1.0	0.0	-	+

Means ± SE are presented; +, positive result; −, negative result; ng, not grown; rhizobacterial strains with the best PGP traits are highlighted in bold

Table S4. Physicochemical properties of the non-sterile soil.

Parameters	Values
pH	6.4
P_2O_5 [mg/kg]	275.0
K_2O [mg/kg]	245.0
Mg [mg/kg]	118
$N\text{-}NH_4$ [mg/kg $_{\text{dry mass}}$]	6.56
$N\text{-}NO_3$ [mg/kg $_{\text{dry mass}}$]	35.31
C_{org} [% per g $_{\text{dry mass}}$]	1.42

Table S5. *Bacillus paralicheniformis* 2R5 genome assemblies and annotations.

Genomes annotations	*Bacillus paralicheniformis* 2R5
Assembly	GCA_025833795.1
WGS	JAOWAK01
Level	Contig
GC	45.9%
Number of reads	491,153
Total length	4.55 Mb
# contigs	78
N50	456,496
L50	4
Gene	4,749
Pseudogene	94

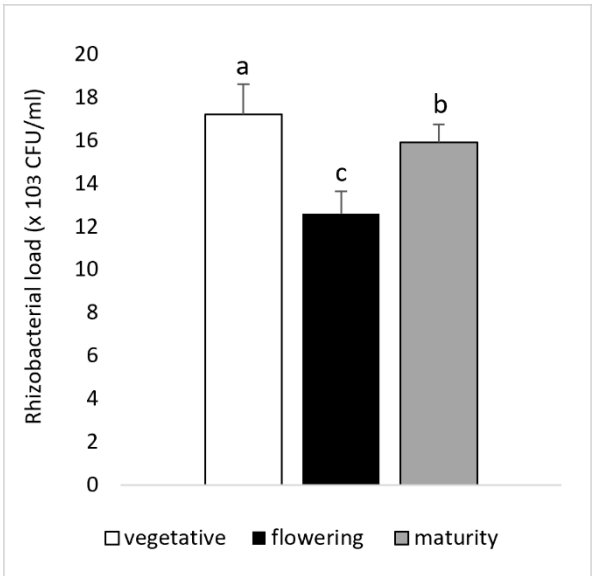

Figure S1. Total viable counts of rhizobacteria from three different canola growth stages; different letters indicate significant differences ($P < 0.05$) between group means (Tukey test as a post-hoc analysis).

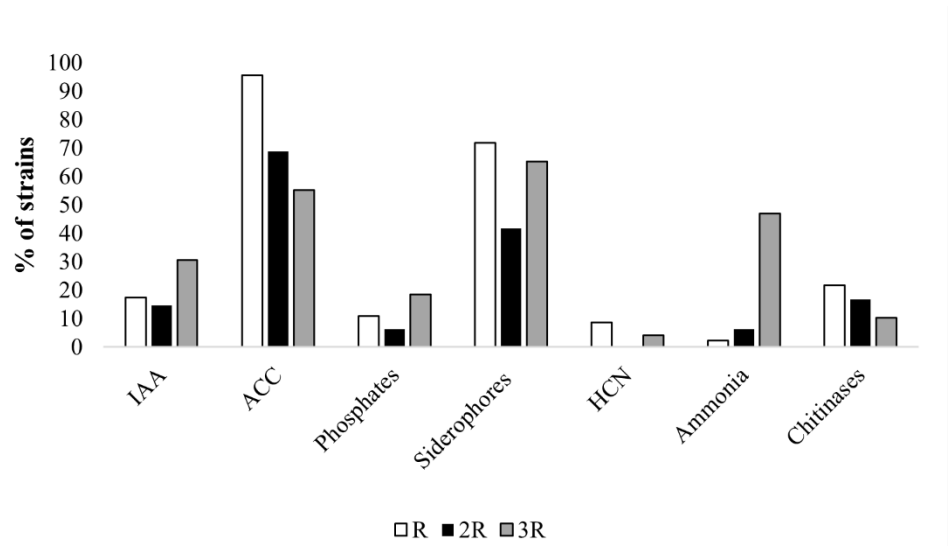

Figure S2. Percentage share of bacteria showing the plant growth – promoting traits in three different canola growth stages: vegetative (R), flowering (2R) and maturity (3R); IAA > 8 μg/ml.

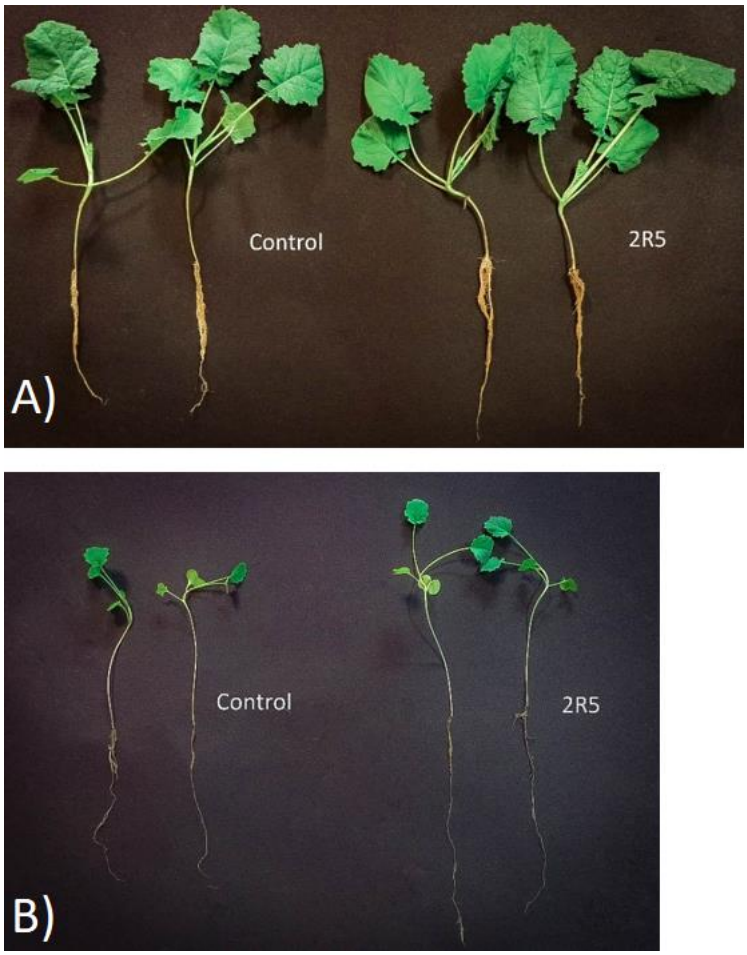

Figure S3. Canola growth – promoting effect of *Bacillus paralicheniformis* 2R5 compared to untreated control: A) under sterile conditions; B) under natural conditions.

Publikacja nr 2

The effect of seed bacterization with *Bacillus paralicheniformis* 2R5 on bacterial and fungal communities in the canola rhizosphere

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The effect of seed bacterization with *Bacillus paralicheniformis* 2R5 on bacterial and fungal communities in the canola rhizosphere

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ABSTRACT

Bacillus sp. is one of the best-studied plant growth-promoting rhizobacteria (PGPR). However, more detailed studies targeting its effect on the rhizosphere microbial community are required for improving management practices regarding its commercial application in the field. Our earlier study showed that PGPR *Bacillus paralicheniformis* 2R5 stimulated canola growth. Hence, this study aimed to assess the time-course impact of *B. paralicheniformis* 2R5 on bacterial and fungal community structure and diversity. The results showed that inoculation with *B. paralicheniformis* 2R5 initially significantly decreased the observed bacterial richness compared to the control, while after 44 days of treatment this alpha diversity metrics increased. A linear discriminant analysis effect size showed that *B. paralicheniformis* 2R5 altered the soil bacterial and fungal community structure by increasing the abundance of plants' beneficial microorganisms such as *Nitrospira*, *Ramlibacter*, *Sphingomonas*, *Massilia*, *Terrimonas* as well as *Solicoccozyma*, *Schizothecium*, *Cyphellophora*, *Fusicolla*, *Humicola*. *B. paralicheniformis* 2R5 seems to be a promising alternative to chemical pesticides and can be considered for practical application in the field. Its ability to alter the rhizosphere microbiome by increasing the diversity and composition of bacterial communities and increasing plants' beneficial groups of fungi, appears to be important in terms of improving canola development. However, further studies on these increased microbial taxa are necessary to confirm their function in promoting canola growth.

1. Introduction

Plant-growth-promoting rhizobacteria (PGPR) have been studied as biofertilizers to enhance crop productivity and induce resistance in plants. PGPRs have been considered important components for promoting sustainable agriculture by reducing the application of fertilizers and pesticides (Bhattacharyya et al., 2018; Patel et al., 2021). In association with the plant rhizosphere, these microbes improve plant growth by secreting phytohormones, solubilizing phosphates, fixing nitrogen, increasing nutrients uptake, and acting as biocontrol agents of pathogens (Ciftci et al., 2021).

The effectiveness of PGPR inoculation is associated with their ability to colonize and survive, as well as their interactions with indigenous bacterial and fungal communities in the rhizosphere (Kong and Liu, 2022). *Bacillus* species are well known to form endospores which favor their survival in soil. *Bacillus* spp. can be harnessed in agricultural technologies as a bioinoculant because its properties result in a long shelf-life of the product before application (Hashmi et al., 2019). However, more detailed studies about the *Bacillus* effect on the rhizosphere microbial community are required to improve management practices regarding its commercial application in the field.

The maintenance of a healthy rhizosphere microbiome is essential for crop development and soil fertility (Santos and Olivares, 2021). The impact of introduced PGPRs on the native bacterial and fungal populations is still underexplored because of the bacterial inoculants' complex roles in the rhizosphere. PGPR can alter the rhizosphere microbiome by eliminating root-dwelling pathogens or removing other non-harmful microorganisms (Bhattacharyya et al., 2018). On the other hand, some native microbes can negatively affect the function and survival of introduced PGPR (Pacheco da Silva et al., 2022).

Our earlier study showed that PGPR *Bacillus paralicheniformis* 2R5 stimulated canola (*Brassica napus* L. var. *napus)* growth under sterile and non-sterile conditions (Świątczak et al., unpublished). Hence, the objective of the present study was to investigate the impact of *B. paralicheniformis* 2R5 on the composition and diversity of bacterial and fungal communities in the canola rhizosphere using next generation

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sequencing (NGS) of the 16S rRNA gene and internal transcribed spacer (ITS2). Additionally, the time-course impact on native microorganisms in the rhizospheres of bacteria-treated and untreated canola plants on the first, twenty-second and forty-fourth day after bacterization was evaluated.

2. Materials and methods

2.1. Seed bacterization and sampling

Bacillus paralicheniformis 2R5 strain was isolated from the canola (*Brassica napus* L. var. *napus*) rhizosphere (Świątczak et al., unpublished). Canola seeds were sterilized with 1 % sodium hypochlorite (NaOCl) for 30 min and washed three times with sterile distilled water, according to the method described by Rudolph et al. (2015). Sterilized seeds were agitated in *B. paralicheniformis* 2R5 suspension (10^8 CFU/ml), and 10 ml LB broth supplemented with 0.5 % carboxymethyl cellulose (CMC) for 30 min, while sterilized seeds agitated with 10 ml LB broth and 0.5 % CMC were the control. The seeds were germinated in pots (four seeds per pot) containing soil taken from the arable soil in Górsk, Poland (53°01′46.1"N18°26′59.4"E). Treated with *B. paralicheniformis* 2R5 and untreated rhizospheres samples (four replicates for each time-point) were collected by scraping the soil from the roots after 0, 22, and 44 days of sowing (total of 24 samples).

2.2. Rhizosphere DNA isolation, PCR, and sequencing

DNA was extracted from 0.25 g of rhizosphere soil using the DNeasy Power Soil Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The purity of the isolated DNA was measured with a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The microbial community was determined through the next-generation-sequencing of bacterial 16S hypervariable region (V3-V4) and fungal internal transcribed spacer (ITS2). Bacterial libraries were obtained with the use of the following primers: 5′ CCTACGGGNGGCWGCAG (forward) and 5′ GACTACHVGGGTATCTAATCC (reverse) (Klindworth et al., 2013), while fungal libraries were obtained using 5′ GCATCGATGAAGAACGCAGC (ITS3 forward, without overhangs) and 5′ TCCTCCGCTTATTGATATGC (ITS4 reverse, without overhangs) primers, according to White et al. (1990). Library preparation was followed by Illumina Support Center (ISC) protocol with a slight modification: 2×Phanta Max Master Mix (Vazyme Biotech, Nanjing City, China) was applied instead of Kapa Hifi Hot Start Ready mix. The PCR products were checked using electrophoretic separation. Libraries were normalized based on band luminescence intensity on a 1.5 % agarose gel, pooled, and sequenced on Illumina MiSeq (Illumina, San Diego, CA, USA) using MiSeq Reagent Kit v2 (500-cycles) in 2 × 250 bps paired-end format.

2.3. Bioinformatic analyses

Sequence sets were merged and mothur v1.44.3 (Schloss et al., 2009) was used for quality processing, taxonomic assignments and OTU picking (at 97 % similarity threshold) of 16S rRNA amplicons based on mothur's MiSeq SOP (Kozich et al., 2013, downloaded at 22th May 2021). Deltaq was adjusted to 10 in the 'make.contigs' command. Ambiguous base calls and reads shorter than 300 nt and longer than 500 nt were excluded from the analysis. Primers were removed from the end of the sequences using trim.seqs(pdiffs=2, checkorient=T). Denoising was carried out with the pre.cluster command. Chimeric reads were filtered out using the mothur implementation of VSEARCH. Singleton reads were discarded from the sequence set according to Kunin et al. (2010). Read alignment and taxonomic assignment were carried out using the ARB-SILVA SSU Ref NR 138 reference database (Quast et al., 2012) with a minimum bootstrap confidence score of 80. Reads assigned to non-primer-specific taxonomic groups ('Chloroplast', 'Mitochondria' and 'unknown') were subsequently removed from the dataset. A random subsampling was performed based on the sample having the lowest sequence number. Richness estimators and diversity indices were also calculated with mothur.

For fungal ITS analysis, initial merging and quality filtering steps were carried out with mothur as described for the 16S rRNA gene amplicons. Subsequently, ITS2 region was extracted from the sequences using the ITSx 1.1-beta software (Bengtsson-Palme et al., 2013) based on the findings of Nilsson et al. (2010). Taxonomic assignment was carried out with mothur's classify.seqs(cutoff = 80) using the UNITE v8.3 database (Abarenkov et al., 2020) as reference. Reads not assigned to any fungal phyla were removed from the read set using the remove.lineage(taxon=k_Fungi_unclassified) command of mothur. ITS2 reads were clustered to OTUs with VSEARCH using a 97 % similarity threshold.

2.4. Exploratory data analyses

Differences in richness estimators and diversity indices between non-treated and *Bacillus paralicheniformis* 2R5 treated plants from 0 to 44 days after bacterization were tested with *t-test* for equal means in Past v 3.08. For non-normally distributed data, a test for equal medians - Mann-Whitney was performed. ANOVA analysis following the Tuckey post hoc test was performed to determine OTU richness, Shannon H′, and Inv Simpson indices changes over time of rhizospheres of the treated and non-treated canola plants. The Shapiro-Wilk test was used to check the assumptions of normality, while Levenés test was used to assess the homogeneity of variances. The Principle Component Analysis (PCoA), ANOSIM analysis, and rarefaction curves were performed in R v 4.0.3. A linear discriminant analysis (LDA) effect size (LEfSe) of 100 most abundant OTUs was used to determine the significant differential abundance ($p < 0.05$) of bacterial and fungal taxonomy groups between the rhizospheres of *Bacillus paralicheniformis* 2R5-treated and untreated canola plants from 0 to 44 days after bacterization (https://huttenhower.sph.harvard.edu/galaxy/, Segata et al., 2011).

2.5. Data availability

The Illumina MiSeq sequences of bacterial 16S rRNA and fungal ITS genes were submitted to GenBank-SRA under Bioproject PRJNA876229.

3. Results

3.1. Sequencing results

Sequencing resulted in 2,687,943 and 2,453,619 high-quality sequence reads (clustered into 16,443 and 2760 OTUs) for bacteria and fungi, respectively. The rarefaction curves showed a high coverage of bacterial and fungal communities in all rhizosphere samples (Fig. S1).

3.2. Changes in bacterial and fungal diversity

The alpha diversity indices of bacterial and fungal communities in the groups of samples were compared in two different ways: i) according to the time (Table S1); ii) between treated with *B. paralicheniformis* 2R5 (Bp) and untreated control (C) samples (Table S2).

Fungal diversity increased with time and significant differences were observed in OTU richness for samples CT0, CT22, CT44. Significant differences were detected in bacterial OTU richness and in Shannon diversity for samples CT0, CT22, CT44, and BpT0, BpT22, BpT44. The Simpson index (1-D) showed no significant differences for CT0, CT22, and CT44, while did show significant differences for BpT0, BpT22, and BpT44. The bacterial communities' mean values in each index were the highest on the 44th day both for the treated and untreated samples (Table S1).

Significant differences were not detected in the fungal indices

between the C samples and samples treated with Bp at T0, T22, and T44. However, bacterial alpha-diversity based on observed OTU richness at T22 and T44, and the Shannon index at T22 showed significant differences. The mean values were significantly lower in Bp samples than in the control at T22, while at T44 mean values of Bp samples were higher (Table S2).

The Principal Coordinate Analysis (PCoA) of bacterial (Fig. 1A) and fungal communities (Fig. 1B) showed a clear separation of C samples and samples treated with Bp at T0, T22, and T44 indicating different microbial community structures, which were also confirmed by ANOSIM (Table S3).

3.3. Changes in the bacterial and fungal community structure

The top 10 bacterial and fungal phyla detected with the highest relative abundance are presented in Fig. 2. The most dominant bacterial phyla were *Proteobacteria*, *Actinobacteriota*, *Acidobacteriota*, and *Bacteroidota*. Whereas, the most abundant fungal phyla were *Ascomycota*, *Mortierellomycota*, *Basidiomycota,* and *Chytridiomycota*. LefSe analysis showed significant differences with time and treatment in bacterial (Fig. 3) and fungal (Fig. 4) communities.

Regarding the bacterial communities, 4 phylotypes were enriched in C and 15 in Bp in the T0 time point. Among cultured representatives of bacteria or bacterial sequences of established taxonomy at the genus level, *Kribbella* and *Nitrosospira* were more abundant in C compared to Bp. For the T22 time point, 25 phylotypes were enriched in C, and 30 were more abundant in Bp treatment. Among cultured bacterial organisms, representatives of *Bradyrhizobium*, *Marmoricola*, *Adhaeribacter*, *Nocardioides*, and *Pseudoxanthomonas* prevailed in C, while *Nitrospira*, *Sphingomonas*, *Terrimonas*, *Massilia,* and *Ramlibacter* were more abundant in the PGPR treated samples. For the T44 time point, 12 phylotypes were enriched in C and 14 in Bp. Among cultured bacterial representatives, *Nocardioides*, *Nakamurella,* and *Microvirga* were the most abundant in C, while *Dongia* prevailed in Bp samples (Fig. 3 and Fig. S2).

Based on ITS2 amplicon sequencing, 11 fungal phylotypes were enriched in C, and 12 were more abundant in Bp treatment in the T0 time point. Among fungal reads of established taxonomy at the species level representatives of *Cladorrhinum foecundissimum*, *Cladorrhinum bulbillosum*, and *Heydenia* sp. prevailed in C, while *Humicola grisea* and *Solicoccozyma terricola* were the most abundant in Bp (Fig. 4, Fig. S3). For the T22 time point, 11 phylotypes were more abundant in Bp treatment. *Solicoccozyma aeria* and *Fusicolla septimanifiniscientiae* were more abundant in Bp compared to C (Fig. 4, Fig. S4). For the T44 time point, 13 phylotypes were enriched in C and 11 in Bp. Fungi *Trichobolus zukalii*, *Heydenia* sp., and *Cephalotrichum stemonitis* were detected with the highest relative abundance in the control, while *Schizothecium* sp., *Plectosphaerella cucumerina*, and *Cyphellophora vermispora* were more abundant in Bp treated samples (Fig. 4, Fig. S5).

4. Discussion

The microbial diversity of a rhizosphere is an important ecological bioindicator in maintaining healthy plants growth (Wang et al., 2022). In the present study, the changes in the bacterial and fungal diversity metrics confirmed the microbiota variability depended on the growth of the plant. As indicated by the Shannon H′ index, the highest bacterial diversity was noted after 44 days in the rhizosphere of both untreated and treated plants. Moreover, bacterial and fungal observed OTU richness was the highest after 44 days in untreated plants. Similarly, Wang et al. (2017) indicated that rhizosphere microbiome structure varied during plant development due to changes in root exudates. Plant roots can produce secondary metabolites such as terpenoids, polyphenols, and nitrogen-containing compounds, which do not directly contribute to growth and development but play a significant role in shaping the structure of the rhizosphere microbiome (Mishra et al., 2022). However, the effect of inoculated PGPR on the rhizosphere microbiome is still underexplored. Our results showed initially decreased OTU bacterial richness in samples treated with *B. paralicheniformis* 2R5 compared to the control, while after 44 days of treatment the alpha diversity index increased. In other research, bacterial inoculation caused changes in *Brassica juncea* rhizosphere microbiome after 8 weeks, suggesting that it takes some time for the inoculated PGPR to survive, actively proliferate, and function (Jeong et al., 2013).

Moreover, there is still limited information on how changes in the rhizosphere microbial communities contribute to improving plant growth and development. It is generally believed that a high abundance of beneficial microorganisms indicates better plant growth, lower plant disease, and higher nutrient and enzyme activities (Wang et al., 2017). Our results showed that the *B. paralicheniformis* 2R5 possibly affected the soil microbial community structure by increasing the abundance of beneficial microorganisms for plants. For example, *Nitrospira* and *Ramlibacter* were more abundant in the *B. paralicheniformis* 2R5 treated soil. Some representatives of *Nitrospira* belong to completely nitrifying 'comammox' bacteria (Daims et al., 2016), while *Ramlibacter* is involved

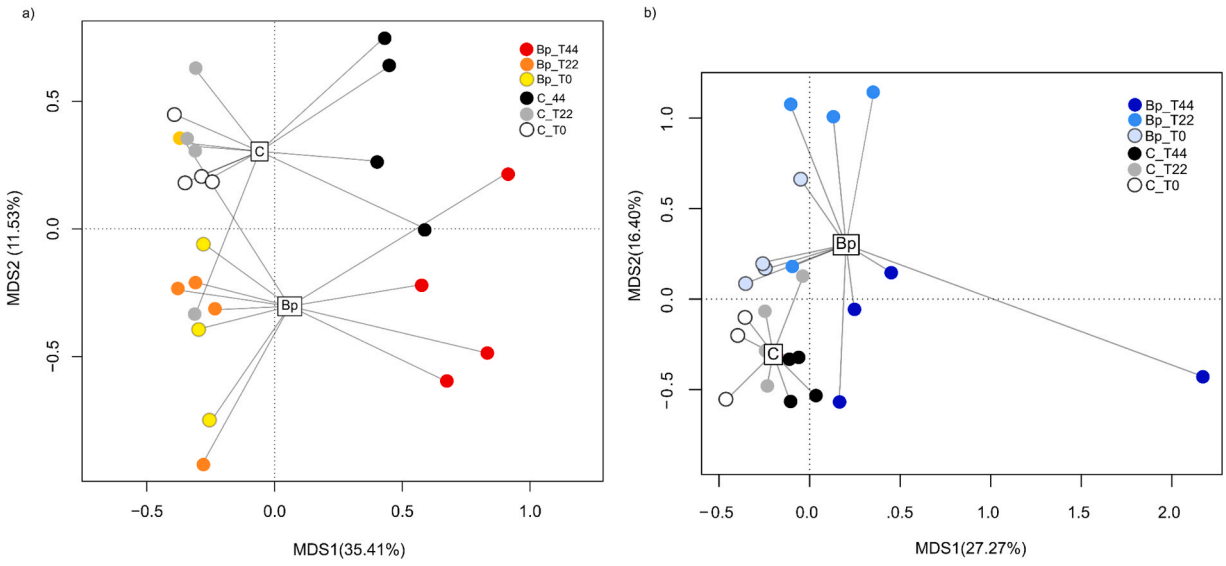

Fig. 1. Principal coordinate analysis (PCoA) of the a) bacterial and b) fungal communities in the rhizospheres of *Bacillus paralicheniformis* 2R5-treated (Bp) and untreated (C) canola plants (n = 4). T0, T22, and T44 – time after bacterization in days.

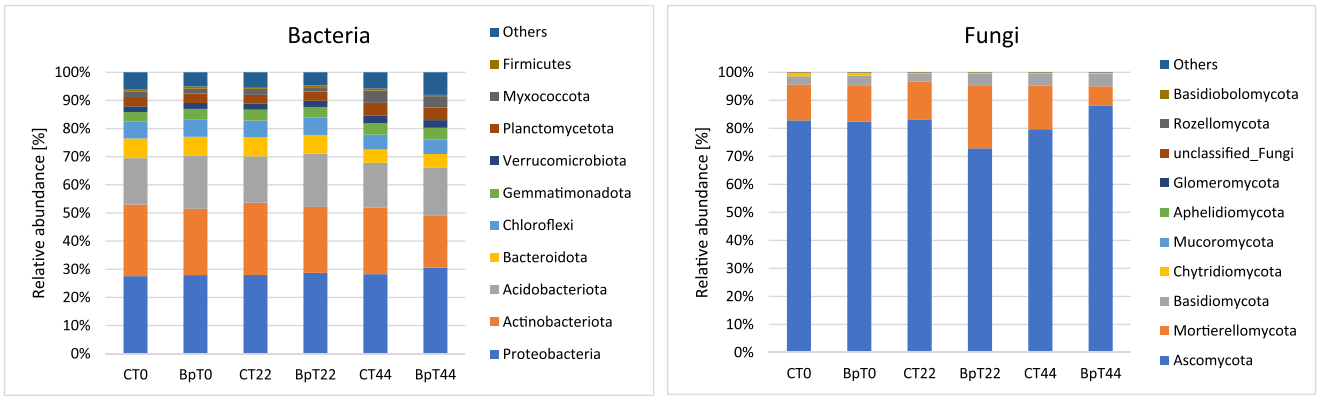

Fig. 2. Mean relative abundance (n = 4) of top 10 most abundant bacterial and fungal phyla in the rhizospheres of *Bacillus paralicheniformis* 2R5-treated (Bp) and untreated (C) canola plants. T0, T22, and T44 – time after bacterization in days.

