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Analysis of composition and potential of microbes associated to the rhizosphere of cotton and wheat

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Summary

Pathogenic fungi and plant-parasitic nematodes threaten the health of crops and thus impair agricultural production. Fungi cause a wide variety of diseases by destroying plant tissue in different ways, disrupting the plant's metabolism, and triggering various defense reactions. Nematodes very often infest the roots of agricultural crops, parasitize or destroy cells, and cause anatomical, morphological, and physiological disorders, resulting in a significant loss of performance and resilience to abiotic stress factors. Since synthetic pesticides often have negative effects on the environment and possibly also on humans, there is a growing need for sustainable approaches to control these pathogens. Plant Growth-Promoting Rhizobacteria (PGPR) offer a promising solution for biological control. PGPR are beneficial bacteria that colonize plant roots and enhance plant growth through various mechanisms. They can suppress fungal and nematode pathogens by producing antimicrobial compounds, triggering systemic resistance in plants, and competing for nutrients and space. Using PGPR as biocontrol agents provides an environmentally friendly and sustainable method for the management of plant diseases, reduces reliance on synthetic chemicals, and promotes healthier ecosystems. This study aims to analyze the PGPR present in the rhizosphere of cotton and wheat under continuous cotton-wheat rotation in Pakistan. It also investigates the biological control potential of these rhizobacteria against the fungus *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) in cotton and against *Heterodera schachtii* in *Arabidopsis thaliana*, as well as their effect on plant growth. **Chapter 2** presents a metagenomic-based investigation of the microbial community, including bacteria and fungi (including all culturable and non-culturable), in the rhizosphere of cotton from four different agroecological sites. The study revealed that the abundance of bacterial and fungal communities varied across the different agroecological sites, even with the same crop and rotation pattern. These differences suggest that specific bacteria exhibit adaptations to their environment and that various other factors beyond the crop itself shape the microbial community. **Chapter 3** presents the investigation of antifungal activity against FOV, biochemical characterization, and genetic diversity of 136 culturable rhizobacteria from five different cotton fields and five different wheat fields. The dendrogram constructed from $(GTG)_5$ and REP-PCR fingerprint profiles indicated greater diversity. Molecular markers used for genetic fingerprinting effectively differentiated the cotton rhizosphere isolates from those obtained from the wheat rhizosphere, indicating a distinct resident bacterial community despite the cotton-wheat rotation. **Chapter 4** presents an investigation of *Bacillus* species isolated from cotton. The eight characterized *Bacillus* isolates all showed biocontrol potential against FOV with different mechanisms of action. **Chapter 5** presents an investigation of the antagonistic

effect of *Bacillus* spp. against *H. schachtii* in *A. thaliana*. Seven *Bacillus* isolates showed anti-nematode activity both *in vitro* and *in vivo,* and also reduced the size of females.

This research revealed that a number of *Bacillus* spp. among 136 isolated and divers rhizobacteria effectively suppressed FOV and *H. schachtii* by different antagonistic mechanisms. These *Bacillus* spp. can be a valuable means as part of an integrated fungal and nematode control strategy. Moreover, this study is the first to report the antagonistic activity of *Bacillus stercoris* against a plant-parasitic nematode.

Zusammenfassung

Pathogene Pilze und pflanzenparasitäre Nematoden gefährden die Gesundheit von Nutzpflanzen und beeinträchtigen dadurch die landwirtschaftliche Produktion. Pilze verursachen eine Vielzahl von Krankheiten, indem sie Pflanzengewebe auf unterschiedliche Weise zerstören, den Stoffwechsel der Pflanzen stören und verschiedene Abwehrreaktionen auslösen. Nematoden befallen sehr häufig die Wurzeln landwirtschaftlicher Kulturpflanzen, parasitieren oder zerstören Zellen und verursachen anatomische, morphologische und physiologische Störungen, was zu erheblichen Leistungseinbußen und einer verminderten Widerstandsfähigkeit gegenüber abiotischen Stressfaktoren führt. Da synthetische Pestizide oft negative Auswirkungen auf die Umwelt und möglicherweise auch auf den Menschen haben, besteht ein wachsender Bedarf an nachhaltigen Ansätzen zur Bekämpfung dieser Krankheitserreger. Pflanzenwachstumsfördernde Rhizobakterien (PGPR) bieten eine vielversprechende Lösung für die biologische Bekämpfung. PGPR sind nützliche Bakterien, die sich an Pflanzenwurzeln ansiedeln und das Pflanzenwachstum durch verschiedene Mechanismen fördern. Sie können pilzliche und nematodale Krankheitserreger unterdrücken, indem sie antimikrobielle Verbindungen produzieren, eine systemische Resistenz in Pflanzen auslösen und um Nährstoffe und Raum konkurrieren. Der Einsatz von PGPR als biologische Bekämpfungsmittel bietet eine umweltfreundliche und nachhaltige Methode zur Bekämpfung von Pflanzenkrankheiten, verringert die Abhängigkeit von synthetischen Chemikalien und fördert gesündere Ökosysteme. Diese Studie zielt darauf ab, die PGPR zu analysieren, die in der Rhizosphäre von Baumwolle und Weizen unter kontinuierlicher Baumwoll-Weizen-Fruchtfolge in Pakistan vorhanden sind. Außerdem wird das biologische Bekämpfungspotenzial dieser Rhizobakterien gegen den Pilz *Fusarium oxysporum* f. sp. vasinfectum (FOV) in Baumwolle und gegen *Heterodera schachtii* in *Arabidopsis thaliana* sowie ihre Wirkung auf das Pflanzenwachstum untersucht. **Kapitel 2** präsentiert eine metagenomische Untersuchung der mikrobiellen Gemeinschaft, einschließlich Bakterien und Pilzen (alle kultivierbaren und nicht kultivierbaren), in der Rhizosphäre von Baumwolle aus vier verschiedenen agroökologischen Standorten. Die Studie ergab, dass die Abundanz der Bakterien- und Pilzgemeinschaften zwischen den verschiedenen agroökologischen Standorten variierte, selbst bei gleicher Kulturpflanze und gleichem Fruchtwechselmuster. Diese Unterschiede deuten darauf hin, dass bestimmte Bakterien Anpassungen an ihre Umgebung zeigen und dass verschiedene andere Faktoren über die Kulturpflanze hinaus die mikrobielle Gemeinschaft prägen. **Kapitel 3** präsentiert die Untersuchung der antifungalen Aktivität gegen FOV, die biochemische Charakterisierung und die genetische Vielfalt von 136 kultivierbaren Rhizobakterien aus fünf verschiedenen Baumwollfeldern und fünf verschiedenen Weizenfeldern. Das aus (GTG)5- und REP-PCR-Fingerprint-Profilen erstellte Dendrogramm zeigte eine größere Vielfalt.

Molekulare Marker, die für den genetischen Fingerprinting verwendet wurden, unterschieden die Isolate aus der Baumwollrhizosphäre wirksam von denen aus der Weizenrhizosphäre, was auf eine unterschiedliche ansässige Bakteriengemeinschaft trotz der Baumwoll-Weizen-Fruchtfolge hindeutet. **Kapitel 4** präsentiert eine Untersuchung von *Bacillus*-Arten, die aus Baumwolle isoliert wurden. Alle acht charakterisierten *Bacillus*-Isolate zeigten ein biologisches Bekämpfungspotenzial gegen FOV mit unterschiedlichen Wirkmechanismen. **Kapitel 5** präsentiert eine Untersuchung der antagonistischen Wirkung von *Bacillus* spp. gegen *H. schachtii* in *A. thaliana*. Sieben *Bacillus*-Isolate zeigten sowohl *in vitro* als auch i*n vivo* eine nematodenbekämpfende Wirkung und reduzierten auch die Größe der Weibchen.

Diese Forschung zeigte, dass eine Reihe von *Bacillus* spp. unter 136 isolierten und diversen Rhizobakterien FOV und *H. schachtii* durch verschiedene antagonistische Mechanismen wirksam unterdrückten. Diese *Bacillus* spp. können ein wertvolles Mittel als Teil einer integrierten Strategie zur Bekämpfung von Pilzen und Nematoden sein. Darüber hinaus ist diese Studie die erste, die über die antagonistische Aktivität von *Bacillus stercoris* gegen einen pflanzenparasitären Nematoden berichtet.

List of abbreviations

Abbreviation	Full Form
ANOVA	Analysis of Variance
APX	Ascorbate Peroxidase
BCA	Biological control agent
CAT	Catalase
CFS	Cell-free supernatant
CFU	Colony-forming unit
Col 0	Colombia 0
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic Acid
DPI	Day-post-inoculation
ERIC	Enterobacterial repetitive intergenic consensus
EPS	Exopolysacchrides
HCN	Hydrogen Cyanide
HSD	Honestly Significant Difference
IAA	Indole acetic acid
IPM	Integrated pest management
J2	Juvenile stage 2
KCN	Potassium Cyanide
KI	Potassium iodide
LB	Luria Bertani
LBC	Living bacterial cell
LSD	Least Significant Difference
MDA	Malondialdehyde
OD 490	Optical density at 490 nm
OD 515	Optical density at 515 nm
OD 590	Optical density at 590 nm
OD 600	Optical density at 600 nm
PCA	Principal component analysis
PCR	Polymerase chain reaction
PGPR	Plant growth-promoting Rhizobacteria
PPN	Plant pathogen nematode

REP	Repetitive extragenic palindromic (DNA sequences)
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
SOD	Superoxide Dismutase
Std	Standard deviation
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
UPGMA	Unweighted pair group method with arithmetic mean
VOC	Volatile organic compound

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1. Introduction

The complex relationships between plants and their associated microbiome play an important role in shaping plant health, productivity and resilience against biotic or abiotic stress. The rhizosphere is directly affected by plant roots. It harbors diverse microbial communities including rhizobacteria, fungi, archaea, and other microorganisms, engaging in a complicated interaction with plants, impacting nutrient acquisition, abiotic and biotic stress resilience, and overall plant development. A group of rhizobacteria known as plant growth-promoting rhizobacteria (PGPR) is increasingly recognized for its contributions to sustainable agriculture and its potential to combat different plant pathogens, including *Fusarium* in various crops. However, their specific role in mitigating *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) in cotton has not been extensively explored. Therefore, the current research project was designed to analyze the rhizobacteria present in the cotton and wheat rhizospheres and to assess the potential of these rhizobacteria to control FOV, a major pathogen impacting cotton production, especially in Pakistan, where cotton significantly contributes to the national income and farmer livelihoods. In this research, the biocontrol potential of isolated rhizobacteria was investigated *in vitro*, and then the best-performing isolates were subjected to *in vivo* experiments to investigate their biocontrol potential against FOV only in cotton, as wheat is typically unaffected by FOV. Wheat fields were included in the study as a source of isolation of beneficial rhizobacteria. This is because cotton and wheat are commonly rotated in Pakistani agricultural systems, and the wheat rhizosphere harbors diverse bacterial communities that may possess antagonistic properties against FOV in cotton. Upon identifying several rhizobacteria with strong antagonistic activity against fungal species, the scope of the investigation expanded to explore their multidimensional biocontrol effects. Given the significant impact of plant-parasitic nematodes on crop yields, we became interested in assessing the potential of these rhizobacteria to suppress nematode parasitism. We used *Heterodera schachtii* as a model nematode and *Arabidopsis thaliana* as a model plant. This investigation reflects a broader goal of developing comprehensive biocontrol strategies that address multiple threats to plant health, promoting sustainable and resilient agricultural practices.

Due to time constraints, the scope of the study was limited to control FOV born Fusarium wilt in cotton. However, future research could investigate the potential of applying consortia of the isolated rhizobacteria for the disease management and for plant growth promotion in both cotton and wheat.

Before going into the further details of fungal and nematode born disease management strategies, the importance of cotton. plant parasitic fungi and plant parasitic nematodes is described in more detail to emphasize the importance of current research project.

Cotton is a globally significant crop and cotton production is considered the backbone of the textile industry. It makes a significant contribution to rural economies and global trade. Cotton accounts for nearly 30% of the world's textile fiber consumption and stands as the most important natural fiber. The global textile market, valued at over USD 1.7 trillion, relies heavily on cotton (Uddin et al., 2023). Beyond textiles, cotton seeds are processed by a wide range of industries into cottonseed oil, an edible oil used in food products, and cottonseed cake, a high-protein feed for livestock. Additionally, cotton by-products are utilized in the production of pharmaceuticals, cosmetics, paper, and bio-based plastics, making it a truly multifunctional crop with economic relevance far beyond clothing. In developing countries, cotton production provides essential cash income for millions of rural households, significantly contributing to agriculture, industry, employment, and export earnings.

Cotton occupies a central role in Pakistan’s agricultural economy, functioning as a vital cash crop and a primary source of livelihood for millions of farming households. Pakistan is one of the world's leading cotton-producing countries. Pakistan’s cotton sector directly supports around 1.5 million farmers and indirectly sustains millions more through the textile value chain. Cotton plays a crucial role in Pakistan's national economy, contributing approximately 0.8–1.0% to the country’s GDP and about 5–6% to the agricultural value (Abubakar et al., 2023). Its importance is further emphasized by the cotton-wheat cropping system, which dominates the agrarian landscape of Punjab and Sindh. While cotton contributes over 65% of the country’s total export earnings through the textile sector, wheat, on the other hand, is the principal staple food, critical for ensuring national food security. Together, cotton and wheat form a strategic alliance that supports Pakistan’s rural economy, export competitiveness, and food self-sufficiency (Sajjad et al., 2018). Cotton produces minimal crop residue, maintaining soil cover through practices like cover cropping, intercropping with high-residue crops, or incorporating wheat residue is crucial for sustainable cotton production; therefore, cotton-wheat rotation is more commonly practiced in Pakistan (Guo, 2022). In Punjab, for example, while the overall cotton acreage for 2025–26 is roughly 3.12 million acres, only 781,000 acres (~25%) are under early sowing, meaning the vast majority is on wheat-cotton rotation. In Sindh, official statistics of the Agriculture Extension Department (shared via All Pakistan Textile Mills Association (APTMA) research wing) show that more than 95% of the 0.94 million acres planted in 2025–26 is traditional wheat-cotton rotation with less diversification because of fewer

substitute winter crops. This trend refers to the close integration of cotton into cereal-based farming systems in Pakistan and indicates little room for rotation-based pest and soil management other than where cropping system diversification is actively pursued. Compared to rice, sugarcane, or even maize which are all far more water-intensive, cotton and wheat are relatively less water-demanding crops, and therefore this rotation is extremely well-suited to Pakistan's chronic water shortages.

Several fungal and nematode-borne diseases can affect cotton and wheat, resulting in significant yield losses. For example, *Fusarium species* are well known to cause disease in both crops (Moya-Elizondo et al., 2011; Cianchetta and Davis, 2015), and cereal cyst nematodes (e.g. *Heterodera avenae*, *Heterodera filipjevi, Heterodera latipons*) are notably recognized for yield losses in wheat (Li et al., 2021).

1.1 Plant-parasitic fungi

Plant diseases, predominantly caused by fungi, pose a significant threat to global food security due to their devastating impact on crop yields. Over 20,000 fungal species are parasitic in nature and capable of causing diseases in crops and plants (Ray et al., 2017). Plant-pathogenic fungi diminish crop productivity and product quality, lead to plant death, and weaken plants' resilience to environmental stressors. These effects result in substantial financial losses for farmers and threaten food security. Approximately 70% of plant diseases are attributed to fungal pathogens, highlighting their importance (Gouda et al., 2025).

Fungi can persist in a dormant state on either living or dead plant tissues until environmental conditions favor their proliferation (Loiko and Islam, 2024). They can invade plant tissues or thrive on the surface. Fungal spores are easily disseminated through wind, water, soil, and animals, facilitating their spread to neighboring fields and potentially damaging entire harvests (Singh, 2023). ***Necrotrophic fungi:*** *Fusarium*, *Colletotrichum*, *Rhizoctonia*, *Alternaria*, *Septoria*, (De Rodriguez et al., 2007) *Botrytis*, *Phytophthora*, *Penicillium*, *Magnaporthe*, *Peronospora*, *Aspergillus*, *Sclerotinia*, *Verticillium*, *Cercospora*, *Cladosporium*, *Pythium*, and *Venturia*; ***biotrophic:*** *Ustilago*, *Puccinia*, *Erysiphe*, are some of the most significant fungal genera containing plant pathogens (Oliver, 2024). These diverse genera encompass species responsible for a wide array of plant diseases affecting various crops worldwide. Both necrotrophic and biotrophic fungi represent contrasting lifestyles in their interactions with host plants. Necrotrophic fungi kill host cells and tissues to obtain nutrients, secreting toxins and enzymes that cause rapid cell death and tissue maceration. This often results in visible symptoms such as blights, rots, and wilts. In contrast, biotrophic fungi establish a long-term, symbiotic relationship with living host cells, extracting nutrients without

causing immediate cell death. Biotrophs often require specialized structures to penetrate and colonize host tissues, and their presence may initially be subtle, causing symptoms like mild chlorosis or altered growth patterns. While both types of fungi can cause significant plant diseases, necrotrophic fungi are generally considered more devastating due to their aggressive and destructive nature, leading to rapid tissue damage and often complete plant death.

1.1.1 *Fusarium*

Fusarium is a large and diverse genus of filamentous fungi. It is widely prevalent in soils and found in association with plants worldwide. It belongs to the division Ascomycota and includes species that act as pathogens, endophytes, and saprophytes. Many *Fusarium* species are significant plant pathogens, causing economically important diseases such as blights, rots, wilts, and cankers on a wide range of crops in both agricultural and natural ecosystems. They can infect various plant parts, including grains, seedlings, stems, and roots, reducing commercial yield and decreasing product quality. Some *Fusarium* species also produce mycotoxins, such as zearalenone, fumonisin, moniliformin, and trichothecenes, which can contaminate grains and pose a threat to animal and human health. *Fusarium oxysporum* is a well-known species that causes Fusarium wilt in many crops.

Pathogenic *Fusarium* species employ several mechanisms to infect plants and cause disease. These include:

- **Penetration:** *Fusarium* can enter plants through wounds, natural openings, or directly penetrate plant tissues (El-Sersawy et al., 2021).
- **Colonization:** Once inside the plant, *Fusarium* colonizes the vascular system and disrupts water and nutrient transport (El-Sersawy et al., 2021).
- **Enzyme production:** *Fusarium* secretes enzymes that degrade plant cell walls, aiding in tissue invasion and nutrient acquisition (Kikot et al., 2009).
- **Effector proteins:** small proteins like those encoded by SIX genes (Secreted in Xylem) manipulate plant immunity and enable the pathogen to colonize the plant successfully (Kaliapan et al., 2024).
- **Toxin production:** Many *Fusarium* species produce mycotoxins that are toxic to plants and animals and contribute to disease development and yield losses (Shabeer et al., 2021).

1.1.2 *Fusarium oxysporum* f. sp. *vasinfectum* (FOV)

Fusarium oxysporum is a widespread soilborne fungus that infects hundreds of plant species. It causes Fusarium wilt, leading to significant economic losses in various crops. The fungus colonizes the vascular system, leading to symptoms such as foliar chlorosis, wilting, and red-brown necrosis of vascular tissue. *F. oxysporum* is divided into different *formae speciales* (f. spp.) based on their host specificity. Some *formae speciales* also cause bulb rot or root rot. An example includes *Fusarium oxysporum* f. sp. *albedinis*. Another well-known example is *Fusarium oxysporum* f. sp. *vasinfectum* (FOV), which is a major concern in cotton production.

FOV was first identified in 1892 in cotton growing in sandy soils in Alabama. The disease was soon discovered in other major cotton-producing areas, including Egypt, India, and later in Tanzania, Sudan, Israel, China, Australia, California, and Brazil (Davis et al., 2006).

1.1.3 Crop yield losses due to FOV

Fusarium wilt is one of the most important and serious diseases caused by FOV. It causes enormous yield losses in various parts of the world and remains a threat to cotton production in the future. The disease is responsible for serious losses to the crop in the United States, China, western and central India, on a considerable scale, and in almost all the cultivated varieties (Davis et al., 2006). Cotton production in Pakistan has suffered a steep decline in recent years, dropping from 14.81 million bales (one bale equals about 170 kg) in 2012-13 to approximately 5.5 million bales in 2022-24. An estimated $4 billion annual loss and a $15 billion reduction in GDP. This crisis is largely due to climate change and the proliferation of Fusarium wilt, which has led to widespread crop failures. The decline of cotton, once a major economic driver, has significantly impacted the country's textile industry and overall financial stability (Aslam et al., 2025).

1.2 Plant-parasitic nematodes

Nematodes, also known as eelworms or threadworms (Greek: *nema* = thread), are unsegmented, ubiquitous roundworms that belong to the phylum Nematoda or Nemata. Plant-parasitic nematodes (PPN) comprise a relatively minor subset within the phylum Nematoda (Kumar et al., 2024). Currently, around 4100 species of PPN have been documented, constituting approximately 15% of the known nematode species (Sorribas et al., 2020). PPN have a considerable impact on agricultural food production

, and cause yield losses of an estimated $175 billion globally each year (Khan, 2023). These nematodes parasitize a wide range of plant species and are a significant constraint on global food security (Lima et al., 2018). A key feature of all PPN is the presence of a hollow, spear-like structure, known as a stylet, used to penetrate plant cells and extract nutrients. The stylet also facilitates the secretion of hormones, proteins, and metabolites that help to parasitize plants. This specialized structure distinguishes PPN from free-living nematodes, such as *Caenorhabditis elegans* (Escobar and Fenoll, 2015).

Sedentary endoparasitic nematodes are the most destructive nematodes in agricultural production. Their larvae penetrate the root and migrate through various tissue layers until they reach the vascular cylinder. Upon reaching there, they use their stylet to puncture individual cells, searching for one that can provide sufficient nutrients for their entire development. These nematodes rely on living host tissues acting as obligate biotrophic organisms. They create a complex relationship with their host. Infection by these sedentary PPN can lead to total yield loss. Root-knot nematodes (*Meloidogyne* spp.) and the cyst nematodes (*Globodera* spp. and *Heterodera* spp.) are the most significant PPN in this group (Clapp et al., 2000).

1.2.1 Cyst nematodes

Cyst nematodes represent a significant group of PPN with substantial economic importance worldwide. Huge yield losses in key crops, including cereals, rice, soybeans, and potatoes are a result of cyst nematodes. All cyst nematodes are sedentary endoparasites, feeding within the roots of their hosts. These nematodes possess cryptobiotic characteristics, enabling them to enter a state of suspended metabolic activity as a survival strategy during unfavorable environmental conditions. Root-knot nematodes are capable of surviving on a broad spectrum of alternate hosts, while cyst nematodes endure by creating a tanned brown cyst as these nematodes exhibit a unique life cycle characterized by the formation of a protective cyst. After invading host plant roots and establishing a feeding site, the female nematode's body swells, and she becomes sedentary. Following fertilization, the female produces numerous eggs, retaining them within her body. When female dies, her body undergoes a remarkable transformation: the cuticle hardens and tans thereby forming a tough, resilient structure known as a cyst. This cyst serves as a durable protective shell for the eggs which protects them from environmental stresses and ensures their survival in the soil for extended periods (Turner and Subbotin, 2006).

The life cycle of cyst nematodes begins when second-stage juveniles (J2) hatch from the cyst, exiting through natural openings or where the female's head has broken away. As a survival strategy, not all juveniles hatch and leave the cyst simultaneously; some J2 remain inside the cyst or in external egg masses. Those juveniles released into the soil migrate to the host, primarily following a chemical gradient produced by the host's root system (Turner and Subbotin, 2006).

Hatching in several cyst nematode species is triggered by chemical stimuli, such as exudates released by host roots, synchronizing their life cycle with the host plant's growth (Perry, 1997). Glycinoeclepin A, isolated from kidney bean roots, was the first characterized hatching factor, inducing hatching of *H. glycines* at very low concentrations. Later, researchers isolated two nortriterpenes with related structures that also affected *H. glycines* in the same way. Solanoeclepin A, a compound released by potato and tomato roots, has also been identified as a hatching stimulant for *Globodera* sp. (Schenk et al., 1999).

Apart from chemical compounds, factors like humidity, soil texture, and temperature are vital in the hatching process of different cyst nematodes and influence their distribution and host rang worldwide. For instance, *H. cruciferae* invades host plants in early spring or winter, while *H. zeae* which is commonly distributed in tropical regions needs a higher optimal temperature of 30°C. In comparison, the sugar beet cyst nematode *H. schachtii* is the most prevalent and economically important nematode in temperate regions. The amount and/or intensity of light during the growth of the host also affected hatching (Turner and Subbotin, 2006).

1.2.1.1 *Heterodera schachtii*

Scientific research on *H. schachtii*, the sugar beet cyst nematode, began in Germany in the mid-19th century. *H. schachtii* was first observed by Hermann schacht in 1859 and later described by Schmidt in 1871 (Stone, 1898). In addition to sugar beet, various other plant such as those in Brassicaceae and Amaranthaceae families are also parasitized by *H. schachtii*. It is a host of more than 200 species belonging to 98 genera (Kim et al., 2016). *H. schachtii* is considered an important model nematode due to its economic impact as a parasite of crops like sugar beets and cruciferous vegetables, making it a relevant subject for developing control strategies. Its relatively short life cycle and ease of laboratory culturing facilitate research on its biology and interactions with host plants. Furthermore, the availability of genetic and genomic resources makes *H. schachtii* a valuable tool for studying nematode-plant interactions at the molecular level.

1.2.1.2 Life cycle of cyst nematode

Cysts hatch and infective J2 move toward the roots of host plants, guided by a gradient of stimuli emanating from the root zone. Some of the stimuli, including amino acids, pH, CO_2, and sugar gradients, serve as general attractants, prompting the nematodes to migrate over considerable distances. This chemotaxis is crucial for the nematodes to locate a suitable host, as they rely on the host for nutrients and reproduction (Perry, 1997).

Upon reaching the host plant, J2 predominantly invade the roots in the elongation zone. They then migrate intracellularly through the cortical tissue towards the pericycle. The J2 use their stylet to pierce cells until they identify an appropriate initial syncytial cell (ISC) (Sijmons et al., 1991). Since the ISC must ensure a continuous flow of nutrients to the nematode, the parasitism process must be carried out carefully. The J2 cautiously insert their stylet into the host cell, avoiding damage to the plasmalemma, and remain immobile for 6-8 hours (Wyss and Zunke, 1986; Wyss and Grundler, 1992). Subsequently, J2 initiates the formation of a feeding tube composed of saliva produced by its pharyngeal glands. This feeding tube acts as a selective membrane and controls the uptake and release of specific molecules from and into the plant cell, regulated by the pharyngeal pump.

After the feeding tube is established, the infected root cell undergoes significant reprogramming. Neighboring cells are incorporated through local cell wall dissolutions, and large, multinucleated, and hypertrophied syncytia are formed. During syncytium formation, several smaller vacuoles replace a large central vacuole. The nuclei undergo hypertrophy, and the number of organelles, including smooth endoplasmic reticulum, increases, particularly, creating a metabolically active nutrient source. During the subsequent two weeks, the syncytium expands, and the nematode progresses through three more molting stages (J3, J4, and adult) to become adult. Interestingly, sex determination in these nematodes appears to be environmentally influenced. Nematodes that establish large feeding sites with ample nutrient access tend to become females, while those inducing smaller syncytia lacking vascular tissue access develop into males. Male cyst nematodes exit the root system in search of sedentary females for reproduction. Following mating, the female's body becomes filled with eggs, eventually transforming into a protective cyst upon her death, safeguarding the eggs form harsh environment (Baldwin and Mundo-Ocampo, 1991; Grundler et al., 1991).

In the current study *H. schachtii* was used as a model nematode along with *Arabidopsis thaliana,* as these are widely used as a model system for the study of plant-nematode interactions (Sijmons et al., 1991). This system offers several advantages. *Arabidopsis* has a small, well-characterized genome, short generation time, efficient transformation methods, and extensive genetic resources, making it ideal for molecular studies (Sijmons et al., 1991). Furthermore, this system is valuable for investigating biological control agents, such as rhizobacteria, that can suppress nematode parasitism (Huang et al., 2021). Researchers can study the mechanisms by which these agents interfere with nematode infection, development, and reproduction. For example, the use of *Bacillus* species has been explored as a biocontrol method.

1.3 Management strategies for plant-parasitic fungi and nematodes

Managing PPN and fungi requires a multifaceted approach, integrating various strategies to minimize crop damage and promote sustainable agriculture. Since nematodes and fungi have developed adaptive strategies to survive, their control is not easy, and eradicating this problem is out of our reach. Therefore, reducing the parasitic population and the damage caused to crops is the most realistic expectation. An overview of some common management strategies is given below.

Cultural practices:

- **Crop rotation:** Many fungal pathogens are host-specific or have a limited host range. By rotating to a non-host crop, the fungus is deprived for food source, causing its population to decline over time (Humphrey, 1891). For example, rotating away from susceptible crops like tomatoes, potatoes, or cotton to non-host crops like corn or small grains can help reduce Fusarium wilt pressure in the soil. Some rotation crops may also have antagonistic effects on fungal pathogens by promoting beneficial soil microbes that suppress the pathogen (Peralta et al., 2018).

  Like fungi, many PPN also have specific host preferences. Rotating to a non-host crop can significantly reduce nematode populations. Some rotation crops may also act as "trap crops," stimulating nematode hatch but preventing them from completing their life cycle. Other rotation crops may release compounds that are toxic to nematodes or promote beneficial nematode-antagonistic organisms. Such as mustard, and rapeseed, produce glucosinolates. When these compounds break down in the soil, they release isothiocyanates, which have nematicidal

properties. The effectiveness depends on the specific *Brassica* species, the concentration of glucosinolates, and soil conditions (Jing, 1994).

Crop rotation, though useful is for managing soilborne disease and insects such as fungi and nematodes, has disadvantages that range from potential decreased profitability from rotating to less valuable crops, planning and logistical challenges in execution, and biological constraints such as lack of efficacy against insects with wide host ranges or the ability to survive for extended periods in the soil. Sophisticated rotations require expert information and forward planning, and may be limited by land availability, climate, and soil type. Solutions to these challenges involve diversifying rotations, cover cropping, integrating with other pest management strategies, providing education and support to farmers, and developing markets for alternative crops (Selim, 2019)

- **Soil solarization:** This involves covering the soil with transparent plastic during the hot summer months to raise the soil temperature and kill nematodes and fungal pathogens (Palti, 2012). It has several limitations such as it is a broad-spectrum method that affects a wide range of soil organisms, both harmful and beneficial, it is climate-dependent and requires sunny and warm conditions for 4-6 weeks and its effectiveness decreases with soil depth. The use of plastic film can also create environmental concerns due to plastic waste, and the cost of materials and labor can be a limiting factor (Wada et al., 2024).

- **Sanitation:** Sanitation is an important aspect of disease management in agriculture. It involves practices that minimize pathogen introduction, survival, and spread (Palti, 2012). Effective sanitation begins with using disease-free seeds and seedlings, followed by diligent removal and destruction of infected plant debris to eliminate sources of inoculum. Weed control is also essential to prevent weeds from serving as alternative hosts for pathogens. Regularly cleaning and disinfecting farm equipment, tools, and machinery helps prevent the mechanical transmission of diseases between fields. Proper storage of harvested crops further reduces the risk of spoilage and pathogen proliferation. Growers can significantly reduce disease pressure and protect their crops from devastating outbreaks by implementing these sanitation measures. This method also has limitations as is labor-intensive and costly to implement. Proper disposal of infected material can be challenging, and incorrect implementation may spread diseases. Some

practices disrupt beneficial organisms and may not be effective against all pathogens, especially airborne ones (Tarr, 1972).

- **Host resistance:** Host resistance is a method of controlling plant diseases that involves using plant varieties that are genetically resistant to specific pathogens. This resistance can be achieved through traditional breeding techniques or through genetic engineering. Planting crop varieties that are resistant to specific nematode or fungal species is an effective way to minimize damage (de Vallavieille-Pope, 2004). Although host resistance is environmentally friendly and cost-effective, developing resistant varieties can be time-consuming, and resistance may break down if pathogens evolve. Additionally, resistant varieties may not be available for all crops or diseases, and can sometimes result in reduced yield potential (Vanderplank, 2012).

Chemical control:

- **Nematicides and fungicides:** Synthetic chemicals can be used to control nematode and fungal populations, but they should be used judiciously due to their potential impact on the environment and human health (Chen et al., 2020). Synthetic fungicides and nematicides, while effective, pose potential risks to human health and the environment. The regulatory restrictions imposed on many effective nematicides due to their adverse environmental impacts have further limited their availability and use and registration of synthetic chemicals for plant disease control in many countries faces hurdles such as extensive data requirements, high costs, lengthy review processes, and stringent safety standards. Public perception, data gaps, lack of regulatory harmonization, residue limit concerns, and impacts on beneficial organisms also pose challenges to registration (Ravichandra, 2018).

Biological control:

Biological control methods use living organisms or their products to suppress or control plant pathogens and diseases, providing an alternative to synthetic chemicals with a more environmentally friendly approach. Certain bacteria, fungi, and other microorganisms can suppress PPN and fungal populations. Biological control offers a more sustainable and environmentally sound approach, reducing fungal and nematode populations by using natural enemies or beneficial microorganisms that produce bioactive molecules to suppress, antagonize, and control plant pathogens. Plant growth-promoting rhizobacteria

(PGPR) are an important part of biological control strategies. While biocontrol may require more time to be effective and its efficacy can vary, it avoids the harmful side effects of synthetic plant protection roducts and contributes to a healthier ecosystem (Verma et al., 2019).

1.3.1 Rhizosphere and plant growth-promoting rhizobacteria

In 1904, Lorenz Hiltner, a German agronomist and plant physiologist, introduced the term "rhizosphere" to describe the interface between plant roots and soil. The term is derived from the Greek word "rhiza" meaning root, refers to the area around a plant root that hosts a unique microbial population influenced by root exudates (chemicals released from the root) (Zodge et al., 2024). Since Hiltner's orignal description, the definition of the rhizosphere has been extended to encompass three zones based on their proximity to and influence from the root: the endorhizosphere, rhizoplane, and ectorhizosphere. The rhizosphere does not possess a definable shape or size due to the inherent complexity and diversity of plant root systems. It is characterized by a gradient in chemical, physical, and biological properties that vary both longitudinally and radially along the root. In the rhizosphere microbial populations are significantly higher than in bulk soil, highlighting the unique interactions that occur in this zone (York et al., 2016).

Diversified species of microorganisms are present in the plant rhizosphere, most of which are known as plant growth-promoting rhizobacteria (PGPR). These PGPR colonize plant roots, facilitate plant development and suppress the growth of plant pathogens, and markedly enhance the crop yield. The proportion of PGPR in the rhizosphere, the soil zone surrounding plant roots, is highly variable and influenced by several factors. These include soil type, plant species, environmental conditions like moisture and temperature, and agricultural practices. Plant roots release several carbon-rich compounds into the rhizosphere, such as amino acids, organic acids, and carbon, resulting in rhizodeposition. The composition of root exudates relies on the plant cultivar, species, soil condition, plant growth stage, and plant growth substrate. Soil microorganisms, together with beneficial bacteria, are attracted towards root exudates to use them as a source of carbon and energy. Secretion of root exudates enhanced the abundance, activity, and diversity of rhizosphere bacteria (Huang et al., 2021).

Several species of *Pseudomonas, Bacillus, Burkholderia, Alcaligens, Klebsiella, Paenibacillus*, *Azospirillum, Enterobacter, Arthrobacter, Azotobacter, Lysobacter, Serratia, Azoarcus, Streptomyces, Rhizobium, Pantoea, Rhodococcus* and *Bradyrhizobium* etc. are well known for their plant growth promoting and

biocontrol potential (Deshwal et al., 2003; Pacwa-Płociniczak et al., 2016; Plucani do Amaral et al., 2023; Ansari et al., 2024; Espinosa-Palomeque et al., 2025; Li et al., 2025; Smith et al., 2025).

1.3.2 *Bacillus* species

Bacillus, discovered by Cohn in 1872, is a genus of heat-resistant, endospore-forming bacteria. Members of this genus are readily isolated from diverse environments, including water, municipal and kitchen waste, soil, and even extreme conditions. *Bacillus* spp. are favored for commercial applications due to their ability to produce various bioactive compounds, their simple growth requirements in ordinary media, and their ease of maintenance and preservation (Miljaković et al., 2020). The *Bacillus* genus encompasses a wide range of species, including plant beneficial ones such as *B. cereus*, *B. pumilus*, *B. subtilis*, *B. stercoris*, *B. amyloliquefaciens*, *B. licheniformis*, *B. thuringiensis*, *B. megaterium*, *B. velezensis*, *B. clausii-halodurans*, and *Bacillus sp.* NRRLB-14911-coahuilensis etc (Mazzola and Freilich, 2017; Korangi Alleluya et al., 2023). In the rhizosphere, *Bacillus* species exhibit dynamic interactions with other bacterial communities, producing plant growth-promoting factors and enhancing soil fertility. Additionally, *Bacillus* species have been found within the internal tissues of plants like *Gossypium barbadense L.* (cotton), and *Vitis vinifera* (grapes) where they colonize their roots, play a role in plant development and biological control against several plant pathogens (Selim et al., 2017).

A key characteristic of the *Bacillus* genus is its ability to endure extreme environments by forming endospores, which are resting-stage structures. These endospores can survive starvation, toxic conditions, extreme temperatures, salinity, and pH levels. Under favorable conditions, the endospores germinate, giving rise to vegetative cells. This resilience makes *Bacillus* species valuable in various biotechnological applications within both industry and agriculture. Several *Bacillus* species, including *B. pumilus*, *B. amyloliquefaciens*, *B. thuringiensis*, *B. subtilis,* and *B. licheniformis* have demonstrated insecticidal activity and are used in agriculture to control insects and other plant pathogens (Villarreal-Delgado et al., 2018). For example, *B. subtilis* strains isolated from *Zea mays* have been shown to strongly inhibit *Botrytis cinerea in vitro* (Bolivar-Anillo et al., 2021). Research has also demonstrated that seed coating or direct soil introduction of *B. subtilis* can effectively antagonize *Rhizoctonia cerealis,* a wheat pathogen. Some *Bacillus* species, such as *B. cereus*, produce metalloproteinases and lipopeptides, which show broad-spectrum activity against PPN (Vasantha-Srinivasan et al., 2025).

Bacillus velezensis CMRP 4489 has been identified and applied as a novel biocontrol agent. Genomic analysis of this strain, including mapping secondary metabolite clusters and examining genes responsible for the formation of biofilm, provides evidence that its biocontrol properties are genetically encoded and stable (Baptista et al., 2022). Among endophytic bacteria, *B. amyloliquefaciens* 3–5, isolated from healthy potato tubers, has been reported to have optimal antagonistic properties (38.90%) against *Streptomyces griseoplanus*, which causes potato scab (Cui et al., 2022).

These *Bacillus* strains also possess plant growth-promoting traits, such as nitrogen fixation and the production of indole-3-acetic acid. Molecular analysis revealed that *B. amyloliquefaciens* 3–5 shows six diverse antibacterial genes, namely *bacAB, ituD*, *bacD*, *ituC*, *sfP*, and *albF,* which contribute to the production of bioactive compounds like iturin, surfactin, bacilysin, and subtilosin. Consequently, the endophytic bacterium *B. amyloliquefaciens* 3–5 shows promise as an environmentally friendly and efficient biocontrol agent for mitigating potato scab (Cui et al., 2022).

1.3.3 Mechanisms of action of PGPR

PGPR can promote plant growth directly or indirectly by antagonizing plant pathogens through different mechanisms

Direct mechanisms of action: PGPR directly promote plant growth by providing synthesized compounds or facilitating nutrient uptake. These mechanisms include:

- **Phytohormone production:** PGPR can synthesize phytohormones such as auxins (e.g., indole-3-acetic acid or IAA), gibberellins, and cytokinins. IAA, for instance, plays a role in root initiation, leaf formation, and fruit development (Ranjan et al., 2024).

- **Nutrient fixation and solubilization:** Certain PGPR can fix atmospheric nitrogen, converting it into usable forms for plants. Others can solubilize phosphorus, potassium, and zinc, making it accessible to plants (Hasan et al., 2024).

Indirect mechanisms of action: PGPR can promote plant growth indirectly by suppressing plant pathogens through several mechanisms. These mechanisms include;

- **Volatile Organic Compounds (VOCs):** PGPR can produce VOCs. In general, VOCs produced by PGPR can indirectly influence plant growth and development (Vejan et al., 2016).These

compounds can directly kill plant pathogens, induce systemic resistance, and enhance stress resistance. *Pseudomonas fluorescens* SS101 produces several VOCs that promote plant growth, including 13-tetradecadien-1-ol, 2-butanone, and 2-methyl-n-1-tridecene (Park et al., 2015).

- **ACC deaminase production:** PGPR can produce ACC deaminase, which lowers ethylene levels in plants, reducing stress and promoting growth (PANWAR, 2024).

- **Siderophore production:** PGPR can produce siderophore compounds. Siderophores are iron-binding ligands that biocontrol agents secrete to thrive in iron-limiting environments like the rhizosphere. These compounds have a particularly high affinity for iron, effectively scavenging it and making it available for the producing organisms, and sometimes the host plants. This is a critical function because iron is an essential nutrient often with limited availability in soil. Many rhizospheric bacteria are known to produce siderophores, and certain plants have developed the ability to use the Fe^{3+} siderophore complexes produced by these bacteria. Interestingly, the role of microbial siderophores is usually associated more with their contribution to biological control mechanisms in the rhizosphere rather than direct competition with plants for nutrients. In agricultural systems, siderophores play a crucial role in suppressing phytopathogens. For example, the ability of *B. velezensis* YL2021 to control *Rhizoctonia solani* and *Magnaporthe oryzae* was correlated with the production of siderophore (Liu et al., 2024). The study of siderophores thus presents promising avenues for developing eco-friendly and effective disease control and nutrient management strategies in various contexts.

- **Hydrolytic enzyme production:** PGPR can produce amylase, urease, pectinase, chitinase, β-1,3-glucanase, protease, and cellulase. These enzymes degrade structural polysaccharides (chitin, glucans) and proteins in fungal cell walls, leading to lysis and death of phytopathogens. Beyond direct pathogen suppression, PGPR hydrolases can break down organic matter (cellulose, starch, proteins), releasing sugars, amino acids, and nutrients into the rhizosphere (Jadhav et al., 2017).

- **Antibiotic production:** PGPR can synthesize antibiotics that can function in different ways to suppress the activities of plant pathogens. These antibiotics may be water-soluble, volatile, or non-volatile compounds. Many of these bacteria-derived compounds are reported to be as effective as chemical pesticides in controlling plant pathogens. *Pseudomonas* and *Bacillus species* are particularly recognized for their ability to produce a wide range of secondary metabolites that

are instrumental in combating plant pathogens. *B. subtilis* strains, for example, are known to synthesize over a dozen antibiotics, including iturin, mycobacillin, bacillomicin, fungistatin, plipastatin, surfactin, bacilysin and fengycin, among these, pyoluteorin, pyrrolnitrin, phenazines, 2,4-diacetylphloroglucinol, biosurfactants, hydrogen cyanide, fengycin, iturin, and surfactin are frequently detected and contribute to the biocontrol efficacy of the *Bacillus* strains (Stein, 2005).

- **Induced resistance:** PGPR can enhance plant defense mechanisms through induced resistance, where plants are primed to defend against abiotic stressors or microbial pathogens. There are two primary forms of induced resistance: localized and systemic induced resistance. Both types offer broad-spectrum protection against a variety of pathogens. Many plants exhibit localized resistance at the site of pathogen attack, while systemic resistance involves a whole-plant response that can protect distant, uninfected parts as well. Phytopathologists are deeply engaged in unraveling the mechanisms underlying induced resistance, examining how non-pathogenic microorganisms exploit these pathways to bolster plant defenses. Systemic acquired resistance (SAR) is a well-characterized form of induced resistance that is mediated by salicylic acid, a compound typically produced in response to pathogen attack, leading to the expression of pathogenesis-related proteins (Vallad and Goodman, 2004). The second form, induced systemic resistance (ISR), is associated with the production of jasmonic acid or ethylene subsequent to contact with certain non-pathogenic rhizobacteria. These pathways can be triggered by treatments or colonization with specific rhizosphere microorganisms, such as strains of *Pseudomonas,* which have been shown to protect plants against numerous pathogens, including *Pseudomonas syringae pv. lachrymans, Colletotrichum gloeosporioides,* and *Aspergillus flavus* (Kamle et al., 2020). PGPR produces various chemical elicitors that can induce SAR and ISR, including lipopolysaccharides, 2,3-butanediol, siderophores, salicylic acid, and an assortment of volatile compounds. These elicitors can stimulate the plant's defense pathways, thereby enhancing resistance against a spectrum of pathogens and playing a pivotal role in the biocontrol strategy.

1.4 Study objectives

This research investigates the microbiome of cotton and wheat rhizosphere under continuous cotton - wheat rotation in Pakistan. Cotton is a major economic crop in Pakistan, and its rotation with wheat is a common agricultural practice. However, the microbial communities in the rhizosphere under continuous cotton - wheat rotation in different fields across Pakistan have not been extensively explored. Therefore, this study aims to address this gap with the following objectives:

1. Analysis of the composition of microbial communities (rhizobacteria and fungi) of the cotton rhizosphere under continuous cotton - wheat rotation
2. Biochemical and genetic characterization of PGPR isolated from cotton and wheat rhizosphere
3. Investigation of the biocontrol potential of isolated cotton rhizobacteria against Fusarium wilt caused by *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) in cotton and their effect on plant growth
4. Investigation of the biocontrol potential of isolated cotton and wheat rhizobacteria against *Heterodera schachtii* parasitism in *Arabidopsis thaliana* and their effect on plant growth

1.5 References

Abubakar, M., Sheeraz, M., Sajid, M., Mehmood, Y., Jamil, H., Irfan, M., et al. (2023). Analysis of Cotton Value Chain in Pakistan: Identifying the Process and Critical Factors in Sustainable Agribusinesses. *Journal of Arable Crops and Marketing* 5(2)**,** 63-74. doi: 10.33687/jacm.005.02.5208.

Ansari, F.A., Ahmad, I., Pichtel, J., and Husain, F.M. (2024). Pantoea agglomerans FAP10: A novel biofilm-producing PGPR strain improves wheat growth and soil resilience under salinity stress. *Environmental and Experimental Botany* 222**,** 105759. doi: 10.1016/j.envexpbot.2024.105759.

Aslam, S., Baber, M., Naqqash, T., Javed, M., Bredenbruch, S., Grundler, F.M.W, et al. (2025). Rhizospheric *Bacillus* isolates control Fusarium wilt on cotton and enhance plant biomass and root development. *Frontiers in Microbiology* 16**,** 1580937. doi: 10.3389/fmicb.2025.1580937.

Baldwin, J.G., and Mundo-Ocampo, M. (1991). "Heteroderinae, cyst-and non-cyst-forming nematodes," in *Manual of agricultural nematology,* ed. W.R. Nickle.), 275-362.

Baptista, J.P., Teixeira, G.M., de Jesus, M.L.A., Bertê, R., Higashi, A., Mosela, M., et al. (2022). Antifungal activity and genomic characterization of the biocontrol agent *Bacillus velezensis* CMRP 4489. *Scientific Reports* 12(1)**,** 17401. doi: 10.1038/s41598-022-22380-0.

Bolivar-Anillo, H.J., González-Rodríguez, V.E., Cantoral, J.M., García-Sánchez, D., Collado, I.G., and Garrido, C. (2021). Endophytic bacteria *Bacillus subtilis*, isolated from Zea mays, as potential biocontrol agent against Botrytis cinerea. *Biology* 10(6)**,** 492. doi: 10.3390/biology10060492.

Chen, J., Li, Q.X., and Song, B. (2020). Chemical nematicides: Recent research progress and outlook. *Journal of Agricultural and Food Chemistry* 68(44)**,** 12175-12188. doi: 10.1021/acs.jafc.0c02871.

Cianchetta, A.N., and Davis, R. (2015). Fusarium wilt of cotton: Management strategies. *Crop Protection* 73**,** 40-44. doi: 10.1016/j.cropro.2015.01.014.

Clapp, J.P., Stoel, C.v.d., and Putten, W.v.d. (2000). Rapid identification of cyst (Heterodera spp., Globodera spp.) and root-knot (Meloidogyne spp.) nematodes on the basis of ITS2 sequence variation detected by PCR-single-strand conformational polymorphism (PCR-SSCP) in cultures and field samples. *Molecular Ecology* 9(9)**,** 1223-1232. doi: 10.1046/j.1365-294x.2000.00995.x.

Cui, L., Yang, C., Wang, Y., Ma, T., Cai, F., Wei, L., et al. (2022). Potential of an endophytic bacteria Bacillus amyloliquefaciens 3–5 as biocontrol agent against potato scab. *Microbial pathogenesis* 163**,** 105382. doi: 10.1016/j.micpath.2021.105382.

Davis, R., Colyer, P., Rothrock, C., and Kochman, J. (2006). Fusarium wilt of cotton: population diversity and implications for management. *Plant Disease* 90(6)**,** 692-703. doi: 10.1094/PD-90-0692.

De Rodriguez, D.J., Hernández-Castillo, D., Angulo-Sánchez, J., Rodríguez-García, R., Quintanilla, J.V., and Lira-Saldivar, R. (2007). Antifungal activity in vitro of Flourensia spp. extracts on Alternaria sp., Rhizoctonia solani, and *Fusarium oxysporum. Industrial Crops and Products* 25(2)**,** 111-116. doi: org/10.1016/j.indcrop.2006.08.007.

de Vallavieille-Pope, C. (2004). Management of disease resistance diversity of cultivars of a species in single fields: controlling epidemics. *Comptes rendus biologies* 327(7)**,** 611-620. doi: 10.1016/j.crvi.2003.11.014.

Deshwal, V., Dubey, R., and Maheshwari, D. (2003). Isolation of plant growth-promoting strains of Bradyrhizobium (Arachis) sp. with biocontrol potential against Macrophomina phaseolina causing charcoal rot of peanut. *Current Science***,** 443-448. doi: org/stable/24107431.

El-Sersawy, M.M., Hassan, S.E.-D., El-Ghamry, A.A., El-Gwad, A.M.A., and Fouda, A. (2021). Implication of plant growth-promoting rhizobacteria of *Bacillus* spp. as biocontrol agents against wilt disease caused by Fusarium oxysporum Schlecht. in Vicia faba L. *Biomolecular Concepts* 12(1)**,** 197-214. doi: 10.1515/bmc-2021-0020.

Escobar, C., and Fenoll, C. (2015). *Plant nematode interactions: a view on compatible interrelationships.* Academic Press.

Espinosa-Palomeque, B., Jiménez-Pérez, O., Ramírez-Gottfried, R.I., Preciado-Rangel, P., Buendía-García, A., Sifuentes, G.Z., et al. (2025). Biocontrol of Phytopathogens Using Plant Growth Promoting Rhizobacteria: Bibliometric Analysis and Systematic Review. *Horticulturae* 11(3)**,** 271. doi: 10.3390/horticulturae11030271.

Gouda, S.A., Hassanein, N.M., and Salah, M. (2025). Antifungal Activity of Boron/Selenium Nanoparticles Irradiated via Gamma Rays Against *Alternaria alternata* and *Fusarium equiseti. Current Microbiology* 82(3)**,** 129. doi: 10.1007/s00284-025-04089-1.

Grundler, F.M.W, Betka, M., and Wyss, U. (1991). Influence of changes in the nurse cell system (syncytium) on sex determination and development of the cyst nematode *Heterodera schachtii*: total amounts of proteins and amino acids. *Phytopathology* 81(1)**,** 70-74.

Guo, D. (2022). A review of sustainable agricultre research in global cotton cropping systems.

Hasan, A., Tabassum, B., Hashim, M., and Khan, N. (2024). Role of plant growth promoting rhizobacteria (PGPR) as a plant growth enhancer for sustainable agriculture: A review. *Bacteria* 3(2)**,** 59-75. doi: 10.3390/bacteria3020005.

Huang, M., Bulut, A., Shrestha, B., Matera, C., Grundler, F.M.W, and Schleker, A.S.S. (2021). *Bacillus firmus* I-1582 promotes plant growth and impairs infection and development of the cyst nematode

Heterodera schachtii over two generations. *Scientific reports* 11(1)**,** 14114. doi: 10.1038/s41598-021-93567-0.

Humphrey, J.E. (1891). Treatment of Fungous Diseases. *Science* (428)**,** 212-213. doi: 10.1126/science.ns-17.428.212.

Jadhav, H., Shaikh, S., and Sayyed, R. (2017). "Role of hydrolytic enzymes of rhizoflora in biocontrol of fungal phytopathogens: an overview," in *Rhizotrophs: Plant growth promotion to bioremediation,* ed. S. Mehnaz. (Singapore: Springer), 183-203.

Jing, G.N. (1994). *Evaluation of the nematicidal properties of cruciferous plants for nematode management in replant orchards.* The Pennsylvania State University.

Kaliapan, K., Mazlin, S.N.A., Chua, K.O., Rejab, N.A., and Mohd-Yusuf, Y. (2024). Secreted in Xylem (SIX) genes in Fusarium oxysporum f. sp. cubense (Foc) unravels the potential biomarkers for early detection of Fusarium wilt disease. *Archives of Microbiology* 206(6)**,** 271. doi: 10.1007/s00203-024-03996-4.

Kamle, M., Borah, R., Bora, H., Jaiswal, A.K., Singh, R.K., and Kumar, P. (2020). "Systemic acquired resistance (SAR) and induced systemic resistance (ISR): role and mechanism of action against phytopathogens," in *Fungal biotechnology and bioengineering,* ed. R.S.U. Abd El-Latif Hesham, Gauri Dutt Sharma, Chakravarthula Manoharachary, Vijai Kumar Gupta. (Cham: Springer), 457-470.

Khan, M.R. (2023). "Nematode pests of agricultural crops, a global overview," in *Novel Biological and Biotechnological Applications in Plant Nematode Management,* ed. M.R. Khan. (Singapore: Springer), 3-45.

Kikot, G.E., Hours, R.A., and Alconada, T.M. (2009). Contribution of cell wall degrading enzymes to pathogenesis of Fusarium graminearum: a review. *Journal of basic microbiology* 49(3)**,** 231-241. doi: 10.102/jobm.200800231.

Kim, D.H., Cho, M.R., Yang, C.Y., Kim, H.H., Kang, T.J., and Yoon, J.B. (2016). Host range screening of the sugar beet nematode, H*eterodera schachtii* Schmidt. *Korean J. Appl. Entomol* 55(4)**,** 389-403. doi: https: 10.5656/KSAE.2016.10.0.057.

Korangi Alleluya, V., Argüelles Arias, A., Ribeiro, B., De Coninck, B., Helmus, C., Delaplace, P., et al. (2023). *Bacillus* lipopeptide-mediated biocontrol of peanut stem rot caused by Athelia rolfsii. *Frontiers in Plant Science* 14**,** 1069971. doi: 10.3389/fpls.2023.1069971.

Kumar, S., Abha, R., Sonkar, V.K., Mitra, D.S., and Meena, D.K. (2024). ROOT-KNOT NEMATODE MANAGEMENT IN VEGETABLE CROPS: A REVIEW. *Plant Archives (09725210)* 24(2). doi: 10.51470/PLANTARCHIVES.2024.v24.no.2.086.

Li, H., Liu, G., Zhang, D.-x., Lin, X., Liu, G., Xu, S., et al. (2021). Wheat Root Protection From Cereal Cyst Nematode (*Heterodera avenae*) by Fluopyram Seed Treatment. *Plant Disease* 105(9)**,** 2466-2471. doi: 10.1094/PDIS-08-20-1851-RE.

Li, X., Pan, C., Wang, H., Shen, Y., Li, Y., and Du, L. (2025). Heterologous Production of Phenazines in the Biocontrol Agent Lysobacter enzymogenes C3. *Journal of Agricultural and Food Chemistry* 73(2)**,** 1345-1355. doi: 10.1021/acs.jafc.4c09518.

Lima, F.S., Mattos, V.S., Silva, E.S., Carvalho, M.A., Teixeira, R.A., Silva, J.C., et al. (2018). *Nematodes affecting potato and sustainable practices for their management.* IntechOpen.

