MUHAMMAD SIDDIQUE AFRIDI

ONE OR MILLIONS: HOW MUCH DOES A MICROBIOLOGICALLY-BUFFERED SOIL WITHSTAND CHEMICAL AND BIOLOGICAL PESTICIDES?

LAVRAS – MG

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Thesis presented to the Universidade Federal de Lavras, as part of the requirements of the Post Graduate Program in Agronomy/ Phytopathology, for the degree of Doctorate.

Prof. Dr. Flávio Henrique Vasconcelos de Medeiros
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ONE OR MILLIONS: HOW MUCH DOES A MICROBIOLOGICALLY-BUFFERED SOIL WITHSTAND CHEMICAL AND BIOLOGICAL PESTICIDES?

UM OU MILHÕES: QUANTO UM SOLO MICROBIOLOGICAMENTE TAMPONADO RESISTE A PESTICIDAS QUÍMICOS E BIOLÓGICOS?

Thesis presented to the Universidade Federal de Lavras, as part of the requirements of the Post Graduate Program in Agronomy/ Phytopathology, for the degree of Doctor in Philosophy.

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Dr. Jorge Teodoro de Souza	UFLA
Dr. Luciana Cordeiro Nascimento	UFPB
Dr. Samuel Júlio Martins	UFL
Dr. Amna	QAU

Prof. Dr. Flávio Henrique Vasconcelos de Medeiros
Supervisor

LAVRAS – MG

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To my parents, without whose efforts,

I wouldn't be able to accomplish this study

I dedicate.

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RESUMO GERAL

O solo supressivo a doenças é definido como um tipo de solo onde o patógeno não pode se estabelecer ou persistir, ou causa apenas danos mínimos às culturas, devido à presença de microrganismos específicos e sua atividade no solo, apesar da persistência de patógenos no solo. Os solos supressivos a doenças contribuem substancialmente para a proteção das plantas contra vários patógenos de plantas habitantes do solo, como bactérias, fungos, oomicetos e nematóides. A base da supressão a doenças específicas na maioria dos solos geralmente está associado às suas comunidades microbianas mas sua relação com a aplicação exógena de produtos biológicos e químicos ao longo do tempo ainda não está clara. Os objetivos da pesquisa atual foram de manipular o microbioma supressivo do solo existente por meio de agentes de controle biológico e agroquímicos para explorar a funcionalidade do microbioma em relação aos nematóides parasitos *Meloidogyne* spp. em tomate e cafeeiro e *Heterodera glycines* em soja. O solo natural reduziu s as galhas^{-1} (14,25%) e massas de ovos (74,85%) de tomateiro em relação ao solo esterilizado. No entanto, o tratamento do solo supressivo a antibiótico (Estreptomicina 100 ppm) , fungicida (Ciproconazol 100 ppm) ou a combinação dos dois também reduziu a funcionalidade do microbioma do solo, sendo o efeito dos antibióticos ou sua combinação com o fungicida os de maior efeito na redução da supressão. De forma semelhante, o solo nativo tratado com *Bacillus velezensis* BMH reduziu sua supressividade, o que não aconteceu no solo esterilizado. O efeito deletério não aconteceu com o tratamento com produtos comerciais registrado para este alvo. Este solo supressivo foi então usado para determinar a contribuição de populações específicas de bactérias na supressão. Um total de 42 cepas bacterianas foram isoladas do solo supressivo e 18 delas foram identificadas com alto potencial para controlar *M. incognita*. Além disso, seis das bactérias promissoras produziram compostos orgânicos voláteis (VOCs) e metabólitos em sobrenadantes livres de células com efeitos de mortalidade a nematóide *M. Incognita* e inibição do crescimento de fungos fitopatogênicos: *Fusarium oxysporum* e *Rhizoctonia solani*. Em campo, os produtos biológicos comerciais a base de *Pochonia chlamydosporua* (CEPA PC-10) (Rizotec), *Bacillus methylotrophicus* UFPEDA 20 (Onix) e *Trichoderma koningiopsis* GF362 (não disponível comercialmente) foram aplicados como tratamento de sementes ou em sulco no plantio. O número total de fêmeas na raiz, cistos, ovos, população J_2 (%) ovos/cisto e população J_2 (%) taxa de mortalidade aos 30 e 60 dias após a semeadura, bem como o rendimento das plantas foram avaliados em dois anos consecutivos, mas não diferenças foram observadas entre os tratamentos controle e bioprodutos aplicados. Além disso, a composição da comunidade de bactérias, fungos e eucariotos no solo da rizosfera de plantas tratadas com bioprodutos e os filos dominantes na comunidade bacteriana, fúngica e eucariótica foram *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Ascomycota*, *Basidiomycota*, *Mortierellomycota* e *Ascomycota*, *Cercozoa* respectivamente em ambos os anos. A rede de coocorrência revelou que espécies bacterianas, fúngicas e eucarióticas formaram uma estrutura de rede de alta complexidade em todos os tratamentos com bioprodutos aplicados. De forma semelhante, em cafeeiro, a aplicação continuada de agentes de biocontrole a base de *Trichoderma asperellum*, *Bacillus subtilis* e *B. Methylotrophicus*, o nematicida químico cadusafós, uma combinação de ambos, resultaram em alterações do microbioma de raízes do cafeeiro. As comunidades predominantes de organismos foram *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Ascomycota*, *Mortierellomycota* e *Ascomycota*, *Cercozoa*, respectivamente em ambos os anos consecutivos e, diferentementedo observado para soja, a rede de ocorrência teve complexidade reduzida comparada ao controle quando da adoção de agentes de biocontrole. Nossas descobertas ajudam a compreender que a introdução de micróbios benéficos exógenos em condições de campo é incapaz de modular a microbiota existente e nenhum impacto significativo foi exercido por eles na remodelação do microbioma da rizosfera e propoõe marcadores que podem ser identificados para ação de recrutamento microbiano em soja e em cafeeiro.

Palavras-chave: Microbioma do solo, 16SrRNA, rotação de culturas, supressividade do solo

GENERAL ABSTRACT

Disease suppressive soil is defined as a type of soil where the pathogen cannot establish or persist, or causes only minimal damage to crops, due to the presence of specific microorganisms and their activity in the soil, despite the persistence of pathogens in the soil. Disease suppressive soils substantially contribute to plant protection against various soil-borne plant pathogens such as bacteria, fungi, oomycetes, and nematodes. The foundation of specific disease suppression in most soils affiliates commonly to soil microbial communities. Therefore, the soil microbiota of suppressive soils is considered one of the radical factors contributing to disease suppressiveness against soil-borne diseases. To date, a multitude of microbial taxa and genes have been documented as central players in participating disease suppressiveness of soils. Still, the dominant genera, their sensitivity to alien biocontrol advocacy, agrochemicals and the complexity of microbiome interactions and their underlying mechanisms remain elusive for most disease suppressive soils. The main objective of the current research is to manipulate the existing suppressive soil microbiome through the introduction of various biological control agents and agrochemicals to explore the microbiome functionality towards soil-born (root-knot) nematodes disease. Suppressive soil assay revealed that suppressive soil significantly reduced galls^{-1} (14.25%) and egg masses (74.85%) in relation to sterilized soil. Intriguingly suppressive soil microbiome manipulation by biological control agent *Bacillus* velezensis strain BMH intervened in the microbial functions and reduced its suppressiveness. BMH inoculated suppressive soil significantly increased the galls^{-1} and eggs^{-1} 32% and 47.96% respectively as compared to un-inoculated suppressive soil. Interestingly, suppressive soil slurry blending with antibiotics (Streptomycin 100 ppm) and fungicide (Cyproconazole 100 ppm) significantly modulated the soil microbiome functionality. Soil slurry mixed with antibiotics (and fungicide significantly increased the number of galls^{-1} 174.23% and 87.79% respectively as compared to the untreated slurry. Following the same pattern, antibiotics and fungicide inoculation significantly increased the number of egg masses by 276.24% and 38.17% respectively as compared to the untreated slurry. Biocontrol based on bacteria such as Quatrzo (*Bacillus subtilis; Bacillus licheniformis*), Biobac (*Bacillus subtilis*), Onix (*Bacillus methylotrophicus*) and Rizos(*Bacillus subtilis*) turbulent the soil microbiome performance and insignificantly increased the galls and eggs mass index in relation to suppressive soil. To understand and explore the intrinsic fundamental candidates of the disease suppressive soil, the research promoted to the next level and recovered the responsible candidates from the reported suppressive soil and deciphered their potential role against root-knot nematode (RKN) *Meloidogyne incognita* in the tomato plant. A total of 42 bacterial strains were isolated from the suppressive soil and 18 of them were identified with high potential to control *M. incognita*. The isolates were sequenced based on 16S rRNA and identified 6 different genera namely *Bacillus, Pseudomonas*, *Leclercia*, *Paenarthrobacter*, *Pantoea*, and *Exiguobacterium*. Eighteen bacteria of six different genera were selected based on preliminary screening. The plant was inoculated with strains *Bacillus* sp. P10, *Bacillus* sp. P16, *Bacillus* sp. P19, and *Bacillus* sp. P21 significantly reduced the root galling 47% and the significant average reduction of egg mass was recorded 75.5% in relation to control. Three *Pseudomonas* sp. P17, *Pseudomonas* sp. X11, and *Pseudomonas* sp. X18 exhibited high biocontrol efficacy and significantly reduced the galls and egg masses 54% and 75% in both trials as compared to the control. The isolates such as *Leclercia* sp. P12, *Leclercia* sp. P18 and *Leclercia* sp. P20 exhibited high potential and consistency in controlling gall and egg biomass index in both trials the significant reduction was observed in root galling 47% and egg biomass index 70% as compared to the untreated plants. The bacterial strain, *Paenarthrobacter* sp. X12 showed consistency and maintained the biocontrol capability and significantly reduced the number of galls and egg biomass 57% and 89% respectively in rlation to uninoculated plant. Additionally, all six genera' volatile organic compounds (VOCs) and metabolites in cell-free supernatants had significant effects against the plant pathogens *M. incognita*, Fusarium *oxysporum*, and *Rhizoctonia solani*, but only five strains *Pseudomonas* sp. P7, *Pseudomonas* sp. X11, *Bacillus* sp. P10, *Bacillus* sp. P21, and *Leclercia* sp. P12 significantly inhibited the growth of *Ralstonia solanacearum*. Moreover, all bacterial isolates inherit nematicidal activities and dramatically reduced the egg hatching. These findings recommend that exogenous biological control agents, biostimulants and agrochemicals massively perturb the microbiome structure, composition, ecological and biological activities and detract or infertile the endogenous microbiota

functionality. The study aimed to evaluate the biocontrol efficacy of biocontrol products against soybean cyst-nematode (SCN) employing two seed or furrow treatments under field conditions. The commercially-available biological products based on *Pochonia chlamydosporua* (CEPA PC-10) (Rizotec), *Bacillus methylotrophicus* UFPEDA 20 (Onix) and *Trichoderma koningiopsis* GF362 (not commercially available) were applied as seed treatment or in-furrow upon planting. The total number of females in root, cysts, eggs, J_2 population (%) eggs/cyst and J_2 population (%) mortality rate at 30 and 60 days after sowing as well as plant yield were assessed in two consecutive years, but no significant differences were observed between control and bioproducts applied treatments. Additionally, we evaluated the diversity and community composition of bacteria, fungi and eukaryotes in the rhizosphere soil of bioproducts treated plants and the dominant phyla in bacterial, fungal and eukaryotic community were *Proteobacteria*, *Acidobacteria* Actinobacteria, *Ascomycota*, *Basidiomycota*, *Mortierellomycota*, and *Ascomycota*, *Cercozoa* respectively in both consecutive years. Overall, no significant difference was observed in bacterial, fungal, and eukaryotic community's diversity in both years of data. The co-occurrence network unearthed that bacterial, fungal and eukaryotic species formed a network structure of high complexity in all bioproducts applied treatments. Our findings suggest that the introduction of exogenous beneficial microbes into field conditions is unable to modulate overall the microbial structure but the selective recruitment of key microbial taxa, some of which is also implicated in the nematode suppressiveness. The aim of this study was to analyze the effects of the biological control agent based bioproducts and chemical nematicides at different combination on root-knot nematodes and the microbial community profiling of the coffee plant rhizomicrobiome in a field trial. All the biological control products and chemical nematicide had not shown significant impact on root-knot nematodes control between control and treatments. The total number of number of galls^{-1} and eggs-1 and plant yield were assessed in two consecutive years, but no significant differences were observed between control and bioproducts applied treatments. Additionally, we evaluated the diversity and community composition of bacteria, fungi and eukaryotes in the rhizosphere soil of bioproducts treated plants and the dominant phyla in bacterial, fungal and community were, *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Ascomycota*, *Mortierellomycota*, and *Ascomycota*, *Cercozoa* respectively in both consecutive years. Overall, no significant difference was observed in bacterial, fungal, and eukaryotic community's diversity in both years of data. The co-occurrence network unearthed that bacterial, fungal and eukaryotic species formed a complicated network structure in all bioproducts applied treatments. Our findings assist in comprehending the introduction of exogenous beneficial microbes into field conditions that exerted selective recruitment implicated in nematode parasitism.

Keywords: Soil microbiome manipulation, soil-borne disease, biological control agent (BCA), Bio-pesticides, Soybean, Coffee, sustainable disease management

SUMMARY

FIRST PART

1 GENERAL INTRODUCTION

Disease-suppressive soils are capable to prevent the specific soil-borne plant pathogens to cause the disease or reduce its limistation of infection in susceptible host though the soil-borne plant pathogens inhabit in surrounding. In most cases, suppressiveness has a microbiological genesis. (BAKER et al., 1975; SIEGEL-HERTZ et al., 2018). The disease suppressive soil categorized into two types (1), General disease suppressive soil, (2) Specific disease suppressive soil. Multitrophic interactions underlie the suppressiveness of soils, which are influenced by soil management techniques that have an effect on overall microbial activity and can be modified (MAZZOLA and GU, 2002; STIRLING et al., 2012). Some soils exhibit a level of plant pathogen-specific suppressiveness that is unique to those soil-borne pathogens. in addition to general suppressiveness. The coordinated actions of specific members of the soil microbial community that cause disruption are linked to specific suppressiveness pathogen's ability to spread disease (ALABOUVETTE, 1999). The best examples of natural plant defense based on microbes are observed in suppressive soils, where plant roots deploy rhizodeposition to enrich, nourish, and promote The first line of defense against soil-borne diseases is soil microorganisms (SCHLATTER et al., 2017).

Characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties (WHIPPS et al.,1988). The term thus not only refers to the microorganisms involved but also encompasses their theatre of activity. Some microbes can be advantageous because they possess genes that enable them to absorb nutrients (BERENDSEN et al., 2012), while other microbes can shield hosts by inhibiting pathogen invasion (SCHLATTER et al., 2017). For instance, a wide range of microorganisms in soils influence the ecology and evolution of plant communities (PUTTEN et al., 2016). Several soil microorganisms form close relationships with plant roots, which may promote plant growth through a variety of mechanisms (MENDES et al., 2015). Suppression of disease is a key mechanism through which soil microbes promote plant fitness and efficiency. In this scenario, a strong and well-functioning soil microbiome act as a plant's first line of defense against soil-borne pathogens within the indigenous soil microbial communities, either direct through antimicrobial substances or parasitism

(MENDES et al., 2013), or indirectly by improving plant immunological reactions (MENDES et al., 2013).

Often, soil microbial communities with the ability to create antimicrobial compounds are linked to plant pathogen suppression. Antibiosis has been specifically connected to the ability to suppress disease, whereby competitive inhibition between antagonistic microorganisms was associated with a reduction in fungal infections (WELLER et al., 2002).

With around 46.5% of the global production of Coffee arabica, or 48.2 million 60-kg green coffee bags in the 2018/2019 harvest, the largest producer and exporter of coffee worldwide is Brazil. (USDA 2020, CONAB 2021). Inadequate nourishment, which makes these plants more vulnerable to infections, is one issue that can lower the productivity of coffee growing, among others (da Silva et al., 2019). One of these, infections with root-knot nematodes (Meloidogyne spp) is a major concern that adversely affects agricultural production across the continent of South America (VALLEJOS-TORRES et al., 2021). Nematode infections negatively affect and have a severe impact on crop production throughout South America and Meloidogyne exigua and Meloidogyne incognita are the most detrimental species to coffee plants (CARNEIRO and COFCEWICZ, 2008).

The root systems of numerous important agricultural crops are damaged by these plant-parasitic nematodes (SCHOUTEDEN et al., 2015). Root-knot nematodes influence the uptake of water and nutrients by causing the development of large cells and root galls (COLLANGE et al., 2011), moreover, to resulting in a mineral shortage that reduce longevity plant life and delays crop production (Anjos et al., 2010). One of the most widely cultivated crop in the world is the soybean. In 2020–2021, over 127 million acres of soybeans were grown, yielding roughly 363 million tons of seeds. (USDA, 2022). Brazil was the top global producer during this time, producing 135 million tons on roughly 38 million acres (CONAB, 2022). A significant crop that offers a sustainable source of protein and oil worldwide is the soybean (Glycine max (L.) Merr.). The production of soybeans is severely hampered by the soybean cyst nematode (Heterodera glycines Ichinohe), a tiny roundworm that feeds on the roots of the bean plant. This nematode is the most economically significant pathogen on soybeans, causing yield losses of more than US$1 billion yearly in the United States alone (LIU et al., 2012).

Heterodera glycines Ichinohe (Nematoda: Heteroderidae) (=SCN), a soybean cyst nematode, is one of the most destructive and challenging to eradicate in this crop. Although it can also reproduce on other Fabaceae, its primary host, the soybean, suffers direct and indirect harm from it (BACK et al., 2002; TRUDGILL and BLOK, 2001). One of the most

severe and challenging pests to eradicate from soybean crops in Brazil and other countries is the soybean cyst nematode (SCN) (BACK et al., 2002; TRUDGILL and BLOK, 2001).

Numerous bacterial species are found in the surrounding region of the root, termed rhizosphere, the region of soil around plant roots, where they produce plant growth regulators, increase nutrient availability, and induce resistance in plants as a first line of defense against soil-borne plant infections. Two of the most studied commercial rhizosphere-colonizing rhizobacterial genera are Pseudomonas and Bacillus (MHATRE et al., 2019). Their colonization in the rhizosphere determines how successfully these rhizospheric bacteria may be used for advantageous tasks including phytostimulation, biofertilization, and biocontrol.