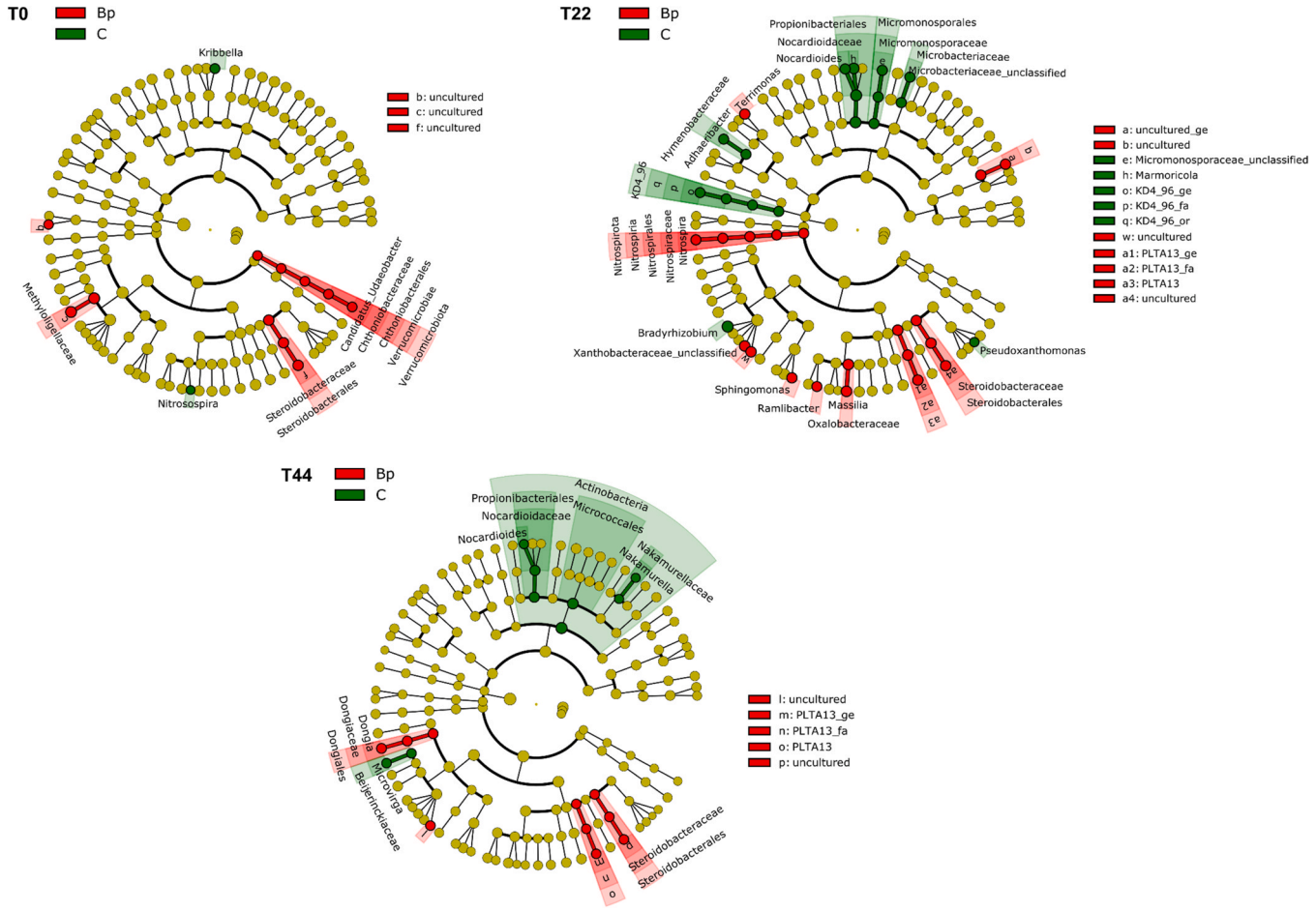

Fig. 3. Cladograms of bacterial top 100 groups in the rhizospheres of *Bacillus paralicheniformis* 2R5-treated (Bp) and untreated (C) canola plants (n = 4). T0, T22, and T44 – time after bacterization in days.

in N-cycling (Milkereit et al., 2021). The increasing abundance of these genera might confirm our earlier results, where *B. paralicheniformis* 2R5 through interactions with soil microbiota increased the number of microorganisms associated with the nitrogen cycle, simultaneously promoting canola growth (Świątczak et al., unpublished). Moreover, relative abundance of other beneficial microorganisms was increased in *B. paralicheniformis* 2R5 treated samples. It was reported that the family of *Xanthobacteraceae* and *Steroidobacteraceae* play the most important role to survive under drought stress conditions in rice (Jang et al., 2020), while the genus *Sphingomonas* have the ability to increase *Solanum pimpinellifolium* growth under salinity stress (Khan et al., 2017). The family of *Gemmatimonadaceae* is known as beneficial bacteria which have a positive effect on plant growth when it is inoculated into the soil (Zhao et al., 2020). Members of the genus *Massilia* can promote plants growth through their ability to solubilize phosphorus and nitrogen fixation (Guo et al., 2019) or through their positive effect on root colonization by arbuscular mycorrhizal fungi (Krishnamoorthy et al., 2016). Genus *Terrimonas* was positively linked with the non-ribosomal peptide synthetase (NRPS) gene – COG 1020 involved in biocontrol of soil-borne plant pathogen *Ralstonia solanacearum* (Michelsen et al., 2015; Wei

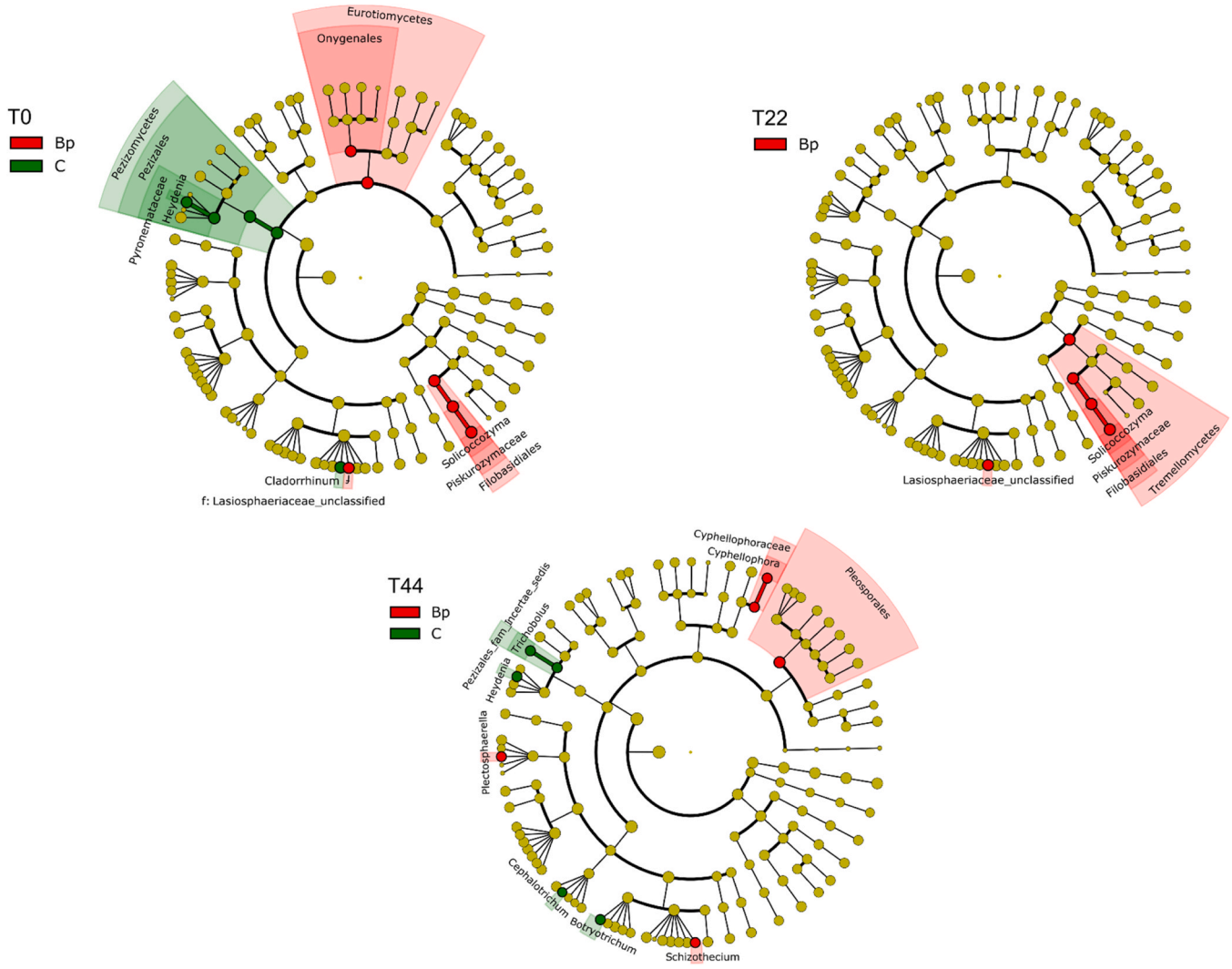

Fig. 4. Cladograms of fungal top 100 groups in the rhizospheres of *Bacillus paralicheniformis* 2R5-treated (Bp) and untreated (C) canola plants (n = 4). T0, T22, and T44 – time after bacterization in days.

et al., 2019). Whereas, "*Candidatus* Udaeobacter" was reported to exhibit multidrug resistance and have the ability to release antibiotics in the soil (Willms et al., 2020). Members of the family *Dongiaceae*, and *Vicinamibacterales* were found to be associated with the roots (Syranidou et al., 2018; Barreto and Alonso, 2021), while families of PLTA13 and *Methyloligellaceae* were previously found in soils, but not much is known about the function of these groups in this environment (Ceja-Navarro et al., 2010; Köberl et al., 2017; Rummel et al., 2020).

Regarding the fungal communities, *Solicoccozyma*, *Schizothecium*, *Cyphellophora*, *Fusicolla,* and *Humicola* were more abundant in the treated rhizosphere. It can have a positive effect on plants because *Solicoccozyma* has the ability to produce indole-3-acetic acid (IAA), which is the most common phytohormone regulating plant development (Nicola et al., 2021). Whereas, *Schizothecium* sp. is well known plant root-associated colonizer (Hugoni et al., 2018) which has antifungal activity towards a number of plant pathogens (Narisawa, 2018; Tymon et al., 2020). Moreover, *C. vermispora* is a saprophyte, isolated from decaying roots, stems, and leaves of plants (Gao et al., 2015), while *F. septimanifiniscientiae* and *H. grisea* are known as soil-inhabiting fungi (White and Downing, 1953; Crous et al., 2021). It should be also noted that the species *Plectosphaerella cucumerina* – a pathogen of several plant species such as radish (Miao et al., 2018), cucurbits, tomatoes, and bell pepper (Carlucci et al., 2012), was more abundant in samples treated with *B. paralicheniformis* 2R5. However, there is no information about *P. cucumerina* as a pathogen causing canola diseases.

Increase in relative abundance of above bacterial and fungal groups could indicate beneficial effect of *B. paralicheniformis* 2R5 treatment on canola plants. However, these increased groups of microorganisms should be further isolated to confirm their ability to promote canola growth as it is presented in Jin et al. (2023) study. The authors found that biochar amendments increased the relative abundance of potential PGPR, e.g. *Sphingomonas*, *Lysobacter* and *Pseudomonas* spp. in tomato rhizosphere. These representative microorganisms were further isolated and characterized to validate their function. A pot experiment showed that specially culturable bacteria i.e. *Sphingomonas* sp. S21, *Lysobacter* sp. L08 and *Pseudomonas* sp. P13 promoted tomato growth and mitigated Cd toxicity (Jin et al., 2023).

5. Conclusion

Bacillus paralicheniformis 2R5 seems to be a promising alternative to chemical pesticides and can be considered for practical application in the field. Its potential ability to alter the rhizosphere microbiome by increasing the diversity and composition of bacterial communities and increasing plants' beneficial groups of fungi appears to be important in terms of improving canola development. However, further studies on these increased microbial taxa are necessary to confirm their function in promoting canola growth.

CRediT authorship contribution statement

JŚ wrote the original draft, made greenhouse experiment and sampling, made isolation of DNA, conducted statistical analyses, and prepared the manuscript editorially. AK made rarefaction curves, LEfSe PCoA, and ANOSIM analysis, coordinated the study, and checked the validity of the original draft. AS carried out the sequence analysis. MS designed and coordinated the study and checked the validity of the original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2023.127448.

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Supplementary Materials

TABLE S1 OTU richness and diversity indices in the rhizosphere of *Bacillus paralicheniformis* 2R5-treated and untreated canola plants according to time.

Sample	Observed OTU richness			Shannon H'			Inv Simpson		
	Mean	*p*	F	Mean	*p*	F	Mean/ Median	*p*	F/H
				Bacteria					
CT0	4369.4±82.67[b]	0.016	6.78	6.94±0.02[b]	0.002	13.81	401.22±10.22	ns	4.21
CT22	4371.6±66.79[b]			6.93±0.02[b]			386.73±9.67		
CT44	4695±65.08[a]			7.08±0.03[a]			437.7±17.13		
BpT0	4308±32.48[b]	0.000	81.66	6.92±0.01[b]	0.000	133.3	396.13±12.14[b]	0.006	9.37
BpT22	3943.5±45.47[c]			6.85±0.02[c]			372.49±13.68[b]		
BpT44	4983.5±84.31[a]			7.13±0.00[a]			466.64±20.82[a]		
				Fungi					
CT0	243.9± 9.08[b]	0.026	5.61	3.38±0.1	ns	1.89	10.61±0.79	ns	0.34
CT22	246.1±6.28[ab]			3.53±0.1			12.11±1.88		
CT44	274.3±5.61[a]			3.63±0.07			11.53±0.93		
BpT0	270.8±10.03	ns	0.45	3.65±0.08	ns	0.17	13.18±0.62[nnd]	ns	0.73[nnd]
BpT22	250±13.31			3.77±0.18			16.80±4.03[nnd]		
BpT44	270.2±25.63			3.61±0.29			12.98±3.56[nnd]		

nnd – not normal distribution (test for equal medians: Kruskal-Wallis); ns – non significant; variants marked with different letters indicate groups significantly different from each other, $p<0.05$ (Tukey's test as post hoc); C – control, untreated plants; Bp – plants treated with *Bacillus paralicheniformis* 2R5; T0, T22 and T44 - time after bacterization in days.

TABLE S2 Differences in OTU richness and diversity indices between the rhizospheres of *Bacillus paralicheniformis* 2R5-treated and untreated canola plants according to treatment.

Sample	Observed OTU richness			Shannon			Inv		
	Mean	*p*	t	Mean/ Median	*p*	t/z	Mean/ Median	*p*	t/z
					Bacteria				
CT0	4369.4	ns	0.69	6.94	ns	0.78	401.22	ns	0.32
BpT0	4308			6.92			396.13		
CT22	4371.6	0.002	5.23	6.93	0.024	3.01	2.75^{nnd}	ns	-1.01^{nnd}
BpT22	3943.5			6.85			1.75^{nnd}		
CT44	4695	0.035	-2.71	7.08	ns	-1.90	437.7	ns	-1.07
BpT44	4983.5			7.13			466.64		
					Fungi				
CT0	243.9	ns	-1.99	1.62*	ns	-1.30^{nnd}	1.37^{nnd}	ns	-1.88
BpT0	270.8			2.87*			3.12^{nnd}		
CT22	246.1	ns	-0.27	3.52	ns	-1.15	12.11	ns	-1.54
BpT22	250.02			3.77			18.96		
CT44	274.3	ns	0.16	3.63	ns	0.09	11.53	ns	-0.57
BpT44	270.2			3.60			13.63		

ns – non significant; nnd – not normal distribution (test for equal medians: Mann-Whitney); C – control, untreated plants; Bp – plants treated with *Bacillus paralicheniformis* 2R5; T0, T22 and T44 - time after bacterization in days.

TABLE S3 ANOSIM results for bacterial and fungal communities in the rhizosphere of *Bacillus paralicheniformis* 2R5-treated and untreated canola plants.

Target	Time	Statistic	Value	
Bacteria	T0	R	0.1979	
		P-value	0.173	ns
	T22	R	0.333	
		P-value	0.025	*
	T44	R	0.4167	
		P-value	0.022	*
Fungi	T0	R	0.2812	
		P-value	0.033	*
	T22	R	0.4375	
		P-value	0.041	*
	T44	R	0.3854	
		P-value	0.025	*

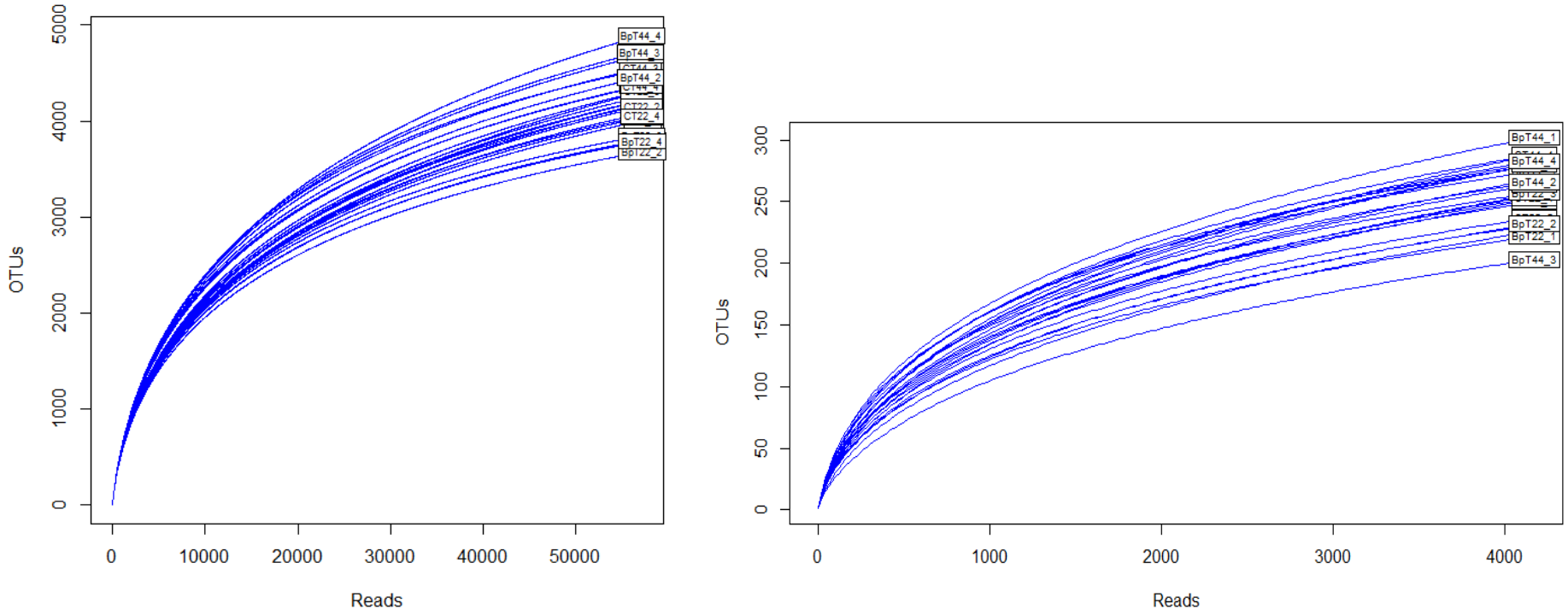

FIGURE S1 Rarefaction curves for the bacterial and fungal reeds.

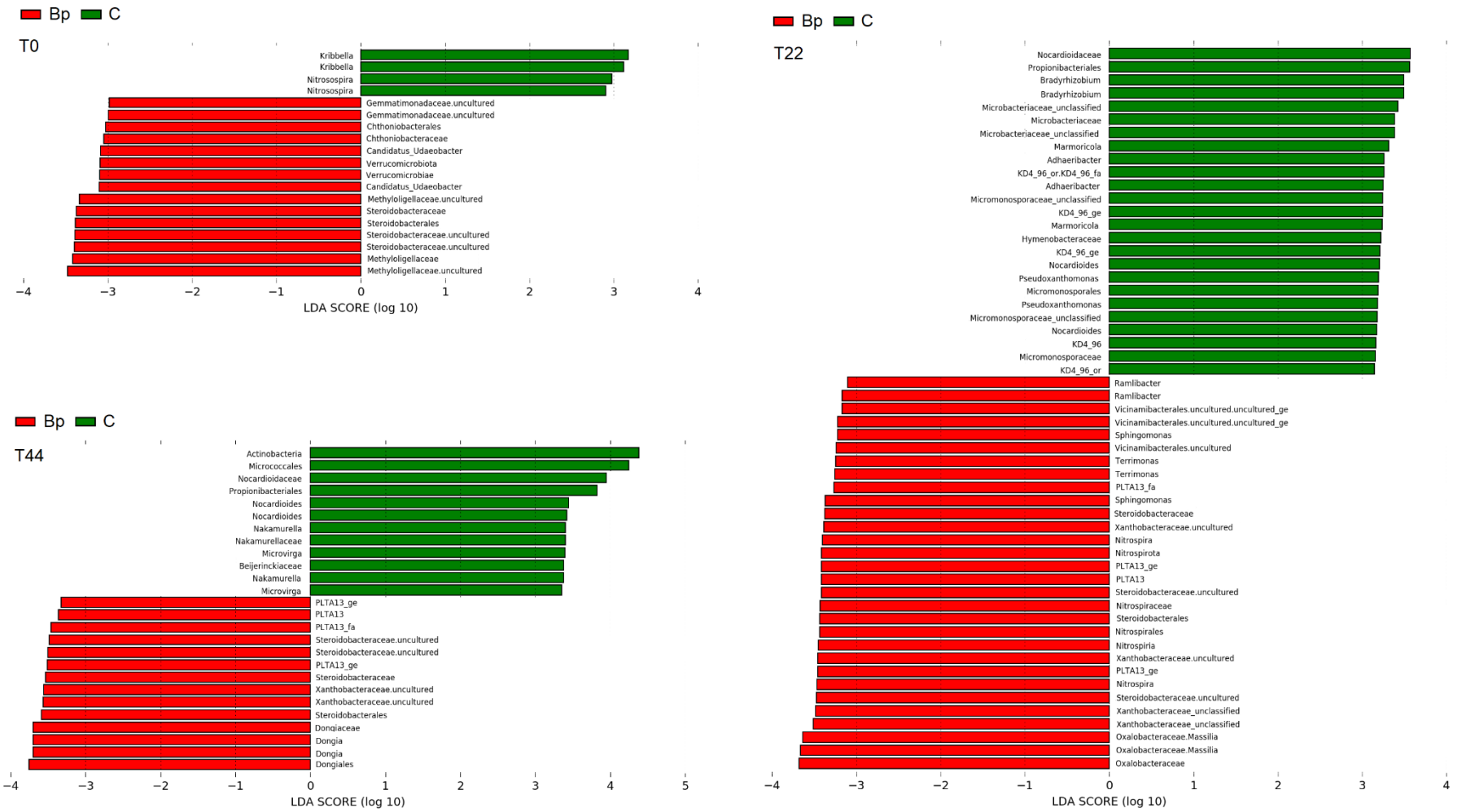

FIGURE S2 LEfSe analysis results indicating the changes in the top 100 bacterial phylotypes after treatment. Legend: (Bp) *Bacillus paralicheniformis* 2R5-treated samples; (C) untreated samples; T0, T22 and T44 – time after bacterization in days (n = 4).

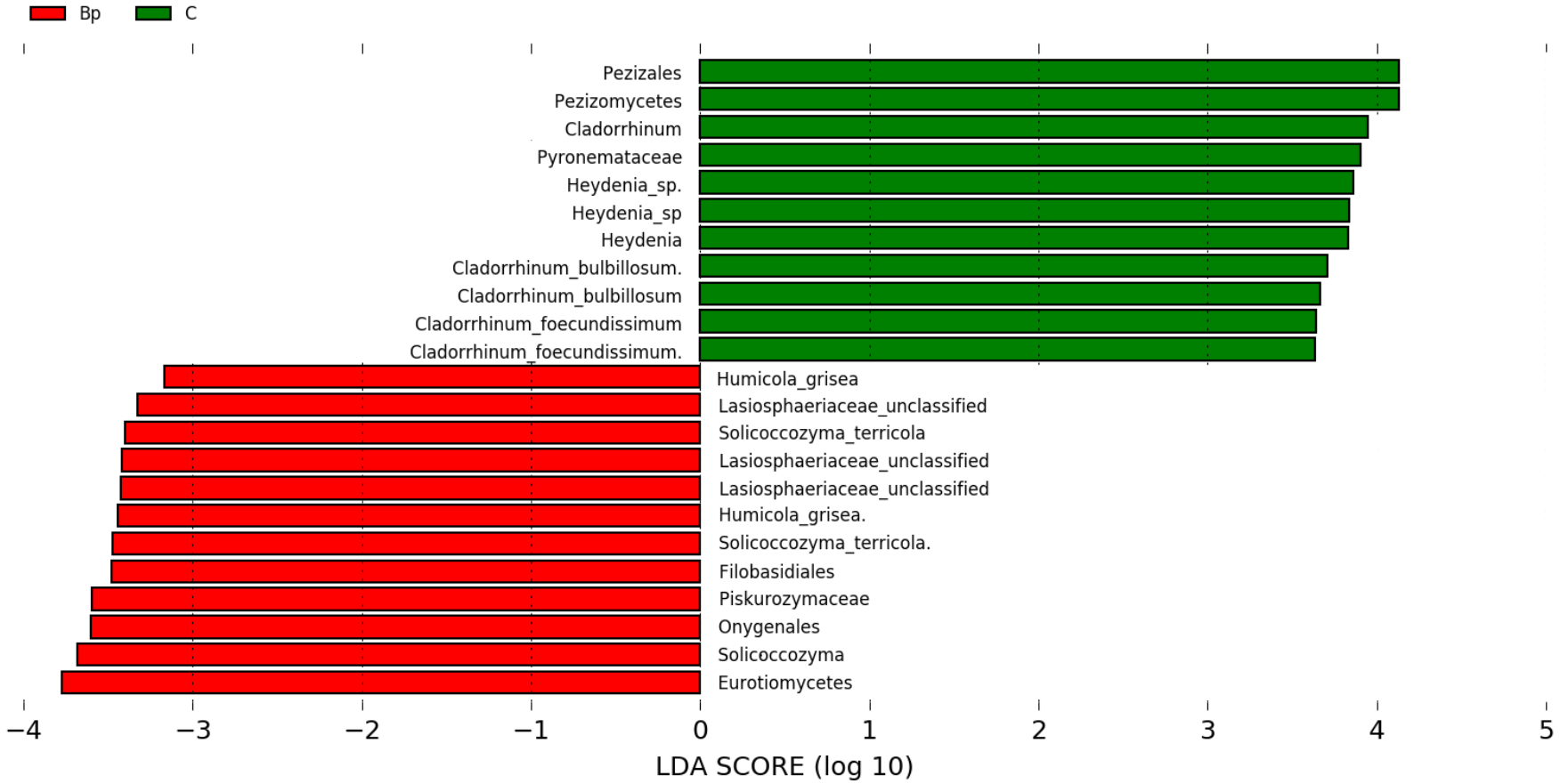

FIGURE S3 LEfSe analysis results indicating the changes in the top 100 fungal phylotypes after treatment at T0. Legend: (Bp) *Bacillus paralicheniformis* 2R5-treated samples; (C) untreated samples.

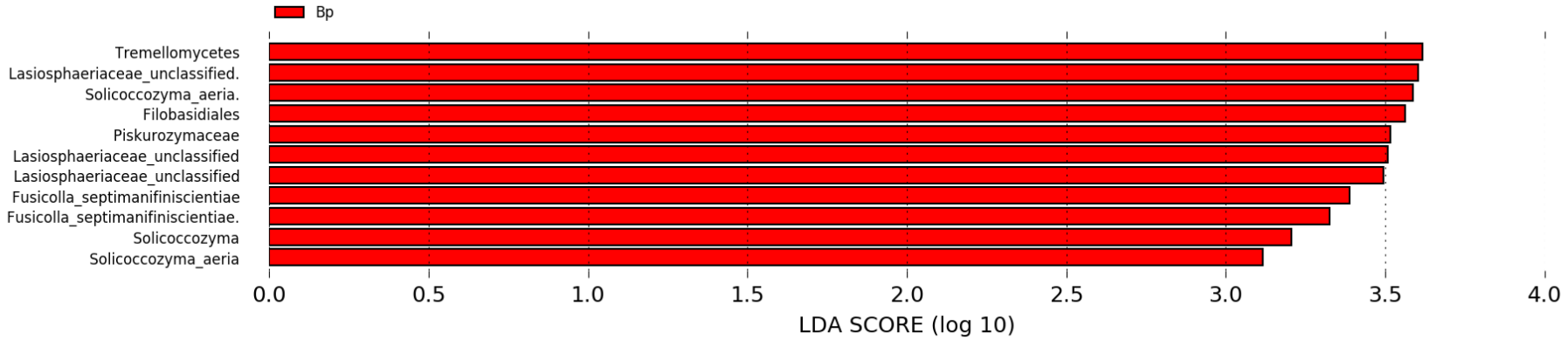

FIGURE S4 LEfSe analysis results indicating the changes in the top 100 fungal phylotypes after treatment at T22. Legend: (Bp) *Bacillus paralicheniformis* 2R5-treated samples; (C) untreated samples.