Liu, Y., Dai, C., Zuo, Y., Qiao, J., Shen, J., Yin, X., et al. (2024). Characterization of Siderophores Produced by *Bacillus velezensis* YL2021 and Its Application in Controlling Rice Sheath Blight and Rice Blast. *Phytopathology®* 114(12)**,** 2491-2501. doi: 10.1094/PHYTO-04-24-0148-R.

Loiko, N., and Islam, M.N. (2024). Plant–soil microbial interaction: Differential adaptations of beneficial vs. pathogenic bacterial and fungal communities to climate-induced drought. *Agronomy* 14(9)**,** 1949. doi: 10.3390/agronomy14091949.

Mazzola, M., and Freilich, S. (2017). Prospects for biological soilborne disease control: application of indigenous versus synthetic microbiomes. *Phytopathology* 107(3)**,** 256-263.

Miljaković, D., Marinković, J., and Balešević-Tubić, S. (2020). The significance of Bacillus spp. in disease suppression and growth promotion of field and vegetable crops. *Microorganisms* 8(7)**,** 1037. doi: 10.3390/microorganisms8071037.

Moya-Elizondo, E.A., Rew, L.J., Jacobsen, B.J., Hogg, A.C., and Dyer, A.T. (2011). Distribution and prevalence of Fusarium crown rot and common root rot pathogens of wheat in Montana. *Plant Disease* 95(9)**,** 1099-1108. doi: 10.1094/PDIS-11-10-0795.

Oliver, R.P. (2024). "Diseases caused by fungi," in *Agrios' Plant Pathology*. Richard P. Oliver ed: Elsevier), 339-427.

Pacwa-Płociniczak, M., Płociniczak, T., Iwan, J., Żarska, M., Chorążewski, M., Dzida, M., et al. (2016). Isolation of hydrocarbon-degrading and biosurfactant-producing bacteria and assessment their plant growth-promoting traits. *Journal of environmental management* 168**,** 175-184. doi: 10.1016/j.jenvman.2015.11.058.

Palti, J. (2012). *Cultural practices and infectious crop diseases.* Springer Science & Business Media.

PANWAR, H. (2024). "CHAPTER TWELVE SIGNIFICANCE OF MICROBIAL 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) DEAMINASE IN MITIGATING SALT STRESS IN CROPS," in *Rhizospheric Interactions for Abiotic Stress Mitigation,* ed. P.C. Arvind Kumar Rai, Nirmalendu Basak, Parul Sundha, Rajender Kumar Yadav. (Newcastle upon Tyne, UK: Cambridge Scholars Publishing), 257.

Park, Y.-S., Dutta, S., Ann, M., Raaijmakers, J.M., and Park, K. (2015). Promotion of plant growth by *Pseudomonas fluorescens* strain SS101 via novel volatile organic compounds. *Biochemical and Biophysical Research Communications* 461(2)**,** 361-365. doi: 10.1016/j.bbrc.2015.04.039.

Peralta, A.L., Sun, Y., McDaniel, M.D., and Lennon, J.T. (2018). Crop rotational diversity increases disease suppressive capacity of soil microbiomes. *Ecosphere* 9(5)**,** e02235. doi: 10.1002/ecs2.2235.

Perry, R.N. (1997). "Plant signals in nematode hatching and attraction," in *Cellular and molecular aspects of plant-nematode interactions,* ed. F.M.W.G. Carmen Fenoll, S. A. Ohl Springer), 38-50.

Plucani do Amaral, F., Wang, J., Williams, J., Tuleski, T.R., Joshi, T., Ferreira, M.A., et al. (2023). Mapping genetic variation in Arabidopsis in response to plant growth-promoting bacterium Azoarcus olearius DQS-4T. *Microorganisms* 11(2)**,** 331. doi: 10.3390/microorganisms11020331.

Ranjan, A., Rajput, V.D., Prazdnova, E.V., Gurnani, M., Sharma, S., Bhardwaj, P., et al. (2024). Augmenting abiotic stress tolerance and root architecture: The function of phytohormone-producing PGPR and their interaction with nanoparticles. *South African Journal of Botany* 167**,** 612-629. doi: 10.1016/j.sajb.2024.02.041.

Ravichandra, N. (2018). *Agrochemicals in plant disease management.* India: Scientific publishers.

Ray, M., Ray, A., Dash, S., Mishra, A., Achary, K.G., Nayak, S., et al. (2017). Fungal disease detection in plants: Traditional assays, novel diagnostic techniques and biosensors. *Biosensors and Bioelectronics* 87**,** 708-723. doi: 10.1016/j.bios.2016.09.032.

Sajjad, A., Anjum, S.A., Ahmad, R., and Waraich, E.A. (2018). Relay cropping of wheat (Triticum aestivum L.) in cotton (Gossypium hirsutum L.) improves the profitability of cotton-wheat cropping system in Punjab, Pakistan. *Environmental Science and Pollution Research* 25**,** 782-789. doi: 10.1007/s11356-017-0422-3.

Schenk, H., Driessen, R.A., de Gelder, R., Goubitz, K., Nieboer, H., Brüggemann-Rotgans, I.E., et al. (1999). Elucidation of the structure of solanoeclepin A, a natural hatching factor of potato and tomato cyst nematodes, by single-crystal X-ray diffraction. *Croatica Chemica Acta* 72(2-3)**,** 593-606.

Selim, H.M., Gomaa, N.M., and Essa, A.M. (2017). Application of endophytic bacteria for the biocontrol of Rhizoctonia solani (Cantharellales: ceratobasidiaceae) damping-off disease in cotton seedlings. *Biocontrol Science and Technology* 27(1)**,** 81-95. doi: 10.1080/09583157.2016.1258452.

Selim, M. (2019). A review of advantages, disadvantages and challenges of crop rotations. *Egyptian Journal of Agronomy* 41(1)**,** 1-10. doi: 10.21608/agro.2019.6606.1139.

Shabeer, S., Tahira, R., and Jamal, A. (2021). Fusarium spp. mycotoxin production, diseases and their management: An overview. *Pakistan Journal of Agricultural Research* 34(2)**,** 278. doi: 10.17582/journal.pjar/2021/34.2.278.293.

Sijmons, P.C., Grundler, F.M.W, von Mende, N., Burrows, P.R., and Wyss, U. (1991). *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *The Plant Journal* 1(2)**,** 245-254. doi: 10.1111/j.1365-313X.1991.00245.x.

Singh, Y. (2023). *A Textbook Of Fungi, Bacteria And Viruses.* Academic Guru Publishing House.

Smith, E., Daniel, A.I., Smith, C., Fisher, S., Nkomo, M., Keyster, M., et al. (2025). Exploring Paenibacillus terrae B6a as a sustainable biocontrol agent for Fusarium proliferatum. *Frontiers in Microbiology* 16**,** 1547571. doi: 10.3389/fmicb.2025.1547571.

Sorribas, F.J., Djian-Caporalino, C., and Mateille, T. (2020). "Nematodes," in *Integrated pest and disease management in greenhouse crops*. Maria Lodovica Gullino, Ramon Albajes, Philippe C. Nicot ed (Cham: Springer), 147-174.

Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular microbiology* 56(4)**,** 845-857. doi: 10.1111/j.1365-2958.2005.04587.x.

Stone, G.E. (1898). *Nematode worms.* Hatch Experiment Station of the Massachusetts Agricultural College.

Tarr, S. (1972). "Disease control by plant sanitation," in *Principles of Plant Pathology*. (London: Springer), 469-477.

Turner, S.J., and Subbotin, S.A. (2006). Cyst nematodes. *Plant nematology***,** 91-122.

Uddin, M.A., Begum, M.S., Ashraf, M., Azad, A.K., Adhikary, A.C., and Hossain, M.S. (2023). Water and chemical consumption in the textile processing industry of Bangladesh. *PLOS Sustainability and Transformation* 2(7)**,** e0000072. doi: 10.1371/journal.pstr.0000072.

Vallad, G.E., and Goodman, R.M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop science* 44(6)**,** 1920-1934. doi: org/10.2135/cropsci2004.1920.

Vanderplank, J.E. (2012). *Disease resistance in plants.* Elsevier.

Vasantha-Srinivasan, P., Park, K.B., Kim, K.Y., Jung, W.-J., and Han, Y.S. (2025). The role of Bacillus species in the management of plant-parasitic nematodes. *Frontiers in Microbiology* 15**,** 1510036. doi: 10.3389/fmicb.2024.1510036.

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., and Nasrulhaq Boyce, A. (2016). Role of plant growth promoting rhizobacteria in agricultural sustainability—a review. *Molecules* 21(5)**,** 573. doi: 10.3390/molecules21050573.

Verma, P.P., Shelake, R.M., Das, S., Sharma, P., and Kim, J.-Y. (2019). "Plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF): potential biological control agents of diseases and pests," in *Microbial Interventions in Agriculture and Environment: Volume 1: Research Trends, Priorities and Prospects,* ed. V.K.G. Dhananjaya Pratap Singh, Ratna Prabha. (Singapore: Springer), 281-311.

Villarreal-Delgado, M.F., Villa-Rodríguez, E.D., Cira-Chávez, L.A., Estrada-Alvarado, M.I., Parra-Cota, F.I., and Santos-Villalobos, S.d.l. (2018). The genus *Bacillus* as a biological control agent and its implications in the agricultural biosecurity. *Revista mexicana de fitopatología* 36(1)**,** 95-130. doi: 10.18781/r.mex.fit.1706-5.

Wada, N., Berry, P.A., Hill, B., Mallory-Smith, C., and Parke, J.L. (2024). Soil solarization as a non-chemical weed control method in tree nursery production systems of the Pacific Northwest, USA. *Frontiers in Agronomy* 5**,** 1321372. doi: 10.3389/fagro.2023.1321372.

Wyss, U., and Grundler, F.M.W (1992). "Feeding behavior of sedentary plant parasitic nematodes," in *Netherlands Journal of Plant Pathology,* ed. J.H.M. Visser.), 165-173.

Wyss, U., and Zunke, U. (1986). Observations on the behaviour of second stage juveniles of Hetero inside host roots. *Rev Nematol* 9**,** 153-165.

York, L.M., Carminati, A., Mooney, S.J., Ritz, K., and Bennett, M.J. (2016). The holistic rhizosphere: integrating zones, processes, and semantics in the soil influenced by roots. *Journal of experimental botany* 67(12)**,** 3629-3643. doi: 10.1093/jxb/erw108.

Zodge, H., Koyande, P., Salaskar, D., and Mehetre, S. (2024). "Soil Inhabitant Bacteria: Journey from Rhizosphere to Eco-Holobiont Approach," in *Industrial Applications of Soil Microbes,* ed. A.G. Shampi Jain, Neeraj Verm. Bentham Science Publishers), 161-170.

Comparative metagenomics of cotton rhizosphere microbial communities from different fields with continuous cotton-wheat rotations

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Abstract

The rhizosphere microbiome plays a crucial role in plant health, nutrient cycling, and stress tolerance. It is significantly influenced by the root exudates of cultivated crops, environmental factors, soil conditions, and crop rotation patterns. This study utilized next-generation sequencing to analyze the rhizosphere microbial communities of cotton (*Gossypium hirsutum*) under a continuous cotton-wheat rotation across four distinct agroecosystem sites (CR1, CR2, CR3, and CR4) in Punjab, Pakistan. The bacterial phyla Actinomycetota, Pseudomonadota, and the fungal phylum Ascomycota were predominant. Notably, CR3 was dominated by Pseudomonadota, a phylum known for its metabolically diverse and plant-beneficial bacteria. Genus-level PCA indicated that CR3 featured a rich consortium of plant-beneficial bacterial genera, including *Brevundimonas, Microvirga, Archangium, Micromonospora, Sphingomonas,* and *Nocardia*, recognized for plant growth promotion, nitrogen fixation, and biocontrol. In contrast, CR2 and CR1 were associated with *Mesorhizobium, Bradyrhizobium*, and antibiotic-producing *Micromonospora* and *Streptomyces*, but exhibited lower diversity and evenness. CR4 had a high abundance of stress-adapted taxa like *Bacillus*, *Salinicoccus,* and *Kocuria,* which are found in extreme environments. CR3 was found with less alpha diversity of rhizobacteria but dominated by several plant beneficial bacteria compared to other soil samples and this sampling site was observed with higher cotton yield.

The overall number of reads for fungal genera compared to bacteria was lower. The relative abundance of all bacterial and fungal species also differed notably among the soil samples. In fungal

genera, both plant beneficial and plant pathogenic fungi, including *Aspergillus*, *Alternaria*, *Fusarium*, *Colletotrichum*, and *Verticillium*, *Beauveria* and *Metarhizium, Rhizophagus*, *Pseudozyma,* and *Trichoderma, Chaetomium* were abundant, more than 0.5% in all fungi. *Aspergillus* was highly abundant in CR2 and CR4.

These findings reveal that rhizosphere soils from repeated cotton–wheat rotation systems are enriched with beneficial bacteria and have reduced levels of pathogenic fungal communities in the rhizosphere. Moreover, specific sampling sites distinctly shape microbial community structures influencing plant health and development, and offer a foundation for microbiome-informed crop management across diverse agroecological settings.

Keywords: rhizosphere microbiome, root exudates, cotton wheat rotation

1. Introduction

The rhizosphere is a narrow zone of soil directly influenced by plant roots, characterized by intense biological activity and complex interactions between plants and microorganisms (York et al., 2016; Ragland et al., 2024). This dynamic interface facilitates the exchange of energy, substances, and signals among plants and the surrounding microbial community, all influenced by interactions with the soil environment. Plant-associated microbial communities exhibit a wide taxonomic diversity, with variations expected across locations and time points (Liu et al., 2020). However, studies suggest the existence of a core microbiota, a subset of microbial lineages that consistently associate with a specific host across diverse environments (Luo et al., 2022). Bacteria and fungi are the most studied and dominant members of these microbiomes. Still, other groups, such as archaea, viruses, protists, and nematodes also play significant roles in regulating plant growth and health.

The rhizosphere microbiome, which includes rhizobacteria and fungi, plays a crucial role in plant growth, development, and stress resistance by influencing nutrient uptake, chemical signaling, and enzymatic processes (Philippot et al., 2024). Plant growth-promoting rhizobacteria exemplify this interaction, directly or indirectly providing essential nutrients such as nitrogen and phosphorus, promoting growth, preventing pathogen colonization, and enhancing plant resistance to both biotic and abiotic stresses (De Mandal et al., 2021).

Plant-related factors, such as crop rotation, developmental stage, genotype, and the composition of root exudates, also contribute to shaping the rhizosphere microbiome (Park et al., 2023). Plants can modulate the rhizosphere environment by altering the input of materials, energy, and signals, thereby shaping the structure of the rhizosphere microbiome (Adeniji et al., 2024; Yang et al., 2025). In

addition, several other factors further contribute to the complexity and dynamics of the rhizobiome. Environmental conditions, such as temperature and moisture, exert selective pressures on microbial communities (Salazar et al., 2022). Soil properties, including physical characteristics like texture (Franzluebbers, 2024) and chemical attributes such as pH and organic matter content, also play a significant role (Zhang et al., 2024). Furthermore, the pre-existing microbial community in the surrounding soil influences the colonization potential and establishment of new microorganisms within the rhizosphere (Wang et al., 2024). The dynamic interplay of these factors determines the structure and function of the microbial communities associated with plant roots (Khan et al., 2019). Hence, there is considerable interest in identifying the taxa associated with specific plant rhizospheres in different soil types and cropping patterns. While some microbes can be cultured in isolation using laboratory-generated media, many cannot, partly because they may rely on symbiotic relationships with other microbes, grow slowly, or require environmental conditions that cannot be replicated in a laboratory setting. Since metagenomics is used to avoid the need for cultivation and is relatively high-throughput, sequence-based phylogenetic profiling of environmental and host-associated microbial samples has become a preferred method for surveying microbial community composition (Simmons et al., 2018).

Metagenomic studies have become increasingly prevalent in characterizing the diverse microbial communities associated with various crops. These studies often focus on the rhizosphere, endosphere, and surrounding soil, aiming to understand the complex interactions between plants and their associated microbes (Dlamini et al., 2023). For instance, researchers have used metagenomics to investigate the diversity and dynamics of bacterial communities in cotton rhizosphere soil across different growth stages (Shi et al., 2020). Wei et al. (2019) showed that the composition of the cotton rhizosphere and endosphere microbiome varies among cultivars, with different cultivars exhibiting distinct associations with beneficial microbes that contribute to resistance against *Verticillium dahliae*. Additionally, researchers are exploring the potential of using metagenomics to identify enzymes involved in cotton biomass degradation (Zhang et al., 2016). Various studies demonstrate the power of metagenomics in unraveling the complex microbial communities associated with cotton and their potential roles in plant health and productivity.

Our study aimed to investigate the structure and diversity of microbial communities in the rhizosphere of cotton grown in different fields under a continuous cotton-wheat rotation system in Punjab, Pakistan. The cotton-wheat cropping system plays a crucial role in the agriculture-dependent economy of Pakistan (Mubeen et al., 2020). Farmers often maintain this cotton-wheat rotation for many years. However, there is a gap in research concerning the microbial community structure and

the accumulation of beneficial and pathogenic bacteria and fungi in the cotton rhizosphere in Pakistan under continuous cotton-wheat rotation, without disruptions from other crops or management practices. Therefore, we were interested to find out how cotton-wheat rotation performed for more than five years affects microbial communities in the cotton rhizosphere. Since the cropping system is known to have an influence on the presence and abundance of microbial species in the soil (Tian et al., 2019). The study was conducted across semi-arid and arid agroecosystems, using the same cotton genotype, *Gossypium hirsutum* commercial cultivar SS-32 to evaluate the influence of the same cropping pattern across different sampling sites on rhizospheric microbial populations. These cotton-producing regions of Punjab, Pakistan, have a hot and dry climate with notable seasonal changes. In semi-arid areas summers is very hot June is recorded as warmest month, the average temperatures of selected areas were recorded 46.95°C (116.51°F) and 45.92°C (114.66°F) in the arid areas in June 2020 (https://weatherandclimate.com/pakistan). The winters are cool and dry, with temperatures sometimes falling to 7°C (44°F). Precipitation is comparatively low, with the majority falling during the monsoon season (late June to mid-September in the region of Punjab, Pakistan). Farmers who were performing the wheat-cotton rotational farming method used a well-designed farming technology for enhancing the yields of crops and the efficient utilization of resources. In 2020 and 2021 when samples were collected from the selected fields, sowing was done at the end of May at an average seeding rate of 8 to 10 kilograms per acre. The standard row spacing was 30 inches with 9 to 12 inches of plant spacing, which gave a plant density of approximately 18,000 to 24,000 plants per acre. Fertilizer was applied as planned: phosphorus (P) and potash (K) at sowing (23 kg/acre each), and nitrogen (N) in five splits of 23 kg/acre starting from the first irrigation and continued through critical growth stages of the crop such as first flower, full flowering, and 50% boll formation. The first irrigation was done after 30 days of sowing as drill sowing was performed. After that further irrigations were performed 10 to 15 days, and the final irrigation was conducted in the second week of October.

This study was conducted to understand the understand the effect of different agroecological sites on the cotton rhizosphere in a continuous cotton-wheat rotation farming system. The knowledge obtained from this study will help to further identify the complexity of the cotton rhizobiome when wheat rotation is performed for more than five years and will show the impact of different sampling sites under same cropping pattern.

2. Materials and methods

2.1 Soil sampling

Rhizospheric soil was collected from four different locations. Sample 1 (CR1) located at latitude 30.041247° N, and longitude at 71.846692° E, sample 2 (CR2) located at latitude 30.319141°, and longitude 71.879716° (CR3) located at latitude 30.371862° N, and longitude 71.807828° E, sample 3 (CR3) were collected from the Khanewal district, while sample 4 (CR4) located at latitude 29.130240° N and longitude 71.757148° E was collected from the Bahawalpur district, Punjab, Pakistan, in 2020 and 2021. In Aslam et al. (2025) these sampling sites have the identifiers SC1 (equals CR1), SC2 (equals CR2), SC3 (equals CR3), and SC5 (equals CR4) and are published along with their physicochemical properties. The sampled fields were all cultivated in continuous cotton – wheat rotations since 2014.

Rhizospheric soil was collected from cotton plants 150 days post sowing (BBCH stage 7-8). For each sampling site, three cotton plants were randomly selected within an appropriate distance of one meter to serve as technical replicates. The sampling process was initiated with the careful excavation of selected cotton plants, ensuring minimal disturbance to the root system and surrounding soil. A sterile shovel was used to dig around each cotton plant, approximately 15-20 cm from the base of the stem, and a block of soil containing the intact root system was carefully excavated. The closely adhered soil was carefully collected from the roots of these three plants and combined to create a composite sample for metagenomic analysis. To ensure the integrity of the samples for downstream metagenomic analysis, the collected rhizosphere soil was immediately placed in sterile zip-lock bags, flash-frozen in liquid nitrogen, and stored at -80°C until DNA extraction.

2.2 DNA extraction

Genomic DNA was meticulously extracted from each collected soil sample (CR1, CR2, CR3, and CR4) using the Qiagen PowerSoil DNA Isolation kit, strictly following the manufacturer's protocol. The PowerSoil kit employs a bead-beating method for efficient cell lysis, followed by chemical purification steps to remove contaminants that can interfere with downstream molecular analyses. The quantity and purity of the extracted DNA were determined using a NanoDrop spectrophotometer (Thermo Scientific US), which measures the absorbance at 260 nm and 280 nm. The DNA concentration was calculated based on the 260 nm absorbance, and the purity was assessed by the 260/280 ratio, with a ratio between 1.8 and 2.0 indicating high-quality, pure DNA.

2.3 Metagenome analysis by Next Generation Sequencing (NGS)

DNA samples CR1, CR2, CR3, and CR4 were sent to Eurofins Genomics Europe Sequencing GmbH in Germany for INVIEW metagenome analysis service including library preparation, sequencing (Illumina technology, 2 x 150 bp paired end, 10 million read pairs per sample) and basic bioinformatics. The main steps of the bioinformatics analysis are given below.

The raw sequencing data was preprocessed to generate high-quality data suitable for downstream analysis. This involved quality control steps to retain only high-quality bases. Adapter trimming was performed to remove adapter sequences. Quality filtering and per-read quality pruning were applied. After quality trimming, the reads were checked for any remaining adapter sequences, which were then removed. Additionally, reads shorter than a specified length (e.g., 30bp) were discarded to ensure that only high-quality sequencing reads were used for downstream analysis. In the case of paired-end reads, both reads had to pass these quality control criteria to be considered for downstream analysis.

Host removal was performed using Kraken (Wood and Salzberg, 2014), a taxonomic classification tool that assigns labels to DNA sequences. Kraken functions by dissecting reads into k-mers and mapping each k-mer to the lowest common ancestor of genomes containing that k-mer within a reference database. This enabled rapid classification of reads based on their taxonomic origin. To further refine the results, KrakenUniq (Breitwieser et al., 2018) was employed to filter out false positives. Subsequently, SeqKit (Shen et al., 2016) was used to filter the fastq files, eliminating sequences identified as originating from the host and retaining only the non-host sequences for downstream analysis. This step was crucial to reduce complexity, improve sensitivity.

2.4 Taxonomic annotation

Taxonomic annotation was performed using MetaPhlAn (Truong et al., 2015), a computational tool designed to characterize the composition of microbial communities from metagenomic shotgun sequencing data with species-level resolution. MetaPhlAn relies on unique clade-specific marker genes identified from ~17,000 reference genomes (~13,500 bacterial and archaeal, ~3,500 viral, and ~110 eukaryotic).

Reads that remained unclassified by MetaPhlAn were subsequently processed using KrakenUniq (Breitwieser et al., 2018). For each read, a classification tree is constructed by pruning the taxonomy and retaining only taxa (including ancestors) associated with k-mers in that read. Each node is

weighted by the number of k-mers mapped to it, and the path from root to leaf with the highest sum of weights is used to classify the read. KrakenUniq then computes the number of unique k-mers observed for each taxon, which helps to filter out false positives.

2.5 Taxa abundance and species diversity

The analysis involved collecting and normalizing read counts of input samples at various taxonomic levels. To enable comparison of species richness across samples with varying sizes, a rarefy function, as implemented in the vegan bioconductor package, was applied (Oksanen et al., 2013). Rarefied read counts facilitate improved comparisons of operational taxonomic unit profiles between samples with different sequencing depths. Abundance, measured as the percentage of OTU-assigned reads from different taxonomic levels, was determined and subsequently used to generate heatmaps and bar plots at the Phylum, Genus, and Species levels. Species indices, including the Simpson Diversity Index, Inverse Simpson Diversity index and Shannon Diversity Index, were also determined based on the species count (Oksanen et al., 2013).

2.6 Programs and software

Fastp 0.20.0 software was used for the preprocessing for FastQ files. Kraken Uniq, 0.5.8 was used for confident and fast metagenomics classification using unique k-mer counts. MetaPhlAn 3.0.7 was used for MetaPhlAn for enhanced metagenomic taxonomic profiling SeqKit 0.12.0 was used as toolkit for FASTA/Q file. R 4.1.3 and Python 3.10.0 were used for statistical computing. Vegan 2.6.4 was used for diversity analysis.

3. Results

The objective of our study was to investigate the composition and diversity of bacteria and fungi associated with cotton rhizosphere and the effect on the microbiome in the cotton rhizosphere when wheat rotation was performed for more than five years without any interruption. To achieve this, we used representative soil samples from three different semi-arid field sites and one arid field site, at 150 days post-sowing, reflecting the microbial communities and nutrient profiles associated with cotton roots during the development of fruits and seeds (approximately BBCH stage 7-8). The yield at the end of cotton picking was different from all the sampling sites. Cotton yield of CR1 site was recorded 873kg/hectare, CR2 806Kg/hectare, CR3 1048kg/hectare and CR4 968kg/hectare.

3.1 Taxonomic annotation

Around 20.9-21.8 million clean read were received per sample and 6% (CR1) to 13% (CR4) were classified (Supplementary table 1). The metagenomic data revealed that bacteria dominated the microbial communities across all four rhizosphere samples, accounting for over 97% of classified reads (Table 1). While bacteria were consistently dominant, the second most abundant group varied across sites, with archaea and fungi showing notable fluctuations in relative abundance. For instance, CR2 exhibited the highest proportions of archaea (0.79%) and fungi (0.69%), whereas CR4 had the lowest relative abundance of archaea (0.21%) and eukaryotes overall (0.04%). Viral sequences were detected at consistently low levels (≤ 0.02%) across all sites.

Table 1. Taxonomic classification of metagenomic reads across domains and major groups in different field sites (CR1, CR2, CR3, and CR4). Number and relative abundance (percentage in parentheses) of reads assigned to Archaea, Bacteria, Fungi, Eukaryota (excluding fungi), and Viruses.

Sample Name	Archaea	Bacteria	Fungi	Eukaryota	Viruses
CR1	4,262 (0.33%)	1,272,436 (97.96%)	5,392 (0.42%)	1,081 (0.08%)	103 (0.01%)
CR2	12,477 (0.79%)	1,534,165 (97.25%)	10,888 (0.69%)	1,702 (0.11%)	169 (0.01%)
CR3	6,820 (0.34%)	1,951,031 (98.35%)	6,586 (0.33%)	2,189 (0.11%)	314 (0.02%)
CR4	6,000 (0.21%)	2,788,785 (98.64%)	6,156 (0.22%)	1,035 (0.04%)	182 (0.01%)

3.2 Bacterial Community

In this study, 46 bacterial phyla were detected across the four soil samples, with 34 phyla found to be common to all (Figure 1A). Bacterial community composition revealed a complex interplay among several phyla in the cotton rhizosphere across four different agricultural fields, primarily dominated by Actinomycetota (formerly known as Actinobacteria), Pseudomonadota (formerly known as Proteobacteria), Bacillota (formerly known as Firmicutes), and Myxococcota (Figure 1B). The prevalence of Actinomycetota in all samples ranged from approximately 37% (CR3) to 77% (CR4), indicating that this phylum was a core component of the microbial community in these environments. In CR3, Actinomycetota proportion was lower with approximately 37% and the Pseudomonadota with

approximately 53% became the dominant phylum. Pseudomonadota also exhibited a strong presence in CR2 (approximately 33%) and CR1 (approximately 27%), while in CR4 (approximately 10%), it decreased compared to other samples. The phylum Bacillota was notably present in CR4 (10%) but in very small proportions in the other samples, ranging from approximately 1.3% (CR1) to 1.6 % (CR3), suggesting that CR4 may provide unique conditions or selective pressures that support its growth. Myxococcota ranked as the third most abundant phylum in CR3 (approximately 7%), showing variation in other samples between 1.7% (CR4) and 3% (CR1). Bacteroidota and Planctomycetota were present with an average relative abundance of ≥ 0.5% among all rhizobacteria in at least one soil sample. Bacteroidota had approximately 0.7% proportion in CR3 of the total reads, while Planctomycetota had approximately 0.6% of the total reads. Other phyla, including Gemmatimonadota, Verrucomicrobiota, Acidobacteriota, Nitrospirota, Spirochaetota, Cyanobacteria, Rhodothermota, Chloroflexota, Proteobacteria, Deinococcota, and Thermomicrobiota, were present in tiny proportions, less than 0.5 %, in all samples (Supplementary Table 2).

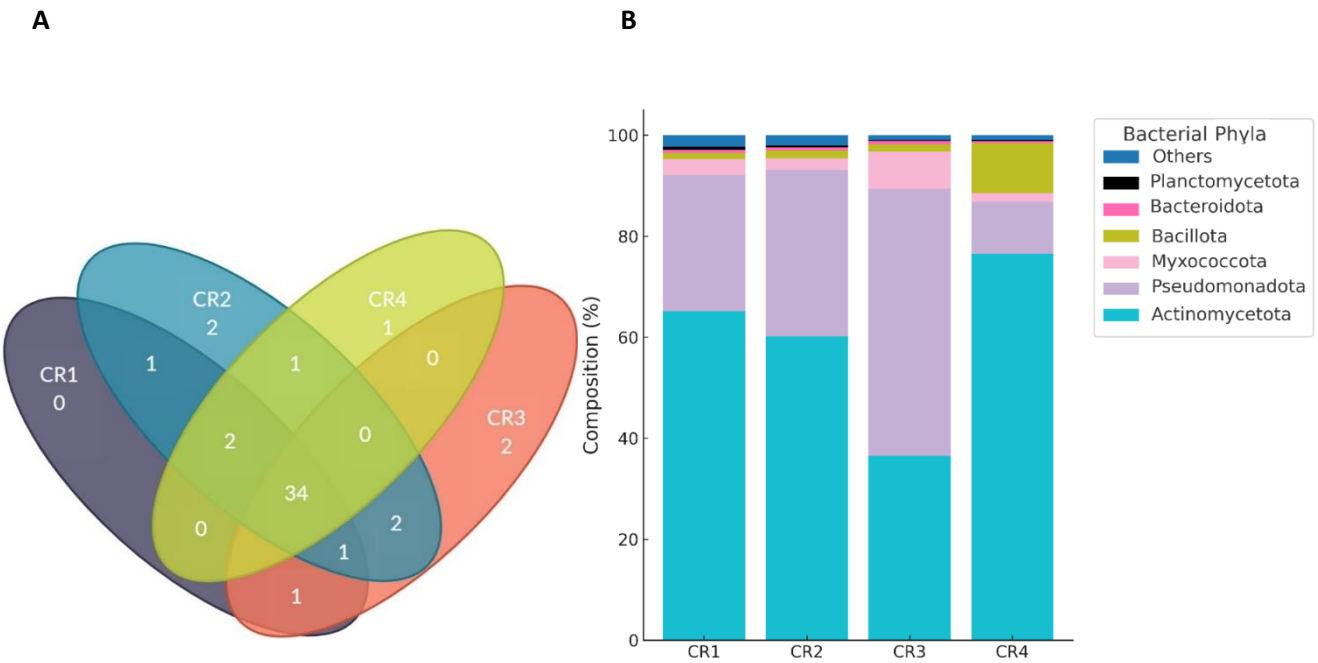


Figure 1. The composition of the rhizobacteria communities at the phylum-level. **(A)** Distribution of all bacterial phyla across soil samples CR1 to CR4, as visualized by a Venn diagram. Overlapping areas indicate shared phyla among the samples; **(B)** The abundance of bacterial phyla (in %) is based on bacteria-assigned read counts within the four cotton rhizosphere samples CR1 to CR4. Phyla with an average relative abundance of ≥ 0.5% in all rhizobacteria among at least one soil sample were included

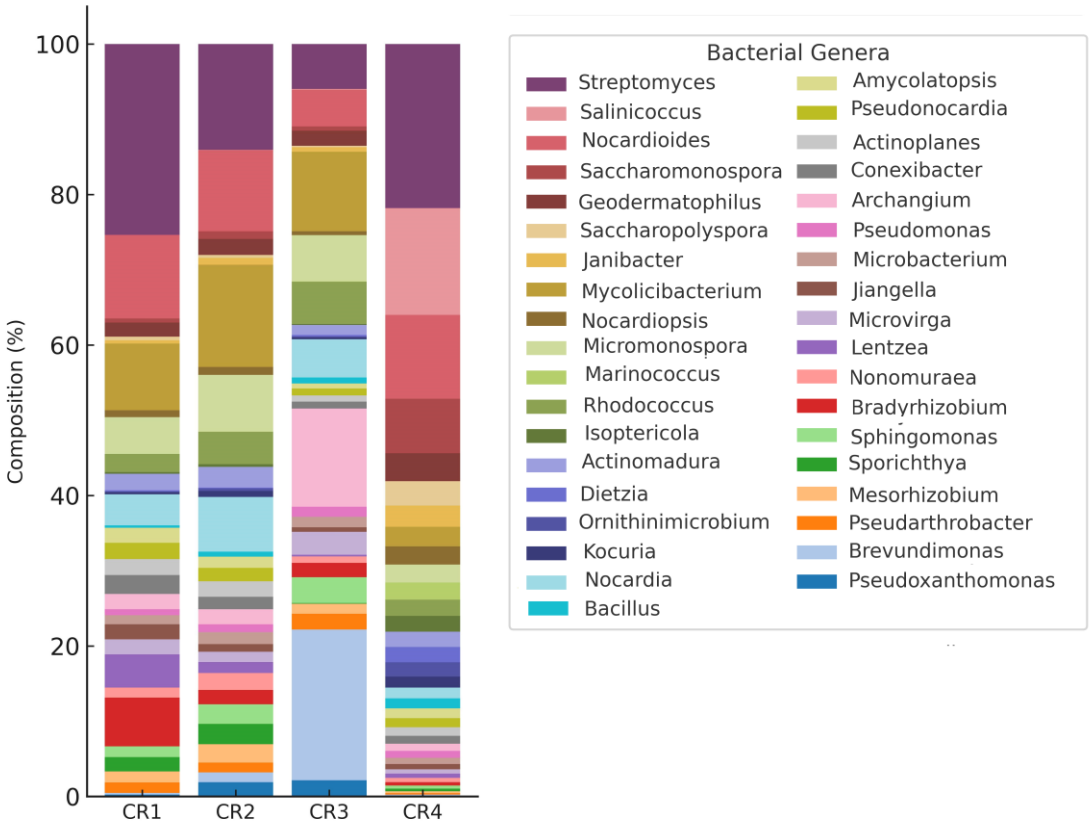
in this bar plot. Phyla with an average relative abundance of < 0.5% in all rhizobacteria among all sample were summarized as “Others”.

3.3. Genus-level distribution of bacteria

3.3.1 Beta diversity

A total of 1645 genera were identified across all four samples. Of these, 1402 genera were common to all samples (Supplementary Figure 1). More than half of the total read counts (57%) belonged to only 37 bacterial genera. They had a relative abundance equal to or greater than 0.7% in at least one of the soil samples. The genus-level microbial community analysis of these 37 genera revealed distinct patterns in taxonomic structure and ecological dynamics (Figure 2). The rhizosphere community of CR3 and CR4 showed the greatest divergence to each other, but also strongly differed from CR1 and CR2 , when comparing the different dominant genera (Figure 2A) and the distinct clustering in the PCA biplot (Figure 2B). The PCA biplot illustrates variation in bacterial community composition among the four environmental samples based on the relative abundance of bacterial genera. The first two principal components account for 89.2% of the dataset's variability, explaining 63.7% (PC1) and 25.5% (PC2) of the total variance, respectively (Figure 2B, Supplementary Table 3). CR1 was separated by greater influence of *Bradyrhizobium* and *Lentzea*. CR3 and CR4 are located at greatest distance from each other, partly resulting from altered genera prevalence. CR3 is characterized by a higher abundance of genera like *Brevundimonas*, *Archangium, Micromonospora*, *Microvirga, sphingomonas, Pseudoxenthonomas*, *Microvigra* whereas CR4 shows a shift to the genera *Salinicoccus*, *Kocuria*, *Janibacter, Bacillus, Pseudomonas, Dietzia, Saccharopolyspora* , *Janibacter*, *Mycolicibacterium* suggesting a markedly different microbial community.

A


Composition (%)
100
80
60
40
20
0
CR1
CR2
CR3
CR4
Bacterial Genera
Streptomyces
Salinicoccus
Nocardioides
Saccharomonospora
Geodermatophilus
Saccharopolyspora
Janibacter
Mycolicibacterium
Nocardiopsis
Micromonospora
Marinococcus
Rhodococcus
Isoptericola
Actinomadura
Dietzia
Ornithinimicrobium
Kocuria
Nocardia
Bacillus
Amycolatopsis
Pseudonocardia
Actinoplanes
Conexibacter
Archangium
Pseudomonas
Microbacterium
Jiangella
Microvirga
Lentzea
Nonomuraea
Bradyrhizobium
Sphingomonas
Sporichthya
Mesorhizobium
Pseudarthrobacter
Brevundimonas
Pseudoxanthomonas

B

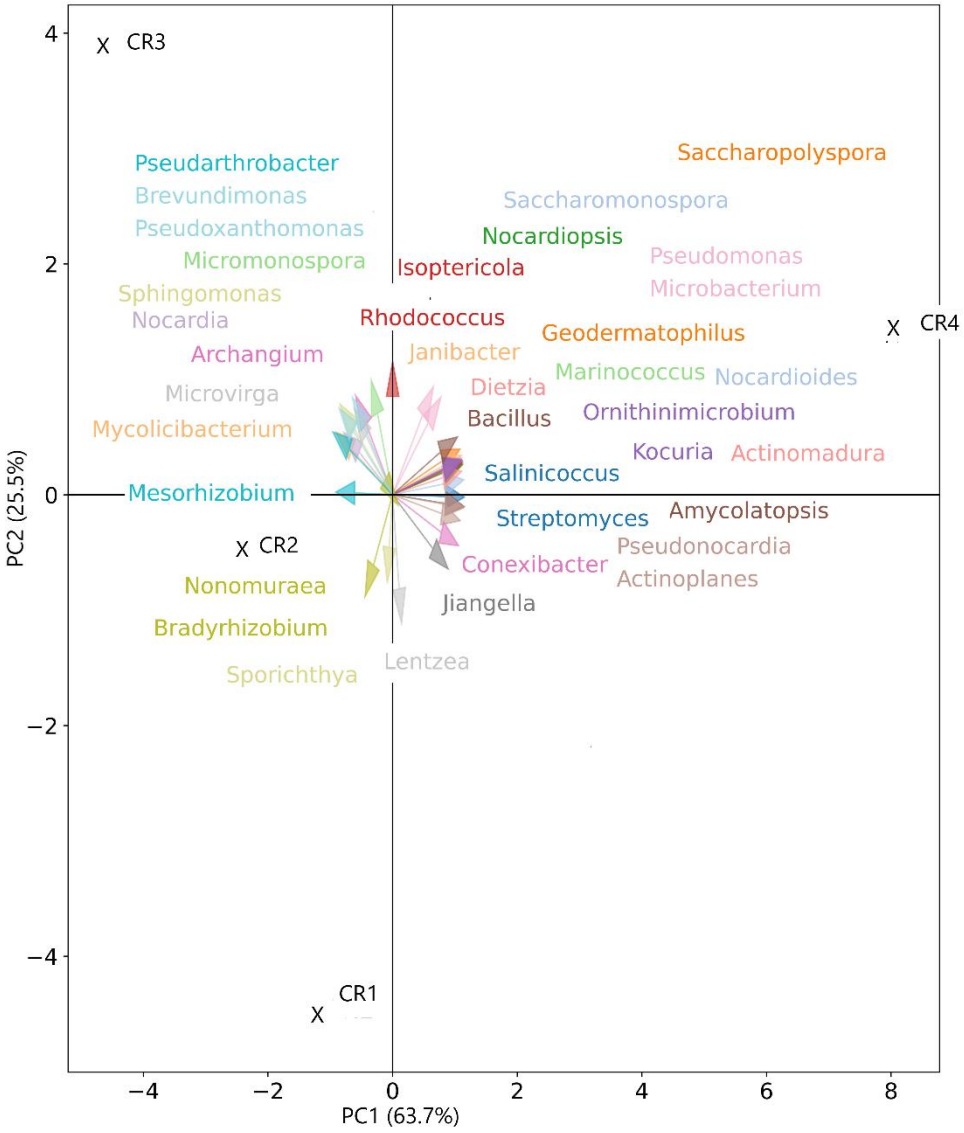


Figure 2. The composition of rhizobacteria communities at genus-level across the four cotton rhizosphere samples, CR1 to CR4. **(A)** Bar plot showing genus-level abundance (in %) based on bacteria-assigned read counts; **(B)** Principal component analysis (PCA) showing the variation in genus-level community composition across samples. Genera with an average relative abundance of ≥ 0.7% in all rhizobacteria among at least one soil sample were included in the analysis.

3.3.2 Alpha diversity

The diversity analysis based on genus-level counts across samples CR1 to CR4 reveals that all samples differed in genus richness and evenness (Figure 3). CR2 exhibits the highest overall diversity compared

to other samples, with the greatest evenness and balance among genera, as reflected in its top score across the Shannon (3.04), and Inverse Simpson (14.1) and lowest score across the Simpson (0.07) indices (Figure 3). In contrast**,** CR3 and CR1 showed almost similar Shannon index 2.83 and 2.84 respectively but, CR3 showed slightly better evenness compared to CR1. CR1, having the highest Simpson index (0.1), had the lowest Inverse Simpson index (9.9), pointing toward a community dominated by a few abundant species likely due to the dominance by Streptomyces accounting for about 25% (Figure 2A) of the cotton rhizosphere, which reduces evenness despite having the same number of genera. CR4 maintains moderate diversity. These results indicate considerable differences in microbial richness across the sampling sites.

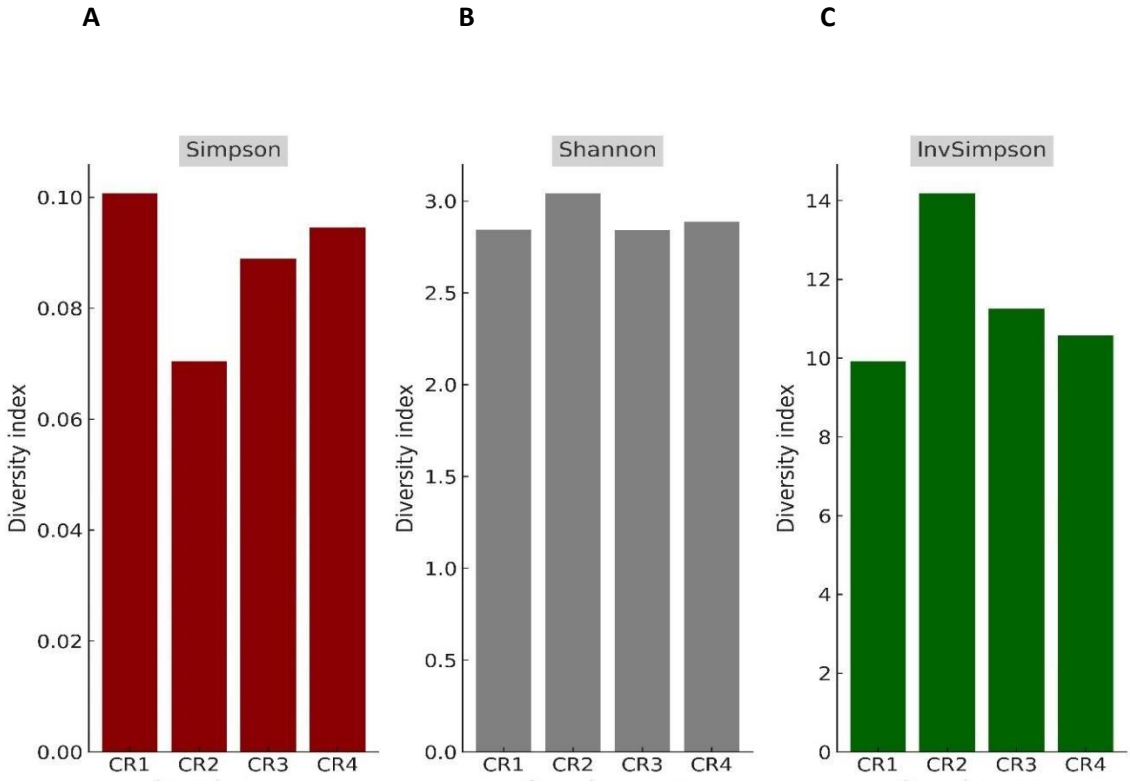


Figure 3. Diversity Indices of rhizobacteria communities of the four cotton rhizosphere samples CR1 to CR4, showing the richness and evenness at the genus level based on the bacteria-assigned read counts. **(A)** Simpson index; **(B)** Shannon index; **(C)** Inverse Simpson index. Genera with an average relative abundance of ≥ 0.7% in rhizobacteria among at least one soil sample were included in the analysis.

3.3.3 Species-level distribution

Across all samples, 4428 species were detected (2,173,871 reads). However, a small subset of 37 species, each exceeding 0.3% abundance in at least one soil sample, comprised a substantial portion of the community. These dominant species accounted for 558,843 reads, representing approximately 25 % of all bacterial species identified. These 37 species revealed that bacterial communities exhibited distinct profiles across samples, characterized by variations in relative abundance. On species level CR1 and CR2 were closest, followed by CR3 and CR4 was most different from all other rhizospheres (Figure 4 and Supplementary Figure 2). CR4 was dominated by *Salinicoccus luteus* (approximately 31%), followed by *Saccharomonospora cyanea* (approximately 12 %). Other considerable members included *Marinococcus halotolerans* (approximately 5%), *Isoptericola variabilis* (approximately 4.5), *Ornithinimicrobium pekingense* (approximately 4.3%), and *Dietzia alimentaria* (approximately 4%), while in other rhizosphere these spices were less than 1%. CR3 was dominated by *Archangium gephyra* (approximately 28%) and *Brevundimonas viscosa* (approximately 23%), while CR2 sample was dominated by *Nocardia cyriacigeorgica* (approximately 12%). CR1 was dominated by *Solirubrobacter soli* (approximately 11%) and *Conexibacter woesei* (approximately 10%).

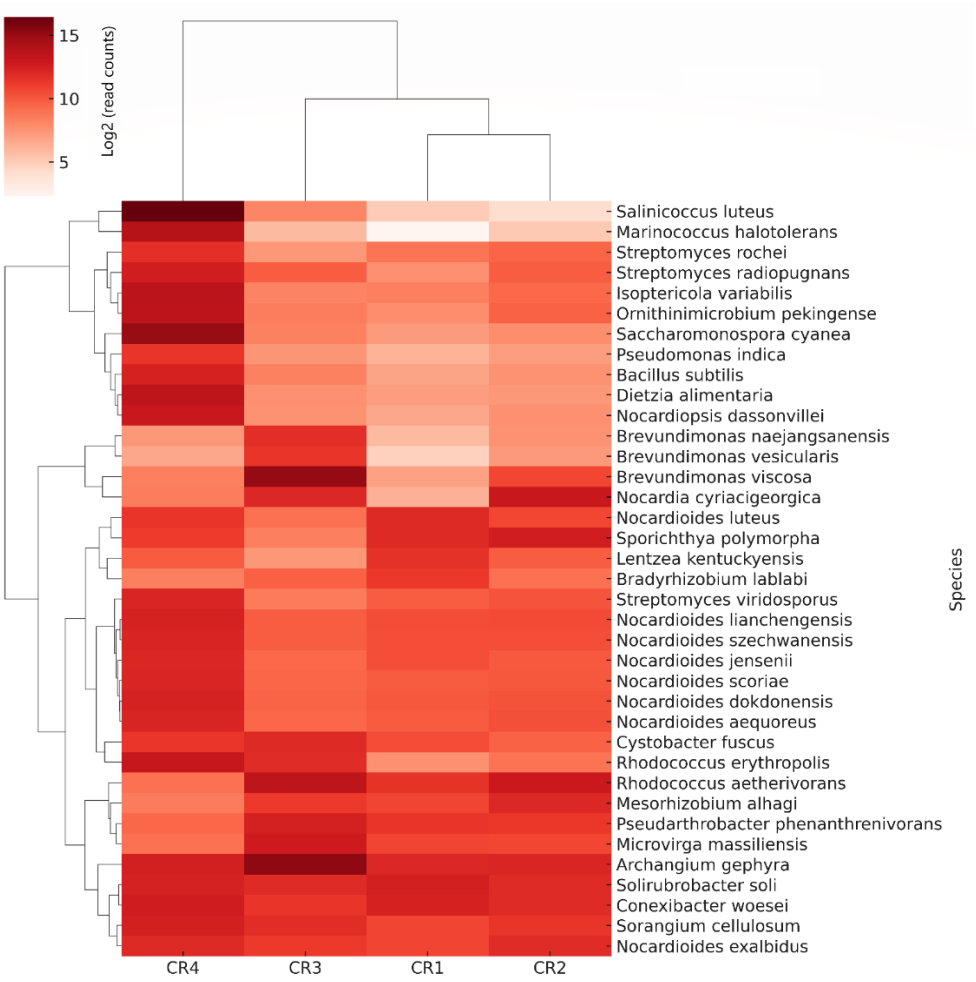


Figure 4. The composition of the rhizobacteria communities at species-level across the four cotton rhizosphere samples CR1 to CR4. A heat map illustrating the taxonomic abundance and their relationships across the samples. A dendrogram, determined by computing hierarchical clustering from the abundance levels, displays the connections between the samples and the species. The abundance levels (number of reads associated with each taxon) are log-transformed to base 2 for clarity. Species with an average relative abundance of ≥ 0.3% in at least one soil sample were included in the analysis.

3.4 Fungal community

A total of five fungal phyla were found, and the community structure was consistently dominated by the phylum Ascomycota across all four cotton soil samples (Figure 5). While Ascomycota was the most abundant of the relative abundance of approximately 73% (CR1) to 90% (CR2), Basidiomycota was the second dominant phylum with the relative abundance of approximately 9% (CR2) to 20% (CR1) and Mucoromycota the third dominant phylum with the relative abundance of 0.7% (CR2) to 7% (CR1).

The other two phyla Chytridiomycota and Microsporidia, were present with very low abundances (Supplementary Table 4)

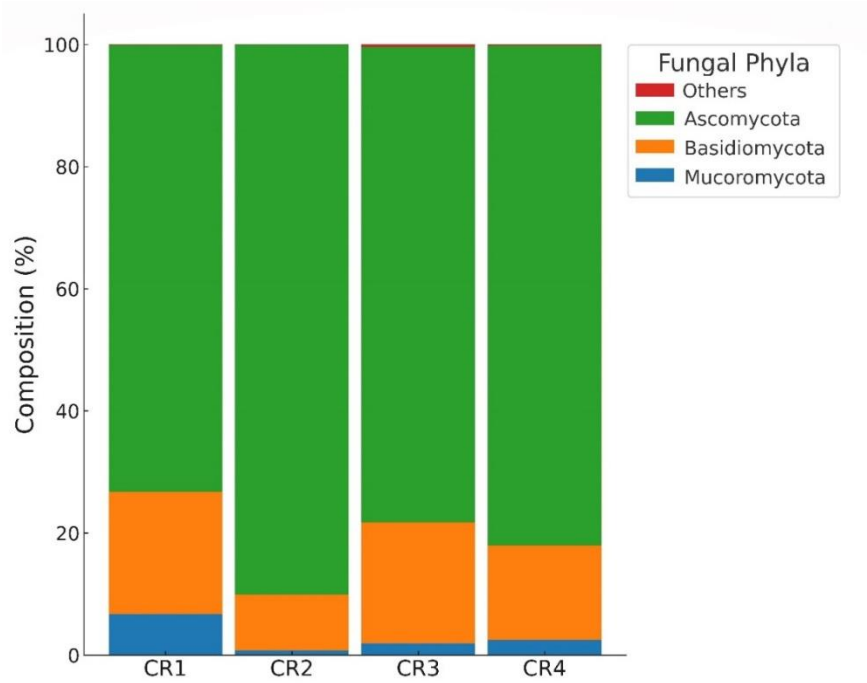


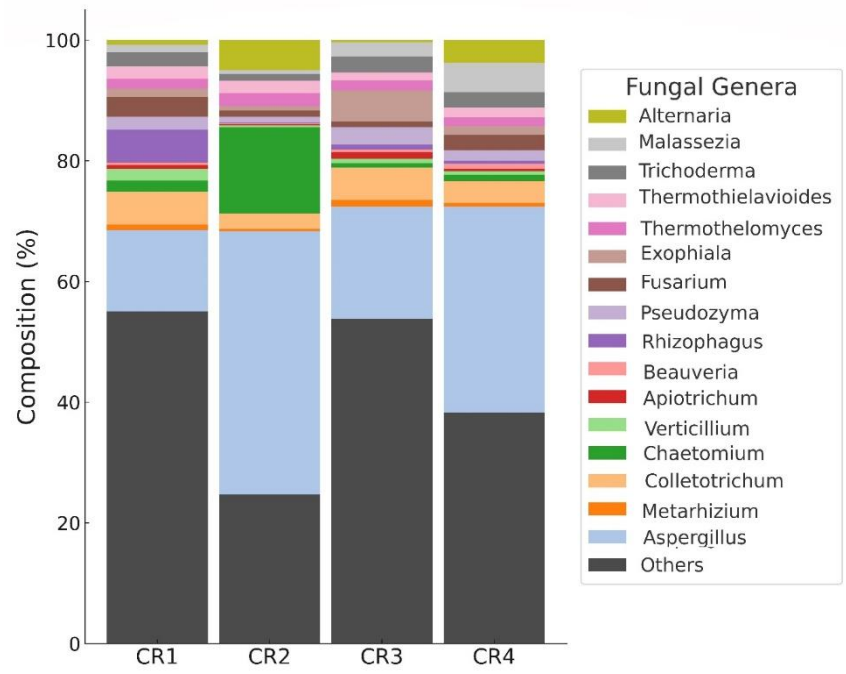
Figure 5. The composition of the fungal communities at the phylum-level. Abundance of fungal phyla is based on fungi-assigned read counts within the four cotton rhizosphere samples CR1 to CR4. Phyla with an average relative abundance of ≥ 0.5% in all fungi among at least one soil sample were included in this bar plot. Phyla with an average relative abundance of < 0.5% in all fungi among at least one soil sample were summarized as "Others".

Across all soil samples, 160 fungal genera were found, and 107 genera were common in all soil samples (Supplementary Figure 3), with 20,253 reads. The 16 most abundant genera (each >0.5% of total reads) accounted for 61.8% of the fungal community at the genus level (12,523 reads). At the genus level, the composition of the fungal community exhibited notable variability across the samples, with distinct dominance patterns among specific genera (Figure 6A). The fungal community in the CR1 rhizosphere was characterized by the higher relative abundance of *Rhizophagus* (approximately 5%). In CR2 *Aspergillus* was the most abundant (approximately 44%), and a high relative abundance of *Chaetomium* (approximately 34%). Other genera present included *Alternaria* (approximately 4%), *Thermothielavioides,* and *Thermothelomyces* (approximately 2% reads each). In contrast, the CR3 sample was characterized by the abundance of *Exophiala* (approximately 5%), *Pseudozyma* (approximately 3%), and *Apiotrichum* (approximately 1%). Finally, the CR4 sample was characterized by the presence of *Malassezia* (approximately 4%), *Trichoderma* (approximately 5%), and *Beauveria*

(approximately 1%). *Aspergillus* was highly abundant in all the rhizospheres, but in CR2 and CR4, its relative abundance was observed highest and close to each other compared to CR1 and CR3.