The interaction between plant growth-promoting substances (PGPR) and plant parasitic nematodes has been extensively investigated in order to manage plant parasitic nematodes successfully. PGPR were reported to be a promising agent to lessen damage caused by plant parasitic nematodes. Rhizospheric microorganisms can promote plant growth by creating a range of chemicals that do so and by destroying phytopathogens and nematodes (RASHAD et al., 2015; TABATABAEI and SAEEDIZADEH, 2017). The primary opponents of PPNs from the rhizosphere are representatives of the aerobic endospore-forming bacterial group, Pseudomonas sp. and Bacillus sp. Numerous Bacillus strains can inhibit nematodes while fostering plant growth. Additionally, Bacillus sp. found a direct antagonistic relationship with PPNs Meloidogyne, Heterodera, and Rotylenchulus (LI et al., 2005). Additionally, Pseudomonas rhizospheric strains have harmful defenses against PPNs (SIDDIQUI et al., 2005). Studies to comprehend the mechanisms were carried out using the synthesis of antibiotics and the development of systemic resistance. underlying the decrease in PPN populations during the interaction between Pseudomonas and PPN (SIDDIQUI and SHAHID SHAUKAT, 2003)

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FIRST PART - ARTICLES

Article 1- New opportunities in plant microbiome engineering for increasing agricultural sustainability under stressful conditions

Muhammad Siddique Afridi [1], Muhammad Ammar Javed [2], Sher Ali [3], Flavio Henrique Vasconcelos De Medeiros [1], Baber Ali [4], Abdul Salam [5], Sumaira [6], Romina Alina Marc [7], Dalal Hussien M. Alkhalifah [8], Samy Selim [9], Gustavo Santoyo [10]*

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1 Department of Plant Pathology, Federal University of Lavras, CP3037,37200-900, Lavras, MG, Brazil
2 Institute of Industrial Biotechnology, Government College University, Lahore, 54000, Pakistan
NMR Lab, Department of Chemistry, Federal University of Paraná, Curitiba 81530-900, PR, Brazil
Department of Plant Sciences, Quaid-i-Azam University, Islamabad 45320, Pakistan
Zhejiang Key Lab of Crop Germplasm, Department of Agronomy, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, China
Department of Biotechnology, Quaid-i-Azam University, Islamabad 45320, Pakistan
Food Engineering Department, Faculty of food Science and Technology, University of Agricultural Science and Veterinary Medicine Cluj-Napoca, 3-5 Calea Mănă ștur Street, 400372 Cluj-Napoca, Romania
Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia
Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Jouf University, Sakaka 72388, Saudi Arabia
Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Mich., 58030, México
*Corresponding Author: Email: gustavo.santoyo@umich.mx

Abstract

Plant microbiome (or phytomicrobiome) engineering (PME) is an anticipated untapped alternative strategy that could be exploited for plant growth, health and productivity under different environmental conditions. It has been proven that the phytomicrobiome has crucial contributions to plant health, pathogen control and tolerance under drastic environmental (a)biotic constraints. Consistent with plant health and safety, in this article we address the fundamental role of plant microbiome and its insights in plant health and productivity. We also explore the potential of plant microbiome under environmental restrictions and the proposition of improving microbial functions that can be supportive for better plant growth and production. Understanding the crucial role of plant associated microbial communities, we propose how the associated microbial actions could be enhanced to improve plant growth-promoting mechanisms, with a particular emphasis on plant beneficial fungi. Additionally, we suggest the possible plant strategies to adapt to a hrsh environment by manipulating plant microbiomes. However, our current understanding of the microbiome is still in its infancy, and the major perturbations, such as anthropocentric actions, are not fully understood. Therefore, this work highlights the importance of manipulating the beneficial plant microbiome to create more sustainable agriculture, particularly under different environmental stressors.

Keywords: Plant microbiome, Fungi, Sustainable agriculture, Biotic and biotic constraints.

Introduction

Different researchers have highlighted that by 2050, it is expected that the world population will reach 10 billion people. The massive surge in population will increase the amount of food necessary for the entire planet to be fed. However, food could be a problem for this drastically increased population. Even today, approximately 9% of the world's population (690 million people) go to bed with an empty stomach each night (Sakschewski et al., 2014). Combining these challenges without compromising the environment and human health is a major issue in the agricultural production sector and the forefront of many plant scientists.

To achieve this goal, it will be obligatory to engage two closely associated goals. The first is to improve crop yield, especially for cereal crops, which can be accomplished through different procedures, such as genetic modification, selective breeding, avoiding waste in irrigation as well as fertilization regimes (Beddington, 2010; Godfray et al., 2010). Second, curtail crop losses due to pests and diseases, which have been causing losses on the order of 20–40%, in addition to the indirect effects on livelihoods and the environment (OERKE, 2006; Beddington, 2010; Godfray et al., 2010; Savary et al., 2012; McDonald and Stukenbrock, 2016). Implementing strategies to attain the latter is challenging, particularly because the elements that corroborate plant maladies are extremely complex and multivariate (Savary et al., 2012). Moreover, cereal crops are affected by several different organisms, e.g., a variety of bacteria, fungi, oomycetes, nematodes and viruses (Dean et al., 2012).

Fungal species competence to survive in soil mainly invade the plant roots, causing various notorious diseases in plants while simultaneously undermining the host plant of its nutrients; this is the case for wheat disease caused by Gaeumannomyces graminis var. triciti, which in some cases can eradicate an entire wheat crop. Thus, worldwide, the take-all of wheat is considered the most important root ailment of wheat (Coombs, 2004; Kwak and Weller, 2013; Cook et al., 2015; Hernández-Restrepo et al., 2016; Ahmad et al., 2022). Plant-parasitic nematodes living in the same vicinity as plant roots are among the most destructive plant pathogens, causing estimated damage of more than US$100 billion per year. An expert-based assessment of crop health listed nematodes as among the most damaging pests and pathogens for different crops (Savary et al., 2012). To avoid crop losses due to maladies, chemical pesticides are routinely applied on crops, with the main goal of eradicating or lessening the disease invasion, infection or severity. However, it is becoming

increasingly clear that long-term chemical pesticide usage poses several adverse effects on the environment and human health (Sanyal and Shrestha, 2008; Kortekamp, 2011). For instance, a myriad of pesticides can cause acute and chronic toxicity in humans, and they are progressively being shown to cause widespread damage to the broader ecosystem, affecting nontarget organisms, such as pollinator species, and soil pollution and water (Arora and Sahni, 2016; Grewal et al., 2017). These nontarget effects can also extend to reduce the beneficial microbial diversity within soil, which in turn refrains and suppresses the available populations of pathogens from competition and elevates the risks of pathogen invasion and colonization of plant tissues (Jacobsen and Hjelmsø, 2014). Additionally, plant pathogen genetic evolution and resistance against various resistant bread crop varieties can be devastating outcomes of the continuous application of pesticides that pathogens can rapidly evoke plant host resistance mechanisms, especially when only a single gene is responsible for resistance. In certain circumstances, there are many crop species for which resistant cultivars are unavailable. For instance, every 2-3 years, rice cultivars that are usually resistant to M. oryzae typically become ineffective. These combined issues have opened up ways to search for another alternative. Plant-associated microbiomes have essential functions in improving plant nutrition acquisition and provide protection against biotic and abiotic stressors.

Nutrient acquisition has been thoroughly studied for plant symbioses with arbuscular mycorrhizal fungi (AMF) and Rhizobium bacteria (Bergelson et al., 2019; Trivedi et al., 2020). Additionally, these diverse microbial communities of plant microbiome perform multiple functions such as nitrogen fixation, nutrient solubilization, protection against devastating plant pathogens and production of phytohormones like indole acetic acid, auxin, gibberellin, abscisic acid, aminocyclopropane-1-carboxylate deaminase, antibiotics, development of induced resistance to pathogens in plants, and promotion of the population of other helpful microorganisms (Afridi et al., 2019; Afridi et al., 2021; Zainab et al., 2021; Mehmood et al., 2021; Jain, 2012). Manipulation of the soil microbiome for plant growth and protection is considered one of the possible avenues in previous decades. The soil microbiome has complex interactions with the plant and its roots, helping to remove contaminants, provide nutrients, and proliferate growth (Liu et al., 2019). Continued research into this subject matter is necessary to elucidate the complex interactions that occur so that manipulating these relations may be used to help feed 10 billion people. Therefore, this review aimed to highlight the beneficial services of the plant-associated microbiome to be

manipulated and optimized, resulting in better agricultural production, even under nonoptimal conditions.

Defining the plant microbiome

Plants are associated with a diverse group of microbes, such as bacteria, oomycetes, fungi, archaea, and viruses, through three major associations, the rhizosphere (root-attached soil), endosphere (internal tissue), and phyllosphere (aboveground parts), which execute significant activities that influence host health and fitness and inhabit a well-defined area of plant microbiome. Among them, the rhizosphere is the most complex and diverse niche of microbial communities (Lakshmanan et al., 2014; Bandyopadhyay et al., 2017).

Plants have evolved to form complex, beneficial relationships with the microorganisms in their surroundings. Although the plant microbiome includes bacteria, fungi, archaea, protists and viruses, the majority of research has focused on bacterial and fungal communities (Trivedi et al., 2020). These organisms play important roles in the health and productivity of crops by forming complex co-association with plants (Fitzpatrick et al., 2018). In particular, plant-associated microbiota and plants form a ‘holobiont’, and evolutionary selection among microbes and plants contributes to the stability of the ecosystem (Hamonts et al., 2018; Xu et al., 2018a). Recently, developed culture-independent high-throughput sequencing has accelerated the identification of microbial communities inhabiting the surrounding spaces, as well as inside tissues and surfaces of plants, and demonstrated the existence of microbial lineage subsets, termed ‘core microbiota’, which reproducibly make contacts with host plants across a wide range of environmental conditions (Bergelson et al., 2019; Roman-reyna et al., 2019).In terms of therapeutic or diagnostic benefits and technical advancements, the study of the microbial community has been a leading interest amongst scientific society. In addition to compensation, all or some of these microbes actively support plant improvement (Parray and Shameem, 2019). In accordance with distribution, these microbes can be found in the phyllosphere (above the ground–stem and tissues), endosphere (underground–tissues within the plant) and rhizosphere (roots alongside growth layers) of the host (Figure 1).

This is because the plant anatomy represents and provides a remarkably suitable environment for these microbes (Schlaeppi and Bulgarelli, 2015a). Over the past decades, individual microbes from these microbiomes have displayed exceptional features (Rani et al.,

2019; Gupta et al., 2020) containing their interactions with the host. The symbiotic association has been determined to be pathogenic and/or nonpathogenic to the host plants, including nitrogen fixation, development, bioremediation and stress tolerability (Chhabra and Dowling, 2017; Roth and Paszkowski, 2017; Li et al., 2019). To overview an extended mutualistic to parasitic and commensalism dealing, plants correlated with the microbiota cover a large portion. Additionally, the study of this connection may lead to in-depth knowledge and could provide appreciative outputs.

According to the growth of the global population, a sustainable environment of high food security is urgently needed, which is achieved mostly by strengthening crop practices. In this regard, the microbial system has been a key technology in such progress. Since ~300 BC, this goal has been founded by the manipulation of the soil microbiome (Vessey, 2003), which is a key to the green revolution (Parnell et al., 2016). It is interesting to note that soil microbiomes are now touted as a cornerstone of the next green revolution.

The plant microbiome at work

The microbiome, as a 'second genome' of organisms, including plants, has a mutualistic relation with health and general well-being. Taking this into consideration, Figure 1 depicts a holistic overview of the plant microbiome with some attributes, signalling and cross-talk between the plant and its relevant biota. This mutualism can be direct and/or indirect; plant–plant, microbe–microbe, plant–microbe, and/or with some microbe–microbe and macrosoil eukaryote interactions (Tarkka et al., 2008). In addition, these interactions could be classified into competition, parasitism, mutualism and commensalism. Being more common, the latter two interactions provide major benefits to one or both interacting species.

Microbial services

Within this context, the manipulation of the phytomicrobiome can be of greater interest to boost diagnostics and therapies in plants, which are extendable to animals and humans in the future (Zmora et al., 2016). However, the phytomicrobiome is generally associated with multiple microorganisms that are major factors for agricultural production and play a critical function. Agricultural sustainability has been a major proposal in the world and has been completed by the implementation of many microorganisms. In fact, some of these microbes colonized the plant roots, improve plant growth and regulate vital

functions against detrimental pathogens and thereby lead to plant productivity (van der Heijden and Hartmann, 2016; Cordovez et al., 2019; Rafique et al., 2019). The world is transitioning to ecologically safe and economically effective approaches that could be used to promote agricultural productivity. Therefore, a balanced farming system is critical in terms of the survival of Earth. In this regard, crop output per unit area of land must be raised to fulfil the demand for food (Doran, 2002). As per recommendations, an equivalent improvement in plant health could be achieved via various strategies. Among them, PGPR, as probiotics for plant roots and prebiotic substrates/additives, can be used to cause compositional alterations in the phytomicrobiome and are termed soil amendments. The plant microbiome has a strong influence on nutrient availability and the growth and development of the host (Carvalhais et al., 2013). Accordingly, plants on the basis of natural exudate recruit and "engineer" a local microbiome (Kumar et al., 2018b; Rojas-Solís et al., 2018) and make this habitat fit to their survival.

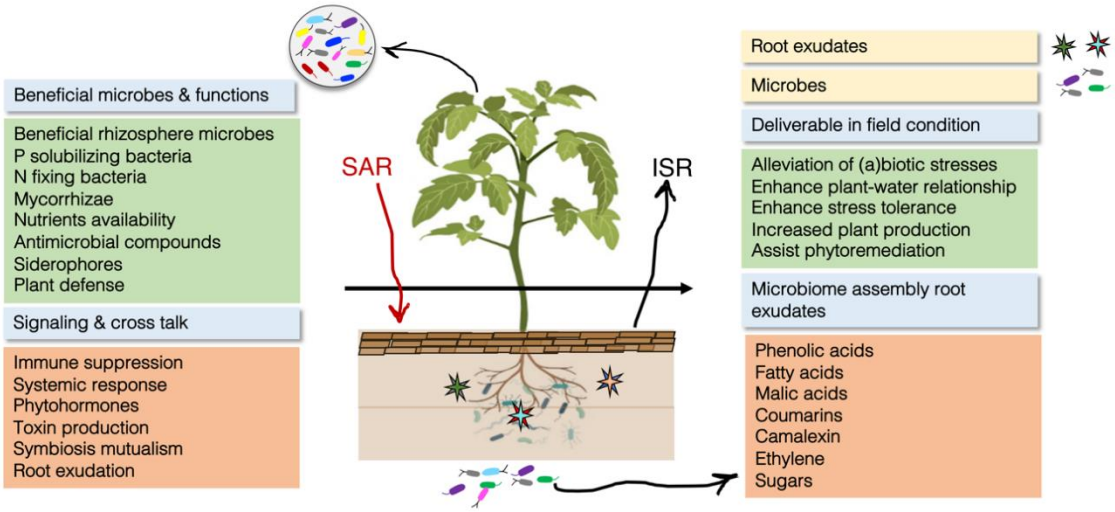

Figure 1. The holistic overview of plant microbiome compositions, the interaction between plant and its associated microbes, function and its positive effect on plant growth and development under extreme conditions. Plant recruit and assembly beneficial microbes via exudation and constitute a healthy and beneficial microbial community. This microbiome improves plant health, alleviates abiotic stresses and provides a safeguard to the host exhibiting various direct and indirect mechanisms

Signalling and Cross-talk

In general, plants of the local habitat are in cross-talk with numerous surrounding stimuli incorporating the microbial communities (Figure 1); therefore, this is termed the homeostatic photomicrobiome. In such a homeostatic phytomicrobiome, plants are allowed to sense and properly respond to any interactive stimulus of the system. However, after microbial substance recognition, they can ultimately lead to mutualism or immunity. Furthermore, communicatory signalling is an important phenomenon responsible for healthy lifestyles and the survival of organisms (Cook et al., 2015; Müller et al., 2016). This communicatory network can be predicted for any of the micro- or macroorganisms living on the planet, such as quorum sensing bacteria (Cornforth et al., 2014), whales (Parks et al., 2015) and those across the tree of life. Such communicating circuitry plays a decisive role in the evolution of the life of associated organisms (West et al., 2015). Overlooking such a communicatory web, chemical signalling is highly vital and participates in perception and modulation in stationary organisms, such as plants. However, plants use these chemical bases as signals to maintain mutual links with presided microbes either on the aerial (trunk, shoots, leaves, etc.) and/or the underground parts (roots). As per estimation, approximately 5–20% photosynthetically fixed carbon has been an active ingredient in plant rhizosphere-inducing microbes for healthier microbial community formation (Horst Marschner, 1995). In addition to carbon, microorganisms discharge many more signalling chemical substances to the rhizosphere. Through them, the most prominent are phytohormones, extracellular enzymes, organic acids, antibiotics, volatile contents and surface factors, e.g., immunomodulatory precursors such as flagellins and lipopolysaccharides in Pseudomonas (PING, 2004; Dangl et al., 2013).

As a signalling molecule, quorum sensing, e.g., N-acyl-homoserine lactones (AHLs), when secreted, is used to regulate gene expression by plant-associated bacteria (Berendsen et al., 2012a). However, AHLs have been major precursors affecting root development in a model plant of Arabidopsis (Ortíz-Castro et al., 2008). Moreover, AHLs have the tendency to elicit “systemic resistance” (ISR) that allows plants to evade lethal pathogens without requiring bacterial factors. This effect can be a systemic mechanism because the roots are inoculated with manifold plant growth-promoting rhizobacteria (PGPR), such as *Pseudomonas, Burkholderia* and *Bacillus* sp. that turn host plants nonsusceptible to invaders (Schuhegger et al., 2006; Choudhary et al., 2007; Tarkka et al., 2008). In line, such a microbial combination is essential and responsible for fitness and plant health and beyond

fulfilling fundamental demands (water, nutrients, etc.), they increase the tolerability of plants against any of the (a)biotic stressors (van der Heijden and Hartmann, 2016; Cordovez et al., 2019). This association provides the main benefits to soil biochemistry to suppress soil-borne diseases and detrimental pathogens. It is noteworthy that these pathogens may still be present but in an inactive state that would not be able to cause soil-borne diseases or damage their resident host, while this setup can be termed "soil suppression".