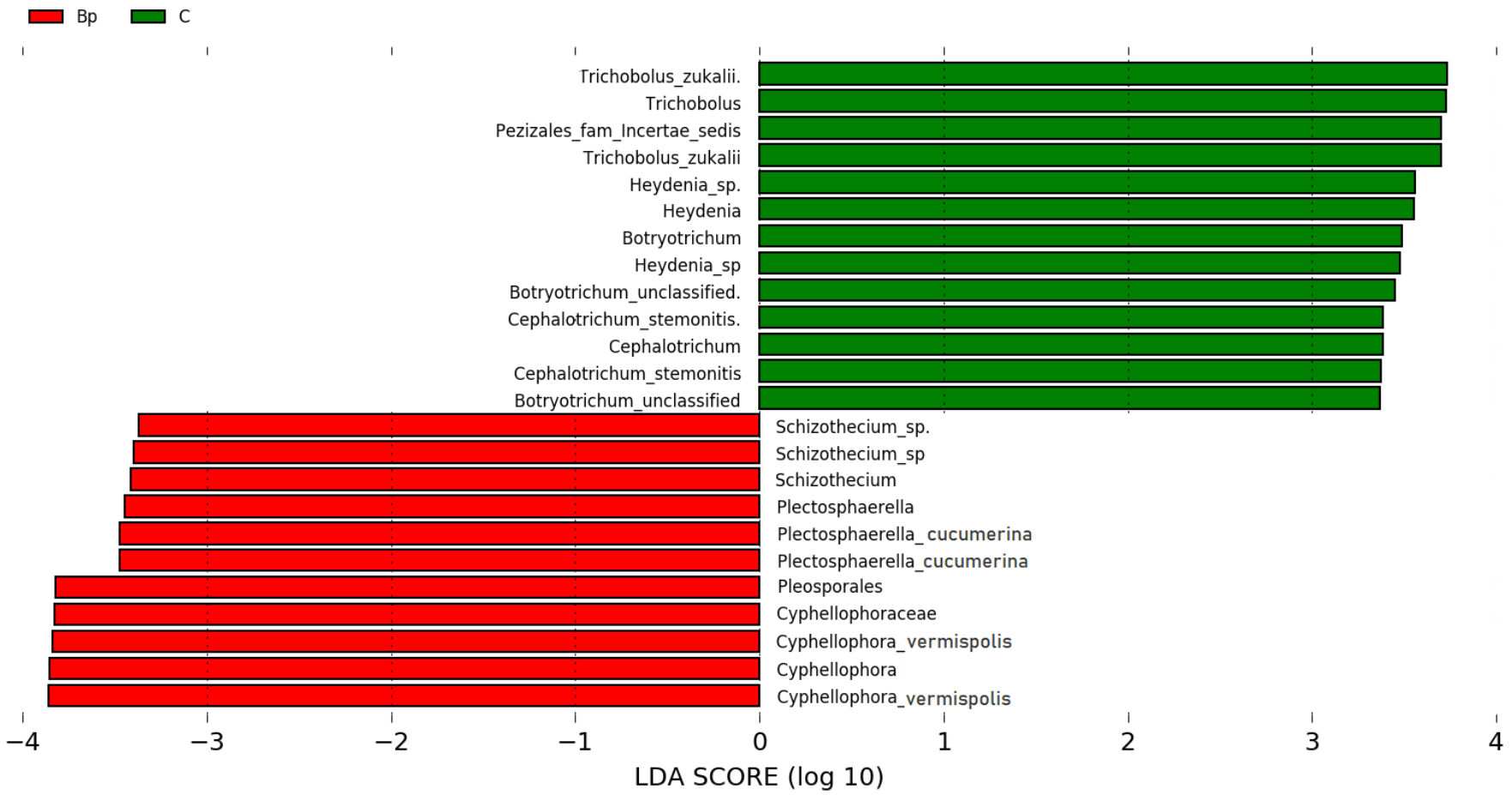

FIGURE S5 LEfSe analysis results indicating the changes in the top 100 fungal phylotypes after treatment at T44. Legend: (Bp) *Bacillus paralicheniformis* 2R5-treated samples; (C) untreated samples.

Publikacja nr 3

Plant growth–promoting rhizobacteria: *Peribacillus frigoritolerans* 2RO30 and *Pseudomonas sivasensis* 2RO45 for their effect on canola growth under controlled as well as natural conditions

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Plant growth–promoting rhizobacteria: *Peribacillus frigoritolerans* 2RO30 and *Pseudomonas sivasensis* 2RO45 for their effect on canola growth under controlled as well as natural conditions

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Even though canola is one of the most important industrial crops worldwide, it has high nutrient requirements and is susceptible to pests and diseases. Therefore, natural methods are sought to support the development of these plants. One of those methods could be a plant growth–promoting rhizobacteria (PGPR) that have a beneficial effect on plant development. The aim of this study was a genomic comparison of two PGPR strains chosen based on their effect on canola growth: *Peribacillus frigoritolerans* 2RO30, which stimulated canola growth only in sterile conditions, and *Pseudomonas sivasensis* 2RO45, which promoted canola growth in both sterile and non-sterile conditions. First of all, six bacterial strains: RO33 (*Pseudomonas* sp.), RO37 (*Pseudomonas poae*), RO45 (*Pseudomonas kairouanensis*), 2RO30 (*Peribacillus frigoritolerans*), 2RO45 (*Pseudomonas sivasensis*), and 3RO30 (*Pseudomonas migulae*), demonstrating best PGP traits *in vitro*, were studied for their stimulating effect on canola growth under sterile conditions. *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45 showed the best promoting effect, significantly improving chlorophyll content index (CCI) and roots length compared to the non-inoculated control and to other inoculated seedlings. Under non-sterile conditions, only *P. sivasensis* 2RO45 promoted the canola growth, significantly increasing CCI compared to the untreated control and to other inoculants. Genome comparison revealed that the genome of *P. sivasensis* 2RO45 was enriched with additional genes responsible for ACC deaminase (*acdA*), IAA (*trpF*, *trpG*), and siderophores production (*fbpA*, *mbtH*, and *acrB*) compared to 2RO30. Moreover, *P. sivasensis* 2RO45 showed antifungal effect against all the tested phytopathogens and harbored six more biosynthetic gene clusters (BGC), namely, syringomycin, pyoverdin, viscosin, arylpolyene, lankacidin C, and enterobactin, than *P. frigoritolerans* 2RO30. These BGCs are well known as antifungal agents; therefore, it can be assumed that these BGCs were responsible for the antifungal activity of *P. sivasensis* 2RO45 against all plant pathogens. This study is the first report describing *P. sivasensis* 2RO45 as a canola growth

promoter, both under controlled and natural conditions, thus suggesting its application in improving canola yield, by improving nutrient availability, enhancing stress tolerance, and reducing environmental impact of farming practices.

KEYWORDS

PGPR, canola, sterile and non-sterile conditions, genome analysis, *Peribacillus frigoritolerans*, *Pseudomonas sivasensis*

1 Introduction

Plant growth–promoting rhizobacteria (PGPR) that colonize plant rhizosphere are group of bacteria exerting a beneficial effect on plant development (Adedeji et al., 2020). The application of these microorganisms can reduce the requirement of synthetic chemical fertilizers and pesticides, which is important for the development of sustainable agriculture (Patel et al., 2021). PGPR can promote plant growth directly or indirectly. Direct plant growth promotion includes mechanisms providing plants with phosphorus, nitrogen, iron, and indol-3-acetic-acid, while indirect promotion consists of preventing phytopathogens due to antimicrobial metabolites and extracellular enzymes (Chouyia et al., 2020; Wang et al., 2023). Many beneficial microorganisms, including certain bacteria and fungi, produce antibiotics as secondary metabolites, inhibiting the growth and development of phytopathogens by disrupting their cell walls, membranes, or metabolic processes. Beneficial microorganisms can outcompete phytopathogens for essential nutrients in the rhizosphere. Additionally, some microbial metabolites can induce systemic resistance in plants, making them more resistant to pathogenic attacks. By breaking down the cell walls of phytopathogens, bacterial enzymes such as chitinases and glucanases inhibit the growth and spread of pathogens. Meanwhile, extracellular proteases and lipases produced by beneficial microorganisms can hydrolyze proteins and lipids in the cell membranes (Kumari et al., 2019; de Andrade et al., 2023).

The genus *Pseudomonas* and *Bacillus* are well known as PGPRs and biocontrol agents due to their ability to solubilize phosphates and produce phytohormones or secondary metabolites such as hydrogen cyanide (HCN), siderophores, and lipopeptides (Sheoran et al., 2015; Lastochkina et al., 2019; Oni et al., 2022). It was reported that bio-inoculations of the three PGPR *Bacillus* strains with the ability to catabolize ACC, increased shoots, and roots length of canola plants (Kashyap et al., 2019). Whereas *Pseudomonas* sp. strain, which was able to produce siderophores and fix nitrogen, affected canola seeds germination and seedlings growth (Jamalzadeh et al., 2021). Ecological applications of *Pseudomonas* and *Bacillus* species as bioinoculants are crucial for maintaining food security (Sah et al., 2021; Etesami et al., 2023).

Peribacillus frigoritolerans is a rod-shaped, Gram-positive bacterium belonging to the family *Bacillaceae* and classified originally as *Brevibacterium frigoritolerans* (Montecillo and Bae, 2022). It is well documented that *Brevibacterium frigoritolerans* stimulates plant growth and suppresses diseases caused by phytopathogens. *B. frigoritolerans* promotes growth of wheat (*Triticum aestivum* L.) increasing roots, shoots, seedlings length, and plant biomass (Tara and Saharan, 2017), while Raufa et al. (2019) found this strain as biocontrol agent in suppression of maize (*Zea mays* L.) stalk rot caused by *Fusarium moniliforme*. Whereas, *Pseudomonas sivasensis* was isolated from farm fisheries in Turkey and described for the first time by Duman et al. (2020). Świątczak et al. (2023a) found that PGPR *Pseudomonas sivasensis* bacterization altered the taxonomic structure of bacterial and fungal communities by increasing the abundance of plant beneficial microorganisms and increasing metabolic activity and functional diversity of microbial communities in the canola rhizosphere.

In our study, we present new insights into the plant growth–promoting (PGP) properties of *Pseudomonas sivasensis* 2RO45, which promoted canola growth under both controlled and natural conditions. Moreover, PGP and biosynthetic cluster genes of *Pseudomonas sivasensis* 2RO45 were compared with other PGPR —*Peribacillus frigoritolerans* 2RO30 that promoted canola growth only in sterile conditions.

2 Materials and methods

2.1 Isolation of plant growth–promoting rhizobacteria

Canola (*Brassica napus* L. var. *napus*) plant samples collected from three following growth stages: vegetative (RO), flowering (2RO), and maturity (3RO) were sourced from farmland in Ostroda, Poland (53°41′38″N 19°57′58″E). To isolate bacteria from the rhizosphere, canola roots were washed with sterile distilled water and cut into small pieces (2 mm). Following the serial dilution process, each 10-fold diluent was spread onto nutrient agar (NA) (Biomaxima, Warsaw, Poland) plates with

amphotericin B (40 µg/mL) as an antifungal agent and incubated for 3 days at 28°C. After incubation, the rhizospheric bacteria were enumerated and 50 colonies from each plant growth stage were isolated and stored in glycerol stocks at −80°C until further studies.

2.2 Assay of plant growth–promoting traits *in vitro*

2.2.1 Qualitative estimations

The rhizobacterial strains were qualitatively screened for PGP traits, namely, phosphates, siderophores, chitinases, hydrogen cyanide (HCN), and ammonia production. Phosphorus solubilization and siderophores sequestration abilities were assessed using Pikovskaya agar plates and Chrome Azurol Sulphonate (CAS) medium, respectively (Pikovskaya, 1948; Alexander and Zuberer, 1991). Pikovskaya agar plates were incubated at 26°C for 7 days for observation of a halo zone around the colonies. The solubilization index (SoI) was evaluated as the ratio of total diameter (colony + halo zone) to colony diameter (Luziatelli et al., 2019). The CAS plates were incubated for 4 days at 26°C for observation of an orange halo zone around the colonies. The siderophores index (SI) was calculated as the ratio of the halo zone diameter to colony diameter. For chitinase production, bacteria were inoculated on medium containing (g/L): peptone 1.0, $FeSO_4 \times 7H_2O$ 0.1, iron gluconate 0.1, yeast extract 0.1, colloidal chitin 7.0 g dry mass, and agar 15.0 (Swiontek Brzezinska et al., 2013). Plates were incubated for 14 days at 22°C to observe clearing zone around the colonies. The colloidal chitin was prepared with the Lingappa and Lockwood method (1962). HCN production was checked using NA plates supplemented with glycine (0.44%) according to Lorck (1948). A sterile filter paper (Whatman No. 1) was soaked in solution of picric acid (0.5%) and sodium carbonate (2%) and was kept on the plate lid. After 4 days of incubation at 28°C, color change of filter paper from yellow to brown was a positive indicator for HCN production. Ammonia production was assessed by inoculating bacteria in nutrient broth (Biomaxima, Warsaw, Poland) for 3 days at 26°C. After adding a Nessler reagent (0.5 mL), the development of orange color was considered as a positive result.

2.2.2 Quantitative estimations

2.2.2.1 Indole acetic acid production

Bacterial indole acetic acid (IAA) may increase roots length and surface, allowing the plant better uptake of soil nutrients and water, which, in turn, can stimulate plant growth (Gilbert et al., 2018).

IAA production was quantified using medium (g/L): peptone 5.0; yeast extract 3.0; and L-tryptophan 1.0 following the modified method of Ehmann (1977). After 4 days of incubation at 28°C, culture suspension was centrifuged at 10080*g* for 10 min. Centrifuged culture suspension supernatant was mixed with Salkowski reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$) and incubated for 30 min in the dark. Intensity of the color was measured at 530 nm using Hitachi U-2500 spectrophotometer.

2.2.2.2 ACC deaminase activity

PGP bacteria that express ACC deaminase activity may increase roots length and facilitate adaptation and survival of plants (del Carmen Orozco-Mosqueda et al., 2020).

Quantitative estimation of ACC was carried out according to the modified method described by Honma and Shimomura (1978). Bacterial cultures were inoculated in nutrient broth medium (Biomaxima, Warsaw, Poland), following incubation at 30°C for 24h in a rotary shaker. Culture suspension was centrifuged at 6000*g* for 10 min (4°C) and Dworkin and Foster (DF) salts minimal medium (5 mL) was added to the pellet (Dworkin and Foster, 1958). Culture suspension was centrifuged again at 6000*g* for 10 min (4°C) and DF salts minimal medium (5 mL) with 0.5 M ACC (30 µL) were added to the pellet. After incubation at 30°C for 24h, culture suspension was centrifuged at 4032*g* for 10 min (4°C) and bacterial cells were washed with 0.1M Tris-HCl (5 mL; pH 7.6). After centrifugation at 10000*g* for 5 min, 0.1 M Tris-HCl (600 µL; pH 8.5), toluene (30 µL), and 0.5 M ACC substrate (20 µL) were added to the pellet following incubation at 30°C for 30 min. 0.56 M HCl (1 ml) was added and culture suspension was centrifuged at 10000*g* for 5 min. Centrifuged culture suspension supernatant (1 mL) was mixed with 0.56 M HCl (800 µL) and 0.2% 2,4-dinitrophenylhydrazine (300 µL). After a 30-min long incubation at 30°C, 2 M NaCl (2 mL) was added and the absorbance was determined at 540 nm using Hitachi U-2500 spectrophotometer. The ACC deaminase was expressed in terms of nanomoles of α-ketobutyrate produced per milligram protein per hour. Protein content was estimated according Bradford (1976) method.

2.3 Greenhouse experiment in sterile and non-steriled conditions

2.3.1 Seed sterilization and inoculum preparation

The winter rapeseed of the Areti variety was selected for the study. It is a hybrid variety characterized by outstanding health and a very high yield potential. Sterilization of canola seeds was performed by disinfection in 1% NaOCl for 30 min and by washing 3 times in sterile distilled water following the method of Rudolph et al. (2015). Fresh bacterial cultures were inoculated in LB broth and incubated for 2 days at 26°C in a shaker incubator. The sterilized seeds were resuspended in bacterial suspension (10 mL of 10^8 CFU/mL) supplemented with 0.05 g carboxymethyl cellulose (CMC) and agitated for 30 min. The control was the seeds resuspended in 10 mL of nutrient broth without bacterial inoculum and supplemented with 0.5% CMC (Rudolph et al., 2015).

2.3.2 Seed treatment

Four canola seeds were sown per pot in eight replicates for each treatment. Seeds resuspended in six bacterial suspension (RO33, RO37, RO45, 2RO30, 2RO45, and 3RO30) were germinated in a sterile sand and vermiculite (1:1). Seeds with 2RO30, 2RO45 strains and their consortium were additionally sown in non-sterile soil taken from the field in Ostroda, Poland (53°41′38″N 19°57′58″E).

Canola seedlings were maintained in a day–night cycle of 16h light (100 µmol/m2/s) and a temperature of 22°C. The plants were moistened with an equal amount of water and were harvested after 44 days (Świątczak et al., 2023b).

2.3.3 Compatibility assay

For non-sterile greenhouse experiment, *in-vitro* antagonism compatibility test of two strains: *Peribacillus frigoritolerans* 2RO30 and *Pseudomonas sivasensis* 2RO45 was performed. Each isolates were resuspended in sterile water (10^8 CFU/mL) and 100 µL of the tested microorganism were spread onto NA plates. A sterile filter paper disc (5 mm in diameter) was placed onto NA plate containing the spread bacteria and 10 µL of bacterial suspension was inoculated on the paper disc. Plates were incubated at 28°C for 48h. When a clear zone of inhibition around the disc was observed, microorganisms were considered incompatible. When inhibition zone was not observed, they were classified as compatible (Widyantoro, 2019; Tabacchioni et al., 2021). Each experiment was performed in triplicate.

2.3.4 Canola parameters determination

Chlorophyll content index (CCI) was measured using a chlorophyll meter CCM-200plus (Opti-Sciences, Hudson, USA). Other canola growth parameters – length of roots, shoots and epicotyl were measured after washing the plant roots with distilled water. Photosynthetic area of leaves was calculated using DigiShape 1.3 software (Moraczewski, 2005). The plant roots, shoots, epicotyl, petioles and leaves were dried at 85°C for 48 hours and dry weight of these plant parts was determined. Furthermore, two following indexes were calculated: specific leaf area (SLA) and leaf weight ratio (LWR). The SLA index was calculated as follows: SLA = assimilation area [cm^2]/leaves dry biomass [g], while LWR index was evaluated as follows: LWR = leaves dry biomass [g]/total plant dry biomass [g] (Piernik et al., 2017).

2.3.5 Soil physicochemical analyses

The physical and chemical parameters of the non-sterile soil were determined at District Chemical and Agricultural Station (Bydgoszcz, Poland). The physicochemical analyses such as pH, phosphorus, potassium, magnesium, ammonium nitrogen, nitrate nitrogen, and organic carbon were evaluated according to PN-ISO10390, 1997; PN-R-04020:1994/Az1, 2004; PN-R-04022, 1996; PN-R-04022:1996/Az1, 2002; PN-R-04028, 1997, and PN-ISO14235, 2003 standards, respectively.

2.4 Statistical analysis

The statistical analysis of the data was performed using Past3 (version 3.25). To determine significant differences between treatments, one-way analysis of variance (ANOVA) was applied, followed by Tuckey post-hoc test. ANOVA assumptions were checked using Shapiro–Wilk test for normality and Levene's test for homogeneity of variances. When not normal distribution occurred, test for equal medians—Mann–Whitney was performed.

2.5 Identification of isolates based on 16S rRNA gene

Six bacterial isolates were identified based on 16S rRNA gene sequence according to Kalwasińska et al. (2020). Total genomic DNA was extracted using GeneMATRIX Bacterial and Yeast Genomic DNA Purification Kit (EURx, Gdańsk, Poland), following the manufacturer's protocol. For polymerase chain reaction (PCR) amplification, the following 20 µL of reaction total volume was used: Taq DNA polymerase, 0.2 mM dNTP mixture, Polbuffer B with 1.5 mM $MgCl_2$, 0.25µM of 27 F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492 R (5-TACGGTTACCTTGTTACGACTT-3) primers, and 1 µL of genomic DNA. The PCR conditions were: initial denaturation (95°C for 3 min), 30 cycles of amplification: denaturation (95°C for 30s), annealing (52°C for 20s), extension (72°C for 1 min 40s) and final extension (72°C for 5 min). PCR amplicons were checked in 1% (w/v) agarose gel stained with Midori Green DNA Stain. Sequencing of PCR products was performed using Big Dye Terminator v 3.1 Cycle Sequencing Kit according to manufacturer's instruction. Capillary electrophoresis was performed by the Sequencing and Oligonucleotides Synthesis Laboratory, IBB (Warsaw, Poland). Nucleotide sequences of RO33, RO37, RO45, 2RO30, 2RO45, and 3RO30 were submitted to GenBank under the accession numbers MW599360, MW599361, MW599362, MW599363, MW599366, and MW599367, respectively. The taxonomy of bacterial isolates were determined using the EzBioCloud database (Yoon et al., 2017).

2.6 Genome sequencing, prediction of PGP genes and biosynthetic gene cluster

Genomic DNA of *Peribacillus frigoritolerans* 2RO30 and *Pseudomonas sivasensis* 2RO45 was extracted and whole genome was sequenced on Illumina HiSeq using 250 bp paired-end protocol at the University of Birmingham (UK). Library construction was prepared with the use of Nextera XT Library Prep kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions with the following modifications: PCR elongation time was increased from 30 s to 1 min, and 2 ng of DNA was used as an input instead of 1 ng. The library preparation and quantification of DNA were done with a Microlab STAR automated liquid handling system (Hamilton, Bonaduz, Switzerland). Quantification of pooled libraries was performed using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 (Bolger et al., 2014). *De-novo* assembly was performed on samples using SPAdes version 3.7 (Bankevich et al., 2012), and contigs were annotated using Prokka 1.11 (Seemann, 2014). Further annotation was performed using NCBI's Prokaryotic Genome Annotation Pipeline (PGAP). The genomic sequences are available in DDBJ/ENA/GenBank under the accession numbers JAOAQM000000000 and JAOAQN000000000, respectively, for *Peribacillus frigoritolerans* 2RO30 and *Pseudomonas sivasensis* 2RO45.

The prediction of PGP genes was obtained from KEGG pathway analysis (https://www.kegg.jp/kegg/mapper/reconstruct.html), while secondary metabolite biosynthesis gene clusters were determined by antiSMASH (version 6.0) (https://antismash.secondarymetabolites.org).

2.7 Assay of plant pathogens growth inhibition *in vitro*

Bacterial isolates were screened for inhibition against fungal pathogens: *Alternaria alternata* 783, *Botrytis cinerea* 873, *Fusarium culmorum* 2333, *Fusarium oxysporum* 872, *Fusarium solani* 25, *Phytophthora cactorum* 1925, and *Phytophthora megasperma* 404, which can affect canola plants (Fernandez, 2007; Krasnow and Hausbeck, 2015; Al-Lami et al., 2019; Romero et al., 2008; Monnier et al., 2018; Ismaiel et al., 2021; Yu et al., 2023). Plant pathogenic fungi were obtained from the Plant Pathogenic Bank of the Institute of Plant Protection in Poznan (Poland). Fungi were cultivated on PDA plates (Biomaxima, Warsaw, Poland) for 5 days at 26°C, while bacteria were cultivated on PDA for 24h at 26°C. After incubation, mycelium agar discs (5 mm in diameter) were inserted onto PDA plate containing spread bacteria. Cultures were incubated for 7 days at 26°C and the diameter of the fungal mycelium was estimated. Pure cultures of each fungus were used as a control. Each experiment was performed in triplicate. The fungal growth inhibition zone was estimated by the following formula:

$$\text{inhibition } \% = (C - B)/C \times 100$$

where C is the diameter of fungi colony in the control plate, B is the diameter of fungi colony that grew in the presence of rhizobacteria (Wonglom et al., 2019).

3 Results

3.1 Assay of plant growth–promoting traits *in vitro*

The total rhizobacterial population was counted from different plant growth stages: the vegetative, flowering, and maturity. This analysis showed that the total bacterial load was the highest in the flowering stage followed by a maturity and vegetative stage (Supplementary Table S1). Fifty canola rhizospheric bacteria were isolated from the three plant growth stages and were tested for PGP traits including IAA, phosphate, ACC deaminase, siderophores, chitinases, HCN, and ammonia production. Ammonia and HCN production by bacterial strains can also have a positive effect on plant growth, for example by elongation of plant roots and shoots (Bhattacharyya et al., 2020). The plant growth promotion assay *in vitro* showed that rhizobacterial strains isolated from different growth stages exhibited various PGP traits (Supplementary Figure S1). Six isolates possessed the best PGP properties (Table 1). All of the strains were able to produce IAA, sequester siderophores and solubilize phosphates. Among these strains 2RO45 was the most effective regarding all three PGP. RO37 demonstrated the highest ACC deaminase activity. Three of the isolates: RO33, RO45, and 3RO30 were able to produce chitinases. Moreover, RO33 and 3RO30 strains were HCN and ammonia producers.

3.2 Greenhouse experiment in sterile and non-steriled conditions

Six bacterial strains identified as *Pseudomonas* sp. RO33, *Pseudomonas poae* RO37, *Pseudomonas kairouanensis* RO45, *Peribacillus frigoritolerans* 2RO30, *Pseudomonas sivasensis* 2RO45, and *Pseudomonas migulae* 3RO30 were used in greenhouse experiment to evaluate their *in-vivo* ability to stimulate canola growth under sterile conditions (Figure 1). The results showed that *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45 significantly increased CCI and roots length compared to the non-inoculated control and to seedlings inoculated with RO33, RO37, RO45, and 3RO30. In addition, these isolates significantly improved shoots length and epicotyl weight compared to the untreated control. Seedlings inoculated with *P. frigoritolerans* 2RO30 showed significantly higher mean value of epicotyl length compared to RO37, RO45, and 3RO30, while for *P. sivasensis* 2RO45, this parameter was higher compared to RO37 and 3RO30 strains. Inoculated canola with 2RO30 and 2RO45 had significantly higher shoots weight compared to non-inoculated plants and those inoculated with RO33, RO37, and RO45 strains. Moreover, *P. sivasensis* 2RO45 induced leaves weight compared to the control and to RO33, RO37, RO45, and 3RO30 treatments while, for petioles weight, significant increase was observed compared to seedlings inoculated with RO33, RO45, and 3RO30, but not to the control. The PGP effect of *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45 under sterile conditions is visible in the Figure 2.

Based on growth promotion effects under sterile conditions *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45 were selected for greenhouse experiment in non-sterile soil. Additionally, as compatibility between these strains was not observed, the consortium of the isolates was used for the experiment (Figure 3).

Our findings demonstrated that *P. sivasensis* 2RO45 significantly improved CCI compared to the untreated control and other inoculant treatments. Moreover, 2RO45 strain significantly increased shoots length compared to the non-inoculated control, but not to the other inoculated seedlings. Seedlings inoculated with the strain showed significantly higher SLA index and weight of roots compared to consortium but not to the control. Significant differences in LWR index and shoots weight between *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45 were observed with higher mean values for *P. sivasensis* 2RO45. Figure 4 showed the PGP effect of *P. sivasensis* 2RO45 compared to untreated control under non-sterile conditions.

The physicochemical analysis of the non-sterile soil (Supplementary Table S2) recorded potassium content of 350 mg/kg, followed by phosphorus content of 310 mg/kg, and magnesium content of 91 mg/kg. The ammonium nitrogen, nitrate nitrogen, and organic carbon contents were 6.57 mg/kg, 79.13 mg/kg, and 1.65%, respectively. The soil had a slightly acidic pH.

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TABLE 1 Plant growth–promoting traits by selected PGPR strains.