Principal Component Analysis (PCA) revealed distinct fungal community profiles across the samples, with PC1 and PC2 explaining 40.9% and 34.8% of the variance, respectively (Figure 6B and Supplementary Table 5). PCA ordination revealed that samples CR2 was separated from other soil samples due to the dominance of *Aspergillus*, *Chaetomium*, *Alternaria*, *Colletotrichum*, *Thermothielavioides* and *Thermothelomyces*. CR1 and CR3 were relatively close to each other while CR4 showed moderate variance from the other samples. Evaluation of alpha diversity indices indicated distinct fungal community structures across the four samples (Figure 7). CR4 and CR2 exhibited the highest fungal diversity with Simpson index (0.27) and (0.28) respectively.

A



B

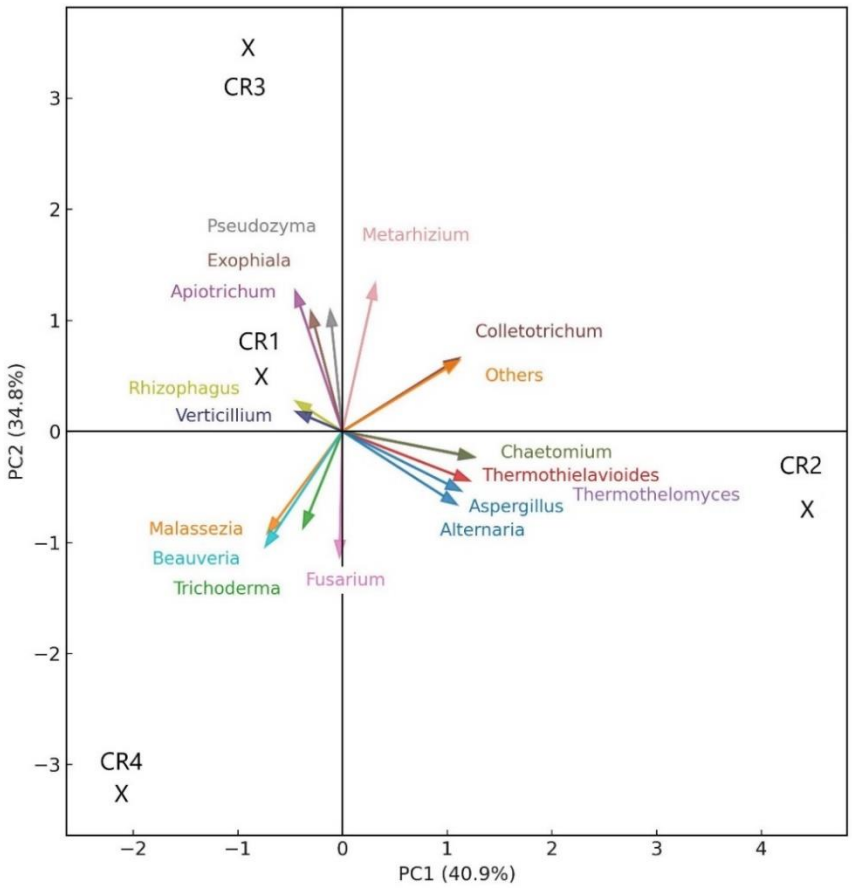


Figure 6. The genus-level fungal communities across the four cotton rhizosphere samples CR1 to CR4. **(A)** genus-level abundance based on read counts; **(B)** Principal component analysis (PCA) showing the variation in genus-level community composition across samples. Genera with an average relative abundance of ≥ 0.5% in all fungi among at least one soil sample were included in the analysis. Genera with < 0.5 % abundance are included as “others”.

while CR3 and CR1showed the lower diversity with Simpson index (0.33). CR4 showed high evenness with Inverse Simpson (3.69) Shannon index (1.78) and contrasting with the lower evenness observed in CR3 with Inverse Simpson (3.00) Shannon index (1.6).

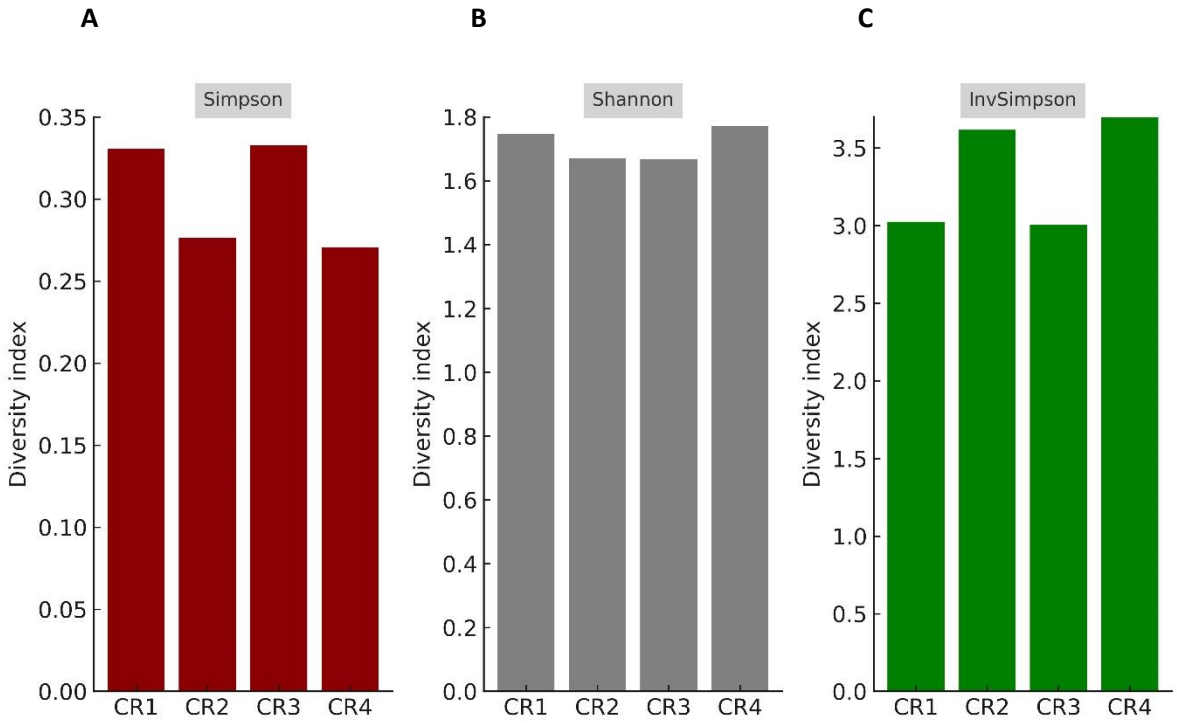


Figure 7. Diversity Indices of fungal communities of the four cotton rhizosphere samples CR-1 to CR-4 showing the richness and evenness at the genus level based on the fungi-assigned read counts. **(A)** Simpson index; **(B)** Shannon index; **(C)** Inverse Simpson index. Genera with an average relative abundance of ≥ 0.5% in all fungi among at least one soil sample were included in the analysis.

At the species level, a total of 128 fungal species were identified from 9570 reads. However, a small subset of 24 species, each representing more than 0.3% of the total reads, accounted for approximately 50% of the observed fungal community. *Aspergillus nidulans* and *Chaetomium globosum* were the most in CR2 with relative abundance of approximately 57% and 22% respectively. *Aspergillus terreus* had high relative abundance in CR4 approximately 20% in CR4 (Figure 8).

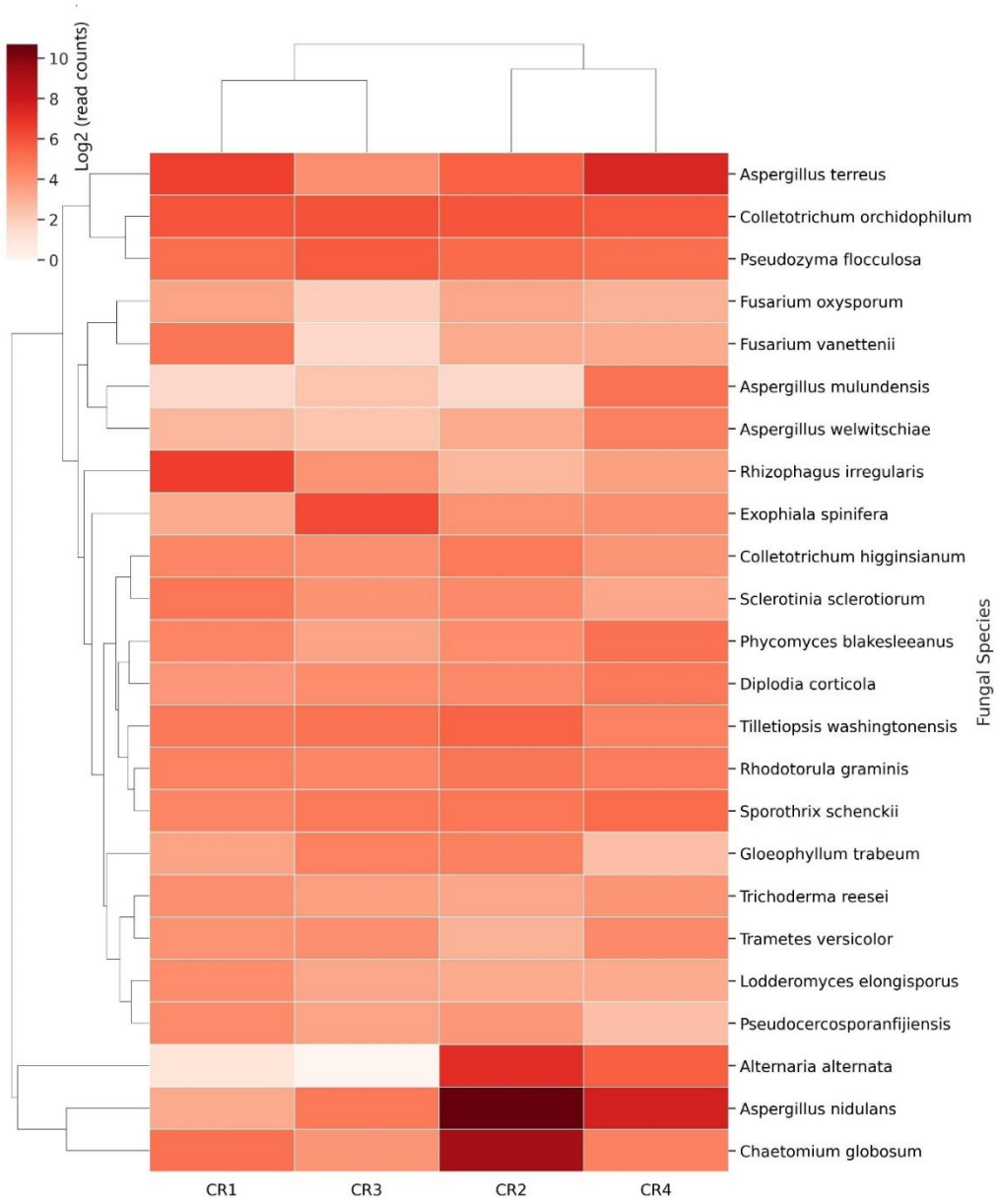


Figure 8. The species-level fungal communities across the four cotton rhizosphere samples CR1 to CR4. **(A)** A bar plot showing fungal abundance based on read counts; **(B)** A heat map illustrating the taxonomic abundance and their relationships across the samples. A dendrogram, determined by computing hierarchical clustering from the abundance levels, displays the connections between the samples and the species. The abundance levels (number of reads associated with each taxon) are log-transformed to base 2 for clarity. Species with an average relative abundance of ≥ 0.3% in all fungi among at least one soil sample were included in the analysis.

4. Discussion

Previous studies revealed that plant root exudates attract microorganisms from the surrounding soil resulting in specific microbial communities that differ significantly among plant species (Finkel et al., 2017). Furthermore, environmental factors including pH, salinity, drought, fertility, soil structure, and cropping pattern exert considerable selective pressure on the structure of these microbial communities (Yan et al., 2015; Verbon and Liberman, 2016; Zhang et al., 2023). Zhang et al. (2023) reported that cotton-onion and intercropping cotton-garlic were effective in managing Verticillium wilt in cotton, as they accumulated beneficial microbial communities including species of *Burkholderia*, *Pseudomonas*, *Penicillium*, and *Chaetomium*, which suppress disease development in cotton.

In our study, we explored the rhizosphere microbiome of cotton, all cultivated in a continuous cotton-wheat rotation system, but at different farming sites with differences in soil type and climatic conditions. Variation in the microbial community compositions at the phylum level was rather small, but increased at the genus level. Additionally, at the species level, we found differences in both microbial community composition and abundance. We found that bacteria overwhelmingly dominated the microbial communities in all four cotton rhizosphere samples (97.25–98.64%), while fungi were represented at lower levels (0.22–0.69%). The highest bacteria-to-fungi ratio was observed in CR4 (~448:1), while the lowest was in CR2 (~141:1), suggesting a relatively higher fungal presence in CR2. These observations align with existing research that generally finds bacterial communities to be more abundant than fungal communities in the cotton rhizosphere (Xi et al., 2019). However, the composition and diversity of these communities can be influenced by several factors, including the plant's developmental stage, soil properties, and genotype (Qiao et al., 2017).

In our study we found in the rhizobacterial community, Actinomycetota was the most prevalent phylum, especially in CR4, CR1, and CR2, indicating its strong dominance. Pseudomonadota was also abundant, with particularly high representation in CR3 and CR2. Myxococcota and Bacillota were moderately abundant, with Myxococcota in CR3 and Bacillota in CR4. Bacteroidota shows a relatively consistent presence in CR1 to CR3 but declines in CR4. Planctomycetota remains at low abundance across all samples, decreasing from CR1 to CR4. The other phyla, including Chloroflexota, Cyanobacteriota, and Gemmatimonadota were relatively abundant in CR1. Deinococcota, Verrucomicrobiota, Rhodothermota, Synergistota, Chlorobiota, and Campylobacterota were abundant in CR2.

While the specific dominant bacterial phyla in the rhizosphere vary among different crops, some are commonly found across plant species. Studies show that Actenomycetota, Bacteroidota, and Pseudomonadota were frequently dominant in the rhizosphere of various plants, including Arabidopsis (Lundberg et al., 2012), watermelon (Ling et al., 2015), barley (Bulgarelli et al., 2015), and cotton (Qiao et al., 2017). This suggests that these phyla may represent a core set of bacterial groups in plant rhizospheres. Qiao et al. (2017), Xi et al. (2019), Lv et al. 2022, and Feng et al. (2024) reported that Pseudomonadota were more abundant in cotton rhizosphere (Qiao et al., 2017; Xi et al., 2019; Lv et al., 2022; Feng et al., 2024),. In the contrary, we found that Actinomycetota dominated the bacterial communities in samples CR1, CR2, and CR4. Pseudomonadota were the second most abundant in these samples, but were dominant in sample CR3, where Actinomycetota were subdominant. Notably, Bacillota and Myxocotta exhibited higher abundance than Bacteroidota in our analysis.

In fungal community composition, Ascomycota consistently exhibited the highest relative abundance in all soil samples. The second most abundant phylum was Basidiomycota, and Mucoromycota was present in smaller proportions, with its most significant contribution in CR1. Other phyla, including Chytridiomycota and Microsporidia, were present in proportions less than 1% of total fungi. The relative abundance of Ascomycota and Basidiomycota in our results was consistent with the findings of Moussa et al. (2017), Song et al. (2024), and Karapareddy et al. (2025), which stated that Ascomycota was the most abundant phylum, followed by Basidiomycota in the cotton rhizosphere (Moussa et al., 2017; Song et al., 2024; Karapareddy et al., 2025).

At the genus level, we observed that most of the rhizobacteria genera present at >0.7% abundance in at least one sample were associated with plant growth promotion, and some were neutral, as no beneficial of pathogenic effect was found in previous studies. *Streptomyces*, known for its biological control potential against plant pathogens and its plant growth-promoting properties (Nazari et al., 2023), was abundant in CR1 and CR4, but its abundance was lower in CR2 and CR3. While CR1 and CR2 exhibited some similarity at the phylum and genus levels, CR3 and CR4 were distinctly different from CR1 and CR2, and also from each other. Each sample displayed a unique profile, with specific genera being most abundant in particular samples. Despite the samples being collected from the rhizosphere of the same crop under the same cropping pattern, distinct patterns of genus abundance emerged, suggesting local environmental influences as. Although CR3 and CR4 differed from CR1 and CR2, the overall composition across all samples was dominated by beneficial genera. This accumulation of beneficial microbes suggests a positive effect of repeated cotton-wheat rotation over time, although each sampling site retained a unique microbial signature. Our results revealed that CR3 exhibited lower bacterial diversity compared to the other samples, with an uneven distribution of rhizobacteria.

Specifically, *Brevundimonas*, *Archangium*, *Nocardia*, and *Sphingomonas*, and *Microvigra* were distinctively abundant in this sample. Notably, various species within these genera are known for their plant growth-promoting capabilities, including the production of phytohormones and the enhancement of abiotic stress tolerance. For example, *Brevundimonas* species have been shown to fix nitrogen, produce IAA, and enhance the growth of both general plants (Naqqash et al., 2020) and Bt cotton (Kumar and Gera, 2014). *Archangium* species have been implicated in heavy metal stress tolerance (Li et al., 2023). *Nocardia* species are also known to produce IAA (Ghodhbane-Gtari et al., 2019), N fixation, and promote plant growth (Alotaibi et al., 2022). *Sphingomonas* species produce gibberellins and IAA, enhancing plant growth (Khan et al., 2014), and nutrient content, including N (Sultana et al., 2024). Furthermore, *Microvigra* species are also known for N fixation and plant growth promotion (Han et al., 2024). Aslam et al. (2025) reported a high N content of 0.63% of the same soil sample, which may be attributed to the presence and activity of these beneficial rhizobacteria in the rhizosphere and the field. This field also gave the highest yield of cotton as it was recorded as 1048kg/ hectare in 2021.

Genera such as *Salinococcus*, *Kocuria*, *Dietzia*, and *Bicillus* associated with CR4 (arid field) were adapted to harsh environment, and many species of these genera play a role in stress tolerance and plant growth promotion. For instance, *Kocuria* species have demonstrated salt tolerance and plant growth-promoting activity (Afridi et al., 2021) as well as heavy metal tolerance (Hansda et al., 2017). *Dietzia* has been shown to exhibit multi-stress tolerance (Gholami and Etemadifar, 2015), while *Marinococcus* is also known for its stress tolerance (Arora et al., 2014), *Salinococcus* is adapted to extreme environments (Usman et al., 2018). *Bacillus* and *Pseudomonas* were also abundant in CR4 compared to other soil samples and, are well-recognized genera for their plant growth-promoting and biocontrol capabilities. Both genera employ multiple mechanisms, including the production of phytohormones and antimicrobial compounds, nutrient mobilization, and the induction of systemic resistance in plants (Santoyo et al., 2012; Sagar et al., 2022). While CR1 and CR2 soil samples had a high influence of *Bradyrhizobium*, *Lentzea,* and *Micromonospora* for this separated them from other samples. While *Rhizobium* is commonly associated with nitrogen fixation in leguminous plants, certain strains are also capable of promoting growth in non-legumes through the production of plant growth-promoting hormones and by antagonizing plant pathogens (Antoun et al., 1998). *Micromonospora* is also known for its nitrogen-fixing and plant growth-promoting abilities (Trujillo et al., 2015; Nouioui et al., 2025).

This study revealed a diverse array of fungal genera with different abundance in each soil sample (CR1-CR4), encompassing plant pathogens such as *Aspergillus* (Nji et al., 2023), *Alternaria* (Logrieco et al.,

2009), *Fusarium* (Summerell, 2019), *Colletotrichum* (Talhinhas and Baroncelli, 2021), and *Verticillium* (Rajendran et al., 2025), alongside beneficial fungi with biocontrol potential. These included the entomopathogens *Beauveria* and *Metarhizium* (Brunner-Mendoza et al., 2019), known for their use in insect pest management (Imoulan et al., 2017), the arbuscular mycorrhizal fungus *Rhizophagus* (Sery et al., 2018), and *Pseudozyma* and *Trichoderma, Chaetomium* (Soytong et al., 2021), other genera employed in biological control strategies (Avis and Bélanger, 2002; Woo et al., 2023). *Aspergillus* was highly abundant in all soil samples, especially in CR2, and *Chaetomium* was the second most abundant genus in CR2, which is known as antagonistic to other pathogenic fungi, then *Rhizophagus* in CR1 and *Pseudozyma*. These differences in microbial community structure and diversity may affect ecosystem processes such as nutrient cycling, plant health, disease suppression and crop yield. Understanding these variations' mechanisms is crucial for developing microbiome-informed crop management practices.

5. Conclusion

Our study reveals that Actinomycetota and Pseudomycota were the most abundant phyla across the four investigated cotton rhizospheres with sampling sites in continuous cotton-wheat rotational fields. Variations in genus-level abundance across sampling sites support the hypothesis that bacterial communities exhibit adaptations to specific soil and environmental conditions. We found that at the genus level, many of the identified genera are known as plant growth-promoting bacteria and are involved in biological control. The abundance of different genera varied across soil samples. The genera most abundant in sample CR4 are known to be adapted to extreme environments, while sample CR3 exhibited a higher prevalence of genera with plant growth-promoting traits. Samples CR1 and CR2 were relatively similar at the phylum and genus levels; however, at the species level, the abundance of species was site-specific. Fewer fungal reads were recovered compared to bacteria, but among the fungal genera identified, many are known to be beneficial to plants through their involvement in the biological control of plant pathogens. From this study, we conclude that continuous cotton-wheat rotation enriches the rhizosphere with plant-beneficial bacterial genera. However, these genera appear to be adapted to their specific sampling sites, as evidenced by their higher abundance in those sites compared to others. These findings emphasize the complex interplay between cropping systems, cropping sites, and the structure of both bacterial and fungal communities in the rhizosphere.

6. Author Contributions

Conceptualization: SA, MB, FMWG, ASSS; Investigation: SA; Data curation and methodology: SA; Project administration: MB, FMWG, ASSS; Resources: FMWG; Software used: MJ; Supervision and validation: SB, ASSS, MB, FMWG; Writing – original draft: SA; Writing - review and editing of the original draft: ASSS, FMWG, SB; Writing - review and editing of final draft: MB, MJ. All authors have read and agreed to the published version of the manuscript.

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8. Declaration of competing interest

The authors state that they have no conflicts of interest, whether financial or personal, that could be perceived as influencing the presented work.

9. References

Adeniji, A., Huang, J., Li, S., Lu, X., and Guo, R. (2024). Hot viewpoint on how soil texture, soil nutrient availability, and root exudates interact to shape microbial dynamics and plant health. *Plant and Soil*, 1-22. doi: 10.1007/s11104-024-07020-y.

Afridi, M.S., Van Hamme, J.d., Bundschuh, J., Sumaira, Khan, M.N., Salam, A., et al. (2021). Biotechnological approaches in agriculture and environmental management-bacterium *Kocuria rhizophila* 14ASP as heavy metal and salt-tolerant plant growth-promoting strain. *Biologia* 76(10), 3091-3105. doi: 10.1007/s11756-021-00826-6.

Alotaibi, F., St-Arnaud, M., and Hijri, M. (2022). In-depth characterization of plant growth promotion potentials of selected alkanes-degrading plant growth-promoting bacterial isolates. *Frontiers in Microbiology* 13, 863702. doi: 10.3389/fmicb.2022.863702.

Antoun, H., Beauchamp, C.J., Goussard, N., Chabot, R., and Lalande, R. (1998). Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: effect on radishes (Raphanus sativus L.). *Plant and soil* 204, 57-67. doi: 10.1023/A:1004326910584.

Arora, S., Vanza, M.J., Mehta, R., Bhuva, C., and Patel, P.N. (2014). Halophilic microbes for bio-remediation of salt affected soils. *Afr J Microbiol Res* 8(33), 3070-3078.

Avis, T.J., and Bélanger, R.R. (2002). Mechanisms and means of detection of biocontrol activity of *Pseudozyma* yeasts against plant-pathogenic fungi. *FEMS Yeast Research* 2(1), 5-8. doi: 10.1111/j.1567-1364.2002.tb00062.x.

Breitwieser, F.P., Baker, D.N., and Salzberg, S.L. (2018). KrakenUniq: confident and fast metagenomics classification using unique k-mer counts. *Genome biology* 19, 1-10.

Brunner-Mendoza, C., Reyes-Montes, M.d.R., Moonjely, S., Bidochka, M.J., and Toriello, C. (2019). A review on the genus Metarhizium as an entomopathogenic microbial biocontrol agent with emphasis on its use and utility in Mexico. *Biocontrol Science and Technology* 29(1), 83-102. doi: 10.1080/09583157.2018.1531111.

Bulgarelli, D., Garrido-Oter, R., Münch, Philipp C., Weiman, A., Dröge, J., Pan, Y., et al. (2015). Structure and Function of the Bacterial Root Microbiota in Wild and Domesticated Barley. *Cell Host & Microbe* 17(3), 392-403. doi: 10.1016/j.chom.2015.01.011.

De Mandal, S., Sonali, Singh, S., Hussain, K., and Hussain, T. (2021). "Plant–microbe association for mutual benefits for plant growth and soil health," in *Current trends in microbial biotechnology for sustainable agriculture,* ed. J.S. Ajar Nath Yadav, Chhatarpal Singh, Neelam Yadav.), 95-121.

Dlamini, S.P., Akanmu, A.O., Fadiji, A.E., and Babalola, O.O. (2023). Maize rhizosphere modulates the microbiome diversity and community structure to enhance plant health. *Saudi Journal of Biological Sciences* 30(1)**,** 103499.

Feng, G., Wu, Y., Yang, C., Zhang, Q., Wang, S., Dong, M., et al. (2024). Effects of coastal saline-alkali soil on rhizosphere microbial community and crop yield of cotton at different growth stages. *Frontiers in Microbiology* 15**,** 1359698. doi: org/10.3389/fmicb.2024.1359698.

Finkel, O.M., Castrillo, G., Paredes, S.H., González, I.S., and Dangl, J.L. (2017). Understanding and exploiting plant beneficial microbes. *Current opinion in plant biology* 38**,** 155-163. doi: 10.1016/j.pbi.2017.04.018.

Franzluebbers, A.J. (2024). Texture and organic matter associations with soil functional properties in crop and conservation land uses in North Carolina. *Soil Science Society of America Journal* 88(2)**,** 449-464.

Ghodhbane-Gtari, F., Nouioui, I., Hezbri, K., Lundstedt, E., D'Angelo, T., McNutt, Z., et al. (2019). The plant-growth-promoting actinobacteria of the genus *Nocardia* induces root nodule formation in Casuarina glauca. *Antonie Van Leeuwenhoek* 112**,** 75-90. doi: 10.1007/s10482-018-1147-0.

Gholami, M., and Etemadifar, Z. (2015). Isolation and characterization of a novel strain of genus Dietzia capable of multiple-extreme resistance. *Microbiology* 84**,** 389-397. doi: 10.1134/S0026261715030054.

Han, H., Kan, D., Tian, M., and Ruan, Y. (2024). Phosphate-solubilizing bacteria reshaped the rhizosphere microbiome and metabolic profile of wheat to inhibit Cd absorption. *Environmental and Experimental Botany* 226**,** 105929. doi: 10.1016/j.envexpbot.2024.105929.

Hansda, A., Kumar, V., and Anshumali (2017). Cu-resistant Kocuria sp. CRB15: a potential PGPR isolated from the dry tailing of Rakha copper mine. *3 Biotech* 7**,** 1-11. doi: 10.1016/j.envexpbot.2020.104023.

Imoulan, A., Hussain, M., Kirk, P.M., El Meziane, A., and Yao, Y.-J. (2017). Entomopathogenic fungus *Beauveria*: Host specificity, ecology and significance of morpho-molecular characterization in accurate taxonomic classification. *Journal of Asia-Pacific Entomology* 20(4)**,** 1204-1212. doi: 10.1016/j.aspen.2017.08.015.

Karapareddy, S., Anche, V.C., Tamatamu, S.R., Janga, M.R., Lawrence, K., Nyochembeng, L.M., et al. (2025). Profiling of rhizosphere-associated microbial communities in North Alabama soils infested with varied levels of reniform nematodes. *Frontiers in Plant Science* 16**,** 1521579. doi: 10.3389/fpls.2025.1521579.

Khan, A.L., Waqas, M., Kang, S.-M., Al-Harrasi, A., Hussain, J., Al-Rawahi, A., et al. (2014). Bacterial endophyte *Sphingomonas* sp. LK11 produces gibberellins and IAA and promotes tomato plant growth. *Journal of Microbiology* 52**,** 689-695. doi: 10.1007/s12275-014-4002-7.

Kumar, V., and Gera, R. (2014). Isolation of a multi-trait plant growth promoting *Brevundimonas* sp. and its effect on the growth of Bt-cotton. *3 Biotech* 4(1)**,** 97-101. doi: 10.1007/s13205-013-0126-4.

Li, X., Wang, Y., Guo, P., Zhang, Z., Cui, X., Hao, B., et al. (2023). Arbuscular mycorrhizal fungi facilitate Astragalus adsurgens growth and stress tolerance in cadmium and lead contaminated saline soil by regulating rhizosphere bacterial community. *Applied soil ecology* 187**,** 104842. doi: 10.1016/j.apsoil.2023.104842.

Ling, N., Song, Y., Raza, W., Huang, Q., Guo, S., and Shen, Q. (2015). The response of root-associated bacterial community to the grafting of watermelon. *Plant and Soil* 391**,** 253-264. doi: 10.1007/s11104-015-2399-3.

Liu, L., Zhu, K., Wurzburger, N., and Zhang, J. (2020). Relationships between plant diversity and soil microbial diversity vary across taxonomic groups and spatial scales. *Ecosphere* 11(1)**,** e02999.

Logrieco, A., Moretti, A., and Solfrizzo, M. (2009). Alternaria toxins and plant diseases: an overview of origin, occurrence and risks. *World Mycotoxin Journal* 2(2)**,** 129-140. doi: 10.3920/WMJ2009.1145.

Lundberg, D.S., Lebeis, S.L., Paredes, S.H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488(7409)**,** 86-90. doi: 10.1038/nature11237.

Luo, J., Gu, S., Guo, X., Liu, Y., Tao, Q., Zhao, H.-P., et al. (2022). Core microbiota in the rhizosphere of heavy metal accumulators and its contribution to plant performance. *Environmental science & technology* 56(18)**,** 12975-12987.

Lv, N., Liu, Y., Guo, T., Liang, P., Li, R., Liang, P., et al. (2022). The influence of Bt cotton cultivation on the structure and functions of the soil bacterial community by soil metagenomics. *Ecotoxicology and Environmental Safety* 236**,** 113452. doi: 10.1016/j.ecoenv.2022.113452.

Moussa, T.A., Al-Zahrani, H.S., Almaghrabi, O.A., Abdelmoneim, T.S., and Fuller, M.P. (2017). Comparative metagenomics approaches to characterize the soil fungal communities of western coastal region, Saudi Arabia. *Plos one* 12(9)**,** e0185096. doi: 10.1371/journal.pone.0185096.

Mubeen, M., Ahmad, A., Hammad, H.M., Awais, M., Farid, H.U., Saleem, M., et al. (2020). Evaluating the climate change impact on water use efficiency of cotton-wheat in semi-arid conditions

using DSSAT model. *Journal of Water and Climate Change* 11(4), 1661-1675. doi: org/10.2166/wcc.2019.179.

Naqqash, T., Imran, A., Hameed, S., Shahid, M., Majeed, A., Iqbal, J., et al. (2020). First report of diazotrophic Brevundimonas spp. as growth enhancer and root colonizer of potato. *Scientific reports* 10(1), 12893. doi: 10.1038/s41598-020-69782-6.

Nazari, M.T., Schommer, V.A., Braun, J.C.A., dos Santos, L.F., Lopes, S.T., Simon, V., et al. (2023). Using *Streptomyces* spp. as plant growth promoters and biocontrol agents. *Rhizosphere* 27, 100741. doi: 10.1016/j.rhisph.2023.100741.

Nji, Q.N., Babalola, O.O., and Mwanza, M. (2023). Soil *Aspergillus* species, pathogenicity and control perspectives. *Journal of Fungi* 9(7), 766. doi: 10.3390/jof9070766.

Nouioui, I., Zimmermann, A., Gomez Escribano, J.P., Jando, M., Pötter, G., Neumann-Schaal, M., et al. (2025). Taxonomic description of Micromonospora reichwaldensis sp. nov. and its biosynthetic and plant growth-promoting potential. *Microbiology Spectrum* 13(4), e02129-02124. doi: 10.1128/spectrum.02129-24.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R., et al. (2013). Package 'vegan'. *Community ecology package, version* 2(9), 1-295.

Park, I., Seo, Y.-S., and Mannaa, M. (2023). Recruitment of the rhizo-microbiome army: assembly determinants and engineering of the rhizosphere microbiome as a key to unlocking plant potential. *Frontiers in Microbiology* 14, 1163832.

Philippot, L., Chenu, C., Kappler, A., Rillig, M.C., and Fierer, N. (2024). The interplay between microbial communities and soil properties. *Nature Reviews Microbiology* 22(4), 226-239.

Qiao, Q., Wang, F., Zhang, J., Chen, Y., Zhang, C., Liu, G., et al. (2017). The variation in the rhizosphere microbiome of cotton with soil type, genotype and developmental stage. *Scientific reports* 7(1), 3940. doi: 10.1038/s41598-017-04213-7.

Ragland, C.J., Shih, K.Y., and Dinneny, J.R. (2024). Choreographing root architecture and rhizosphere interactions through synthetic biology. *Nature Communications* 15(1), 1370.

Rajendran, L., Jothini Varsha, I., Saravanakumari, K., and Durgadevi, D. (2025). "*Verticillium*," in *Compendium of Phytopathogenic Microbes in Agro-Ecology,* ed. K.K. Natarajan Amaresan. (Cham: Springer), 957-966.

Sagar, A., Yadav, S., Sayyed, R., Sharma, S., and Ramteke, P. (2022). "*Bacillus subtilis*: a multifarious plant growth promoter, biocontrol agent, and bioalleviator of abiotic stress," in *Bacilli in agrobiotechnology: Plant stress tolerance, bioremediation, and bioprospecting,* ed. M.R. Md. Tahir Islam, Pradeep Pandey. (Cham: Springer), 561-580.

Salazar, A., Warshan, D., Vasquez-Mejia, C., and Andrésson, Ó.S. (2022). Environmental change alters nitrogen fixation rates and microbial parameters in a subarctic biological soil crust. *Oikos* 2022(11)**,** e09239.

Santoyo, G., Orozco-Mosqueda, M.d.C., and Govindappa, M. (2012). Mechanisms of biocontrol and plant growth-promoting activity in soil bacterial species of Bacillus and Pseudomonas: a review. *Biocontrol Science and Technology* 22(8)**,** 855-872. doi: 10.1080/09583157.2012.694413.

Sery, D.J.-M., Van Tuinen, D., Drain, A., Mounier, A., and Zézé, A. (2018). The genus *Rhizophagus* dominates arbuscular mycorrhizal fungi communities in contrasted cassava field soils in Côte d'Ivoire. *Rhizosphere* 7**,** 8-17. doi: 10.1016/j.rhisph.2018.06.007.

Shen, W., Le, S., Li, Y., and Hu, F. (2016). SeqKit: a cross-platform and ultrafast toolkit for FASTA/Q file manipulation. *PloS one* 11(10)**,** e0163962.

Shi, Y., Yang, H., Chu, M., Niu, X., Huo, X., Gao, Y., et al. (2020). Diversity and space–time dynamics of the bacterial communities in cotton (*Gossypium hirsutum*) rhizosphere soil. *Canadian journal of microbiology* 66(3)**,** 228-242.

Simmons, T., Caddell, D.F., Deng, S., and Coleman-Derr, D. (2018). Exploring the root microbiome: extracting bacterial community data from the soil, rhizosphere, and root endosphere. *Journal of visualized experiments: JoVE* (135)**,** 57561.

Song, W., Wu, H., Xiang, Z., Fan, Y., Wang, S., and Guo, J. (2024). Effects of Plastic Mulch Residue on Soil Fungal Communities in Cotton. *Agriculture* 14(8)**,** 1365. doi: 10.3390/agriculture14081365.

Soytong, K., Kahonokmedhakul, S., Song, J., and Tongon, R. (2021). "Chaetomium application in agriculture," in *Technology in Agriculture,* ed. M.S. Fiaz Ahmad. (Singapore: IntechOpen).

Sultana, R., Islam, S.M.N., Shuvo, S.B., Ehsan, G.M.A., Saha, P., Khan, M.M.R., et al. (2024). Endophytic bacterium *Sphingomonas panaciterrae* NB5 influences soil properties and improves growth, nutrient contents, and yield of red amaranth (Amaranthus tricolor L.). *Current Plant Biology* 39**,** 100372. doi: https://doi.org/10.1016/j.cpb.2024.100372.

Summerell, B.A. (2019). Resolving *Fusarium*: Current status of the genus. *Annual review of phytopathology* 57(1)**,** 323-339. doi: 10.1146/annurev-phyto-082718-100204.

Talhinhas, P., and Baroncelli, R. (2021). Colletotrichum species and complexes: geographic distribution, host range and conservation status. *Fungal Diversity* 110(1)**,** 109-198. doi: 10.1007/s13225-021-00491-9.

Tian, X.-l., Wang, C.-b., Bao, X.-g., Wang, P., Li, X.-f., Yang, S.-c., et al. (2019). Crop diversity facilitates soil aggregation in relation to soil microbial community composition driven by intercropping. *Plant and Soil* 436**,** 173-192. doi: 10.1007/s11104-018-03924-8.

Trujillo, M.E., Riesco, R., Benito, P., and Carro, L. (2015). Endophytic actinobacteria and the interaction of Micromonospora and nitrogen fixing plants. *Frontiers in Microbiology* 6**,** 1341. doi: 10.3389/fmicb.2015.01341.

Truong, D.T., Franzosa, E.A., Tickle, T.L., Scholz, M., Weingart, G., Pasolli, E., et al. (2015). MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nature methods* 12(10)**,** 902-903.

Usman, H., Farouq, A., Baki, A., Abdulkadir, N., and Mustapha, G. (2018). Production and characterization of orange pigment produced by Halophilic bacterium *Salinococcus roseus* isolated from Abattoir soil. *J Microbiol Exp* 6(6)**,** 238-243. doi: 10.15406/jmen.2018.06.00222.

Verbon, E.H., and Liberman, L.M. (2016). Beneficial microbes affect endogenous mechanisms controlling root development. *Trends in plant science* 21(3)**,** 218-229. doi: 10.1016/j.tplants.2016.01.013.

Wang, X., Wang, S., Huang, M., He, Y., Guo, S., Yang, K., et al. (2024). Phages enhance both phytopathogen density control and rhizosphere microbiome suppressiveness. *Mbio* 15(6)**,** e03016-03023.

Woo, S.L., Hermosa, R., Lorito, M., and Monte, E. (2023). Trichoderma: a multipurpose, plant-beneficial microorganism for eco-sustainable agriculture. *Nature Reviews Microbiology* 21(5)**,** 312-326. doi: 10.1038/s41579-022-00819-5.

Wood, D.E., and Salzberg, S.L. (2014). Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome biology* 15**,** 1-12.

Xi, H., Shen, J., Qu, Z., Yang, D., Liu, S., Nie, X., et al. (2019). Effects of long-term cotton continuous cropping on soil microbiome. *Scientific Reports* 9(1)**,** 18297. doi: 10.1038/s41598-019-54771-1.

Yan, N., Marschner, P., Cao, W., Zuo, C., and Qin, W. (2015). Influence of salinity and water content on soil microorganisms. *International soil and water conservation Research* 3(4)**,** 316-323. doi: 10.1016/j.iswcr.2015.11.003.

Yang, C.-X., Chen, S.-J., Hong, X.-Y., Wang, L.-Z., Wu, H.-M., Tang, Y.-Y., et al. (2025). Plant Exudates Driven Microbiome Recruitment and Assembly Facilitates Plant Health Management. *FEMS Microbiology Reviews***,** fuaf008.

York, L.M., Carminati, A., Mooney, S.J., Ritz, K., and Bennett, M.J. (2016). The holistic rhizosphere: integrating zones, processes, and semantics in the soil influenced by roots. *Journal of experimental botany* 67(12)**,** 3629-3643.

Zhang, G., Liu, P., Zhang, L., Wei, W., Wang, X., Wei, D., et al. (2016). Bioprospecting metagenomics of a microbial community on cotton degradation: Mining for new glycoside hydrolases. *Journal of Biotechnology* 234**,** 35-42.

Zhang, H., Liu, W., Xiong, Y., Li, G., Cui, J., Zhao, C., et al. (2024). Effects of dissolved organic matter on distribution characteristics of heavy metals and their interactions with microorganisms in soil under long-term exogenous effects. *Science of The Total Environment* 947**,** 174565.

Zhang, Y., Yang, Y., Lu, X., Wang, A., Xue, C., Zhao, M., et al. (2023). The effects and interrelationships of intercropping on Cotton Verticillium wilt and soil microbial communities. *BMC microbiology* 23(1)**,** 41. doi: 10.1186/s12866-023-02780.

Supplementary data

Table 1. Summary of classified and unclassified reads obtained from metagenomic sequencing. The table presents the total number of reads, the number and percentage of reads successfully taxonomically classified, and those remaining unclassified after quality filtering and bioinformatics analysis.

Sample Name	Reads	Classified reads	Classified reads %	Unclassified reads	Unclassified reads %
CR1	21,094,542	1,298,994	6.16 %	19,795,548	93.84 %
CR2	21,703,372	1,577,619	7.27 %	20,125,733	92.73 %
CR3	21,877,366	1,983,732	9.07 %	19,893,634	90.93 %
CR4	20,903, 870	2,827,216	13.52 %	18,076,654	86.84 %

Table 2. Other Bacterial Phyla (along with their respective reeds) with an average relative abundance of < 0.5% in all rhizobacteria among all rhizosphere soil samples are summarized.

Other phyla	CR1	CR2	CR3	CR4
Deinococcota	1787	2161	0	1707
Acidobacteriota	1745	1636	1320	0
Verrucomicrobiota	1362	1462	1001	784
Chloroflexota	1350	1082	834	813
Cyanobacteriota	1347	952	958	1015
Gemmatimonadota	586	475	476	365
Thermomicrobiota	570	625	559	949
Nitrospirota	467	324	323	191
Rhodothermota	222	467	274	210
Spirochaetota	204	199	180	107
Synergistota	156	218	159	184
Chlorobiota	129	199	160	112
Campylobacterota	78	186	97	106
Armatimonadota	74	68	56	50
Kiritimatiellota	63	74	58	41

Thermotogota	46	32	28	19
Aquificota	40	45	9	20
Chlamydiota	37	63	34	23
Fibrobacterota	36	11	10	15
Mycoplasmatota	32	24	29	16
Nitrospinota	30	37	37	51
Balneolota	26	33	10	41
Bdellovibrionota	24	20	30	22
Chrysiogenota	21	27	32	15
Chloroflexi	14	6	4	36
Ignavibacteriota	13	33	14	13
Deferribacterota	13	32	19	5
Fusobacteriota	11	19	13	10
Elusimicrobiota	6	5	1	1
Calditrichota	5	8	9	7
Coprothermobacterota	3	4	0	1
Dictyoglomota	3	3	0	0
Proteobacteria	0	917	0	0
Verrucomicrobia	0	2	0	0
Nitrospirae	0	1	2	0
Thermodesulfobiota	0	1	0	1
Lentisphaerota	0	0	1	0
Thermodesulfobacteriota	0	0	0	1961

Table 3. PC1 and PC2 loadings of genera**.** The table presents the loadings of each genus on Principal Components 1 and 2, indicating their contributions to the variance captured by each component in the PCA analysis. Higher absolute loading values reflect greater influence of the genus in differentiating the samples along the respective principal component axes.

Rhizobacteria Genera	PC1 Loading	PC2 Loading
Streptomyces	1.152229996	-0.02166
Salinicoccus	1.116760651	0.291198
Nocardioides	1.143558374	0.128899
Saccharomonospora	1.115039733	0.299776
Geodermatophilus	1.084093604	0.396592
Saccharopolyspora	1.122930027	0.266915
Janibacter	1.103352312	0.339901
Mycolicibacterium	-0.908785941	0.543835
Nocardiopsis	1.124465794	0.261742
Micromonospora	-0.339311441	0.98846
Marinococcus	1.116703437	0.291689
Rhodococcus	0.001644726	1.151631
Isoptericola	1.120005843	0.280925
Actinomadura	1.109480377	0.203025
Dietzia	1.109598145	0.315599
Ornithinimicrobium	1.115848198	0.296713
Kocuria	1.107084457	0.305721
Nocardia	-0.784542583	0.539034
Bacillus	1.040708235	0.499914
Amycolatopsis	1.149585605	-0.10388
Pseudonocardia	1.146450558	-0.11373
Actinoplanes	1.084092181	-0.22547
Conexibacter	1.060627072	-0.44294
Archangium	-0.592043624	0.858025
Pseudomonas	0.776201218	0.845635
Microbacterium	0.655707172	0.936839
Jiangella	0.891974626	-0.64386

Microvirga	-0.615065981	0.746924
Lentzea	0.144432786	-1.11363
Nonomuraea	0.088183644	-0.08913
Bradyrhizobium	-0.439942276	-0.88064
Sphingomonas	-0.844921176	0.785633
Sporichthya	-0.085977985	-0.75441
Mesorhizobium	-0.90955731	0.022757
Pseudarthrobacter	-0.956803384	0.542917
Brevundimonas	-0.641741277	0.842129

Table 4. Other fungal Phyla, along with their respective reads with an average relative abundance of < 0.5% in all rhizobacteria among all rhizosphere soil samples, are summarized.

Other phyla	**CR1**	**CR2**	**CR3**	**CR4**
Chytridiomycota	6	6	46	20
Microsporidia	4	3	0	3

Table 5. PC1 and PC2 loadings of fungal genera**.** The table presents the loadings of each genus on Principal Components 1 and 2, indicating their contributions to the variance captured by each component in the PCA analysis. Higher absolute loading values reflect greater influence of the genus in differentiating the samples along the respective principal component axes.

Fungal Genera	**PC1 Loading**	**PC2 Loading**
Alternaria	0.982666	-0.54696
Malassezia	-0.63679	-0.75134
Trichoderma	-0.32232	-0.68785
Thermothielavioides	1.093871	-0.36899
Thermothelomyces	1.128257	-0.19618
Exophiala	-0.26767	0.880946
Fusarium	-0.02471	-0.92003
Pseudozyma	-0.10294	0.88588
Rhizophagus	-0.32728	0.183779

Beauveria	-0.66159	-0.85931
Apiotrichum	-0.41006	1.057892
Verticillium	-0.31604	0.11589
Chaetomium	1.134631	-0.19178
Colletotrichum	1.006081	0.548282
Metarhizium	0.284213	1.114088
Aspergillus	1.018365	-0.44414
Others	1.004697	0.527778

Figure 1

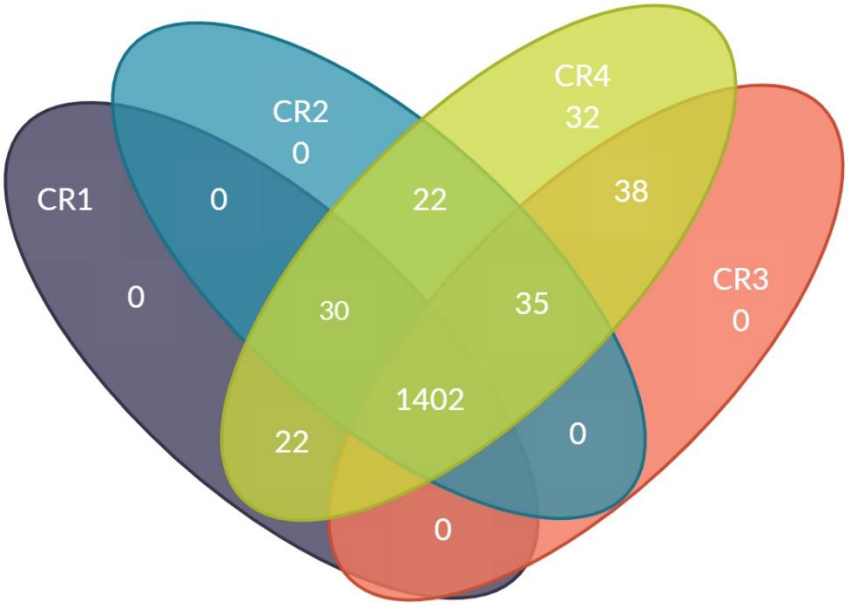

Figure 1: Distribution of all rhizobacteria Genera across soil samples CR1 to CR4, as visualized by a Venn diagram. Overlapping areas indicate shared bacterial genera among the samples.

Figure 2

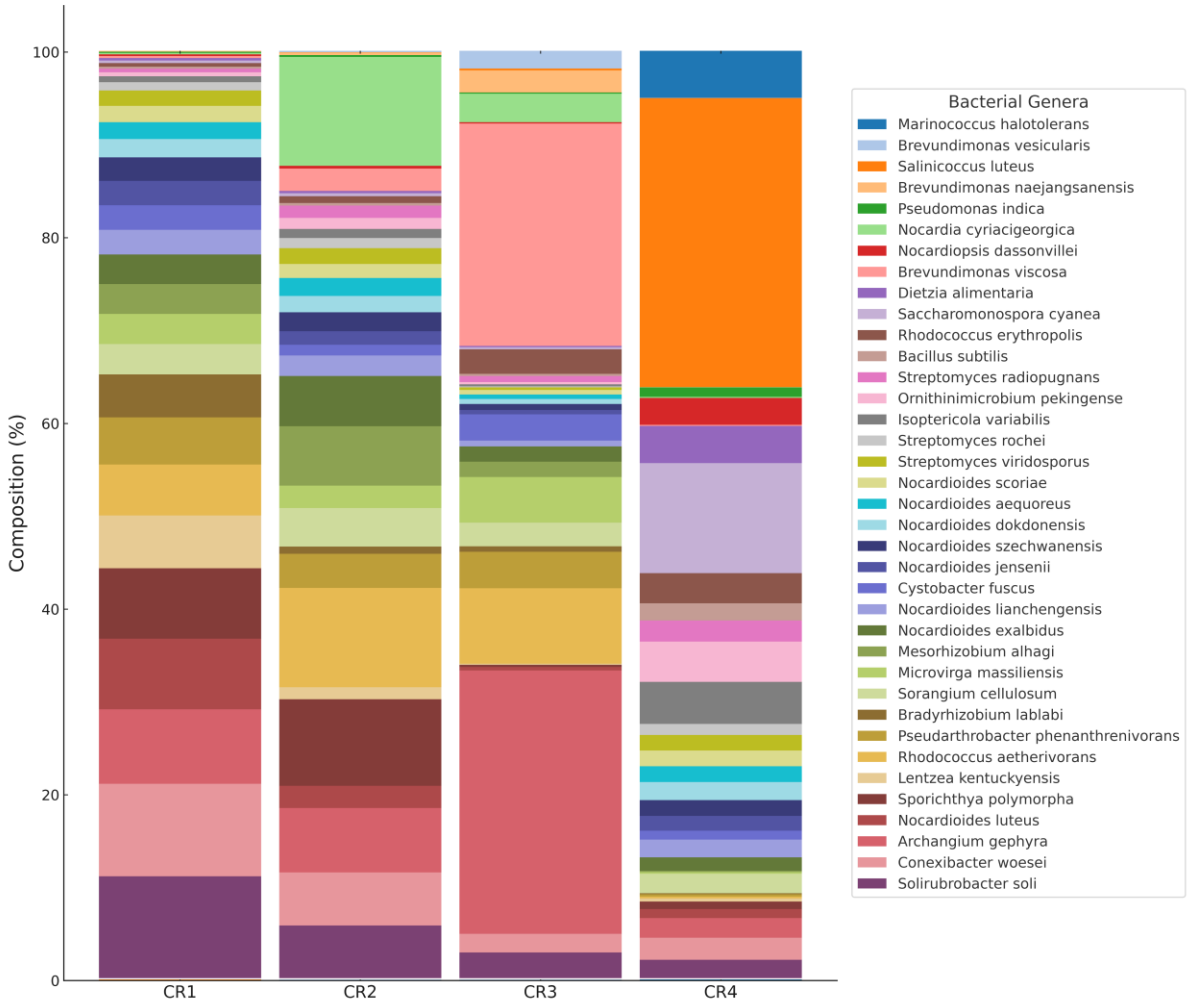

Figure 2. The composition of the rhizobacteria communities at species-level across the four cotton rhizosphere samples, CR1 to CR4. A bar plot showing rhizobacteria abundance based on read counts. Species with an average relative abundance of ≥ 0.3% in at least one soil sample were included.

Figure 3

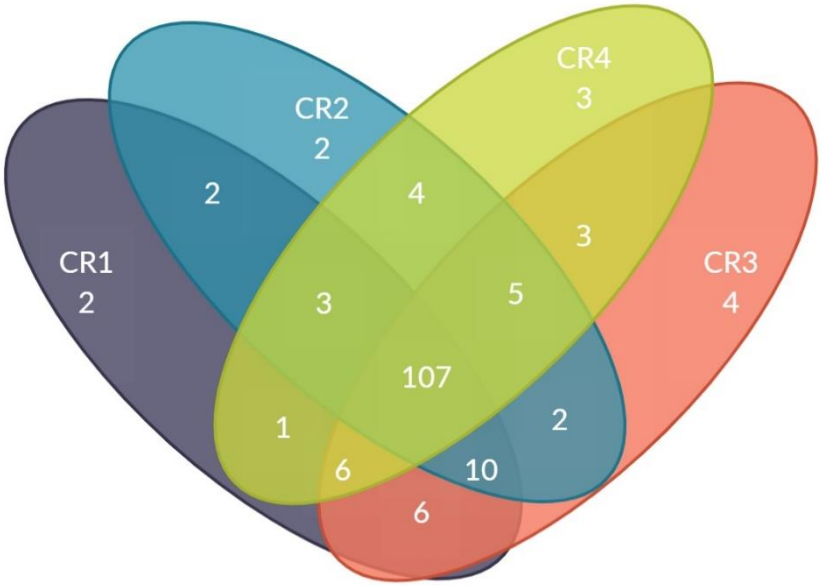

Figure 3: Distribution of all Fungal Genera across soil samples CR1to CR4, as visualized by a Venn diagram. Overlapping areas indicate shared fungal genera among the samples.

Rhizobacterial diversity exhibiting biotic stress tolerance in association with wheat-cotton crop rotation: Implications for plant–microbe interactions and agroecosystem resilience

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ORIGINAL PAPER

Rhizobacterial diversity exhibiting biotic stress tolerance in association with wheat-cotton crop rotation: Implications for plant–microbe interactions and agroecosystem resilience

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Abstract
Biotic stress, particularly from fungal diseases, significantly impedes agricultural productivity worldwide. To meet the increasing demand for sustainable food production, environment-friendly and cost-effective solutions are needed. Plant growth-promoting rhizobacteria (PGPR) provide a sustainable alternative by enhancing plant health and acting as biocontrol agents. This study aimed to investigate the genetic diversity and biocontrol potential of biotic stress-tolerant rhizobacteria isolated from the rhizosphere of cotton and wheat plants infected with fungi in a cotton-wheat rotation area. A total of 136 rhizobacteria were isolated and screened for their *in-vitro* antifungal activity against *Fusarium oxysporum*. Among these, 108 isolates demonstrated antifungal activity against *F. oxysporum*. Additionally, various biocontrol-linked traits were assessed, including hydrogen cyanide (HCN) production, starch hydrolysis, exopolysaccharide (EPS) production, enzyme production (pectinase, protease, gelatinase, catalase) and biofilm formation. The results showed that 88 isolates exhibited pectinase activity, 105 showed biofilm formation and EPS production, 20 demonstrated protease production, 93 showed starch hydrolysis activity. Only three isolates produced hydrogen cyanide. Gelatinase activity was observed in 124 isolates, while catalase activity was detected in 87 isolates. Genetic diversity analysis of the tolerant rhizobacteria was performed using REP, ERIC, and $(GTG)_5$-PCR fingerprinting. The dendrogram constructed from $(GTG)_5$ and REP-PCR fingerprint profiles indicated greater diversity. Moreover, all three PCR-primers effectively differentiated the cotton rhizosphere isolates from those obtained from the wheat rhizosphere, indicating a distinct resident bacterial community despite the cotton-wheat rotation. These findings suggest the presence of diverse, biotic stress-tolerant rhizobacteria in the cotton–wheat rotation area, which could be utilized as potential biocontrol agents against fungal plant diseases. However, further research is required to explore the pathways underlying their antifungal potential and to develop sustainable and efficient bio-formulations for field applications.