The relevant role of plant-associated fungi and bacteria

Plants can be associated with an immense diversity of microorganisms, including fungi. There is sufficient evidence that some fungi, such as arbuscular mycorrhizal fungi (AMF), can provide broad benefits to the plant in a type of symbiotic interaction. AMF are obligate biotrophic organisms that supply mineral nutrients to the host plant and, in return, receive carbon derived from photosynthesis. In this same sense, AMF can modulate carbon distribution in plants by modifying the expression and activity of key enzymes for the synthesis, transport and/or catabolism of carbon compounds, such as sucrose. Since sucrose can be essential for the maintenance of all metabolic and physiological processes, the modifications addressed by AMF can significantly affect plant development and responses to stress. Additionally, the interaction between AMF and plants can also host lipid biosynthesis to acquire storage reserves and generate biomass.

Other fungal species that provide various services to the plant are Trichoderma spp. richoderma (teleomorph Hypocrea) is a fungal genus that inhabits many ecosystems, including those involved in agricultural and production practices. There are several examples of how Trichoderma is part of microbial bioinoculants, either individually or carrying out synergistic interactions with other microorganisms, such as plant growth-promoting bacteria or PGPB. *Trichoderma* species, such as *T. harzianum*, *T. viride*, and *T. virens*, among many more species, can ameliorate the severity of plant diseases by inhibiting the growth of phytopathogens in the soil (mainly), since they exhibit antagonistic and mycoparasitic activities. Additionally, it has been reported that *Trichoderma* spp. It is also capable of interacting directly with the roots, which leads to promoting the growth and development of vegetable crops, as well as of course, stimulating resistance to diseases and tolerance to multiple types of environmental stress, such as salinity or drought to name a few. To further explore topics on the importance of plant-associated fungi and their beneficial role, readers

are directed literature (Santoyo et al., 2021).

The root surfaces tightly adhering to the rhizosphere's soil interface colonize these plant growth-promoting rhizobacteria (PGPR) (Jain, 2016). PGPR-mediated biocontrol processes are wide-ranging, like availability of nutrients and ecological niches, synthesis of allelochemicals including enzymes and antibiotics, development of induced resistance to pathogens in plants, and promotion of the population of other helpful microorganisms (Table 1) (Jain, 2012). The best-known PGPR that colonizes in the rhizosphere strains are *Bacillus, Rhizobium, Acinetobacter, Alcaligenes, , Arthrobacter, Enterobacter, Pseudomonas, Serratia,* and *Burkholderia* (Vinayarani and Prakash, 2018; Mehmood et a., 2021) successfully induce disease resistance against the bacterial pathogen in plants, including *R. solanacearum* (Cao et al., 2018), *E. carotovora* (Chandrasekaran and Chun, 2016), *D. solani*, *E. amylovora,* and *P. carotovorum* (Vega et al., 2019). Both growth promotion and biological control can regulate by the same strain of PGPR. Generally, biological control of these bacteria relies on direct or indirect modes of action; however, all these mechanisms are highly influenced by the type of host plants (Dey et al., 2004; Singh et al., 2019). In direct mechanism, pathogens directly affected by the production of metabolites, for instance, antibiotics, hydrogen cyanide (HCN), iron-chelating siderophores, pyoluteorin, tensin, 2,4-diacetylphloroglucinol, phenazines, viscosinamide, and other cell wall-degrading enzymes, while another mechanism is known as induced systemic resistance, this happens by the intervention of an inducing agent that systemically stimulates the chemical or physical defensive mechanisms of the host plant, resulting in decreased symptoms of pathogens that invade tissues distal to the inducer (Table 1) (Khatoon et al., 2020; Raj et al., 2020).

Why engineer the Plant microbiome?

In light of the intensification of cropping practices and changing climatic conditions, nourishing a growing global population requires optimizing environmental sustainability and reducing the ecosystem impacts of food production. The use of microbiological systems to ameliorate agricultural production in a sustainable and eco-friendly way is widely accepted as a future key technology. The manipulation of soil microbiomes to optimize crop productivity is an ancient practice; records can be traced to ~ 300 BC (Vessey, 2003). It is interesting to note that soil microbiomes are now touted as a cornerstone of the next green revolution (Parnell et al., 2016). In addition, the continuous growth of the world population demands that the global availability of food be one of the major concerns in the near future.

According to the projected data (DESA, 2019), if this increment continues, in turn, the demands for food will reciprocally increase by 8.5 billion in 2030, 9.7 billion in 2050, and 11 billion by 2100. However, the fulfilment of such demand must be ascertained with green and innovative technologies incorporating plant and microbial resources.

Environmental stressors have caused major alterations in plant physiology and biochemistry that lead to significant reductions in plant yield and production. In accordance with previous reports (Kumar et al., 2018a), 30-50% of agricultural losses have been impacted by unfavourable environmental conditions. Agronomic loss coupled with continual population growth demands at least a 60% boost in agrarian production to meet food demand on a larger scale (Wild, 2003). Often, the agricultural production has mostly been supplemented with pesticides. Consistently, approximately 2 million tons of pesticides are globally administered to reduce causative pests, aiming for maximum crop production (Foong et al., 2020). Concurrently, the use of agrochemicals influences biodiversity and soil fertility, biochemistry, agricultural sustainability, food safety and nutritional security, among others. However, excessive use of pesticides not only produces environmental pollution, but over time, their drastic chemical substances can cause diseases in humans and livestock (Sharma et al., 2010; Fu et al., 2022). Additionally, they kill beneficial microbes and reduce nutrient availability, which are essential elements for plant growth and productivity (Meena et al., 2020). Thus, the plant microbiome contributes to the basic functions of microbial ecosystem services in agriculture, plant production and performance, nutrition, improved quality of the soil, and tolerance to (a)biotic stresses (Figure 1) (Quiza et al., 2015; Vandenkoornhuyse et al., 2015; Enebe and Babalola, 2018; Ojuederie et al., 2019). The plant microbiome supports plants through the mechanisms of regulating hormones, specific antagonistic metabolite (rhizobitoxine) production that induces resistance against drastic pathogens, suppression of soil-borne disease, antibiosis, and competition for nutrients in the rhizosphere (Choudhary et al., 2007; Penton et al., 2014; Reitz et al., 2015; Zhang et al., 2019; Rodríguez et al., 2020).

Therefore, plant microbiome engineering is an alternative but an untapped strategy that can be exploited for plant health, growth, and productivity under extreme conditions. Recently, a number of accessible approaches have been proposed for plant microbiome engineering (Figure 2). (Arif et al., 2020; Kumar and Dubey, 2020). An interesting avenue is to harness variations in exudation patterns to enhance the beneficial rhizosphere microbiome (Quiza et al., 2015). The microbiome can be engineered by traditionally amending soil with

(in)organic supplementation and agricultural practices to promote microbial diversity, functions and interactions with the targeted host (Figure 2) (Sankar Ganesh et al., 2017; Saeid and Chojnacka, 2019). Therefore, the living components of the rhizosphere can be engineered to promote plant health and growth, two features that strongly depend upon the interactions of living organisms with their environment (Dessaux et al., 2016). Thus, aiming at viable agronomic production, several innovative tools could play a central role by improving microbial bioengineering that is beneficial to replace lethal agrochemicalsubstance

Table.1 lant growth promoting microbes underpinning plant growth and enhance tolerance against biotic and abiotic stresses employing various mechanisms

Host species	PGPR	Functions/Response	Reference
Arabidopsis thaliana	*B. phytofirmans* PsJN	Abscisic acid signalling, proline and ROS production	Pinedo et al. (2015a)
Arabidopsis thaliana	*B. subtilis* GB03	Import of Sodium ions in root	Wang et al. (2016)
Arabidopsis thaliana	*P. yonginensis* DCY84T	ROS Detoxification , Sodium ion homeostasis	Sukweenadhi et al. (2015)
Abelmoschus esculentus	*Enterobacter* sp. UPMR18	ROS pathway Antioxidant enzymes production	Habib et al. (2016a)
Glycine max	*P. simiae strain* AU	Antioxidant enzymes Production	(Vaishnav et al. (2016a)
Glycine max	*B. firmus* SW5	Production of antioxidant enzymes, salinity tolerance,	El-Esawi et al. (2018)
Gossypium hirsutum	*Brucella* sp. PS4	Pesticide degradation	Ahmad et al. (2022)
Puccinellia tenuiflora	*B. subtilis* GB03	Modulation of Na^+ homeostasis	Niu et al. (2016a)
Saccharum officinarum	*B. xiamenensis*	Phytoremediation	Zainab et al. (2021)
Solanum lycopersicum	*B. megaterium*	Metallothionein Glutathione reductase enzyme synthesis	Zameer et al. (2016a)
Solanum lycopersicum	*E. cloacae* PM23	ROS Detoxification , Sodium ion homeostasis	Ali et al. (2022 b)
Solanum lycopersicum	B. safensis (SCAL1)	Heat Stress	Mukhtar et al. (2022)
Solanum lycopersicum	*B. anthracis* PM21	Phytoremediation	Ali et al. (2021)
Solanum tuberosum	*B. subtilis* PM32	Fungal diseases biocontrol	Mehmood et al. (2021)
Solanum tuberosum	*B. mycoides* PM35	Proline production, and ROS scavenging	Ali et al. (2022a)
Solanum lycopersicum L.	B. safensis Strain SCAL1	Produced exopolysaccharide and ACC deaminase	Mukhtar et al. (2022)
Zea mays L.	*B. amyloliquefaciens* SQR9	Photosynthesis, Na^+ export, and sequestration	Chen et al. (2016b)
Lettuce microcosms	*T. hamatum GD12*	*N-acetyl-β-d*-glucosaminidase genes	Ryder et al. (2012)
Curcuma longa L	*T. harzianum* TharDOB-31	Indole-3-acetic acid hydrogen cyanide production	Vinayarani et al. (2018)
Solanum lycopersicum	*A. pullulans 490*	Produces biosurfactants, biocontrol activity	Köhl et al. (2020)
Solanum lycopersicum	*C. rosea 016*	Produces biosurfactants, biocontrol activity	Köhl et al. (2020)
Capsicum annuum L	*Beauveria bassiana*	Niche or resources and antibiosis	Jaber and Alananbeh (2018)
Pinus radiata	*F. circinatum*	Antagonism,	Martínez et al. (2016)
Poncirus trifoliata	*F. mosseae*	Drought stress, Hyphal water absorption rate	Zhang et al. (2018)
Triticum aestivum	*G. mosseae*	Drought stress, osmotic potential, antioxidant enzymes	Rani (2016)
Triticum aestivum L.	*R. irregularis*	Heat stress, nutrient allocation nutrient composition in root	Cabral et a. (2016)

Zea mays	*R. intraradices*	High temperature, enhanced transpiration photosynthetic rate	Mathur et al. (2016)
Solanum lycopersicum	*R. irregulari*	High temperature, Enhanced photosynthetic phosphorylation	Calvo-Polanco et al. (2016)
Cucumis sativus L.	*G. intraradices*	Salinity stress, enhanced antioxidant enzymes, biomass	Hashem et al. (2018)
Solanum lycopersicum L.	*R. irregularis*	Salinity stress, Enhanced biomass and growth hormones	Khalloufi et al. (2017)

Engineering the plant microbiome for green agricultural production

In addition to protection, plant microbiomes provide key benefits regarding better health, with improved growth and production and plant environmental adaptation (Haney et al., 2015; Berg et al., 2016). Most microorganisms are found in such a biome that they tend to cause physiological alterations and allow plants to survive detrimental invasions (Dubey et al., 2019; Santoyo et al., 2021). Within the microbiome, these microbes are clustered on the surface and tissues of the host plants. The bimodal association thereby allows nutrient acquisition, promoting the growth and resilience of the host against environmental stressors (van der Heijden and Hartmann, 2016; Cordovez et al., 2019).

The traits displayed by the microbiome community are of high relevance to plant health, yet they are influenced by microbial diversity, unwanted conditions and even host plant species (Jain et al., 2020). The entire microbiome is not involved in corresponding functions; however, they are performed by unique microbial species because of synergistic effects between two or more strains (Rojas-Solís et al., 2018). The manipulation of the bacterial microbiome and the production of bioinoculants have enabled scientists to control and properly monitor plant health and production (Adesemoye et al., 2009). In this regard, several strategies, including soil amendment, artificial microbial consortia and host-dependent microbiome engineering, have been proposed that could strengthen stress tolerance, disease resistance and nutrient acquisition in host plants (Figure 2) A traditional method of soil engineering or amendments is adding (in)organic substances directly to soil or using alternative agricultural tools. Any of these sources guide farmers to manipulate plant–microbiome interactions to increase crop production (Wang et al., 2015; Sankar Ganesh et al., 2017).Conforming reported data, a host-mediated microbiome engineering approach is a host-based indirect selection of proper microbes and leveraging out those that are influential to the microbiome in context (Mueller and Sachs, 2015). In addition, an “artificial microbial consortium (AMC)” has also been used in microbiome engineering.

A recent example of biostimulant consortium application in phytomicrobiome for enhancement productivity of chickpea and soil health was conducted by (Mukherjee etal., 2022). These experiments were carried out in two different locations like Banaras Hindu University Varanasi, and Sarai Dangri village, Uttar Pradesh India. Microbial strains BHUJPCS-15 and BHUJPVCRS-1 were isolated from chickpea seedand chickpea rhizosphere soil respectively. This study depicts that consortium significantly increased yield

NPK, microbial counts & soil enzymes. Interestingly, the results showed that microbiome manipulation via potential biostimulant consortium directly influenced the yields and soil health. Recently Bernard et al (2021) explored in their article that mostly plant attract and beneficial microbes.

This study further highlighted that bacterial consortia assist plants in various ways such as promoting plant growth and providing protection to hosts from a wide range of direct and indirect environmental stresses. This study also suggests that the microbiome could be engineered by engineering plant seeds to contain desired bacterial strains. It is unquestioned that Phytomicrobiome is an untapped source which might be potentially resolved the current and future challenges of sustainable agriculture and food security. But at the same time biotic and abiotic constraints substantially imbalance the functionality of phytomicrobiome and we are unable to overlook them (Chouhan et al., 2021). This study also recommends and shaded light on the potential of Culturable PGPR and endophytes that could be harnessed for resilient microbiome engineering.

However, in this functional consortium, an established complex interactive network of different microbes in the rhizosphere environment has been essential (Kumar et al., 2018b). Other than the rhizosphere, microbes can also be found in the root part that permits only useful microbes to access plants as endophytes (Rojas-Solís et al., 2018). As a key benefit, AMC via microbiome engineering can be used to modify the respective phytomicrobiome. An ideal AMC fabrication is based on a systematic method that can contain a series of crucial steps. Similarly, active microbe selection and regulation of their mutual interactions, excavation along the culturing core microbiota to evaluate consortium efficacy (Kong et al., 2018), are major parts of the process utilized in AMC production (Figure 2). Additionally, genotype-dependent host microbiome engineering has been harnessed for microbiome engineering to enhance host functions and induce resistance in diverse environments. The genetic bases of plants are fundamental for the shaping and functioning of microcosms (Arif et al., 2020), such as Pseudomonas simiae WCS417r, for improved biomass production in Arabidopsis (Wintermans et al., 2016). This indicates a genetic relation of Arabidopsis loci (controlling plant defence and cell wall integrity) with phyllospheric bacteria (Horton et al., 2014). It has also been proven that plants can expel bacterial species into the rhizosphere, but the mechanisms by which useful or harmful microbes exchange with related holobionts are unknown.

Improving plant growth-promoting mechanisms

The microbiome is composed of several different types of organisms, including bacteria, fungi, protozoa, archaea, and viruses (Mueller and Sachs, 2015). This array of microbial communities plays a pivotal role in the functioning of plants by influencing their physiology and development (Mendes et al., 2013). Plant microbiomes can play a beneficial role, protecting the plant from potential pathogens, improving plant growth and fitness and inducing tolerance to abiotic stresses (Haney et al., 2015; Berg et al., 2016).

Unsurprisingly, the rhizosphere microbiome also inherits soil-borne plant pathogens that colonize plant roots and successfully hack plant innate immunity by breaking the preventive microbial shield of beneficial microbes and causing disease (Mendes et al., 2013). However, it has been proven in various studies that plants secrete small molecules for the recruitment of actively beneficial microflora to assist their conformation under extreme conditions (Busby et al., 2014). It is well known that plants and associated microbes establish symbiotic relationships that facilitate nutrient acquisition and induce resistance in unfavourable environments. However, the plant unable to distinguish beneficial microbes and restrict the formation of pathogenic associations is still unknown (Zipfel and Oldroyd, 2017). It is well documented that the interactions between plants and their microbiomes are mediated by metabolic signalling. Plant release 20-35% photosynthetic carbon into the rhizosphere in the form of metabolites that recruit beneficial microbes (Figure 1). These microbes symbiotically associate with host plants and underpin them under adverse conditions (Arif et al., 2020; Trivedi et al., 2020).

However, concomitantly, the rhizosphere is also a playground and battlefield for soil-borne pathogens that establish parasitic relationships with host plants. Moreover, the diversity and population ratio of plant pathogens and beneficial microorganisms are linked to the amount and quality of plant root exudates and microbial interactions in the rhizosphere (Somers et al., 2004; Raaijmakers et al., 2009). For example, the model plant Sorghum bicolor secretes specific metabolites, which facilitates bacterial ATP-binding cassette transporter gene expression and, in turn, modifies the root-associated microbiome composition by promoting the abundance and activity of monoderm bacteria, which has a positive impact on the growth and development of Sorghum bicolor plants facing drought stress (Xu et al., 2018b). This is a potential blueprint for developing SynComs from such plant-associated microbiomes to increase crop productivity in arid areas with low precipitation and poor irrigation systems. Understanding the substantial role of metabolites

and biotechnological approaches might help to unravel the mechanisms underlying beneficial microbe recruitment for microbiome engineering.