Plant growth–promoting traits	Isolates					
	RO33	RO37	RO45	2RO30	2RO45	3RO30
IAA (μg/mL)	19.59 ± 1.25^{b}	16.62 ± 1.33^{c}	17.85 ± 1.56^{c}	22.57 ± 1.11^{b}	25.84 ± 1.21^{a}	13.04 ± 1.56^{d}
P- solubilization (SoI)	1.0 ± 0.11^{d}	3.0 ± 0.34^{c}	3.0 ± 0.27^{c}	8.0 ± 0.89^{b}	10.0 ± 1.21^{a}	2.0 ± 0.17^{d}
ACC (nmol α-ketobutyrate/mg protein/h)	278.2 ± 13.5^{c}	1846.6 ± 29.4$_{a}$	115.8 ± 5.5^{e}	178.7 ± 9.69^{d}	1198.1 ± 23.6^{b}	0.0^{f}
Siderophore (d_{halo}/d_{colony})	2.0 ± 0.19^{c}	3.8 ± 0.50^{b}	4.3 ± 0.64^{a}	2.0 ± 0.15^{c}	4.8 ± 0.66^{a}	3.0 ± 0.38^{b}
Chitinase (clear zone in mm)	1.0 ± 0.14^{c}	0.0^{d}	2.0 ± 0.18^{a}	0.0^{d}	0.0^{d}	1.5 ± 0.15^{b}
HCN	+	–	–	–	–	+
Ammonia	+	–	–	–	–	+

Values are mean ± SE of three replicates; (+) positive result; (–) negative result. Different letters indicate significant differences based on the Tuckey HSD test, p< 0.05.

3.3 Genome sequencing, prediction of PGP genes, and biosynthetic gene clusters

Annotation of *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45 genomes identified 5,435 and 5,797 coding genes, respectively, and 120 and 77 pseudogenes, respectively. In total, 1,877,684 and 2,803,949 reads were obtained from the whole-genome sequencing of 2RO30 and 2RO45 and assembled into 85 and 92 contigs with GC content of 40.19% and 59.63%, respectively (Supplementary Table S3).

Whole genome sequencing of the *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45 revealed differences in identified PGP genes between strains (Figure 5 and Supplementary Table S4). 2RO30 genome harbored *pstA*, and *pstB*, while 2RO45 genome coded for *pstS*, genes, which are responsible for phosphate transport. The genomes of 2RO30 and 2RO45 included genes involving in ACC

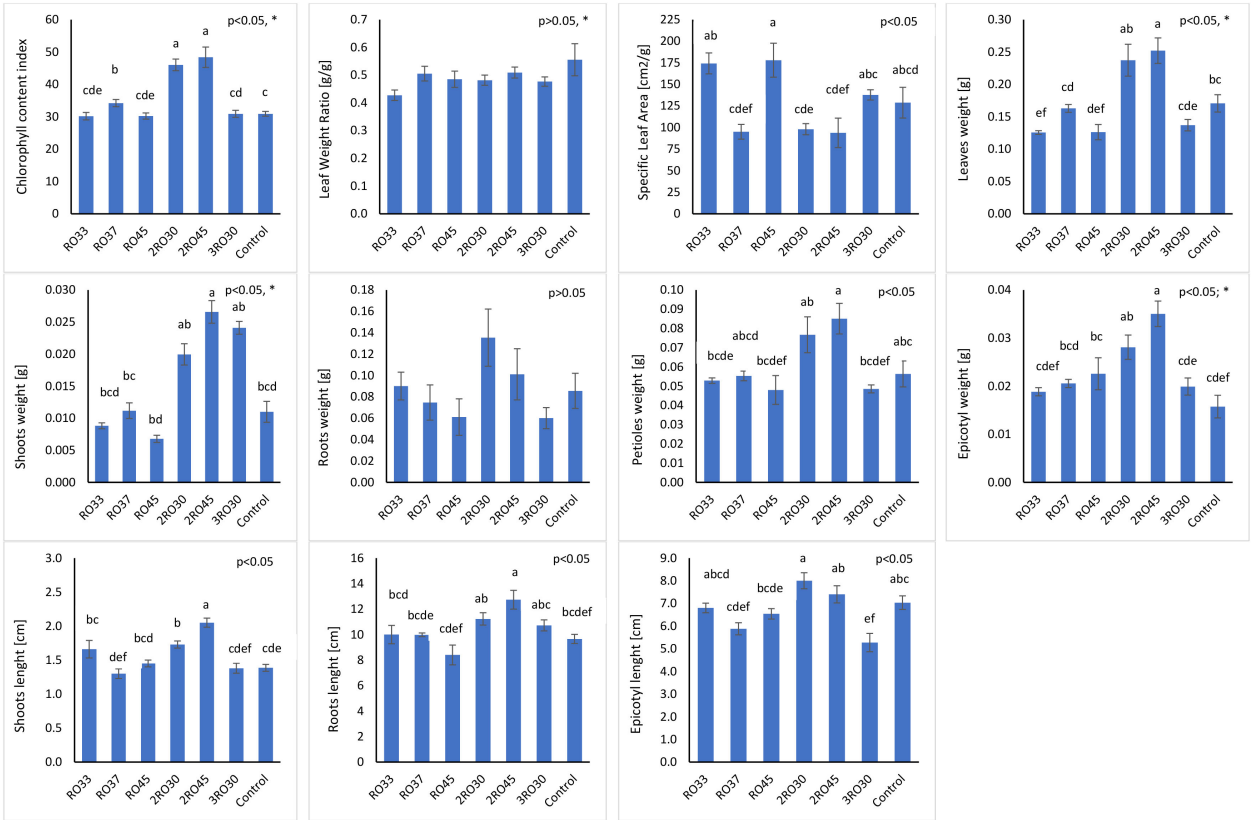

FIGURE 1
Plant growth parameters in sterile soil. Different letters indicate significant differences based on Tukey test as a *post hoc*, $p< 0.05$; *not normal distribution (test for equal medians: Mann–Whitney).

FIGURE 2
Peribacillus frigoritolerans 2RO30 and *Pseudomonas sivasensis* 2RO45 effect on canola growth compared to untreated control under sterile conditions.

deaminase (*dcyD*) and IAA (*trpA,B,C,D,E*) production. However, additional genes contributing to ACC deaminase (*acdA*) and IAA (*trpF, trpG*) were detected in *P. sivasensis* 2RO45 genome. Among 2RO30 and 2RO45 genomes, only 2RO45 genome contained genes involved in siderophores sequestration (*fbpA*, *mbtH*, and *acrB*). Moreover, genes related to acetoin and butanediol synthesis were found in 2RO30 and 2RO45 genomes. 2RO30 genome coded for *budC* gene, while 2RO45 coded for *poxB*.

The analysis of biosynthetic gene clusters (BGC) revealed the presence of betalactone (fengycin) in both *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45 genomes. Moreover, four additional type of genes, including nonribosomal peptide synthetases (NRPS) (viscosin, syringomycin, and pyoverdin), terpene (enterobactin), arylpolyene (APE Vf), and redox-cofactor (lankacidin C) were detected in 2RO45 genome. The abovementioned BGCs of *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45 are present in the Figure 6 and Supplementary Table S5.

3.4 Assay of plant pathogens growth inhibition *in vitro*

The phytopathogens inhibition assay revealed that *P. sivasensis* 2RO45 had antifungal effect against all tested fungi. 2RO45 strain

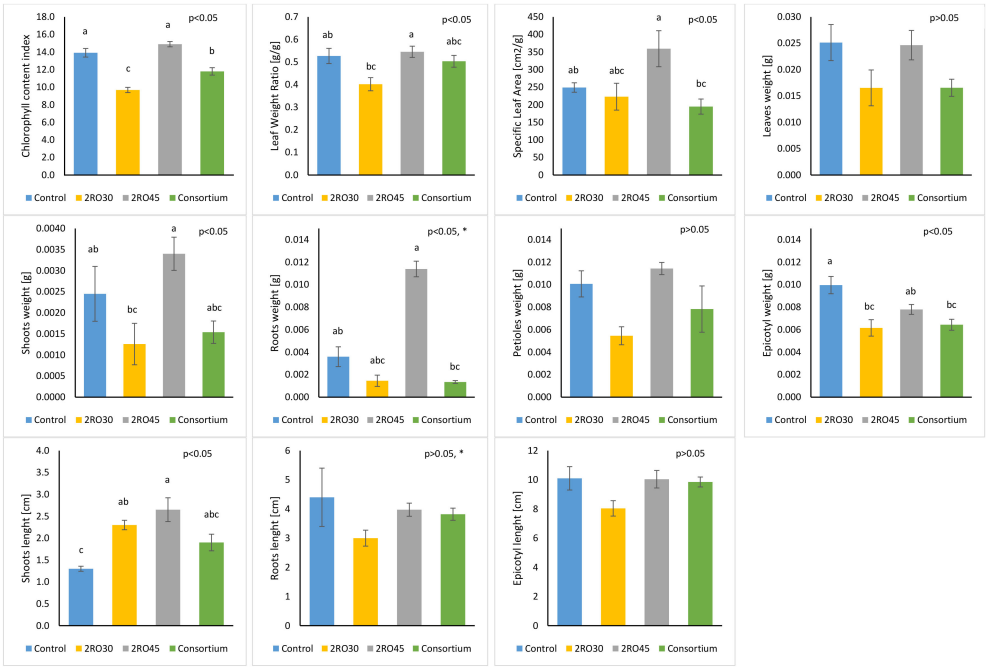

FIGURE 3
Plant growth parameters in non-sterile soil. Different letters indicate significant differences based on Tukey test as a *post hoc*, $p < 0.05$; *not normal distribution (test for equal medians: Mann–Whitney).

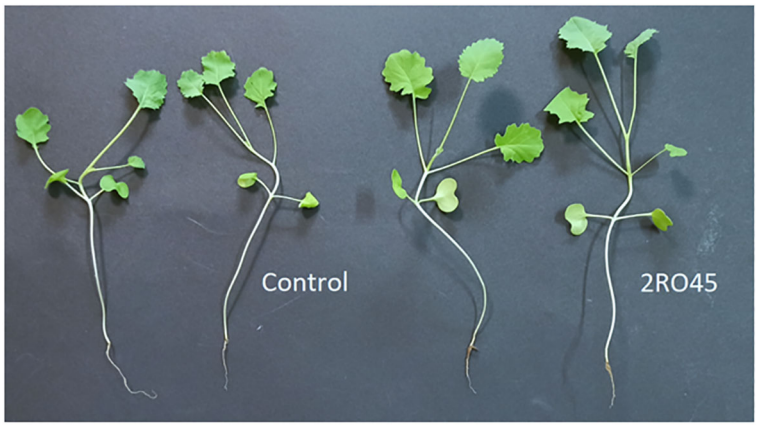

FIGURE 4
Pseudomonas sivasensis 2RO45 effect on canola growth under non-sterile conditions.

inhibited the mycelial growth of six plant pathogens (*A. alternata*, *B. cinerea*, *F. culmorum*, *F. oxysporum*, *F. solani*, and *P. cactorum*) with the inhibition rate of more than 10%. Whereas, *P. frigoritolerans* 2RO30 antagonistic effect in the rate greater than or equal to 10% was observed only against *B. cinerea* and *F. oxysporum* (Figure 7).

4 Discussion

PGPR can promote plant growth by direct and indirect mechanisms, which can be active simultaneously or independently at different plant growth stages (Kumar et al., 2012). In our study, the bacterial community was enumerated from three canola growth stages: vegetative, flowering and maturity. The results showed that the total bacterial load was the highest in the flowering stage followed by a maturity and vegetative stage. It can be explained by the fact that, when a plant grows, rhizospheric bacteria enter the plant system at vegetative stage and multiply during flowering stage but again start declining at maturity stage, for example, due to water stress, nutrient deficiency or free radical formation (Marag and Suman, 2018). Our results are in agreement with other studies. Marag and Suman (2018) enumerated endophytic population from different maize growth stages and found that the maximum bacterial loads were at the flowering stage. However, it is not known whether there is any association between the isolation of bacteria from different growth stages and their plant growth promoting (PGP) characteristics. Our study showed that rhizobacterial strains isolated from different periodic growth stages exhibited various PGP traits. However, a higher number of strains with the following PGP activities: IAA, ACC, phosphates, siderophores, and ammonia production were isolated from the flowering stage. This could be explained by much higher bacterial loads at the flowering stage than at the vegetative and maturity stages.

In addition, our results showed that two strains isolated from flowering stage growth: *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45 were the most effective in stimulating canola growth in sterile conditions. According to Pérez-Montaño et al. (2014) when PGPR promote plant growth under sterile conditions, their effect on plant development should be evaluated also under non-sterile conditions. Therefore, to demonstrate the plant growth promotion ability of *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45, a greenhouse experiment in non-sterile conditions was performed. In addition to testing the PGP effect of *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45, a consortium of these two strains was used in the non-sterile experiment. Swiontek Brzezinska et al. (2022) showed that co-inoculation of three rhizobacterial strains from different taxa (*Pseudomonas*, *Sphingobacterium* and *Microbacterium*) enhanced canola growth in comparison to single-inoculant treatments. However, our results showed that canola growth parameters increased only when plants were inoculated with *P. sivasensis* 2RO45. Interestingly, *P. frigoritolerans* 2RO30, which promoted plant growth under sterile conditions, did not promote canola under non-sterile conditions. Beneficial effects of PGPR on plant development are highly variable under natural conditions due to the native rhizosphere microbial communities' presence (Pacheco da Silva et al., 2022). According to Bhattacharyya et al. (2018), certain indigenous microbes might negatively affect the function and survival of introduced PGPR. Our results could be explained by that *P. frigoritolerans* 2RO30 failed in competition with soil natural microbiota and therefore it did not promote in non-sterile conditions. Moreover, it could be associated with differences in PGP characteristics *in vitro* and PGP genes between *P. frigoritolerans* 2RO30 and *P. sivasensis* 2RO45 strains.

High IAA production and phosphates solubilization features responsible for promoting canola growth under sterile conditions have been confirmed by the presence of appropriate genes in the genomes of these strains. However, *P. sivasensis* 2RO45 coded for many additional genes related to ACC and IAA production, such as *acdA* and *trpF,G*, which could have contributed to better canola growth promotion both

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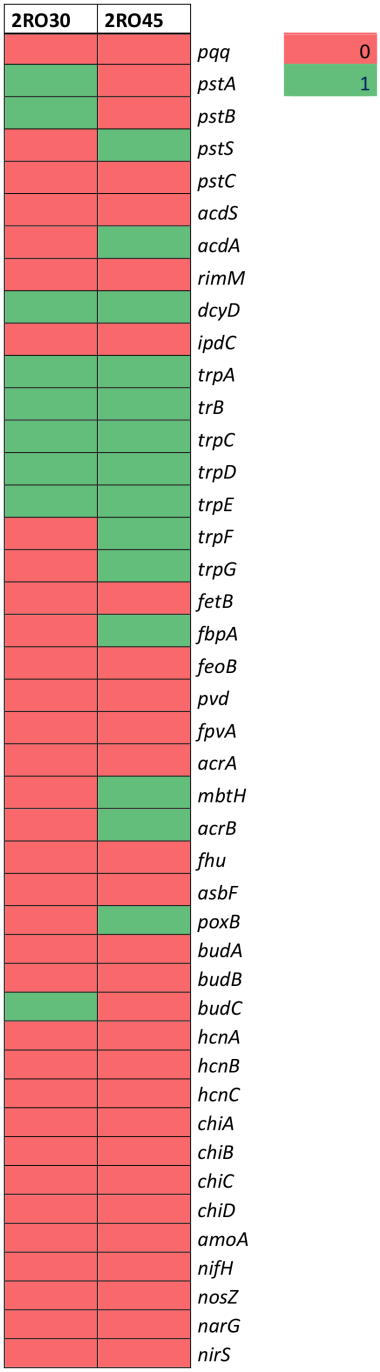

FIGURE 5
Genes responsible for plant growth–promoting characteristics.

in sterile and non-sterile soil. PGPR that produce IAA may increase root biomass, allowing the plant better access to soil nutrients and water uptake, which in turn can stimulate plant growth (Gilbert et al., 2018). Whereas, ACC deaminase can facilitate adaptation and survival of plants and increase roots length, thereby promote plant growth (del Carmen Orozco-Mosqueda et al., 2020). Świątczak et al. (2023c) found that the presence of *trpA,B,C,D,E,F,S* and *pqq*, *pstAB* genes in *Brevibacillus laterosporus* K75 genome and its high IAA and phosphates activity *in vitro* contributed to maize growth promotion under sterile and non-sterile conditions.

Apart from the genes mentioned above, 2RO45 genome contained *fbpA*, *mbtH*, *acrB* genes involved in siderophores production. BGC analysis revealed the presence of siderophore–enterobactin in its genome. Moreover, *P. sivasensis* 2RO45 showed the highest siderophores production in the PGP assay *in vitro*. It was reported that siderophores are responsible for plant growth promotion indirectly by inhibition of plant pathogens. However, siderophores can also stimulate plant species directly by enhancing iron (Fe) uptake in plants (dos Santos et al., 2020). Therefore, we assumed that high siderophores activity *in vitro* and the presence of many genes related to siderophores production in comparison to *P. frigoritolerans* 2RO30 are also responsible for *P. sivasensis* 2RO45 PGP effect under non-sterile conditions.

Moreover, *P. sivasensis* 2RO45 inhibited the mycelial growth of all fungi and with higher inhibition rates than *P. frigoritolerans* 2RO30. These observations are confirmed by genome mining and BGC analysis where *P. sivasensis* 2RO45 harbored six more secondary metabolite cluster genes than *P. frigoritolerans* 2RO30. The founded NRPS: syringomycin, pyoverdine, and viscosin are well known as antifungal agents (Bensaci et al., 2011; Alsohim et al., 2014; Sass et al., 2021). Fengycin is a lipopeptide producing by *Bacillus amyloliquefaciens* PPL, which contributes to antifungal activity against *Fusarium oxysporum* (Kang et al., 2020). Arylpolyene from *Vibrio fischeri* ES114 (APE Vf) was found in many *Pseudomonas* species (Gaete et al., 2022; Han et al., 2022) and according to Dutta et al. (2020) this lipid influences the antifungal effect of *P. fluorescens* NBC275. Strain *Streptomyces rochei* FS18 producing polyketide antibiotic–lankacidin and its derivatives, showed significant antifungal effect against *Aspergillus niger* (Cao et al., 2015). It can be assumed that these BGCs were responsible for the antifungal activity of *P. sivasensis* 2RO45 against plant pathogens, and if it has more possibilities of defense against pathogens, it promotes plants more effectively.

5 Conclusions

Pseudomonas sivasensis 2RO45 and *Peribacillus frigoritolerans* 2RO30 possessing the highest IAA production and phosphates solubilization, showed the best promoting effect on canola growth under sterile conditions. Under non-sterile conditions, only *Pseudomonas sivasensis* 2RO45 promoted canola growth, which can be associated with the presence of additional genes responsible for ACC deaminase (*acdA*), IAA (*trpF, trpG*), and siderophores production (*fbpA*, *mbtH*, and *acrB*) in its genome. Our study is the first report describing *Pseudomonas sivasensis* 2RO45 as a plant growth promoter, both under controlled and natural conditions, thus suggesting its application in improving canola yield, by improving nutrient availability, enhancing stress tolerance, and reducing the environmental impact of farming practices. Its application

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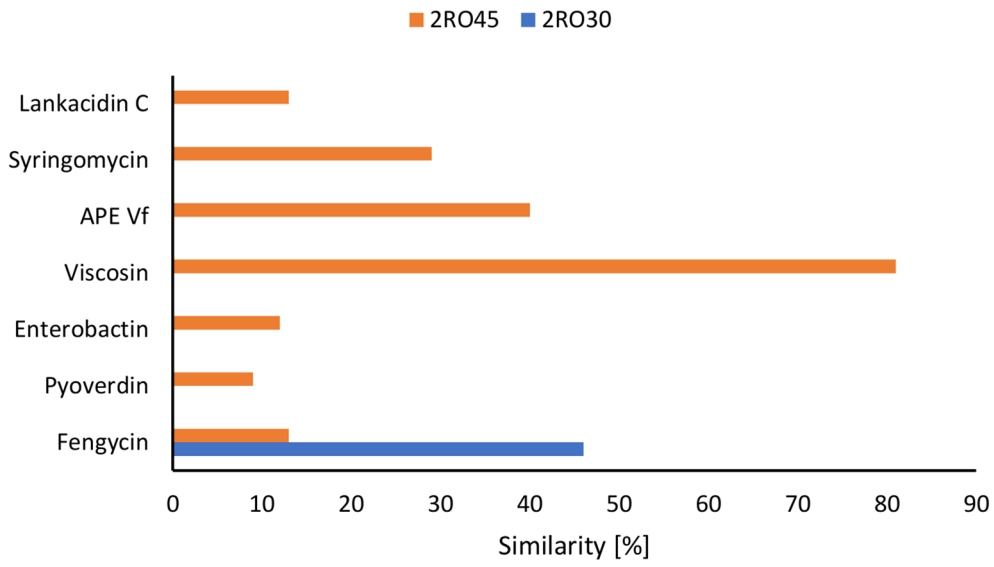

FIGURE 6
Detection of the secondary metabolite genes in *Peribacillus frigoritolerans* 2RO30 and *Pseudomonas sivasensis* 2RO45 genomes.

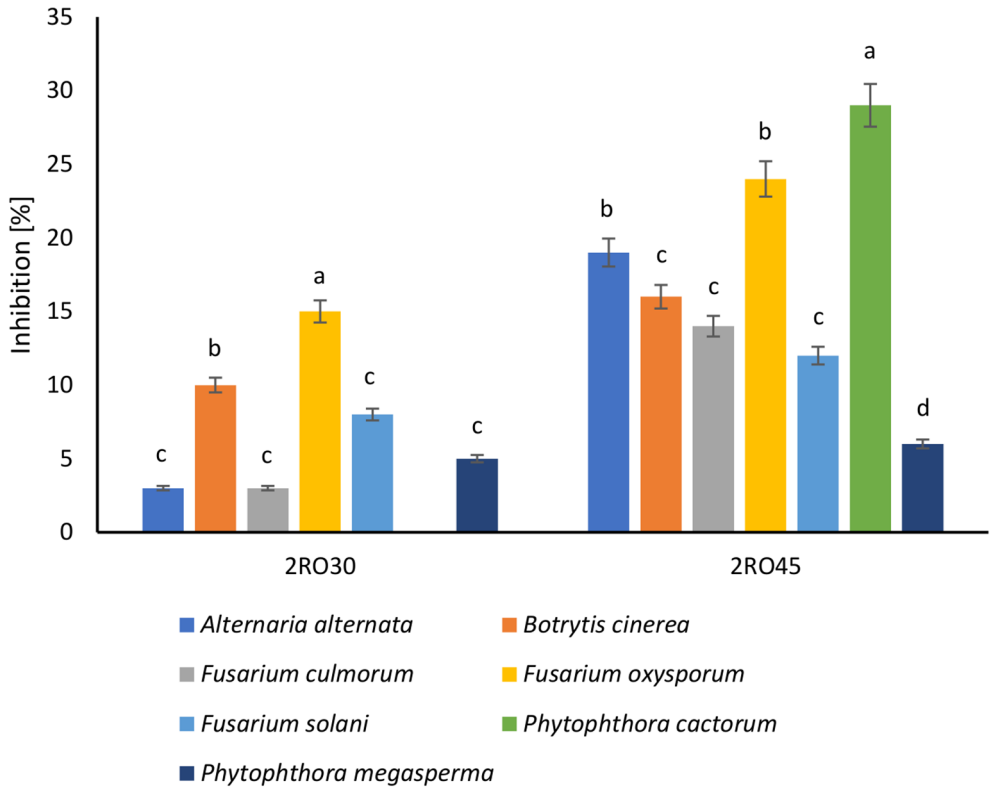

FIGURE 7
Inhibition of plant pathogens by *Peribacillus frigoritolerans* 2RO30 and *Pseudomonas sivasensis* 2RO45. Different letters indicate significant differences based on the Tuckey HSD test, $p < 0.05$.

aligns with the principles of environmentally friendly and economically viable agriculture. However, more research about how this strain competes with native soil microbes is necessary.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

JŚ wrote the original draft, made plant growth – promoting assays *in vitro* and *in vivo*, conducted statistical data and genome analysis, and prepared the manuscript editorially; AK coordinated the study, and checked the validity of the original draft; MS designed and coordinated the study, checked the validity of the original draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1233237/full#supplementary-material

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Supplementary Materials

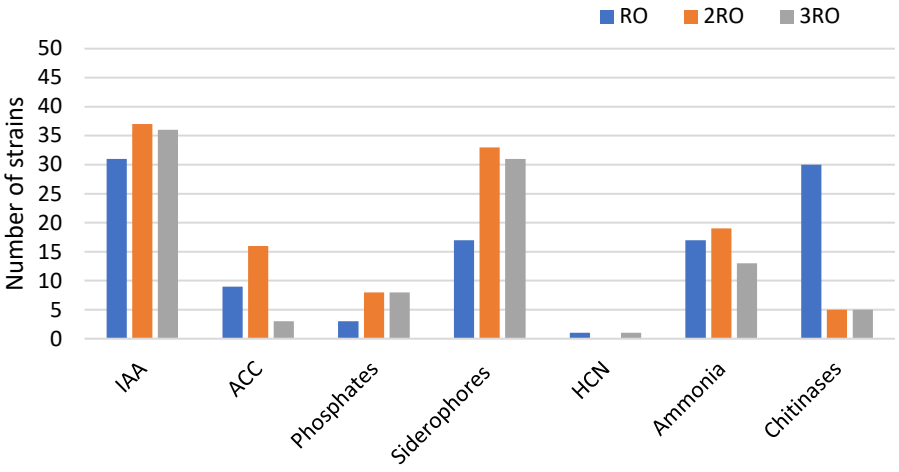

Figure S1 Number of stains showing PGP properties isolated from different plant growth stages: vegetative (RO), flowering (2RO) and maturity (3RO)

Table S1 Total rhizobacterial load from different canola growth stages

Plant growth stage	**Total bacterial load (x 10^3 CFU/mL)**
Vegetative	9.8
Flowering	12.3
Maturity	11.1

Table S2 The physical and chemical analysis of the non-sterile soil

Parameter	**Result**
pH	6.5
Phosphorus [mg/kg]	310.0
Potassium [mg/kg]	350.0
Magnesium [mg/kg]	91
Ammonium nitrogen [mg/kg]	6.57
Nitrate nitrogen [mg/kg]	79.13
Organic carbon [%]	1.65

Table S3 Genomes assemblies and annotations

	***Peribacillus frigoritolerans* 2RO30**	***Pseudomonas sivasensis* 2RO45**
Bioproject	PRJNA876229	PRJNA876229
Biosample	SAMN30648207	SAMN30648208
Assembly	GCA_025209795.1	GCA_025209875.1
Level	Contig	Contig
GC [%]	40.19	59.63
WGS	JAOAQM01	JAOAQN01
Genes	5,435	5,797
Pseudogenes	120	77
Number of reads	1,877,684	2,803,949
Total sequence length	5,517,502	6,309,106
Number of contigs	85	92
Contig N50	2,937,248	460,348
Contig L50	1	5

Table S4 Genes responsible for plant growth – promoting characteristics

Pathway	**2RO30**	**2RO45**	**Genes**
Degradation of phosphonates; phosphate transport	-	-	*pqq*
	+	-	*pstA, pstB*
	-	+	*pstS*
	-	-	*pstC*
ACC deaminase production	-	-	*acdS*
	-	+	*acdA*
	-	-	*rimM*
	+	+	*dcyD*
IAA production; L-tryptophan production	-	-	*ipdC*
	+	+	*trpE, trpA, trpD, trpC, trpB*
	-	+	*trpF, trpG*
Siderophore transport	-	-	*fetB*
	-	+	*fbpA*
	-	-	*feoB*
	-	-	*pvd, fpvA, acrA*
	-	+	*mbtH*
	-	+	*acrB*
	-	-	*fhu*
	-	-	*asbF*
Acetoin and butanediol synthesis	-	+	*poxB*
	-	-	*budA, budB*
	+	-	*budC*
HCN production	-	-	*hcnA, hcnC, hcnB*
Chitinase production	-	-	*chiA, chiB, chiC, chiD*
Nitrogen cycle	-	-	*amoA, nifH, nosZ, narG, nirS*

Table S5 Biosynthetic cluster genes in *Peribacillus frigoritolerans* 2RO30 and *Pseudomonas sivasensis* 2RO45 genomes

Region	From	To	Type	Most similar gene cluster	Similarity [%]
***Peribacillus frigoritolerans* 2RO30**					
Region 1.2	2,130,359	2,154,527	betalactone	fengycin	46
***Pseudomonas sivasensis* 2RO45**					
Region 1.1	276,750	329,661	NRPS	pyoverdin	9
Region 1.2	335,507	357,081	terpene	enterobactin	12
Region 5.1	328,232	441,499	NRPS	viscosin	81
Region 6.1	282,883	326,458	arylpolyene	APE Vf	40
Region 9.1	34,055	62,398	betalactone	fengycin	13
Region 9.2	181,413	211,716	NRPS	syringomycin	29
Region 12.1	45,779	67,926	redox-cofactor	lankacidin C	13

Publikacja nr 4

Pseudomonas sivasensis 2RO45 inoculation alters the taxonomic structure and functioning of the canola rhizosphere microbial community

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Pseudomonas sivasensis 2RO45 inoculation alters the taxonomic structure and functioning of the canola rhizosphere microbial community

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Inoculation with plant growth-promoting rhizobacteria (PGPR) is an eco-friendly sustainable strategy for improving crop productivity in diverse environments under different conditions. Our earlier study demonstrated that *Pseudomonas sivasensis* 2RO45 significantly stimulated canola (*Brassica napus* L. var. *napus*) growth. The aim of the present study was to investigate the structural and functional dynamics in the canola rhizosphere microbiome after inoculation with PGPR *P. sivasensis* 2RO45. The results based on alpha diversity metrics showed that *P. sivasensis* 2RO45 did not significantly alter the diversity of the native soil microbiota. However, the introduced strain modified the taxonomic structure of microbial communities, increasing the abundance of plant beneficial microorganisms, e.g., bacteria affiliated with families *Comamonadaceae*, *Vicinamibacteraceae,* genus *Streptomyces,* and fungi assigned to *Nectriaceae, Didymellaceae, Exophiala, Cyphellophora vermispora*, and *Mortierella minutissima*. The analysis of community level physiological profiling (CLPP) revealed that microbial communities in the *P. sivasensis* 2RO45 treated canola rhizospheres were more metabolically active than those in the non-treated canola rhizosphere. Four carbon sources (phenols, polymers, carboxylic acids, and amino acids) were better metabolized by the microbial communities from the rhizosphere of plants inoculated with the *P. sivasensis* 2RO45 than non-inoculated canola rhizospheres. Based on the community-level physiological profiles, the functional diversity of the rhizosphere microbiome was altered by the *P. sivasensis* 2RO45 inoculation. Substrate utilization Shannon diversity (H) index and evenness (E) index were significantly increased in the treated canola plants. The study provides new insight into PGPR-canola interactions for sustainable agriculture development.