Keywords Rhizobacteria · Antifungal activity · Genetic diversity · Rhizosphere · *Fusarium oxysporum*

Introduction

In plants, the term "stress" refers to environmental factors that negatively affect their growth, development, or productivity. These stressors can cause various changes in the plants, such as altered growth rates, gene expression, crop yields, and cellular metabolism. Biotic stress, which arises from a range of biotic agents such as fungi, viruses, insects, and bacteria, is a major limiting factor affecting plant productivity (Umar et al. 2021). Biotic stress accounts for an estimated 30% of crop losses worldwide, posing a significant challenge to food security worldwide (Kumar and Verma 2018). In addition, the utilisation of pesticides and inorganic

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fertilisers in agriculture further degrades soil fertility and contributes to environmental pollution (Kumar et al. 2019).

To address this challenge, plant growth-promoting rhizobacteria (PGPR) serve as a sustainable and eco-friendly solution to enhance plant tolerance to biotic stress (Bukhat et al. 2020). These beneficial rhizobacteria contribute to plant health and growth by providing a range of services, such as nutrient acquisition, stress tolerance, and disease resistance (Hakim et al. 2021). Among these services, the role of rhizobacteria in enhancing plant tolerance to biotic stress has gained particular attention due to its potential to reduce reliance on synthetic pesticides and fungicides in agriculture by triggering systemic resistance and improving growth (Hakim et al. 2021; Saeed et al. 2021).

Several studies have demonstrated that PGPR employ a multi-faceted approach to combat plant diseases. These beneficial bacteria produce a range of bioactive molecules, including volatile organic compounds, antibiotics, siderophores, and lytic enzymes, which can inhibit fungal growth and reduce disease severity (Elnahal et al. 2022). Additionally, PGPR can modulate ethylene levels in plants, potentially influencing defence responses by altering the cell wall through lignin deposition (Khoshru et al. 2023). Furthermore, research suggests that plants activate overlapping signalling pathways in response to both pathogenic microorganisms and PGPR, indicating a complex and finely tuned regulatory network (Oleńska et al. 2020). The intricate interplay between induced-systemic resistance and systemic acquired resistance pathways is mediated by hormonal regulation. Recent advancements have facilitated the identification of a substantial number of regulatory molecules coordinating these pathways. Moreover, some PGPR can solubilise and mobilise nutrients in soil, promoting their uptake by host plant and enhancing their nutritional status (Bukhat et al. 2020). Therefore, the application of PGPR in agriculture can lead to a reduction in the use of synthetic/artificial pesticides and fertilisers, mitigating their harmful effects on the human health and ecosystem.

Crop rotation is a fundamental agricultural technique that provides various benefits to crop production systems. It not only mitigates the impact of pests and diseases but also plays a critical role in maintaining fertility of soil (Tahir et al. 2015). This is achieved through the release of exudates from the roots of different crops, which stimulates the growth and population of beneficial microbes, enhances nitrogen fixation, and increases the availability of essential nutrients. In Pakistan, wheat–rice and wheat–cotton rotations are more prevalent (Tahir et al. 2015). These rotations have profound influence on the richness, composition, and diversity of the soil microbial communities (Mishra et al. 2019). The rhizosphere soil under crop rotation and continuous cultivation exhibits distinct microbial community profiles (Kour et al. 2019; Ghani et al. 2022).

The diversity and abundance of soil microbes has been a subject of great interest for scientists. Recent advancements in technology, which includes both culture-dependent methods and molecular analysis, have considerably enhanced our understanding of the taxonomic diversity of soil microorganisms (Garza and Dutilh 2015). Moreover, various techniques including enterobacterial repetitive intergenic consensus (ERIC)-PCR, $(GTG)_5$-PCR and repetitive extragenic palindromic (REP)-PCR analysis have enabled researchers to investigate the effect of climatic factors on the abundance and diversity of specific microbial groups within soil. These techniques provide valuable perspective into the composition of microbial communities both locally and globally (Youseif 2018). For example, a study identified novel soil microbiota with soybean growth-promoting capabilities through genetic fingerprinting. GTG marker-based genetic fingerprinting revealed unique band patterns, suggesting their effectiveness in differentiating isolates (Gonçalves et al. 2023). Furthermore, genomic fingerprints generated in sugarcane plants by amplifying $(GTG)_5$ fragments identified the phylogenetic relationships and genetic diversity of endophytic bacteria associated with their roots (Wang et al. 2020).

In the context of wheat-cotton rotation, the selection of biotic stress-tolerant rhizobacteria using these techniques can have a positive impact on crops, as they share common soil, pest, and disease challenges. Several studies have reported the characterization and isolation of rhizobacteria from wheat and cotton rhizosphere with beneficial traits (Zain et al. 2019; Naqqash et al. 2022). The most extensively studied biotic stress-tolerant rhizobacteria include species belonging to the genera of *Bacillus*, *Azospirillum*, *Streptomyces*, and *Pseudomonas* (Bukhat et al. 2020; Bhat et al. 2022). For example, within the rhizospheres of corn plants, *Pseudomonas fluorescens* has been shown to inhibit the growth of *Bipolaris maydis* through competition for glucose (Mohamed and Caunter 1995). However, limited research has been conducted to explore the genetic diversity and biocontrol potential of rhizobacteria in wheat-cotton rotations. Therefore, this research aims to investigate these aspects of biotic stress-tolerant rhizobacteria associated with this cropping system, with a focus on their potential application against fungal plant diseases.

Materials and methods

Collection of soil sample

Soil samples were collected from the rhizosphere of fungal-infected cotton and wheat plants in five different cotton-wheat rotational areas of Punjab, Pakistan: Multan, Alipur, Muzaffargarh, Khanewal, and Yazman. The soil samples were then bagged in plastic (25 × 30 cm) and frozen in dry

ice for subsequent analysis and experimentation. All the experiments were conducted in triplicates with appropriate controls.

Isolation of rhizobacteria

The bacteria present in rhizospheric soil samples were identified using the serial dilution technique. Briefly, rhizospheric soil (one gram) was suspended in 9 ml (0.89%) solution of sterile saline in glass test tubes (Somasegaran and Hoben 1994). Serial dilutions of 100µL were then plated onto LB agar plates to isolate bacteria from the rhizospheric soil following incubation at 28 ± 2 °C for 48 h. Colonies with varying morphology were identified, selected and purified for further analysis.

Physio-chemical analysis

Soil samples were analysed for different physio-chemical properties, such as electrical conductivity, soil texture, soil saturation, pH, sodium adsorption ratio (SAR), organic matter, available nitrogen, phosphorus and potassium (Naqqash et al. 2020). Briefly, soil was dried for 24 h, at 40 °C and sieved using a 2 mm mesh for subsequent analysis. Soil texture analysis was performed using the Bouyoucous hydrometer method. Approximately 50 g soil sample was dispersed in a solution of 1% sodium hexametaphosphate (40 mL) and deionized water (150 mL). Readings were taken after 40 s and 2 h of stirring, and the soil texture was classified according to the international textural classification system (Mwendwa 2022). A 250 g soil-deionized water paste was prepared and allowed to equilibrate for 1 h. The pH of the paste was then measured using a pH meter (JENCO Model-671) (Ryti 1965). Soil electrical conductivity was measured using the Jenway EC meter from a clear extract obtained by vacuum filtration of the soil paste (Corwin and Lesch 2005). Soil organic matter was determined by Walkley–Black method. Soil samples (1 g) were used to prepare a solution with 20 ml of sulfuric acid, 10 mL of potassium dichromate, 25 mL of ferrous sulphate, and 150 mL of water. This mixture was then titrated using 10 mL of potassium permanganate to a pink endpoint (De Vos et al. 2007). Total nitrogen (N) was determined by Kjeldahl digestion (Bremner 1965). Ten grams of soil were digested with 30 mL of sulfuric acid and a digestion mixture [$CuSO_4$ (0.5):K_2SO_4 (10):$FeSO_4$ (1)]. The distillate was collected after ammonia separation and titrated with sulfuric acid using a micro-Kjeldahl apparatus. Extractable phosphorus of soil was estimated using standard protocol, with quantification performed by a spectrophotometer (Pierzynski 2000). Approximately 5 g of soil sample was used to prepare a soil solution with 1 N ammonium acetate solution. The volume of the solution was adjusted to 100 mL. Following shaking, the extractable potassium concentration in the filtered extract (Whatman filter paper No. 1) was determined using a flame photometer.

In-vitro antifungal assay

All isolated rhizobacteria were tested for their *in-vitro* antifungal potential against *Fusarium oxysporum* using a dual-culture assay on potato-dextrose-agar plates (Sakthivel and Gnanamanickam 1986). Briefly, fungal inoculum was spotted in the centre of plates and subsequent streaking of bacterial isolates around the fungal spot. The plates were then subjected to an incubation period of 5–7 days at a temperature of 28 ± 2 °C. Additionally, a positive control was established through spot inoculation of fungus at the centre of a separate Petri dish, which was also subjected to the same incubation conditions. Antifungal activity of test isolates was calculated by following formula:

$$\text{Inhibition}\,(\%) = \{(1 - (\text{Fungal growth} \div \text{Control growth}))\} \times 100$$

HCN production

Rhizobacterial isolates were inoculated in LB broth supplemented with 4.4 g/L glycine. The strips of filter paper saturated in solution of picric acid (0.05% solution prepared in 2% of sodium carbonate) were then suspended in each test tube. Following, incubation for 48 h at 30 °C, the tubes were observed for the colour change. A shift from yellow into red-brownish colouration on the filter strips indicated the positive result for production of HCN (Nowosad and MacVicar 1940).

Starch hydrolysis

Isolated strains were streaked on LB plates containing starch (20 g/L), for the detection of amylase production. These LB plates were then incubated for 24–48 h at 28 ± 2 °C. Following the incubation, the test plates were immersed in a Lugol solution comprising KI (1%) and I_2 (0.5%). These plates were subsequently examined for starch hydrolysis (Dunican and Seeley 1962). Clear zones around the isolated strains colonies indicated positive amylase activity.

Detection of protease and gelatinase production

Skim-milk agar media was utilised for estimating protease activity (O'Sullivan et al. 1991), while nutrient gelatine medium containing peptone (5 g/L), gelatin (120 g/L), and beef extract (3 g/L) was used for the detection of gelatinase production (Holding and Collee 1971).

Detection of pectinase production

Isolates were streaked on agar media plates containing pectin and were incubated at 28 ± 2 °C for 2 days. Following incubation, these plates were flooded with KI solution. Production of pectinase enzyme was estimated by clear zone formation around bacterial colonies (Beg et al. 2000).

Biofilm production

The *in vitro* biofilm formation capacity of all isolated strains was evaluated using a microtiter plate method (Davey and O'toole 2000). Briefly, the strains were allowed to form biofilms in the wells of a microplate. Following incubation, the biofilms were stained using 1% crystal violet solution for ½ h. Subsequently, the wells were washed 2–3 times with phosphate-buffered saline (pH 7.2) to remove unbound stain. To quantify the biofilm biomass, ethanol (70%) was added to each well, resulting in solubilisation of the crystal violet dye incorporated within the biofilm matrix. The absorbance of the solubilised dye was then taken at 590 nm using ELISA reader.

Exopolysaccharide (EPS) production

The production of EPS by the isolated strains was quantified using the phenol–sulfuric acid method (Titus et al. 1995). Briefly, cell-free supernatants were obtained by centrifuging overnight cultures of the isolates at 8000 rpm. The sterilised supernatants were then treated with equal volumes of 9.8 M sulfuric acid and 0.5 M phenol solution. The absorbance of the resulting solution was taken at 490 nm using a UV–visible spectrophotometer to estimate the amount of EPS present.

Catalase test

Isolated bacteria were inoculated onto nutrient agar slants following incubation at 30 °C for a period of 24 h. After incubation, 100 µl of each culture was placed on a glass slide, then, 50 µl of H_2O_2 was added in these cultures. The bubble formation was observed for positive catalase activity (Pine et al. 1984).

Genetic diversity studies

Bacterial genomic DNA was extracted by CTAB method (William et al. 2012). The DNA quality, integrity and quantity were checked using 0.8% agarose gel electrophoresis and UV spectrophotometer (Voytas 1992). For diversity analysis of isolated rhizobacteria, polymerase chain reactions were performed using ERIC (Forward: 5′-ATGTAAGCTCCTGGGGATTCAC-3′; Reverse: 5′-AAGTAAGTGACTGGGGTGAGCG-3′), REP (Forward: 5′-IIIICGICGICATCIGGC-3′; Reverse: 5′-ICGICTTATCIGGCCTAC-3′) and $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) primer (Li et al. 2017; Lanoot et al. 2004). ERIC and REP PCR were performed in Gene Amp® system (Applied BioSystems) thermocycler using 20 µl of reaction mixture containing: 3 µl of bacterial DNA template, 12 µl of Vazyme Taq master mix, 2 µl of PCR water and 1.5 µl of each primer. For GTG_5-PCR, 20 µl reaction mixture was prepared by using 3 µl of bacterial DNA template, 12 µl of Vazyme Taq master mix, 0.5 µl of PCR water, 1.5 µl of primer and 1.5 µl of DMSO. A total of 35 cycles were performed using previously reported reaction conditions with minor modifications. The program included an initial denaturation at 95 °C for 5 min, followed by final denaturation for each cycle at 94 °C for 30 s. Annealing temperatures were set at 48 °C for ERIC, 41.8 °C for REP and at 50 °C for $(GTG)_5$ primer, each lasting for 1.5 min. The extension step was set at 70 °C for 1 min per cycle and final extension at 70 °C for 10 min (Di Giovanni, 1999). 2% agarose gel was used for the conformation of PCR products (Bilung et al., 2018).

After the confirmation of PCR amplification on agarose gel electrophoresis, Polyacrylamide Gel Electrophoresis (PAGE) was performed for the separation of products on the base of product size. 6 µl of each PCR product were loaded in the PAGE and samples were allowed to run for two hours at 100 V. A ladder of 100 bp was also run in the gel for the comparison of products size. Following electrophoresis, the gel was treated with a fixative solution (450 ml distilled water, 5 ml absolute ethanol and 3 ml of acetic acid) to preserve the bands. Subsequently, a silver nitrate stainer and developer (6 g NaOH and 4 ml of formaldehyde) were used for visualisation of bands (Muiru et al. 2010).

Data analysis

The visible bands on the gel were scored using 0 to indicate the absence of an allele, and 1 to represent the presence of an allele. Polymorphism, which reflects genetic diversity within isolates, was calculated based on the presence and absence of alleles. To construct a phylogenetic tree of the 136 rhizobacterial isolates based on genetic distances, the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) was employed (Dallwitz 1988). To assess statistical differences of different physio-chemical properties of all soil samples, analysis of variance (ANOVA) was performed using Statistix 8.1 software. Comparisons of between different samples were performed using Fisher's Least Significant Difference (LSD) test at a significance level of $\alpha = 0.05$.

Results

Physio-chemical properties of soil samples

The texture of rhizospheric soil samples collected from five different fungal-infected cotton and wheat plants varied, with loamy, clay loamy and sandy loamy textures observed. The electrical conductivity (EC) of the cotton samples ranged from 1.15 to 3.90, while the wheat samples ranged from 1.30 to 3.70. Both crops exhibited saturation levels between 25 and 48%, with a pH range of 7.8–9.0. The sodium adsorption ratio (SAR) was higher in the cotton samples (1.2–4.2) compared to the wheat samples (1.5–2.2). The percentage of organic matter and total nitrogen in the cotton samples varied between 0.49–0.71% and 0.024–0.630%, respectively. In contrast, the wheat samples exhibited a range of 0.48–0.83% for organic matter and 0.024%-0.313% for total nitrogen. Total mineral phosphorus was higher in the cotton samples (5–20 ppm) compared to the wheat samples (5–9 ppm), whereas both types of samples showed a similar range for total mineral potassium ranging between 153–290 ppm. All the measured soil physio-chemical properties are enlisted in Table 1.

Isolation of rhizobacterial strains, *in-vitro* antifungal assay and biocontrol traits

A total of 136 rhizobacteria were isolated from the rotational areas of cotton-wheat crops using the serial dilution method and subsequently screened for antifungal activity against *F. oxysporum*. Out of these 136 rhizobacterial isolates, 108 exhibited antifungal activity (Fig. 1; Table 2). The isolated rhizobacteria were further screened for different biocontrol traits including HCN production, gelatinase, catalase, pectinase and protease activity, biofilm formation and production of exopolysaccharides (Figs. 2 & 3). Notably, only three strains (SW3, SW30 and SW31) isolated from the wheat rhizosphere produced HCN. Among the isolates, gelatinase activity was performed by 124 isolates, catalase activity was performed by 87 isolates and protease activity was performed by 28 isolates. Biofilm formation and exopolysaccharides were observed in 105 isolates. Moreover, 93 isolates showed starch hydrolysis activity, while 88 isolates showed pectinase activity (Fig. 3). Supplementary Table 1 represents the results of all the isolates regarding the biocontrol and antifungal activity performed in this study.

Genetic diversity

In the present study, REP, ERIC, and $(GTG)_5$ fingerprints were utilised to investigate the genetic diversity of selected

Table 1 Physio-chemical analysis of soil samples from where the rhizobacteria were isolated

Properties	SC1	SC2	SC3	SC4	SC5	SW1	SW2	SW3	SW4	SW5
EC (d S m^{-1})	1.15 ± 0.03^{g}	3.90 ± 0.20^{a}	2.34 ± 0.24^{b}	2.26 ± 0.09^{c}	2.29 ± 0.07^{c}	1.30 ± 0.05^{f}	1.32 ± 0.06^{f}	3.70 ± 0.22^{a}	2.11 ± 0.06^{d}	1.45 ± 0.03^{e}
Soil texture	Loam	Clay loam	Loam	Loam	Sandy loam	Loam	Loam	Clay loam	Loam	Loam
Saturation (%)	36 ± 3.21^{d}	48 ± 4.21^{a}	35 ± 3.55^{d}	37 ± 2.43^{d}	25 ± 2.84^{e}	37 ± 3.15^{d}	41 ± 3.44^{c}	44 ± 3.61^{b}	37 ± 2.56^{d}	35 ± 2.75^{d}
Soil pH 1:1	8.2 ± 0.20^{b}	7.8 ± 0.22^{c}	8.1 ± 0.25^{b}	8.8 ± 0.33^{a}	9 ± 0.35^{a}	8.2 ± 0.20^{b}	8.1 ± 0.24^{b}	7.8 ± 0.21^{c}	8.1 ± 0.22^{b}	8.2 ± 0.22^{b}
SAR	2 ± 0.11^{d}	1.2 ± 0.10^{f}	1.2 ± 0.09^{f}	4 ± 0.15^{b}	4.2 ± 0.15^{a}	1.4 ± 0.03^{e}	1.9 ± 0.05^{d}	1.5 ± 0.03^{e}	2 ± 0.11^{d}	2.2 ± 0.09^{c}
Organic matter (%)	0.49 ± 0.002^{f}	0.49 ± 0.003^{f}	0.61 ± 0.005^{d}	0.71 ± 0.005^{b}	0.62 ± 0.009^{d}	0.55 ± 0.005^{e}	0.62 ± 0.002^{d}	0.83 ± 0.008^{a}	0.48 ± 0.005^{g}	0.69 ± 0.005^{c}
Total N (%)	0.0245 ± 0.0002^{f}	0.025 ± 0.0001^{f}	0.630 ± 0.002^{a}	0.035 ± 0.0001^{e}	0.312 ± 0.005^{b}	0.275 ± 0.003^{c}	0.313 ± 0.005^{b}	0.042 ± 0.0001^{d}	0.024 ± 0.0001^{f}	0.035 ± 0.0001^{e}
Available Phosphorus (ppm)	5 ± 0.43^{e}	20 ± 0.37^{a}	7 ± 0.50^{c}	5 ± 0.44^{e}	6 ± 0.38^{d}	6 ± 0.13^{d}	6 ± 0.23^{d}	9 ± 0.57^{b}	7 ± 0.49^{c}	5 ± 0.20^{e}
Available Potash (ppm)	153 ± 2.00^{f}	290 ± 3.10^{a}	220 ± 2.78^{c}	162 ± 3.85^{e}	125 ± 4.57^{g}	290 ± 4.55^{a}	250 ± 5.89^{b}	157 ± 2.78^{f}	250 ± 5.55^{b}	210 ± 2.65^{d}

SC cotton samples; *SW* wheat samples; *SAR* sodium adsorption ratio

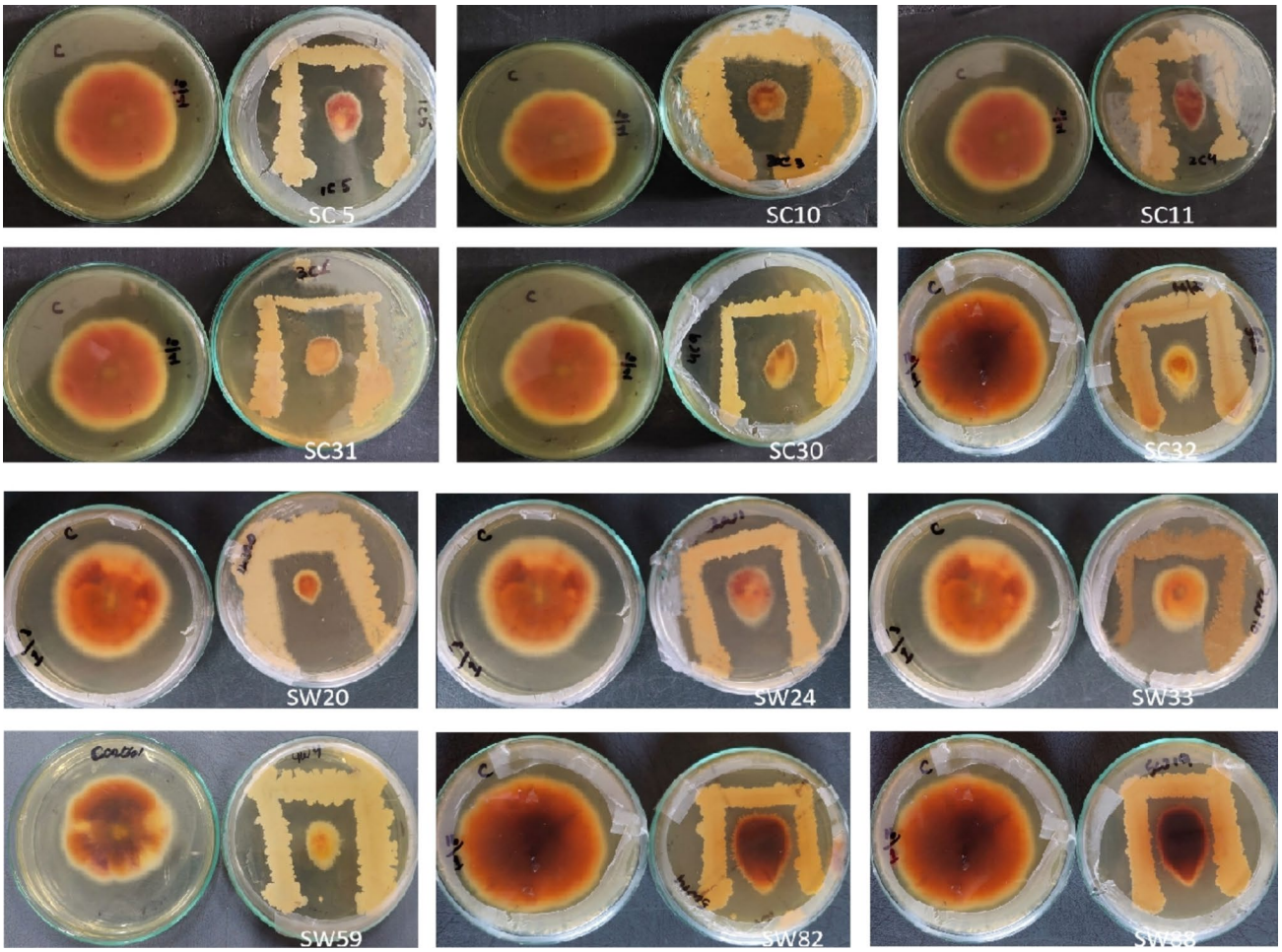


Fig. 1 Representative figures of antifungal activity of different rhizobacteria isolated from cotton wheat rotation areas

rhizobacteria isolated from the cotton and wheat rhizosphere. Various polymorphic bands ware observed ranging from 50 bp to above 2 kb, which facilitated clear differentiation among the isolates. High-quality DNA fingerprint profiles were generated for all 136 isolates using each primer set. REP, ERIC, and $(GTG)_5$-PCR generated highly complex fingerprint patterns due to the large number of polymorphic bands amplified from each isolate. Banding patterns on the agarose gels were visually analysed to estimate variability among the strains. $(GTG)_5$-PCR produced the highest number of bands (Supplementary Fig. 1), indicating significant genetic diversity among all 136 isolates. In contrast, ERIC-PCR produced a simpler profile compared to $(GTG)_5$ (Supplementary Fig. 2) and REP-PCR profile (Supplementary Fig. 3).

Dendrograms were constructed using REP, ERIC, and (GTG)5-PCR fingerprints to determine the relatedness of all isolated bacterial strains (Fig. 4). For data analysis, Jaccard similarity coefficients and a clustering method based on pairwise similarity coefficients using UPGMA were employed to analyse the data and explore the relationships among the rhizobacterial isolates. The dendrogram generated from $(GTG)_5$-PCR fingerprints showed four major clusters. Cluster I contained 48 isolates and was further subdivided into various sub-clusters. Similarly, cluster II, which contained 21 isolates, was also sub-divided into various sub-clusters. Cluster III and IV consisting of 55 and 12 isolates, respectively, were also subdivided into various sub-clusters (Fig. 4A). The dendrogram generated by this marker suggests a higher degree of similarity among isolates from the cotton rhizosphere, which tend to cluster together more closely compared to the bacterial strains isolated from the wheat rhizosphere.

The dendrogram produced through REP-PCR fingerprints exhibits two major clusters. Cluster I contained 83 isolates, while Cluster II contained 53 isolates. Both clusters were further divided into various sub-clusters (Fig. 4B). Similar to the results obtained from $(GTG)_5$-PCR, the dendrogram generated by this marker revealed that isolates from the cotton rhizosphere are more similar to each other compared

Table 2 Antifungal activity of rhizobacteria isolated from cotton wheat rotation areas

Isolates	Antifungal activity	% inhibition
SC1	+	20.51
SC2	+	41.03
SC3	+	12.82
SC4	+	10.26
SC5	+	61.54
SC6	+	33.33
SC7	−	0.00
SC8	+	23.08
SC9	+	25.64
SC10	+	33.33
SC11	+	51.28
SC12	+	23.08
SC13	−	0.00
SC14	+	56.41
SC15	+	41.03
SC16	+	23.08
SC17	+	5.13
SC18	+	2.56
SC19	−	0.00
SC20	+	64.10
SC21	+	7.69
SC22	−	0.00
SC23	+	32.65
SC24	+	34.69
SC25	+	28.57
SC26	+	28.57
SC27	−	0.00
SC28	+	34.78
SC29	−	0.00
SC30	+	46.94
SC31	+	22.45
SC32	+	48.98
SC33	+	24.49
SC34	−	0.00
SC35	+	38.78
SC36	−	0.00
SC37	−	-4.35
SC38	−	0.00
SC39	+	44.90
SC40	−	0.00
SC41	+	44.90
SC42	+	34.69
SC43	−	0.00
SW1	+	13.64
SW2	+	13.64
SW3	+	11.36
SW4	+	31.82
SW5	+	27.27
SW6	+	4.35
SW7	+	18.18
SW8	+	2.17
SW9	−	0.00
SW10	+	2.17
SW11	+	13.64
SW12	+	43.18
SW13	−	0.00
SW14	+	43.18
SW15	−	0.00
SW16	+	45.45
SW17	+	43.18
SW18	+	50.00
SW19	−	0.00
SW20	+	45.45
SW21	−	0.00
SW22	+	45.45
SW23	−	0.00
SW24	+	43.18
SW25	+	31.81
SW26	+	25.00
SW27	+	29.55
SW28	+	50.00
SW29	+	25.00
SW30	+	29.50
SW31	+	2.17
SW32	+	6.82
SW33	+	50.00
SW34	+	43.18
SW35	+	27.27
SW36	+	2.17
SW37	+	9.09
SW38	+	22.73
SW39	+	35.29
SW40	+	56.86
SW41	+	9.80
SW42	−	0.00
SW43	+	49.02
SW44	+	21.57
SW45	−	0.00
SW46	−	0.00
SW47	+	58.82
SW48	+	60.78
SW49	−	0.00
SW50	+	21.57
SW51	+	13.04
SW52	+	31.37
SW53	+	56.86
SW54	+	11.76
SW55	+	23.53
SW56	+	2.17

Table 2 (continued)

Isolates	Antifungal activity	% inhibition
SW57	−	0.00
SW58	+	56.86
SW59	+	56.86
SW60	+	9.80
SW61	+	29.41
SW62	+	11.76
SW63	+	27.45
SW64	+	3.92
SW65	+	31.37
SW66	+	3.92
SW67	+	41.18
SW68	−	0.00
SW69	+	60.78
SW70	+	13.56
SW71	−	0.00
SW72	−	0.00
SW73	+	56.52
SW74	+	45.76
SW75	+	2.17
SW76	+	44.07
SW77	+	15.25
SW78	+	44.07
SW79	+	66.10
SW80	+	49.15
SW81	+	52.54
SW82	+	16.95
SW83	+	57.63
SW84	+	28.81
SW85	+	33.90
SW86	+	10.17
SW87	+	61.02
SW88	+	49.15
SW89	+	32.20
SW90	+	28.81
SW91	−	0.00
SW92	+	32.20
SW93	+	64.41

+positive activity; − no activity

to the bacterial isolates from the wheat rhizosphere as they were grouped within the same cluster.

The dendrogram constructed from ERIC-PCR fingerprints also exhibited two major clusters. Cluster I and II Contain 123 and 13, isolates respectively, which were further subdivided into various sub-clusters (Fig. 4C). Additionally, the dendrogram based on the biochemical properties of all the isolates was generated using UPGMA. This dendrogram revealed three major clusters (I, II and III). Cluster I consisting of 106 isolates was subdivided into various sub-clusters. Cluster II consists of only four isolates, while Cluster III includes 26 isolates (Fig. 4D). The dendrogram based on biochemical lacked distinct separation between clusters, suggesting lower diversity among the rhizobacteria isolated from both cotton and wheat crops.

Discussion

Fungal diseases pose a significant challenge to crop productivity worldwide. However, numerous studies have demonstrated the efficacy of PGPR in controlling these diseases caused by fungal pathogens (Shaikh and Sayyed 2015). The identification of rhizobacteria with antifungal properties is an initial step in developing bio-formulations for the biocontrol of plant fungal diseases (Walsh et al. 2001). The present research reports the characterisation and isolation of 136 rhizobacteria from the rhizospheres of wheat and cotton crops. These isolates were screened for their antifungal potential against *Fusarium oxysporum* and evaluated for other biocontrol traits. REP, ERIC, and $(GTG)_5$-PCR fingerprints were employed to analyse genetic diversity of these bacterial strains.

Fungal-infected cotton and wheat rhizospheric soil samples were investigated to identify potential antifungal rhizobacteria. The results demonstrated variations in the percentage of total mineral nitrogen, organic matter, and total mineral phosphorus across the different soil samples, indicating differences in soil fertility and nutrient availability. The variation in soil properties might potentially influence the growth and composition of resident microbial communities, including rhizobacteria (Li et al. 2021; Arshad et al. 2022). The successful isolation of 136 rhizobacteria from the collected soil samples indicated the presence of a diverse bacterial community within the rhizosphere of these plants. The isolation of rhizobacteria from the rhizosphere of plants is a crucial step in developing PGPR for the biocontrol of fungal pathogenic microbes (Chaiharn et al. 2009). Moreover, the current study also investigated the antifungal potential of isolated rhizobacterial strains from cotton and wheat rhizospheric soil against *F. oxysporum*, a fungal pathogen responsible for wilt and root rot diseases in several economically important crops (Bahadur 2021). Our results demonstrated that a significant portion (108 out of 136) of the isolated rhizobacteria inhibited the growth of *F. oxysporum,* indicating their potential as biocontrol agents against this fungus in cotton and wheat crops. These findings are consistent with previous studies that highlight the efficacy of PGPR in controlling fungal pathogens (Devkota et al. 2020; Vignesh et al. 2021).

Rhizobacteria were screened for various biocontrol traits, including HCN production, gelatinase, catalase, pectinase, protease activity, biofilm formation, and the

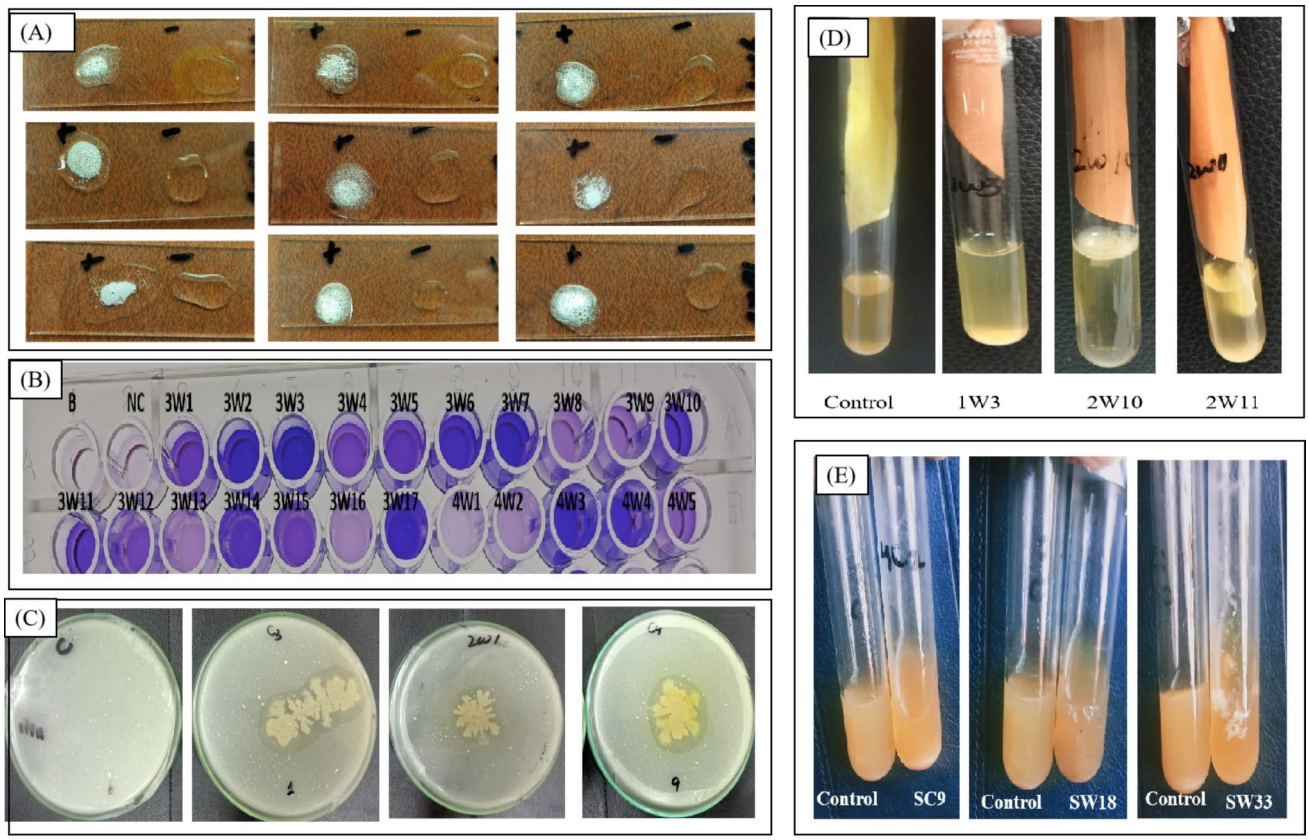


Fig. 2 Representative figures of biochemical characterization of rhizobacteria isolated from cotton wheat rotation areas **A** Catalase activity; **B** Biofilm production; **C** Protease activity; **D** HCN production and; **E** Gelatinase activity

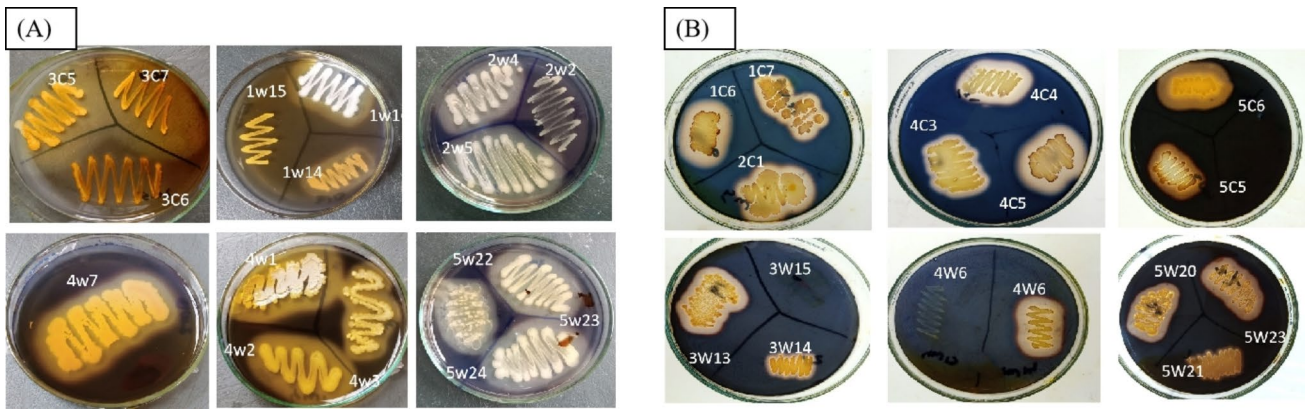


Fig. 3 Representative figures of (**A**) Starch hydrolysis and; (**B**) Pectinase activity of different rhizobacteria isolated from cotton wheat rotation areas

production of exopolysaccharides. These traits have been shown to be important for rhizobacterial ability as biocontrol agents against fungal diseases (Benaissa 2023). HCN production is reported to be a common trait of rhizobacteria with biocontrol activity against fungal phytopathogens (Goswami et al. 2015). In our study, only three isolates from the wheat rhizosphere (SW3, SW33, and SW34) were able to produce HCN. Hydrolytic enzymes like gelatinase, catalase, amylase, and protease may potentially enable these isolates to degrade extracellular components of fungal pathogens (Jadhav et al. 2017). Biofilm formation and exopolysaccharide production are crucial for rhizobacteria

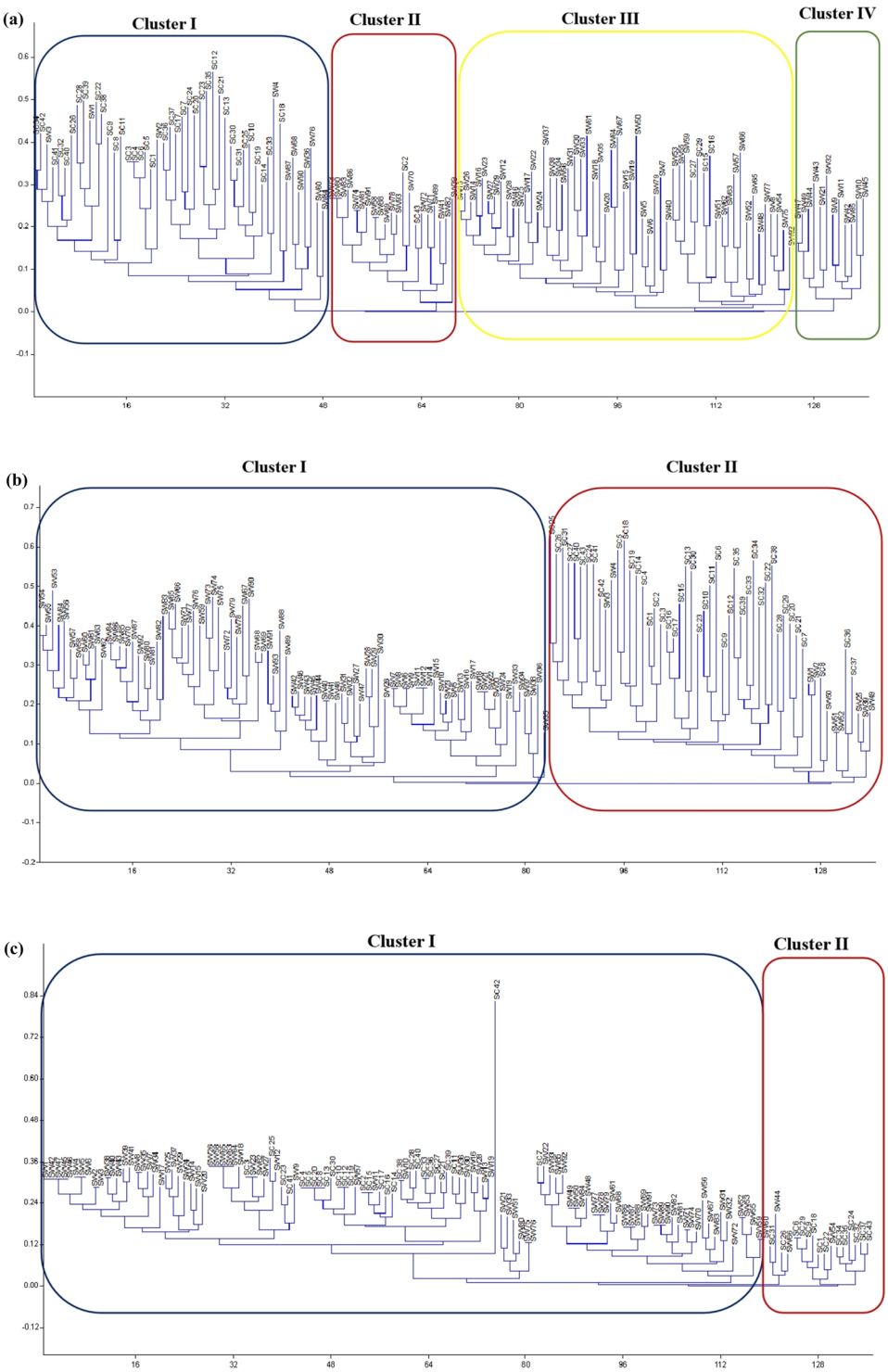
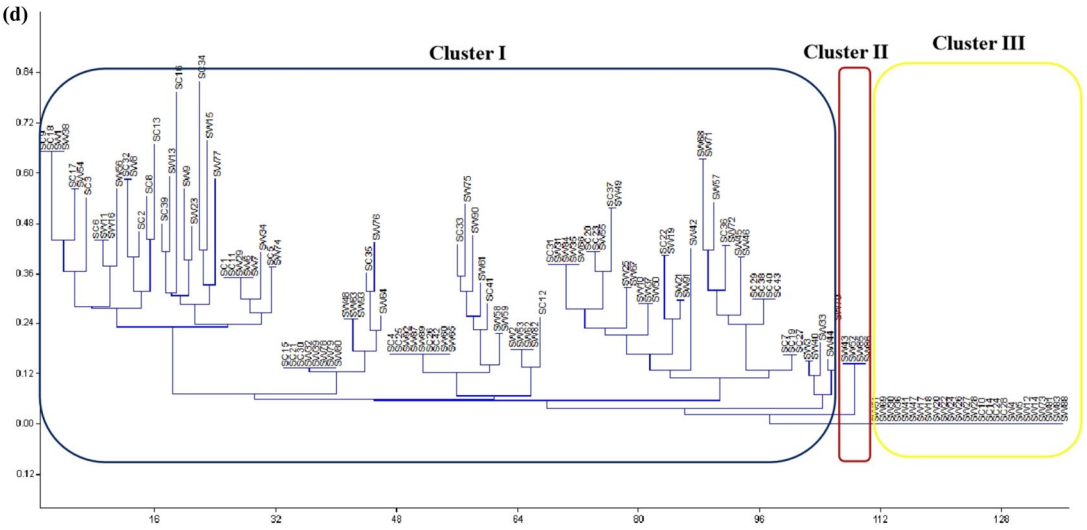


Fig. 4 **A**: Cluster analysis of $(GTG)_5$-PCR fingerprints revealing the genotypic diversity of all the isolated rhizobacteria from cotton and wheat rhizosphere. Similarity coefficient calculations were used to obtain dendrogram and clustering was done using unweighted pair-grouping method based on arithmetic averages (UPGMA) using NTSYS software and the Jaccard coefficient. **B**: Cluster analysis of REP-PCR fingerprints revealing the genotypic diversity of all the isolated rhizobacteria from cotton and wheat rhizosphere. Similarity coefficient calculations were used to obtain dendrogram and clustering was done using unweighted pair-grouping method based on arithmetic averages (UPGMA) using NTSYS software and the Jaccard coefficient. **C**: Cluster analysis of ERIC-PCR fingerprints revealing the genotypic diversity of all the isolated rhizobacteria from cotton and wheat rhizosphere. Similarity coefficient calculations were used to obtain dendrogram and clustering was done using unweighted pair-grouping method based on arithmetic averages (UPGMA) using NTSYS software and the Jaccard coefficient. **D**: Cluster analysis based on biochemical assays revealing the diversity of all the isolated rhizobacteria from cotton and wheat rhizosphere. Similarity coefficient calculations were used to obtain dendrogram and clustering was done using unweighted pair-grouping method based on arithmetic averages (UPGMA) using NTSYS software and the Jaccard coefficient

to colonise plant roots and compete effectively with fungal pathogens (Zhu et al. 2020). Therefore, investigating the distribution, growth, and colonisation of bacterial biofilms within the rhizosphere of plants is of great significance. In this study, an *in-vitro* biofilm formation assay showed that out of 136 isolates, 105 showed biofilm formation.

The genetic diversity of the isolated rhizobacteria was analysed using REP, ERIC, and $(GTG)_5$ fingerprinting methods. ERIC sequences are 127-bp long imperfect palindromes that exist in multiple copies within bacterial genomes; they were initially discovered in *E. coli* by (Hulton et al. 1991; Wilson and Sharp 2006). However,

Fig. 4 (continued)



ERIC-PCR produced a lex complex profile as compared to the $(GTG)_5$ and REP-PCR profiles in this study. REP sequences are shorter (33 bp) and can be found in multiple copies at a single position in the chromosome (Becerril et al. 1985). $(GTG)_5$-PCR fingerprinting, an established genotyping technique that uses a single poly-trinucleotide (GTG) primer to target the conserved and repetitive poly GTG sequences found in bacterial genomes (Wang et al. 2020; Gonçalves et al. 2023). This technique has been shown to be effective for differentiating bacterial strain compared to other molecular typing analyses (Ranjbar and Afshar 2019).

Analysis using REP and $(GTG)_5$-PCR fingerprinting showed a high degree of variability among the bacterial strains, generating the highest number of bands and indicating the considerable genetic diversity among the isolates. These findings are consistent with previous studies that have reported similar high genetic diversity among rhizobacteria isolated from different crops. For example, Marasco et al. (2013) found that rhizobacteria isolated from grapevine exhibited considerable diversity, and that PCR-based fingerprinting methods were effective in characterising this diversity. Similarly, Li et al. (2017) reported high levels of genetic diversity among rhizobacteria isolated from sugarcane plants, using a combination of 16S rRNA gene sequencing and PCR fingerprinting.

Moreover, dendrograms analysis of $(GTG)_5$-PCR and REP-PCR fingerprints revealed greater similarity among cotton rhizosphere isolates compared to those from the wheat rhizosphere. This finding support the fact that interactions among microbes and plants in the rhizosphere are extremely dynamic shaped by co-evolutionary pressure (Garcia and Kao-Kniffin 2018). This finding is in line with previous research suggesting that different crops may harbour distinct rhizosphere microbiomes, and that microbes exhibit various behaviours in the rhizosphere environment (Edwards et al. 2015; Hu et al. 2018).

The identification of repetitive DNA elements within bacterial genomes, including those belonging to the REP, ERIC, and BOX families, has facilitated the development of repetitive-sequence PCR fingerprinting techniques (Louws et al. 1999). This method offers a relatively simple and efficient approach for bacterial strain characterisation at the genomic or genus level. Supporting the findings presented here, Trindade et al. (2005) employed REP-, ERIC-, and BOX-PCR fingerprinting to characterise *Xanthomonas campestris*, yielding 16, 19, and 12 bands, respectively. Additionally, Bilung et al. (2018) further investigated the potential of these techniques by characterising 29 different *Leptospira* strains using ERIC-PCR and BOX-PCR. Their work revealed a higher primer discriminatory index for BOX-PCR (0.809).

Advancements in PCR-based molecular approaches have led to the development of rapid, cost-effective, and user-friendly methods for characterisation of microbes at both the species and genus level (Albuquerque et al. 2009). Among these techniques, repetitive-sequence PCR fingerprinting offers a powerful tool for generating unique genetic profiles of individual microbial strains or entire microbial communities through the direct analysis of amplified DNA fragments (amplicons). Compared to traditional methods like DNA sequencing, PCR-based DNA fingerprinting techniques offer moderate-to-high sensitivity, cost-effective, and speed, allowing the simultaneous analysis of numerous samples (Levin et al. 2018). Since, DNA fingerprinting techniques can reveal the composition and diversity of microbial communities in a single step. However, it is important to acknowledge that these techniques may not always provide detailed phylogenetic information (Buszewski et al. 2017). This inherent limitation underscores the need of further research using high-throughput

characterisation methods to establish detailed phylogenetic relationship among biotic-stress tolerant rhizobacteria.

Conclusions for future biology

In conclusion, this study provides valuable insights into the genetic diversity and biocontrol potential of rhizobacteria isolated from the cotton and wheat rhizospheres. The PCR fingerprinting methods revealed considerable variability among the isolates, with ERIC-PCR producing a less complex profile as compared to $(GTG)_5$ and REP-PCR profiles. Furthermore, the screening of these isolates demonstrated their efficacy as biocontrol agents against plant fungal pathogens. In the context of wheat-cotton rotation, understanding the genetic diversity and functional traits of rhizobacteria associated with both crops can led to the development of effective biocontrol strategies against fungal plant diseases. These strategies hold promise for sustainable and cost-effective management of plant fungal diseases, ultimately contributing to global food security. Overall, these findings have important implications for the advancements of sustainable and environmentally friendly approaches to crop protection. However, further research is needed to evaluate the effectiveness of these isolated strains under field or greenhouse conditions, considering natural factors like competition with native microbiota and soil composition.

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References

Albuquerque P, Mendes MV, Santos CL, Moradas-Ferreira P, Tavares F (2009) DNA signature-based approaches for bacterial detection and identification. Sci Total Environ 407(12):3641–3651

Arshad M, Naqqash T, Tahir M, Leveau JH, Zaheer A, Tahira SA, Saeed NA, Asad S, Sajid M (2022) Comparison of bacterial diversity, root exudates and soil enzymatic activities in the rhizosphere of AVP1-transgenic and nontransgenic wheat (*Triticum aestivum* L.). J Appl Microbiol 133(5):3094–3112

Bahadur A (2021) Current status of fusarium and their management strategies. In: Fusarium-An Overview of the Genus. IntechOpen

Becerril B, Valle F, Merino E, Riba L, Bolivar F (1985) Repetitive extragenic palindromic (REP) sequences in the Escherichia coli gdhA gene. Gene 37(1–3):53–62

Beg Q, Bhushan B, Kapoor M, Hoondal G (2000) Production and characterization of thermostable xylanase and pectinase from Streptomyces sp. QG-11–3. J Indus Microbiol Biotechnol 24(6):396–402

Benaissa A (2023) Plant beneficial bacteria from rhizosphere as biocontrol agents of phytopathogens. Vegetos:1–4

Bhat BA, Tariq L, Nissar S, Islam ST, Islam SU, Mangral Z, Ilyas N, Sayyed RZ, Muthusamy G, Kim W (2022) The role of plant-associated rhizobacteria in plant growth, biocontrol and abiotic stress management. J Appl Microbiol 133(5):2717–2741

Bilung LM, Pui CF, Su'ut L, Apun K (2018) Evaluation of BOX-PCR and ERIC-PCR as molecular typing tools for pathogenic Leptospira. Disease markers 2018

Bremner J (1965) Total nitrogen. Methods of soil analysis: part 2 chemical and microbiological properties 9:1149–1178

Bukhat S, Imran A, Javaid S, Shahid M, Majeed A, Naqqash T (2020) Communication of plants with microbial world: exploring the regulatory networks for PGPR mediated defense signaling. Microbiol Res 238:126486

Buszewski B, Rogowska A, Pomastowski P, Złoch M, Railean-Plugaru V (2017) Identification of microorganisms by modern analytical techniques. J AOAC Int 100(6):1607–1623

Chaiharn M, Chunhaleuchanon S, Lumyong S (2009) Screening siderophore producing bacteria as potential biological control agent for fungal rice pathogens in Thailand. World J Microbiol Biotechnol 25:1919–1928

Corwin DL, Lesch SM (2005) Apparent soil electrical conductivity measurements in agriculture. Comput Electron Agric 46(1–3):11–43

Dallwitz M (1988) A flexible clustering method based on UPGMA and ISS.