Enhancing phytoremediation activities

Phytoremediation is an environmentally friendly, solar-powered and cost-effective soil remediation technology. Based on plant ability, this technology has to do with the already existing contamination in the system biome, where it intercepts, takes up, accumulates and translocates contaminants (Pilon-Smits, 2005). The efficiency of phytoremediation depends on plants (Vangronsveld et al., 2009), contaminant concentration, soil pH, nutrients and oxidoreduction (Sessitsch et al., 2013) as well as those microorganisms that are associated with soil and plants, respectively. Phytoremediation, instead of a better technology, has often been observed with nonuniform results at the field scale, slow and incomplete degradation, and long clean-up processes (Vangronsveld et al., 2009; Stephenson and Black, 2014). To date, the improvement in soil, contaminant availability and accessibility (de La Torre-Roche et al., 2012), plant growth (Sessitsch et al., 2013), and exploration for the exploitation of soil and plant-associated organisms in phytoremediation (Barac et al., 2004a; Abhilash et al., 2012) have been main topics of interest.

In recent decades, many approaches have been focused on individual organisms rather than on integrated meta-organisms, while in such regards, the potential impact has been limited. Improved phytoremediation necessitates a central understanding of plant–microbe interactions, and responses to pollutants can be of high relevance. In line, the comprehension of how the host combines the beneficial microbiome and its function under contaminant stress is unavoidable. Molecular data and ecological models in this regard have clarified the assemblage of fewer insects (Scheuring and Yu, 2012), respectively.

Beyond plants and related microorganisms, the metaorganism has shown successful improvement in agriculture practices (Mendes et al., 2013; Berg et al., 2014) and disease mitigation (Berendsen et al., 2012a) and has uncovered mutual interactions between plants and unlimited degradative microbial taxa. It has been declared that the plant microbiome can be helpful in extending the functional potential of targeted hosts. Therefore, such a microbiome enables regulation of the expression of traits in plants, thus strengthening physiological state and tolerance (Mendes et al., 2013). However, it can be emphasized that the phytoremediation is microbiome dependent. Moreover, it is accepted that hosts assemble non-random sets of microbial symbionts with a higher proportion of beneficial microbes than

expected. With respect to polluted soil, a host plant is free to choose microbes with degradative genes within a pool of candidates in bulk soil (Siciliano et al., 2001a), but a full understanding of how hosts carry the process is lacking. Expressively, hosts can be found with a mutualistic symbiosis of plant growth-promoting rhizobacteria (PGPR) and mycorrhizal fungi. Within this symbiotic association, plants provide root exudates and produce a microbial habitat, while PGPR degradative bacteria and mycorrhiza sponsor plant growth and detoxify the environment. In the presence of contamination, the rhizosphere and root microbial communities are strongly damaged (Siciliano et al., 2001a).

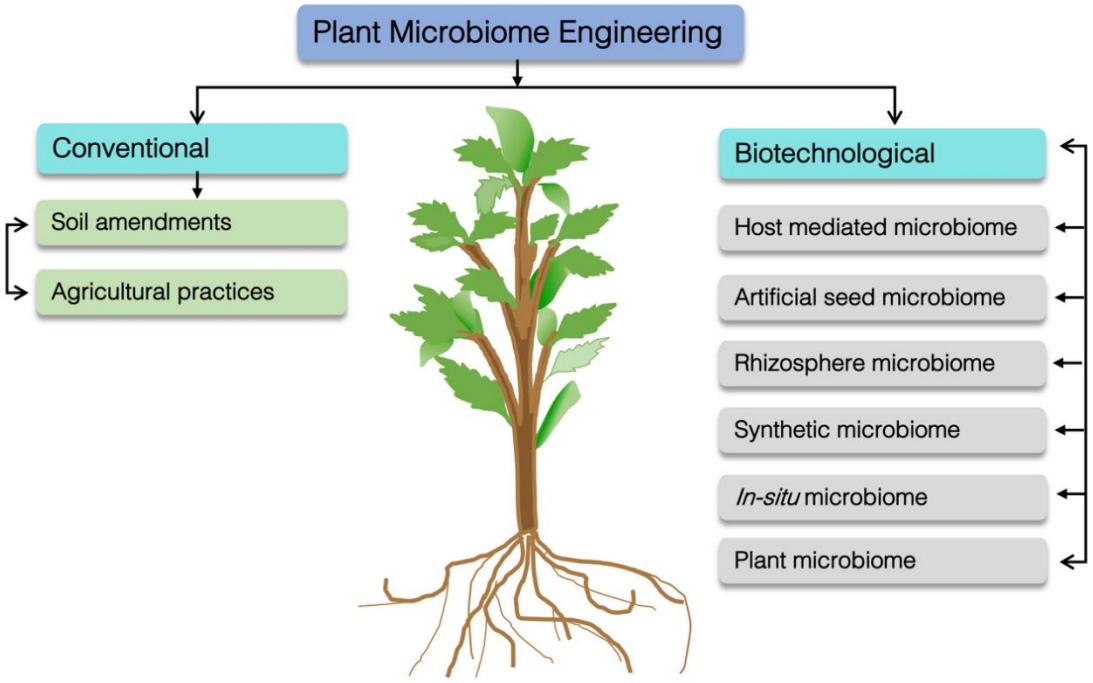

Figure 2. Plant microbiome engineering via biotechnological and conventional approaches**.** Host-mediated microbiome (indirectly selection of microbiome through utilization of host phenotype), artificial seed microbiome (artificial selection of microbiome and its integration/inoculation with seeds. This establish microbiome may evolve during the development and germination that consequently impact plant microbiome structure and function), Rhizosphere microbiome (bacterial competitiveness engineering) Synthetic microbiome **(**genetically engineered microbe's **i**noculation to host plant) In-situ microbiome (manipulation of native microbial community in their native context) Plant mycobiome (optimization and improvement of beneficial plant-fungal interactions

Ameliorating plant stress

The plant microbiome presents a complex interrelationship among many environmental factors and bacterial communities. In particular, under open field conditions, the possible bias in laboratory experiments is emphasized due to the lack of variability in environmental changes. Extreme environmental stresses, mainly climatic changes, can influence microbial communities. The soil microbiome can be affected by these stresses directly from drought-, salt- or heat-tolerant taxa (Martiny et al., 2017; Naylor et al., 2017) and indirectly by altering soil chemistry or diffusion rates (Liptzin et al., 2011).

The impact of salinity can be alleviated by the implementation of halo-tolerant synthetic microbiomes in saline soil systems. As the majority of microbes are halo-sensitive, some halophytic plant-associated members are halotolerant and can be considered potential targets for developing synthetic microbiomes. It has been demonstrated that inoculated halotolerant rhizobacteria improve the native microbial community's resilience to salinity stress and, as a result, can improve plant growth and stability in saline states (Bharti et al., 2015; Ali et al., 2022c). An engineered microbiome approach is recommended for use in areas with saline water irrigation systems.

Drought is among the worst obstacles to agricultural productivity. Plant stress tolerance must be improved to allow acceptable crop production in limited resources of water under drought situations (Liu et al., 2019; Salam et al., 2022). Drought stress tolerance in plants based on root-associated bacteria has also been reported. In addition, molecular compositions (such as root exudates) have shown promising potential in the relevant scenario of plant microbiome perturbations. Studies have better explored an example of the biosynthetic salicylic acid in *A. thaliana* that collects a normal root microbiome (Lundberg et al., 2012). This study has shown that central regulators in the immune system of plants have an impact on root microbiome composition. Moreover, such regulators can be adapted to amend the microbial community, which, in addition to improved productivity, can increase resilience against unwanted stressors.

Most studies on the plant microbiome have considered model plants, particularly *A. thaliana*. All information attained could be extrapolated to other plant communities. Therefore, more effort should be directed to microbiome engineering to enhance crop characteristics, such as tolerance against drought and diseases, thus allowing sustainable agricultural production. However, this technology has recently demonstrated its potential for the root microbiome of S. bicolour, for which drought conditions have caused the enrichment

of a set of root microbes. Drought-based induced upgradation with metabolic shift was observed for the plants and microbes, revealing it to be a potential blueprint in handling the microbiome to strengthen crop fitness and upsurge production (Xu et al., 2018).

Stimulating antagonistic and biocontrol activities

Plant diseases are the cause of major economic losses for farmers worldwide. The FAO estimated that pests and diseases are responsible for approximately 25% of crop loss (Dean et al., 2005). There are regional differences reported: it is estimated that diseases typically reduce crop yields by 10% every year in more developed countries, but yield loss due to diseases often exceeds 20% in less developed areas. To avoid crop losses due to maladies, chemical pesticides are routinely applied on crops, with the main goal of eradicating or lessening the disease invasion, infection, or severity (McMichael et al., 2007). However, it is becoming increasingly clear that long-term chemical pesticide usage poses several adverse effects on the environment and human health (Rani et al., 2021).

Plants harbour a diverse array of microbes in the rhizosphere that establish beneficial relationships with their hosts, guarding from plant pathogens and influencing their health and fitness through direct and indirect mechanisms. Competition, hyperparasitism, antibiosis, production of extracellular enzymes, and induction of resistance are well documented mechanisms (Figure 1) (Raymaekers et al., 2020). All these beneficial microbes associated with the roots of crop plants exert beneficial effects on their hosts and are referred to as plant growth-promoting biocontrol agents. Various studies have proven that plants secrete small molecules for the recruitment of actively beneficial microflora to assist their conformation under extreme conditions (Busby et al., 2014). This array of microbes possesses various biological control traits, such as competition for food space and colonization (Hunziker et al., 2015; Lloyd and Allen, 2015; Santhanam et al., 2015), antibiosis (Gómez Expósito et al., 2017), hyperparasitism (McNeely et al., 2017) and the production of degradative enzymes. In addition, these microorganisms associated with plants form a mutual association that impacts the host plant-associated microbiome and hosts an innumerable wealth of bacterial taxa, many of which promote tolerance to abiotic and biotic stresses and plant growth, suppress plant diseases, degrade xenobiotic compounds, and positively affect yields (Berg et al., 2016). This immense microbial diversity can be a target of manipulation by employing artificial microbial consortia, providing new synergistic opportunities for enhancing disease management (Poudel et al., 2016).

Current challenges

Difficulties in isolating and characterizing microbiomes

Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria are the major rhizobacterial phyla that are compliant with cultivation. Several studies have been conducted for their isolation, genome sequencing and characterization of their phenotypes (Bai et al., 2015; Mauchline et al., 2015; Levy et al., 2018). Experiments are performed in laboratories mimicking their natural interaction with plants to find the key features of plant–microbe relations. These studies enable scientists to understand the microbial recruitment behaviour in the rhizosphere as microorganisms take part in the growth and tolerance of the plant (Bai et al., 2015; Niu et al., 2017).

Isolation makes the assembly and sequencing of individual genomes simpler. Moreover, it provides more resolved data compared to assembling metagenomes. Furthermore, the isolation step also confirmed the presence of isolates in the rhizospheric community and their interaction with the host plant (Levy et al., 2018). After isolation, strains can be easily detected for key enzymes and molecular mechanisms involved, e.g., the proteomic or transcriptomic response of a single fungus or bacterium to nutrient stress or the plant microbiome enlightens the plant growth promoting (PGP) potential of microorganisms. This helps discover novel traits of the microorganisms related to their PGP activities (Bruto et al., 2014; Lidbury et al., 2016). The phenotypes embarked with the plants for PGP traits are not revealed by in vitro screening methods. In this regard, fast and large-scale screening can be performed by genome sequencing, which also encourages the discovery of novel PGP traits or genes (Finkel et al., 2017). The knowledge of interactions between plants and microorganisms and the role of PGP traits or genes in enabling these interactions can be improved by combining these strategies with complementary molecular approaches, i.e., bioreporter and mutagenic expression systems (Wetmore et al., 2015).

Efforts to assign functions to microbes

The task of assigning a specific function to an individual microbiome or a group of microbiomes is often challenging, as a completely different lifestyle is evident in species of even a particular genus. It varies from mutualist to pathogen and vice versa depending upon the transfer of functional genes between distantly related species or the environmental conditions (Qiu et al., 2009; Hacquard et al., 2016). The desired traits, such as phosphate

mobilization in microbial phenotypes, are altered by this changeability (Lidbury et al., 2016). Therefore, there is a need to find more sensitive methods for the characterization of bacterial species beyond the genus level, and large-scale throughput methods are required for better functional characterization of each species (Schlaeppi and Bulgarelli, 2015b).

Advancement in technology, combined with modelling/computational techniques, can be very auspicious. For example, a combination of metagenomics products with the environment i.e., The adaptation of metagenomics to metaphenomics takes into account all the parameters that may sway the plant-microbiome interaction within a community or environment (Jansson and Hofmockel, 2018). This transition makes metagenomics more powerful and widens its functional capabilities, such as carbohydrate utilization or secondary metabolite production (Bulgarelli et al., 2015). Moreover, these new advancements also enable researchers to gain more specific insights into the specific taxa responsible for imparting key functional characteristics. Ready-to-use commercial kits facilitate DNA extraction from a sample easily (Prosser, 2015). In soils, most of the microbial biomass (>90%) is dormant or inactive (Fierer, 2017), but in the rhizosphere, this number drops significantly as most of the microorganisms are made metabolically active in these habitats by plant-mediated factors (Bulgarelli et al., 2013).

Microorganisms from these niches have been isolated, and their RNA is extracted to identify the mechanisms involved in inducing responses to microbial or plant stimuli (Yergeau et al., 2014). Similarly, 13C-labelled CO_2 enrichment is combined with metatranscriptomics to study the response of microorganisms to plant exudates released in the rhizosphere and to better understand the plant-microbiome relation (Haichar et al., 2016). Exoproteins are more stable in the environment than RNA, which has short turnover times, reducing the robustness and simplicity of sampling efficiency and making sampling more prone to errors (Prosser, 2015). Metaproteomics also enables an intriguing possibility of studying metabolic activities, as it gives the profiles of expressed proteins (Heyer et al., 2015). The ecologically important proteins for nutrient uptake and microbial-host and microbial-microbial relationships (e.g., transporter systems and extracellular hydrolytic enzymes) are enriched by exometaproteomics or exoproteomics (Lidbury et al., 2016). However, the need for enough starting material (up to 100 g of soil) (Johnson-Rollings et al., 2014), accurate peptide profiles, and adequate computational power limit the applications of metaproteomics (Muth et al., 2016). These might be the reasons that restrict the use of meta(exo)proteomics in rhizosphere research.

Omics approaches to unveil plant-associated microbiota

Recently, the advent of omics tools, gene-editing techniques, and sequencing technology has allowed us to unravel the entangled webs of plant-microbes interactions, enhancing plant fitness and tolerance to biotic and abiotic challenges. Genomics is an effective tool for studying and predicting the interactions of microbes and plants and developing pathogen stress tolerance in plants (Frantzeskakis et al., 2020).

High genetic variability in the soil microbiome can be confirmed by multiple sequencing methodologies, such as prokaryotic16S, fungal ITS (internal transcribed spacer regions), and/or metagenomic analysis. Describing who is associated with the plant is relevant to unveiling their functions, so these microorganisms can become the extended genome partner of the host (Berendsen et al., 2012b). More reports on genome engineering, gene editing, and advanced plant-microbe interaction technologies have been discussed (Frantzeskakis et al., 2019; Sharma et al., 2020). The microbiome composition can be altered by environmental factors such as soil conditions and temperature. However, plant biochemistry and the immune system also play key roles in determining the variability of the microbiome (Turner et al., 2013). Although plants bring beneficial microorganisms, such as PGPR and disease-suppressing microorganisms, it has been evident that they can also bring phytopathogens as well as human pathogenic bacteria. These harmful bacteria may enter the food chain, can cause plant disease, and can alter the entire microbiome composition (Gorshkov et al., 2020). Therefore, tools such as metagenomics, for example, offer a promising strategy to diagnose these phytopathogens (Chiu and Miller, 2019).

Currently, nanopore sequencing using Oxford Nanopore Technologies (ONT) is the most encouraging technology for the identification of pathogens by metagenome sequencing (Jain et al., 2016). It is fast and is a direct sequencing method requiring no amplification step. It can be used even if we lack any prior knowledge of pathogens, as it can directly detect and identify all pathogens except RNA viruses. Moreover, it can also reconstruct the functional pathways in the microbiome and can foresee its composition. A high error rate limits the use of ONT (Rang et al., 2018). Therefore, it can be combined with Illumina technologies to enhance the sequence assembly quality (Sevim et al., 2019). MinON™ has already been used for metagenomics sequencing of bacterial, fungal and viral pathogens on several crops (Jongman et al., 2020; Mechan Llontop et al., 2020). Low sequencing cost and high quality suggest that direct sequencing is likely to be the future of metagenomics (Ciuffreda et al., 2021). An increasing number of propositions are becoming feasible because of the expanding

information in metagenomics.

It was first proposed that the initial molecular assessment of the soil and soil microbiome could help in the improvement of agricultural treatments (Schlaeppi and Bulgarelli, 2015b). Conversely, the complimentary response of the host towards beneficial microbes should also be a part of the engineering program because the host is also involved in bringing the interaction. It would enable the plant cultivars to interact efficaciously with natural as well as acquired microorganisms (Bulgarelli et al., 2013). The drawback of genomic analysis is that it does not provide knowledge about the functional states of biological objects; therefore, a metagenomics approach can be used in combination with a transcriptomic approach to evaluate key traits in plant-microbiome interactions.