KEYWORDS

PGPR, *Pseudomonas*, rhizosphere diversity, metabolic functions, canola microbiome

1. Introduction

The rhizosphere is a hotspot around the roots where numerous important processes related to the nutrition, growth, and fitness of plants occur (Sugiyama et al., 2014; Zuluaga et al., 2021). Rhizosphere microorganisms, such as PGPR, are regarded as prominent components of sustainable agriculture due to their positive influence on plant growth through alleviation of biotic and abiotic stresses, providing nutrients and secretion of phytohormones (Zuluaga et al., 2021).

The native rhizosphere microbial communities are altered by numerous interactions, including interfering in root exudation patterns, exchange of genetic material, and transformation of nutrients. Rhizosphere microbiome may be also modified by PGPR inoculation (Bhattacharyya and Lee, 2016). Therefore, before the use of PGPR as bioinoculants in the field, it is necessary to determine the changes occurring in the resident soil microorganisms structure after PGPR inoculation. It was reported that PGPR inoculation can lead to transient or even permanent alterations in the abundance of microbial communities and their function, which can finally contribute to promoting plant growth and its fitness (Bhattacharyya and Lee, 2016; Kong and Liu, 2022).

Because rhizosphere microbial communities are important for plant growth promotion; an increasing number of studies pay more attention to the next generation sequencing (NGS) methods to better understand how PGPR inoculation affects the structure of rhizosphere microbiomes (Sugiyama et al., 2014). Moreover, it was reported that the effect of PGPR inocula on the functional diversity of the native rhizosphere microbial community should receive more attention (Di Salvo et al., 2018a). Some researchers analyzed the changes in microbial communities' metabolic activity and function after PGPR inoculation using the community-level physiological profiling (CLPP) method. However, the analyses on different crops, such as rice, tomato, maize and wheat have been performed (Naiman et al., 2009; de Salamone et al., 2010; Di Salvo et al., 2018a; Zuluaga et al., 2021).

Our earlier results showed that the bacterization of canola (*Brassica napus* L. var. *napus*) seeds with *Pseudomonas sivasensis* 2RO45 significantly promoted growth of plant (Świątczak et al., unpublished). This study aimed to determine whether the bacterization of canola seeds with *P. sivasensis* 2RO45 alters the structural and functional diversity of microbial communities in the rhizosphere. The taxonomic structure changes in bacterial and fungal communities were evaluated using NGS, while the function of the microbial community was investigated by CLPP method using Biolog EcoPlates. The analyzes of interactions between canola plants and their associated microbiota after PGPR inoculation can provide new approaches for canola growth management.

2. Materials and methods

2.1. Rhizobacterium *Pseudomonas sivasensis* 2RO45

The *Pseudomonas sivasensis* 2RO45 was originally isolated from the canola (*Brassica napus* L. var. *napus*) rhizosphere taken from the field in Ostroda, Poland (53°41′38″N 19°57′58″E). The 2RO45 was selected based on its PGP traits, and its ability to promote canola growth under sterile and non-sterile conditions. The 2RO45 was able to produce indole-3-acetic acid (IAA), sequester siderophores, solubilize phosphates, and to produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase.

2.2. Experimental design and rhizospheric soil sampling

Pseudomonas sivasensis 2RO45 cells were harvested in LB broth (10 ml) with 0.05 g carboxymethyl cellulose (CMC) to yield 10^8 colony forming units (CFU)/ml. Seeds of canola were sterilized by soaking for 30 min in 1% sodium hypochlorite, followed by three rinses with sterile distilled water and incubation with 2RO45 inoculum for 30 min in a shaker incubator. Control seeds were incubated in LB broth (10 ml) with 0.05 g CMC under the same conditions (Rudolph et al., 2015). Four seeds were sowed in pots containing soil taken from the field in Ostroda, Poland (53°41′38″N 19°57′58″E). The bacterized and non-bacterized rhizospheres samples (total of 24 samples) were collected from the canola roots by scraping the adhering soil after 0 (T0), 22 (T22), and 44 days (T44). Four replicate pots were maintained for each time-point (**Supplementary Figure 1**). The "time" was considered as a main factor for establishing the groups of samples, in which the changes in microbiota structure and function were evaluated.

2.3. Colonization efficiency of *Pseudomonas sivasensis* 2RO45

To test if P. *sivasensis* 2RO45 can colonize the canola rhizosphere, sterilized canola seeds were inoculated with 2RO45 (10^8 CFU/ml) under sterile soil conditions. Canola plants were maintained in a growth chamber in a day-night cycle of 16 h light (100 μmol/m2/s) and a temperature of 22°C. Root samples were collected after 0 (T0), 22 (T22), and 44 days (T44). The plant roots were vortexed for 30 min in PBS solution (Ke et al., 2019) and the number of P. *sivasensis* 2RO45 were counted on PCA medium after incubating at 28°C for 48 h.

2.4. DNA extraction and sequencing

Rhizosphere samples collected from the replicate pots of each treatment were pooled separately (**Supplementary Figure 1**; Bhattacharyya et al., 2018) and total genomic DNA was extracted from 0.25 g of fresh rhizosphere samples using a DNeasy Power Soil Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. DNA concentration was determined using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, each sample was sent for (NGS) at the University of Łódź (Biobank), Poland.

Bacterial and fungal communities were determined by the amplification of V3-V4 hyper-variable regions of the

16S rRNA gene and Internal Transcribed Spacer (ITS2), respectively. For bacteria, the following primers were used: 5′ CCTACGGGNGGCWGCAG (forward) and 5′ GACTACHVGGGTATCTAATCC (reverse) (Klindworth et al., 2013). For fungi, a forward primer was used: 5′GCATCGATGAAGAACGCAGC (ITS3, without overhangs) with the reverse primer 5′ TCCTCCGCTTATTGATATGC (ITS4, without overhangs) (White et al., 1990). The methodology for library preparation followed the protocol available from Illumina Support Center (ISC) with a slight modification (2 × Phanta Max Master Mix, Vazyme Biotech, Nanjing City, China was applied instead of Kapa Hifi Hot Start Ready mix). The products were verified after each PCR using the electrophoretic separation. Libraries were normalized based on band luminescence intensity on a 1.5% agarose gel and pooled. Sequencing was performed on a MiSeq (Illumina, San Diego, CA, USA) using MiSeq Reagent Kit v2 (500-cycles) 2 × 250 bps paired-end format.

2.5. Amplicon sequence analysis

In the present study, the merging of sequence sets was performed, and the analysis of 16S rRNA amplicons was carried out using mothur v1.44.3 (Schloss et al., 2009). The quality processing, taxonomic assignments, and operational taxonomic unit (OTU) picking were done according to the MiSeq SOP of mothur (Kozich et al., 2013) with a 97% similarity threshold. The 'make.contigs' command was executed with a deltaq value of 10, and the analysis excluded ambiguous base calls and reads shorter than 300 nt or longer than 500 nt. The end of the sequences was trimmed to remove the primers using trim.seqs (pdiffs = 2, checkorient = T). The pre.cluster command was utilized for denoising, and chimeric reads were filtered out using VSEARCH in mothur. The sequence set was further processed by discarding the singleton reads as per Kunin et al. (2010). The read alignment and taxonomic assignment were carried out using the ARB-SILVA SSU Ref NR 138 reference database (Quast et al., 2013) with a minimum bootstrap confidence score of 80. Reads assigned to non-primer-specific taxonomic groups ("Chloroplast," "Mitochondria," and "unknown") were excluded from the dataset. Finally, a random subsampling was performed based on the sample with the lowest sequence number.

Fungal ITS analysis was performed through the following steps. The merging and quality filtering of sequences were carried out using mothur, as described for the 16S rRNA gene amplicons. The ITS2 region was extracted from the sequences with the ITSx 1.1-beta software (Bengtsson-Palme et al., 2013) based on the findings of Nilsson et al. (2010). Taxonomic assignment was performed using mothur's classify.seqs (cutoff = 80) and the UNITE v8.3 database (Abarenkov et al., 2020) as reference. Sequences that were not assigned to any fungal phyla were discarded from the amplicon set with the remove.lineage (taxon = k__Fungi_unclassified) command of mothur. Finally, the clustering of ITS2 reads into OTUs was performed using VSEARCH with a 97% similarity threshold.

The sequences were uploaded to the GenBank-Sequence Read Archive under Bioproject PRJNA876229.

2.6. Functional potential of microbial communities

The effect of *Pseudomonas sivasensis* 2RO45 inoculation on the microbial community function and diversity in the canola rhizosphere were analyzed by the CLPP method using Biolog EcoPlates (Biolog Inc., Hayward, CA, USA). Rhizosphere samples (10 g) were collected after 44 days of *P. sivasensis* 2RO45 inoculation. Samples were added to 90 ml of 0.85% sterile saline solution and shaken for 30 min at 200 rpm (Sun et al., 2010). Samples were diluted to a 10^{-2} gradient and 150 µl suspension of the dilution obtained from each rhizosphere sample was applied to Biolog EcoPlates wells. The plates were incubated at 28°C and the optical density (OD) was measured at 595 nm using a microplate absorbance spectrophotometry (Multiskan EX, Thermofisher Scientific, Waltham, MA, USA) at 24 h intervals up to 7 days incubation. Three replicates were performed for each treatment.

The microbial activity was calculated by the average well color development (AWCD) using the following equation: AWCD = Σ (C–R)/31, where C is the absorbance value of each well, and R is the absorbance value of the control (Bhattacharyya and Lee, 2016). The 31 substrates were divided into six kinds of carbon sources: phenols (pyruvic acid methyl ester, glucose phosphate, glycerol phosphate), polymers (tween 40, tween 80, cyclodextrin), carbohydrates (cellobiose, lactose, b-methyl-d-glucoside, d-xylose, erythritol, mannitol), carboxylic acids (glucosaminic acid, b-galactonic acid, galacturonic acid, hydroxybenzoic acid, 4 hydroxybenzoic acid, hydroxybutyric acid, itaconic acid, keto butyric acid, malic acid), amino acids (L-arginine, asparagine, phenylalanine, serine, threonine. glycyl glutamic acid) and amines/amides (phenylethylamine, putrescine). The AWCD for each carbon substrate group was determined using the following formula: AWCD = Σ (C–R)/N, where C is the absorbance value of each well, R is the absorbance value of the control and N is the number of substrates in the category (Bhattacharyya and Lee, 2016; Zuluaga et al., 2021). The functional diversity of microbial communities was expressed as the substrate utilization Shannon diversity index (H), substrate utilization Shannon evenness index (E) and substrate utilization Simpson diversity index (D) (Ge et al., 2018; Koner et al., 2021). The absorbance values on the 7th day of incubation were used for the calculation of metabolic functional diversity indices.

2.7. Microbial community structure

Richness estimators and diversity indices were calculated with mothur. LEfSe (Linear discriminant analysis effect size) pipeline, available at http://huttenhower.sph.harvard.edu/galaxy/ was used to identify taxonomic bacterial and fungal groups that were differentially abundant ($p < 0.05$) in the untreated and *Pseudomonas sivasensis* 2RO45 treated plants at each time of the inoculation (T0, T22, and T44). This analysis was performed for the 50 most abundant OTUs.

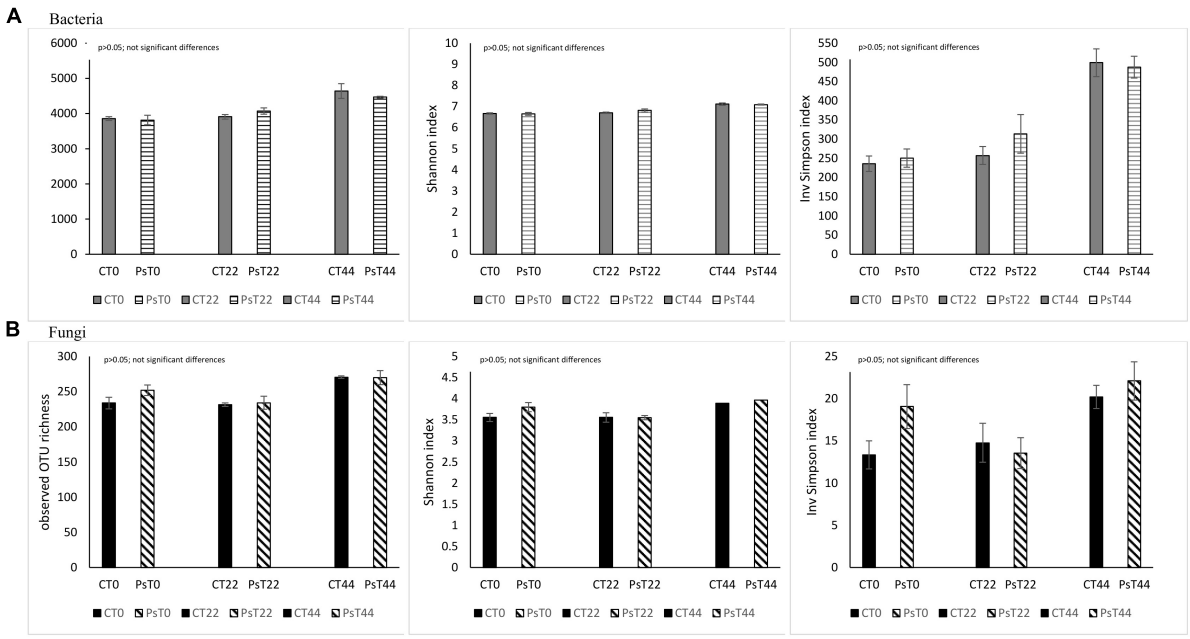

FIGURE 1
OTU richness and diversity indices (Shannon H' and Inv Simpson) for the bacterial **(A)** and fungal **(B)** communities in *Pseudomonas sivasensis* 2RO45 treated canola rhizospheres and untreated rhizospheres samples according to treatment based on NGS sequencing; vertical bars represent standard deviation (n = 3).

2.8. Data analysis and statistics

Rarefaction curves were generated using phyloseq in R v 4.0.3. Differences in microbial alpha diversity [observed OTU richness, Shannon (H'), and Simpson index (1-D)] were estimated using Past v 3.08. Two different statistical analyses in terms of microbial alpha diversity were performed: (i) *t-test* for equal means to detect differences between non-treated and *Pseudomonas sivasensis* 2RO45 treated canola plants; (ii) one-way analysis of variance (ANOVA) followed by *post-hoc* test (Tukey's HSD test; p-values ≤ 0.05) to evaluate differences in the rhizosphere of *Pseudomonas sivasensis* 2RO45 treated and untreated plants according to time. Normality and homogeneity of variance assumptions were performed by the Shapiro-Wilk and Levene's tests. Principal coordinates analysis (PCoA) and analysis of similarities (ANOSIM) were performed using R (v 4.0.3).

3. Result and discussion

The bioinformatic data processing resulted in 2,198,786 and 2,121,080 high quality bacterial and fungal sequence reads, respectively. Datasets covered 16,443 bacterial and 2,760 fungal OTUs. The rarefaction curves indicated a high coverage of the estimated bacterial and fungal diversity in the canola rhizosphere samples (**Supplementary Figure 2**).

Different alpha biodiversity indices for bacteria, e.g., OTU richness, Shannon, and Inv Simpson significantly increased over time, both in non-treated and treated with *Pseudomonas sivasensis* 2RO45 rhizosphere samples (**Supplementary Table 1**). Whereas, fungal alpha-diversity based on the OTU richness and Shannon diversity index were the highest in non-treated samples on 44th day (**Supplementary Table 2**). Rhizosphere bacterial and fungal communities were altered by various environmental factors such as climate and soil properties (Li et al., 2021), as well as host factors such as plant genotype and plant age (Fazal et al., 2021). Our results indicated that microbial richness and diversity increase with time and plant development. Previous studies also confirmed that the abundance and structure of the soil microbial community can differ depending on the plant growth stage (Liu et al., 2015; Wang et al., 2017).

Plant growth-promoting rhizobacteria inoculants can be a suitable option to developing sustainable agriculture, reducing or even eliminating the use of agrochemicals without yield loss (Kong and Liu, 2022). Nonetheless, understanding the effect of PGPR inoculation on the structure and functional dynamics in the rhizosphere microbiome is essential for better exploitation of PGPR potential in improving plant health and fitness (Zuluaga et al., 2021; Kong and Liu, 2022). Therefore, we studied biodiversity of the microbial community in the canola rhizosphere after inoculation with *Pseudomonas sivasensis* 2RO45 which features the ability to improve canola growth.

There were no significant differences, given the alpha diversity metrics between non-treated and *P. sivasensis* 2RO45 treated canola plants (**Figure 1**), indicating that the 2RO45 strain did not significantly alter the diversity of the native soil microbiota. These results were also supported by PCoA (**Figure 2**) and ANOSIM (**Table 1**). No significant bacterial, archaeal, and diazotrophic enrichments were observed in the rhizosphere of maize (*Zea mays* L.) after *Pseudomonas stutzeri* A1501 inoculation (Ke et al., 2019). There are reports demonstrating that PGPR inoculation increases the microbial diversity in the rhizosphere, simultaneously

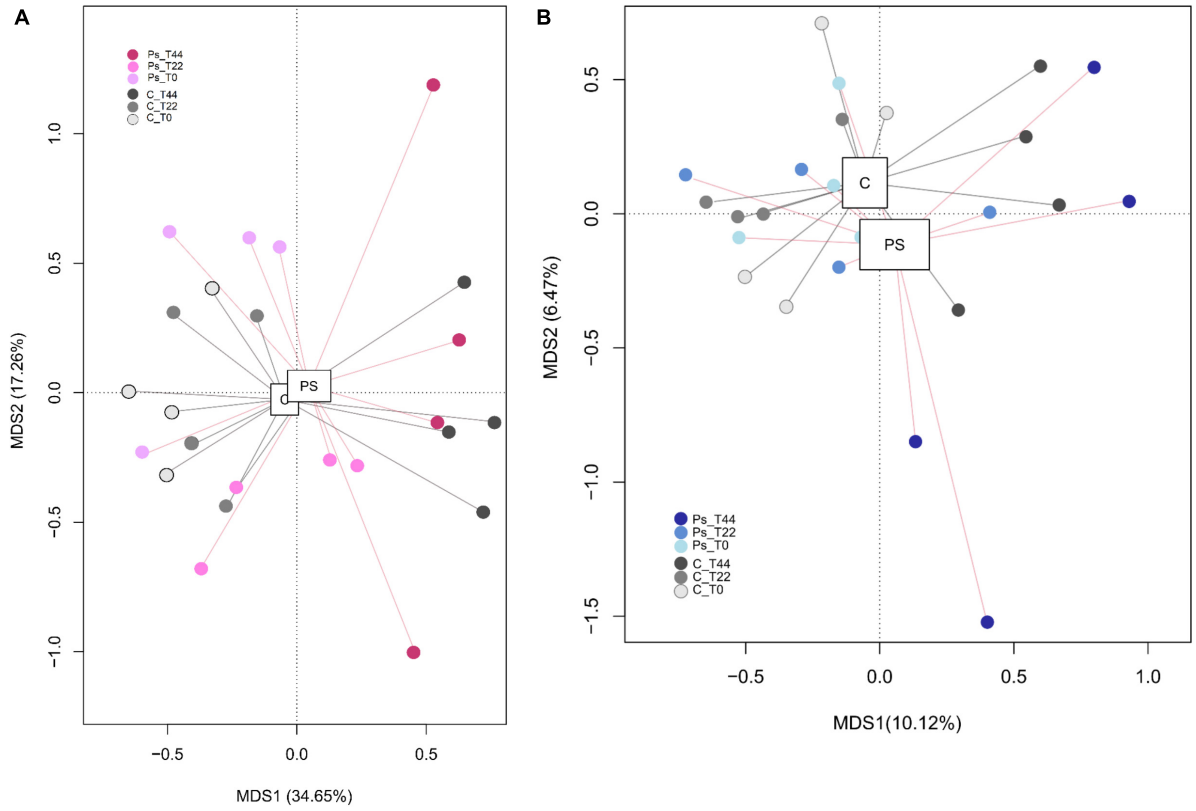

FIGURE 2
Principal coordinates analysis of bacterial **(A)** and fungal **(B)** distributions in canola plants inoculated with *Pseudomonas sivasensis* 2RO45 (PS) or uninoculated (C); T0, T22, and T44–time after inoculation in days.

TABLE 1 Analysis of similarities (ANOSIM) for microbial communities in the canola rhizosphere.

Time	ANOSIM statistic R	Significance
Bacteria		
T0	0.219	0.100
T22	0.208	0.113
T44	0.083	0.216
Fungi		
T0	0.073	0.461
T22	0.042	0.443
T44	0.198	0.150

promoting Kimchi cabbage (*Brassica rapa* L. ssp. *pekinensis*) growth (Yu and Lee, 2013; Bhattacharyya et al., 2018). However, if the PGPR inoculants are unable to survive in the competition with indigenous bacterial communities, minor or no changes would happen in terms of rhizosphere microbiome structure or diversity (Piromyou et al., 2011; Touceda-González et al., 2015). Although PGPR inoculation caused only minor changes in the rhizosphere microbial community, plant growth promotion has been observed (Piromyou et al., 2011; Touceda-González et al., 2015). Jiménez et al. (2020) showed that PGPR *Pseudomonas fluorescens* LBUM677 influences the rhizosphere microbiome of three different oilseed crops: canola (*Brassica napus* L.), soybean (*Glycine max* L.), and corn gromwell (*Buglossoides arvensis* L.). Interestingly, *P. fluorescens* LBUM677 inoculation decreased diversity of these rhizosphere microbiomes (Jiménez et al., 2020). According to Kong and Liu (2022) the effectiveness of inoculated PGPR is associated with their ability to being efficient rhizosphere colonizers. Our results showed that based on the relative abundance of the *Pseudomonas* phylotype (OTU00038) in the total bacterial community (**Supplementary Figure 3**) one can conclude that some *Pseudomonas* bacteria were already present in the soil before the treatment, and the inoculation of *Pseudomonas sivasensis* 2RO45 did not necessarily cause the substantial proliferation of this bacteria in the soil. Moreover, colonization of the inoculated *Pseudomonas sivasensis* 2RO45 in the rhizosphere in sterile conditions, measured as CFU was determined. The results showed that the strain was able to survive up to 44 days (**Supplementary Table 3**). The viability of the strain ranged from 6.6×10^6 CFU/ml at T0 to 5.5×10^6 CFU/ml at T44. Nevertheless, according to Chen et al. (2022), neither the colonization of roots by PGPR inoculants, nor modifications in the rhizosphere microbiome were necessary for the plant growth promotion process. The authors demonstrated that PGPR-induced DNA methylation modifications in roots mediated the long-term impact on plant growth promotion (Chen et al., 2022).