Davey ME, O'toole GA (2000) Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 64(4):847–867

De Vos B, Lettens S, Muys B, Deckers JA (2007) Walkley-Black analysis of forest soil organic carbon: recovery, limitations and uncertainty. Soil Use Manag 23(3):221–229

Devkota P, Kloepper JW, Enebak SA, Eckhardt LG (2020) Towards biocontrol of ophiostomatoid fungi by plant growth-promoting rhizobacteria. Biocontrol Sci Tech 30(1):19–32

Dunican LK, Seeley HW (1962) Starch hydrolysis by Streptococcus equinus. J Bacteriol 83(2):264–269

Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, Eisen JA, Sundaresan V (2015) Structure, variation, and assembly of the root-associated microbiomes of rice. Proc Natl Acad Sci 112(8):E911–E920

Elnahal AS, El-Saadony MT, Saad AM, Desoky E-SM, El-Tahan AM, Rady MM, AbuQamar SF, El-Tarabily KA (2022) The use of microbial inoculants for biological control, plant growth promotion, and sustainable agriculture: a review. Eur J Plant Pathol 162(4):759–792

Garcia J, Kao-Kniffin J (2018) Microbial group dynamics in plant rhizospheres and their implications on nutrient cycling. Front Microbiol 9:1516

Garza DR, Dutilh BE (2015) From cultured to uncultured genome sequences: metagenomics and modeling microbial ecosystems. Cell Mol Life Sci 72:4287–4308

Ghani MI, Ali A, Atif MJ, Pathan SI, Pietramellara G, Ali M, Amin B, Cheng Z (2022) Diversified crop rotation improves continuous

monocropping eggplant production by altering the soil microbial community and biochemical properties. Plant and Soil: 1–22
Gonçalves OS, Souza TS, Gonçalves GdC, Fernandes AS, Veloso TGR, Tupy SM, Garcia EA, Santana MF (2023) Harnessing novel soil bacteria for beneficial interactions with soybean. Microorganisms 11(2):300
Goswami D, Patel K, Parmar S, Vaghela H, Muley N, Dhandhukia P, Thakker JN (2015) Elucidating multifaceted urease producing marine Pseudomonas aeruginosa BG as a cogent PGPR and biocontrol agent. Plant Growth Regul 75(1):253–263
Hakim S, Naqqash T, Nawaz MS, Laraib I, Siddique MJ, Zia R, Mirza MS, Imran A (2021) Rhizosphere engineering with plant growth-promoting microorganisms for agriculture and ecological sustainability. Front Sustain Food Syst 5:617157
Holding A, Collee J (1971) Chapter I Routine biochemical tests. In: Methods in microbiology, vol 6. Elsevier, pp 1–32
Hu L, Robert CA, Cadot S, Zhang X, Ye M, Li B, Manzo D, Chervet N, Steinger T, Van Der Heijden MG (2018) Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. Nat Commun 9(1):2738
Hulton C, Higgins C, Sharp P (1991) ERIC sequences: a novel family of repetitive elements in the genomes of Escherichia coli, Salmonella typhimurium and other enterobacteria. Mol Microbiol 5(4):825–834
Jadhav H, Shaikh S, Sayyed R (2017) Role of hydrolytic enzymes of rhizoflora in biocontrol of fungal phytopathogens: an overview. Rhizotrophs: Plant growth promotion to bioremediation: 183–203
Khoshru B, Mitra D, Joshi K, Adhikari P, Rion MSI, Fadiji AE, Alizadeh M, Priyadarshini A, Senapati A, Sarikhani MR (2023) Decrypting the multi-functional biological activators and inducers of defense responses against biotic stresses in plants. Heliyon 9(3)
Kour D, Rana KL, Yadav N, Yadav AN, Kumar A, Meena VS, Singh B, Chauhan VS, Dhaliwal HS, Saxena AK (2019) Rhizospheric microbiomes: biodiversity, mechanisms of plant growth promotion, and biotechnological applications for sustainable agriculture. In: Plant Growth Promoting Rhizobacteria for Agricultural Sustainability. Springer, pp 19–65
Kumar A, Verma JP (2018) Does plant—microbe interaction confer stress tolerance in plants: a review? Microbiol Res 207:41–52
Kumar R, Kumar R, Prakash O (2019) Chapter-5 the impact of chemical fertilizers on our environment and ecosystem. Chief Ed 35:69
Lanoot B, Vancanneyt M, Dawyndt P, Cnockaert M, Zhang J, Huang Y, Liu Z, Swings J (2004) BOX-PCR fingerprinting as a powerful tool to reveal synonymous names in the genus Streptomyces. Emended descriptions are proposed for the species Streptomyces cinereorectus, S. fradiae, S. tricolor, S. colombiensis, S. filamentosus, S. vinaceus and S. phaeopurpureus. Systematic and applied microbiology, 27 (1):84–92
Levin RE, Ekezie F-GC, Sun D-W (2018) DNA-based technique: Polymerase chain reaction (PCR). In: Modern techniques for food authentication. Elsevier, pp 527–616
Li H-B, Singh RK, Singh P, Song Q-Q, Xing Y-X, Yang L-T, Li Y-R (2017) Genetic diversity of nitrogen-fixing and plant growth promoting Pseudomonas species isolated from sugarcane rhizosphere. Front Microbiol 8:1268
Li T, Li Y, Shi Z, Wang S, Wang Z, Liu Y, Wen X, Mo F, Han J, Liao Y (2021) Crop development has more influence on shaping rhizobacteria of wheat than tillage practice and crop rotation pattern in an arid agroecosystem. Appl Soil Ecol 165:104016
Louws F, Rademaker J, De Bruijn F (1999) The three Ds of PCR-based genomic analysis of phytobacteria: diversity, detection, and disease diagnosis. Annu Rev Phytopathol 37(1):81–125
Marasco R, Rolli E, Fusi M, Cherif A, Abou-Hadid A, El-Bahairy U, Borin S, Sorlini C, Daffonchio D (2013) Plant growth promotion potential is equally represented in diverse grapevine root-associated bacterial communities from different biopedoclimatic environments. BioMed research international 2013
Mishra P, Singh PP, Singh SK, Verma H (2019) Sustainable agriculture and benefits of organic farming to special emphasis on PGPR. In: Role of Plant Growth Promoting Microorganisms in Sustainable Agriculture and Nanotechnology. Elsevier, pp 75–87
Mohamed S, Caunter I (1995) Isolation and characterization of a Pseudomonas fluorescens strain suppressive to Bipolaris maydis. J Phytopathol 143(2):111–114
Muiru W, Koopmann B, Tiedemann A, Mutitu E, Kimenju J (2010) Use of repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) and BOX sequences to fingerprint Exserohilum turcicum isolates. J Appl Biosci 30:1828–1838
Mwendwa S (2022) Revisiting soil texture analysis: Practices towards a more accurate Bouyoucos method. Heliyon 8 (5)
Naqqash T, Imran A, Hameed S, Shahid M, Majeed A, Iqbal J, Hanif MK, Ejaz S, Malik KA (2020) First report of diazotrophic Brevundimonas spp. as growth enhancer and root colonizer of potato. Scientif Rep 10(1):1–14
Naqqash T, Fatima M, Bukhat S, Shahid M, Shabir G, Tahir M, Arshad M, Babar M (2022) Plant Growth-Promoting Rhizobacteria Significantly Improves Growth Attributes and Photosynthetic Machinery in Wheat. Journal of Plant Growth Regulation: 1–15
Nowosad F, MacVicar R (1940) Adaptation of the "picric-acid test" method for selecting HCN-free lines in Sudan grass. Scientif Agric 20(10):566–569
Oleńska E, Małek W, Wójcik M, Swiecicka I, Thijs S, Vangronsveld J (2020) Beneficial features of plant growth-promoting rhizobacteria for improving plant growth and health in challenging conditions: a methodical review. Sci Total Environ 743:140682
O'Sullivan M, Stephens PM, O'Gara F (1991) Extracellular protease production by fluorescent Psevdomonas SPP and the colonization of sugarbeet roots and soil. Soil Biol Biochem 23(7):623–627
Pierzynski GM (2000) Methods of phosphorus analysis for soils, sediments, residuals, and waters. North Carolina State University Raleigh
Pine L, Hoffman P, Malcolm G, Benson R, Keen M (1984) Determination of catalase, peroxidase, and superoxide dismutase within the genus Legionella. J Clin Microbiol 20(3):421–429
Ranjbar R, Afshar D (2019) Evaluation of (GTG) 5-PCR for genotyping of Klebsiella pneumonia strains isolated from patients with urinary tract infections. Iranian Journal of Public Health, 48(10): 1879
Ryti R (1965) On the determination of soil pH. Agric Food Sci 37(1):51–60
Saeed Q, Xiukang W, Haider FU, Kučerik J, Mumtaz MZ, Holatko J, Naseem M, Kintl A, Ejaz M, Naveed M (2021) Rhizosphere bacteria in plant growth promotion, biocontrol, and bioremediation of contaminated sites: a comprehensive review of effects and mechanisms. Int J Mol Sci 22(19):10529
Sakthivel N, Gnanamanickam S (1986) Toxicity of pseudomonas fluorescens towards rice sheath-rot pathogen Acrocylindrium oryzae Saw. Current Sci India 55(2):106–107
Shaikh S, Sayyed R (2015) Role of plant growth-promoting rhizobacteria and their formulation in biocontrol of plant diseases. In: Plant microbes symbiosis: Applied facets. Springer, pp 337–351
Somasegaran P, Hoben HJ (1994) Quantifying the growth of rhizobia. In: Handbook for rhizobia. Springer, pp 47–57
Tahir M, Mirza MS, Hameed S, Dimitrov MR, Smidt H (2015) Cultivation-based and molecular assessment of bacterial diversity in the rhizosheath of wheat under different crop rotations. PLoS ONE 10(6):e0130030
Titus S, Gaonkar S, Srivastava R, Karande A (1995) Exopolymer production by a fouling marine bacterium Pseudomonas alcaligenes

Trindade LC, Lima MF, Ferreira MA (2005) Molecular characterization of Brazilian strains of *Xanthomonas campestris* pv. viticola by rep-PCR fingerprinting. Fitopatol Bras 30:46–54

Umar OB, Ranti LA, Abdulbaki AS, Bola A, Abdulhamid A, Biola M, Victor K (2021) Stresses in plants: Biotic and abiotic. Current Trends in Wheat Research

Vignesh K, Rajamohan K, Balabaskar P, Anandan R (2021) In vitro efficacy of PGPR Pseudomonas fluorescens against Fusarium wilt of tomato caused by *Fusarium oxysporum* f. sp. Lycopersici. Plant Arch 21(1):430–436

Voytas D (1992) Agarose gel electrophoresis. Current protocols in immunology 2 (1):10.14. 11–10.14. 18

Walsh UF, Morrissey JP, O'Gara F (2001) Pseudomonas for biocontrol of phytopathogens: from functional genomics to commercial exploitation. Curr Opin Biotechnol 12(3):289–295

Wang Z, Yu ZX, Solanki M, Yang LT, Xing YX, Dong DF, Li YR (2020) Diversity of sugarcane root-associated endophytic Bacillus and their activities in enhancing plant growth. J Appl Microbiol 128(3):814–827

William S, Feil H, Copeland A (2012) Bacterial genomic DNA isolation using CTAB. Sigma 50 (6876)

Wilson LA, Sharp PM (2006) Enterobacterial repetitive intergenic consensus (ERIC) sequences in Escherichia coli: evolution and implications for ERIC-PCR. Mol Biol Evol 23(6):1156–1168

Youseif SH (2018) Genetic diversity of plant growth promoting rhizobacteria and their effects on the growth of maize plants under greenhouse conditions. Ann Agric Sci 63(1):25–35

Zain M, Yasmin S, Hafeez FY (2019) Isolation and characterization of plant growth promoting antagonistic bacteria from cotton and sugarcane plants for suppression of phytopathogenic Fusarium species. Iranian journal of biotechnology 17 (2)

Zhu M-L, Wu X-Q, Wang Y-H, Dai Y (2020) Role of biofilm formation by Bacillus pumilus HR10 in biocontrol against pine seedling damping-off disease caused by Rhizoctonia solani. Forests 11(6):652

Rhizospheric *Bacillus* isolates control Fusarium wilt on cotton and enhance plant biomass and root development

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Rhizospheric *Bacillus* isolates control Fusarium wilt on cotton and enhance plant biomass and root development

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Cotton is a globally significant crop, serving as a source of natural fiber for the textile industry and contributing to various other products. Its economic importance is substantial, impacting livelihoods and international trade. However, cotton production faces numerous challenges, including Fusarium wilt caused by *Fusarium oxysporum* f. sp. *vasinfectum* (Fov), which can lead to significant yield and fiber quality losses. Plants alter their root exudate profiles in response to pathogens, often selectively enriching for beneficial rhizobacteria with antagonistic activity and plant growth-promoting traits. This study thus aims to characterize bacteria isolated from the rhizosphere of diseased cotton plants. The antifungal activity of 43 isolates was assessed against Fov *in vitro*. Eight of these inhibited Fov growth by 68.4 to 76.9%. 16S rRNA sequencing confirmed these isolates as *Bacillus* species. These eight *Bacillus* strains were further examined for their different modes of action *in vitro*, and their effect on cotton plants in greenhouse experiments challenged with Fov. All eight strains produced chitinases and pectinases, seven demonstrated cellulase and three protease activity, six produced urease, and five siderophores. Only *B. subtilis* SC11 exhibited phosphate solubilization activity. Seed treatments revealed that *B. subtilis* SC10 and *B. subtilis* SC11 were the standout treatments reducing Fov-caused symptoms by ~83% compared to Fov-inoculated control plants and most significantly improved plant growth and antioxidant activity. In detail, *B. subtilis* SC11 increased shoot and root dry weight by 160 and 250%, respectively. *B. subtilis* SC10 increased peroxidase activity by ~143% and ascorbate peroxidase activity by ~60%, while in *B. subtilis* SC11 treated plants superoxide dismutase activity increased by ~100%. *Bacillus* treatments effectively mitigated lipid peroxidation, achieving up to 91.4% reduction (*B. subtilis* SC10, *B. halotolerans* SC15), and decreased H_2O_2 accumulation by up to 58.4% (*B. halotolerans* SC32) compared to the Fov control. Principle component analysis revealed that regarding plant growth parameters, the treatments, and controls were distributed differentially across PC1 and PC2, with 60.30 and 15.62% data variance, respectively, showing the effectiveness of *Bacillus* isolates in greenhouse experiments. The findings of this study will contribute to the development of sustainable biocontrol strategies for managing Fusarium wilt in cotton.

KEYWORDS

antifungal activity, hydrolytic enzymes, siderophore, plant growth-promoting rhizobacteria, *Bacillus*

1 Introduction

Pests and diseases lead to significant agricultural losses globally equaling about 550 billion dollars each year. Of these losses, 60% are due to pests, weather conditions, and weeds, while plant diseases, particularly those caused by fungal pathogens, are responsible for the remaining 40% (Etesami et al., 2023). Fungi alone are culpable for the destruction of over 125 million tons of key crops annually, including cotton wheat, maize, rice, potatoes, and soybeans (Shukla et al., 2022). Over the past decade, Pakistan's cotton production has sharply declined from 14.81 million bales (one bale equals about 170 kg) in 2012–13 to just 5.5 million bales in 2022–24, causing an annual direct loss of approximately $4 billion and at least $15 billion in GDP. This drastic reduction is primarily attributed to climate change and the devastating impact of Fusarium wilt outbreaks during the monsoon season, leading to widespread crop failure. The persistent decline in cotton, often referred to as "white gold," has severely affected the country's textile industry and overall economic stability. "We thank M. Javed (personal communication, March 2025) for noting this ambiguity." Current strategies for managing phytopathogens typically involve the use of synthetic pesticides. The potential detrimental environmental consequences of such agrochemicals, which may include biodiversity loss and pollution through toxicity and bioaccumulation, underscore the urgency to develop more sustainable alternative biocontrol methods, that utilize natural predators and antagonists of phytopathogens. These offer a promising alternative to chemical agents for protecting crops and ensuring plant health. Biological control agents especially plant growth-promoting rhizobacteria (PGPR) are becoming more prevalent in sustainable agriculture, displaying notable benefits for plant health across various regions (Landa et al., 2012).

Amongst PGPR the genera *Pseudomonas*, *Bacillus*, *Azoarcus*, *Klebsiella*, *Azospirillum*, *Enterobacter*, *Azotobacter, Serratia,* and *Rhizobium* are the most common and well-studied. They support plant growth through various mechanisms such as nitrogen fixation, solubilization of phosphate and other minerals, production of plant hormones, and suppression of pathogens through antagonistic actions. The effectiveness of PGPR can differ markedly due to variations in soil type, plant species, and availability of nutrients (Etesami and Adl, 2020). There is significant diversity within rhizobacteria species and genera; not all strains exhibit the same plant growth-promoting traits or abilities. The diverse range of characteristics and capabilities is owed to differences in the organisms' genetic makeup and metabolic capacities. For practical agricultural use, PGPRs are processed into microbial formulations. These can be applied directly to seeds or in the soil to enhance the plant rhizosphere's beneficial microbial population. When applied, PGPR can colonize plant roots and deliver direct nutrition to the plant or provide protection against soil-borne diseases (Abdelaziz et al., 2023). Using PGPR in crop production is considered a safer and more environmentally friendly alternative compared to synthetic chemicals. Their application leads to various plant growth improvements, such as increased seed germination rates, enhanced development of plant shoots and roots, and higher plant biomass, as well as to plant health improvements due to their antagonistic properties (Raklami et al., 2019). PGPRs can suppress phytopathogenic soil microbes through competition for nutrients and space, antibiotics, siderophore that sequester iron and make it less available to the phytopathogens, enzymes that degrade the cell walls of fungi, and induction of plant defense responses including systemic resistance (Nadeem et al., 2014).

Bacillus spp. stand out as exceptionally effective PGPR due to their multifaceted roles in promoting plant health and resilience. Their versatility stems from their ability of enhancing nutrient availability, serving as biocontrol agents by suppressing diseases, and improving overall soil health through various mechanisms. They exhibit diverse modes of action, including nitrogen fixation, phosphate solubilization, production of plant hormones, induction of systemic resistance, and synthesis of antimicrobial compounds. Furthermore, their capacity to withstand environmental stresses and form spores ensures their survival and consistent performance in diverse agricultural settings. The capacity of PGPR strains to colonize the plant root is also paramount. These combined attributes make *Bacillus* spp. invaluable tools for sustainable agriculture, offering a holistic approach to crop production while minimizing reliance on synthetic chemicals (Fira et al., 2018). Many strains of *Bacillus* spp. are well-known PGPR with strong antagonistic activity against *F. oxysporum*. This fungus is a member of the Ascomycota phylum, has a wide host range and is responsible for a variety of plant diseases including vascular wilts, root rots, head blights, and patch diseases (Liu and Zhang, 2021). It attacks plants by infiltrating mainly seedling roots, facilitated by wounds, before colonizing the vascular system of the host. This pathogen leads to substantial crop losses in many economically valuable plant species, such as cotton, cereals, potatoes, tomatoes, ornamental flowers, date palms, oil palms, and bananas (Zaim et al., 2016).

PGPR use for controlling Fusarium wilt in cotton holds great promise. Additionally, integrating PGPR usage with other crop management practices such as the use of disease-resistant varieties, crop rotation, and optimal fertilization can lead to a more robust integrated disease management system (Fu et al., 2017). Hence, promoting the use of antagonistic PGPR can be an essential part of a strategy for maintaining the health of cotton crops and ensuring high yields, thereby supporting the livelihoods of cotton farmers and the textile industry at large.

Further research is crucial to explore the effects of antagonistic PGPR on cotton crop development and their capacity for endurance under fungal stress. So far, the microbiome of the cotton rhizosphere from continuous cotton-wheat rotation systems was not specifically explored in this respect. Therefore, our research aims to investigate PGPR isolated from the cotton rhizosphere of continuous cotton-wheat rotation fields and evaluate their antagonistic effect against the phytopathogenic fungus *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) both *in vitro* and in the greenhouse as well as the isolates' plant growth promoting properties. This approach is significant because PGPR strains isolated from this specific environment are more likely to be adapted to the local soil conditions and exhibit enhanced biocontrol potential against Fov in this particular cropping system. This research can provide valuable insights into developing tailored biocontrol strategies for cotton-wheat rotations. Fov was selected for this study due to its prevalence worldwide, including the United States, India, China, Pakistan, and Africa as well as the limited efficacy of current control strategies, which cause substantial economic losses in major cotton-producing regions worldwide.

2 Materials and methods

2.1 Bacterial isolates

Rhizobacteria used in this study were isolated, purified, and preliminary screened for their plant growth-promoting and biocontrol

traits at the Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Pakistan (Aslam et al. unpublished data). Fields infected with Fov were selected for rhizobacteria sampling. Cotton plants exhibiting relatively mild Fov disease symptoms were chosen based on the hypothesis that Fov infection alters root exudate profiles, potentially enriching for rhizobacteria with antagonistic activity against Fov. Rhizosphere soil tightly adhered to the roots was collected, and bacterial isolates were obtained using serial dilution plating (Juhnke et al., 1987). 43 rhizobacteria isolated from five distinct cotton rhizosphere samples were subsequently tested for their *in vitro* antifungal activity against Fov. Further biochemical assays for best performing isolates were performed to elucidate the biocontrol capabilities and plant growth-promoting properties of these rhizobacteria.

2.2 *In vitro* antifungal assay

Fusarium oxysporum f. sp. *vasinfectum* (Fov) was obtained from the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan, where it was originally isolated from the rhizosphere of cotton. *Fusarium graminearum* (CSB 5–15), *Leptosphaeria maculans* (DSM 62910) and *Cercospora beticola* (DSM 621607) were obtained from INRES-Molecular Phytomedicine University of Bonn, Germany. Each fungal culture was taken from a glycerol stock (20%) and inoculated on potato-dextrose agar (PDA) in the Petri dishes (20 g dextrose, 15 g agar, and 4 g potato starch in 1 L dH_2O) for further use in dual culture assays. All 43 isolates from relevant glycerol stocks were streaked on LB agar (5 g yeast extract, 5 g NaCl, 10 g trypton, and 15 g agar-agar in 1 L dH_2O) for further use. All 43 isolated rhizobacteria were evaluated for *in vitro* antifungal activity by performing a dual-culture assay on PDA in Petri dishes against Fov (Sakthivel and Gnanamanickam, 1986). The eight best performing isolates were then used to check antifungal activity against *F. graminearum*, *L. maculans* and *C. beticola* in order to get an idea on their specificity. Biocontrol agents exhibiting broad-spectrum antimicrobial activity are generally more advantageous for field applications compared to agents with activity limited to one or a few pathogens. This broader efficacy can offer more robust and comprehensive disease control in complex field environments where multiple pathogens may be present (Ali et al., 2014). A fungal (≈5 mm^2) plug was placed at the center of the plates, followed by streaking of freshly grown bacterial isolates 3 cm away from fungal plug. The plates were then incubated at 28 ± 2°C for 5 to 7 days in the dark. In addition, a control was set up by inoculating fungus onto the center of a Petri dish under the same incubation conditions but without bacterial treatment. Three biological replicates each with three technical replicates were performed. To determine antifungal activity, the following formula was used.

$$\text{Fungal growth inhibition (\%)} = \left[1 - \left(\frac{\text{Fungal growth}_{\text{Treated variant}}}{\text{Fungal growth}_{\text{Control}}}\right)\right] \times 100$$

2.3 Identification of the most potent rhizobacteria isolates

Based on the observed antifungal performance of the 43 rhizobacteria, 8 isolates designated as SC5, SC10, SC11, SC15, SC30, SC32, SC41, and SC42 were selected and subjected to 16S rRNA gene amplicon sequencing. Therefore, the isolates were cultured on LB agar plates and incubated at 28 ± 2°C for 24 h in the dark. An individual colony was transferred into 100 µL of sterile nuclease-free H_2O, incubated for 20 min at 99°C and then centrifuged (4000xg) for 2 min. Subsequently, the supernatant containing bacterial DNA was used in a PCR with the universal primer pair fD1 (AGAGTTTGATCC TGGCTCAG) and rD1 (AAGGAGGTGATCCAGC) (Weisburg et al., 1991). Fifty microliters of the total reaction mixture were prepared using DreamTaq PCR Master Mix (Thermo Fisher Scientific) (24 µL), nuclease-free water (16 µL), 10 µM forward primer (2 µL), 10 µM reverse primer (2 µL), and bacterial DNA (6 µL). PCR cycling conditions were 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 56°C for 30 s, 72°C for 1 min, and final extension at 72°C for 10 min. The PCR product was run on a 1% TAE-agarose gel for 50 min at a constant 80 V. Subsequently, the required DNA fragments of ~1.5 kbp were cut and transferred into autoclaved Eppendorf tubes. DNA was purified from the gel by using a DNA purification kit (Thermo Fisher Scientific) according to the manufacturer's instructions and sent to Eurofins Genomics (Germany) for sequencing. NCBI BLAST was used to analyze the obtained sequences by comparison to already deposited sequences in the Gene Bank database. After the initial blast against the whole NCBI database to get an idea of the identity of the isolates, the blast analysis was done with all type strains of a particular group to exclude incorrectly annotated entries (including several type strains utilized in the analysis and the selected group). ClustalW in Molecular Evolutionary Genetics Analysis Version 11 (MEGA 11) software was used to align highly homologous sequences and generate neighbor-joining trees. Phylogenetic tree nodes were statistically supported by bootstrap replication with 1000 replicates using MEGA 11 (Ki et al., 2009).

2.4 Extracellular enzymatic activities

The eight isolates of rhizobacteria (SC5, SC10, SC11, SC15, SC30, SC32, SC41, and SC42) that performed high antifungal activity were investigated for the production of chitinase, cellulase, pectinase, protease, and urease activities. All assays to identify extracellular enzymatic activities represented a qualitative approach. To determine chitinase, cellulase, and pectinase activities, the bacteria were inoculated on mineral salt (MS) agar media (1 g KH_2PO_4, 5 g $NaNO_3$, 2 g K_2HPO_4, 0.1 g $CaCl_2$, 0.1 g KCl, 0.5 g $MgSO_4$, $7H_2O$, 0.02 g $FeSO_4$, $7H_2O$, 15 g agar in 1 L dH_2O) supplemented with 1% colloidal chitin [prepared by treating chitin from crab shells with HCl following the protocol described by Li et al. (2019)] 1% carboxy-methylcellulose (CMC), and 1% pectin, respectively, (Chaiharn et al., 2019). A negative control without bacterial inoculation on supplemented MS agar was also used. After inoculation of a single pure colony of the respective bacterial isolate, plates were incubated at 28 ± 2°C for 48 h. Then, for better visualization plates were immersed in a Lugol solution comprising potassium iodide (KI;1% w/v) and Iodine (I_2;0.5% w/v) in dH_2O. Skim milk medium (10 g skim milk powder, 5 g tryptone, 2.5 g yeast extract, 1 g dextrose, and 15 g agar in 1 L dH_2O) was used for the determination of protease activity. The formation of a zone of clearance around bacterial colonies indicated extracellular enzymatic activities (O'Sullivan et al., 1991).

For the determination of urease activity, urea agar base supplemented with 40% urea was used. The change in color from yellow to pink represented urea hydrolysis (Goswami et al., 2015). Three biological replicates each with three technical replicates were performed.

2.5 Siderophore production

2.5.1 Qualitative assay

Siderophore secretion was qualitatively determined by using an LB agar medium containing Chrome Azurol S (CAS) as an indicator dye, Fe^{3+} solution, and hexadecyl trimethyl ammonium bromide (HDTMA). A single pure colony of the respective rhizobacteria isolate was inoculated onto a CAS agar plate and incubated for 72 h at 28 ± 2°C. The appearance of an orange-yellow zone around the bacterial colony represents the production of siderophore (Schwyn and Neilands, 1987). This experiment was performed in three biological replicates each with three technical replicates.

2.5.2 Quantitative assay

For siderophore quantification, a single pure colony of each rhizobacteria from overnight bacteria culture was inoculated in 100 mL LB broth in a 500 mL conical flask and after 72 h of shaking (150 rpm) incubation at 28 ± 2°C supernatant was collected followed by centrifugation (9,000xg, 5 min). 100 µL supernatant of each bacterial culture was transferred into a separate well of a 96-well microtiter plate followed by the addition of an equal volume of CAS reagent. The optical density of each sample was recorded at 630 nm followed by incubation (Arora and Verma, 2017). Three biological and four technical replicates were used and siderophore production was calculated in percent siderophore unit (psu) (Schwyn and Neilands, 1987).

2.6 P-solubilization

A qualitative assay for P-solubilization activity of rhizobacteria was performed by using Pikovskaya's agar media following the protocol described by Pikovskaya (1948). Fresh bacteria culture was inoculated in the center of Pikovskaya's agar plates. Formation of the zone of clearance around the bacterial growth after 3 to 5 days of incubation at 28 ± 2°C represented the P-solubilization activity of bacteria. Three biological replicates each with three technical replicates were performed.

2.7 Indole 3-acetic acid production

To determine indole-3-acetic acid (IAA) production by rhizobacteria, a single colony of each isolate was inoculated into 50 mL of LB broth media both without and amended with tryptophan (100 mg /L) in 250 mL conical flasks and incubated at 28 ± 2°C for 72 h. After incubation, the cultures were centrifuged, and 150 µL of supernatant was mixed with an equal volume of Salkowski's reagent in a 96 well plate. The cultures were incubated in the dark at 28 ± 2°C for 30 min. Development of a pink color indicates successful IAA production (Glickmann and Dessaux, 1995). Three biological replicates each with three technical replicates were performed.

2.8 Greenhouse experiment

2.8.1 Experimental design

A pot experiment using a completely randomized design and eight technical and two biological replicates for each treatment was conducted to evaluate the efficacy of the 8 best performing isolates against Fov in cotton plants in the greenhouse of the Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan. Soil for this experiment was collected from a nursery in Multan. The physicochemical analysis of the soil used in this experiment is given in Supplementary Table 1. Cotton seeds of the commercially available MNH-1020 (Fov susceptible) variety were used. MNH-1020 is a commercial cultivar developed by the Cotton Research Institute (CRI) Multan, Pakistan. Ten treatments were designed as illustrated below:

$$T1 = \text{No treatment} - \text{Fov}\left(\text{uninfected control}\right)$$

$$T2 = \text{No treatment} + \text{Fov}\left(\text{infected control}\right)$$

$$T3 = SC5 + \text{Fov}$$

$$T4 = SC10 + \text{Fov}$$

$$T5 = SC11 + \text{Fov}$$

$$T6 = SC15 + \text{Fov}$$

$$T7 = SC30 + \text{Fov}$$

$$T8 = SC32 + \text{Fov}$$

$$T9 = SC41 + \text{Fov}$$

$$T10 = SC42 + \text{Fov}$$

In all 10 treatments sterilized cotton seeds were used. One-week post sowing, plant thinning was performed so that only one healthy plant in each pot remained. Throughout the experiment, greenhouse condition was constantly maintained at a temperature ranging from 25 to 30°C, 16 h photoperiod, and 60% humidity.

2.8.2 Bacterial inoculum

A pure colony of each of the 8 isolates (already growing on LB agar plate) was separately inoculated into 100 mL LB media in a conical flask (250 mL) and incubated for 48 h at 28 ± 2°C in a shaking incubator at 120 rpm. The cultures were centrifuged in 50 mL falcon tubes for 10 min at 4000xg, the supernatant was discarded and the pellet was suspended in sterile dH_2O. 1×10^8 CFU mL^{-1} of each isolate was used for cotton seed treatment (Eddin et al., 2007). In T3 to T10

sterilized cotton seeds were soaked in the respective isolate inoculum for 15 min. Then three seeds from each treatment were sown in individual pots in eight replicates. In the T1 and T2 only sterilized seeds were planted.

2.8.3 Fungal inoculum

Fov (loop full culture) was inoculated in 700 mL of potato dextrose broth (PDB) (20 g dextrose, and 4 g potato starch in 1 L dH_2O) in 1 L conical flask (four 1 L conical flasks each with 700 mL PDB) and incubated for 15 days at 28 ± 2° C in the shaking incubator at 120 rpm. After 15 days, PDB with growing fungus was centrifuged in a 50 mL falcon tube at 4000xg for 10 min to obtain fungus pellets. The fungal pellets were suspended in sterile dH_2O to obtain a density of 3×10^7 spores/ml (Dilfuza and Dilfuza, 2011). This fungal inoculum was injected into soil near plant roots to infect soil (3×10^7 spores/kg of soil) of treatments T2 to T10 2 weeks post sowing. Treatment T1 (without bacterial and fungal inoculation) was used as a control in this experiment. T2 (only with fungal inoculation) was used as a control to evaluate the infection severity. The plants were properly irrigated and daily observed for symptoms of a fungal infection.

The disease severity of Fusarium wilt was calculated 45 days after Fov inoculation based on leaf yellowing and root discoloration on a scale of 0 to 5, representing increasing severity: 0 indicates no symptoms; 1 indicates 1–20% light brown vascular discoloration and yellowing leaves; 2 indicates 20–40% light brown vascular discoloration and yellowing leaves; 3 indicates 40–60% dark brown vascular discoloration and yellowing leaves; 4 indicates 60–80% dark brown vascular discoloration and yellowing leaves; and 5 indicates 80–100% yellowish leaves and dark brown or dead vascular systems. Disease severity was then calculated using the formula:

$$\text{Disease severity} = \Sigma\left[\begin{matrix}(N \times 0)+(N \times 1)+(N \times 2)+\\(N \times 3)+(N \times 4)+(N \times 5)\end{matrix}\right] / 5 \times T$$

where N = number of plants at each symptom grade 0 to 5, and T = total number of plants (multiplied by maximum symptom grade 5).

The percentage reduction in Fusarium wilt incidence was calculated using the following formula:

$$\text{Reduction percentages}(\%) = (A - B) / A \times 100$$

where A represents the disease incidence (disease severity) of the positive control and B represents the disease incidence of the treatment (El-Sersawy et al., 2021).

2.8.4 Determination of growth parameters

After 45 days post inoculation (DPI) of Fov cotton plants were uprooted, main root and shoot length as well as root and shoot fresh weight were determined after washing and carefully drying the roots with tissue paper. In order to obtain the dry weight, roots and shoots half of plants ($n = 4$) from each biological replicate were oven-dried for 2 weeks at 65°C before weighing. Half of plants ($n = 4$) from each biological replicate were preserved in liquid nitrogen and stored at – 80°C for the measurement of oxidative stress and antioxidants.

2.9 Measuring oxidative stress

2.9.1 Lipid peroxidation

Malondialdehyde (MDA) content was measured to determine lipid peroxidation. Fifty-nine days old cotton plants (T1 to T10) as describes in 2.7.2, were uprooted at 45 DPI with Fov, except for T1, which served as a non-inoculated control. The plants were immediately preserved in liquid nitrogen at – 80°C for further analysis. Three leaf samples were collected separately from each plant. Five milliliters of 0.1% trichloroacetic acid (TCA) solution were used to homogenize 200 g of cotton leaves that were preserved in liquid nitrogen and the homogenized solution was centrifuged for 5 min at 10,000 rpm. One milliliter of supernatant was mixed with 3 mL of 5% TCA and 1% TBA (thiobarbituric acid) solution. After heating for 30 min in a water bath at 95°C the final mixture was centrifuged for 5 min at 5000 rpm or 30 min. The supernatant (300 µL) was collected in a 96 well plate to measure the absorbance of samples at 532 and 600 nm by using a spectrophotometer. The extinction coefficient value (155 μM^{-1} cm^{-1}) was used to estimate the actual value of samples (Heath and Packer, 1968). This experiment was performed in two independent biological replicates each with 12 technical replicates per treatment.

2.9.2 Hydrogen peroxide

To determine the H_2O_2, 0.2 grams of leaves from each treatment as described in 2.8.1 were grounded in 0.1% TCA (2 mL), followed by centrifugation at 10,000 rpm for 15 min. Subsequently, 500 µL of 10 mM potassium phosphate (KP) buffer (pH 7.0) and 1 mL of 1 M Potassium Iodide (KI) were added in 500 µL of supernatant. The optical density (OD) of the mixture was then measured at 390 nm (Velikova et al., 2000). This experiment was performed in two independent biological replicates each with 12 technical replicates per treatment.

2.10 Determination of antioxidant activity

2.10.1 Preparation of enzyme extract from cotton leaves

Leaves samples as described in section 2.8.1 were collected. 50 mM KP buffer was used to homogenize cotton leaf samples for the estimation of enzymatic antioxidant activity. Pre-chilled pestle and mortars were used for grinding. The reaction mixture was centrifuged at 12,000 rpm for 20 min at 4°C. Supernatant was collected for antioxidant analysis.

2.10.2 Catalase

Catalase (CAT) activity was assessed by monitoring the decomposition of H_2O_2 at 240 nm. The reaction mixture consisted of 1 mL of 50 mM KP buffer, 450 µL of 5.9 mM H_2O_2, and 50 µL of enzyme extract. Spectrophotometer was used to measure the absorbance at 240 nm after the intervals of 20 s up to 2 min (Aebi, 1984). The experiment was performed in two independent biological replicates each with 12 technical replicates per treatment.

2.10.3 Ascorbate peroxidase

For Ascorbate peroxidase (APX) reaction mixture consists of 207 µL of 50 mM KP buffer, 7.6 µL of 300 mM H_2O_2, 7.6 µL of ascorbic

acid and 7.6 µL of enzyme extract. Absorbance was measured at 290 nm (Dhindsa and Matowe, 1981). This experiment was performed in two independent biological replicates each with 12 technical replicates per treatment.

2.10.4 Peroxidase

Peroxidase (POD) activity was assessed according to the method outlined by Maehly (1954) with slight modifications. The reaction mixture was comprised of 50 mM KP buffer (pH 7.8), 50 µL of enzyme extract, and 0.2 mL of guaiacol (20 mM). 0.25 mL of H_2O_2 (40 mM) was added in the reaction mixture to initiate the reaction. Absorbance readings were taken at 470 nm using a spectrometer at 20-s intervals for 2 min. This experiment was performed in two independent biological replicates each with 12 technical replicates per treatment.

2.10.5 Superoxide dismutase

The activity of superoxide dismutase (SOD) was measured using the nitro blue tetrazolium (NBT) photoreduction method developed by Dhindsa and Matowe (1981). The reaction mixture consisted of 100 µL of enzyme extract, 50 µL of 33 mM NBT, 50 µL of 0.003 mM riboflavin 100 µL of 10 mM L-methionine, and 250 µL of 50 mM KP buffer. This mixture was exposed to a 15 W lamp for 30 min and then the lamp was turned off to stop the reaction. Control experiments were conducted with illuminated and non-illuminated reactions without enzyme extract. The absorbance was measured at 560 nm. This experiment was performed in two independent biological replicates each with 12 technical replicates per treatment.

2.11 Statistical analysis

The data collected from pot experiments were statistically analyzed using the analysis of variance method, based on the completely randomized design layout and programmed in the software Statistix 8.1. Differences between the means of each treatment were tested using the least significant difference LSD test at a 5% level of significance. The data were also analyzed using principal component analysis (PCA) to determine the relationship between the growth parameters and treatment using XLSTAT (Garcia et al., 2019).

3 Results

3.1 Screening for antifungal activity

Out of 43 isolates 31 isolates performed antifungal activity by inhibiting Fov colony growth by 3.5 to 76.9% in dual culture plate assay (Supplementary Table 1, Supplementary Figure 1). Eight bacterial isolates exhibiting the highest antifungal activity against Fov, ranging from 66.7 to 76.9% inhibition, were evaluated for their broader antifungal activity against three additional fungal strains. These isolates demonstrated a wider range of inhibition against *F. graminearum*, *L. maculans*, and *C. beticola*, ranging from 62.9 to 93% (Table 1). The eight isolates were selected for molecular characterization and further experiments were performed for the analysis of their biocontrol and plant growth-promoting potential.

3.2 16S rRNA identification of the most potent rhizobacteria isolates

The eight rhizobacteria isolates designated SC5, SC10, SC11, SC15, SC30, SC32, SC41, and SC42 that possessed high antifungal activity against 4 phytopathogenic fungi were identified on molecular level by 16S rRNA sequencing. Sequence analysis revealed that all eight isolates are a member of the genus *Bacillus*. SC5 was highly similar to *B. stercoris*. SC10, SC11 SC41, and SC4 were highly similar to *B. subtilis*. SC15, SC30, and SC32 were highly similar to *B. halotolerans* (Figure 1). The phylogenetic tree contains the type strains that are more closely related to the isolates under investigation. The sequences are available at GenBank under the accession numbers PV147758 to PV147765.

3.3 Extracellular enzymatic activities

The production of extracellular enzymes is a key trait of many successful biocontrol agents, contributing significantly to their ability to suppress fungal pathogens and promote plant health. All isolates performed low to strong chitinase activity. *B. subtilis* isolates SC10, SC11, SC41, and SC42 performed low chitinase activity, *B. halotolerans* isolates SC15, SC30 and SC32 performed strong chitinase activity and

TABLE 1 *In vitro* antagonistic potential (% inhibition of fungal growth) of eight rhizobacteria against four fungal phytopathogens.

Isolates	*Fusarium oxysporum* f.sp. *vasinfectum* (% growth inhibition)	*Fusarium graminearum* (% growth inhibition)	*Leptosphaeria maculans* (% growth inhibition)	*Cercospora beticola* (% growth inhibition)
B. stercoris SC5	72.2 ± 6.3	68.7 ± 3.4	91.7 ± 1.9	84.4 ± 3.9
B. subtilis SC10	70.3 ± 3.5	67.2 ± 2.5	83.1 ± 6.2	68.1 ± 3.9
B. subtilis SC11	68.9 ± 3.7	79.2 ± 8.9	90.0 ± 2.5	76.5 ± 3.3
B. halotolerans SC15	75.9 ± 3.8	62.9 ± 6.1	88.3 ± 3.1	77.0 ± 5.5
B. halotolerans SC30	70.3 ± 4.7	73.1 ± 2.5	93.0 ± 1.0	79.2 ± 2.6
B. halotolerans SC32	76.9 ± 4.6	67.3 ± 11.2	92.1 ± 1.5	80.1 ± 4.9
B. subtilis SC41	68.5 ± 3.9	77.0 ± 5.9	87.3 ± 1.0	75.6 ± 2.5
B. subtilis SC42	66.7 ± 5.7	70.8 ± 5.7	91.7 ± 3.2	77.8 ± 1.3

A dual culture experiment was performed with three biological replicates each with three technical replicates.

B. stercoris SC5 performed very strong chitinase activity (Table 2, Figure 2A). *B. stercoris* SC5, *B. subtilis* isolates SC10, SC11, SC42, and *B. halotolerans* isolates SC15, SC30, SC32 performed strong pectinase activity and only *B. subtilis* SC41 performed low pectinase activity (Table 2, Figure 2B). All isolates but *B. halotolerans* SC30 performed cellulase activity, with *B. subtilis* isolates SC10, SC11, and *B. halotolerans* SC15 even performing very strong activity (Table 2, Figure 2C). The three isolates *B. stercoris* SC5, *B. halotolerans* SC15, and *B. halotolerans* SC30 performed strong protease activity (Table 2, Figure 2D). Enzymatic activities of all above mentioned enzymes were categorized based on the width of zone clearance. The four isolates *B. stercoris* SC5, *B. subtilis* isolates SC10, SC42, and *B. halotolerans* SC30 performed low, and the two isolates *B. subtilis* SC11 and *B. halotolerans* SC32 strong urease activity (Table 2). Urease activity was categorized based on the change in color of media from yellow to dark pink color (Figure 2E). All traits exhibited by these bacteria are summarized in Table 2.

3.4 Siderophore production, P-solubilization and indole 3-acetic acid production

Out of the eight isolates, five isolates performed siderophore production in CAS agar media as indicated by a change of the medium color from blue to yellow-orange in the qualitative assay (Figure 3A). The quantitative assay indicated siderophore production ranging from 17.4 to 33.6% (Table 2). Only isolate *B. subtilis* SC11 was capable of P-solubilization in Pikovskaya's agar medium (Table 1). P- solubilization was categorized based on the area of zone of clearance (Figure 3B). None of the eight bacterial isolates produced IAA.

3.5 Biocontrol of fusarium wilt on cotton plant using rhizospheric bacterial isolates

In order to validate the biocontrol potential of the eight *Bacillus* isolates, greenhouse pot experiments were conducted to determine how well these bacteria reduce the severity of Fusarium wilt caused by Fov on cotton plants. Additionally, the isolates' impact on cotton plant growth was investigated under Fov infection pressure. We found that all bacterial treatments reduced disease severity and additionally improved plant growth parameters significantly compared to plants treated with Fov alone. The disease severity percentage in treatments with *Bacillus* isolates *B. stercoris* SC5, *B. subtilis* SC10, SC11, SC41 and *B. halotolerans* SC15, SC32 presented themselves as considerably lower (12.5 ± 5 to 21.25 ± 2.5) compared to the control infected only with Fov (72.5 ± 5) (Figure 4). Moreover, *B. subtilis* SC10 and SC11, in particular, showed the overall highest enhancement in both shoot and root growth parameters (Figure 5). Compared to the Fov-inoculated control plants, application of *B. subtilis* SC10 and SC11 resulted in 15.8 and 5.3% longer shoots, 60 and 53.3% longer roots, 150 and 160% more shoot dry weight, 125 and 250% more root dry weight, respectively. Even compared to the uninoculated control, both isolates significantly increased shoot and root growth of Fov-inoculated plants. Although *B. stercoris* SC5, *B. halotolerans* SC15, and *B. subtilis* SC41 display a positive effect on plant health by combating Fov infestation, their performance does not surpass that of

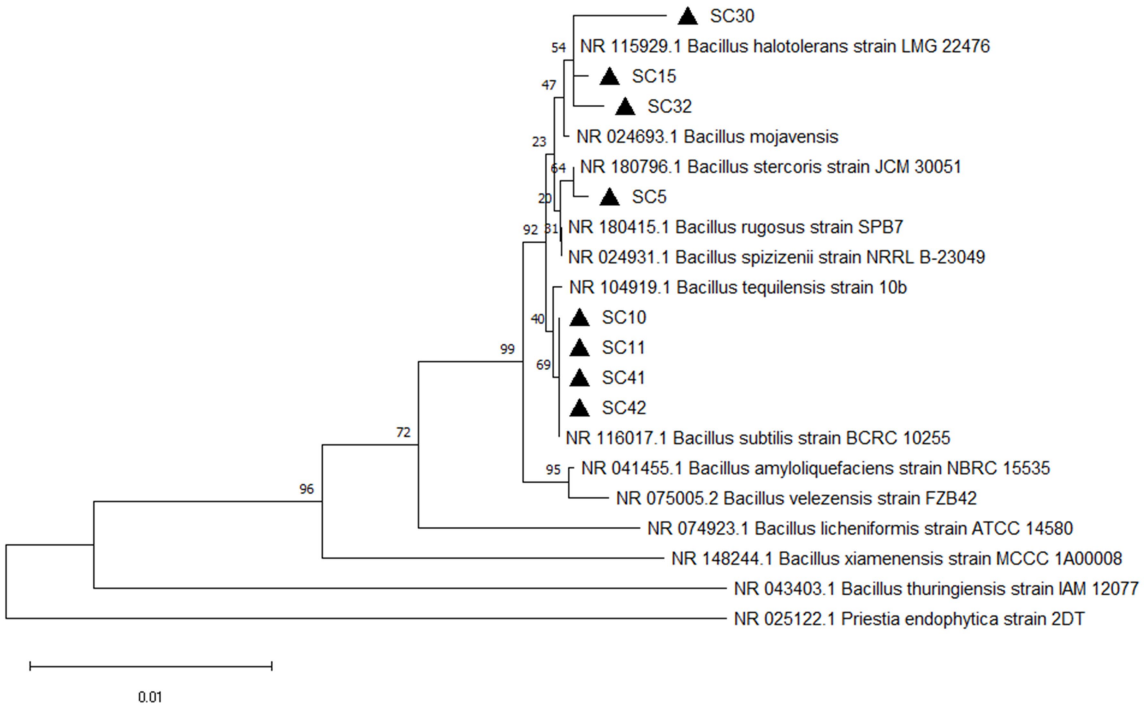

FIGURE 1
Phylogenetic tree of isolates *Bacillus stercoris* SC5, *Bacillus subtilis* (SC10, SC11, SC40, SC42), *Bacillus halotolerans* (SC15, SC30, SC32) and their closest relatives based on 16S rRNA sequence. The phylogenetic tree was constructed using the neighbor-joining (method in MEGA11 software). The bootstrap values are shown at the branch points.

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TABLE 2 Biocontrol and plant growth promoting traits of bacterial isolates characterized based on their activity.

Isolates	Chitinase activity	Cellulase activity	Pectinase activity	Protease activity	Urease activity	Siderophore production (psu)	P-solubilization
B. stercoris SC5	+++	++	++	++	+	–	–
B. subtilis SC10	+	+++	++	–	+	$31.1^{B} \pm 1.3$	–
B. subtilis SC11	+	+++	++	–	++	$33.6^{A} \pm 1.4$	+
B. halotolerans SC15	++	+++	++	++	–	$30.6^{B} \pm 1.6$	–
B. halotolerans SC30	++	–	++	++	+	–	–
B. halotolerans SC32	++	+	++	–	++	–	–
B. subtilis SC41	+	++	+	–	–	$18.9^{C} \pm 1.2$	–
B. subtilis SC42	+	++	++	–	+	$17.4^{C} \pm 1.1$	–

+++ (with 2.1 cm to 2.5 cm zone width) very strong activity; ++ (1.1 cm to 2 cm zone width) strong activity; + (up to 1 cm zone width) low activity; – no activity. All qualitative experiments were performed in three biological replicates each with three technical replicates. Siderophore was quantified by using four technical and three biological replicates. Values represent the average + standard deviation of three biological replicates. Significant differences between treatments are indicated by different letters.

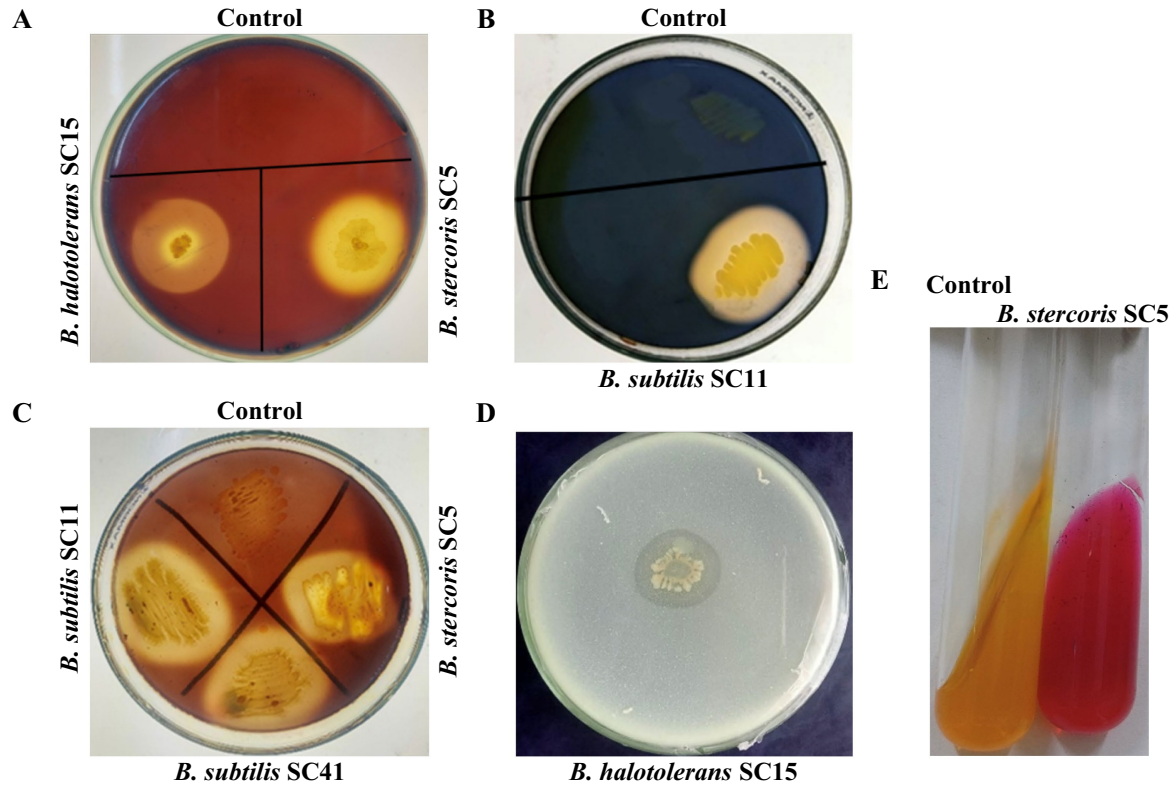

FIGURE 2
Plate assays for screening of enzymatic activities of antagonistic rhizobacteria. Representative examples. **(A)** Chitinase enzyme production on medium amended with 1% colloidal chitin indicated by the development of a halo zone around the bacterial colony. **(B)** Cellulase production on medium amended by 1% carboxymethyl cellulose (CMC) indicated the development of a halo zone around the bacterial colony. **(C)** Pectinase enzyme production on medium amended by 1% pectin indicated by the development of halo zone around bacterial colony. **(D)** Production of protease on skim milk medium indicated by development of halo zone around bacteria colony. **(E)** Urease production indicated a change in the color of the medium.

the most successful *B*. sublitis isolates SC10 and SC11. However, these strains' ability to substantially decrease Fov severity while promoting better plant growth compared to the Fov-inoculated control suggests that they are also promising candidates for biocontrol applications against Fusarium wilt.

Conversely, treatments *B. halotolerans* SC30 and *B subtilis* SC42 showed marginal biocontrol effects with a disease severity percentage of 42.5 ± 5 and 41.25 ± 6, respectively. Although this equals a reduction of 41.38% (*B. halotolerans* SC30) and 43.10% (*B subtilis* SC42) compared to the Fov-inoculated control plants, it underlines the variability in *Bacillus* strains' capacity to function as biocontrol agents.

3.6 Effect on oxidative stress and antioxidant activities

Next, oxidative stress markers were investigated to obtain insights into the physiological impact of Fov infection in plants, particularly

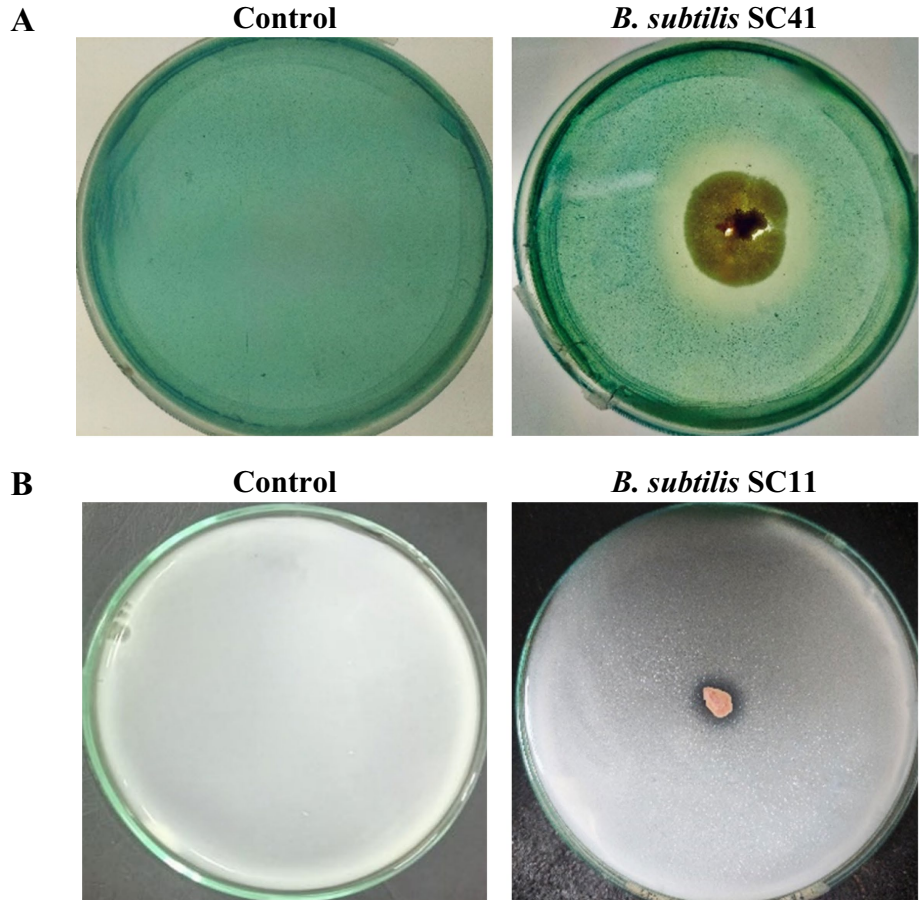

FIGURE 3
Representative examples. **(A)** Plate assay for the production of siderophore indicated by the change in medium color from blue to yellow/orange due to iron removal from CAS/HDTMA-Fe^{+3}. **(B)** P-solubilization indicated by formation of a halo zone around the bacterial colony in Pikovskaya's agar medium.

when treated with *Bacillus* strains. The analysis of oxidative stress markers in plants subjected to Fov infection revealed a significant elevation in the levels of H_2O_2 and MDA, which are indicative of cellular damage due to oxidative stress. Specifically, the Fov infected plant exhibited higher concentrations of MDA and H_2O_2 with an approximate increase of 455 and 69%, respectively as compared to the control plants treated with water only (Figure 6). Plants within the WC group maintained baseline levels of H_2O_2 and MDA, reflecting the absence of (pathogenic) stress and indicating normal physiological conditions. In contrast, the increase of H_2O_2 and MDA in the Fov group suggests that the fungal infection prompted an oxidative burst, a typical plant response that typically stimulates antioxidant enzyme activities as a part of the plant's defensive response to plant pathogens and oxidative stress.

This is validated by the observed increase in POD, SOD, CAT, and APX activities by 6.9, 16.6, 8.1, and 24.4%, respectively in the infected control compared to the water-treated control.