NGS-based transcriptomics is another approach used to unravel the molecular mechanisms involved in plant-microbiome interactions. It is usually applied in plant pathology and stress studies. It reveals the physiological response of plants to pathogens and characterizes the signalling events taking place in the rhizosphere. Although we can predict community function from multi-omics data alone to some extent, validation of interactions requires the complementary work with cultured isolates that can be interrogated in the laboratory (Terekhov et al., Kehe, J. et al.,2019). For example, the resistance of barley to *Bulumeria graminis* by the gene network has been uncovered by NGS (Li et al., 2020). It also revealed the underlying mechanism of resistance against *Pectobacterium atrosepticum* (Tsers et al., 2020). Moreover, the characteristic interactions between *Phytophthora infestans* and potato plants have been revealed by gene expression patterns or NGS (Duan et al., 2020). NGS can also be used to study plant interactions with noninfectious microbes and plant responses to abiotic stresses. For example, the tolerance of tomato to hypoxia (Safavi-Rizi et al., 2020), changes in the gene expression pattern of orchard grass due to short-term flooding (Qiao et al., 2020), and gene expression changes in Arabidopsis because of high ultraviolet stress (Huang et al., 2019) have been revealed by NGS. However, the vast data profiles generated by NGS are too enormous to be efficaciously translated into simple language. This makes the interpretation of NGS transcriptomic data difficult for higher plants (Murat et al., 2012). Moreover, in most cases, the expression level is not restricted to a single gene (Das et al., 2020). Therefore, the focus of transcriptomic studies has shifted from the individual gene level to the gene set level. Significant impact of anthropogenic activities on the plant microbiome

Over the past few decades, industrialization and urbanization have caused an increase

in carbon dioxide and temperature, which affect the climate globally. These changes cause erratic events worldwide, such as a decrease in moisture level, an increase in temperature, excessive greenhouse gas emissions, and an increase in snowfall and rainfall. Climate change, range shift and urbanization are key factors that affect plant microbial interactions in the rhizosphere (Figure 3). Soil microbial community determines the soil, and plant health and prerequisite for external constraints. Soil microbial ecosystem functions and diversity are significantly influenced by anthropogenic activities These activities produce a diverse array of hazardous substances including pesticides, heavy metals and organic pollutants and put tremendous pressure on soil microbiomes. Heavy metals notoriously imbalance the microbial population, diversity and seriously decline their activities (Abdu et al., 2017; Fajardo et al., 2019; Li et al., 2020).

Climate change

Abrupt changes in climate and weather patterns have become a global dilemma among researchers and farmers (Amna et al., 2021; Wahab et al., 2022). Anthropogenic activities such as global warming, deforestation, the greenhouse effect, and urbanization have made these climate changes inevitable. Excessive fertilizer and pesticide use, livestock farming, nitrous oxide emissions, and fossil fuel combustion are the other contributors to climate change. The development of plants is affected by different climatic factors, such as CO_2 levels in the atmosphere, temperature (Saeed et al., 2022), drought (Wahab et al., 2022), salinity (Hussain et al., 2022; Mehmood et al., 2021a), heavy metsla (Nawaz et al., 2022) and rainfall patterns. However, the impact of climate change on the variety of microfauna is also worthy of attention because microorganisms are also influenced by these changes as they perform carbon and nutrient cycling. Abrupt changes in climate can disrupt the microbial population above and below ground and can have a negative impact on plant development. For example, global warming affects microbial respiration and therefore directly alters the microbial composition (Classen et al., 2015).

Temperature plays a key role in defining the microbial community of plants and is also decisive in plant phenological characteristics and development (Kashyap et al., 2017). In the past few decades, emissions of greenhouse gases (CO_2, CH_4, water vapour, etc.) due to rapid urbanization and industrialization has elevated the temperature. According to Compant et al. (Compant et al., 2010), the average temperature is expected to rise by 1.8-3.6 °C by 2100, which would lead to water scarcity and droughts. Several studies have been performed to

describe the effects of elevated temperature on plant morphology (Chen et al., 2021). Similarly, elevated temperature also influences the activities and composition of microorganisms in the rhizosphere. An increase in temperature increases the growth rate of microorganisms with altered respiration (Figure 3) (Classen et al., 2015). Karhu et al. (Karhu et al., 2014) reported an exponential increase in soil respiration with increasing temperature. Additionally, organic matter utilization by microorganisms is also dependent on temperature (Frey et al., 2013). Temperature alterations are also correlated with the pathogenicity of microbes. Increased temperature increases the growth of *Glomus mossae* and *Glomus intraradices* (Monz et al., 1994).

Disease incidences in plants by certain seed-borne microbes that degrade cell walls and *Pectobacterium atrosepticum* causing soft rot can be increased by an increase in temperature (Hasegawa et al., 2005). Drought conditions pose a threat to plant carbohydrate exchange and nutrient uptake in the rhizosphere by arbuscular mycorrhizal fungi (AFM) (Newsham et al., 1995). In mountainous soil, the warming effect is amplified when heat waves combined with elevated temperature increase the C and N cycling of microorganisms (Donhauser et al., 2021). However, other factors, such as UV radiation and moisture, also affect microbial communities. AFM cannot colonize plants under drought conditions (Staddon et al., 2004). The bacterial population is also reduced in the rhizosphere of sorghum roots under drought conditions (Xu et al., 2018c). The allocation of carbon in the rhizosphere is regulated by atmospheric carbon dioxide. Therefore, atmospheric CO_2 regulates the root exudate composition in soil, which defines the microbial community in the rhizosphere (Williams et al., 2018). Microorganisms are the key factors in the net exchange of carbon in soil. They perform this function in various ways by altering the nutrient status of the soil, forming symbiotic or pathogenic interactions with plants, respiration and organic matter decomposition. Therefore, high levels of CO_2 can alter the microbial population directly or indirectly by altering plant physiology and metabolism. Elevated CO_2 levels alter the root exudate composition and nutrient availability in soil (Compant et al., 2010). Some fungi have the potential to assimilate more carbon than bacteria; therefore, they can store carbon than mobilization. Thus, the microbial population in soil is stimulated by excessive emission of carbon by roots. This microbial propagation eventually reduces nitrogen availability for plants because of nitrogen immobilization in the soil. Soil respiration is also increased by elevated CO_2 levels. Microorganisms respond differently to elevated CO_2 levels in soil. No significant effect was observed by Gavito et al. (Gavito et al., 2000) in AMF of *Pisum*

sativum with an increase of 700 ppm of CO_2, while only with an increase of 7 ppm CO_2 was an increase in mycorrhizal colonization observed by Tang et al. (Tang et al., 2009) in Barnyard grass.

The 18S RNA sequencing-based Illumina MiSeq technique revealed a significant decrease in the populations of Glomus and Claroideoglomus species after long-term CO_2 (550 ppm) exposure in paddy fields (Panneerselvam et al., 2020). In *Pinus strobus* and *Boswellia papyrifera* plants, an increase in CO_2 (700 ppm) concentration increased the ectomycorrhizal fungi (ECM) population (Godbold and Berntson, 1997). Similarly, a 3-fold increase in ECM mycelia was observed in *P. sylvestris* with an increase in CO_2 concentration (Fransson et al., 2005). PGPB are also influenced by the CO_2 concentrations in the soil. Several studies have been performed to observe the effect of CO_2 elevation on plant microbe interactions (Thakur et al., 2019; Yu and Chen, 2019; Prescott et al., 2020; Terrer et al., 2021). A 3-fold increase in *R. leguminosarum* was observed by Schortemeyer in the rhizosphere of white clover by an increase in CO_2 (600 ppm) concentration (Schortemeyer et al., 1996). In addition, more efforts are required to understand the behaviour of plant-microbial interactions under elevated CO_2 levels to engineer the desired beneficial microorganisms for plant development.

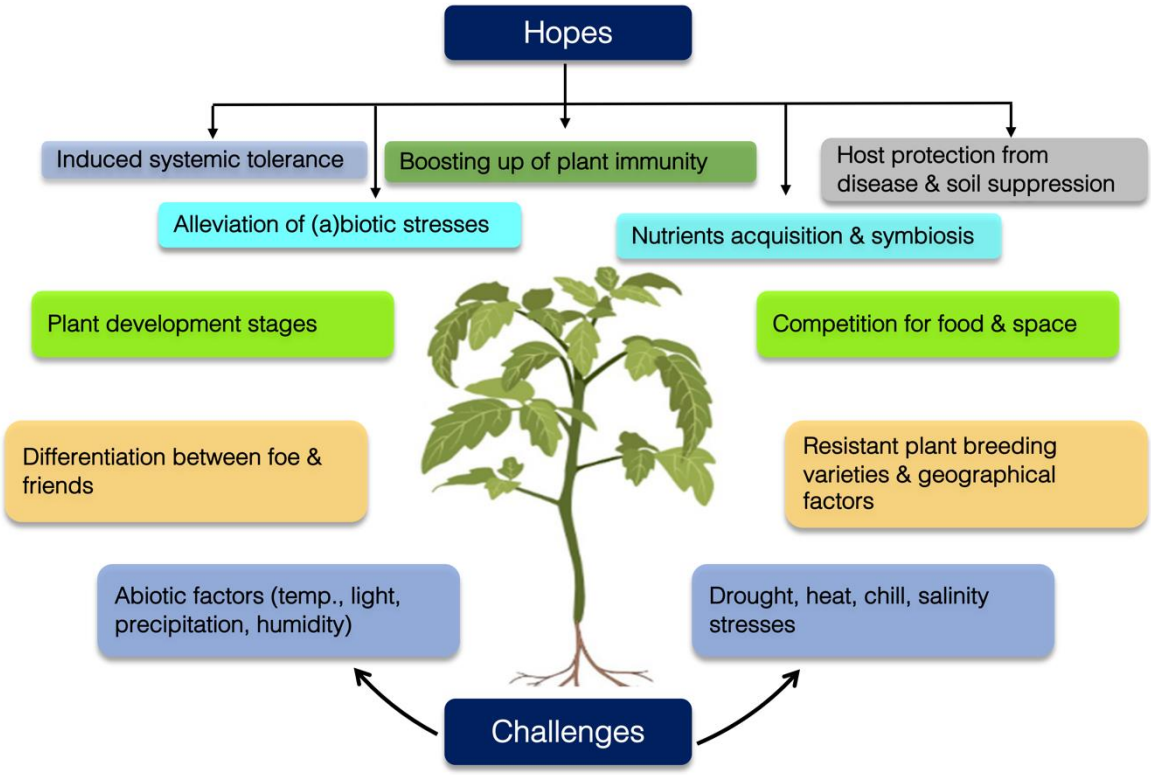

Figure 3. Plant Microbiome provides key functions for plant health and its protection. Plant microbiome offers vital services for plant health. It facilitates biogeochemical cycling of plant nutrients, assist plant growth under biotic and abiotic conditions, induces systemic acquired resistance (SAR) and induces systemic resistance (ISR) in plant against plant pathogen. Inversely, Plant microbiome synchronously encounters biotic and abiotic stresses which are the substantial drivers that influence or alter microbiome diversity and functionality

Range shifts

Human activities have introduced new species to the new habitats (Essl et al., 2011) and have caused environmental warming that expands the potential survival capabilities of these species in the habitats where they could never survive before or have contracted their habitat (Walther et al., 2009). These two reasons have triggered the shifting ranges. Plant-microbial interactions have gone through unforeseen impacts because of these range shifts. The elevation gradient provides a practical system to evaluate the effect of abiotic and biotic factors on plant–microbe interactions, microbial composition and distribution. Cobian et al. (Cobian et al., 2019) revealed that a parabolic relationship was followed by leaf fungal endophyte specialization, where specialization was maximum at the centre of tree species

ranges and reduced towards edges. Balsam poplars' fungal community has higher diversity when relocated to the upper edges of the elevation gradient because they experience higher abiotic stresses (Bálint et al., 2015). Compared to fungi, leaf bacterial communities are less affected by changes in elevation gradients because fungi are more sensitive to temperature changes (Vacher et al., 2016). Along the elevation gradient, plant community dynamics also face a turn from competition to facilitation. However, a vast majority of research is required to study the positive and negative effects of elevation gradient shifts on plant-microbial interactions. Plant–soil feedback (PSF) is a mechanism by which plants influence abiotic and biotic factors in soil, and feedbacks influence their development and growth (van der Putten et al., 2016). PSF and microorganisms negatively affect native species (Bever, 2003).

Previously established communities of microorganisms are reestablished by the novel soil biota through species range expansion, e.g., negative interactions develop between the soil biota and *Centaurea maculosa* in native ranges, while in North America, they develop positive interactions with microorganisms in soil (Callaway et al., 2004). The survival of nonnative species in novel environments is favoured by the dearth of natural enemies. A significant reduction in foliar and floral pathogens has been evident in invasive plants (Ramirez et al., 2019). In comparison, seed germination of *Acer saccharum was* reduced in soil beyond its native range limits even though the abiotic conditions were sufficient (Carteron et al., 2020). A variety of microbial interactions can influence species range shifts; however, thorough research is needed in this sector to evaluate the contrasting roles of microorganisms in driving plant range shifts.

Urbanization

Urbanization has been a source of various airborne pollutants. The use of chemicals and micro- and macronutrients influences local vegetation, eventually altering plant-microbial interactions (Annamalai and Namasivayam, 2015). Moreover, these anthropogenic activities also have the impact of the microbial population, which has the potential to remediate air pollution. The phyllosphere communities of bacteria and fungi are distinct in rural and urban trees (Smets et al., 2016; Laforest-Lapointe et al., 2017). A 10% increase in alpha-bacteria was observed by Laforest-Lapointe et al. (Laforest-Lapointe et al., 2017) and Imperato et al. (Imperato et al., 2019) in urban tree leaves. Espenshade et al. (Espenshade et al., 2019) also observed the impact of traffic patterns and urban density on the bacterial composition of tree leaves, which was associated with black carbon and ultrafine particulate

matter. A lower diversity of fungi was observed on urban trees by Jumpponen and Jones (Jumpponen and Jones, 2010). However, a higher fungal load was observed by Imperato et al. (Imperato et al., 2019). Moreover, traffic levels also influenced the phyllosphere community of bacteria (Smets et al., 2016). These findings enable the need to better understand the elements that bring changes in the phyllosphere of urban trees and to check the varying changes that take place within microbial functions. Recent investigations have started to generate a link between the impact of urbanization on the genetic and functional changes of the phyllosphere microbiota. For instance, a higher number of bacteria was observed in urban trees. These bacteria have genes encoding enzymes for aromatic degradation that impart PGP traits to plants (Imperato et al., 2019). Additionally, it has also been observed that hydrocarbon-degrading bacteria are selected by plants when hydrocarbon levels increase in the atmosphere (Gandolfi et al., 2017).

This phenomenon is termed phytoremediation, and plant-microbial interactions play a pivotal role in efficacious phytoremediation. Endophytes can remediate soil and water contaminants and promote the growth of plants (Siciliano et al., 2001b; Mukhtar et al., 2018). Soil contaminants increase the prevalence of catabolic genes in endophytes, and this phenomenon can be artificially introduced in bacteria. The introduction of toluene-degrading genes in endophytic bacteria can enhance toluene degradation in soil, thus reducing phytotoxicity and toluene evapotranspiration through the leaves by up to 70% (Barac et al., 2004b). A number of studies have been performed to evaluate the contaminant-degrading capabilities of bacteria (Hong et al., 2018; Undugoda et al., 2018; Dekeukeleire, 2019; Ben-Israel, 2020). However, the true potential of microorganisms and plants in degrading air and soil pollutants has yet to be discovered. In addition, these findings suggest that we need to determine the influence of urbanization on plant-microbial interactions if we want to engineer the microbiome of plants.

Conclusions

Chemical fertilizers and pesticides have been used for a long time among agricultural platforms. The goal of using such sources is to attain better crop production as per the demand of the growing human population. Excessive implementation of these chemical means may not be an acceptable choice for sustainable ecosystems. In such a way, this review, in addition to unveiling the complexities of the plant-microbiome interactions, as well as the wide possibilities to manipulate them under stressful conditions, has unravelled

vital factors that are relevant to generate sustainable agriculture. Therefore, the engineering of the microbiome is a highly fundamental approach dedicated to the betterment of the health, growth and functions of plants. Studies aiming to grasp this interplay at the community level can enhance the understanding of factors that control the microbiome assemblage with its relevant feedback to a host plant. Such goals are obtainable with the support of modern tools such as “omics”, yet combining such an innovative approach with additional efforts in rhizosphere microbiome engineering can interestingly provide new insights. Similarly, an optimized phytomicrobiome meta-organism may result in a sustainable ecosystem with better agricultural production and can similarly diminish greenhouse gas emissions and soil pollution. As the microbes in the rhizosphere are scarcely investigated, further efforts are required to monitor and engineer the arrangement and activities of this microbiome. A large body of research covered the various aspects of phytomicrobiome engineering. In the last decade, massive progress has been made in plant microbiome studies but some gaps are still needed to address and fulfilled. Understanding the importance of the plant microbiome, (1) the influence of secondary metabolites of microorganisms on beneficial microbes of the plant microbiome, (2) The alteration of continuous environmental condition and their impact on the host and its associated microbial communities, (3) to investigate the ability of host plant to refrain pathogenic microbes, (4) the integration of agronomic practices with synthetic biology and their optimization and compatibility to each other.

As per demand, further elaboration can support the comprehension of the mutual association of many microbes with their host plant based on their molecular and genetic basis under any environmental constraints, which beyond can open up new avenues to advance biological and ecological practices. Future studies are directed to explore the identified gaps and, based on current knowledge, should mainly focus on classifying those biotic and abiotic factors that responsibly influence the diversity, functions and association of the microbial communities with hosts in extreme habitats. Therefore, novel findings can lead us to better understand the ecological connections between plant and underground microbes.

AUTHOR CONTRIBUTIONS

MSA planned and designed this review manuscript. MSA and MA wrote this review paper. MSA, BA, and SA help to draw the figures. GS, FHVM, AS and S, helped to write and improve the manuscript writing. GS contributed to the critically revising of the manuscript.

All the authors have reviewed, edited, and approved the manuscript before submission

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CONFLICT OF INTEREST

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

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ARTICLE 2 – One or millions: How much does a microbiologically-buffered soil withstand chemical and biological pesticides?