Pseudomonas sivasensis 2RO45 treatment did not alter the overall microbial diversity, but changed the proportions of some bacterial (**Figure 3**) and fungal (**Figure 4**) taxa. The introduced strain modified microbial communities, particularly

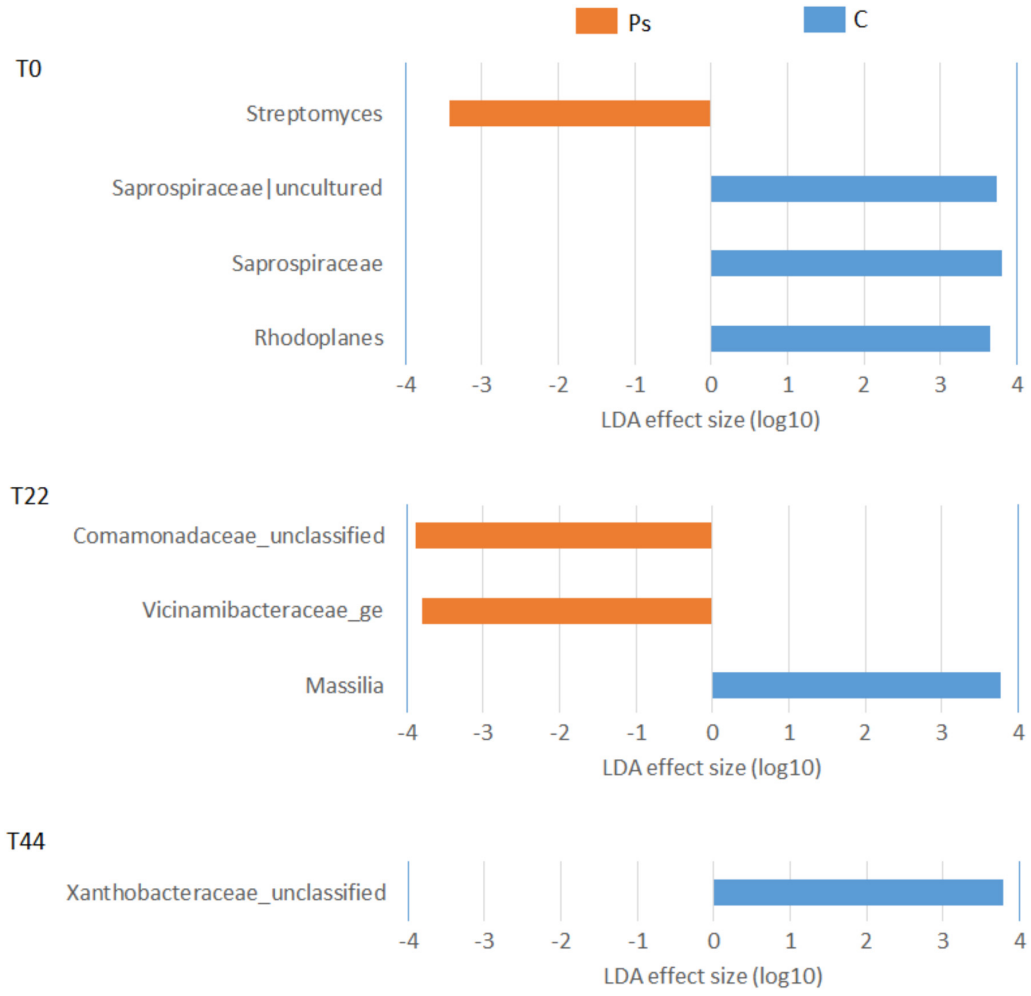

FIGURE 3
Linear discriminant analysis effect size (LEfSe) analysis showing bacterial taxonomy changes between canola plants inoculated with *Pseudomonas sivasensis* 2RO45 (PS) and uninoculated (C); T0, T22, and T44–time after inoculation in days.

increasing the abundance of microorganisms beneficial for a plant growth. Initially at T0, *P. sivasensis,* 2RO45 increased the abundance of genus *Streptomyces,* then at T22 bacterial families *Comamonadaceae* and *Vicinamibacteraceae* were more abundant in the *P. sivasensis* 2RO45 treated soil. Whereas, at T44 *P. sivasensis* 2RO45 strain did not cause any changes in the bacterial community structure. Numerous studies reported that *Streptomyces* has beneficial associations with plants, improving their growth and protecting them against bacterial and fungal diseases through the production of antibiotics and bioactive compounds (Amaresan et al., 2018; Suárez-Moreno et al., 2019; Vergnes et al., 2020; Le et al., 2021). Bacteria belonging to the *Vicinamibacteraceae* family are degraders of organic matter and chitin (Whitton et al., 2022), while members of the family *Comamonadaceae* are able to control *Fusarium* wilt disease by secreting more organic acids (Wen et al., 2020). The changes in taxonomic structure until 22 days after *P. sivasensis* 2RO45 bacterization indicated a transient and short-term perturbation in the taxonomic structure of bacterial communities. The results also indicate that the taxonomic structure of bacterial communities in young canola plants was more dynamically influenced by *P. sivasensis* 2RO45 bacterization compared to that in older plants. Similar results were obtained by Bhattacharyya and Lee (2016).

After *P. sivasensis* 2RO45 inoculation into the soil, the highest changes in fungal communities in comparison to the control were observed at T22. Initially at T0, *Cyphellophora vermispora* was more abundant in *P. sivasensis* 2RO45 treated soil, then at T22 *P. sivasensis* 2RO45 increased the abundance of the fungal family of *Nectriaceae*, genus *Exophiala* and species *Mortierella minutissima*. Whereas, at T44 *P. sivasensis* 2RO45 increased the abundance of the *Didymellaceae* family. Species of *Didymellaceae* were found as plant fungal pathogens causing fruit, leaf, stem, and root lesions on a wide variety of crops (Hou et al., 2020). However, *Didymellaceae* is the largest family within the order *Pleosporales* with more than 5 400 taxon names, including also saprobic, endophytic, and clinically relevant species (Hou et al., 2020; Yuan et al., 2021). *Exophiala* has been effectively used in a agricultural biotechnology. For instance, *Exophiala pisciphila* stimulated maize growth (Xu et al., 2020) and suppressed Fusarium-wilt disease in strawberries (Harsonowati et al., 2020), while *Exophiala* sp. promoted cucumber growth under abiotic stresses (Khan et al., 2011). *Cyphellophora vermispora* was isolated from a natural environment such as plant stems, roots,

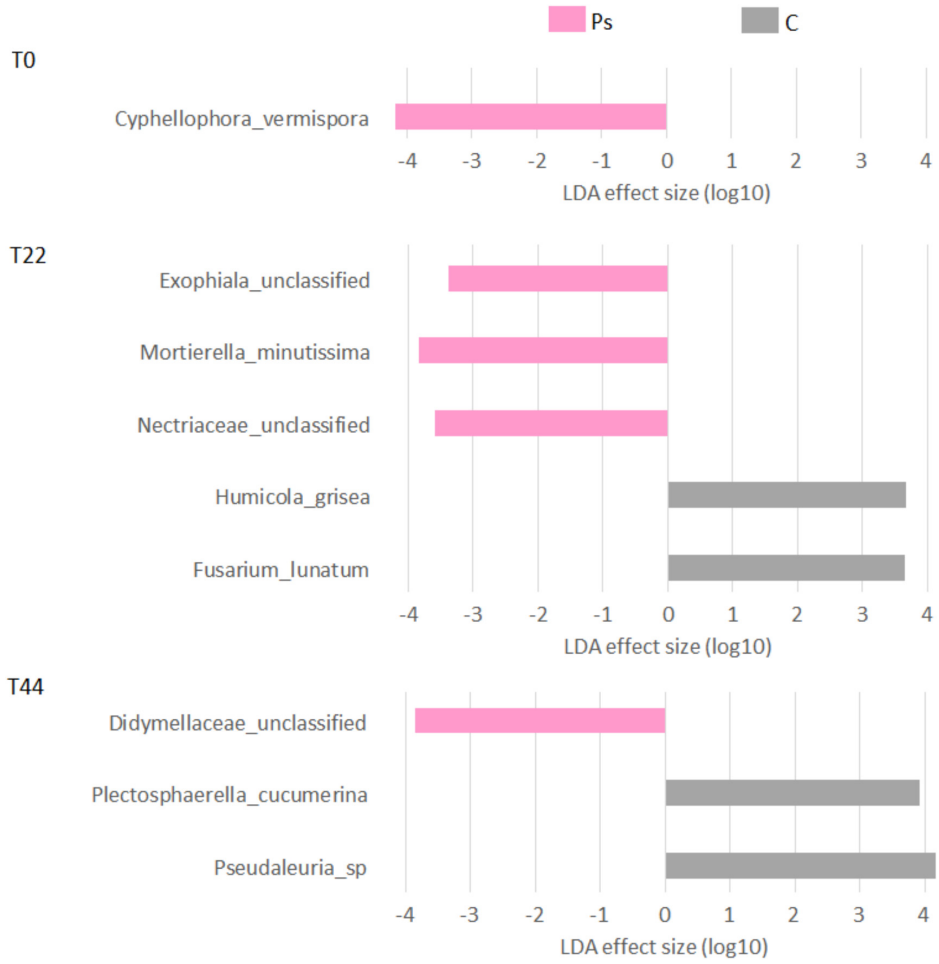

FIGURE 4
Linear discriminant analysis effect size (LEfSe) analysis showing fungal taxonomy changes between canola plants inoculated with *Pseudomonas sivasensis* 2RO45 (PS) and uninoculated (C); T0, T22, and T44–time after inoculation in days.

and leaves (Gao et al., 2015). Members of the *Nectriaceae* family have been reported as plant opportunistic pathogens; however, several species belonging to the *Nectriaceae* have been also used as biocontrol agents and biodegraders for developing sustainable agriculture (Lombard et al., 2015). The genus *Mortierella* includes numerous PGP fungi degrading biopolymers, e.g., *M. minutissima* isolated from the root surface had a strong chitinolytic activity (Ozimek and Hanaka, 2020).

The community-level physiological profiling (CLPP) using Biolog EcoPlates was estimated to analyze the changes in the canola rhizosphere metabolic profile and functional diversity in response to *P. sivasensis* 2RO45 introduction. The alterations in the metabolic profile of microbial communities from non-treated and *P. sivasensis* 2RO45 treated rhizospheres were analyzed on the 44th day. This time point was chosen to reveal if *P. sivasensis* 2RO45 inoculation has a long-term impact on the functional diversity of the canola rhizosphere microbiome.

The metabolic functional diversity indices, except substrate utilization Simpson index (D), had significant differences ($p < 0.05$) between non-treated and *P. sivasensis* 2RO45 treated canola rhizospheres (**Table 2**). Substrate utilization Shannon diversity (H) index and evenness (E) were the highest in canola plants

TABLE 2 Functional diversity indices based on Biolog EcoPlates results on the 7th day of incubation for the canola rhizosphere microbial communities.

Sample	Shannon diversity index (H)	Shannon evenness index (E)	Simpson diversity index (D)
C	3.01 ± 0.05*	0.89 ± 0.02*	0.92 ± 0.01
Ps	3.12 ± 0.03*	0.91 ± 0.01*	0.92 ± 0.01

Ps–canola plants inoculated with *Pseudomonas sivasensis* 2RO45, C–control, uninoculated canola plants; significant differences (*$p < 0.05$) according to *t*-test.

inoculated with *P. sivasensis* 2RO45, indicating that the functional diversity of the canola rhizosphere microbial community was altered by bacterization. The changes in the metabolic profiles of soil microbial communities after PGPR introduction is in line with previous studies on plants, such as wheat (Di Salvo et al., 2018b), maize (Di Salvo et al., 2018a), tomato (Zuluaga et al., 2021), and rice (de Salamone et al., 2010).

The average well color development (AWCD) curve of all carbon sources was plotted to check the metabolic activity of microbial communities (**Figure 5**). The microbial communities of the *P. sivasensis* 2RO45 treated canola rhizospheres samples

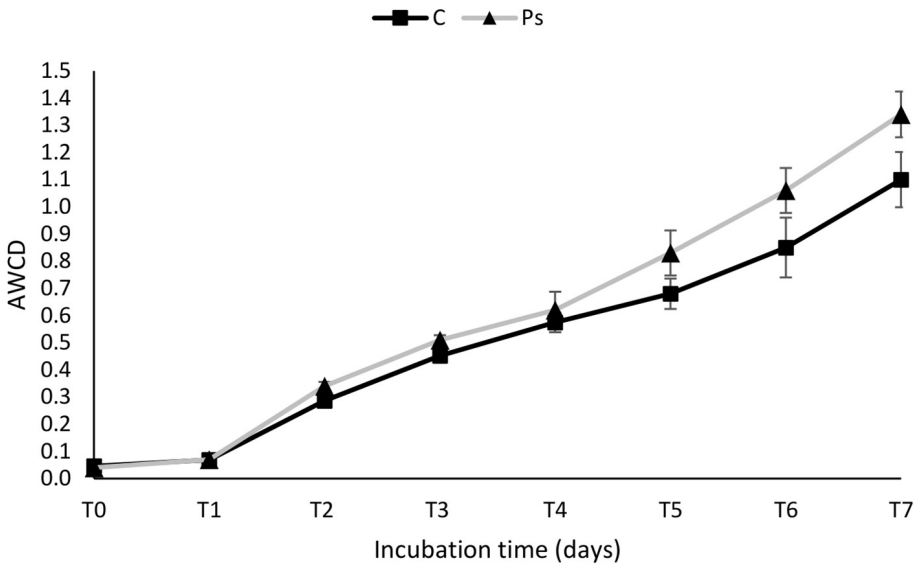

FIGURE 5
The average well color development (AWCD) of all carbon sources in *Pseudomonas sivasensis* 2RO45 treated and non-treated rhizosphere samples according to incubation time; vertical bars represent standard deviation (n = 3).

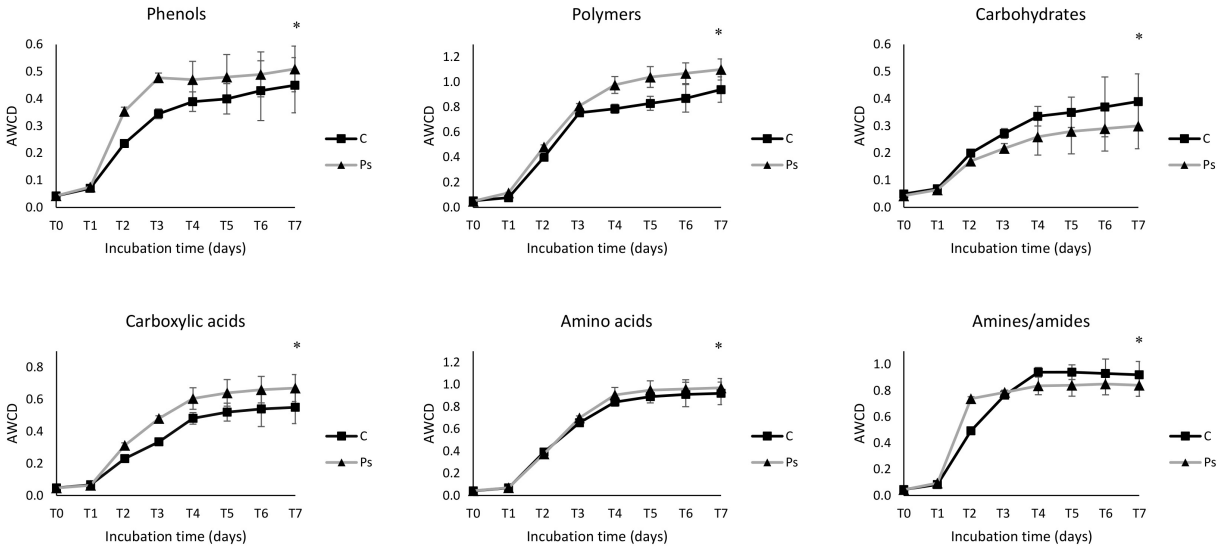

FIGURE 6
The average well color development (AWCD) of six carbon source groups, including phenols, polymers, carbohydrates, carboxylic acids, amino acids, and amines/amides in *Pseudomonas sivasensis* 2RO45 treated and non-treated rhizosphere samples; vertical bars represent standard deviation (n = 3); significant differences (*$p < 0.05$).

exhibited higher AWCD values than the control samples, suggesting that microbiota associated with *P. sivasensis* 2RO45 were more active in their use of different types of carbon substrates during cell growth than the indigenous microbiota and the overall metabolic activity was increased by bacterization. Furthermore, the microbial activity in the canola rhizospheres was calculated by AWCD for six categories of carbon sources: phenols, carbohydrates, amino acids, carboxylic acids, polymers, and amines (**Figure 6**). The microbial communities in the *P. sivasensis* 2RO45 treated canola rhizospheres samples were found to utilize phenols, polymers, carboxylic acids, and amino acids more intensively than those in the non-treated samples. Whereas, carbohydrates and amines were better metabolized by the rhizosphere community from non-treated canola plants. The results are similar to the findings reported by previous research, where the metabolic profile of microbial communities was modified due to PGPR bacterization (Bhattacharyya and Lee, 2016; Zuluaga et al., 2021). According to Bhattacharyya and Lee (2016), an increase in the utilization of amino acids and a reduction in the utilization of carbohydrates in the bacterized rhizosphere could have a beneficial effect on Kimchi cabbage growth. Whereas,

Zuluaga et al. (2021) found that carbohydrates, amines, polymers, and phenolic compounds were the main carbon substrate groups that contribute to the rhizosphere microbiome function between rhizosphere plants inoculated with PGPR *Pseudomonas* sp. and non-inoculated plants. The authors suggested that rhizobacterial inoculants can modulate the rhizosphere microbiome function by affecting the root exudation profile, consequently interfering in the plant-soil feedback and the shaping of the plant-associated microbial communities (Zuluaga et al., 2021).

4. Conclusion

Plant growth-promoting rhizobacteria *Pseudomonas sivasensis* 2RO45 inoculation altered the taxonomic structure of canola rhizosphere microbial communities by increasing the abundance of plant beneficial microorganisms: bacteria affiliated with families *Comamonadaceae*, *Vicinamibacteraceae,* genus *Streptomyces,* and fungi assigned to the *Nectriaceae, Didymellaceae, Exophiala, Cyphellophora vermispora*, and *Mortierella minutissima*. Moreover, *P. sivasensis* 2RO45 induced perturbations in the rhizosphere microbiome by increasing metabolic activity and functional diversity of microbial communities. Phenols, polymers, carboxylic acids, and amino acids were the major classes of carbon substrates that contributed to the function of the rhizosphere microbiome after inoculation with *P. sivasensis* 2RO45. The results provide new insight and future perspectives into PGPR-canola interactions for sustainable agriculture development. The introduction of *P. sivasensis* 2RO45 is beneficial to development of a sustainable agriculture because it changes the native microbiota crucial for the proper functioning of the soil. The minor changes that are observed are positive, especially the increase of metabolic activity which may increase the possibility of detoxification of the environment (decomposition of phenols and polymers) or the elements circulation (e.g., carbon and nitrogen).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary material**.

Author contributions

JŚ wrote the original draft, made greenhouse experiment and sampling, made isolation of DNA, made community-level physiological profiles (CLPP) analysis using Biolog EcoPlates, conducted statistical analyses, and prepared the manuscript editorially. AK made rarefaction curves, LEfSe PCoA, and ANOSIM analysis, coordinated the study, and checked the validity of the original draft. AS carried out the sequence analysis. MS designed and coordinated the study and checked the validity of the original draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1168907/full#supplementary-material

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Supplementary Materials

Table S1 OTU richness and diversity indices (Shannon H' and Inv Simpson) for the bacterial community in *Pseudomonas sivasensis* 2RO45 treated canola rhizospheres and untreated rhizospheres samples according to time based on NGS sequencing.

Sample	Observed OTU richness	Mean/ Median	*P*-value	Shannon H'	Mean	*P*-value	Inv Simpson	Mean/ Median	*P*-value
CT0_1	3807.99	3834.69[nnd]		6.62	6.66		202.69	235.76	
CT0_2	3861.39			6.66			233.10		
CT0_3	3745.34			6.63			214.20		
CT0_4	4009.30			6.75			293.04		
CT22_1	3884.38	3907.03[nnd]		6.63	6.70		217.32	257.16	
CT22_2	4075.07		0.02	6.79		0.00	316.38		0.00
CT22_3	3929.69			6.72			272.67		
CT22_4	3743.00			6.64			222.25		
CT44_1	5082.35	4682.86[nnd]		7.22	7.11		556.83	499.29	
CT44_2	4866.79			7.18			555.09		
CT44_3	4498.94			7.09			480.03		
CT44_4	4127.62			6.95			405.20		
PsT0_1	3750.54	3813.61		6.60	6.65		241.66	257.21[nnd]	
PsT0_2	4033.27			6.78			299.99		
PsT0_3	4012.88			6.75			272.77		
PsT0_4	3457.76			6.47			188.80		
PsT22_1	4261.21	4067.82		6.94	6.81		406.15	321.70[nnd]	
PsT22_2	4057.99			6.75			250.91		
PsT22_3	3836.12		0.00	6.64		0.00	204.59		0.02
PsT22_4	4115.96			6.91			392.48		
PsT44_1	4490.12	4468.30		7.13	7.09		530.12	485.90[nnd]	
PsT44_2	4479.74			7.14			541.79		
PsT44_3	4399.89			7.05			441.68		
PsT44_4	4503.44			7.03			436.80		

nnd – not normal distribution (test for equal medians: Kruskal-Wallis); ns – non significant; variants marked with different letters indicate groups significantly different from each other, $p<0.05$ (Tukey's test as post hoc); C – control, untreated plants; Ps – plants treated with *Pseudomonas sivasensis* 2RO45; T0, T22 and T44 - time after bacterization in days.

Table S2 OTU richness and diversity indices (Shannon H' and Inv Simpson) for the fungal community in *Pseudomonas sivasensis* 2RO45 treated canola rhizospheres and untreated rhizospheres samples according to time based on NGS sequencing.

Sample	Observed OTU richness	Mean/ Median	*P*-value	Shannon H'	Mean	*P*-value	Inv Simpson	Mean/ Median	*P*-value
CT0_1	219.58	231.89[nnd]		3.48	3.55		12.30	13.34	
CT0_2	251.54			3.72			15.76		
CT0_3	243.26			3.70			16.20		
CT0_4	220.52			3.31			9.12		
CT22_1	233.55	230.59[nnd]		3.57	3.55		14.89	14.76	
CT22_2	227.63		0.02	3.78		0.02	21.24		ns
CT22_3	227.61			3.38			11.49		
CT22_4	237.23			3.48			11.43		
CT44_1	268.94	269.29[nnd]		3.89	3.89		20.81	20.22	
CT44_2	276.08			4.02			23.66		
CT44_3	267.78			3.77			17.09		
CT44_4	269.65			3.87			19.30		
PsT0_1	254.01	251.90		3.80	3.80		17.64	19.06	
PsT0_2	271.84			4.11			26.80		
PsT0_3	243.99			3.64			15.94		
PsT0_4	237.78			3.66			15.88		
PsT22_1	260.56	234.17		3.81	3.55		17.66	13.54	
PsT22_2	230.43		ns	3.66		ns	15.49		ns
PsT22_3	226.00			3.40			11.29		
PsT22_4	219.70			3.34			9.74		
PsT44_1	286.28	270.00		4.07	3.96		24.92	22.10	
PsT44_2	287.76			4.14			26.73		
PsT44_3	255.85			3.87			19.98		
PsT44_4	250.10			3.77			16.78		

nnd – not normal distribution (test for equal medians: Kruskal-Wallis); ns – non significant; variants marked with different letters indicate groups significantly different from each other, $p<0.05$ (Tukey's test as post hoc); C – control, untreated plants; Ps – plants treated with *Pseudomonas sivasensis* 2RO45; T0, T22 and T44 - time after bacterization in days.

Table S3 *Pseudomonas sivasensis* 2RO45 load at different time.

Time	***Pseudomonas sivasensis* 2RO45 load (x 10^6 CFU/ml)**
T0	6.6
T22	5.7
T44	5.5

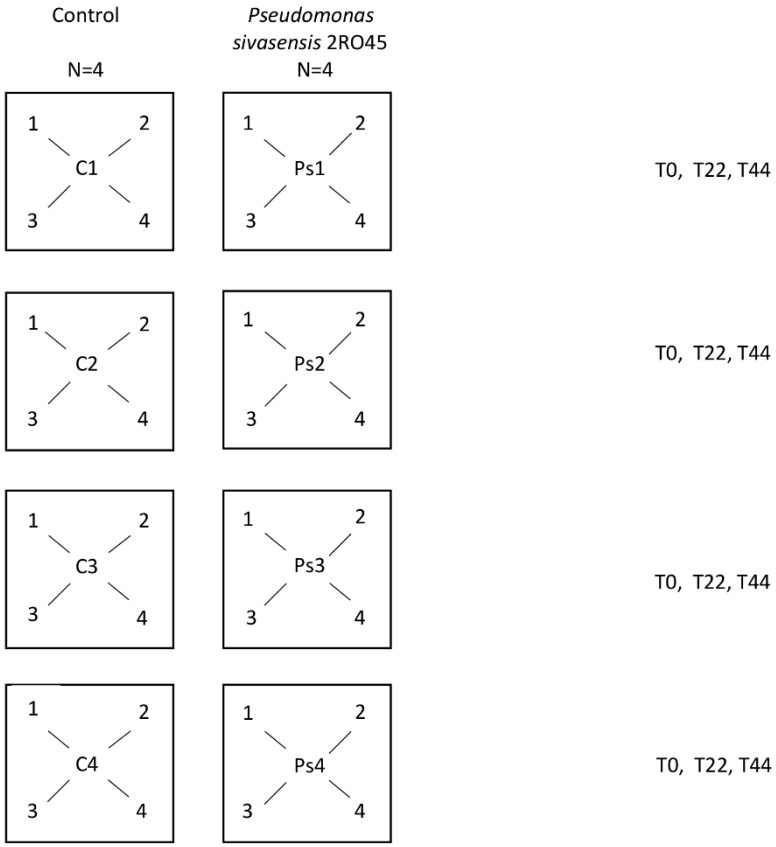
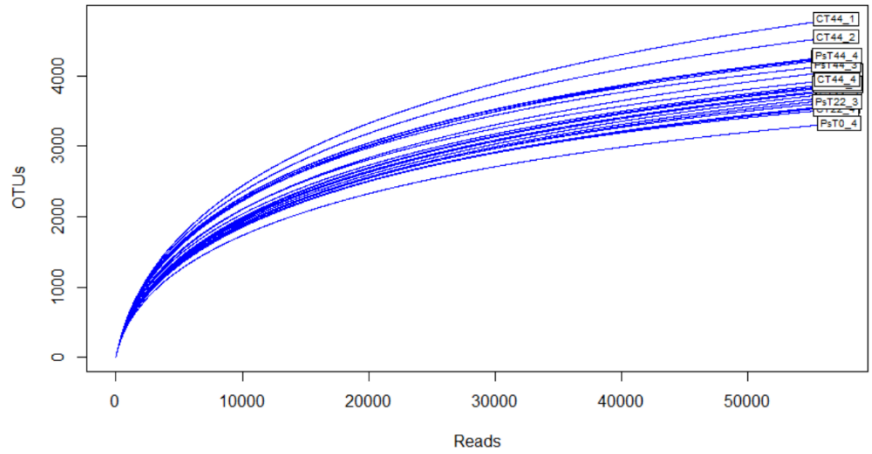


Figure S1 Canola rhizosphere sampling procedure. Pooled samples (N=4) were taken from the canola rhizosphere at the time of introducing the *Pseudomonas sivasensis* 2RO45 (T0), after 22 days (T22), and after 44 days (T44).

A)



B)

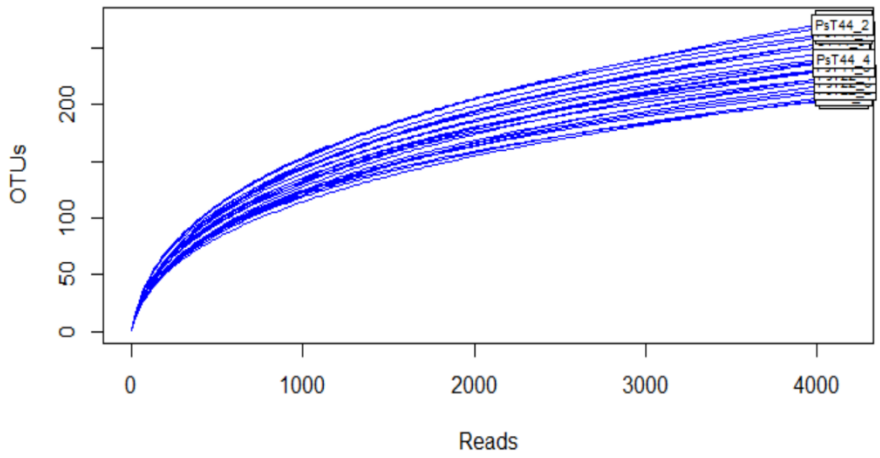


Figure S2 Rarefaction curves for the (A) bacterial (B) fungal soil 16S rRNA gene sequences.

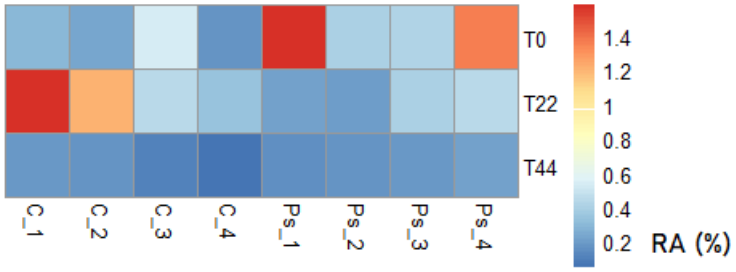


Figure S3 Relative abundance (%) of *Pseudomonas* phylotype (OTU00038) in total bacterial community.