Application of the eight *Bacillus* strains to Fov-inoculated cotton plants significantly reduced lipid peroxidation (74.4 to 91.4% reduction compared to the Fov control) and H_2O_2 production (29.4 to 58.4% reduction compared to the Fov control) while causing a considerable increase in POD in some treatments including *B. stercoris* SC5, *B. subtilis* SC10 (with highest increase of 142.61% compared to Fov control), *B. halotilerans* SC15, SC30, SC32, *B. subtilis* SC42 and reduction in *B. subtilis* SC11 (16.58%) and SC42 (17.69%) compared to Fov control. SOD was increased in all *Bacillus* treatments (66.63% in SC32 + Fov to 100% in SC11 + Fov compared to Fov control). A moderate increase in CAT (8.39% in SC11 + Fov to 23.53% in SC15 + Fov compared to Fov control) and APX (10.17% in SC41 + Fov to 59.75% in SC10 + Fov) antioxidant activities was observed compared to the Fov-inoculated control plants.

These elevated antioxidant levels suggest that all the *Bacillus* treatments greatly enhanced the defensive response of the plants against oxidative stress caused by the fungal pathogen. Data of all agronomical parameters, ROS, and antioxidants are also represented in Figure 7 using principle component analysis biplot to explore relationships between different treatments and parameters. The treatments appear to cluster into distinct groups. The no-treatment WC is separated from the Fov-infected control and most of the *Bacillus* treatments. The PCA biplot illustrates the relationships between different treatments and physiological parameters in cotton plants under Fov infection. The first two principal components (F1 = 60.30%, F2 = 15.62%) together explain 75.92% of the total variance. The *Fov*-infected plants show a strong association with oxidative stress markers (H_2O_2 and MDA), indicating enhanced lipid peroxidation and ROS accumulation. In contrast, disease protection,

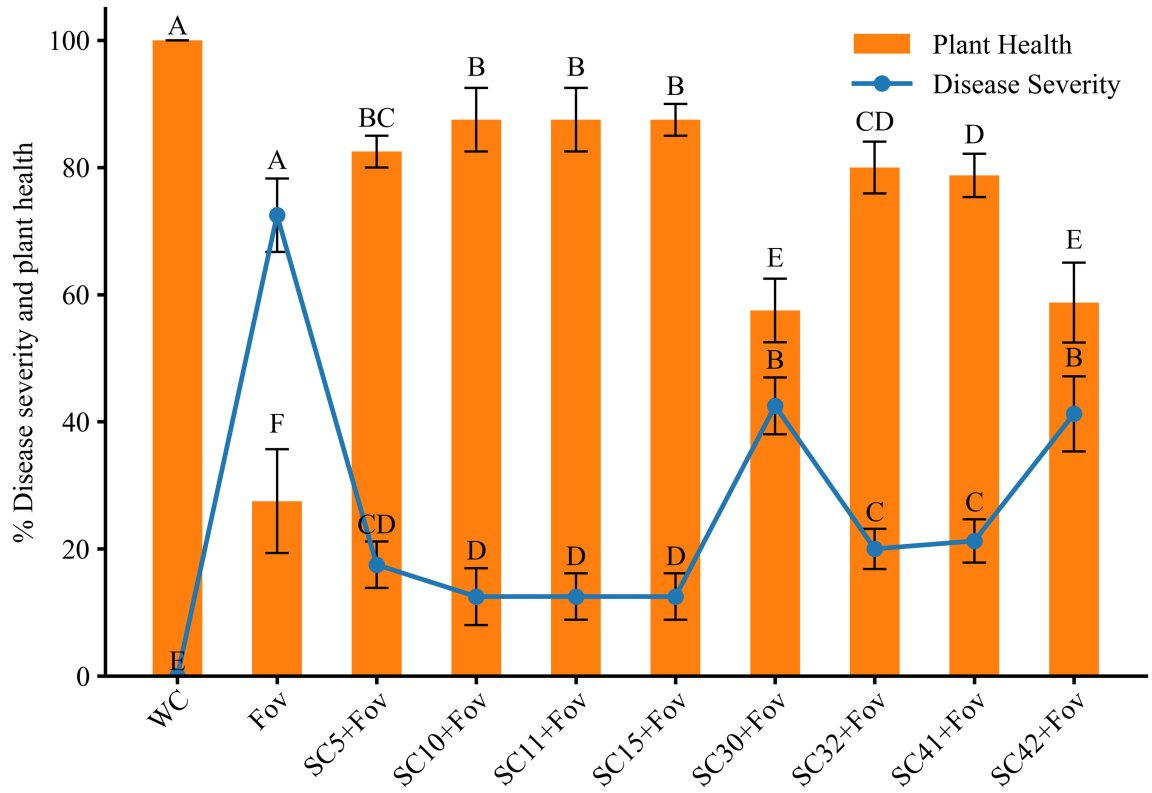

FIGURE 4
Effects of *Bacillus* isolates (SC5, SC10, SC11, SC15, SC30, SC32, SC41, and SC42) inoculation on disease severity and plant health in cotton under Fov stress. Values represent the average + standard deviation of two biological replicates (n = 16 in total). Significant differences between treatments are indicated by different letters. WC, water control; Fov, *Fusarium oxysporum* f.sp. *vasinfectum*.

root fresh weight, shoot dry weight, and root dry weight are positively correlated, suggesting that these traits contribute to plant resistance. Antioxidant enzymes (APX, SOD, and CAT) cluster together, highlighting their role in mitigating oxidative stress. Treatments such as SC10 + Fov and SC11 + Fov are closely associated with root and shoot growth parameters, indicating a potential resistance effect, while WC remains distant from stress markers, signifying a healthier state. The analysis reveals that *Fov* infection negatively affects plant growth through oxidative damage, while specific treatments promote tolerance by enhancing antioxidant defense and maintaining biomass production. This suggests that the *Bacillus* treatments have a measurable effect on the plant's response to Fov infection.

4 Discussion

The results of this study highlight the significant biocontrol potential of *Bacillus* species against Fusarium wilt, a debilitating disease that severely impacts the productivity of a wide range of economically important crops. This study adds significantly to the growing body of data supporting the use of biological control agents as a sustainable and environmentally friendly alternative to conventional chemical fungicides. In this study, we evaluated the biocontrol potential of rhizobacteria isolated from the rhizosphere of cotton plants infected with fungal pathogens. We succeeded in discovering *Bacillus* isolates with antagonistic activity against Fov and additionally against *F. graminearum*, *L. maculans* and *C. becticola* *in vitro* and biocontrol activity against Fov in greenhouse pot experiments with cotton plants. Eight isolates showed the highest antifungal activity, reducing fungal growth by up to 93% *in vitro* (Table 1). Molecular characterization based on 16S rRNA revealed that all eight isolates belonged to the genus *Bacillus*. Members of the genus *Bacillus* stand out as the main group of rhizospheric bacteria utilized for the biological control of plant-pathogenic fungi (Boulahouat et al., 2023). The effectiveness of these bacteria in biocontrol is due to their production of diverse secondary metabolites and their capacity for swift propagation, both of which allow for their effective incorporation into biocontrol approaches (Kiesewalter et al., 2021). The prevalence of *Bacillus* isolates with biocontrol potential can be attributed to several factors:, their ubiquity and adaptability, their established role as biocontrol agents producing various antimicrobial compounds, LB's non-selectivity favoring fast-growing bacteria, Bacillus' robust spore-forming ability and other potential sampling bias (Fira et al., 2018). The use of different isolation methods and media could reveal additional biocontrol agents.

The eight isolates were further evaluated for biocontrol and plant growth promoting activities such as hydrolytic enzyme production, siderophore, P solubilization, and IAA. In hydrolytic enzymes, chitinase can break down chitin, a major structural component of fungal cell walls (Veliz et al., 2017). By degrading pectin, pectinase enzymes can help the bacteria colonize plant tissues and potentially compete with fungi for space and nutrients

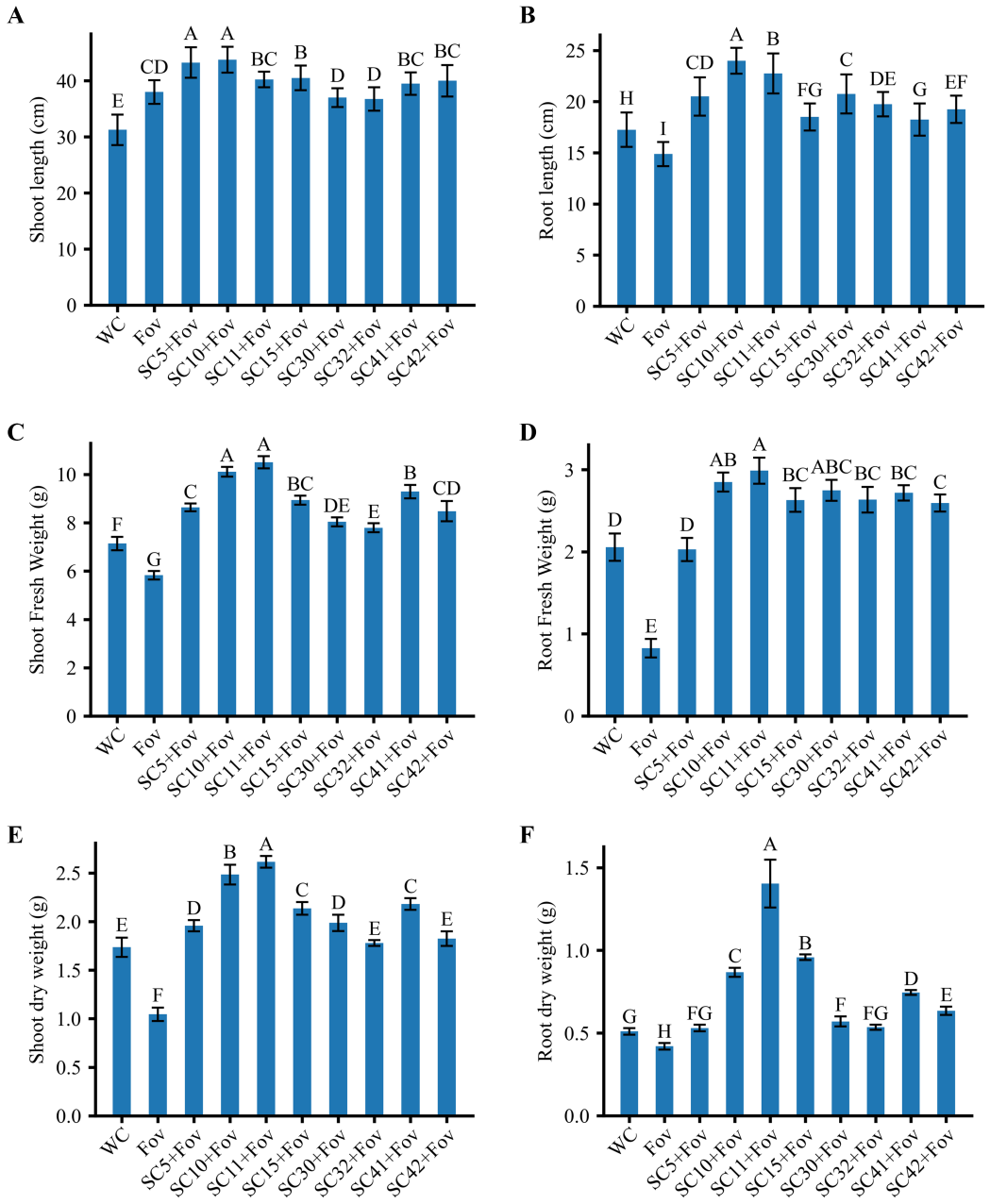

FIGURE 5
Effects of *Bacillus* isolates (SC5, SC10, SC11, SC15, SC30, SC32, SC41, and SC42) inoculation on cotton plants with Fov stress; **(A)** shoot length (n = 16), **(B)** root length (n = 16), **(C)** shoot fresh weight (n = 16), **(D)** root fresh weight (n = 16), **(E)** shoot dry weight (n = 8), and **(F)** root dry weight (n = 16). Values represent the average + standard deviation of two biological replicates (n = 8 or 16 in total). Significant differences between treatments are indicated by different letters. WC, water control; Fov, *Fusarium oxysporum* f.sp. *vasinfectum*.

(Riseh et al., 2024). While cellulose is not a major component of most fungal cell walls, some fungi produce cellulose in certain structures. Cellulase can degrade these structures, potentially affecting fungal growth and development (Brown Jr, 2004). PGPR cellulases primarily target dead organic matter, which increases nutrient availability for plants, and generally do not degrade intact plant cell walls. This is because plant cellulose is often protected by lignin and other structural components that PGPR cellulases cannot easily break down (McKee and Inman, 2019). Protease enzymes can target fungal cell wall proteins, contributing to cell wall weakening and disruption (Dimkić et al., 2022). Urease activity in bacteria plays several crucial roles, primarily related to nitrogen metabolism, pathogenicity, and environmental interactions (Pei et al., 2024). Siderophores are iron-chelating compounds produced by bacteria that contribute to the biocontrol of fungal pathogens by sequestering iron making it unavailable to fungi, but improving iron availability for plants and thus promoting plant growth. Furthermore, in some cases, they have direct antifungal activity, (by) and act synergistically with other biocontrol mechanisms (Elshahat et al., 2016). Improved P nutrition can strengthen plant defense mechanisms, making them

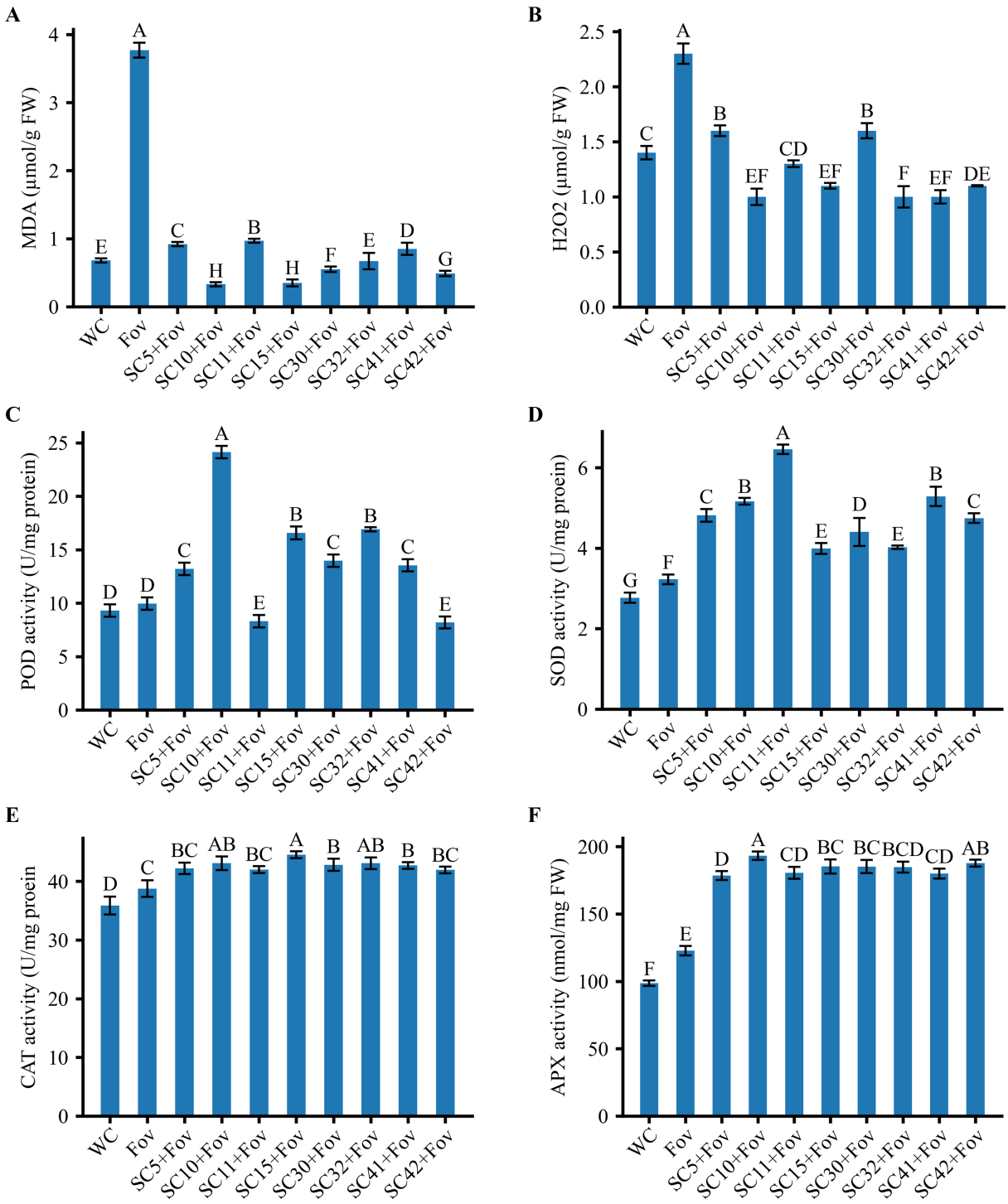

FIGURE 6
Effect of *Bacillus* isolates (SC5, SC10, SC11, SC15, SC30, SC32, SC41, and SC42) on ROS and antioxidant activities of cotton **(A)** malondialdehyde (MDA), **(B)** H_2O_2, **(C)** Peroxidase (POD) activity, **(D)** Superoxide dismutase (SOD) activity, **(E)** Catalase (CAT) activity, **(F)** Ascorbate peroxidase (APX) activity. Values represent the average + standard deviation of two biological replicates (n = 24 in total). Significant differences between the treatments are indicated by different letters. WC, water control; Fov, *Fusarium oxysporum* f.sp. *vasinfectum*.

more resistant to fungal pathogens (Huber and Haneklaus, 2007). A healthy, vigorously growing plant is generally better equipped to withstand pathogen attacks. The production of IAA, a key auxin hormone, is commonly associated with the ability of plant growth-promoting rhizobacteria to enhance plant root growth and overall vigor. Bacteria with antagonistic activity are known to play a crucial role in regulating plant growth and development through different mechanisms (Meena et al., 2020). In our study, no rhizobacteria was producing IAA although these rhizobacteria are good antagonists of fungi.

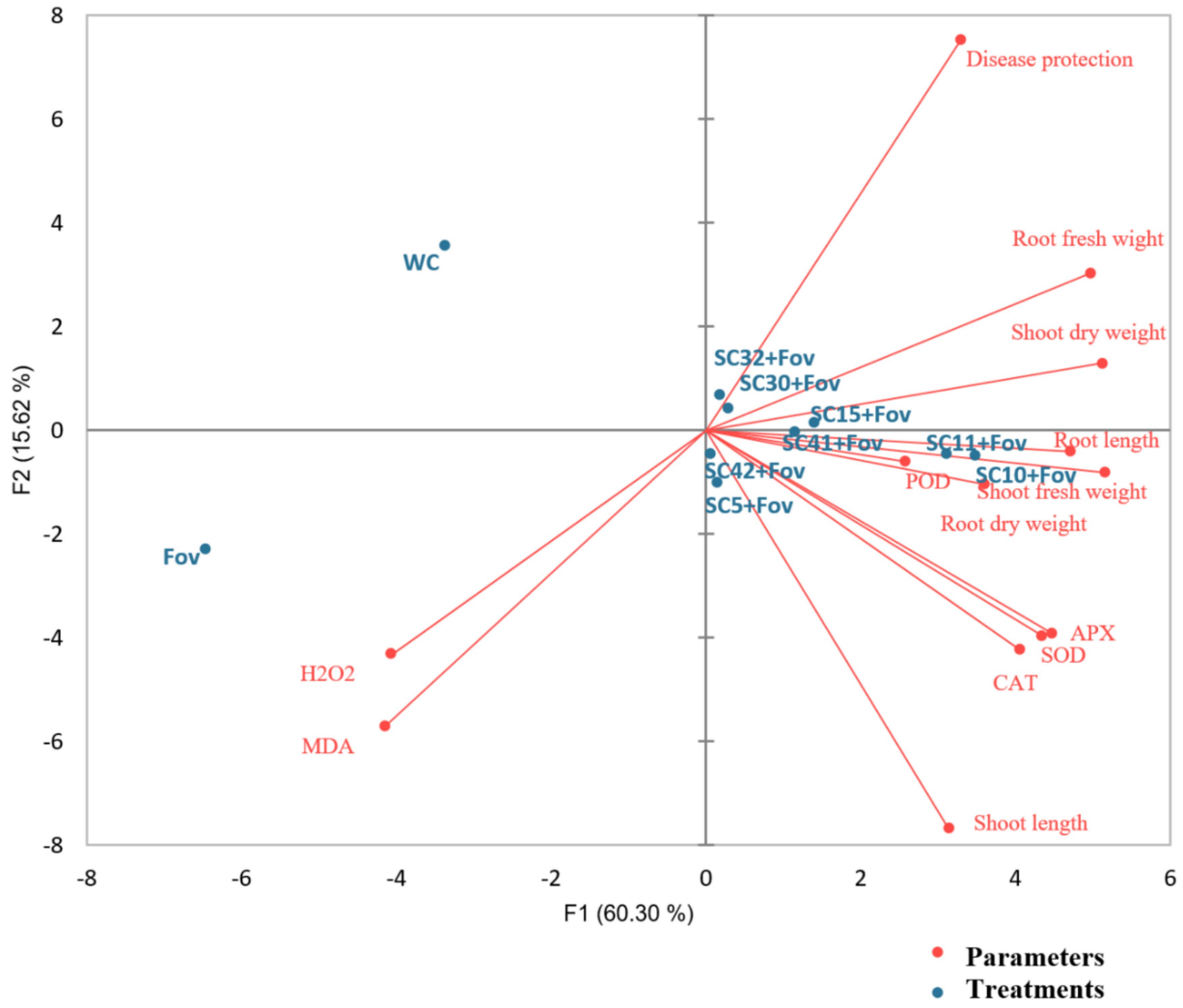

FIGURE 7
Principal component analysis biplot of the effects of the *Bacillus* isolates (SC5, SC10, SC11, SC15, SC30, SC32, SC41, and SC42) on reactive oxygen species (ROS), antioxidants, and growth parameters of cotton plants when applied during *Fusarium oxysporum* f.sp. *vasinfectum* (Fov) stress. WC, water control; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; POD, peroxidase; APX, ascorbate peroxidase; SOD, superoxide dismutase; CAT, catalase.

Although *in vitro* assays are valid in screening for antifungal capabilities, it is crucial to evaluate the biocontrol efficacy of PGPR through *in vivo* studies. Therefore, for validation, a greenhouse experiment was conducted using all *Bacillus* isolates to counteract Fov infection of cotton plants. The results showed that Fov infection decreased root and shoot fresh and dry weights compared to the uninfected control. All isolates suppressed Fusarium wilt from 41.30 to 82.7%. In particular, *B. subtilis* isolates SC10 and SC11 showed outstanding performance in increasing biomass and root development of cotton plants. Our findings on *B. subtilis* SC11, which possesses high siderophore production, strong urease activity, and P solubilization capabilities, indicate its potential as a highly effective biocontrol agent for plant growth promotion. The combined effects of siderophore production, urease activity, and P solubilization can act synergistically to promote plant growth and suppress fungal pathogens (Sritongon et al., 2023). For example, iron and phosphorus deficiency can weaken plants, making them more susceptible to disease. By increasing the availability of these nutrients, SC11 can improve plant health and make plants more resistant to fungal attack. Our results also indicate that Fov-mediated oxidative stress affects the activity of antioxidant enzymes in cotton plants. The observed increase in MDA levels indicated oxidative stress-induced membrane damage caused by Fusarium wilt in cotton plants. This increase in MDA, a marker of oxidative stress and cell membrane damage, is consistent with previous research. Notably, cotton plants inoculated with antagonistic *Bacillus* strains exhibited significantly reduced MDA levels compared to those inoculated with Fov alone, suggesting that these rhizobacterial strains mitigate the deleterious effects of Fov. The reduction in MDA levels due to inoculation resulted in reduced oxidative damage, thereby promoting plant growth.

In this study, Fov appeared to activate antioxidant enzymes such as POD, SOD, CAT, and APX in exposed cotton plants, revealing a protective response to Fov-mediated oxidative stress. This is consistent with previous research, in which was found that different plants increased their antioxidant enzyme activities to scavenge ROS

generated by fungal stress (Zhang et al., 2004). After the application of PGPR, there was a further increase in the activities of these enzymes in Fov-infected plants, indicating the beneficial role of plant-microbe interactions under fungal stress. These interactions help plants to cope with the harmful effects of Fov by enhancing their antioxidant defense mechanisms.

Antioxidant enzymes, including SOD, POD, CAT, and APX play a critical role in protecting plants from oxidative damage caused by ROS. SOD catalyzes the conversion of superoxide radicals to H_2O_2 and oxygen, thereby reducing the damaging effects of ROS. CAT then rapidly breaks down H_2O_2 into water and oxygen, preventing its accumulation. Similarly, APX uses ascorbate as an electron donor to reduce H_2O_2 to water, thereby maintaining cellular redox balance. POD also contributes to using various electron donors to scavenge H_2O_2. Together, these enzymes form an integrated defense system to mitigate oxidative stress, protect cellular components, and ensure metabolic homeostasis (Hasanuzzaman et al., 2020). In our study cotton plants inoculated with Fov and treated with *Bacillus* isolates showed higher SOD, POD (except SC11 and SC42), CAT, and APX activity compared to those without treatment (WC) and those inoculated with Fov alone. Our results are in line with studies in so far that an increase in various antioxidants is a significant plant-resistance feature against plant pathogens as elevated levels of antioxidant enzyme activity protected cotton plants from the oxidative damage induced by fungal infection and mitigated the adverse effects of lipid peroxidation caused by ROS (De Gara et al., 2003). Therefore, it can be concluded that the described *Bacillus* isolates combat Fov-induced stress and have the ability to induce plant defense mechanisms.

Future field trials will validate whether the isolates identified in this study, particularly *B. subtilis* SC10 and SC11, effectively control Fov and promote plant growth in practical agricultural conditions. An interesting aspect to investigate as well is whether an individual isolate is sufficiently effective or whether a consortium of different bacteria is preferable. Recently, a consortium of three *Bacillus* isolates was shown to promote plant grown of *Vicia faba* better and to control *Fusarium oxysporum* more efficiently than the individual strains (El-Sersawy et al., 2021). Moreover, a comparison between the performance of the bacteria and a commercially available synthetic chemical seed treatment is an interesting aspect to look into and necessary to gain acceptance.

5 Conclusion

The experimental data reveal that all eight bacterial isolates SC5, SC10, SC11, SC11, SC30, SC32, SC41, and SC42 that were originally isolated from the cotton rhizosphere of Fov and subsequently selected for their fungal activity against Fov also show beneficial traits associated with plant growth promotion and plant protection against other fungi. Particularly, *B. subtilis* SC10 and *B. subtilis* SC11, when applied to cotton plants against Fov, significantly reduced Fov infection, enhanced plant growth, and strengthened the defense system against Fusarium wilt. The *B. subtilis* SC10 treatment stands out with the highest shoot growth and biomass enhancement, suggesting an overall improvement in aboveground plant vitality. *B. subtilis* SC11 showed exceptional performance in promoting root growth and had the highest activity of CAT, APX, and SOD key antioxidant enzymes, which are critical for mitigating oxidative stress caused by pathogen attacks. The lower disease severity observed for the treatments with the *Bacillus* isolates, particularly *B. subtilis* SC10 and SC11, further supports their potential as effective biocontrol agents. The lower MDA and higher antioxidant levels in these treatments provide additional support for their protective effects. These isolates are good candidates for the generation of versatile bio-formulations with the capacity to enhance plant growth and ensure plant health. These formulations could then be commercially leveraged for integrated plant nutrient and pathogen management, thereby endorsing practices that facilitate sustainable agriculture.

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 below: GenBank accession numbers PV147758 to PV147765. https://www.ncbi.nlm.nih.gov/nuccore/?term=PV147758:PV147765[accn].

Author contributions

SA: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. MB: Conceptualization, Project administration, Resources, Supervision, Validation, Writing – review & editing. TN: Conceptualization, Project administration, Supervision, Validation, Writing – review & editing. MJ: Formal analysis, Writing – review & editing. SB: Supervision, Validation, Writing – review & editing. FG: Conceptualization, Project administration, Supervision, Validation, Writing – review & editing. AS: Conceptualization, Project administration, Supervision, Validation, Writing – review & editing.

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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/fmicb.2025.1580937/full#supplementary-material

References

Abdelaziz, A. M., Hashem, A. H., El-Sayyad, G. S., El-Wakil, D. A., Selim, S., Alkhalifah, D. H., et al. (2023). Biocontrol of soil borne diseases by plant growth promoting rhizobacteria. *Trop. Plant Pathol.* 48, 105–127. doi: 10.1007/s40858-022-00544-7

Aebi, H. (1984). "Catalase *in vitro*" in Methods in enzymology. ed. L. Packer (New York: Academic Press), 121–126.

Ali, S., Hameed, S., Imran, A., Iqbal, M., and Lazarovits, G. (2014). Genetic, physiological and biochemical characterization of *Bacillus* sp. strain RMB7 exhibiting plant growth promoting and broad spectrum antifungal activities. *Microb. Cell Factories* 13, 144–115. doi: 10.1186/PREACCEPT-6657919731258908

Arora, N. K., and Verma, M. (2017). Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. *3 Biotech* 7:381. doi: 10.1007/s13205-017-1008-y

Boulahouat, S., Cherif-Silini, H., Silini, A., Bouket, A. C., Luptakova, L., Alenezi, F. N., et al. (2023). Biocontrol efficiency of rhizospheric *Bacillus* against the plant pathogen *Fusarium oxysporum*: a promising approach for sustainable agriculture. *Microbiol. Res.* 14, 892–908. doi: 10.3390/microbiolres14030062

Brown, R. M. Jr. (2004). Cellulose structure and biosynthesis: what is in store for the 21st century? *J. Polym. Sci. A Polym. Chem.* 42, 487–495. doi: 10.1002/pola.10877

Chaiharn, C., Sujada, N., Pathom-aree, W., and Lumyong, S. (2019). Biological control of Rigidoporus microporus the cause of white root disease in rubber using PGPRs in vivo. *Chiang Mai J. Sci.* 46, 850–866.

De Gara, L., de Pinto, M. C., and Tommasi, F. (2003). The antioxidant systems Vis-à-Vis reactive oxygen species during plant–pathogen interaction. *Plant Physiol. Biochem.* 41, 863–870. doi: 10.1016/S0981-9428(03)00135-9

Dhindsa, R. S., and Matowe, W. (1981). Drought tolerance in two mosses: correlated with enzymatic defence against lipid peroxidation. *J. Exp. Bot.* 32, 79–91. doi: 10.1093/jxb/32.1.79

Dilfuza, J. D. E., and Dilfuza, D. (2011). Evaluation of root associated bacteria for control of cotton root rot caused by *Fusarium oxysporum* in salinated soils. *Igdır Univ. J. Inst. Sci. Technol.* 1, 37–42.

Dimkić, I., Janakiev, T., Petrović, M., Degrassi, G., and Fira, D. (2022). Plant-associated *Bacillus* and *Pseudomonas* antimicrobial activities in plant disease suppression via biological control mechanisms-a review. *Physiol. Mol. Plant Pathol.* 117:101754. doi: 10.1016/j.pmpp.2021.101754

Eddin, K. S., Marimuthu, T., Ladhalakshmi, D., and Velazhahan, R. (2007). Biological control of bacterial blight of cotton caused by *Xanthomonas axonopodis* pv. *malvacearum* with *Pseudomonas fluorescens*. *Arch. Phytopathol. Plant Protect.* 40, 291–300. doi: 10.1080/03235400600587557

El-Sersawy, M. M., Hassan, S. E.-D., El-Ghamry, A. A., El-Gwad, A. M. A., and Fouda, A. (2021). Implication of plant growth-promoting rhizobacteria of *Bacillus* spp. as biocontrol agents against wilt disease caused by *Fusarium oxysporum* Schlecht. In *Vicia faba* L. *Biomol. Concepts* 12, 197–214. doi: 10.1515/bmc-2021-0020

Elshahat, M. R., Ahmed, A. A., Enas, A. H., and Fekria, M. S. (2016). Plant growth promoting rhizobacteria and their potential for biocontrol of phytopathogens. *Afr. J. Microbiol. Res.* 10, 486–504. doi: 10.5897/AJMR2015.7714

Etesami, H., and Adl, S. M. (2020). "Plant growth-promoting rhizobacteria (PGPR) and their action mechanisms in availability of nutrients to plants" in Phyto-microbiome in stress regulation. eds. M. Kumar, V. Kumar and R. Prasad (Singapore: Springer), 147–203.

Etesami, H., Jeong, B. R., and Glick, B. R. (2023). Biocontrol of plant diseases by *Bacillus* spp. *Physiol. Mol. Plant Pathol.* 126:102048. doi: 10.1016/j.pmpp.2023.102048

Fira, D., Dimkić, I., Berić, T., Lozo, J., and Stanković, S. (2018). Biological control of plant pathogens by *Bacillus* species. *J. Biotechnol.* 285, 44–55. doi: 10.1016/j.jbiotec.2018.07.044

Fu, L., Penton, C. R., Ruan, Y., Shen, Z., Xue, C., Li, R., et al. (2017). Inducing the rhizosphere microbiome by biofertilizer application to suppress banana fusarium wilt disease. *Soil Biol. Biochem.* 104, 39–48. doi: 10.1016/j.soilbio.2016.10.008

Garcia, D. P., Caraschi, J. C., Ventorim, G., Vieira, F. H. A., and de Paula Protásio, T. (2019). Assessment of plant biomass for pellet production using multivariate statistics (PCA and HCA). *Renew. Energy* 139, 796–805. doi: 10.1016/j.renene.2019.02.103

Glickmann, E., and Dessaux, Y. (1995). A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Appl. Environ. Microbiol.* 61, 793–796. doi: 10.1128/aem.61.2.793-796.1995

Goswami, D., Patel, K., Parmar, S., Vaghela, H., Muley, N., Dhandhukia, P., et al. (2015). Elucidating multifaceted urease producing marine *Pseudomonas aeruginosa* BG as a cogent PGPR and bio-control agent. *Plant Growth Regul.* 75, 253–263. doi: 10.1007/s10725-014-9949-1

Hasanuzzaman, M., Bhuyan, M. B., Zulfiqar, F., Raza, A., Mohsin, S. M., Mahmud, J. A., et al. (2020). Reactive oxygen species and antioxidant defense in plants under abiotic stress: revisiting the crucial role of a universal defense regulator. *Antioxidants* 9:681. doi: 10.3390/antiox9080681

Heath, R. L., and Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* 125, 189–198. doi: 10.1016/0003-9861(68)90654-1

Huber, D. M., and Haneklaus, S. (2007). Managing nutrition to control plant disease. *Landbauforschung Volkenrode* 57:313.

Juhnke, M. E., Mathre, D., and Sands, D. (1987). Identification and characterization of rhizosphere-competent bacteria of wheat. *Appl. Environ. Microbiol.* 53, 2793–2799. doi: 10.1128/aem.53.12.2793-2799.1987

Ki, J.-S., Zhang, W., and Qian, P.-Y. (2009). Discovery of marine *Bacillus* species by 16S rRNA and rpoB comparisons and their usefulness for species identification. *J. Microbiol. Methods* 77, 48–57. doi: 10.1016/j.mimet.2009.01.003

Kiesewalter, H. T., Lozano-Andrade, C. N., Wibowo, M., Strube, M. L., Maróti, G., Snyder, D., et al. (2021). Genomic and chemical diversity of *Bacillus subtilis* secondary metabolites against plant pathogenic fungi. *Msystems* 6:e00770-20. doi: 10.1128/mSystems.00770-20

Landa, B. B., Montes-Borrego, M., and Navas-Cortés, J. A. (2012). "Use of PGPR for controlling soilborne fungal pathogens: assessing the factors influencing its efficacy" in Bacteria in agrobiology: Disease management. ed. D. K. Maheshwari (Berlin, Heidelberg: Springer), 259–292.

Li, J., Huang, W.-C., Gao, L., Sun, J., Liu, Z., and Mao, X. (2019). Efficient enzymatic hydrolysis of ionic liquid pretreated chitin and its dissolution mechanism. *Carbohydr. Polym.* 211, 329–335. doi: 10.1016/j.carbpol.2019.02.027

Liu, X., and Zhang, Y. (2021). Exploring the communities of bacteria, fungi and ammonia oxidizers in rhizosphere of Fusarium-diseased greenhouse cucumber. *Appl. Soil Ecol.* 161:103832. doi: 10.1016/j.apsoil.2020.103832

Maehly, A. (1954). "The assay of catalases and peroxidases" in Methods of biochemical analysis. ed. D. Glick (New York: Interscience Publishers, Inc.), 357–424.

McKee, L. S., and Inman, A. R. (2019). "Secreted microbial enzymes for organic compound degradation" in Microbes and enzymes in soil health and bioremediation, 225–254.

Meena, M., Swapnil, P., Divyanshu, K., Kumar, S., Harish, Tripathi, Y. N., et al. (2020). PGPR-mediated induction of systemic resistance and physiochemical alterations in plants against the pathogens: current perspectives. *J. Basic Microbiol.* 60, 828–861. doi: 10.1002/jobm.202000370

Nadeem, S. M., Ahmad, M., Zahir, Z. A., Javaid, A., and Ashraf, M. (2014). The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. *Biotechnol. Adv.* 32, 429–448. doi: 10.1016/j.biotechadv.2013.12.005

O'Sullivan, M., Stephens, P. M., and O'Gara, F. (1991). Extracellular protease production by fluorescent *Psevdomonas* SPP and the colonization of sugarbeet roots and soil. *Soil Biol. Biochem.* 23, 623–627. doi: 10.1016/0038-0717(91)90074-T

Pei, P., Aslam, M., Wang, H., Ye, P., Li, T., Liang, H., et al. (2024). Diversity and ecological function of urease-producing bacteria in the cultivation environment of *Gracilariopsis lemaneiformis*. *Microb. Ecol.* 87:35. doi: 10.1007/s00248-023-02339-y

Pikovskaya, R. (1948). Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Mikrobiologiya* 17, 362–370.

Raklami, A., Bechtaoui, N., Tahiri, A.-I., Anli, M., Meddich, A., and Oufdou, K. (2019). Use of rhizobacteria and mycorrhizae consortium in the open field as a strategy for improving crop nutrition, productivity and soil fertility. *Front. Microbiol.* 10:1106. doi: 10.3389/fmicb.2019.01106

Riseh, R. S., Vatankhah, M., Hassanisaadi, M., and Barka, E. A. (2024). Unveiling the role of hydrolytic enzymes from soil biocontrol bacteria in sustainable phytopathogen management. *Front. Biosci.* 29:105. doi: 10.31083/j.fbl2903105

Sakthivel, N., and Gnanamanickam, S. (1986). Toxicity of *Pseudomonas fluorescens* towards rice sheath-rot pathogen *Acrocylindrium oryzae saw*. *Curr. Sci.* 55, 106–107.

Schwyn, B., and Neilands, J. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56. doi: 10.1016/0003-2697(87)90612-9

Shukla, S., Upadhyay, D., Mishra, A., Jindal, T., and Shukla, K. (2022). "Challenges faced by farmers in crops production due to fungal pathogens and their effect on Indian economy" in Fungal diversity, ecology and control management. eds. I. S. V. R. Rajpal and S. S. Navi (Singapore: Springer), 495–505.

Sritongon, N., Boonlue, S., Mongkolthanaruk, W., Jogloy, S., and Riddech, N. (2023). The combination of multiple plant growth promotion and hydrolytic enzyme producing rhizobacteria and their effect on Jerusalem artichoke growth improvement. *Sci. Rep.* 13:5917. doi: 10.1038/s41598-023-33099-x

Velikova, V., Yordanov, I., and Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Sci.* 151, 59–66. doi: 10.1016/S0168-9452(99)00197-1

Veliz, E. A., Martínez-Hidalgo, P., and Hirsch, A. M. (2017). Chitinase-producing bacteria and their role in biocontrol. *AIMS Microbiol.* 3, 689–705. doi: 10.3934/microbiol.2017.3.689

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703. doi: 10.1128/jb.173.2.697-703.1991

Zaim, S., Belabid, L., Bayaa, B., and Bekkar, A. A. (2016). "Biological control of chickpea fusarium wilts using rhizobacteria "PGPR"" in Microbial-mediated induced systemic resistance in plants. eds. A. V. Devendra and K. Choudhary (Singapore: Springer), 147–162.

Zhang, Z., Henderson, C., and Gurr, S. J. (2004). *Blumeria graminis* secretes an extracellular catalase during infection of barley: potential role in suppression of host defence. *Mol. Plant Pathol.* 5, 537–547. doi: 10.1111/j.1364-3703.2004.00251.x

Bacillus subtilis and *Bacillus stercoris* antagonize the plant-parasitic nematode *Heterodera schachtii* and enhance plant growth of *Arabidopsis thaliana*

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Abstract

Plant-parasitic nematodes like *Heterodera schachtii* reduce crop yields. Chemical control if available raises environmental concerns, highlighting the need for alternatives. Certain plant growth-promoting rhizobacteria (PGPR) offer promising solutions by enhancing plant growth and antagonizing nematodes. Thus, this study investigated the potential of seven rhizobacteria isolates (SC11, SW33, SW44, SW53, SW69, SW73, and SW83) to control *H. schachtii*. *In vitro* assessment of the nematicidal activity of living bacterial cells (LBC) or their cell-free supernatants (CFS) against *H. schachtii* infective stage juveniles (J2) revealed that all treatments significantly increased J2 mortality compared to the control ($p < 0.05$). LBC of SW44 and SW73, and CFS of SW33 caused the highest J2 mortality of up to ~48% and ~64%, respectively. *In vivo* experiments with *Arabidopsis thaliana* in a nematode infection assay further confirmed the biocontrol potential of these rhizobacteria. All bacterial treatments reduced *H. schachtii* parasitism. SW83 showed the most substantial reduction in nematode infestation, with a ~69% decrease in developed females and a ~58% decrease in the total number of nematodes. Moreover, we found that the bacteria significantly reduced the invasion of J2 into the plant roots by up to ~46% (SC11). The development of invaded J2 into adults was further reduced significantly in SW33 (~26%), SW73 (~34%) and SW83 (~36%), and female size was reduced by 12% (SC11) to 18% (SW44 and SW83). In addition, these isolates also demonstrated positive effects on plant development, with SW33, SW53, SW69, and SW73 significantly enhancing plant growth.

16S rRNA gene sequencing identified high homology of SC11, SW33, and SW53 to *B. subtilis*, and of SW44, SW69, SW73, and SW83 to *B. stercoris*. Biochemical characterization revealed that (i) all isolates produced biofilm and siderophore, (ii) SW44, SW73, and SW83 secreted chitinase, (iii) SW33 generated HCN, and (iv) SW33, and SW44 performed P-solubilization.
Our study shows that *Bacillus* isolates effectively suppress nematodes both directly and in the presence of host plants, and thus, can be a valuable means as part of an integrated nematode control strategy. Moreover, this study is the first to report the antagonistic activity of the *B. stercoris* against a plant-parasitic nematode.

Keywords: rhizobacteria, plant-parasitic nematode, antagonism, plant growth promotion, *Bacillus subtilis*, *Bacillus stercoris*, *Heterodera schachtii*

1. Introduction

Plant-parasitic nematodes (PPN) significantly threaten agricultural productivity across diverse crop systems (Phani et al., 2021). These nematodes, primarily residing in the soil, target the underground plant structures, making their detection, identification, and management a significant challenge. Nematode infestations have a two-fold detrimental impact on plants: they directly impede growth and reduce crop yields, and some PPN species act as vectors for plant viruses or facilitate infections by microbial root pathogens (Huang et al., 2021). Common nematode-borne diseases are caused by root-knot nematodes (*Meloidogyne* spp.), cyst nematodes (*Heterodera* and *Globodera* spp.), and lesion nematodes (*Pratylenchus* spp.), all of which pose serious threats to global food security and production (Messa et al., 2019).

Cyst nematodes, including *H. schachtii* (sugar beet cyst nematode), *H. glycines* (soybean cyst nematode), and *Globodera rostochiensis* (golden potato cyst nematode), present significant challenges for agriculture, particularly in temperate regions (He et al., 2022). Nematode management strategies are implemented to mitigate nematode damage and maintain crop health and productivity (Elango et al., 2020). Measures like crop rotation, soil solarization, and synthetic nematicide application are widely used, but they face limitations regarding their long-term effectiveness and sustainability. Cultural practices like crop rotation often demand significant knowledge and labor, and can produce inconsistent results due to environmental fluctuations. Additionally, the cost of implementing such practices and the potential development of nematode resistance can hinder their long-term success (Vasantha-Srinivasan et al., 2025). Synthetic nematicides offer rapid and effective nematode control, but their use carries significant risks. Due to increasing concerns regarding their

broad toxicity, environmental impact (Jeevan et al., 2025), and potential harm to non-target organisms, many synthetic nematicides have faced restrictions or are banned in many countries(Ankit et al., 2020). Consequently, there is a need for alternatives in PPN control that are not only effective, but also sustainable and minimize the risks to human health and the environment.

The rhizosphere, a rich and diverse microbial ecosystem, is a promising source for developing such alternatives. Plant growth-promoting rhizobacteria (PGPR) have emerged as particularly strong candidates for biocontrol (Jian et al., 2024). These beneficial bacteria colonize plant roots, and beyond stimulating plant growth, they were observed to be able to also suppress plant diseases. Their application minimizes ecological disruption, supports biodiversity, and maintains the integrity of soil and water systems. By promoting natural plant defense mechanisms and reducing reliance on chemical interventions, PGPR-based biocontrol strategies contribute to maintaining a healthy environment.

PGPR *Bacillus* spp. employ a variety of direct and indirect mechanisms to promote plant growth and suppress pathogens, offering a multifaceted approach to nematode control. Direct mechanisms of plant growth promotion by *Bacillus* spp. include the production of phytohormones, such as auxins, cytokinins, and gibberellins, which can stimulate root and shoot growth, and the increase of the bioavailability of essential nutrients, such as phosphorus (P) and micronutrients, through solubilization or mineralization processes (Jalal et al., 2024). Indirect mechanisms of *Bacillus* spp. to promote plant growth mainly involve biocontrol activity against various plant pathogens, for instance by the production of antibiotics (Wang et al., 2024), lytic enzymes (Reddy et al., 2022), or volatile organic compounds that inhibit the growth of phytopathogens (Toral et al., 2021). They can also induce systemic resistance in the host plant, enhancing its ability to defend itself against various pathogens (Messa, 2021). Furthermore, *Bacillus* spp. can compete with deleterious microorganisms for nutrients and space in the rhizosphere, thereby reducing the impact of these harmful organisms on plant health (Saeed et al., 2021).

Numerous studies have highlighted the significant role of rhizobacteria in the biological control of PPN. In our study, we used *H. schachtii,* an established model PPN to study the impact of rhizobacteria on nematode parasitism (Huang et al., 2021). *H. schachtii* is widely used in nematology research due to its relatively simple life cycle, ease of raring, and interaction with host plants including *Arabidopsis thaliana* (Matera et al., 2021). Our study focused on the detailed characterization of seven bacteria previously isolated form the rhizosphere of cotton and wheat plants from fields in Pakistan with respect to the following points: (i) molecular identification of rhizobacteria isolates, (ii) evaluation of

the *in vitro* effects of both living bacterial cells (LBC) and their metabolites on the mortality of *H. schachtii* second-stage juveniles (J2), (iii) the impact of LBC on *H. schachtii* parasitism in *A. thaliana* plants, (iv) impact of LBC on plant growth in nematode-infected *A. thaliana*, and (v) the biochemical characterization of the isolates.

2. Material and methods

2.1 Bacterial isolates

Twenty-five rhizobacteria were obtained from the Institute of Molecular Biology and Biotechnology at Bahauddin Zakariya University in Multan, Pakistan, previously isolated from the rhizosphere of cotton or wheat (Aslam et al., 2025a). These isolates were preliminarily screened for their ability to antagonize *H. schachtii* parasitism in *A. thaliana* plants (Aslam et al., unpublished data). Seven were found to be effective in reducing *H. schachtii* infection in *A. thaliana*: SC11 isolated from the cotton rhizosphere, and SW33, SW44, SW53, SW69, SW73, and SW83 isolated from wheat rhizospheres of four distinct fields located in Punjab, Pakistan.

2.2 Molecular identification of isolates based on 16S rRNA gene sequencing

SC11 was already identified as highly homologous to *B. subtilis*(Aslam et al., 2025b). 16S rRNA gene amplicon sequencing was performed for the identification of the other six isolates. A single colony from each isolate was suspended into 100 µL of sterile nuclease-free H_2O, incubated at 99 °C for 20 min, and then centrifuged for 2 min at 4000 × g The resulting supernatant, containing bacterial genomic DNA, was utilized for polymerase chain reaction (PCR) amplification using the universal primer pair fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGC-3′) (Weisburg et al., 1991). PCR reactions were prepared in a total volume of 50 µL, consisting of 16 µL nuclease-free H_2O, 24 µL DreamTaq PCR Master Mix (Thermo Fisher Scientific), 2 µL of 10 µM reverse primer rD1, 2 µL of 10 µM forward primer fD1, and 6 µL of bacterial DNA template. The amplification protocol included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 30 seconds, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min.

The PCR products were electrophoresed on a 1% TAE-agarose gel at a constant voltage of 80 V for 50 minutes. DNA fragments of approximately ˜1.5 k bp were excised from the gel and transferred into autoclaved Eppendorf tubes. Purification was performed using a DNA purification kit (Thermo Fisher Scientific) as per the manufacturer's protocol, and the purified DNA was sent to Eurofins Genomics

(Germany) for sequencing. The obtained sequences were analyzed using the NCBI BLAST tool by comparison with deposited sequences in the GenBank database. Initial BLAST analysis was conducted against the entire NCBI database to determine the preliminary identity of the isolates. A subsequent BLAST analysis was performed against type strains within the relevant taxonomic group to eliminate misannotated entries. Sequence alignment of highly homologous regions was conducted using ClustalW in Molecular Evolutionary Genetics Analysis Version 11 (MEGA 11) software. Phylogenetic trees were generated using the neighbor-joining method, and node support was assessed via bootstrap analysis with 1000 replicates (Ki et al., 2009).

2.3 Impact of bacterial cells and secondary metabolites on nematode mortality

2.3.1 Nematode preparation

Approximately 300 cysts of the plant-parasitic nematode *H. schachtii* were harvested from aseptically grown mustard (*Sinapsis alba* cv. Albatros) roots and submerged in a sterile 3 mM $ZnCl_2$ solution within a Baermann funnel (Kadlecová et al., 2024). J2 were collected after 7 days, $ZnCl_2$ was washed off four times with sterile tap H_2O, and J2 were immediately used for further analysis.

2.3.2 Bacterial cell culture and secondary metabolite preparation

All rhizobacteria isolates were kept in 20% glycerol stock cultures in LB broth (10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 L dH_2O) at −80 °C. Cultivation was started by streaking 3 µL of the glycerol stock on LB agar (LB broth with 15 g agar in 1 L dH_2O) plates and incubation at 28 ± 2 °C for 24 h. Single colonies were inoculated into 500 mL conical flasks containing 100 mL of LB broth and incubated at 28 ± 2 °C with shaking at 150 rpm (Edmund Buhler GmbH, Germany) overnight. The following morning, the main cultures were prepared by diluting in LB broth to an optical density (OD) of 0.1 at 600 nm (OD_{600}) followed by another incubation at 28 ± 2 °C with shaking at 150 rpm for 72 h. Subsequently, the bacterial cultures were collected in 15 mL falcon tubes and centrifuged (4000 ×g) for 5 min. The supernatant was collected into a separate sterile 15 mL falcon tube. The supernatant of each respective rhizobacterium was then filtered through a 0.22 µm sterile filter to obtain cell-free supernatant (CFS) containing bacterial secondary metabolites. Rhizobacteria cell pellets were washed once with sterile tap H_2O and suspended in sterile tap H_2O. The OD_{600} was measured, and rhizobacteria cells were used for further studies by adjusting to the required OD_{600} depending on subsequent applications.

2.3.3 Impact of bacterial cells and secondary metabolites on nematode mortality

The effect of living rhizobacteria cells on the mortality of *H. schachtii* J2was determined in 24-well plates. As treatment, the OD_{600} of each cell culture was adjusted with 1/3 strength LB broth to 0.1 and 1 mL of bacteria culture was added per well, together with 5 µL suspension containing approximately 60 J2 suspended in sterile tap H_2O. 1/3 strength LB broth without bacteria culture was used as a negative control. To determine the effect of secondary metabolites on all seven isolates, two different concentrations of secondary metabolites (100% pure filtrate and 50% diluted filtrate with sterile tap H_2O) were used against *H. schachtii* of J2. Approximately 60 J2 suspended in 5 µL of sterile tap H_2O were added to each well of 96-well plates, which contained either 100 µl of undiluted or 50% diluted CFS Controls included standard LB broth (undiluted and 50% diluted).

After preparation, all plates were incubated at 25 °C, and nematode mortality was assessed at 96 h post-treatment by adding 10 µL of 1M NaOH solution (Matera et al., 2021). Nematodes that did not respond to the addition of NaOH were considered dead. Mortality (%) was calculated by using the following formula;

Mortality % = [(number of dead nematodes) / (number of total nematodes)] ×100

All treatments were performed with four technical replicates per variant and three independent biological replicates.

2.4 Impact of bacterial cells on nematode parasitism and plant growth

A. thaliana Col-0 seeds were surface sterilized with 0.7% sodium hypochlorite (5 min), followed by 70% ethanol (1 min), and five rinses with sterile dH_2O. These sterilized seeds were dried in a laminar flow hood (4 h) and stored in the dark at 4°C. Then these seeds were cultivated in a modified Knop medium (Sijmons et al., 1991). Since all rhizobacteria used in this study showed the highest growth rate at pH 7.5 (Aslam *et al.,* unpublished data) Knop medium was prepared with a pH value of 7.5, but without sucrose to avoid bacterial overgrowth. First, Petri dishes (90 mm diameter) were filled with the modified Knop medium and then two 50 µL droplets of standard modified Knop medium (+ sucroseand a pH value of 6.4) were addedon the dried medium as spots for sowing the sterilized *A. thaliana* seeds. Plates were placed horizontally in a climate chamber under a red/blue light with a 16/8 light/dark photoperiod at 24 °C. After 4 days, plates were tilted at an angle of 60° to facilitate surface growth of the roots. At 8 days post-seeding, root tips of *A. thaliana* seedlings were inoculated with 7 µL of bacterial suspension (OD_{600} = 0.1) prepared from the 3-day-old bacterial culture as described

in chapter 2.3.2. Seedlings inoculated with sterile tap H_2O alone served as control. Seven days post treatment of rhizobacteria, each plant was inoculated with 70-80 *H. schachtii* J2. The infection assay was conducted to analyze nematode parasitism on *A. thaliana* plants (Huang et al., 2021). This involved counting the numbers of male and female nematodes at 12days post inoculation (dpi) using a stereomicroscope (Leica, Germany), as well as measuring the size of female nematodes at 14dpi using a digital stereomicroscope (Leica, Germany) equipped with Leica Application Suite (LAS) software. The impact of rhizobacteria on plant growth was evaluated 21 days post-treatment for aboveground-related parameters (shoot diameter, weight, and leaf numbers) and underground-related parameters (total root length) using Epson Perfection V700 Photo scanner [Epson, Japan] equipped with the image analysis software WinRHIZO. All treatments were performed with 15 technical replicates per variant and three independent biological replicates.

2.4.1 Impact of bacterial cells on nematode invasion

To understand whether changes in nematode parasitism as a result of a rhizobacteria treatment are related to the timing of early nematode infection, the rhizobacterial impact on the root invasion rate of *H. schachtii* was investigated. The experiments were prepared as described in 2.4, but the number of *H. schachtii* J2 inside the roots was counted after 1, 2, and 3 dpi. The invasion rate was calculated in percentage by using the following formula:

Invasion rate % = (no. of invaded J2 in root / total no. of inoculated J2) ×100

At 12 dpi, the number of adult nematodes was counted, and development rate after the invasion was calculated as percentage using the following formula:

Development % = (total no. of adult nematodes at 12dpi / total no. of invaded J2 at 3dpi) × 100

Three independent biological replicates of all treatments with 8 technical replicates each were performed (n = 24).