Abstract

Disease-suppressive soils contribute to plant protection against various soil-borne plant diseases. Disease-suppressive soils contribute in plant protection against various soil-borne plant diseases. The current research aimed to 1) investigate the stability of disease suppressiveness through the introduction of biocontrol agents and agrochemicals 2) evaluate the functionality of the soil microbiome towards root-knot nematodes (RKN) disease and 3) to isolate, screen and characterize the responsible candidates of soil-suppressiveness of the established suppressive soil microbiome. Suppressive soil assay revealed that suppressive soil significantly reduced galls (11%) and egg masses (42%) in relation to sterilized soil. Suppressive soil slurry experiment treated with antibiotics (streptomycin) or fungicide (cyproconazole) modulated the disease suppressiveness and microbiome functionality. The number of galls significant increase was 168% with antibiotics and 86% with fungicides as compared to the untreated slurry. Likewise, suppressive soil slurry treated with antibiotic or fungicide significantly increased the number of egg masses by 273% and 36% respectively as compared to the untreated slurry. Intriguingly suppressive soil microbiome manipulation by biological control agent *Bacillus velezensis* BMH intervened in the microbial functions and reduced its suppressiveness by significantly increasing the galls and eggs 32% and 48% respectively as compared to un-inoculated suppressive soil. However, commercially available biocontrol based on bacteria such as Quatrzo (*Bacillus subtilis* FMCH002(DSM32155)*; Bacillus licheniformis* FMCH001(DSM32154), Biobac (*Bacillus subtilis* Y1336), Onix (*Bacillus methylotrophicus* UFPEDA 20) and Rizos (*Bacillus subtilis* UFPEDA 764) had no significant effect on the soil suppressiveness. A total of 42 bacterial strains were isolated from the suppressive soil and 18 of them were identified with nematode biocontrol potential. The isolates were sequenced based on 16S rRNA and identified 6 different genera: *Bacillus*, *Pseudomonas*, *Leclercia*, *Paenarthrobacter*, *Pantoea*, and *Exiguobacterium*. Therefore, understanding the status of the soil in terms of suppressiveness and the key role of bacteria and fungi is postulated as important in order to recommend biocontrol products. Bacteria are the most important and various species can be used as sentinels to monitor the natural suppressiveness of the soil to the gall nematode and preserve such community from exogenous bactericidal activity is as important.

Keywords: Soil microbiome manipulation, soil-borne disease, biological control agent (BCA), Bio-pesticides, sustainable disease management

Introduction

Plants harbor a multitude of groups of microorganisms by performing various functions in a complex ecosystem. Microbial communities associated with plants, particularly in the below ecosystem, carry out many activities in favor of their host's growth and protection. Unfortunately, diverse groups of plant pathogens inhabit the same ecosystem and interact with host plants for food acquisition and survival. Among them, plant parasitic nematodes e.g RKN belonging to Meloidogynes pp are the most devastating plant pathogens that infect a wide range of plant species, including vegetables (Talavera et al., 2012).

Plant-parasitic nematodes are some of the most devastating plant pests which have been crop losses of 21.3% overall, estimated to cause annual economic losses of $157 billion worldwide. Among the plant-parasitic nematodes, root-knot (RKNs) (Meloidogyne spp.) are soil-borne pathogens that can cause serious diseases and have a considerable detrimental effect on plant growth and production (Elhady et al., 2018; Kumar et al., 2020). It is assumed that these drastic agents, if allowed to continue, can infest over 30% of the vegetable different crops (Sikora and Fernández, 2005). Deep-rooted perennial crops with established root-knot nematodes make it challenging to be controlled, mainly if limited options are available.

Nematode management approaches mostly rely on the adoption of resistant cultivars, crop rotation and chemical or solar soil sterilization (DiLegge et al., 2019). So far, the exploitation of the resistance genes is limited due to the availability of few RKN-resistance genes and the difficulties in transferring them into susceptible crops, the introduction of resistance genes is restricted or unfeasible in annual crops Additionally, due to the very large host range of RKN, crop rotation is not always effective (Saucet et al., 2016). Thus, the primary strategy for controlling RKN in the current scenario is still chemical nematicides. Due to their extreme toxicity, the usage of a number of synthetic nematicides has been prohibited, though, as a result of growing concerns about human and environmental safety (Liu et al., 2020). This necessitates the employment of environmentally acceptable management measures. Applying non-chemical and environmentally friendly approaches, root-knot nematode maintenance management is currently being carried out. These include sanitation, fertilization, soil and organic supplements, thermal strategies, and biocontrol techniques to maintain worldwide vegetable productivity. Overall, the biocontrol has been a commonly deployed management

strategy (Choi et al., 2020; Shakeel et al., 2022; Stouvenakers et al., 2019).

RKNs have been repeatedly subjected to various attempts to control using antagonistic bacteria and fungi (Forghani and Hajihassani, 2020). Root-knot nematode damage could be reduced by applying microorganisms or natural biologically active compounds produced by these microbes that are highly detrimental to *Meloidogyne* spp. (DiLegge et al., 2019; Diyapoglu et al., 2022). It has been consistently demonstrated that antagonistic bacteria provide hope as potential biological pest remedies for plant-parasitic nematodes (Subedi et al., 2020). As a result of the identification of several *Pseudomonas* species as plant growth-promoting rhizobacteria (PGPR), the environment for root growth has been improved by the decrease in harmful and pathogenic rhizosphere microorganisms. These organisms also produce antibiotics, hydrogen cyanide, and iron-chelating siderophores (Ali et al., 2022).

Our previous research studies investiagted the disease suppressive soil against RKN and the soil suppression was of biological prigin (Silva et al., 2022). This study therefore put forth to determine its stability by manipulating chemical and biological pesticides and to isolate and characterize the genera that crutially contribute to *M. incognita* suppression

Materials and Methods

Soil collection site and soil sampling

The soil samples were collected from the sweet pepper-cropped (*Capsicum annuum* cv. ‘Magali’) cultivation system at Universidade Federal de Lavras (UFLA)-Hortagro (51°18′40″N, 6°12′10″E) property in Ingai, Minas Gerais, Brazil. This horticultural area has been cultivated with sweet pepper (*Capsicum annuum* cv. ‘Magali’) continuously for since last four (4) years. using (2-3 cycles per year) applying organic practices. After proper investigation, it was revealed that this area was highly populated with *M. incognita* nematodes and the production of susceptible sweet pepper to *M. incognita* was not influenced by these invaders (Silva et al., 2022). The soil was sampled between harvesting and new planting from the upper layer (0-0.2 m) of the pepper cultivation system randomly selected at 10 different points (each point was distant 10m).

Each soil sample was properly air-dried and mixed before being put in a plastic bag and brought to the lab in a cool box. This process was done to exclude plant debris and stones. The soil was mixed and homogenized properly before planting the plants. The soil samples

collections were consecutively repeated two times at the end of each sweet pepper cycle for the experiment's repetition.

Physicochemical characteristics of soil

Soil samples were collected at 0-15 cm depth from the Ingai a municipality in Minas Gerais-Brazil. A portion (500 g) of each soil sample was applied for the analysis of the physicochemical characteristics (Table.1). The soil samples were processed for the measurement of total macro and micronutrients in the Soil Analysis Laboratory, Department of Soil Sciences, Federal University of Lavras, MG, Brazil (UFLA).

Soil sterilization and inoculum preparation

The soil was divided into two parts. Natura soil and sterilized soil. The soil was sterilized by autoclaving at 121 °C, (0.1 Mpa) for 1 h, three times repeated, after 24 h intervals. The tomato plant (*Solanum lycopersicum* L. 'SantaClara'), was grown for the inoculum preparation and multiplication, containing a purely *Meloidogyne incognita* population in the greenhouse of the Department of Plant Pathology Federal University of Lavras (UFLA) MG, Brazil. After 60 days, the infected root samples of tomato plants were collected, rinsed under running water, cut into 1-2 cm pieces, then blended for 30 seconds with a 0.5% sodium hypochlorite (NaOCl) solution. cut into 1-2 cm pieces, rinsed under running water, then blended for 30 seconds with a 0.5% sodium hypochlorite (NaOCl) solution. The material was loaded into a 200-mesh screen, thoroughly cleaned, then collected from a 500-mesh screen, washed properly, and collected the retained eggs in water collected from a 500-mesh sieve (Boneti and Ferraz, 1981). The 48 and 72-h old eggs and second-stage juveniles (J_2) were used for all *in vitro* assays and experiments (Hussey, 1973).

Evaluation of the sensitivity of suppressive soil microbiome to antibiotics and fungicides

The evaluation of suppressive soil was performed to understand the sensitivity of soil suppressiveness against antibiotics and fungicides. To prepare soil slurries containing the soil microbiomes we applied the techniques of Zhou et al. (2019) and Elhady et al. (2018) with minor modifications. For that, 15 g of each suppressive soil sample were extracted in a blender three times with 15 ml sterile 0.85% NaCl at high speed for 60 s. Soil particles were sedimented and the microbial suspensions of the supernatant were passed through a 5 mm

sieve to remove remaining particles, nematodes, and root debris. The microbes were pelleted for 10 min at 4000 g and resuspended in 45 ml sterile water. Each 100 ml plastic cup was filled with autoclaved substrate and received a 20-days-old tomato seedling. Then each cup received 0, 5, 10 or 15 ml of the soil slurries per cup (0, 5, 10 or 15 %). The concentration of 0 % consisted of 15 ml sterile water representing the control. The transplanted microbiomes were established for 5 days. Then, a suspension with 200 J2 of *M. incognita* was inoculated into the roots of each cup. The tomato plants received irrigation and fertilization according to technical recommendations for 45 days. The galls were counted and the eggs were extracted and also counted. At the end the number of galls and eggs were estimated per gram of root system.

Manipulation of suppressive Soil Microbiome

The aim of the experiment was to measure the effect of exogenous PGPR on suppressive soil microbiome functionality. The normal (Suppressive soil) was divided into two parts. One part was sterilized and the other remained normal. The sterilization method was carried out according to the methodology described above. The 500mL pots were filled with normal and sterilized soil and a 20-day-old susceptible tomato (*Solanum Lycopersicum* cv. Santa Clara) seedlings were transplanted into each pot. The plant-growth-promoting biocontrol agent *Bacillus* strain BMH was inoculated and un-inoculated to each treatment after 6 days of tomatoes plants transplantation. Successively after the 3 days of *Bacillus* strain BMH inoculation, the total amount of 500 second-stage juveniles (J_2) of *M. incognita* were inoculated in all treatments. Seedlings were grown in a greenhouse at 25◦C to 28◦C for 45 days. Each treatment was carried out in four pots (replicates), and then all were placed on the workbench in a randomized complete design. After 45 days, the tomato plants were harvested and counted the galls, g^{-1}, and eggs, g^{-1}, of root densities.

Commercial Bio-based nematicidal products application on suppressive soil

The trial was implemented in a greenhouse to determine the antagonism effect of biocontrol on root-knot nematodes and soil suppressiveness. The four local commercial bioproducts Quartzo® (*Bacillus subtilis*; *Bacillus licheniformis)*, Biobac® (*Bacillus subtilis* strain Y1336), Onix ® (*Bacillus methylotrophicus*) and Rizos® (*Bacillus subtilis)* were used in this experiment. Sterilized soil was employed to fill the 500mL pots. and a 20-day-old

susceptible tomato (*Solanum Lycopersicum* cv. Santa Clara) seedlings were transplanted into each pot. The biocontrol was applied as a soil drench in all treatments after 6 days of tomatoes plants transplantation except for the control. Successively after the 3 days of bio-pesticides application, the total amount of 500 second-stage juveniles (J_2) of *M. incognita* was inoculated in all treatments. Seedlings were grown in a greenhouse at 26±2◦C for 45 days. Each treatment was carried out in eight pots (replicates), and then all placed on the workbench in a randomized complete design. After 45 days, the tomato plants were harvested in order to count the galls, g^{-1}, and eggs, g^{-1}, of root densities.

Experiment 2

Dissecting the biological nature of suppressiveness

After the screening of suppressive soil microbiome suppressiveness, we decided to investigate the responsible candidate of root-knot nematodes *M. incognita* disease suppression. The screened suppressive soil samples were collected from the pepper-cropped cultivation system at Federal University of Lavras (UFLA)- Centro de Desenvolvimento e Transferência de Tecnologia (CDTT) (51°18′40″N, 6°12′10″E) Property in Ingai, Minas Gerais-Brazil. Bacterial strains were isolated by the serial dilution method. Ten grams of rhizosphere soil in 90 mL of sterile saline (0.85%) was diluted to10^{-7} (Bharathi et al., 2004). An aliquot of 100 µL of each dilution was distributed with the aid of the handle Drigalsky in Petri dishes containing nutrient agar culture medium (Kado and Heskett, 1970) and incubated at 28 °C for 24 h. Morphologically distinct colonies were picked up, sub cultured and replicated to get the purified isolates. A total of fifty (50) bacterial isolates were isolated and stored in cryogenic tubes at -20 ^{0}Cfor further study.

Screening and selection of potential biocontrol bacterial isolates against M. incognita
Seedling tray experiment

One plastic tray containing 50 holes filled each one with sterilized soil, each one containing a ratio of soil and sand (3:1, v: v) respectively. A mesh of 2 mm was utilized to sieve the soil, and autoclaved for 3 days with 24 h intervals as mentioned above. The susceptible Soybean seed variety M6410 (Provided by the department of seed, Federal University of Lavras, MG, Brazil) was used in the experiment. sodium hypochlorite (1%) was used for the all seeds

surface sterilization, kept for five (5) minutes and washed with double-ionized distilled water consecutively three (3) times.

Next, Nutrient broth (NB) media were used to cultivate the bacterial trains, adjusted the optical density of 1 (10^8 CFU/ml) at 600nm. All the sterilized seeds were soaked in the bacterial suspension for 2-3 h, the seeds were kept for few minutes to air-dry and then each seed was individually transplanted to a tray of length: 55 cm, width: 27 cm, height: is 9 cm and have the capacity: 5 000 ml. The tray contains total of 50 cells and each height: is 9 cm with a 5 cm upper and 2 cm bottom diameter with the capacity of 100 ml (Fanyu Company, Shenyang, China). Seeds were sown in each hole of tray. After 15 days of the germination, 3 ml bacterial suspension of each isolated strain was inoculated to each treatment adjusted optical density 1 (10^8 CFU/ml) at 600nm. (Zhao et al., 2018, Afridi et al., 2019). After 3 days of bacterial strains inoculation, each hole possessing one soybean plant seedling was infested with 2500 *M. incognita* eggs at the first true leaf stage (V1). Each treatment was represented by one plant and each treatment had four replicates, and were randomly distributed on a workbench. After 45 days, all of the treatments were harvested, and the root gall index (GI) and eggs index (EI) were determined following Barker (1985) 's recommendations.

Experiment 3

Evaluation of biological control potential of selected bacterial strains against M. incognita (J2) under greenhouse conditions

Pot experiment

To evaluate the biocontrol potential of selected bacterial isolates, the pot experiment was conducted under greenhouse conditions. The tomato seedlings were transplanted into pot containing 600g soil and each treatment has 6 replicates. After 28 days of germination, 5 mL bacterial suspension of each isolated strain was inoculated to each treatment adjusting its OD 1 at 600nm. (Zhao et al., 2018, Afridi et al., 2019). After 3 days after bacterial strain inoculation, each pot possessing one tomato plant seedling was inoculated with 6000 *M. incognita* eggs at the V1 stage. A total of six replicates of one plant were maintained in each treatment and the trial was performed twice using a complete randomized design. All the treatments were harvested after 45 days and the root gall index (GI) and eggs index (EI) were determined by following the (Barker, 1985)'s protocol.

DNA Extraction PCR Amplification and 16S rRNA Sequencing

All the screened bacterial strains that showed potential and bio-controlled significantly the root-knot nematodes in the greenhouse experiment were selected and extracted their Genomic DNA. Polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene using the universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′) according to the previously described protocol (De Souza et al., 2021; Leite et al., 2013). Sequences were evaluated using the BLASTN algorithm available in GenBank (http://www.ncbi.nlm.nih.gov)(Altschul, 1997). With the use of the Clustal W software, sequences were aligned. The alignment and phylogeny analysis was performed using MEGA software(Kumar et al., 2016). The maximum likelihood approach was used to construct the phylogenetic tree. All the other 16S rRNA sequences were type strains used in the phylogenetic tree construction and retrieved from the List of Prokaryotic names with Standing in Nomenclature (LPSN) (Parte et al., 2020).

Measurement of growth parameters

The experiment was harvested after 45 days and the roots were separated from the shoots. The roots were shaken gently, washed with tap water and dried with a paper towel. The plant's root mass was measured by using a standard measuring scale. All the treatments possessed eight replicates and the experiment was performed twice at different times.

Galls and Eggs Quantification

The plants were harvested after 45 days of nematodes infestation and quantified the total galls and eggs mass index. To determine the effects of each treatment, the roots of tomato plants (*Solanum Lycopersicum* L. 'Santa Clara') were retrieved, rinsed with tap water, cut into 1 cm pieces, and blended for 40 seconds with a 0.5% NaOCl solution. The total eggs retained on a 25 µm sieve were transferred to a vile and counted under the microscope (Hussey, 1973).

Data analysis

All experiments were performed with different replicates and in a completely randomized design. The data sets were subjected to preliminary analyses of normality (Shapiro–Wilk) and variance homogeneity (Bartlett). The data was transformed when preliminary analyses of

normality (Shapiro–Wilk) were not passed. Analysis of variance (ANOVA) with the F-test was performed when the data followed a normal distribution. When the significance level ($P < 0.05$) occurred, using the *ExpDes.pt* package of the R statistical program (R Core Team, 2017) performed the Duncan's Means Test. To use the joint analysis, the following formula was used(Pimentel-Gomes and Garcia, 2002): QM1/QM2 < 7 the test will not be significant at the 5% level. If the covariance of the errors is unknown and consistent, the generalized least squares (GLS) estimator model was used. It is concluded that, in the case of groups of similar experiments, in which all treatments have the same number of repetitions, joint analysis can be performed if the set between the largest and smallest residual mean squares is less than 7. To analyze reproduction and infectivity a two-way analysis of variance (ANOVA) was performed in a factorial scheme with two types of soil (sterilized soil and suppressive soil) at four replicates, while the mean between treatments were calculated for significance test by Duncan's multiple range test at $P = 0.05$.

Results

Biological assessment of soil samples for disease suppression

Sensitivity of suppressive soil microbiome to antibiotic and fungicides

The manipulation of suppressive soil microbiome (soil slurry) with antibiotic and fungicide perturb the microbiome functions and reduction was observed in suppressiveness against root-knot nematodes (Fig. 2) ($P < 0.05$). The manipulated soil microbiome showed a significant upsurge of the nematodes in relation to the suppressive soil microbiome but the impact was different according to the considered product. The soil slurry treated with the fungicide and antibiotic increased the number of eggs by 88%, 174%, 38.17% and 276.24% with significant effects for both variables only for the antibiotic amendment. The combination of the amendment of both products to the soil slurry completely reduced the suppression to the gall, i.e. the effect was similar to the autoclaved soil but not to the number of eggs since there was no difference between the antibiotic amended or the combination of fungicide + antibiotic. (Fig. 1).