IV Dyskusja i podsumowanie uzyskanych wyników

1. Oznaczenie liczebności hodowlanych ryzobakterii i charakterystyka ich właściwości promujących wzrost roślin

W niniejszej pracy doktorskiej, przeprowadzono analizę określenia liczebności ryzobakterii hodowlanych z różnych faz wzrostu rzepaku (wegetatywnej, kwitnienia i dojrzewania), uprawianego w Górsku w województwie kujawsko-pomorskim (publikacja nr 1) i w Ostródzie w województwie warmińsko-mazurskim (publikacja nr 3). Powyższe badania wykazały złożoną dynamikę zbiorowisk drobnoustrojów, ujawniając różnice w liczebności hodowlanych ryzobakterii na różnych etapach wzrostu rzepaku. W przypadku ryzosfery rzepaku pobranego z Górska, populacja hodowlanych ryzobakterii była najwyższa w fazie wegetatywnej, zmniejszyła się w fazie kwitnienia, a następnie zwiększyła się w fazie dojrzałości rośliny (publikacja nr 1). Natomiast w ryzosferze rzepaku pobranego z Ostródy, liczebność hodowlanych bakterii ryzosferowych była najwyższa w fazie kwitnienia, po czym zmniejszyła się kolejno w fazie dojrzałości i wegetatywnej rośliny (publikacja nr 3). Uzyskane w niniejszej pracy doktorskiej wyniki można wytłumaczyć zróżnicowanym wytwarzaniem wydzielin korzeniowych, co w konsekwencji prowadzi do zmiany struktury mikrobiomu ryzosfery. Korzenie roślin wydzielają do gleby specyficzne substancje organiczne, tzw. wydzieliny korzeniowe, które są cennym źródłem wzrostu drobnoustrojów. Proces ten sprzyja zatem zwiększeniu liczebności drobnoustrojów w pobliżu korzeni, a także ich aktywności (Zboralski i Filion, 2020). Doniesienia literaturowe podają, że ilość i jakość wydzielin korzeniowych zależy od gatunku i wieku rośliny, a także od warunków środowiskowych (Ma i in., 2022). W badaniach Marag i in. (2018) porównano populację hodowlanych bakterii endofitycznych z trzech różnych faz wzrostu kukurydzy i zaobserwowano największą liczebność bakterii w fazie kwitnienia rośliny. Według autorów, bakterie intensywniej rozmnażały się w fazie kwitnienia rośliny, po czym w fazie dojrzałości ich liczebność spadała z powodu niedoboru składników odżywczych i wody czy tworzenia się wolnych rodników.

W ramach niniejszej pracy doktorskiej, wyizolowano 300 izolatów bakteryjnych: 150 izolatów z ryzosfery korzeni rzepaku uprawianego w Górsku, w tym po 50 izolatów z fazy wegetatywnej, kwitnienia i dojrzewania (publikacja nr 1) oraz 150 izolatów z ryzosfery korzeni rzepaku uprawianego w Ostródzie, w tym po 50 izolatów również z fazy wegetatywnej, kwitnienia i dojrzewania (publikacja nr 3). Następnie, przeprowadzono charakterystykę ich

właściwości promujących wzrost roślin (PGP – ang. Plant Growth Promoting), w tym produkcję kwasu indolilo-3-octowego (IAA), fosforanów, deaminazy ACC, sideroforów, chitynaz, cyjanowodoru (HCN) i amoniaku. Badanie miało na celu sprawdzenie czy istnieje związek między liczebnością ryzobakterii izolowanych z różnych faz wzrostu rośliny, a ich właściwościami PGP. W ryzosferze rzepaku uprawianego w Górsku, największą populację hodowlanych ryzobakterii stwierdzono w fazie wegetatywnej rzepaku, a cztery z siedmiu badanych cech PGP, takich jak: produkcja deaminazy ACC, sideroforów, HCN i chitynaz, były wytwarzane przez najwyższy procent izolatów również w fazie wegetatywnej rośliny (publikacja nr 1). W przypadku ryzosfery rzepaku uprawianego w Ostródzie, najwyższą liczebność hodowlanych bakterii ryzosferowych odnotowano w próbach pobranych w fazie kwitnienia rzepaku i również z tej fazy wzrostu rośliny wyizolowano najwięcej szczepów zdolnych do produkcji IAA, ACC deaminazy, fosforanów, sideroforów oraz amoniaku (publikacja nr 3).

Według doniesień literaturowych, badanie właściwości PGP ryzobakterii pozwala na ich skuteczną selekcję w kontekście zwiększenia wzrostu i rozwoju roślin (Ahemad i Kribet, 2014). Właściwości te obejmują: produkcję auksyn i sideroforów, wiązanie azotu, antagonizm przeciwko fitopatogenon roślinnym, cyjanogenezę, solubilizację fosforanów i wytwarzanie ACC deaminazy (Ahemad i Kribet, 2014). Według Ansari i Ahmad (2019), inokulacja szczepem bakteryjnym posiadającym wiele cech PGP jest bardziej korzystna dla wzrostu roślin niż inokulacja szczepem posiadającym tylko jedną właściwość PGP. W niniejszej rozprawie doktorskiej, analiza właściwości PGP ryzobakterii umożliwiła wybór 12 izolatów do dalszych badań. Spośród wszystkich izolatów, 6 izolatów wyizolowanych z ryzosfery rzepaku uprawianego w Górsku: R27, R38, R44, 2R5, 3R27 i 3R43 (publikacja nr 1) oraz 6 izolatów wyizolowanych z ryzosfery rzepaku uprawianego w Ostródzie: RO33, RO37, 2RO24, 2RO30, 2RO45 i 3RO30 (publikacja nr 3) wykazywały wysokie aktywności co najmniej czterech z siedmiu badanych cech. W niniejszej pracy, wytypowane szczepy pochodzące z ryzosfery rzepaku uprawianego w Górsku wykazywały zdolność do produkcji IAA, ACC deaminazy i sideroforów. Izolaty R27, R44, 3R27 i 3R43 były zdolne do solubilizacji fosforanów. Dodatkowo, izolaty R27 i R38 syntetyzowały cyjanowodór, a 2R5 chitynazy (publikacja nr 1). Natomiast, wytypowane szczepy pochodzące z ryzosfery rzepaku w Ostródzie były zdolne do produkcji IAA, solubilizacji fosforanów i sekwestracji sideroforów. Izolaty RO33, RO37, RO45, 2RO30 i 2RO45 wykazywały aktywność ACC deaminazy. Izolaty RO33, RO45 i

3RO30 były zdolne do produkcji chitynaz, a RO33 i 3RO30 wytwarzały cyjanowodór i amoniak (publikacja nr 3).

2. Identyfikacja taksonomiczna ryzobakterii i doświadczenia wazonowe

Ryzobakterie wykazujące najlepsze właściwości PGP *in vitro*, zidentyfikowano w oparciu o sekwencję nukleotydową genu 16S rybosomalnego RNA (rRNA) i wybrano do doświadczenia doniczkowego, w celu zbadania ich zdolności promujących wzrost rzepaku *in vivo* w warunkach sterylnych (publikacja nr 1 i nr 3). Szczepy wyizolowane z ryzosfery rzepaku uprawianego w Górsku należały do rodzaju *Pseudomonas* i *Bacillus* (*Pseudomonas* sp. R27, *Pseudomonas atacamensis* R38, *Pseudomonas* sp. R44, *Bacillus paralicheniformis* 2R5, *Pseudomonas piscium* 3R27, *Pseudomonas brenneri* 3R43) (publikacja nr 1), natomiast szczepy wyizolowane z ryzosfery rzepaku uprawianego w Ostródzie należały do rodzaju *Pseudomonas* i *Peribacillus* (*Pseudomonas* sp. RO33, *Pseudomonas poae* RO37, *Pseudomonas kairouanensis* RO45, *Peribacillus frigoritolerans* 2RO30, *Pseudomonas sivasensis* 2RO45, *Pseudomonas migulae* 3RO30) (publikacja nr 3). Bakterie z rodzaju *Bacillus* i *Pseudomonas* stanowią najliczniejszą grupę PGPR. Izolaty z tych grup taksonomicznych wykazują szeroki zakres korzystnych właściwości dla roślin, takich jak wydajna kolonizacja roślin, promowanie wzrostu roślin, biologiczna kontrola fitopatogenów i indukcja tolerancji roślin na stres abiotyczny, poprzez mechanizmy obejmujące produkcję fitohormonów, związków antybiotykowych i zwiększenie biodostępności składników odżywczych (Hol i in., 2013; Aloo i in., 2019) .

Badania wazonowe w warunkach sterylnych wykazały, że tylko trzy szczepy promowały wzrost rzepaku: *Bacillus paralicheniformis* 2R5 wyizolowany z ryzosfery rzepaku w Górsku (publikacja nr 1) oraz *Peribacillus frigoritolerans* 2RO30 i *Pseudomonas sivasensis* 2RO45 pochodzące z ryzosfery rzepaku w Ostródzie (publikacja nr 3). Inokulacja szczepem *B. paralicheniformis* 2R5 istotnie zwiększyła zawartość chlorofilu (CCI – ang. Chlorophyll Content Index), masę liści i pędów oraz wskaźnik wagowy liści (LWR – ang. Leaf Weight Ratio) w porównaniu z kontrolą (publikacja nr 1). Natomiast, inokulacja *P. frigoritolerans* 2RO30 i *P. sivasensis* 2RO45 istotnie zwiększyła CCI w roślinie, długość korzeni i pędów oraz masę epikotylu i pędów w porównaniu z kontrolą. Ponadto, inokulacja *P. sivasensis* 2RO45 istotnie zwiększyła masę liści w porównaniu z kontrolą (publikacja nr 3).

Wpływ PGPR na rozwój roślin jest bardzo zmienny w naturalnych warunkach, głównie ze względu na obecność rodzimych zbiorowisk drobnoustrojów ryzosfery (Pacheco da Silva i in., 2022). Z tego powodu, jeśli PGPR efektywnie promują wzrost roślin w warunkach sterylnych, to ich wpływ na rozwój roślin powinien być dodatkowo weryfikowany w warunkach niesterylnych, w glebie zawierającej rodzime mikroorganizmy (Péreza-Montaño i in., 2014). W niniejszej rozprawie doktorskiej, aby ocenić czy wytypowane izolaty mogą promować wzrost rzepaku w warunkach naturalnych, przeprowadzono dodatkowy eksperyment szklarniowy w glebie niesterylnej pobranej z Górska (publikacja nr 1) oraz w glebie niesterylnej pobranej z Ostródy (publikacja nr 3). Dodatkowo, w niniejszej pracy oznaczono parametry fizykochemiczne gleb. Badane gleby wykazały podobne właściwości fizykochemiczne. W przypadku próbek gleby pobranej z Górska, analiza fizykochemiczna wykazała zawartość potasu na poziomie 245 mg/kg, fosforu 275 mg/kg, oraz magnezu 118 mg/kg. Stężenie azotu amonowego i azotanowego w glebie wynosiło odpowiednio 6,56 mg/kg i 35,31 mg/kg, natomiast zawartość węgla organicznego osiągnęła poziom 1,42% (publikacja nr 1). Z kolei, analiza fizykochemiczna gleby pobranej z Ostródy wykazała zawartość potasu na poziomie 350 mg/kg, fosforu 310 mg/kg, oraz magnezu 91 mg/kg. Zawartość azotu amonowego, azotu azotanowego oraz węgla organicznego wynosiła odpowiednio 6,57 mg/kg, 79,13 mg/kg i 1,65% (publikacja nr 3). Próbki gleb miały lekko kwaśny odczyn, wykazując pH 6,4 w glebie pobranej z Górska (publikacja nr 1) i pH 6,5 w glebie pobranej z Ostródy (publikacja nr 3).

Inokulacja pojedynczym szczepem bakteryjnym może przyczyniać się do mniejszej wydajności bionawozów rolniczych, dlatego stosuje się konsorcjum drobnoustrojów (Khan i in., 2022). Przygotowanie konsorcjum składającego się z kompatybilnych szczepów bakteryjnych pozwala na synergistyczną interakcję między nimi, redukując ilość inhibitorów wzrostu i zapewniając lepsze odżywianie roślin. Taka synergia może przyczynić się do poprawy wzrostu i rozwoju roślin w różnorodnych warunkach środowiskowych (Wani i in., 2007; Thomloudi i in., 2019). W niniejszej rozprawie doktorskiej, badania wazonowe w glebie niesterylnej prowadzono wykorzystując pojedyncze szczepy *B. paralicheniformis* 2R5 (publikacja nr 1), *P. frigoritolerans* 2RO30, *P. sivasensis* 2RO45, ale także konsorcjum składające się z dwóch szczepów: *P. frigoritolerans* 2RO30 i *P. sivasensis* 2RO45, przy czym wcześniej sprawdzono czy istnieje między nimi antagonizm (publikacja nr 3).

Badania wazonowe w niesterylnej glebie wykazały, że tylko pojedyncze szczepy *B. paralicheniformis* 2R5 i *P. sivasensis* 2RO45 promowały wzrost rzepaku. Inokulacja szczepem

B. paralicheniformis 2R5 istotnie zwiększyła CCI w roślinie, długość korzeni oraz masę korzeni i liści w porównaniu z kontrolą (publikacja nr 1). Natomiast, inokulacja *P. sivasensis* 2RO45 zwiększyła CCI i długość pędów w porównaniu z kontrolą (publikacja nr 3). Badania przedstawione w niniejszej rozprawie doktorskiej po raz pierwszy opisują gatunek *Bacillus paralicheniformis* (publikacja nr 1) i *Pseudomonas sivasensis* (publikacja nr 3), jako ryzobakterie promujące wzrost rzepaku. Inni autorzy, wykazali, że *Bacillus paralicheniformis* może stymulować wzrost rzodkiewnika pospolitego (*Arabidopsis thaliana* L.) (Gagat i Mackiewicz, 2017) lub pszenicy (*Triticum turgidum* subsp. *durum*) (Chaparro-Encinas i in., 2022). **Badania przedstawione w niniejszej rozprawie doktorskiej potwierdzają również hipotezę 1, w której założono, że ryzosfera korzeni rzepaku uprawianego w Górsku i w Ostródzie stanowi źródło gatunków bakterii promujących wzrost rzepaku**.

Ponadto, powyższe badania szklarniowe wykazały, że inokulacja konsorcjum *P. frigoritolerans* 2RO30 i *P. sivasensis* 2RO45 nie wpłynęła na wzrost roślin rzepaku (publikacja nr 3). W literaturze naukowej istnieją różne stanowiska na temat efektywności inokulacji konsorcjów mikroorganizmów w porównaniu z pojedynczymi mikroorganizmami w kontekście promowania rozwoju roślin. Z jednej strony, inokulacja konsorcjum bakterii promujących wzrost roślin ma korzystniejszy wpływ na rozwój roślin (Kumar i in., 2017; Olanrewaju i Babalola, 2019), z drugiej zaś strony nie ma pewności, że konsorcja drobnoustrojów będą lepszym rozwiązaniem niż pojedyncze inokulanty (Zhao i in., 2018; Duncker i in., 2021). Według Woo i Pepe (2018) interakcje metaboliczne odgrywają znaczącą rolę w efektywności zastosowania konsorcjum ryzobakteryjnego. Wydzielane przez poszczególne gatunki związki metaboliczne mogą stymulować lub hamować wzrost innych gatunków, wprowadzając zmiany w ich wzajemnych relacjach, jak również oddziaływać na funkcjonowanie całej społeczności mikrobiologicznej (Berg i in., 2020).

Należy również wziąć pod uwagę, że w powyższych badaniach nie zaobserwowano efektu promowania wzrostu rzepaku przez szczep *P. frigoritolerans* 2RO30 w niesterylnej glebie. Przeprowadzone doświadczenie dowodzi o istotności wykonania eksperymentów w glebie sterylnej i niesterylnej by w późniejszym etapie móc zastosować izolaty jako szczepionki w środowisku naturalnym. Według Bhattacharyyi i in. (2018) niektóre rodzime drobnoustroje mogą negatywnie wpływać na funkcję i przeżycie inokulowanego PGPR. Uzyskane w niniejszej rozprawie doktorskiej wyniki wskazują, iż *P. frigoritolerans* 2RO30 nie był w stanie konkurować z naturalną mikroflorą glebową, stąd też nie promował wzrostu rzepaku w niesterylnej glebie. Prawdopodobnie został wyeliminowany na skutek wydzielanych do

środowiska metabolitów hamujących wzrost, np. antybiotyków produkowanych przez promieniowce. W związku z powyższym, szczep *P. frigoritolerans* 2RO30 nie został opisany w dalszych badaniach niniejszej rozprawy doktorskiej.

3. Biokontrola przeciwko patogenom roślin

Ryzobakterie mają zdolność do produkcji antybiotyków jako metabolitów wtórnych. Ta zdolność odgrywa kluczową rolę w hamowaniu wzrostu i rozwoju fitopatogenów, poprzez zakłócanie integralności ich ścian i błon komórkowych, a także ingerencję w ich procesy metaboliczne. Ponadto, mikroorganizmy te są w stanie efektywnie rywalizować z fitopatogenami o dostęp do składników odżywczych w ryzosferze, co dodatkowo ogranicza możliwość rozwoju patogenów. Istotnym aspektem działania niektórych bakterii ryzosferowych jest również indukowanie systemowej odporności w roślinach, czyniąc je bardziej odpornymi na działanie patogenów. Ponadto, produkowane przez ryzobakterie enzymy lityczne, takie jak chitynazy i glukanazy, hamują wzrost fitopatogenów poprzez degradację ich ścian komórkowych (de Andrade i in., 2023).

Na podstawie badań wazonowych, dwa szczepy promujące wzrost rzepaku w niesterylnej glebie: *Bacillus paralicheniformis* 2R5 (publikacja nr 1) oraz *Pseudomonas sivasensis* 2RO45 (publikacja nr 3) przebadano pod względem ich aktywności antagonistycznej wobec siedmiu patogenów roślin: *Alternaria alternata* 783, *Botrytis cinerea* 873, *Fusarium culmorum* 2333, *Fusarium oxysporum* 872, *Fusarium solani* 25, *Phytophthora cactorum* 1925 i *Phytophthora megasperma* 404. Według doniesień literaturowych, rzepak jest atakowany przez różne gatunki grzybów, powodujące zgniliznę korzeni, plamistość i więdnięcie liści (Krasnow i Hausbeck, 2015; Monnier i in., 2018; Al-Lami i in., 2019; Ismaiel i in., 2021; Yu i in., 2023). W niniejszej rozprawie doktorskiej, *B. paralicheniformis* 2R5 wykazywał zdolność hamowania wzrostu sześciu badanych fitopatogenów, w tym pięciu patogenów: *A. alternata*, *F. culmorum*, *F. oxysporum*, *F. solani* oraz *P. cactorum* w stopniu wyższym niż 15% (publikacja nr 1). Natomiast *P. sivasensis* 2R045 hamował wzrost grzybni siedmiu patogenów roślinnych, w tym czterech patogenów: *A. alternata, B. cinerea, F. oxysporum* i *P. cactorum* w stopniu wyższym niż 15% (publikacja nr 3).

4. Analiza genomów ryzobakterii promujących wzrost rzepaku

W ramach niniejszej rozprawy doktorskiej szczepy promujące wzrost rzepaku, *Bacillus paralicheniformis* 2R5 (publikacja nr 1) oraz *Pseudomonas sivasensis* 2RO45 (publikacja nr 3), poddano szczegółowej analizie polegającej na sekwencjonowaniu ich genomów. Analizę tę wykonano w celu znalezienia genów odpowiedzialnych za ich zdolność do promowania wzrostu rzepaku w glebie niesterylnej oraz genów zaangażowanych w biologiczną kontrolę patogenów roślin.

4.1. Poszukiwanie genów odpowiedzialnych za promowanie wzrostu roślin

Analiza sekwencjonowania genomu ryzobakterii umożliwia identyfikację genów sprzyjających wzrostowi roślin. Metoda ta pozwala na zrozumienie molekularnych i funkcjonalnych mechanizmów, dzięki którym ryzobakterie wpływają na wzrost roślin, w tym m. in. na ich zdolność lepszego przyswajania składników odżywczych, zwiększenia odporności na stresy środowiskowe, a także poprawę struktury gleby (Huang i in., 2022). Według Bloemberga i Lugtenberga (2001) poznanie mechanizmów działania PGPR może przyczynić się do opracowania nowych, bardziej skutecznych i ekologicznych metod stymulowania wzrostu roślin w rolnictwie.

Analiza genomu *B. paralicheniformis* 2R5 wykazała obecność genów odpowiedzialnych za produkcję IAA (*trpA,B,C,D,E,F*) oraz solubilizację fosforanów (*pstA,S*) (publikacja nr 1). Natomiast w genomie *P. sivasensis* 2RO45 zidentyfikowano geny odpowiedzialne za produkcję IAA (*trpA,B,C,D,E,F,G*), solubilizację fosforanów (*pstS*) oraz produkcję ACC deaminazy (*dcyD* i *acdA*) (publikacja nr 3). Geny *pst* są odpowiedzialne za akumulację fosforanów, gdy komórki znajdują się w środowiskach o ograniczonej zawartości fosforanów (Hudek i in., 2016). Geny *trp* są zaangażowanie w biosyntezę tryptofanu, który jest głównym prekursorem IAA (Idris i in., 2007; López-Gómez i in., 2022). Z kolei, geny *acdA*, które kodują enzym deaminazę ACC, mogą pełnić funkcję prekursora w procesie obniżania poziomu etylenu w roślinach (Adeleke i in., 2021).

4.2. Poszukiwanie genów i biosyntetycznych klastrów genów odpowiedzialnych za biokontrolę patogenów roślin

Obecność biosyntetycznych klastrów genów (BGC – ang. Biosynthetic Gene Clusters) w genomach ryzobakterii może odgrywać znaczącą rolę w ich aktywności przeciwgrzybowej. Klastry te kodują szlaki metaboliczne niezbędne do wytwarzania różnorodnych związków bioaktywnych, w tym antybiotyków oraz innych metabolitów wtórnych zdolnych do inhibicji rozwoju patogenów grzybowych. W konsekwencji, ryzobakterie zdolne są do skutecznej rywalizacji z grzybami o dostęp do składników odżywczych w ryzosferze. Analiza i badanie biosyntetycznych klastrów genowych może dostarczyć cennych informacji na temat potencjalnych mechanizmów działania tych drobnoustrojów (Winter, 2021).

Analiza genomu *B. paralicheniformis* 2R5 wykazała obecność wielu klastrów genów odpowiedzialnych za syntezę biologicznie czynnych metabolitów wtórnych, wśród których dominowały klastry genów kodujące syntetazy nierybosomalnych peptydów (bacylibaktyna, fengycyna), tiopeptydy (butirozyna A, butirozyna B) i lantipeptydy (haloduracyna β, haloduracyna α) (publikacja nr 1). W genomie *B. paralicheniformis* 2R5 stwierdzono również występowanie klastrów genowych, których sekwencje są identyczne z sekwencjami kodującymi antybiotyk bacytracynę oraz lichenizynę (publikacja nr 1), wykazującą działanie przeciwnowotworowe i przeciwdrobnoustrojowe (Gudiña i Teixeira, 2022). Uzyskane wyniki są zgodne z badaniami Du i in. (2019). Według autorów, *B. paralicheniformis* posiada klastry genów związanych z metabolitami wtórnymi, takimi jak lichenizyna, fengycyna, bacytracyna i bacylibaktyna. Ponadto, w niniejszej rozprawie doktorskiej wykryto geny zaangażowane w sekwestrację sideroforów (*mbtH*) i produkcję chitynaz (*chiA*) w genomie *B. paralicheniformis* 2R5 (publikacja nr 1). Według Chen i in. (2004) *Bacillus subtilis* F29-3, wykazujący ekspresję genu *chiA*, posiadał zwiększoną aktywność przeciwgrzybową w porównaniu ze szczepem kontrolnym *Bacillus subtilis*. Podczas gdy, białko *mbtH* bierze udział w biosyntezie sideroforu mykobaktyny (Mbt) u *Mycobacterium tuberculosis* i odgrywa kluczową rolę w funkcjonowaniu syntetaz nierybosomalnych peptydów, które są dobrze znane z działania przeciwdrobnoustrojowego, immunosupresyjnego, cytostatycznego i przeciwwirusowego (Zwahlen i in., 2019).

Analiza genomu *P. sivasensis* 2RO45 wykazała obecność biosyntetycznych klastrów genów kodujących syntetazy nierybosomalnych peptydów (syringomycyna, piowerdyna), terpeny (enterobaktyna), arylopolieny (arylopolien VF), betalaktony (fengycyna) i kofaktor

redoks (lankacydyna C) (publikacja nr 3). Według doniesień literaturowych związki te wykazują działanie przeciwgrzybowe (Bensaci i in., 2011; Cao i in., 2015; Dutta i in., 2020; Kang i in., 2020; Khan i in., 2021; Sass i in., 2021). W genomie *P. sivasensis* 2RO45 wykryto również klaster genowy, którego sekwencja była w 81% podobna do wiskozyny, wykazującej działanie przeciwgrzybowe (Alsohim i in., 2014). Ponadto, w niniejszej rozprawie doktorskiej wykryto obecność genów zaangażowanych w sekwestrację sideroforów: *fbpA* (Banerjee i in., 2014), *mbtH* (Li i in., 2020) oraz *acrB* (Horiyama i Nishino, 2014) w genomie *P. sivasensis* 2RO45 (publikacja nr 3).

Podsumowując, w genomach ryzobakterii promujących wzrost rzepaku, *B. paralicheniformis* 2R5 oraz *P. sivasensis* 2RO45, zidentyfikowano wiele genów odpowiedzialnych za stymulowanie wzrostu i rozwoju roślin oraz wiele biosyntetycznych klastrów genów zaangażowanych w biokontrolę patogenów roślinnych, co potwierdza przedstawioną w niniejszej rozprawie doktorskiej hipotezę 2.

5. Analiza ilościowa mikroorganizmów związanych z obiegiem azotu w ryzosferze

W genomie *Bacillus paralicheniformis* 2R5 znaleziono geny związane z obiegiem azotu (N), takie jak *narG* i *nosZ*. Z tego względu, sprawdzono czy inokulacja *B. paralicheniformis* 2R5 może mieć wpływ na liczbę kopii tych genów w ryzosferze rzepaku. Ponadto, dokonano oznaczenia liczby kopii innych ważnych genów związanych z obiegiem azotu, takich jak *nifH*, *nirS* i *amoA*. Badania wykazały, że inokulacja szczepem *B. paralicheniformis* 2R5 znacząco zwiększyła liczbę kopii genów, takich jak *narG*, *nosZ*, *nifH* i *nirS* w ryzosferze rzepaku (publikacja nr 1). Według doniesień literaturowych, podwyższony poziom genów zaangażowanych w cykl azotowy może być korzystny dla wzrostu i rozwoju roślin ze względu na wzrost dostępnego azotu (Bhattacharyya i in., 2018). Jednakże ważne jest, aby wziąć pod uwagę specyficzne działania poszczególnych genów. Gen *nifH* umożliwia bakteriom wiązanie azotu atmosferycznego (N_2) w amoniak (NH_3), który jest łatwo przyswajalny przez rośliny (Gresshoff i in., 2015; Dasgupta i in., 2021). Natomiast, geny *narG*, *nirS* i *nosZ* biorą udział w procesie denitryfikacji, który przyczynia się do utraty azotu w glebach rolniczych (Igiehon i Babalola, 2018; Zhu i in., 2018). W niniejszej rozprawie doktorskiej, obecność genów *narG* i *nosZ* w genomie *B. paralicheniformis* 2R5, była zbieżna z istotnymi zmianami w liczbie kopii genów związanych z cyklem N w ryzosferze. Inokulacja *B. paralicheniformis* 2R5

spowodowała zarówno wzrost liczby kopii genów *narG* i *nosZ*, które występowały w genomie szczepu 2R5, jak i wzrost liczby kopii innych genów, takich jak *nifH* i *nirS*. Warto również zauważyć, że liczba kopii genów cyklu azotowego wzrosła z czasem w ryzosferze nieinokulowanej *B. paralicheniformis* 2R5. Poprzednie badania wykazały, że rośliny poprzez wydzieliny korzeniowe kształtują mikrobiom ryzosfery, wpływając tym samym na liczebność i aktywność drobnoustrojów cyklu N, w tym na bakterie redukujące azotany i bakterie denitrifikujące (Henry i in., 2008; Weng i in., 2013).