2.5 Biochemical characterization of bacteria

2.5.1 Chitinase production

Chitinase production of the rhizobacterial isolates was determined by inoculating a single bacteria colony from overnight fresh culture on chitin agar. The chitin agar composition was as follows: 10 g colloidal chitin, 1 g KH_2PO_4, 2 g K_2HPO_4, 0.5 g $MgSO_4 .7H_2O$, 5 g $NaNO_3$, 0.1 g $CaCl_2$, 0.1 g KCl, 0.02 g $FeSO_4 .7H_2O$, 15 g agar in 1 L dH_2O (Chaiharn et al., 2019).

Following a 72 h incubation period at 28 ± 2 °C isolates exhibiting a clear zone around their colonies were identified as positive results. Three biological replicates, each with three technical replicates were used.

2.5.2 Siderophore production

Siderophore production of the isolates was determined both qualitatively and quantitatively as described by Schwyan and Neilands in 1987.

Chrome Azurol S (CAS) agar was used for the qualitative assay. The medium comprised LB agar supplemented with CAS indicator dye, ferric ions (Fe3+), and hexadecyl trimethyl ammonium bromide. Single colonies of rhizobacteria isolates were cultured on CAS agar plates and incubated at 28 ± 2 °C for 72 h. Siderophore production was indicated by the formation of an orange-yellow halo zone surrounding the bacterial colony.

Siderophore quantification was performed using a microplate assay. Rhizobacterial isolates were grown in 100 mL LB broth in 500 mL Erlenmeyer flasks, with shaking at 150 rpm (Edmund Buhler GmbH, Germany) at 28 ± 2°C in shaker incubator for 72 hours. Following incubation, the bacterial cultures were centrifuged at 9000 ×g for 5 min to obtain cell-free supernatants. Aliquots (100 µL) of each supernatant were transferred to individual wells of a 96-well microtiter plate, followed by the addition of an equal volume of CAS reagent. The absorbance of each sample was measured at 630 nm after incubation of 30 min in dark at room temperature, and calculated as percent siderophore units (psu) (Schwyn and Neilands, 1987). Siderophore production was determined using three biological replicates, each with four technical replicates.

2.5.3 Hydrogen cyanide production

Hydrogen cyanide (HCN) production was determined by using LB broth containing 4.4 g/L glycine. A single colony from an overnight rhizobacteria culture was inoculated in this medium. Strips of Whatman No. 1 filter paper soaked in alkaline picrate solution (0.5% picric acid in 2% sodium carbonate) were dried and placed in test tubes containing 5 mL of bacterial culture. The tubes were plugged with cotton to prevent volatilization and incubated at 28 ± 2 °C with shaking at 150 rpm (Edmund Buhler GmbH, Germany) for 3-5 days. HCN production was indicated by a color change in the filter paper from yellow to light brown or reddish-brown. To quantify HCN, at the 3rd day of incubation at 28 ± 2 °C, the filter paper strips were transferred to fresh tubes with 10 mL of distilled water and vortexed. The optical density of the resulting solutions was measured at 515 nm, and HCN concentration was determined using a standard curve generated with potassium cyanide (KCN)

concentrations of 20, 40, 60, 80, and 100 µg/mL (Vasant et al., 2023). Three biological replicates, each with three technical replicates were used.

2.5.4 P-solubilization

P-solubilization by rhizobacteria was qualitatively evaluated using Pikovskaya's agar (Pikovskaya, 1948). One fresh bacterial colony of each isolate was spot-inoculated at the center of Pikovskaya's agar plates. Following incubation at 28 ± 2°C for 3-5 days, P-solubilization was determined by a clear zone surrounding the bacterial growth. Three biological replicates each with three technical replicates were used.

2.5.5 Indole 3- acetic acid production

To determine indole-3-acetic acid (IAA) production by rhizobacterial isolates, a single colony of each was cultured in 50 mL of LB broth medium, both with and without the addition of tryptophan, in 250 mL conical flasks. These cultures were incubated with shaking at 150 rpm (Edmund Buhler GmbH, Germany) at 28 ± 2°C for 72 h. Following incubation, the cultures were centrifuged, and 150 µL of the supernatant was combined with an equal volume of Salkowski's reagent in a 96-well plate. The samples were then incubated in the dark at 28 ± 2°C for 30 min. The development of a pink color indicates successful IAA production (Naureen et al., 2022). Three biological replicates each with three technical replicates were used.

2.5.6 Biofilm formation

Single colonies of overnight fresh cultures of rhizobacteria isolates were inoculated in 50 mL LB broth in a 250 mL flask. The cultures were incubated at 28 ± 2 ˚C with shaking at 150 rpm for 24 h. Microtiter plates were used to determine the biofilm formation. 200 µL of LB broth supplemented with 2% glucose was added in each well, and then each well was inoculated with 2 µL of freshly prepared culture of the respective rhizobacteria isolates (with OD_{600} = 2 to maintain the final working culture with OD_{600} = 0.02). 200 µL of LB broth supplemented with 2% glucose was used as a control. These microtiter plates were incubated at 28 ± 2 ˚C with shaking at 50 rpm for 48 h, then the broth was poured off and the plates were washed with phosphate buffer saline (PBS) (8.0 g NaCl**,** 0.2 g KCl**,** 1.44 g Na_2HPO_4**,** and 0.24 g KH_2PO_4 in 1 L of dH_2O) followed by dH_2O. 250 µL of crystal violet stain (1%) was added to each well for 45 min, then the excess stain was removed. Ethanol (95%) was added to dissolve the stain bound to the biofilm, and the resulting solution's optical density was measured at 570 nm using a spectrophotometer which correlates with biofilm quantity (Stepanović et al., 2007).

2.6 Statistical analysis

All experiments were performed in three biological replicates and analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$) was performed for data analysis using the software Statistix 8.1.

3. Results

3.1 Identification of selected isolates based on 16S rRNA sequencing

The seven rhizobacteria isolates designated SC11, SW33, SW44, SW53, SW69, SW73, and SW83 were selected following an initial screening for their ability to inhibit *H. schachtii* infection. One isolate SC11 was already reported as *B. subtilis (*Accession: PV147760.1)(Aslam et al., 2025b). Molecular identification through 16S rRNA gene sequencing and ncbi BLAST analysis revealed that the other six isolates also belong to the *Bacillus* genus. Subsequent type strain comparison uncovered that, SW33, and SW53 were closely related to the *B. subtilis* strain BCRC 10255, while SW44, SW69, SW73, and SW83 were closely related to the *B. stercoris* strain JCM 30051 (Figure 1). These 16S rRNA gene sequences have been submitted to GenBank and have the accession numbers PV905101 to PV905106.

3.2 Impact of bacterial cells and secondary metabolites on nematode mortality

To assess the direct impact of living rhizobacteria on *H. schachtii* J2 mortality, freshly hatched J2 were incubated for 96 hours in a bacterial suspension (initial OD_{600} = 0.1, 1/3 strength LB broth). The number of dead and alive nematodes was counted after 96 h and the mortality rate was calculated. All treatments showed significantly increased mortality compared to the control. Control mortality was approximately 25%, while the mortality in treatments ranged from approximately 39% (*B. subtilis* SW53) to 48% (*B. stercoris* SW44 and SW74) based on the arithmetic mean (Figure 2A). This suggests that LBC has a nematicidal effect. Similarly, the effect of both undiluted and 50% diluted CFS of each rhizobacteria on the mortality of *H.* schachtii J2 was assessed in an *in vitro* experiment. Both undiluted CFS and diluted CFS increased the mortality rate significantly. Undiluted CFS caused higher mortality in J2, ranging from 47% (*B. subtilis* SW53) to 64% (*B. subtilis* SW33) compared to the control (24%). Diluting the CFS by 50% reduced J2 mortality, ranging from 32% (*B. subtilis* SW53) to 38% (*B. subtilis* SW33), but it was still higher than the control (21%) (Figure 2B). This suggests that CFS has a nematicidal effect, and the mortality rate is dose-dependent.

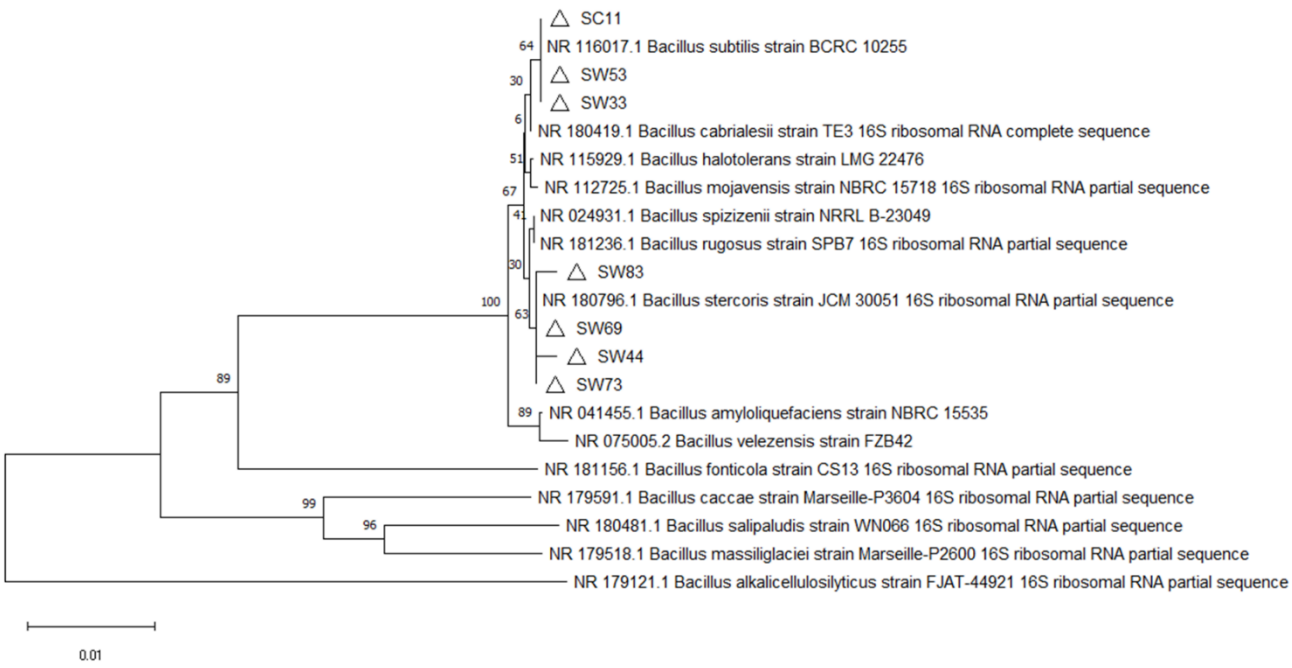


Figure 1. Phylogenetic tree of *Bacillus* isolates SC11, SW33, SW53, SW44, SW69, SW73, and SW83 with focus on type strain comparison based on16S rRNA gene sequence. The phylogenetic tree was constructed using the neighbor-joining method in MEGA11 software. The bootstrap values are shown at the branch points.

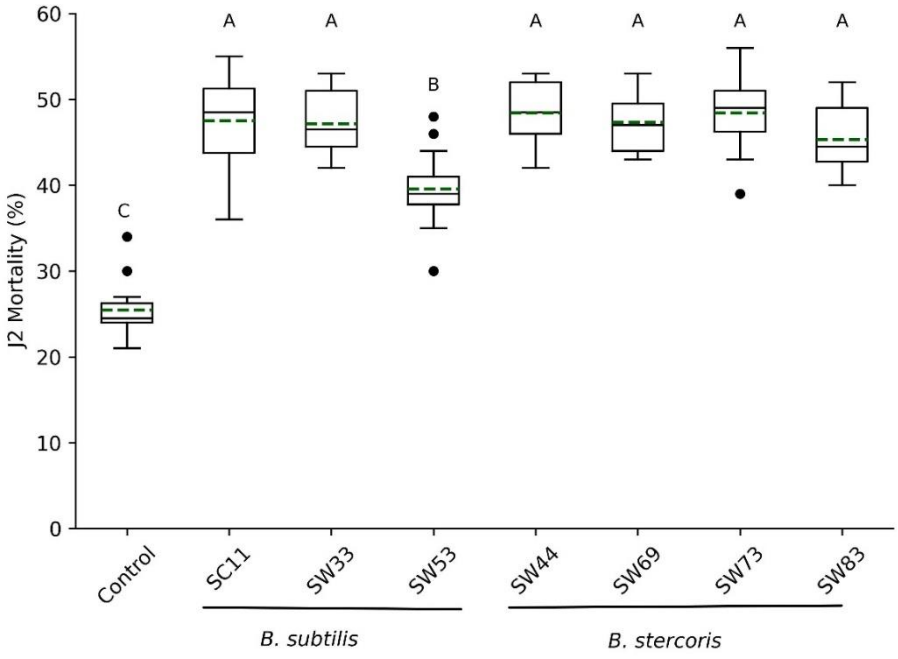
3.3 Impact of rhizobacteria on nematode parasitism and plant growth

The impact of each rhizobacterium on *H. schachtii* parasitism was assessed in an *in vivo* experiment on *A. thaliana*. The results revealed that the treatments significantly reduced the numbers of female, male, and total nematodes compared to the control. The number of females decreased by 27% (*B. subtilis* SW53) to 69% (*B. stercoris* SW83), while the number of males decreased by 34% (*B. subtilis* SW53) to 49% (*B. subtilis* SW83) (Figure 3A). Similarly, the total number of nematodes also reduced by 30% (*B. subtilis* SW53) to 58% (*B. stercoris* SW83) (Figure 3B). Furthermore, the size of the females at 14 dpi was significantly smaller in all treatments, with a reduction by ~12% (*B. subtilis* SC11) to 18% (*B. stercoris* SW83) compared to the size of the females in the control (Figure 3C).

In addition to the effect of the rhizobacteria on nematode parasitism, their impact on host plant development under infection pressure was evaluated. The aboveground-related parameters (shoot

diameter and weight, and number of leaves) and underground-related parameters (total root length) were measured 28 days post-sowing. Most bacterial treatments affected the shoot diameter and shoot fresh weight positively compared to the control. The shoot diameter increased significantly in *B. subtilis* isolates SC11 and SW53 by approximately 24.6% and 29.3% respectively, and in SW69, SW73 and SW83 by approximately 31.4%, 22.1% and 31.4 % respectively (Figure 4A) while shoot fresh weight increased significantly in *B. subtilis* isolates SC11 and SW53 by approximately 49.7%, 36.5% respectively and in *B. stercoris* isolates SW73, and SW83 by approximately 38.6% and 45.9% respectively compared to control (Figure 4B). Similarly, the number of leaves increased significantly in *B. subtilis* isolates SC11 and SW53 by approximately 14% and 30% respectively and *B. stercoris* isolates SW69, SW83 by approximately 19% and SW83 by approximately 17% compared to the control (Figure 4C) and total root length was significantly increased in *B. subtilis* SC11 and *B. stercoris* SW73 by approximately 24% and 31% (Figure 4D).

A



B

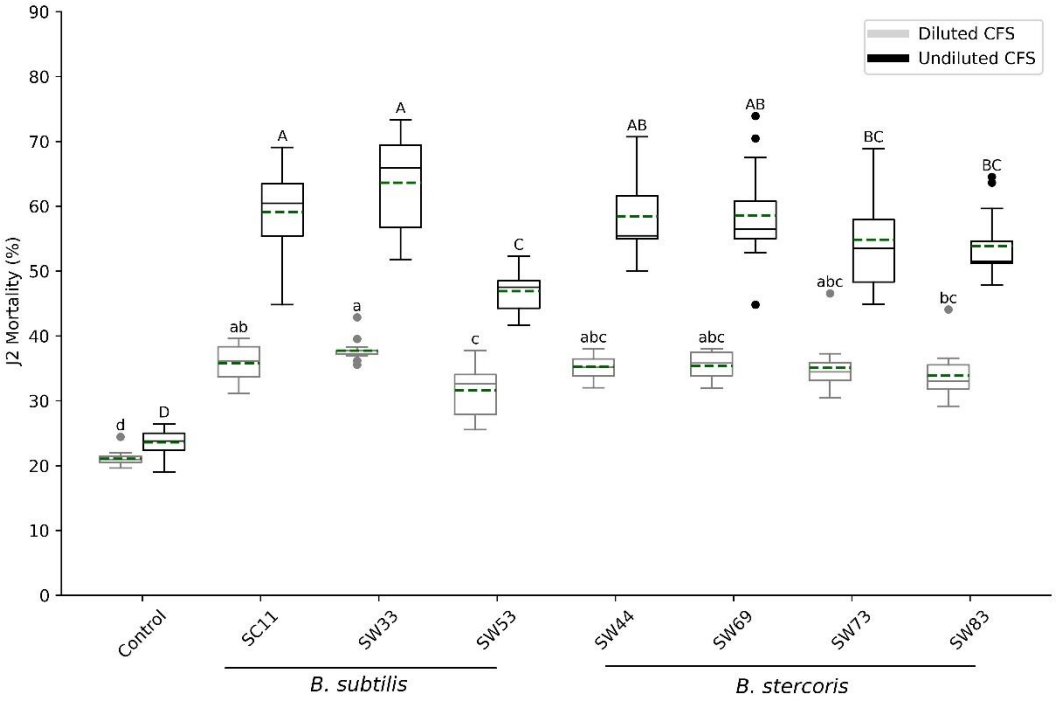


Figure 2. Nematicidal activity of *Bacillus* isolates (**A**) Effect of living bacteria cells (LBC) on *Heterodera schachtii* J2; **(B)** Effect of cell-free supernatant (CFS) on *H. schachtii* J2. Results are expressed in a boxplot as median (black line) with 25 %- and 75 %-quartile additionally including the arithmetic mean (green dashed line) of three independent biological replicates with total n = 12; Different letters indicate statistically significant differences among treatments according to Turkey's HSD test ($p < 0.05$).

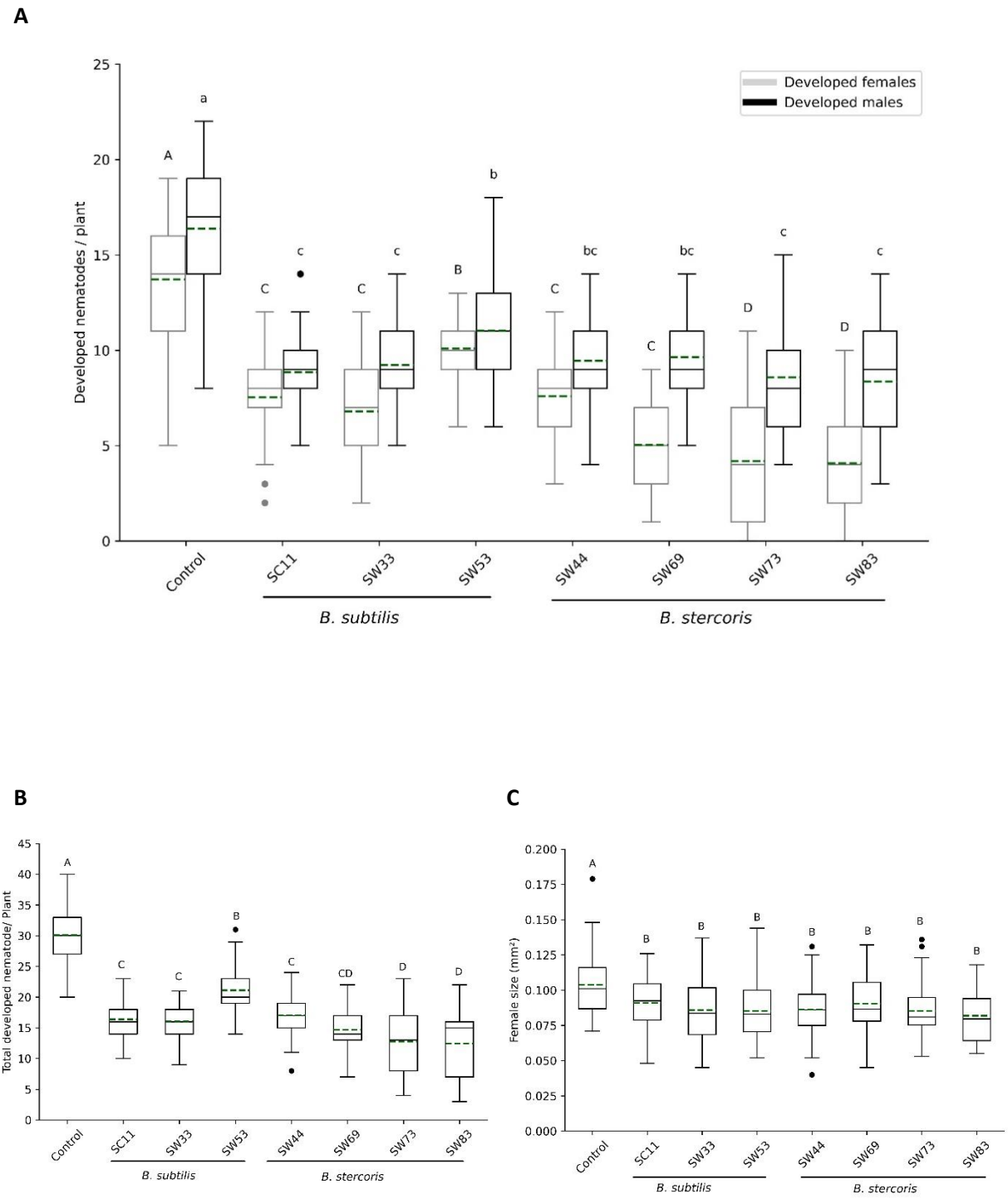


Figure 3. Effect of living bacterial cells (LBC) of *Bacillus* isolates on the development of adults of *Heterodera schachtii* in *Arabidopsis thaliana* roots. **(A)** Number of developed female and male nematodes per plant at 12 days post inoculation (dpi) in the presence of *Bacillus* LBC; **(B)** Number of total developed nematodes per plant at 12 dpi; **(C)** size of female per plant at 14 dpi. Results are expressed as the mean (green dashed line) and median (black line) with 25 %- and 75 %-quartile of

three independent biological replicates with total 45 ≤ n ≤ 51; Different letters indicate statistically significant differences among treatments according to Turkey's HSD test ($p < 0.05$).

3.3.1 Impact of rhizobacteria on nematode invasion

Since we found that a root treatment with the isolates reduced the number of adult nematodes compared to the control, we aimed to determine at what stage during plant-nematode interaction the antagonistic effect of the bacterial isolates becomes apparent. Therefore, we first investigated the impact of the rhizobacteria on J2 invasion into the plant root tissue. Already at 1 dpi, the invasion rate decreased significantly in all treatments compared to the control. It remained significantly lower also at 2 dpi and 3 dpi. In the control group, the *H. schachtii* invasion rate reached approximately 46% at 3 dpi, whereas in the treatment groups, the invasion rate ranged from approximately 25% (*B. subtilis* SC11) to 32% (*B. stercoris* SW83) which equals a maximum reduction of approximately 46% (*B. subtilis* SC11) compared to the number of successful invasion events in the control (Figure 5).

At 12 dpi, males and females were counted, and the ratio of adult nematodes (J4) (12 dpi) and invaded J2 (3 dpi) was calculated (Figure 6). We found that J2 development into males and females in the untreated control was 61% and that it was significantly reduced by approximately 26%, 34%, and 36% base on arithmetic mean in the treatments with *B. subtilis* SW33, *B. stercoris* SW73, and *B. stercoris* SW83, respectively compared to the control. Except for *B. subtilis* SW53, all other isolates in tendency negatively affect the nematode development rate.

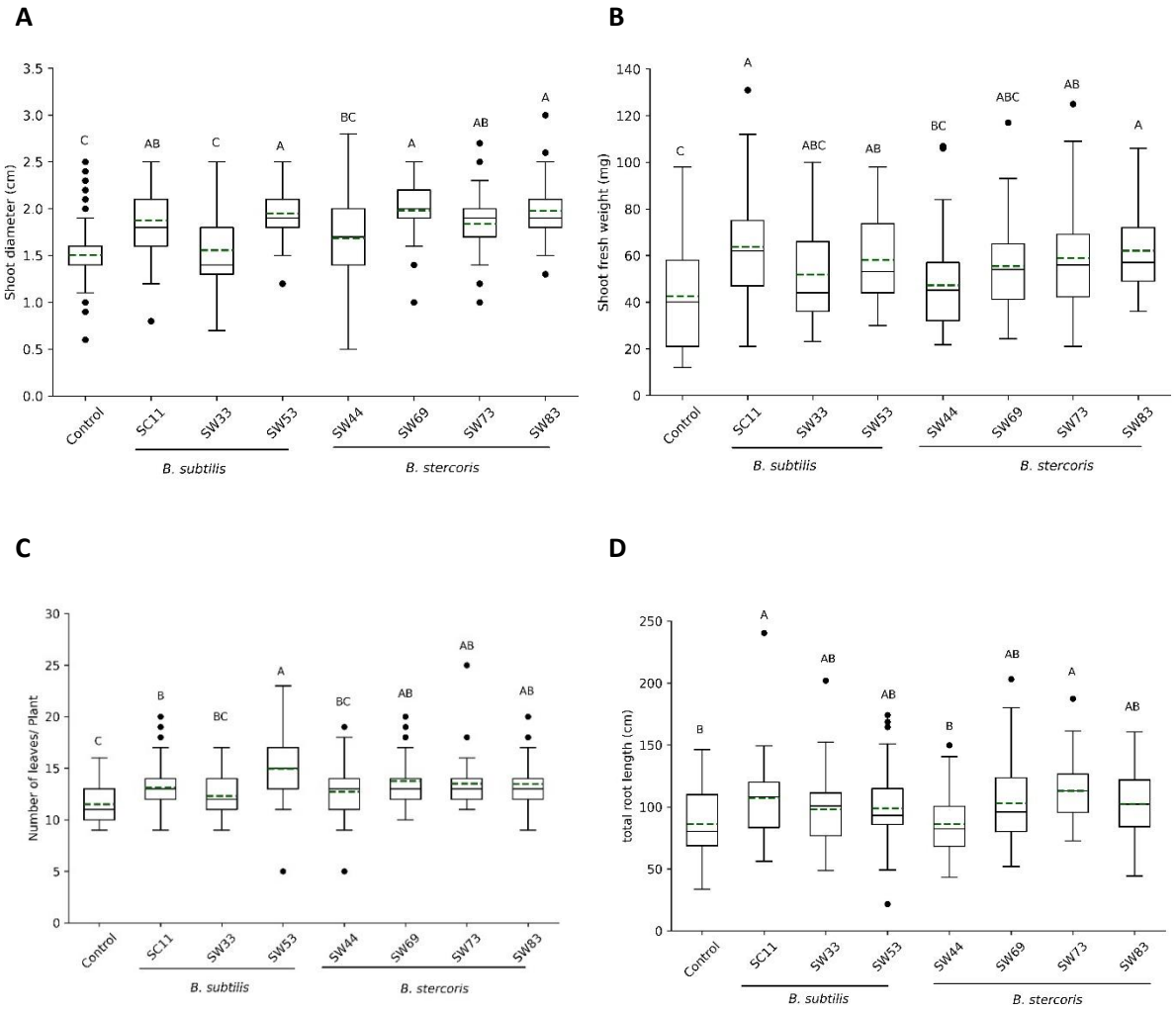


Figure 4. Effect of living bacterial cells (LBC) of *Bacillus* isolates on *Arabidopsis thaliana* development. (A) Shoot diameter; **(B)** Shoot fresh weight; **(C)** Number of leaves **(D)**, Total root length. Results are expressed as the mean (green dashed line) and median (black line) with 25 %- and 75 %-quartile of three independent biological replicates with total n = 45; Different letters indicate statistically significant differences among treatments according to Turkey's HSD test ($p < 0.05$).

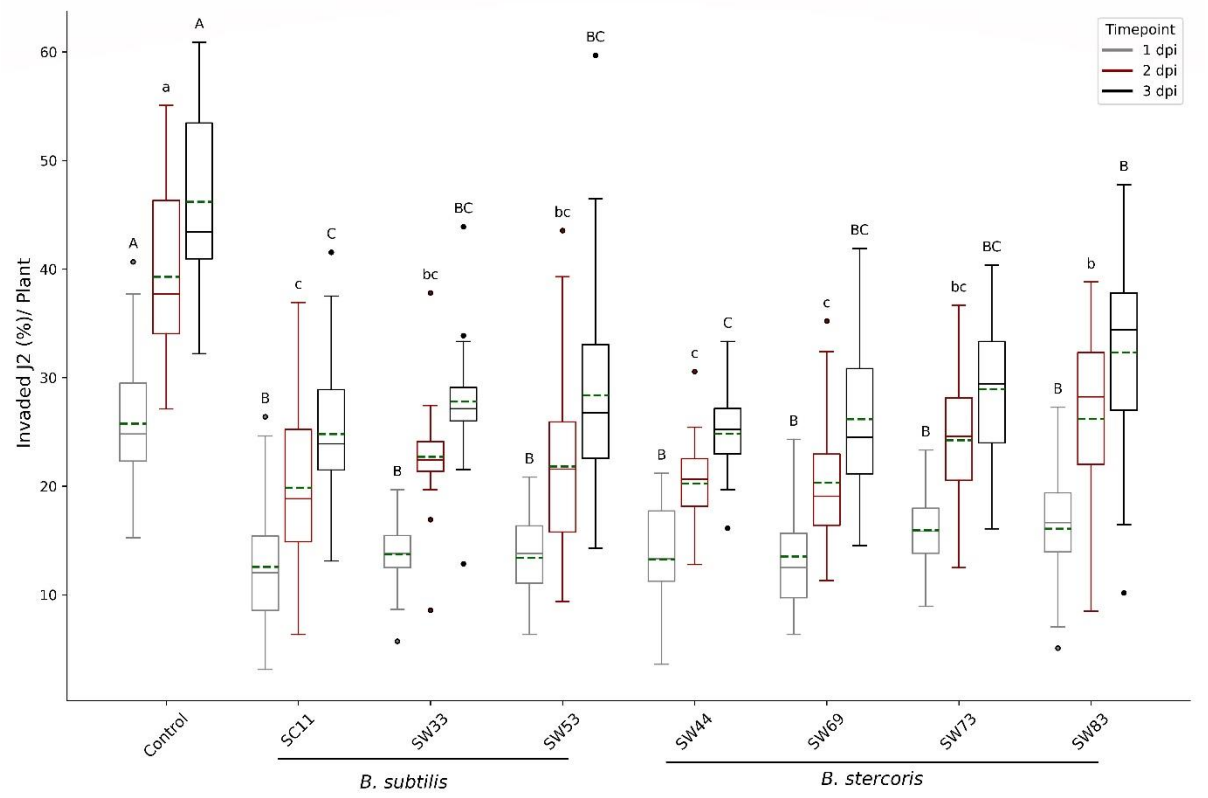


Figure 5. Effect of living bacterial cells (LBC) of *Bacillus* isolates on the invasion rate of *Heterodera schachtii* J2 into the roots of *Arabidopsis thaliana* 1, 2, and 3 days post inoculation (dpi). Results are expressed as the mean (green dashed line) and median (black line) with 25 %- and 75 %-quartile of three independent biological replicates with total n = 24; Different letters indicate statistically significant differences among treatments according to Turkey's HSD test ($p < 0.05$).

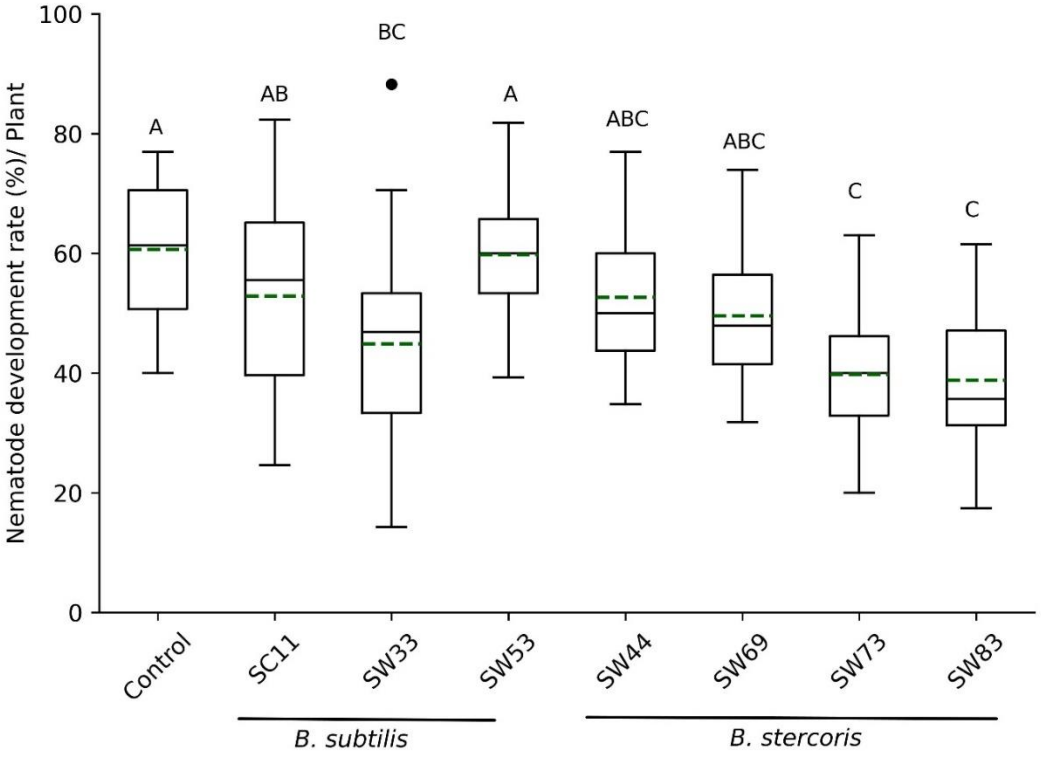


Figure 6. Effect of living bacterial cells (LBC) *Bacillus* isolates, on the ratio of development of *Heterodera schachtii* J2 into the adult male and female at *Arabidopsis thaliana*. Results are expressed as the mean (green dashed line) and median (black line) with 25 %- and 75 %-quartile of three independent biological replicates with total n = 24; Different letters indicate statistically significant differences among treatments according to Turkey's HSD test ($p < 0.05$).

3.4 Biochemical characterization

The ability of rhizobacteria to produce chitinase, siderophore, HCN and IAA and to solubilize P was assessed. Aslam et al. 2025b already reported that *B. subtilis* SC11 was producing chitinase and siderophore. Chitinase enzyme production could also be confirmed for the three *B. stercoris* isolates SW44, SW73, and SW83, while all six isolates also produced siderophores, approximately 18 - 27psu. Aslam et al. 2025b reported that *B. subtilis* SC11 was performing P-solubilization. This could also be confirmed for *B. subtilis* SW33 and *B. stercoris* SW44. None of the isolates produced IAA (Table 1). As already reported by Aslam et al. 2025a, none of the isolates produced protease and only one isolate, *B. subtilis* SW33, produced HCN. In the current investigation, HCN production of SW33 was quantified to be approximately 47µg/mL (Table 1) (Supplementary Figure 1).

Table 1. Biochemical characterization of *Bacillus* isolates: ++ strong activity (width of clearance zone around bacteria colony 1.1 to 2 cm); + low activity (width of clearance zone around bacteria colony up to 1cm); - no activity. All experiments were performed in three independent biological replicates, each with three technical replicates. Results for Chitinase, siderophore, IAA and P-solubilization for *B. subtilis* SC11 are already published (Aslam et al., 2025b) and displayed here for better comparison.

Isolates		**Chitinase Activity**	**Siderophore production** (psu)	**IAA production**	**P-solubilization**	**HCN** (µg/mL)
Bacillus subtilis	SC11	**+**	34 ± 1.4^{A}	-	+	-
	SW33	**-**	24 ± 1.5^{BC}	-	**+**	47 ± 7.4
	SW53	**-**	20 ± 2.4^{C}	-	-	-
Bacillus stercoris	SW44	**++**	18 ± 2.1^{C}	-	**+**	-
	SW69	**-**	20 ± 3.2^{C}	-	-	-
	SW73	**++**	24 ± 2.4^{BC}	-	-	-
	SW83	**++**	27 ± 3.1^{B}	-	-	-

3.5 Biofilm formation

We observed that all seven rhizobacteria produced biofilm. Biofilm formation was quantified by measuring the OD at 590 nm and revealed that the absorbance in the treatment groups was significantly higher than in the control (LB broth supplemented with 2% glucose). *B. subtilis* SC11 and *B. stercoris* SW44 exhibited the highest OD_{590} of approximately 2.7 (Figure 7).

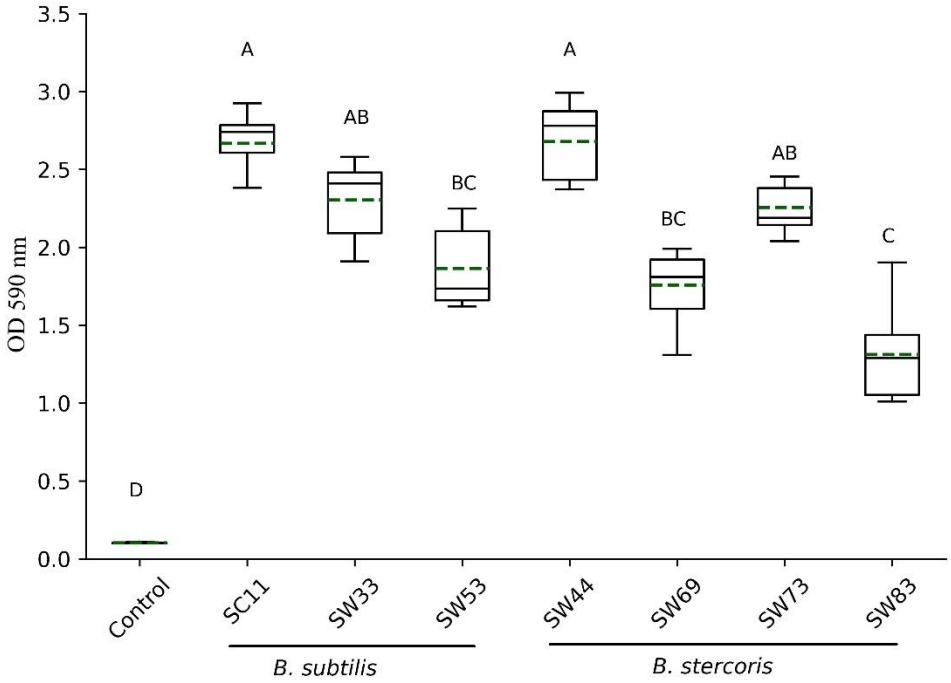


Figure 7. Biofilm mass quantification for *Bacillus* isolates measured in 200 µl at 590 nm. Results are expressed as the mean (green dashed line) and median (black line) with 25 %- and 75 %-quartile of three independent biological replicates with total n = 9; Different letters indicate statistically significant differences among treatments according to Turkey's HSD test ($p < 0.05$).

4. Discussion

Our findings demonstrate that all seven rhizobacteria exhibited strong antagonistic effects against *H. schachtii*, and isolates SC11, SW53, SW69, SW73, and SW83 significantly enhanced plant growth under infection pressure, supporting the potential of PGPR as sustainable plant growth-promoting and biocontrol agents in general. Phylogenetic analysis revealed that isolates SC11, SW33, and SW53 belong to the species *B. subtilis,* and SW44, SW69, SW73, and SW83 belong to the species *B. stercoris*. In our research, all culturable and sequenced rhizobacteria belonged to *Bacillus*, which may have been limited by our exclusive use of LB media for isolation. The fast-growing nature of *Bacillus* on LB could mask the presence of other rhizobacteria with different nutritional needs. Future studies should incorporate a wider range of media to capture a more comprehensive picture of the rhizobacterial population.

Bacillus spp. have been reported to promote plant growth and combat with plant pathogens acting as biocontrol agent (Shahid et al., 2021). Different *Bacillus* spp. including *B. subtilis* were already described to be effective in the management of PPN (Vasantha-Srinivasan et al., 2025). The dual

functionality of plant growth promotion and pathogen suppression has already been reported for certain rhizobacteria isolates (Cetintas et al., 2018). The mechanisms underlying these effects may include improved nutrient uptake (Shabaan et al., 2022), hydrolytic enzymes production (Jadhav et al., 2017), siderophore production (Verma et al., 2019), HCN production (Khaing et al., 2021), phytohormones production including IAA (Gao et al., 2023), antibiosis (Mandaliya et al., 2025), root colonization, or induced systemic resistance (Meena et al., 2020).

In our study, the tested isolates enhanced plant growth increasing shoot biomass up to 49.7% (*B. subtilis* SC11), root length up to 31% (*B. stercoris* SW73), and leaf number up to 29.9% (*B. subtilis* SW53) under PPN infection pressure compared to the infected control. The observed improvement in plant phenotype under infection pressure can be attributed to a combination of factors. The reduction in infection severity directly alleviates stress on the plant, allowing for a greater allocation of resources towards growth and development. Furthermore, the plant PGPR may induce systemic resistance , priming the plant's defense mechanisms and further contributing to the observed positive effects (Monson et al., 2022).

Previous studies reported that P availability is often a limiting factor in plant growth, thus, P-solubilization capability can be crucial for enhancing nutrient uptake and improving overall plant development (Bechtaoui et al., 2024). In our study, *B. subtilis* SC11 and *B. stercoris* isolates SW33 and SW44 exhibit significant P-solubilization activity, highlighting their potential as PGPR.

IAA is a phytohormone produced by many PGPR. It plays a crucial role in plant development, influencing processes such as cell division, root elongation, and overall plant growth. PGPR-produced IAA can enhance root development, leading to increased nutrient uptake and improved plant vigor (Maheshwari et al., 2015). Lwin et al. (2012) highlighted the variability in IAA production among different rhizobacterial species and strains, and demonstrated the potential of selected isolates to promote maize growth in pot trials, even though they didn't significantly improve seed germination (Lwin et al., 2012). In our study, although *Bacillus* isolates did not exhibit detectable IAA production, we observed a significant positive effect on plant growth under nematode infection. This suggests that other PGPR mechanisms, independent of IAA, were responsible for the observed effects. These mechanisms may include the production of nematicidal compounds, the induction of systemic resistance, or the enhancement of nutrient availability in the rhizosphere. It is also possible that the isolates produced low levels of IAA, below the detection limit of our colorimetric assay, but still sufficient to elicit a plant response (Duca et al., 2014). Further research is needed to elucidate the specific mechanisms by which these isolates promote plant growth and suppress nematode activity.

Siderophores are iron-chelating compounds produced by various microorganisms, including beneficial PGPR. They contribute to biocontrol by limiting iron availability for plant pathogens. PGPR siderophores scavenge iron, forming stable complexes that are taken up by the bacteria, depriving pathogens of this essential nutrient. This can inhibit pathogen growth and virulence through iron sequestration, competition for iron, and sometimes direct antimicrobial activity. The effectiveness of siderophore-mediated biocontrol depends on factors like siderophore type and quantity, pathogen iron requirements, and environmental conditions (Fgaier and Eberl, 2011). Proença et al. (2019) repoted that siderophores from certain bacterial species were chelating iron and leading to the death of *Caenorhabditis elegans* (Proença et al., 2019).In our study all isolates are producing siderophore and their CFS exhibited nematicidal activity, indicating a strong association between siderophore production and nematode suppression. Our isolates also can play a role in nematode suppression when used in greenhouse or field conditions. In the presented *in vitro* assays, siderophore production likely does not affect the results as iron should not be a limiting factor in the knop medium. Although the role of siderophores in nematode control is less studied than their role in fungal disease suppression, the available evidence suggests that siderophore-producing bacteria can be valuable tools for managing PPNs in agriculture.

In vitro investigation revealed that the mortality rate of *H. schachtii* J2 increased approximately 1.9-fold compared to the control after being exposed to LBC for 96 h. Undiluted CFS increased J2 mortality by 2.7-fold (*B. subtilis* SW33 and SC11) compared to the control. A 50% dilution of the CFS resulted in an approximate 1.8-fold (*B. subtilis* SW33) increase in J2 mortality. These results indicate that the nematicidal activity of the CFS is concentration-dependent. Previous studies have reported that chitinases can degrade the nematode cuticle, leading to increased mortality (Chen et al., 2015). The presence of chitinase activity in some of our isolates, particularly *B. subtilis* SC11 and *B. stercoris* isolates SW44, SW73, and SW83, may also have contributed to the determined nematicidal effect. In another study, it was reported that some *Bacillus* species produce lytic enzymes, volatile organic compounds, and antibiotics that disrupt nematode physiology (Vasantha-Srinivasan et al., 2025) further supporting the nematicidal properties observed for the *B. subtilis* and *B. stercoris* strains in this study. The comparatively lower nematicidal activity of the LBC may be attributed to the growth conditions. Using 1/3 strength LB broth, intended to prevent bacterial overgrowth, decelerated bacterial growth and thus, compromised the production of secondary metabolites. However, a lower concentration of bacterial metabolites was clearly less effective to induce nematode mortality. The data indicate that the nematicidal effect of the tested *Bacillus* spp. can be rather linked to their exo-secretions, than to direct interaction between both organisms as observed in other studies (Topalović et al., 2019; Topalović et al., 2020).

Beshah et al. repoted that the primary role of HCN in biocontrol is its toxicity to many plant pathogens, including fungi, bacteria, and nematodes. HCN disrupts cellular respiration by inhibiting cytochrome c oxidase, a key enzyme in the electron transport chain. This inhibition leads to energy depletion and ultimately cell death in the target organism (Beshah et al., 2024). In our study the isolate *B. subtilis* SW33 was producing approximately 47 µg/mL HCN. This HCN might be a part of the nematicidal activity of SW33.

Proteases, or proteolytic enzymes, also play a significant role in the nematicidal activity. Proteases contribute to nematicidal activity by directly attacking nematode structures or interfering with their physiological processes. These enzymes are produced by various bacteria with known nematicidal capabilities (Lian et al., 2007). Lian et al. (2020) highlighted that proteases play a role in the suppression of PPN. In our study, none of the investigated *Bacillus* isolates produced proteases. Even though they did not exhibit protease production *in vitro* but both LBC and CFS increase mortality in *in vitro* and suppress *H. schachtii* infection *in vivo* indicating that our isolates are employing alternative mechanisms to suppress the nematode infection.

In vivo experiments demonstrated that all tested *Bacillus* isolates significantly reduced nematode infection rates and development in *A. thaliana*. *B. stercoris* SW83 displayed the highest efficacy, reducing female nematodes by approximately 69%, total nematode numbers by approximately 58%, and the size of developed females by approximately 18% compared to the untreated control. Further investigation of the nematode interaction with *A. thaliana* and the bacteria revealed that isolates *B. subtilis* SC11 and *B. stercoris* SW44 strongly interfered with nematode invasion into *A. thaliana* roots. Both isolates were producing the highest amount of biofilm among all seven isolates as observed in the *in vitro* biofilm formation assay. Additionally, on *A. thaliana* roots inoculated with SC11 and SW44, a biofilm could be seen along plant roots using a stereomicroscope (Leica, Germany) (supplementary figure 2). Bacteria colonize plant root and form a biofilm matrix that can act as a physical barrier, preventing pathogens from accessing the plant root surface and establishing an infection (Knights et al., 2021). PGPR within the biofilm can outcompete pathogens for resources such as nutrients and space, limiting pathogen growth and colonization (Seneviratne et al., 2011). Our study also supports this fact as it was observed that the two isolates *B. subtilis* SC11 and *B. stercoris* SW44 with higher biofilm production showed a maximum reduction of approximately 46% of the invasion of J2 *H. schachtii* into the plant root.

The two *B. stercoris* isolates SW73 and SW83 strongly interfered with the development of J2 into male or female adults after invasion of J2 into roots. The observed decrease by 18% (*B. stercoris* SW83) in

female size shows that the infection is not only impaired during early stages, but at least from the invasion to the development of the adults themselves. Our findings align with Huang et al. (2021) where *B. firmus* I-1582 colonization of plant roots led to decreased *H. schachtii* parasitism and reduction in female size. There are various studies with the evidences that rhizobacteria are antagonists of PPN, for example, one study reported that a *B.* cereus strain that was producing chitinase and protease reduced female development of *H avenae* (Ahmed et al., 2019). Xiang et al. (2017) reported that *B. velezensis* Bve2, *B. mojavensis* Bmo3, and Mixture 1 (Bve2 + Bal13) show potential for *H. glycines* management in soybean (Xiang et al., 2017). Singh and Saddiqi (2010) reported that isolates Pa8 and Pa9 of *Pseudomonas aeruginosa*, which effectively colonize plant roots and produce high quantity of siderophores, HCN, and IAA, exhibit strong biocontrol potential against root-knot nematodes in tomato compared to other isolates of *P aeruginosa* used in same study (Singh and Siddiqui, 2010).

Our study provides strong evidence that *B. subtilis* and *B. stercoris* can effectively suppress *H. schachtii* through direct nematicidal activity and competition for resources. Moreover, the isolates positively affect plant development under PPN infection pressure. Furthermore, we show for the first time that *B. stercoris* has nematicidal properties since this bacterium has not yet been reported to antagonize PPN.

Future research should focus on identifying specific bioactive compounds responsible for the nematicidal effects, optimizing bacterial application methods, and assessing their field efficacy across different crops. Additionally, investigating interactions between these *Bacillus* isolates and other soil microbes could provide valuable insights into their role within the broader soil ecosystem.

5. Conclusion

This study demonstrates that all tested *Bacillus* isolates (SC11, SW333, SW44, SW53, SW69, SW73, SW83) effectively antagonized *H. schachtii*. The top three best-performing isolates (SW83, SW73, SW69) belong to *B. stercoris* and cause the highest impact on *H. schachtii* parasitism. Notably, isolates *B. stercoris* SW69, SW73, and *B. subtilis* SC11, SW53 also promoted plant growth during nematode infection pressure. While *B. stercoris* is known for its biocontrol potential against various plant pathogens, this is the first report of its efficacy against a PPN. As biocontrol agents, *B. subtilis* and *B. stercoris* offer several advantages, including sustainability, environmental safety, and potential plant growth promotion. These findings pave the way for further greenhouse and field experiments to validate its antagonistic potential in future studies. If consistent results are observed in future

greenhouse or field experiments, these isolates could serve as effective, eco-friendly biocontrol agents for practical agricultural applications.

6. Author Contributions

Conceptualization: SA, MB, FMWG, ASSS; Investigation: SA; Data curation and methodology: SA; Project administration: MB, FMWG, ASSS; Resources: MB, FMWG; Statistical analysis of experimental data: MJ; Supervision and validation: SB, ASSS, MB, FMWG; Writing original draft: SA; Writing - review and editing of the original draft: ASSS, FMWG, SB; Writing - review and editing of final draft: MB, MJ. All authors have read and agreed to the published version of the manuscript.

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9. Declaration of competing interest

The authors state that they have no conflicts of interest, whether financial or personal, that could be perceived as influencing the presented work.

10. References

Ahmed, S., Liu, Q., and Jian, H. (2019). Bacillus cereus a potential strain infested cereal cyst nematode (Heterodera avenae). *Pak. J. Nematol* 37**,** 53-61. doi: 10.18681/pjn.v37.i01.p53-61.

Ankit, Saha, L., Kishor, V., and Bauddh, K. (2020). "Impacts of synthetic pesticides on soil health and non-targeted flora and fauna," in *Ecological and practical applications for sustainable agriculture,* ed. S.K. Kuldeep Bauddh , Rana Pratap Singh, John Korstad. (Singapore: Springer), 65-88.

Aslam, S., Babar, M., Shabir, G., Naqqash, T., and Grundler, F.M.W. (2025a). Rhizobacterial diversity exhibiting biotic stress tolerance in association with wheat-cotton crop rotation: Implications for plant–microbe interactions and agroecosystem resilience. *Biologia Futura*. doi: 10.1007/s42977-025-00269-z.

Aslam, S., Baber, M., Naqqash, T., Javed, M., Bredenbruch, S., Grundler, F.M.W, et al. (2025b). Rhizospheric *Bacillus* isolates control Fusarium wilt on cotton and enhance plant biomass and root development. *Frontiers in Microbiology* 16**,** 1580937. doi: 10.3389/fmicb.2025.1580937.

Bechtaoui, N., Raklami, A., Tahiri, A.-i., Benidire, L., Göttfert, M., and Oufdou, K. (2024). Phosphate-solubilizing rhizobacteria and their effects on the growth and phosphorus uptake by wheat plants. *Journal of Plant Nutrition* 47(17)**,** 2811-2823. doi: 10.1080/01904167.2024.2369069.

Beshah, A., Muleta, D., Legese, G., and Assefa, F. (2024). Exploring stress-tolerant plant growth-promoting rhizobacteria from groundnut rhizosphere soil in semi-arid regions of Ethiopia. *Plant Signaling & Behavior* 19(1)**,** 2365574. doi: 10.1080/15592324.2024.2365574.

Cetintas, R., Kusek, M., and Fateh, S.A. (2018). Effect of some plant growth-promoting rhizobacteria strains on root-knot nematode, Meloidogyne incognita, on tomatoes. *Egyptian Journal of Biological Pest Control* 28**,** 1-5. doi: 10.1186/s41938-017-0008-x.

Chaiharn, C., Sujada, N., Pathom-aree, W., and Lumyong, S. (2019). Biological control of Rigidoporus microporus the casue of white root disease in rubber using PGPRs in vivo. *Chiang Mai J. Sci* 46**,** 850-866.

Chen, L., Jiang, H., Cheng, Q., Chen, J., Wu, G., Kumar, A., et al. (2015). Enhanced nematicidal potential of the chitinase pachi from *Pseudomonas aeruginosa* in association with Cry21Aa. *Scientific Reports* 5(1)**,** 14395. doi: 10.1038/srep14395.

Duca, D., Lorv, J., Patten, C.L., Rose, D., and Glick, B.R. (2014). Indole-3-acetic acid in plant–microbe interactions. *Antonie Van Leeuwenhoek* 106**,** 85-125. doi: 10.1007/s10482-013-0095-y.

Elango, K., Sobhana, E., Sujithra, P., Bharath, D., and Ahuja, A. (2020). Traditional agricultural practices as a tool for management of insects and nematode pests of crops: An overview. *Journal of Entomology and Zoology Studies* 8(3)**,** 237-245.

Fgaier, H., and Eberl, H.J. (2011). Antagonistic control of microbial pathogens under iron limitations by siderophore producing bacteria in a chemostat setup. *Journal of theoretical biology* 273(1)**,** 103-114. doi: 10.1016/j.jtbi.2010.12.034.

Gao, X., Liu, Y., Liu, C., Guo, C., Zhang, Y., Ma, C., et al. (2023). Individual and combined effects of arbuscular mycorrhizal fungi and phytohormones on the growth and physiobiochemical characteristics of tea cutting seedlings. *Frontiers in Plant Science* 14**,** 1140267. doi: 10.3389/fpls.2023.1140267.

He, Y., Wang, R., Zhao, H., Ren, Y., Agarwal, M., Zheng, D., et al. (2022). Predicting potential global distribution and risk regions for potato cyst nematodes (Globodera rostochiensis and Globodera pallida). *Scientific reports* 12(1)**,** 21843. doi: 10.1038/s41598-022-26443-0.

Huang, M., Bulut, A., Shrestha, B., Matera, C., Grundler, F.M.W, and Schleker, A.S.S. (2021). *Bacillus firmus* I-1582 promotes plant growth and impairs infection and development of the cyst nematode Heterodera schachtii over two generations. *Scientific reports* 11(1)**,** 14114. doi: 10.1038/s41598-021-93567-0.

Jadhav, H., Shaikh, S., and Sayyed, R. (2017). "Role of hydrolytic enzymes of rhizoflora in biocontrol of fungal phytopathogens: an overview," in *Rhizotrophs: Plant growth promotion to bioremediation,* ed. S. Mehnaz. (Singapore: Springer), 183-203.

Jalal, A., Oliveira, C.E.d.S., Gato, I.M.B., Moreira, V.d.A., de Lima, B.H., Bastos, A.d.C., et al. (2024). Interaction of Mineral Nutrients and Plant Growth-Promoting Microbes for Biofortification of Different Cropping Systems. *Journal of Plant Growth Regulation***,** 1-17. doi: 10.1007/s00344-024-11380-1.