The Bacillus strain BMH perturb the suppressive soil microbiome functionality

The soil was divided into autoclave-sterilized soil and naturally-suppressive soil and conducted the experiment in greenhouse conditions. The bioassay of the soil suppressiveness

against *M. incognita* in greenhouse conditions showed that the suppressive soil (suppressive soil), significantly decreased the number of galls. g^{-1} and eggs. g^{-1} and reduction occurred 14% and 75% respectively as compared to sterilized soil ($P < 0.05$) (Table. 3) The PGPR *Bacillus* BMH inoculated to both sterilized and suppressive soil. Significant differences were detected between sterilized and suppressive soil with and without inoculation. Sterilized soil inoculated with *Bacillus* strain BMH reduced galls and eggs 34% and 33% respectively in relation to un-inoculated sterilized soil ($P < 0.05$) (Table. 3). Intriguingly the total number of galls. g^{-1} and eggs. g^{-1} were significantly higher in suppressive soil treated with *Bacillus* strain BMH. with increases of 32%, and 47.96% respectively ($P < 0.05$) (Table. 3).After the experiment was harvested, the root fresh weight was measured. The significant difference was only observed in sterilized soil inoculated with *Bacillus* strain BMH in relation to all treatments. The root fresh weight was increased 74% in sterilized soil inoculated with *Bacillus* strain BMH ($P < 0.05$) (Table. 4).

Bio-control influence the suppressive soil microbiome functions

The application of biocontrol products in suppressive soil did not have a significant effect on nematode control ($P < 0.05$). All biocontrol products had a number of galls and eggs per gram of root similar to the naturally suppressive soil, i.e., there was no additive protective effect of the biocontrol. (Fig. 2).

Isolation and preliminary screening, seedling bioassay of isolated bacterial isolates against root-not nematodes

Since the microbial community more likely involved in the biocontrol was of bacterial nature (Figure 2), we have dived into the culturable bacteria that would have a role in the soil suppressiveness to the nematode. A total of 42 bacterial strains were isolated and purified based on using the serial dilution technique, from the root-knot nematode's suppressive soil. In a small tray experiment, all these strains were preliminarily screened against *M. incognita*. Bacterial isolates, which showed significant differences in terms of both parameters (galls, eggs) were selected and processed for further study. Among them, 18 were chosen for their controlling potential of galls g^{-1} and eggs g^{-1} of root (Supplementary Fig. S1, S2). Generally, 32.30 % of the bacterial strains only reduced the galls g^{-1} while 25.80% reduced the eggs g^{-1} in plant root.

Bacterial strains that show high potential for controlling both galls g^{-1} and eggs g^{-1} were observed 41.90% of the total isolates (Fig. 3).

Molecular identification of bacterial isolates

All the bacterial isolates were identified based on the 16S rRNA gene sequence. All bacterial 16S rRNA gene sequences were deposited to NCBI BLAST and phylogenetic analysis was performed via MEGA 11 software. After performing BLAST homologous analysis in GenBank. Seven isolates, B1, B3, B7, P10, P16, P19, and P21 showed 100% similarity with *Bacillus* spp and five isolates such as P7, P17 and X2 showed 100% and X2, X11, and X18 showed 99.93% similarity with *Pseudomonas* spp. Three isolates like P12, P18, P20 showed 99.90, 99.80, 99.80% showed similarity with *Leclercia* spp. X5, showed 99.85% similarity with *Pantoea* spp, while, X12 showed 99.50% with *Paenarthrobacter* spp and X14, showed 100% identity with *Exiguobacterium* spp. (Fig. 4).

Effect and biocontrol potential of Bacillus strains on nematodes control under greenhouse conditions

The experiment was conducted under greenhouse conditions and all the Bacillus sp applied to the tomato plant in the pot experiment significantly reduced the number of galls of *M. incognita* compared with the infested plant after 45 days of inoculation in the first trial ($P < 0.05$) (Fig. 5). The plants inoculated with strains *Bacillus* sp. B1, *Bacillus* sp. B3, *Bacillus* sp. B7, *Bacillus* sp. P10, *Bacillus* sp. P16, *Bacillus* sp. P19, *Bacillus* sp. P21 significantly ($P < 0.05$) reduced the root galling 62%, 66%, 72%, 55%, 52%, 37%, and 59%, respectively, in relation to control. Regarding the egg mass reduction, only *Bacillus* sp. B1 showed a significant difference ($P < 0.05$) in controlling eggs as compared to control (Fig. 8). Subsequently, the trial was repeated and plant inoculated with strains *Bacillus* sp. P10(48%), *Bacillus* sp. P16(54%), *Bacillus* sp. P19 (45%) and *Bacillus* sp. P21 (63%) significantly ($P < 0.05$) reduced the root galling in relation to control. Regarding the egg mass, all the bacterial strains of *Bacillus* sp. B1(72%), *Bacillus* sp. B3(65%), *Bacillus* sp. B7(69%), *Bacillus* sp. P10(67%), *Bacillus* sp. P16(69%), *Bacillus* sp. P19(79%) and *Bacillus* sp. P21 87% significantly ($P < 0.05$) reduced the mass of eggs as compared to the un-inoculated plants (Fig. 8). The strains *Bacillus* sp. P10, *Bacillus* sp. P16, *Bacillus* sp. P19 and *Bacillus* sp. P21 exhibited consistency in reducing both galls index and egg mass in both trials.

The Pseudomonas strains effectively paralyzed the Meloidogyne incognita population and reduced their infection in tomato plants

The five (5) bacterial strains were identified as *Pseudomonas* spp from the isolated bacterial strains. They were coded as *Pseudomonas* sp. P7, *Pseudomonas* sp. P17, *Pseudomonas* sp. X2, *Pseudomonas* sp. X11, *Pseudomonas* sp. X18, and inoculated with tomato plant with the J_2 of *M. incognita* under greenhouse conditions. All the bacterial isolates (P7, P17, X2, X11, X18) significantly controlled the gall index (70%, 55%, 61%, 60%, 47%) respectively in relation to the untreated plant (P <0.05) (Fig. 6), while only P7 showed the efficacy and significantly (P <0.05) reduced the number of eggs per gram of root (79%) as compared to control plant (Fig. 6). In the second trial, the isolates P17, reduced 54%, X11, 51 and X18 (57%), showed consistency and significantly controlled the gall index when compared to the control (P <0.05) (Fig. 6). The *Pseudomonas* sp. P7, *Pseudomonas* sp. P17, *Pseudomonas* sp. X11, and *Pseudomonas* sp. X18 exhibited high biocontrol efficacy and significant reduction was observed. The *Pseudomonas* sp. P7, reduced 68%, *Pseudomonas* sp. P17, 59%, *Pseudomonas* sp. X11, 81, and *Pseudomonas* sp. X18,86% and controlled the egg biomass and exhibited high biocontrol efficacy in relation to the un-inoculated control plant (Fig. 6)

Efficiency of Leclercia spp. in suppressing root-knot disease of tomato plant

The data from the pot experiment conducted under greenhouse conditions elucidated that *Leclercia* spp, P12, P18, and P20 inoculated to tomato plants significantly controlled the nematode infection (P <0.05) (Fig. 7). All the isolates such as *Leclercia* sp. P12, *Leclercia* sp. P18 and *Leclercia* sp. P20 exhibited high potential in controlling gall and egg biomass index per gram of tomato plant root (P <0.05) (Fig. 7). On inoculation of bacterial isolates, P12, P18, P20, 50%, 48% and 45% reduction was observed respectively in the gall production in tomato root when compared to control (P <0.05) (Fig. 7). Consequently, the eggs biomass also reduced significantly 71%, 64% and 76% on inoculation of *Leclercia* sp. P12, *Leclercia* sp. P18 and *Leclercia* sp. P20 to tomato plants in relation to the control (P <0.05) (Fig. 10). Interestingly, on repetition of the experiment, these isolates showed consistency and reduced significantly the number of galls and eggs per gram of root on the same pattern except for *Leclercia* sp. P20 (P <0.05) (Fig. 7).

Biocontrol control efficiency of Paenarthrobacter, Pantoea and Exiguobacterium spp. in suppressing root-knot disease of tomato plant

Tomato plants infested with root-knot nematodes then inoculated with the bacterial *Pantoea* sp. X5, *Paenarthrobacter* sp. X12 and *Exiguobacterium* sp. X14 exhibited a significant reduction in the galling index and egg masses, respectively (P <0.05) (Fig. 8). The bacterial strains *Pantoea* sp. X5, *Paenarthrobacter* sp. X12 significantly controlled the galls per gram of root at 71%, and 52% respectively. The bacterial strain, *Paenarthrobacter* sp. X12 maintained its biocontrol capability and significantly reduced 63% the number of galls per gram of root as compared to the control in the second trial. The egg mass was also significantly reduced by the inoculation of *Pantoja* sp. X5, *Paenarthrobacter* sp. X12 and *Exiguobacterium* sp. 14, 59, respectively %, %89 and 59 %, respectively (P <0.05) (Fig. 8).

Discussion

Fungicide and antibiotics alone or combined dwindle the antagonism of soil microbiota against root-knot nematodes

Manipulation of suppressive soil microbiome was implemented in this study via ciproconazole and streptomycin amendment. The manipulation of suppressive soil microbiome (soil slurry) with such fungicide and antibiotic disturbed the microbiome functions and significant ($P < 0.05$) reduction was observed in soil suppressiveness against RKNs (Fig. 2). Recent studies revealed that suppressive soil microbiomes foster special microbial groups that are involved in the suppression of soil-borne diseases (Cha et al., 2016; Chapelle et al., 2016). The indiscriminate application of agrochemicals leads to the demolishing of the normal ecological process of beneficial soil microbial population in the soil ecosystem (Shahid and Khan, 2022). A huge research body of studies on the severe effect of fungicides have been documented as these studies revealed that biocontrol agents that were effective against Fusarium wilt disease lost their potential (Ali et al., 2022; Fravel et al., 2005). These agrochemicals might be deleterious to long-term soil bacterial community (Meena et al., 2020) and inhibit the growth of soil bacteria that produce active metabolites against soil-borne pathogens (Fig. 1) (Meena et al., 2020; Pal et al., 2005).

Biological control agent introduction reduces suppressive soil microbiome functions

Plants harbor a multitude of groups of microorganisms by performing various functions in a complex ecosystem. Microbial communities associated with plants, particularly in the below ecosystem, carry out many activities in favor of their host's growth and protection.

This study was conducted to investigate the soil suppressiveness against root-knot nematodes (*Meloidogyne* spp), influence of biological control agents, fungicide and antibiotic on its suppressiveness (suppressive soil microbiome), and their antagonistic effect on (RKNs nematodes). We also aimed to explore the interaction of beneficial microbes-based bioproducts (positive or negative) with their host and their biocontrol efficacy of root-knot nematodes. The soil was screened for its suppressiveness and the suppression against root-knot nematodes was significantly observed against RKN. The suppressive soil significantly controlled the RKN when compared to control sterilized soil ($P < 0.05$) (Table. 3). This suppression of soil against soil-borne disease is of microbiological origin in nature, and an array of dominant microbial taxa own this credit (Table. 3) (Mazzola, 2007; Svenningsen et al., 2018; Weller et al., 2002). Microbial communities associated with the suppressive soil release multitude of secondary metabolites that consequently sterilize the activeness of plant pathogens, and are unable to infect plant tissues (Compant et al., 2013; Khan et al., 2020). The suppressive soil is treated with *Bacillus* BMH, alters the functions of pre-existing microbiota of suppressive soil and dramatically significantly ($P < 0.05$) (Table. 3) increased the nematodes population densities when compared to un-inoculated suppressive soil. Suppressive soil fosters bacteria, archaea and fungi and they collectively contribute to soil suppressiveness by tailoring the number of mechanisms (Afridi et al., 2022). Gu et al., (Gu et al., 2017) documented in his research that *B. amyloliquefaciens* strain FZB42 own antagonism trait and produces various antifungal secondary metabolites such as lipopeptides, surfactins, bacillomycin D, and fengycins, which might inhibit the activities of beneficial bacteria and fungi associated with soil suppression in the screened suppressive soil. The evaluated strain had previously been demonstrated as having potential for root knot nematode management and promote root growth but fail to sustain plant growth when combined with another *B. velezensis* strain (Cruz-Magalhaes et al., 2022). However, such deleterious effect of a *Bacillus* strain was more likely an exception than a rule since similar experiment with *Bacillus*-based, commercially-available biocontrol products have not resulted in a deleterious effect on the suppressive soil suppressiveness to the RKN (Figure 4). Although we still have not evidence to explain such results, in a stepwise development of a biocontrol product, two important steps before launching a biocontrol product is the establishment of the ideal dose

for the biocontrol product and a formulation that assure its competitive advantage (Bettiol et al., 2022). Hence, in a proof-of-concept stage where scientists propose a biocontrol agent that has been tested under controlled conditions, the determination of the appropriate amendment rate and formulation may result in similar or better than the obtained by the evaluated commercial biocontrol agents.

Noteworthy, if the amendment of the biocontrol agent does not result in a complementary benefit in protection against RKN, what would be the advantage of their adoption? The distribution of not only RKN but all soil-borne diseases of various etiological nature is not uniform, i.e., the soil suppressiveness is not widespread and the role of the biocontrol amendment throughout the whole field relies on the management where the product would be required to cope with the lack of a biocontrol ecological service, while fine-tuned delivery strategies such as the one proposed by Mitchel (2021) is not a common sense.

Introduction of biological control agents into established microbiome encounter various challenges

Deploying bacterial biological control agents individually or as consortia into suppressive soil owing the aggressive behavior against the indigenous microflora and gradually undermines the functions of pre-existing natural microbiota of the rhizosphere. This might be due to the colonization of two or more populations of microbes same ecological niche, resulting in competition for nutritional resources and ecological niche (Castledine et al., 2020).

Identification of potential biocontrol agents and their harnessing for plant protection against plant parasitic nematodes (PPNs) is an emerging topic in sustainable and eco-friendly disease management approaches. The advent of sustainable, environment-friendly and protective measurements implementation against PPNs diseases must be prioritized in both in *in vivo* and *in vitro* conditions across Brazil. Hence, the last part of this study was to investigate and isolate the potential biocontrol agents inhabiting the suppressive soil and causing soil suppression against RKNs. Thus eighteen (18) isolates from 42 bacterial strains that exhibited biocontrol efficacy against RKNs were chosen for further analysis (Fig. 3). 16S rRNA identification revealed 6 different genera namely *Bacillus*, *Pseudomonas, Leclercia*, *Paenarthrobacter*, *Pantoea*, and *Exiguobacterium* (Fig. 4). Some bacterial strains have the capacity to inhibit *M. incognita*, about which no previous knowledge has been published.

Responsible bacterial communities for soil-borne disease suppression in suppressive soil microbiome

A large body of research has been witnessed on numerous microbe's role as biocontrol agents against RKN in greenhouse and field conditions and they have been successfully bio-controlled the RKNs employing an arsenal of mechanisms (Akhtar and Panwar, 2013; Wei et al., 2014; Zhou et al., 2013). Antagonistic strains achieve range of strategies to attenuate nematodes infection intensity and significantly escalate J_2 mortality, reduce egg hatching in a controlled environment (Wei et al., 2010).

The study, which we conducted in a greenhouse, inoculating potential screened antagonists exhibited a significant reduction in root gall^{-1} g and egg^{-1} g of *M. incognita* in tomato plants. Biocontrol agents achieve various mechanisms to biocontrol plant pathogens and plant parasitic nematodes. The decrease in *M.incognita* egg masses and root galling observed by (Choi et al., 2020) which likely happen due to the production of nematotoxic compounds or some *B. pumilus* synthesize various extracellular hydrolytic enzymes which destroy the cuticle of juvenile or nematode eggshell. In our studies, overall all the *Bacillus* spp suppressed the *M. incognita* J_2 infection in tomatoes resulting in the reduction of root galling and egg mass which has been proven in various studies (Fig. 5) (Chauhan et al., 2015). *Bacillus* spp. also employ other biocontrol mechanisms such as perturbation in recognition, toxins production, and competition for food and induction of systemic resistance (Gu et al., 2017). The most popular natural insecticides in agriculture are the toxin proteins generated by *Bacillus thuringiensis* (Bt). It is commonly utilized to control PPNs because *B. thuringiensis* (Bt) has nematicidal crystal proteins. For instance, BtCR371 was evaluated and proven to be effective in both *in vitro* and *in vivo* settings next to the model nematodes *Caenorhabditis elegans* (Ramalakshmi et al., 2020; Zuckerman et al., 1993). Researchers also clarified how Cry proteins govern nematodes. It is known that crystal proteins of various molecular weights can enter through the stylet of nematodes, create apertures in the esophagus, and kill the nematodes as a result. *Bacillus thuringiensis* which is naturally isolated frequently works well as a site-specific control agent. The bacterium and the nematode may have coevolved in that specific ecology, which may be the cause. Location-specific *Bacillus thuringiensis* is consistently reported to have effective toxicity, and more new proteins might be anticipated from them.

Among 18, five *Pseudomonas* spp. were identified (Fig. 9), and overall, they showed the potential of biocontrolling of root galls and eggs mass per gram of tomatoes roots as reported in various studies (Khan et al., 2016; Khatamidoost et al., 2015; Zhao et al., 2021). They protect

plants from nematodes by producing diverse metabolites including antibiotics, siderophores and HCN and enhance plant growth resulting in reducing the nematode galling in plant roots. (Akhtar and Panwar, 2013). *Pseudomonas* sp. also synthesizes variety of enzymes that are capable to modulate plant hormones concentration and additionally produce the siderophore which reduces the availability of iron. The antibiotics that produce *Pseudomonas* spp. also causes mortality in plant pest (Ali et al., 2022). Induction of systemic resistance against the plant pathogen is one of the common indirect approaches tailored by various plant biocontrol agents including *Pseudomonas* spp (Elsharkawy et al., 2022).