6. Wpływ inokulacji ryzobakterii promujących wzrost rzepaku na bioróżnorodność strukturalną mikroorganizmów ryzosferowych

W niniejszej rozprawie doktorskiej, ryzobakterie promujące wzrost rzepaku: *Bacillus paralicheniformis* 2R5 (publikacja nr 2) i *Pseudomonas sivasensis* 2RO45 (publikacja nr 4) wybrano do określenia ich wpływu na bioróżnorodność strukturalną mikroorganizmów ryzosferowych. Według Martínez-Hidalgo i in. (2019), szczegółowe badania pozwalające określić zmiany zachodzące w strukturze taksonomicznej mikroorganizmów glebowych po inokulacji bakteryjnej są niezbędne przed komercyjnym zastosowaniem PGPR.

6.1. Analiza wskaźników bioróżnorodności strukturalnej

Analiza wskaźników bioróżnorodności strukturalnej wykazała, iż różnorodność społeczności drobnoustrojów była zależna od wzrostu i rozwoju rośliny. Badania Wang i in. (2017) potwierdzają, że struktura mikrobiomu ryzosfery zmieniała się w trakcie rozwoju roślin ze względu na zmiany w wydzielinach korzeniowych. Korzenie roślin uwalniają do gleby metabolity wtórne, takie jak terpenoidy, polifenole i związki zawierające azot, które nie przyczyniają się bezpośrednio do wzrostu i rozwoju, ale odgrywają znaczącą rolę w kształtowaniu struktury mikrobiomu ryzosfery (Mishra i in., 2022).

Niemniej jednak nadal jest niejasne, w jaki sposób inokulacja PGPR wpływa na mikrobiom ryzosfery i w jaki sposób zmiany w mikrobiomie ryzosfery przyczyniają się do poprawy wzrostu roślin (Kong i Liu, 2022). W niniejszej rozprawie doktorskiej zaobserwowano, iż inokulacja *P. sivasensis* 2RO45 nie wpłynęła istotnie na wskaźniki alfa-

bioróżnorodności (publikacja nr 4). Jednak należy podkreślić, że niektóre rodzaje PGPR są w stanie wspierać wzrost roślin, wykazując przy tym tylko niewielkie zmiany w strukturze społeczności drobnoustrojów obecnych w ryzosferze (Piromyou i in., 2011; Touceda-González i in., 2015). Według Piromyou i in. (2011) oraz Touceda-González i in. (2015), brak zmian w strukturze mikrobiomu ryzosfery może wystąpić kiedy wprowadzone szczepy PGPR nie są w stanie przetrwać i konkurować z rodzimymi zbiorowiskami mikroorganizmów. Z drugiej strony, Chowdhury i in. (2013) dowiedli, że *Bacillus amyloliquefaciens* może z powodzeniem zkolonizować ryzosferę, nie wykazując trwałego wpływu na strukturę społeczności bakteryjnej. W niniejszej rozprawie doktorskiej, na podstawie względnej obfitości filotypu *Pseudomonas* (OTU00038) w obrębie całej społeczności bakteryjnej, można zauważyć, że pewne bakterie z rodzaju *Pseudomonas* były obecne w ryzosferze już przed inokulacją. W związku z tym, inokulacja *P. sivasensis* 2RO45 nie spowodowała trwałego wzrostu liczebności tych bakterii w ryzosferze. Jednak warto podkreślić, że *P. sivasensis* 2RO45 zkolonizował ryzosferę w warunkach sterylnych, wykazując zdolność przetrwania do 44 dni (publikacja nr 4).

Ponadto, w niniejszej rozprawie doktorskiej zaobserwowano, że inokulacja *B. paralicheniformis* 2R5 początkowo zmniejszyła bogactwo gatunkowe społeczności bakteryjnych, podczas gdy po 44 dniach inokulacji wskaźnik alfa-bioróżnorodności wzrósł (publikacja nr 2). Inni autorzy dowiedli, że PGPR powodował zmiany w mikrobiomie ryzosfery dopiero po ośmiu tygodniach od inokulacji, sugerując, że wprowadzony do ryzosfery PGPR wymaga czasu, aby przetrwać i aktywnie się rozmnażać wykazując różnorodne funkcje (Jeong i in., 2013). W badaniach Yu i Lee (2013) oraz Bhattacharyya i in. (2018), inokulacja PGPR zwiększyła strukturę mikroorganizmów w ryzosferze, jednocześnie promując wzrost kapusty pekińskiej (*Brassica rapa* L. ssp. *pekinensis*). Niemniej jednak, według Chen i in. (2022), ani kolonizacja korzeni przez PGPR, ani modyfikacje w mikrobiomie ryzosfery nie były konieczne dla procesu promocji wzrostu roślin. Autorzy wykazali, że indukowane przez PGPR modyfikacje metylacji DNA w korzeniach pośredniczą w długotrwałym wpływie na promocję wzrostu roślin.

6.2. Analiza zmian składu taksonomicznego natywnej mikrobioty po inokulacji PGPR

Powszechnie uważa się, że duży udział pożytecznych mikroorganizmów w ryzosferze oznacza lepszy wzrost roślin, niższą zachorowalność roślin na choroby oraz wyższą aktywność

składników odżywczych i enzymów (Wang i in., 2017). W niniejszej pracy doktorskiej, analiza LEfSe wykazała, że zarówno inokulacja szczepem *B. paralicheniformis* 2R5 (publikacja nr 2) jak i *P. sivasensis* 2RO45 (publikacja nr 4) zwiększyła liczbę odczytów korzystnych dla wzrostu roślin grup taksonomicznych bakterii i grzybów.

Inokulacja szczepem *B. paralicheniformis* 2R5 początkowo nie wpłynęła na liczbę odczytów bakterii, ale spowodowała wzrost udziału grzybów *Humicola grisea* i *Solicoccozyma terricola* (publikacja nr 2). Grzyby z rodzaju *Solicoccozyma* posiadają zdolność wytwarzania kwasu indolo-3-octowego, który jest głównym fitohormonem regulującym rozwój roślin (Nicola i in., 2021). Podczas gdy, *Humicola grisea* są znane jako grzyby zamieszkujące glebę (White i Downing, 1953). W niniejszej rozprawie doktorskiej, po 22 dniach inokulacji *B. paralicheniformis* 2R5 zaobserwowano wzrost udziału bakterii z rodzaju *Nitrospira, Sphingomonas, Terrimonas, Massilia,* i *Ramlibacter* oraz grzybów *Solicoccozyma aeria* i *Fusicolla septimanifiniscientiae* (publikacja nr 2). *Fusicolla septimanifiniscientiae* są znane jako grzyby zasiedlające środowisko glebowe (Crous i in., 2022). Ponadto, bakterie z rodzaju *Sphingomonas* mają zdolność promowania wzrostu pomidorów (*Solanum pimpinellifolium*) w warunkach stresu solonego (Khan i in., 2017). Z kolei bakterie z rodzaju *Massilia* mogą promować wzrost i rozwój roślin poprzez ich zdolność do rozpuszczania fosforu i azotu (Guo i in., 2019), a bakterie z rodzaju *Terrimonas* ułatwiają roślinie pobieranie składników odżywczych (Visioli i in., 2018). Bakterie rodzaju *Nitrospira,* tzw. bakterie „comammox" uznawane są za mikroorganizmy zdolne do prowadzenia pełnej nitryfikacji (Daims i in., 2016), natomiast bakterie z rodzaju *Ramlibacter* biorą udział w obiegu N (Milkereit i in., 2021). Zwiększona liczba odczytów tych rodzajów bakterii w ryzosferze inokulowanej *B. paralicheniformis* 2R5 potwierdza wcześniejsze badania, w których inokulacja *B. paralicheniformis* 2R5 spowodowała wzrost liczby kopii genów związanych z obiegiem N w ryzosferze rzepaku (publikacja nr 1). W niniejszej rozprawie doktorskiej zaobserwowano również wzrost udziału bakterii z rodzaju *Dongia* oraz grzybów *Schizothecium* sp., *Plectosphaerella cucumerina* i *Cyphellophora vermispora* po 44 dniach inokulacji szczepem *B. paralicheniformis* 2R5 (publikacja nr 2). Według Nditasari i in. (2023) bakterie z rodzaju *Dongia* promują wzrost roślin trawiastych. Grzyby z gatunku *Schizothecium* sp. efektywnie kolonizują korzenie roślin (Hugoni i in., 2018) i wykazują aktywność przeciwgrzybową wobec wielu patogenów roślin (Narisawa, 2018; Tymon i in., 2020). Z kolei, *Cyphellophora vermispora* jest saprofitem izolowanym z korzeni, łodyg i liści roślin (Gao i in., 2015). Należy również wziąć pod uwagę, że *Plectosphaerella cucumerina* to patogen wielu roślin, w tym

rzodkiewki (Miao i in., 2018), roślin dyniowatych, pomidorów i pieprzu (Carlucci i in., 2012). Jednak nie ma danych literaturowych mówiących o tym, aby ten gatunek grzyba wywoływał choroby rzepaku.

Inokulacja szczepem *P. sivasensis* 2RO45 początkowo spowodowała wzrost udziału promieniowców z rodzaju *Streptomyces* oraz grzybów *Cyphellophora vermispora* (publikacja nr 4). Liczne badania wykazały, że promieniowce z rodzaju *Streptomyces* poprawiają wzrost roślin i chronią je przed patogenami, produkując antybiotyki i związki bioaktywne (Amaresan i in., 2018; Suárez-Moreno i in., 2019; Vergnes i in., 2020; Le i in., 2021). W prezentowanej rozprawie doktorskiej, po 22 dniach inokulacji szczepem *P. sivasensis* 2RO45, obserwowano wzrost liczby odczytów bakterii z rodziny *Comamonadaceae* i *Vicinamibacteraceae* oraz grzybów z rodziny *Nectriaceae,* rodzaju *Exophiala* i gatunku *Mortierella minutissima* (publikacja nr 4). Bakterie należące do rodziny *Vicinamibacteraceae* degradują materię organiczną i chitynę (Whitton i in., 2022), natomiast bakterie z rodziny *Comamonadaceae* kontrolują fuzaryjne więdnięcię roślin poprzez wydzielanie większej ilości kwasów organicznych (Wen i in., 2020). Grzyby z rodziny *Nectriaceae* zostały uznane za oportunistyczne patogeny roślin, jednakże kilka gatunków należących do tej rodziny jest stosowanych w biokontroli roślin (Lombard i in., 2015). Z kolei grzyby z rodzaju *Exophiala* są szeroko wykorzystywane w rolnictwie, na przykład *Exophiala pisciphila* stymuluje wzrost kukurydzy (Xu i in., 2020) i zapobiega rozwojowi fuzariozy truskawek (Harsonowati i in., 2020), a *Exophiala* sp. promuje wzrost ogórka w warunkach stresów abiotycznych (Khan i in., 2011). Grzyby z rodzaju *Mortierella* obejmują liczne gatunki grzybów, które promują wzrost roślin degradując biopolimery, na przykład *M. minutissima* wyizolowany z powierzchni korzenia wykazywał silne działanie chitynolityczne (Ozimek i Hanaka, 2020). W ramach niniejszej pracy doktorskiej stwierdzono brak zmian w liczbie odczytów bakterii i wzrost udziału grzybów z rodziny *Didymellaceae* po 44 dniach inokulacji szczepem *P. sivasensis* 2RO45 (publikacja nr 4). Wiele gatunków z rodziny *Didymellaceae* to patogeny grzybowe powodujące uszkodzenia owoców, liści, łodyg i korzeni różnorodnych roślin uprawnych (Hou i in., 2020). Jednakże *Didymellaceae* to największa rodzina w obrębie rzędu *Pleosporales* licząca ponad 5 400 gatunków, w tym także gatunki saprobowe, endofityczne i kliniczne (Hou i in., 2020; Yuan i in., 2021).

Warto zwrócić uwagę na to, że inokulacja szczepem *P. sivasensis* 2RO45 spowodowała jedynie przejściowe i krótkotrwałe zmiany (do 22 dni po inokulacji) w składzie taksonomicznym bakterii (publikacja nr 4). Podobne wyniki uzyskali Bhattacharyya i Lee

(2016). Według autorów inokulacja PGPR zmieniała strukturę zbiorowisk bakteryjnych bardziej dynamicznie u młodych roślin. Ponadto, w niniejszej rozprawie doktorskiej zaobserwowano, że inokulacja *B. paralicheniformis* 2R5 początkowo nie zmieniła składu taksonomicznego bakterii, dopiero po 22 dniach inokulacji liczba odczytów korzystnych dla wzrostu roślin grup taksonomicznych bakterii zwiększyła się (publikacja nr 2). Najprawdopodobniej izolat *B. paralicheniformis* 2R5 wymaga czasu, aby aktywnie się rozmnażać wykazując różnorodne funkcje (Jeong i in., 2013).

7. Wpływ inokulacji *Pseudomonas sivasensis* 2RO45 na bioróżnorodność funkcjonalną mikroorganizmów ryzosferowych

Według doniesień literaturowych, warto poświęcić więcej uwagi badaniom pozwalającym określić wpływ inokulacji PGPR na bioróżnorodność funkcjonalną rodzimej społeczności drobnoustrojów ryzosfery (Di Salvo i in., 2018). W niniejszej pracy doktorskiej, przeanalizowano wpływ inokulacji *Pseudomonas sivasensis* 2RO45 na aktywność metaboliczną i bioróżnorodność funkcjonalną mikroorganizmów ryzosferowych rzepaku (publikacja nr 4). Zachodzące zmiany analizowano w 44 dniu inokulacji. Ten punkt czasowy wybrano aby sprawdzić, czy inokulacja *P. sivasensis* 2RO45 ma długoterminowy wpływ na różnorodność funkcjonalną mikrobiomu ryzosfery rzepaku.

Badania wykazały, że inokulacja szczepem *P. sivasensis* 2RO45 przyczyniła się do wzrostu ogólnej aktywności metabolicznej drobnoustrojów ryzosferowych (publikacja nr 4). Ponadto, w niniejszej rozprawie doktorskiej obliczono aktywność metaboliczną drobnoustrojów dla sześciu konkretnych grup biochemicznych substratów węglowych, takich jak: fenole, węglowodany, aminokwasy, kwasy karboksylowe, polimery i aminy. Badania wykazały, że cztery źródła węgla, w tym fenole, polimery, kwasy karboksylowe i aminokwasy były lepiej metabolizowane przez zespół mikroorganizmów ryzosfery inokulowanej *P. sivasensis* 2RO45 niż w próbach kontrolnych. Podczas gdy, węglowodany i aminy były lepiej metabolizowane przez społeczność drobnoustrojów ryzosfery nieinokulowanej (publikacja nr 4). Podobne wyniki otrzymali Bhattacharyya i Lee (2016) badając wpływ inokulacji PGPR na profil metaboliczny zbiorowisk drobnoustrojów. Według autorów, drobnoustroje w inokulowanej ryzosferze wykazywały zwiększoną aktywność metabolizowania aminokwasów

i mniejszą aktywność wykorzystania węglowodanów, co korzystnie wpłynęło na wzrost kapusty.

W oparciu o uzyskane profile rozkładu substratów węglowych obliczono wskaźniki różnorodności funkcjonalnej. Badania wykazały, że inokulacja *P. sivasensis* 2RO45 spowodowała wzrost różnorodności funkcjonalnej mikrobiomu ryzosfery rzepaku mierzonej wskaźnikiem bioróżnorodności Shannona-Wienera (*H'*) oraz indeksem równocenności Shannona-Wienera (*E*) (publikacja nr 4). Inni autorzy również zaobserwowali zmiany w bioróżnorodności funkcjonalnej mikroorganizmów ryzosferowych po inokulacji PGPR. Jednak analizy zostały przeprowadzone na innych roślinach uprawnych, takich jak ryż, pomidor, kukurydza i pszenica (Naiman i in., 2009; de Salamone i in., 2010; Di Salvo i in., 2018; Zuluaga i in., 2021).

Powyższe badania potwierdzają przedstawioną w niniejszej rozprawie doktorskiej hipotezę 3, w której założono, że bakterie promujące wzrost roślin mają wpływ na bioróżnorodność rodzimych społeczności drobnoustrojów ryzosfery.

V Wnioski

Przeprowadzone w niniejszej rozprawie doktorskiej badania poszerzają wiedzę na temat ryzobakterii promujących wzrost roślin i ich wpływie na rodzime drobnoustroje w ryzosferze oraz pozwoliły zweryfikować postawione hipotezy badawcze.

1. Ryzosfera korzeni rzepaku uprawianego w gospodarstwie rolnym w Górsku i w Ostródzie jest siedliskiem gatunków bakterii promujących wzrost rzepaku: *Bacillus paralicheniformis* 2R5 oraz *Pseudomonas sivasensis* 2RO45. Gatunki tych ryzobakterii są znane z innych właściwości, jednak jako promujące wzrost rzepaku zostały po raz pierwszy opisane w niniejszej rozprawie.
2. Analiza genomów szczepów stymulujących wzrost rzepaku, *Bacillus paralicheniformis* 2R5 oraz *Pseudomonas sivasensis* 2RO45, potwierdziła występowanie licznych genów PGP, a także biosyntetycznych klastrów genów, co może potencjalnie wpływać na zdolność tych bakterii do promowania wzrostu rzepaku i aktywność przeciwko patogenom grzybowym.
3. Ryzobakterie promujące wzrost rzepaku, *Bacillus paralicheniformis* 2R5 oraz *Pseudomonas sivasensis* 2RO45, wykazują pozytywny wpływ na bioróżnorodność strukturalną w ryzosferze, istotnie zwiększając udział korzystnych dla wzrostu roślin grup bakterii i grzybów, co może być ważne z punktu widzenia poprawy jakości i wydajności upraw rzepaku. Ponadto, *P. sivasensis* 2RO45 wykazuje zdolność do zwiększenia aktywności metabolicznej i różnorodności funkcjonalnej zbiorowisk drobnoustrojów w ryzosferze. To z kolei może prowadzić do zwiększenia możliwości detoksykacji środowiska, na przykład poprzez rozkład fenoli i polimerów.
4. Inokulacja roślin rzepaku szczepem *Bacillus paralicheniformis* 2R5 lub *Pseudomonas sivasensis* 2RO45 może stanowić istotny element w strategii poprawy wydajności upraw oraz w dążeniu do bardziej zrównoważonego zarządzania ekosystemami rolnymi.

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VII Dorobek naukowy

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Wykształcenie:

od 2018	Studia doktoranckie z zakresu nauk biologicznych, Wydział Nauk Biologicznych i Weterynaryjnych, Uniwersytet Mikołaja Kopernika w Toruniu
2015-2018	Studia stacjonarne drugiego stopnia: Biologia molekularna, Wydział Nauk Biologicznych, Uniwersytet Kazimierza Wielkiego w Bydgoszczy
2014-2017	Studia stacjonarne drugiego stopnia: Biotechnologia, Wydział Biologii i Ochrony Środowiska, Uniwersytet Mikołaja Kopernika w Toruniu
2011-2014	Studia stacjonarne pierwszego stopnia: Biotechnologia, Wydział Nauk Biologicznych, Uniwersytet Kazimierza Wielkiego w Bydgoszczy

Projekty badawcze:

- Grant „Mikrobiologia w konserwacji zabytków” w ramach programu „Inicjatywa Doskonałości – Uczelnia Badawcza”, finansowanego przez Uniwersytet Mikołaja Kopernika w Toruniu, 2020-2022, wykonawca
- Grant “Effect of plant growth-promoting rhizobacteria on rape growth and their influence on native microbial population” w ramach IBUB – Debiuty, finansowanego przez Uniwersytet Mikołaja Kopernika w Toruniu, 2020-2021, wykonawca

Nagrody i stypendia:

- stypendium Rektora dla najlepszych doktorantów (rok akademicki 2019/2020, 2020/2021, 2021/2022, 2022/2023, 2023/2024)
- stypendium naukowe dla doktorantów (rok akademicki 2018/2019, 2019/2020, 2020/2021, 2021/2022, 2022/2023, 2023/2024)
- stypendium projakościowe (rok akademicki 2018/2019, 2019/2020, 2020/2021, 2021/2022, 2022/2023, 2023/2024)
- ITC Conference Grant (COST Action: CA15114) na czynne uczestnictwo w konferencji: AMiCI Final Conference, Kraków, Polska, 19-20.04.2020
- ITC Conference Grant (COST Action: CA15114) na czynne uczestnictwo w konferencji: International Conference on Polymer Science and Technology, Barcelona, Spain, 5-6.11.2020

Szkolenia naukowe:

- szkolenie naukowe „Metody oznaczania aktywności chitynaz oraz β-1,3-glukanaz, techniki izolacji białek z komórek bakteryjnych, chromatografia jonowymienna niskociśnieniowa oraz analiza otrzymanych frakcji", Katedra Biochemii i Mikrobiologii, Szkoła Główna Gospodarstwa Wiejskiego w Warszawie, 26-28.02.2020.
- Training School "Molecular typing of Xanthomonadaceae – from epidemiological surveillance to outbreak investigation", Zurich University of Applied Sciences, Wadenswil, Switzerland, 07.2019
- Training School "Antimicrobial Coating Innovations to Prevent Infectious Diseases", University of Amsterdam, The Netherlands, 04.2019

Staże naukowe:

- University of Eötvös Loránd, Budapest, Hungary w ramach programu PROM – Międzynarodowa Wymiana Stypendialna Doktorantów i Kadry akademickiej, Narodowej Agencji Wymiany Akademickiej (NAWA), 06.2022

Dorobek naukowy:

1. Kumar, S.B., Kalwasińska, A., **Świątczak, J.,** Swiontek Brzezinska, M., Kęsy, J., 2024. Characterization of salt-tolerant diazotrophs with plant growth-promoting potential isolated from soda industry-affected technosoils. Plant and Soil, pp.1-14.
2. **Świątczak, J**., Kalwasińska, A., Niemcewicz, P., Chylińska, M., Ogińska, A., Swiontek Brzezinska, M., 2023. Sandstone conservation of polish tomb chapel and its effect on microbial diversity. International Journal of Architectural Heritage, pp.1-10.
3. **Świątczak, J**., Kalwasińska, A., Wojciechowska, A., Swiontek Brzezinska, M, 2023. Physiological properties and genomic insights into the plant growth-promoting rhizobacterium Brevibacillus laterosporus K75 isolated from maize rhizosphere. Journal of the Science of Food and Agriculture, 103(3), pp.1432-1441.
4. Swiontek Brzezinska, M., Pałubicka, K., Latos, M., L., Janik, A., Tarnawska, P., Krajnik, K., Burkowska-But, A., **Świątczak J.**, et al., 2023. Natural compounds derived from Brassicaceae plants as an alternative to synthetic fungicides and their influence on soil fungus diversity. Journal of the Science of Food and Agriculture, 103(1), pp.317-327.
5. Swiontek Brzezinska, M., **Świątczak, J**., Wojciechowska, A., Burkowska-But, A., Kalwasińska, A., 2022. Consortium of plant growth-promoting rhizobacteria enhances oilseed rape (Brassica napus L.) growth under normal and saline conditions. Archives of Microbiology, 204(7), p.393.
6. Swiontek Brzezinka, M., Richert, A., Kalwasińska, A., **Świątczak, J.**, Deja-Sikora, E., Walczak, M., Michalska-Sionkowska, M., Piekarska, K., Kaczmarek-Szczepańska, B., 2021. Microbial degradation of polyhydroxybutyrate with embedded polyhexamethylene guanidine derivatives. International Journal of Biological Macromolecules, 187, pp.309-318.
7. Kaczmarek-Szczepańska, B., Sionkowska, M.M., Mazur, O., **Świątczak, J.**, Swiontek Brzezinska, M., 2021. The role of microorganisms in biodegradation of chitosan/tannic acid materials. International Journal of Biological Macromolecules, 184, pp.584-592.
8. Swiontek Brzezinska, M., Walczak, M., Kalwasińska, A., Richert, A., **Świątczak, J**., Deja-Sikora, E., Burkowska-But, A., 2020. Biofilm formation during biodegradation of polylactide, poly (3, 4 hydroxybutyrate) and poly (ε-caprolactone) in activated sludge. International Journal of Biological Macromolecules, 159, pp.539-546.

9. Swiontek Brzezinska, M., Kalwasińska, A., **Świątczak, J.,** Żero, K., Jankiewicz, U., 2020. Exploring the properties of chitinolytic Bacillus isolates for the pathogens biological control. Microbial Pathogenesis, 148, p.104462.
10. Swiontek Brzezinska, M., Jankiewicz, U., Kalwasińska, A., **Świątczak, J.**, Żero, K., 2019. Characterization of chitinase from Streptomyces luridiscabiei U05 and its antagonist potential against fungal plant pathogens. Journal of Phytopathology, 167(7-8), pp.404-412.
11. Swiontek Brzezinska, M., Walczak, M., Burkowska-But, A., Chylińska, M., Kalwasińska, A., **Świątczak, J.**, 2019. Antifungal Activity of Polyhexamethyleneguanidine Derivatives Introduced into Biodegradable Polymers. Journal of Polymers and the Environment, 27, pp.1760-1769.

Udział w konferencjach:

1. ECCO XL New Horizons in Accessing Microbial Diversity, Referat: "Microbial diversity evolution in sandstone tomb chapel following a bio-conservation treatment". 09.2022
2. Second Edition of Virtual International Conference „Plant productivity and food safety: Soil science, Microbiology, Agricultural Genetics and Food quality", Poster: "Complete genome sequencing of plant growth – promoting rhizobacterium - Pseudomonas extremaustralis 2RO45 and its effect on canola growth". 09.2022
3. 10th PSEPB Conference "Experimental plant biology at various scales: from molecules to the environment.", Virtual Conference, Poster: "Evaluating antifungal activity of preparation based on phenolic compounds: carvacrol and eugenol and its potential use in plant protection as an alternative to synthetic fungicides", 09.2021
4. 10th PSEPB Conference "Experimental plant biology at various scales: from molecules to the environment.", Virtual Conference, Referat: "Genome sequencing and assessment of plant growth-promoting properties of Brevibacillus laterosporus 75 strain isolated from maize". 09.2021
5. The EuroXanth COST Action 4th Annual Conference "Integrating Science on Xanthomonadaceae for integrated plant disease management in Europe", Virtual Conference, Poster: "Antibacterial and antibiofilm activity of phenolic compounds against Xanthomonas campestris pv. campestris", 06.2021

6. 3rd Annual Conference of EuroXanth COST Action, Lednice, Czech Republic, Poster: “Comparison of the effect of Stenotrophomonas rhizophila strains isolated from different plant rhizospheres on plant growth promotion”, 09.2019
7. Konferencja HYDROMICRO 2019, 20 lat hydromikrobiologii- dokonania i perspektywy, Toruń, Referat: „Biofilmy powstające na powierzchni polimerów w trakcie procesu biodegradacji”, 06.2019
8. Workshop for Early Career Investigators and Short Term Scientific Missions, Riga, Latvia, Poster: “Antimicrobial activity of natural phenolic compounds and their potential application”, 03.2019

Działalność dydaktyczna:

- Mikrobiologia ogólna (S1)
- Mikrobiologia przemysłowa (S1)
- Podstawy chemii dla biologów (S1)
- Mikroorganizmy na zwłokach kręgowców (S1)
- Pracownia magisterska (S2)

Działalność popularyzatorska i organizacyjna:

- członek komitetu organizacyjnego Konferencji HYDROMICRO 2019 - 20 lat hydromikrobiologii- dokonania i perspektywy w Toruniu, 06.2019
- recenzent podczas IX Kopernikańskiego Sympozjum Studentów Nauk Przyrodniczych, Uniwersytet Mikołaja Kopernika w Toruniu, 09.2020
- prowadzenie zajęć w ramach „Nocy Biologów, Uniwersytet Mikołaja Kopernika w Toruniu, 11.01.2019 i 10.01.2020

Doświadczenie zawodowe:

- zatrudnienie na stanowisku biolog w Zakładzie Mikrobiologii Środowiskowej i Biotechnologii, Uniwersytet Mikołaja Kopernika w Toruniu, 10.2018-09.2019