Jeevan, H., Patidar, R.K., Kadam, V., Dutta, P., Nongbri, E., Gouda, M.R., et al. (2025). Implementing sustainable practices to combat root knot nematode infestation in tomato farming from Meghalaya. *Scientific Reports* 15(1)**,** 602. doi: 10.1038/s41598-024-84292-5.

Jian, Q., Zhang, T., Wang, Y., Guan, L., Li, L., Wu, L., et al. (2024). Biocontrol potential of plant growth-promoting rhizobacteria against plant disease and insect pest. *Antonie Van Leeuwenhoek* 117(1)**,** 92. doi: 10.1007/s10482-024-01975-9.

Kadlecová, A., Hendrychová, R., Jirsa, T., Čermák, V., Huang, M., Grundler, F.M.W, et al. (2024). Advanced screening methods for assessing motility and hatching in plant-parasitic nematodes. *Plant Methods* 20(1)**,** 108. doi: 10.1186/s13007-024-01233-z.

Khaing, A., Theint, W., Thi, O., and Fu, P. (2021). Antagonistic activity of indigenous rhizobacteria through biosynthesis of indole-3-acetic acid (IAA), hydrogen cyanide (HCN), and siderophores. *Aust. J. Biotechnol. Bioeng* 8(1110)**,** 10.26420.

Ki, J.-S., Zhang, W., and Qian, P.-Y. (2009). Discovery of marine *Bacillus* species by 16S rRNA and rpoB comparisons and their usefulness for species identification. *Journal of Microbiological Methods* 77(1)**,** 48-57. doi: 10.1016/j.mimet.2009.01.003.

Knights, H.E., Jorrin, B., Haskett, T.L., and Poole, P.S. (2021). Deciphering bacterial mechanisms of root colonization. *Environmental Microbiology Reports* 13(4)**,** 428-444. doi: 10.1111/1758-2229.12934.

Lian, L., Tian, B., Xiong, R., Zhu, M., Xu, J., and Zhang, K. (2007). Proteases from *Bacillus*: a new insight into the mechanism of action for rhizobacterial suppression of nematode populations. *Letters in Applied Microbiology* 45(3)**,** 262-269. doi: 10.1111/j.1472-765X.2007.02184.x.

Lwin, K.M., Myint, M.M., Tar, T., and Aung, W.Z.M. (2012). Isolation of plant hormone (indole-3-acetic acid-IAA) producing rhizobacteria and study on their effects on maize seedling. *Engineering Journal* 16(5)**,** 137-144. doi: 10.4186/ej.2012.16.5.137.

Maheshwari, D.K., Dheeman, S., and Agarwal, M. (2015). "Phytohormone-producing PGPR for sustainable agriculture," in *Bacterial metabolites in sustainable agroecosystem,* ed. D.K. Maheshwari. (Cham: Springer), 159-182.

Mandaliya, V.B., Mangroliya, U.C., and Patel, H.R. (2025). "Broad-Spectrum Disease Resistance in Plants by Bio-Control Agents," in *Bio-control Agents for Sustainable Agriculture: Diversity, Mechanisms and Applications,* ed. S.d.l.S.V. Debasis Mitra , Anju Rani · Beatriz Elena Guerra Sierra ,Snežana Andjelković (Singapore: Springer), 293-309.

Matera, C., Grundler, F.M.W, and Schleker, A.S.S. (2021). Sublethal fluazaindolizine doses inhibit development of the cyst nematode *Heterodera schachtii* during sedentary parasitism. *Pest Management Science* 77(7)**,** 3571-3580.

Meena, M., Swapnil, P., Divyanshu, K., Kumar, S., Harish, Tripathi, Y.N., et al. (2020). PGPR-mediated induction of systemic resistance and physiochemical alterations in plants against the pathogens: Current perspectives. *Journal of Basic Microbiology* 60(10)**,** 828-861. doi: 10.1002/jobm.202000370.

Messa, V., Nunes, J., and Mattei, D. (2019). Seed Treatment with *Bacillus amyloliquefaciens* for the control of Meloidogyne javanica" in vivo" bean culture and its direct effect on the motility, mortality and hatching of M. javanica" in vitro". *Agronomy Science and Biotechnology* 5(2)**,** 59-59. doi: 10.33158/ASB.2019v5i2p59.

Messa, V.R. (2021). Biocontrol by induced systemic resistance using plant growth promoting rhizobacteria. *Rhizosphere* 17**,** 100323. doi: 10.1016/j.rhisph.2021.100323.

Monson, R.K., Trowbridge, A.M., Lindroth, R.L., and Lerdau, M.T. (2022). Coordinated resource allocation to plant growth–defense tradeoffs. *New Phytologist* 233(3)**,** 1051-1066. doi: 10.1111/nph.17773.

Naureen, A., Nasim, F.-u.H., Choudhary, M.S., Ashraf, M., Grundler, F.M.W, and Schleker, A.S.S. (2022). A new endophytic fungus CJAN1179 isolated from the Cholistan desert promotes lateral root growth in *Arabidopsis* and produces IAA through tryptophan-dependent pathway. *Archives of Microbiology* 204(3)**,** 181. doi: 10.1007/s00203-022-02768-2.

Phani, V., Khan, M.R., and Dutta, T.K. (2021). Plant-parasitic nematodes as a potential threat to protected agriculture: Current status and management options. *Crop Protection* 144**,** 105573. doi: 10.1016/j.cropro.2021.105573.

Pikovskaya, R. (1948). Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Mikrobiologiya* 17**,** 362–370.

Proença, D.N., Heine, T., Senges, C.H., Bandow, J.E., Morais, P.V., and Tischler, D. (2019). Bacterial metabolites produced under iron limitation kill pinewood nematode and attract Caenorhabditis elegans. *Frontiers in microbiology* 10**,** 2166. doi: 10.3389/fmicb.2019.02166.

Reddy, E.C., Reddy, G.S., Goudar, V., Sriramula, A., Swarnalatha, G.V., Al Tawaha, A.R.M., et al. (2022). "Hydrolytic enzyme producing plant growth-promoting rhizobacteria (PGPR) in plant growth promotion and biocontrol," in *Secondary metabolites and volatiles of PGPR in plant-growth promotion,* ed. V.G.U. R. Z. Sayyed. (Cham: Springer), 303-312.

Saeed, Q., Xiukang, W., Haider, F.U., Kučerik, J., Mumtaz, M.Z., Holatko, J., et al. (2021). Rhizosphere bacteria in plant growth promotion, biocontrol, and bioremediation of contaminated sites: a comprehensive review of effects and mechanisms. *International journal of molecular sciences* 22(19)**,** 10529. doi: 10.3390/ijms221910529.

Schwyn, B., and Neilands, J. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* 160(1)**,** 47-56. doi: 10.1016/0003-2697(87)90612-9.

Seneviratne, G., Weerasekara, M., Seneviratne, K., Zavahir, J., Kecskés, M., and Kennedy, I. (2011). "Importance of biofilm formation in plant growth promoting rhizobacterial action," in *Plant growth and health promoting bacteria,* ed. D.K. Maheshwari. (Berlin, Heidelberg: Springer), 81-95.

Shabaan, M., Asghar, H.N., Zahir, Z.A., Zhang, X., Sardar, M.F., and Li, H. (2022). Salt-tolerant PGPR confer salt tolerance to maize through enhanced soil biological health, enzymatic activities, nutrient uptake and antioxidant defense. *Frontiers in Microbiology* 13**,** 901865. doi: 10.3389/fmicb.2022.901865.

Shahid, I., Han, J., Hanooq, S., Malik, K.A., Borchers, C.H., and Mehnaz, S. (2021). Profiling of metabolites of *Bacillus* spp. and their application in sustainable plant growth promotion and biocontrol. *Frontiers in Sustainable Food Systems* 5**,** 605195. doi: 10.3389/fsufs.2021.605195.

Sijmons, P.C., Grundler, F.M.W, von Mende, N., Burrows, P.R., and Wyss, U. (1991). *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *The Plant Journal* 1(2)**,** 245-254. doi: 10.1111/j.1365-313X.1991.00245.x.

Singh, P., and Siddiqui, Z.A. (2010). Biocontrol of root-knot nematode Meloidogyne incognita by the isolates of *Pseudomonas* on tomato. *Archives of Phytopathology and Plant Protection* 43(14)**,** 1423-1434. doi: http://www.tandfonline.com/loi/gapp20.

Stepanović, S., Vuković, D., Hola, V., Bonaventura, G.D., Djukić, S., Ćirković, I., et al. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by *staphylococci*. *Apmis* 115(8)**,** 891-899. doi: 10.1111/j.1600-0463.2007.apm_630.x.

Topalović, O., Bredenbruch, S., Schleker, A.S.S., and Heuer, H. (2020). Microbes attaching to endoparasitic phytonematodes in soil trigger plant defense upon root penetration by the nematode. *Frontiers in Plant Science* 11**,** 138. doi: 10.3389/fpls.2020.00138.

Topalović, O., Elhady, A., Hallmann, J., Richert-Pöggeler, K.R., and Heuer, H. (2019). Bacteria isolated from the cuticle of plant-parasitic nematodes attached to and antagonized the root-knot nematode *Meloidogyne hapla*. *Scientific reports* 9(1)**,** 11477. doi: 10.1038/s41598-019-47942-7.

Toral, L., Rodríguez, M., Martínez-Checa, F., Montaño, A., Cortés-Delgado, A., Smolinska, A., et al. (2021). Identification of volatile organic compounds in extremophilic bacteria and their effective use in biocontrol of postharvest fungal phytopathogens. *Frontiers in microbiology* 12**,** 773092. doi: 10.3389/fmicb.2021.773092.

Vasant, G., Bhatt, S., and Raghav, R. (2023). Isolation and Molecular Characterization of Plant Growth Promoting Rhizobacteria from Groundnut (Arachis Hypogaea L.) Rhizosphere. *Current Agriculture Research Journal* 11(1).

Vasantha-Srinivasan, P., Park, K.B., Kim, K.Y., Jung, W.-J., and Han, Y.S. (2025). The role of Bacillus species in the management of plant-parasitic nematodes. *Frontiers in Microbiology* 15**,** 1510036.

Verma, P.P., Shelake, R.M., Das, S., Sharma, P., and Kim, J.-Y. (2019). "Plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF): potential biological control agents of diseases and pests," in *Microbial Interventions in Agriculture and Environment: Volume 1: Research Trends,*

Priorities and Prospects, ed. V.K.G. Dhanajaya Pratap Singh, Ratna Prahba. (Singapore: Springer), 281-311.

Wang, Y., Pei, Y., Wang, X., Dai, X., and Zhu, M. (2024). Antimicrobial metabolites produced by the plant growth-promoting rhizobacteria (PGPR): *Bacillus* and *Pseudomonas*. *Advanced Agrochem*. doi: 10.1016/j.aac.2024.07.007.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology* 173(2)**,** 697-703. doi: 10.1128/jb.173.2.697-703.1991.

Xiang, N., Lawrence, K.S., Kloepper, J.W., Donald, P.A., and McInroy, J.A. (2017). Biological control of *Heterodera glycine*s by spore-forming plant growth-promoting rhizobacteria (PGPR) on soybean. *PloS one* 12(7)**,** e0181201. doi: 10.1371/journal.pone.0181201.

Supplementary data

Figure 1

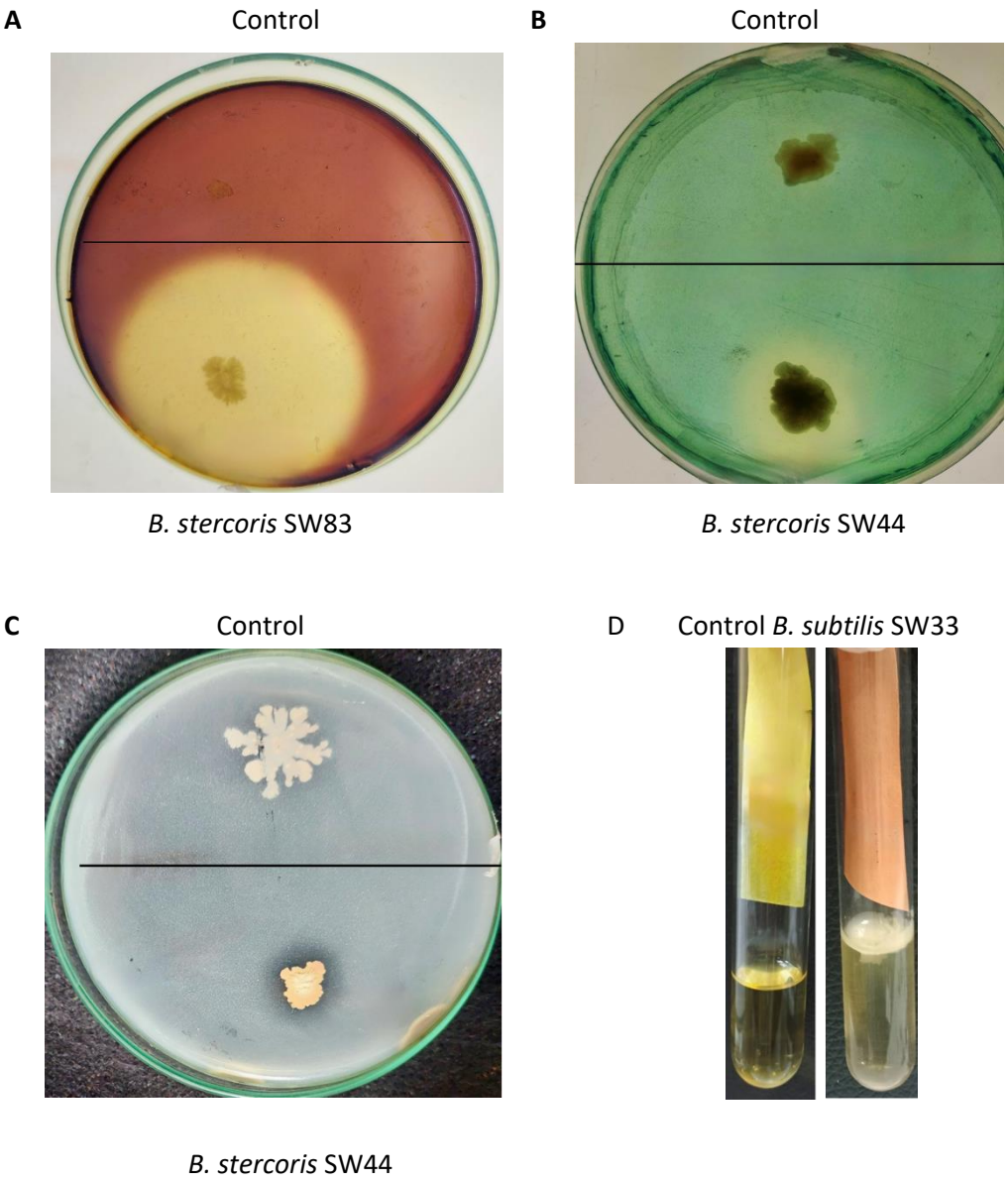

Figure 1: Biochemical characterization of *Bacillus* isolates: representative examples. (A) Chitinase production was indicated by a clear zone surrounding bacterial colonies grown on a medium supplemented with 1% colloidal chitin. **(B)** Siderophore production was visually confirmed by the formation of a yellow/orange halo in CAS agar medium, resulting from iron removal. **(C)** Phosphate solubilization was observed as a zone of clearance around bacterial colonies on Pikovskaya's agar. **(D)** Hydrogen cyanide (HCN) production was detected by a reddish-brown discoloration of filter paper in LB broth containing glycine.

Figure 2

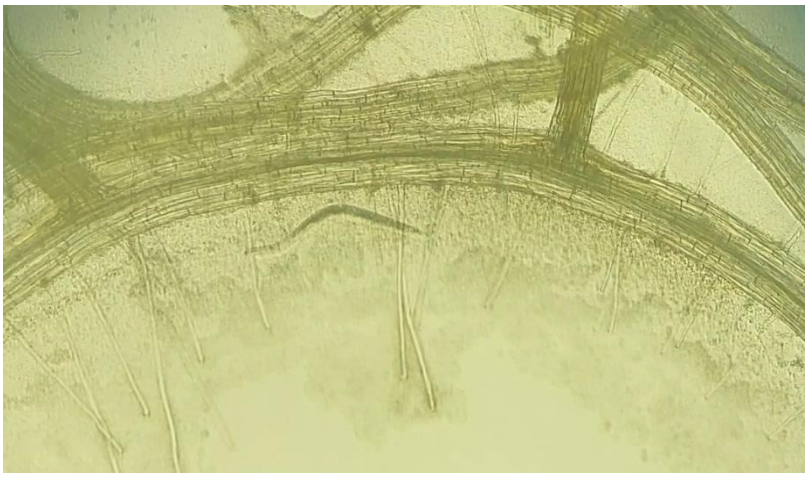

Figure 2: Colonization of *Bacillus subtilis* SC11 around the root of Arabidopsis thaliana at 10 days post-treatment of isolate and 3 days post inoculation (3 dpi) of *Heterodera* schachtii.

6. Discussion

Plant growth-promoting rhizobacteria (PGPR) are a potential source of biological control for plant pathogens and represent a developing area of sustainable agricultural practices. These beneficial rhizobacteria colonize the rhizosphere and exhibit a multifaceted approach to protect plants from disease. PGPR are considered valuable components of integrated pest management strategies (Zill-e-Huma Aftab et al., 2025).

Plants attract specific PGPR through root exudates, which are a mixture of chemical substances released from the roots (Vives-Peris et al., 2020). The composition varies depending on the plant species, growth stage, and environmental conditions. Different components of these exudates attract various PGPR (Singh and Mukerji, 2006). Different components of these exudates attract various PGPR (Singh and Mukerji, 2006). These bacteria can detect specific chemicals in the soil's root exudates and move toward the roots through chemotaxis, a process often described as a "smell and follow" mechanism where bacteria sense a gradient of a particular chemical and migrate accordingly. Some exudate chemicals serve as signals for specific PGPR strains; for example, plants may secrete flavonoids to attract nitrogen-fixing Rhizobia, although Rhizobia are more host-specific than PGPR (Singh et al., 2022). Other chemicals can stimulate PGPR to produce particular enzymes or exhibit other beneficial traits. Once in the rhizosphere, PGPR communicate through quorum sensing, which involves producing and sensing signal molecules that help coordinate their activities and form biofilms on the root surface (Chamkhi et al., 2020). Additionally, plants can recognize certain molecules secreted by PGPR that promote growth or activate defense mechanisms. Mutual recognition between plants and microbes is crucial for a successful interaction.

The findings of Bouffaud et al. (2014) also demonstrate that the microbial community composition differs among different crops, as they found that root microbiome communities in wheat, sorghum, and maize showed distinct community compositions. Several studies demonstrated that while the specific dominant bacterial phyla in the rhizosphere vary among different crops, some are commonly found across plant species. Studies show that Actinomycetota, Bacteroidota, and Pseudomonadota were frequently dominant in the rhizosphere of various plants, including Arabidopsis (Lundberg et al., 2012), barley (Bulgarelli et al., 2015), watermelon (Ling et al., 2015), and cotton (Qiao et al., 2017). This suggests that these phyla may represent a core set of bacterial groups in plant rhizospheres.

In the current study, although the primary objective was to investigate the biocontrol potential of culturable PGPR isolated from the cotton-wheat rhizosphere against Fusarium wilt in cotton caused by *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) and the plant growth-promoting capabilities, we also explored the microbiome composition of cotton rhizospheres in different agroecosystems, specifically semi-arid and arid fields. Studying the microbiome in different agroecosystems (arid vs. semi-arid, as you've done) provides insights into how environmental factors shape microbial communities and influence plant performance. This knowledge is crucial for developing tailored management strategies for different regions.

Our results revealed distinct bacterial and fungal community structures between these environments. Analysis of the bacterial communities revealed considerable differences in the rhizosphere of all sites. However, two rhizospheres, CR1 and CR2, were relatively close to each other compared to the other two rhizospheres, CR3 and CR4. Most studies, including those by Qiao et al. (2017), Xi et al. (2019), Lv et al. (2022), and Feng et al. (2024), reported that Pseudomonadota were more abundant in the cotton rhizosphere. In contrast, we found that Actinomycetota dominated the bacterial communities in samples CR1, CR2, and CR4. Pseudomonadota were the second most abundant in these samples, but were dominant in sample CR3, where Actinomycetota were subdominant. Most of the bacterial genera with a relative abundance of more than 0.7% among the studied rhizosphere are known as plant-beneficial genera.

Fungal communities were present in very low abundance compared to bacterial communities, and Ascomycota and Basidiomycota were highly abundant in all rhizospheres. The relative abundance of Ascomycota and Basidiomycota in our results was aligned with the findings of Moussa et al. (2017), Song et al. (2024), and Karapareddy et al. (2025), which indicated that Ascomycota was the most abundant phylum, followed by Basidiomycota in the cotton rhizosphere (Moussa et al., 2017; Song et al., 2024; Karapareddy et al., 2025). At the genus level, we found that there was a combination of plant-beneficial and plant-pathogenic fungi. Overall, our findings demonstrate that, despite cultivation of the same crop, microbial community composition varied considerably, particularly at the genus and species levels, indicating local adaptation to specific site conditions and align with Ramirez et al. (2014) findings that environmental factors and geographic location play a significant role in shaping bacterial community composition (Ramirez et al., 2014).

To further investigate the microbiome, additional rhizosphere samples were collected from a cotton field and five distinct wheat fields. A total of 136 rhizobacteria were obtained from the rhizospheres (five cotton rhizospheres and five wheat rhizospheres).

Genetic diversity among 136 rhizobacteria from these samples was then determined using ERIC, REP, and $(GTG)_5$ PCR fingerprinting. This molecular approach aimed to elucidate the differences between cotton and wheat rhizospheres. Our analysis revealed considerable genetic diversity between the bacterial communities associated with the two crops, as evidenced by these molecular markers. This finding aligns with previous research suggesting that different crops may harbor distinct rhizosphere microbiomes, and that microbes exhibit various behaviors in the rhizosphere environment (Ali et al., 2017).

Isolated rhizobacteria were investigated for their biological control potential against different fungal strains *in vitro*, and Fusarium wilt in cotton and nematode *Heterodera schachtii* in *Arabidopsis thaliana* (nematode-plant model system). Fungal and nematode-borne diseases represent significant challenges in agriculture, resulting in substantial yield losses and economic damage (Ray et al., 2017; Sorribas et al., 2020; Khan, 2023). These pathogens disrupt plant growth, reduce nutrient uptake, and increase susceptibility to other diseases. PGPR offer a promising avenue for combating these diseases sustainably and can also increase plant growth. PGPR employ various mechanisms such as induced systemic resistance, direct antagonism through antimicrobial compounds, competition for resources, nematode egg hatching inhibition, and overall enhancement of plant growth, to protect plants from these pathogens and foster healthier, more resilient crops. Therefore, all 136 rhizobacteria isolates were initially screened for antifungal activity against two *Fusarium oxysporum* strains, including FOV. The most effective isolates were then selected for further investigation of their antifungal activity against *Fusarium graminearum*, *Leptosphaeria maculans*, and *Cercospora beticola* to determine their broad-spectrum antifungal activity against several fungi. Eight isolates SC5, SC10, SC11, SC15, SC30, SC32, SC41, and SC42, from the cotton rhizosphere showed good antifungal activity *in vitro* and *in vivo* against the Fusarium wilt in cotton. Many isolates from cotton and wheat were also screened for the nematicidal effect of the bacterial living cell (LBC) and cell-free supernatant (CFS) directly on the juvenile stage 2 (J2) of *H. schachtii* and parasitism in the *A. thaliana*. Seven isolates (SW33, SW44, SW53, SW69, SW73, SW83 SC11) with one common isolate SC11 were found good antagonists of *H. schachtii*. 16S rRNA sequencing revealed that all 12 isolates belong to *Bacillus*. These findings align with several studies where in PGPR, *Bacillus* spp. have been reported to promote plant growth and combat plant pathogens, acting as a biocontrol agent (Shahid et al., 2021). *Bacillus* spp. exhibit a broad spectrum of activity, producing diverse antimicrobial compounds that inhibit the growth of pathogens. They can also form endospores, enhancing their resilience in adverse environmental conditions. Different *Bacillus* spp. including *B. subtilis,* were already described to be effective in the management of fungal-borne diseases and PPN (Cawoy et al., 2011; Vasantha-Srinivasan et al., 2025). The dual functionality of plant growth promotion and pathogen suppression has already been reported for

certain rhizobacteria isolates (Cetintas et al., 2018). The mechanisms underlying these effects may include improved nutrient uptake (Shabaan et al., 2022), hydrolytic enzymes production (Jadhav et al., 2017), siderophore production (Verma et al., 2019), hydrogen cyanide (HCN) production (Khaing et al., 2021), phytohormones production including indole acetic acid (IAA) (Gao et al., 2023), antibiosis (Mandaliya et al., 2025), root colonization, biofilm formation, or induced systemic resistance (Meena et al., 2020). The prevalence of *Bacillus* isolates also could be due to the use of only LB media, which non-selectivity favors fast-growing bacteria, *Bacillus*' robust spore-forming ability, and other potential sampling biases (Fira et al., 2018) as metagenomic in this study revealed a very small proportion of *Bacillus* in cotton rhizospheres but among culturable best performing 12 isolates all belonged to *Bacillus*. The use of different isolation methods and media could reveal additional biocontrol agents.

In this study, the different mechanisms of action of the selected isolates involved in biocontrol and plant growth promotion were also investigated, including the production of biofilm, hydrolytic enzymes, HCN, siderophore, IAA, and P-solubilization.

Most of the isolates were forming Biofilm and were good antagonists. These results align with previous studies, that PGPR colonize plant roots and form a biofilm matrix that can act as a physical barrier, preventing pathogens from accessing the plant root surface and establishing an infection (Knights et al., 2021). PGPR within the biofilm can outcompete pathogens for resources such as nutrients and space, limiting pathogen growth and colonization (Seneviratne et al., 2011).

Only one Isolate, SW33 was producing HCN and was a good antagonist of *H. schachtii.* Our results align with previous studies in which Beshah et al. reported that the primary role of HCN in biocontrol is its toxicity to many plant pathogens, including fungi, bacteria, and nematodes. HCN disrupts cellular respiration by inhibiting cytochrome c oxidase, a key enzyme in the electron transport chain. This inhibition leads to energy depletion and ultimately cell death in the target organism.

Most of the isolates were producing hydrolytic enzymes and were acting as good biological control agent against Fusarium wilt and *H. schachtii* parasitism. Previous studies reported that in hydrolytic enzymes, chitinase can break down chitin, a major structural component of the cell wall of fungi (Veliz et al., 2017). This enzymatic degradation can inhibit fungal growth and development, potentially leading to cell lysis in severe cases by weakening cell walls and making the fungus more susceptible to environmental stresses. Several bacterial species, including *Bacillus*, *Streptomyces*, and *Pseudomonas*, produce chitinases that exhibit nematicidal activity. These enzymes disrupt nematode development by degrading eggshells, preventing hatching, and damaging the nematode cuticle, affecting molting,

feeding, and reproduction. Research has shown that chitinases from *Bacillus thuringiensis* are effective against root-knot nematodes by degrading eggshells, while *Streptomyces* chitinases disrupt nematode cuticles, leading to immobilization and death. Protease enzymes are produced by various bacteria and can target fungal cell wall proteins, contributing to cell wall weakening and disruption (Dimkić et al., 2022) and are also known to have nematicidal capabilities and play a role in the suppression of PPN (Lian et al., 2007). While cellulose is not a major component of most fungal cell walls; however, some fungi create cellulose in certain structures. PGPR cellulase is capable of degrading these structures, possibly impacting fungal growth and development (Brown Jr, 2004). The primary function of PGPR cellulases is to target dead organic matter, leading to increased nutrient availability for plants; they generally do not break down intact plant cell walls. This selectivity is due to the protection of plant cellulose by lignin and other structural components that PGPR cellulases cannot readily degrade (McKee and Inman, 2019). Pectinase enzymes break down pectin, which can assist bacteria in colonizing plant tissues and potentially competing with fungi for resources like space and nutrients (Riseh et al., 2024). Urease activity in bacteria plays several crucial roles, primarily related to pathogenicity, nitrogen metabolism, and environmental interactions (Pei et al., 2024).

In this study, most of the isolates produced siderophore and also performed well against FOV and *H. schachtii* parasitism. These findings align with previous studies that reported that siderophores, iron-chelating compounds produced by PGPR, represent a potent tool for biocontrol in agricultural systems. Their primary function is to limit the availability of iron, a vital nutrient, to plant pathogens. PGPR siderophores accomplish this by scavenging iron and forming stable complexes that the bacteria readily utilize, effectively starving pathogens. This can inhibit pathogen growth and virulence through direct iron sequestration, active competition for iron, and, in some cases, direct antimicrobial activity. Understanding the factors that influence siderophore-mediated biocontrol, such as the type and quantity of siderophores, alongside the pathogen's iron requirements, is crucial for optimizing their application in sustainable pest management strategies. Several studies have reported that siderophores from certain bacterial species chelate iron, leading to the death of *Caenorhabditis elegans* and fungi (Jayaprakashvel et al., 2019; Proença et al., 2019).

In this study three isolates, SC11, SW33 and SW44 were performing P-solubilization and isolate SC11 treatment highly increased the plant vigor in the *in vivo* experiment in cotton. There findings align with previous studies which reported that P availability is often a limiting factor in plant growth, thus, P-solubilization capability can be crucial for enhancing nutrient uptake and improving overall plant development (Bechtaoui et al., 2024).

In this *in vivo* study, we observed that under FOV stress in cotton, treatment with certain isolates led to elevated levels of antioxidants in the cotton leaves. Notably, these treated plants exhibited a significantly lower disease incidence compared to the positive control, which was inoculated with FOV alone. Furthermore, the treated plants displayed healthy growth and vigor, suggesting a protective effect of the isolates against FOV. These findings align with previous studies suggesting that PGPR can also significantly enhance antioxidant activity in plants during pathogen defense. By inducing systemic resistance, PGPR primes plants for enhanced defense responses, including the activation of antioxidant systems. Certain PGPR strains produce antioxidant compounds, directly boosting the plant's antioxidant capacity (Begum et al., 2022). Furthermore, PGPR modulates the expression of plant antioxidant genes, increasing levels of antioxidant enzymes and compounds. During fungal infection, plants produce reactive oxygen species as a defense mechanism, but excessive ROS can cause oxidative damage. PGPR helps scavenge these ROS, reducing oxidative stress and protecting plant tissues, thus playing a crucial role in the plant's defense against plant pathogens (Begum et al., 2022).

In this study, we identified eight PGPR isolates demonstrating promising biocontrol potential against fungal pathogens and seven PGPR isolates against nematodes. These isolates exhibited diverse antagonistic mechanisms, suggesting their utility in managing fungal diseases and nematode infestations. Further investigation is needed to evaluate their efficacy under field conditions for fungal disease control, as well as in greenhouse and field trials for nematode management. Introducing these PGPR into the rhizosphere can be a beneficial strategy for sustainable agriculture, offering the potential to reduce reliance on synthetic inputs, improve plant health, and enhance nutrient availability. Research has demonstrated positive effects of PGPR inoculation on plant growth, disease suppression, and nutrient uptake, highlighting their value in improving agricultural practices. However, the persistence and long-term effectiveness of PGPRs can be influenced by factors such as soil type, environmental conditions, competition with native microbes, and plant species. While plants may exhibit compensatory mechanisms in response to PGPR inoculation, such as changes in root architecture, enhanced photosynthetic activity, and activation of defense mechanisms, these may not fully offset the initial effects of the PGPRs. Therefore, continued monitoring and management may be necessary to ensure optimal plant health and productivity. If these isolates maintain their effectiveness at the field level, they could be considered for formulation and subsequent registration for commercial application.

After the inoculation of PGPR, the long-term stability of inoculated PGPR remains a significant challenge in sustainable agriculture. Several studies demonstrated that while rhizosphere

manipulation, such as inoculation with PGPR, offers short-term benefits, its effects are often not permanent due to the influence of native soil communities, environmental fluctuations, and plant development stages, leading to a reversion toward the original microbiome composition. Factors like tillage, fertilization, irrigation, and crop rotation further influence the stability of these manipulations, and even emerging technologies like gene editing and synthetic biology require further evaluation to determine their long-term efficacy under field conditions. The plant itself actively shapes its rhizosphere microbiome, selecting for microbes that best support its growth and stress tolerance, which can limit the establishment of externally introduced microbes unless they offer a clear advantage or fit well within the existing community (Mahmud et al., 2021; Che et al., 2022; Sharma et al., 2024).

Integrated strategies, such as combining microbial inoculants with plant breeding for compatible traits, adaptive management, and the application of advanced biotechnologies to predict and control rhizosphere dynamics, may be necessary to achieve durable changes. Integrated Pest Management (IPM) provides a holistic framework for implementing biological control strategies sustainably and effectively. IPM emphasizes a comprehensive approach that combines monitoring, prevention, and biological control tactics, with chemical interventions reserved as a last resort. By carefully considering the ecological context and employing a combination of compatible control methods, IPM minimizes the reliance on synthetic pesticides and promotes a more balanced and resilient agroecosystem (Angon et al., 2023; Zhou et al., 2024).

By embracing biological control and IPM, we can move towards a more sustainable and resilient agricultural system that protects human health, preserves biodiversity, and ensures food security for future generations. Further research should focus on the synergistic effects of combining different biological control agents and optimizing their integration into IPM programs. Additionally, the long-term economic and environmental benefits of adopting these sustainable pest management strategies need to be carefully evaluated to inform policy decisions and promote widespread adoption (Angon et al., 2023; Zhou et al., 2024).

Several studies also revealed that breeding offers potential for reducing dependence on PGPR treatments. Significant genetic variation exists among plant varieties in their responsiveness to beneficial microbes, and some modern breeding practices may have inadvertently reduced reliance on certain microbial symbioses. Identifying QTLs linked to beneficial plant-microbe interactions enables the breeding of crops that are more efficient at recruiting or benefiting from native PGPR, with high-throughput phenotyping and genotyping platforms facilitating the selection of plant

genotypes with enhanced microbiome responsiveness. However, the effectiveness of this approach depends on the environment, as traits supporting beneficial microbial associations may only be advantageous under specific soil or nutrient conditions. While complete independence from microbial assistance is unlikely, breeding can optimize the synergy between plant genetics and the soil microbiome (Marco et al., 2022). Furthermore, the studies also show that Biostimulants derived from PGPR, such as metabolites, extracts, or formulations containing microbial byproducts, can stimulate plant growth and stress tolerance in ways similar to live bacteria, improving nutrient uptake, hormone regulation, and resistance to biotic and abiotic stresses. Biostimulants offer longer shelf life, stability, and easier integration into existing agricultural practices, and they often face fewer regulatory hurdles and safety concerns than live microbial products. However, biostimulants may not fully replicate the complex and adaptive interactions of live PGPR, which can dynamically interact with the plant and soil microbiome, potentially offering more sustained or context-specific benefits. While the performance of both biostimulants and live PGPR can vary under field conditions, integrated approaches that combine plant breeding, biostimulant use, and microbial inoculation may offer the most robust and sustainable outcomes (Castiglione et al., 2021; Bartucca et al., 2022; Kumari et al., 2022).

In conclusion, while plant breeding efforts can decrease the dependence on PGPR treatments by strengthening inherent plant-microbe relationships, and biostimulants present practical and regulatory benefits, neither strategy alone provides a universally optimal solution. Instead, the most promising path towards sustainable agriculture lies in an integrated approach, combining plant breeding, biostimulant applications, and microbial inoculations, carefully tailored to the specific crop, environmental conditions, and management practices. Our study provides a foundation for more broad-spectrum research, as it has isolated effective antagonistic *Bacillus* isolates.

6.1 Conclusion and Outlook

In conclusion, this study provides valuable insights into the composition, diversity, and biocontrol potential of rhizobacterial communities associated with cotton under continuous cotton-wheat rotation. The identification of site-specific microbial communities and the discovery of *Bacillus* isolates exhibiting broad-spectrum antagonistic activity against both fungal pathogens and nematodes highlight the potential of these microorganisms as sustainable biocontrol agents. The effectiveness of specific isolates, particularly SC11, against both *Fusarium oxysporum f. sp. vasinfectum* and *Heterodera schachtii* underscores the possibility of developing multi-target biocontrol strategies for enhanced crop protection. In short

- **Rhizosphere microbial communities:** The microbial communities of cotton rhizospheres at sites CR1 and CR2 exhibited comparative similarity at each taxonomic level. However, sites CR3 and CR4 displayed high dissimilarity, suggesting that factors beyond cropping patterns significantly influence microbial community structure.
- **Genetic diversity:** Molecular marker analysis revealed considerable genetic diversity among the 136 culturable isolates obtained from different cotton and wheat rhizospheres across various sites.
- **Antagonistic mechanisms:** Several isolates, including SC30, SC15, SC32 (*B. halotolerans*), SC10, SC11, SC32, SC41, SC42, SW33, SW53 (*B. subtilis*), SC5, SW44, SW69, SW73, and SW83 (*B. stercoris*), exhibited key antagonistic mechanisms. These included biofilm formation, production of hydrolytic enzymes, and siderophore production. Notably, SW33 produced HCN at approximately 47 µg/mL. Isolates SC11, SW33, and SW44 also solubilized phosphorus, which is known to promote plant growth.
- **Biocontrol of Fusarium wilt:** Isolates SC30, SC15, SW32 (*B. halotolerans*), SC10, SC11, SC41, and SC42 (*B. subtilis*) demonstrated effective biocontrol activity against Fusarium wilt in cotton, reducing disease incidence in the presence of *Fusarium oxysporum f. sp. vasinfectum* and enhancing plant growth. SC10 and SC11 showed the highest biocontrol potential and plant growth promotion.
- **Antagonistic activity against *H. schachtii***: Isolates SC11, SW33, SW53 (*B. subtilis*), SW44, SW69, SW73, and SW83 (*B. stercoris*) displayed significant antagonistic activity against *Heterodera schachtii* parasitism in *Arabidopsis thaliana*. Cell-free supernatant (CFS) and living bacterial cells of these isolates had a direct effect on J2 nematodes, with the effect of CFS being concentration-dependent. The top three best-performing isolates SW83, SW73, SW69 (*B. stercoris)* showed the highest impact on *H. schachtii* parasitism. Notably, isolates *B. stercoris* SW69, SW73, and *B. subtilis* SC11, SW53 also promoted plant growth during nematode infection.
- ***Bacillus stercoris*:** This study is the first to report the effectiveness of *Bacillus stercoris* against *H. schachtii* parasitism.

To translate these findings into practical applications and maximize their impact, several avenues for future research should be explored:

- **Field studies:** Comprehensive field trials to evaluate the efficacy of the identified *Bacillus* isolates under real-world agricultural conditions are compulsory. These include assessing their

ability to reduce disease and nematode incidence, improve crop yield, and persist in the rhizosphere over time.

- **Optimization of application methods:** Investigation of optimal methods for delivering these biocontrol agents to the rhizosphere, considering factors such as formulation, timing, and dosage.
- **Synergistic interactions:** investigation of the potential for synergistic interactions between different *Bacillus* isolates or in combination with other biocontrol agents or compatible agricultural practices.
- **Understanding mechanisms of action:** Further investigation of the molecular mechanisms underlying the antagonistic activity of these *Bacillus* isolates against fungal pathogens and nematodes. This knowledge can inform the development of more targeted and effective biocontrol strategies.
- **Expanding host range:** Investigation of the potential of these isolates to control diseases and pests in other economically important crops, particularly those affected by *Fusarium* and *Heterodera* species.
- **Addressing environmental concerns:** Assessment of the environmental impact of these biocontrol agents, ensuring their safety and sustainability for long-term use in agriculture.

By pursuing these research directions, we can harness the biocontrol potential of these beneficial rhizobacteria to develop environmentally friendly and sustainable solutions for crop protection, reducing our reliance on synthetic pesticides and promoting healthier agricultural ecosystems.

6.2 References

Ali, M.A., Naveed, M., Mustafa, A., and Abbas, A. (2017). "The good, the bad, and the ugly of rhizosphere microbiome," in *Probiotics and plant health,* ed. M.K. Vivek Kumar, Shivesh Sharma, Ram Prasad.), 253-290.

Angon, P.B., Mondal, S., Jahan, I., Datto, M., Antu, U.B., Ayshi, F.J., et al. (2023). Integrated pest management (IPM) in agriculture and its role in maintaining ecological balance and biodiversity. *Advances in Agriculture* 2023(1)**,** 5546373. doi: 10.1155/2023/5546373.

Bartucca, M.L., Cerri, M., Del Buono, D., and Forni, C. (2022). Use of biostimulants as a new approach for the improvement of phytoremediation performance—A Review. *Plants* 11(15)**,** 1946. doi: 10.3390/plants11151946.

Bechtaoui, N., Raklami, A., Tahiri, A.-i., Benidire, L., Göttfert, M., and Oufdou, K. (2024). Phosphatesolubilizing rhizobacteria and their effects on the growth and phosphorus uptake by wheat plants. *Journal of Plant Nutrition* 47(17)**,** 2811-2823. doi: 10.1080/01904167.2024.2369069.

Begum, N., Wang, L., Ahmad, H., Akhtar, K., Roy, R., Khan, M.I., et al. (2022). Co-inoculation of arbuscular mycorrhizal fungi and the plant growth-promoting rhizobacteria improve growth and photosynthesis in tobacco under drought stress by up-regulating antioxidant and mineral nutrition metabolism. *Microbial ecology***,** 1-18. doi: 10.1007/s00248-021-01815-7.

Beshah, A., Muleta, D., Legese, G., and Assefa, F. (2024). Exploring stress-tolerant plant growthpromoting rhizobacteria from groundnut rhizosphere soil in semi-arid regions of Ethiopia. *Plant Signaling & Behavior* 19(1)**,** 2365574. doi: 10.1080/15592324.2024.2365574.

Bouffaud, M.L., Poirier, M.A., Muller, D., and Moënne-Loccoz, Y. (2014). Root microbiome relates to plant host evolution in maize and other Poaceae. *Environmental microbiology* 16(9)**,** 28042814. doi: 10.1111/1462-2920.12442.

Brown Jr, R.M. (2004). Cellulose structure and biosynthesis: what is in store for the 21st century? *Journal of Polymer Science Part A: Polymer Chemistry* 42(3)**,** 487-495.

Bulgarelli, D., Garrido-Oter, R., Münch, Philipp C., Weiman, A., Dröge, J., Pan, Y., et al. (2015). Structure and Function of the Bacterial Root Microbiota in Wild and Domesticated Barley. *Cell Host & Microbe* 17(3)**,** 392-403. doi: 10.1016/j.chom.2015.01.011.

Castiglione, A.M., Mannino, G., Contartese, V., Bertea, C.M., and Ertani, A. (2021). Microbial biostimulants as response to modern agriculture needs: Composition, role and application of these innovative products. *Plants* 10(8)**,** 1533. doi: 10.3390/plants10081533.

Cawoy, H., Bettiol, W., Fickers, P., and Ongena, M. (2011). *Bacillus-based biological control of plant diseases.* IntechOpen London, UK.

Cetintas, R., Kusek, M., and Fateh, S.A. (2018). Effect of some plant growth-promoting rhizobacteria strains on root-knot nematode, Meloidogyne incognita, on tomatoes. *Egyptian Journal of Biological Pest Control* 28**,** 1-5. doi: 10.1186/s41938-017-0008-x.

Chamkhi, I., El Omari, N., Benali, T., and Bouyahya, A. (2020). "Quorum sensing and plant-bacteria interaction: role of quorum sensing in the rhizobacterial community colonization in the rhizosphere," in *Quorum Sensing: Microbial Rules of Life*. ACS Publications), 139-153.

Che, J., Wu, Y., Yang, H., Wang, S., Wu, W., Lyu, L., et al. (2022). Long-term cultivation drives dynamic changes in the rhizosphere microbial community of blueberry. *Frontiers in Plant Science* 13**,** 962759. doi: 10.3389/fpls.2022.962759.

Dimkić, I., Janakiev, T., Petrović, M., Degrassi, G., and Fira, D. (2022). Plant-associated *Bacillus* and *Pseudomonas* antimicrobial activities in plant disease suppression via biological control mechanisms-A review. *Physiological and Molecular Plant Pathology* 117**,** 101754.

Feng, G., Wu, Y., Yang, C., Zhang, Q., Wang, S., Dong, M., et al. (2024). Effects of coastal saline-alkali soil on rhizosphere microbial community and crop yield of cotton at different growth stages. *Frontiers in Microbiology* 15**,** 1359698. doi: 10.3389/fmicb.2024.1359698.

Fira, D., Dimkić, I., Berić, T., Lozo, J., and Stanković, S. (2018). Biological control of plant pathogens by *Bacillus* species. *Journal of Biotechnology* 285**,** 44-55. doi: 10.1016/j.jbiotec.2018.07.044.

Gao, X., Liu, Y., Liu, C., Guo, C., Zhang, Y., Ma, C., et al. (2023). Individual and combined effects of arbuscular mycorrhizal fungi and phytohormones on the growth and physiobiochemical characteristics of tea cutting seedlings. *Frontiers in Plant Science* 14**,** 1140267. doi: 10.3389/fpls.2023.1140267.

Jadhav, H., Shaikh, S., and Sayyed, R. (2017). "Role of hydrolytic enzymes of rhizoflora in biocontrol of fungal phytopathogens: an overview," in *Rhizotrophs: Plant growth promotion to bioremediation,* ed. S. Mehnaz. (Singapore: Springer), 183-203.

Jayaprakashvel, M., Chitra, C., and Mathivanan, N. (2019). "Metabolites of plant growth-promoting rhizobacteria for the management of soilborne pathogenic fungi in crops," in *Secondary Metabolites of Plant Growth Promoting Rhizomicroorganisms: Discovery and Applications,* ed. C.K. Harikesh Bahadur Singh, M. S. Reddy, Edgar Sansinenea, Carlos García-Estrada. (Singapore: Springer), 293-315.

Karapareddy, S., Anche, V.C., Tamatamu, S.R., Janga, M.R., Lawrence, K., Nyochembeng, L.M., et al. (2025). Profiling of rhizosphere-associated microbial communities in North Alabama soils infested with varied levels of reniform nematodes. *Frontiers in Plant Science* 16**,** 1521579. doi:

10.3389/fpls.2025.1521579.

Khaing, A., Theint, W., Thi, O., and Fu, P. (2021). Antagonistic activity of indigenous rhizobacteria through biosynthesis of indole-3-acetic acid (IAA), hydrogen cyanide (HCN), and siderophores. *Aust. J. Biotechnol. Bioeng* 8(1110)**,** 10.26420.

Khan, M.R. (2023). "Nematode pests of agricultural crops, a global overview," in *Novel Biological and Biotechnological Applications in Plant Nematode Management,* ed. M.R. Khan. (Singapore: Springer), 3-45.

Knights, H.E., Jorrin, B., Haskett, T.L., and Poole, P.S. (2021). Deciphering bacterial mechanisms of root colonization. *Environmental Microbiology Reports* 13(4)**,** 428-444. doi: 10.1111/17582229.12934.

Kumari, M., Swarupa, P., Kesari, K.K., and Kumar, A. (2022). Microbial inoculants as plant biostimulants: A review on risk status. *Life* 13(1)**,** 12. doi: org/10.3390/life13010012.

Lian, L., Tian, B., Xiong, R., Zhu, M., Xu, J., and Zhang, K. (2007). Proteases from *Bacillus*: a new insight into the mechanism of action for rhizobacterial suppression of nematode populations. *Letters in Applied Microbiology* 45(3)**,** 262-269. doi: 10.1111/j.1472-765X.2007.02184.x.

Ling, N., Song, Y., Raza, W., Huang, Q., Guo, S., and Shen, Q. (2015). The response of root-associated bacterial community to the grafting of watermelon. *Plant and Soil* 391**,** 253-264. doi: 10.1007/s11104-015-2399-3.

Lundberg, D.S., Lebeis, S.L., Paredes, S.H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488(7409)**,** 86-90. doi: 10.1038/nature11237.

Lv, N., Liu, Y., Guo, T., Liang, P., Li, R., Liang, P., et al. (2022). The influence of Bt cotton cultivation on the structure and functions of the soil bacterial community by soil metagenomics. *Ecotoxicology and Environmental Safety* 236**,** 113452. doi: 10.1016/j.ecoenv.2022.113452.

Mahmud, K., Missaoui, A., Lee, K., Ghimire, B., Presley, H.W., and Makaju, S. (2021). Rhizosphere microbiome manipulation for sustainable crop production. *Current Plant Biology* 27**,** 100210. doi: 10.1016/j.cpb.2021.100210.

Mandaliya, V.B., Mangroliya, U.C., and Patel, H.R. (2025). "Broad-Spectrum Disease Resistance in Plants by Bio-Control Agents," in *Bio-control Agents for Sustainable Agriculture: Diversity, Mechanisms and Applications,* ed. S.d.l.S.V. Debasis Mitra, Anju Rani · Beatriz Elena Guerra Sierra, Snežana Andjelković (Singapore: Springer), 293-309.

Marco, S., Loredana, M., Riccardo, V., Raffaella, B., Walter, C., and Luca, N. (2022). Microbe-assisted crop improvement: a sustainable weapon to restore holobiont functionality and resilience. *Horticulture research* 9**,** uhac160. doi: 10.1093/hr/uhac160.

McKee, L.S., and Inman, A.R. (2019). Secreted microbial enzymes for organic compound degradation. *Microbes and Enzymes in Soil Health and Bioremediation***,** 225-254.

Meena, M., Swapnil, P., Divyanshu, K., Kumar, S., Harish, Tripathi, Y.N., et al. (2020). PGPR-mediated induction of systemic resistance and physiochemical alterations in plants against the pathogens: Current perspectives. *Journal of Basic Microbiology* 60(10)**,** 828-861. doi: 10.1002/jobm.202000370.

Moussa, T.A., Al-Zahrani, H.S., Almaghrabi, O.A., Abdelmoneim, T.S., and Fuller, M.P. (2017). Comparative metagenomics approaches to characterize the soil fungal communities of western coastal region, Saudi Arabia. *Plos one* 12(9)**,** e0185096. doi: 10.1371/journal.pone.0185096.

Pei, P., Aslam, M., Wang, H., Ye, P., Li, T., Liang, H., et al. (2024). Diversity and ecological function of urease-producing bacteria in the cultivation environment of Gracilariopsis lemaneiformis. *Microbial Ecology* 87(1)**,** 35.

Proença, D.N., Heine, T., Senges, C.H., Bandow, J.E., Morais, P.V., and Tischler, D. (2019). Bacterial metabolites produced under iron limitation kill pinewood nematode and attract *Caenorhabditis elegans*. *Frontiers in microbiology* 10**,** 2166. doi: 10.3389/fmicb.2019.02166.

Qiao, Q., Wang, F., Zhang, J., Chen, Y., Zhang, C., Liu, G., et al. (2017). The variation in the rhizosphere microbiome of cotton with soil type, genotype and developmental stage. *Scientific reports* 7(1)**,** 3940. doi: 10.1038/s41598-017-04213-7.

Ramirez, K.S., Leff, J.W., Barberán, A., Bates, S.T., Betley, J., Crowther, T.W., et al. (2014). Biogeographic patterns in below-ground diversity in New York City's Central Park are similar to those observed globally. *Proceedings of the royal society B: biological sciences* 281(1795)**,** 20141988. doi: 10.1098/rspb.2014.1988.

Ray, M., Ray, A., Dash, S., Mishra, A., Achary, K.G., Nayak, S., et al. (2017). Fungal disease detection in plants: Traditional assays, novel diagnostic techniques and biosensors. *Biosensors and Bioelectronics* 87**,** 708-723. doi: 10.1016/j.bios.2016.09.032.

Riseh, R.S., Vatankhah, M., Hassanisaadi, M., and Barka, E.A. (2024). Unveiling the Role of Hydrolytic Enzymes from Soil Biocontrol Bacteria in Sustainable Phytopathogen Management. *Frontiers in Bioscience-Landmark* 29(3)**,** 105.

Seneviratne, G., Weerasekara, M., Seneviratne, K., Zavahir, J., Kecskés, M., and Kennedy, I. (2011). "Importance of biofilm formation in plant growth promoting rhizobacterial action," in *Plant growth and health promoting bacteria,* ed. D.K. Maheshwari. (Berlin, Heidelberg: Springer), 81-95.

Shabaan, M., Asghar, H.N., Zahir, Z.A., Zhang, X., Sardar, M.F., and Li, H. (2022). Salt-tolerant PGPR confer salt tolerance to maize through enhanced soil biological health, enzymatic activities, nutrient uptake and antioxidant defense. *Frontiers in Microbiology* 13**,** 901865. doi: 10.3389/fmicb.2022.901865.

Shahid, I., Han, J., Hanooq, S., Malik, K.A., Borchers, C.H., and Mehnaz, S. (2021). Profiling of metabolites of *Bacillus* spp. and their application in sustainable plant growth promotion and biocontrol. *Frontiers in Sustainable Food Systems* 5**,** 605195. doi: 10.3389/fsufs.2021.605195.

Sharma, I.P., Giri, K., and Kumar, N. (2024). "Rhizosphere Manipulation for Sustainable Agriculture". Frontiers Media SA).

Singh, G., and Mukerji, K.G. (2006). "Root exudates as determinant of rhizospheric microbial biodiversity," in *Microbial activity in the rhizoshere,* ed. C.M. K.G. Mukerji, J. Singh. (Berlin, Heidelberg: Springer), 39-53.

Singh, K., Chandra, R., and Purchase, D. (2022). Unraveling the secrets of rhizobacteria signaling in rhizosphere. *Rhizosphere* 21**,** 100484. doi: 10.1016/j.rhisph.2022.100484.

Song, W., Wu, H., Xiang, Z., Fan, Y., Wang, S., and Guo, J. (2024). Effects of Plastic Mulch Residue on Soil Fungal Communities in Cotton. *Agriculture* 14(8)**,** 1365. doi: 10.3390/agriculture14081365.

Sorribas, F.J., Djian-Caporalino, C., and Mateille, T. (2020). "Nematodes," in *Integrated pest and disease management in greenhouse crops*. Maria Lodovica Gullino, Ramon Albajes, Philippe C. Nicot ed (Cham: Springer), 147-174.

Vasantha-Srinivasan, P., Park, K.B., Kim, K.Y., Jung, W.-J., and Han, Y.S. (2025). The role of Bacillus species in the management of plant-parasitic nematodes. *Frontiers in Microbiology* 15**,** 1510036.

Veliz, E.A., Martínez-Hidalgo, P., and Hirsch, A.M. (2017). Chitinase-producing bacteria and their role in biocontrol. *AIMS microbiology* 3(3)**,** 689.

Verma, P.P., Shelake, R.M., Das, S., Sharma, P., and Kim, J.-Y. (2019). "Plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF): potential biological control agents of diseases and pests," in *Microbial Interventions in Agriculture and Environment: Volume 1: Research Trends, Priorities and Prospects,* ed. V.K.G. Dhanajaya Pratap Singh, Ratna Prahba. (Singapore: Springer), 281-311.

Vives-Peris, V., De Ollas, C., Gómez-Cadenas, A., and Pérez-Clemente, R.M. (2020). Root exudates: from plant to rhizosphere and beyond. *Plant cell reports* 39(1)**,** 3-17. doi: 10.1007/s00299019-02447-5.

Xi, H., Shen, J., Qu, Z., Yang, D., Liu, S., Nie, X., et al. (2019). Effects of long-term cotton continuous cropping on soil microbiome. *Scientific Reports* 9(1)**,** 18297. doi: 10.1038/s41598-019-54771-1.

Zhou, W., Arcot, Y., Medina, R.F., Bernal, J., Cisneros-Zevallos, L., and Akbulut, M.E.S. (2024). Integrated Pest Management: An Update on the Sustainability Approach to Crop Protection. *ACS Omega* 9(40)**,** 41130-41147. doi: 10.1021/acsomega.4c06628.

Zill-e-Huma Aftab, T.A., Akram, W., Bokhari, N.A., Rehman, A., and Sharif, S. (2025). "Biological Pest Control and Sustainable Agroecosystems," in *Sustainable Agroecosystems-Principles and Practices: Principles and Practices,* ed. R.S.B. Vijay Singh Meena, Ram Kishor Fagodia, Mohammad Hasanian. (London: IntechOpen), 239.