Leclercia spp showed consistency in both trials and significantly controlled the number of galls and egg biomass in a greenhouse experiment (Fig. 10). *Leclercia adecarboxylata* has been associated with very few plant niches, even though the bacterium is often found in the gut habitats of humans and other animals. *Leclercia adecarboxylata* and *Bradyrhizobium* species, however, have lately been associated with an increase nodulation in soybean (Kumawat et al., 2019). It is a well-known fact that *Leclercia adecarboxylata* has a rare association with plants and its presence is regularly seen in the gut of both humans and animals. However, Bradyrhizobium sp. and Leclercia adecarboxylata have lately coexisted. reported and it improve nodulation in soybean (Kumawat et al., 2019; Sahu et al., 2021). Although, *Leclercia* spp have been studied for multifaceted plant growth promoting traits and bio-compatibility in order to develop consortium biofertilizer for a specific region (Kumawat et al., 2019).

For instance, a recent study revealed how *Leclercia adecarboxylata* promotes plant growth and demonstrates how it can reduce salinity stress by producing phytohormones, enzymes, and controlling secondary metabolites (Kang et al., 2019). These characteristics promote plant health, which ultimately leads to a defensive mechanism against parasitic plants. Though, according to the best of our knowledge, we are reporting here for the first time *Leclercia* spp as an antagonist against root-knot nematodes (Fig. 7). *Paenarthrobacter nicotinovorans* CRS-30 significantly colonized wheat plant roots, developed a biofilm in the root matrix, and improved seed germination (%). It also possessed several PGPR characteristics, solubilized zinc, phosphorus, and potassium. Other than that It's interesting to notice that roots under microcosm conditions showed the highest expression of eight zinc transporter (TaZIP) genes, which led to an accumulation of increased zinc content in the plant that had been inoculated with bacterial strains (Yadav et al., 2022). According to (Yuan et al., 2022).

PGPB and biocontrol agent known as *Paenarthrobacter* has been used extensively to combat plant diseases and as biofertilizers. *Paenarthrobacter* spp generally inhabits abundantly

in bulk soil and rhizospheric soil and is reported plant growth promoter in various studies (Guo et al., 2020; Yuan et al., 2022). To combat several crop diseases, the fungicide iprodione, also known as 3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazoli dine-1-carboximide, has been developed. In Taiwan, it is advised for managing fungal diseases in strawberry, tobacco, and pear plants. Numerous data suggest that iprodione may prevent environmental organisms from growing. Therefore, the diversity and functionality of beneficial microorganisms associated with plants may be impeded, which could ultimately have an effect on the health and productivity of the plants. Differnt studies have documented *Paenarthrobacter* sp degradation's of iprodione (Katsoula et al., 2020; Zhang et al., 2021). They bioremediate agricultural soil using carboxylic acid imide fungicides like iprodione and vinclozolin. The *Paenarthrobacter* strain, which was able to break down iprodione, also suggested an unusual Arthrobacter specialty in the breakdown of this fungicide.

Dimethachlon, a representative of the dicarboxamide fungicide class, exhibits better efficacy in controlling *Sclerotinia* spp stem rot and Sclerotinia blight on tobacco, lettuce, rice, cucumber, and oilseed rape (Duan et al., 2013; Wang et al., 2009). Dimethachlon, a broad-spectrum dicarboximide fungicide, poses a risk to both human and environmental safety due to its residue in the environment. A promising option for bioremediation of dimethachlon-contaminated environments is *Paenarthrobacter* sp. JH, which has the capacity to metabolize dimethachlon and utilize it for growth (Zhang et al., 2022). In a series of greenhouse pot trials, the pantoea strain with the highest nematicidal activity in the *in vitro* experiment was assessed for root-knot biocontrol ability (Fig. 8). The pantoea strain reduced disease incidence and disease index in both of the repeated trials in which the strain was applied as soil drenches in comparison to the no-bacteria (nematode-only) control (Fig. 8). The tomato plant's defenses have been strengthened with pantoea to be more effective against the nematode *Meloidogyne incognita*. On tomato plants, *M. incognita* is known to cause root knots. When applied as a soil drench, *Pantoea agglomerans* MK-29 has been shown to increase the tomato plant's systemic resistance and reduced the ability of juvenile nematodes to penetrate and prevent the formation of root knots (Munif et al., 2001).

Pantoea agglomerans 1-7 was the most efficient strain, showing a reduction in disease incidence of almost 58% in relation to un-inoculated control, according to (Zhou et al., 2016). The bean seedling was heavily colonized by *Pantoea agglomerans* from root to apical stem, which potentially controlled bacterial wilt of bean by reducing the disease's severity (Hsieh et al., 2005). Under nitrogen-limited situations, *Pantoea* promote plant development by facilitating

nitrogen fixation in plant roots. Some Pantoea species are particularly appealing for bioremediation and biocontrol. A full hand trains of *Pantoea* have been used as biocontrol agents in commercial bioproducts that protect plants from disease by combating infections and, in some conditions, by triggering the plant's natural defense mechanisms. *Pantoea* sp also contains distinctive biodegradative properties, such as the cellular metabolism that degrade pesticides and other hazardous compounds, enabling the development and marketing of beneficial products (Walterson and Stavrinides, 2015).

There are several species in the genus *Exiguobacterium* that are known to have extremophilic characteristics (Fig. 8). In one of the research that are currently accessible, in disease-suppressive soils, *Pantoea, Exiguobacterium*, and *Microbacteria* interacted to suppress *R. solani* AG-8 on wheat, according to research by (Barnett et al., 2006). *Exiguobacterium* has been shown in supressing development and growth of *Pythium*, *Rhizoctoniasolani*, *Sclerotium rolfsii, and Fusarium oxysporum* in as well as having the ability to produce siderophore and HCN. R. solani, *S. rolfsii, Pythium, and F. oxysporu*m's hyphal development was shown to be most effectively inhibited by the compound the bacterium produced (Selvakumar et al., 2009). Exiguobacterium sp. KRL4 generates compounds with antioxidant and siderophore properties, according to biological assays. Additionally, intracellular extracts demonstrated nematocidal activity against *C. elegans*, indicating that strain KRL4 is a source of anthelmintic compounds (Tedesco et al., 2021)

To the best of our knowledge, this is the first report on the antagonistic property of *Exiguobacterium* spp. against plant parasitic nematodes, *M. incognita*.

It is obvious that many antagonistic bacteria are still unexplored when one considers the diversity of microorganisms observed in the soils of diverse agroecosystems all around the world. To discover novel microflora to be employed in bio-control programs, concerted searches and screening techniques are crucial. We suggest that this cold-tolerant strain of *Exiguobacterium acetylicum* strain is a possible antagonistic bacterium where location-specific biological control agents are needed to achieve the desired consequences of inoculation.

Conclusion

Plant-parasitic nematodes highly damage vegetables and other agronomic crops and cause unbearable loss to the agricultural economy across the world. Plant-associated microbes contribute in engineering the soil's suppression against soil-borne pathogens specifically PPN and their contribution has been proven in both culture-dependent and culture-independent

techniques. Our findings concluded that soil suppressiveness has been credited to the specific microbial communities. Introducing the plant-beneficial isolated microbes into conducive field soil often fail to maintain their reproduction and functions. In this studies, we report that the introduction of invasive plant growth promoting biological control agents into suppressive soil also interfere the microbiome functions and significantly reduce its disease suppression. Suppressive soil microbiota shows sensitivity to fungicides and antibiotics and influenced negatively their functionality that led reduction in soil suppression. Our findings revealed that bacterial communities comparatively play vital role in soil suppression as compared to fungal populations. The isolated microbes of suppressive soil controlled *Meloidogyne incognita*, suggesting that microbes are the origin of soil suppression. To understand the insights of plant microbes and microbes nematodes interactions and functions in the rhizosphere microbiome, metagenomics studies could be harnessed in future to elucidate the mechanisms and mode of actions of the existed microbiota

Declaration of competing interest

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

Data availability

Data will be made available on request.

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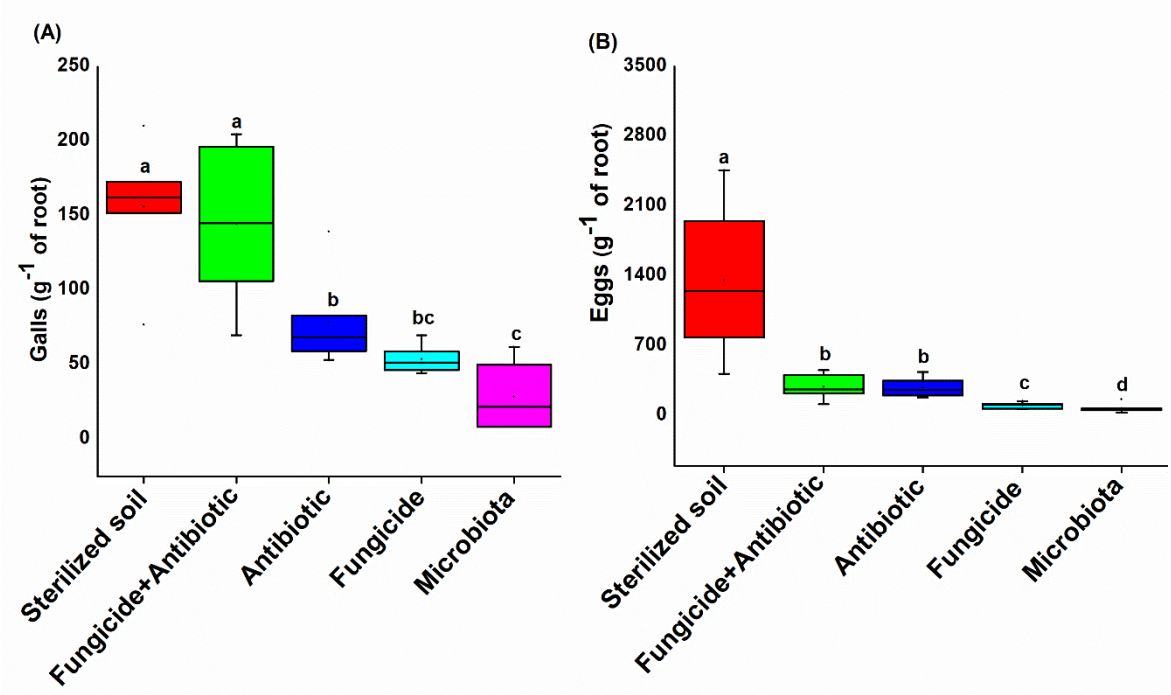


Fig. 1. Influence of fungicide (Cyproconazole at 100ppm) and antibiotic (Streptomycin at 100 ppm) on soil microbiome solution on the (A) Number of galls and (B) Number of eggs of *Meloidogyne incognita*. Microbiota is the microrganisms extracted from the suppressive soil. The number of galls and eggs were quantified 45 days after the infestation of 200 J_2 on tomato root (*Solanum lycopersicum* 'Santa Clara'). Six replicates were used in each treatment and bars with different letters indicate significant differences between different treatments by Duncan's test ($P < 0.05$)

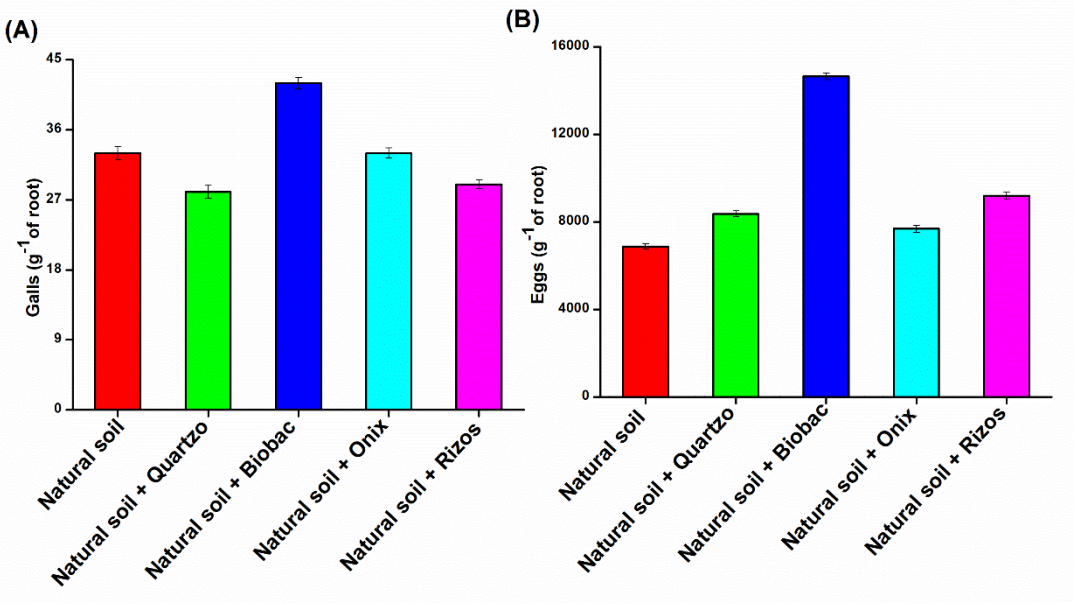


Fig. 2. Evaluation of galls and eggs of tomato plant roots (*Solanum lycopersicum* 'Santa Clara') 45 days after the infestation of 500 J2 suppressive soil. The commercially-available bioproducts products applied and determined their influence on biocontrol of M. incognita and soil microbiota activities. A) Number of galls B) Number of eggs. Eight replicates were used in each treatment and bars with different letters indicate significant differences between different treatments by Duncan's test (P <0.05)

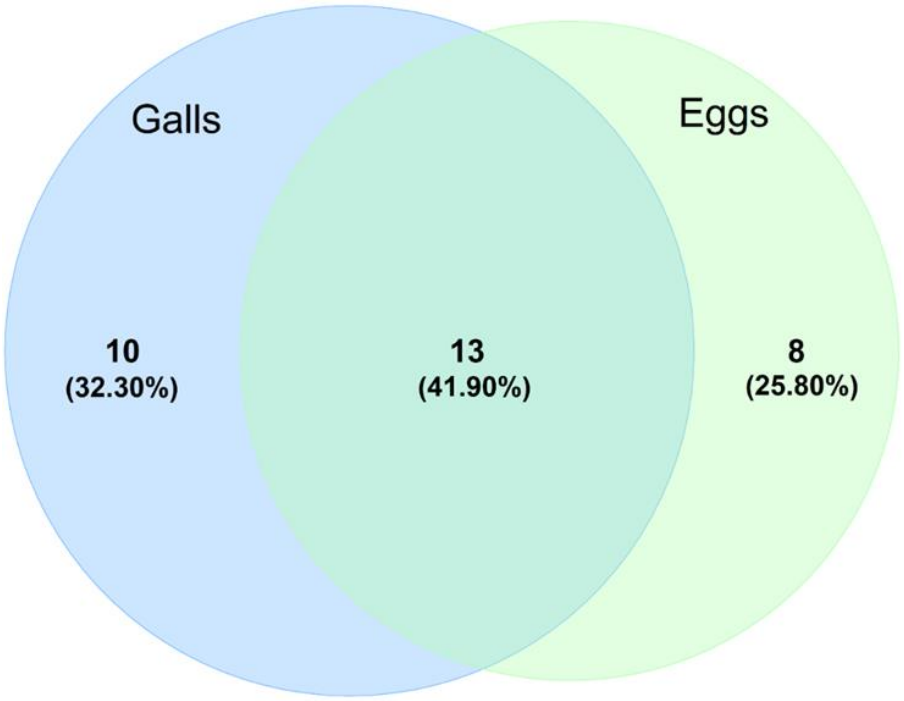
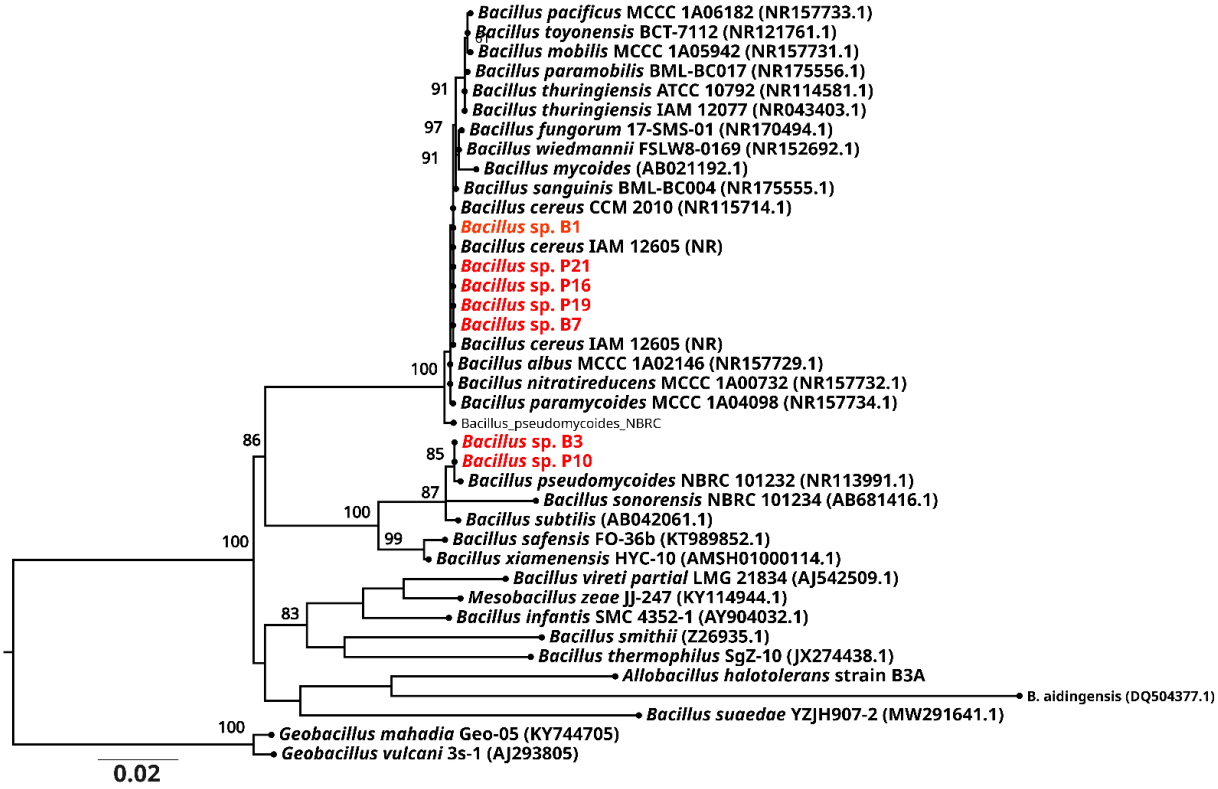
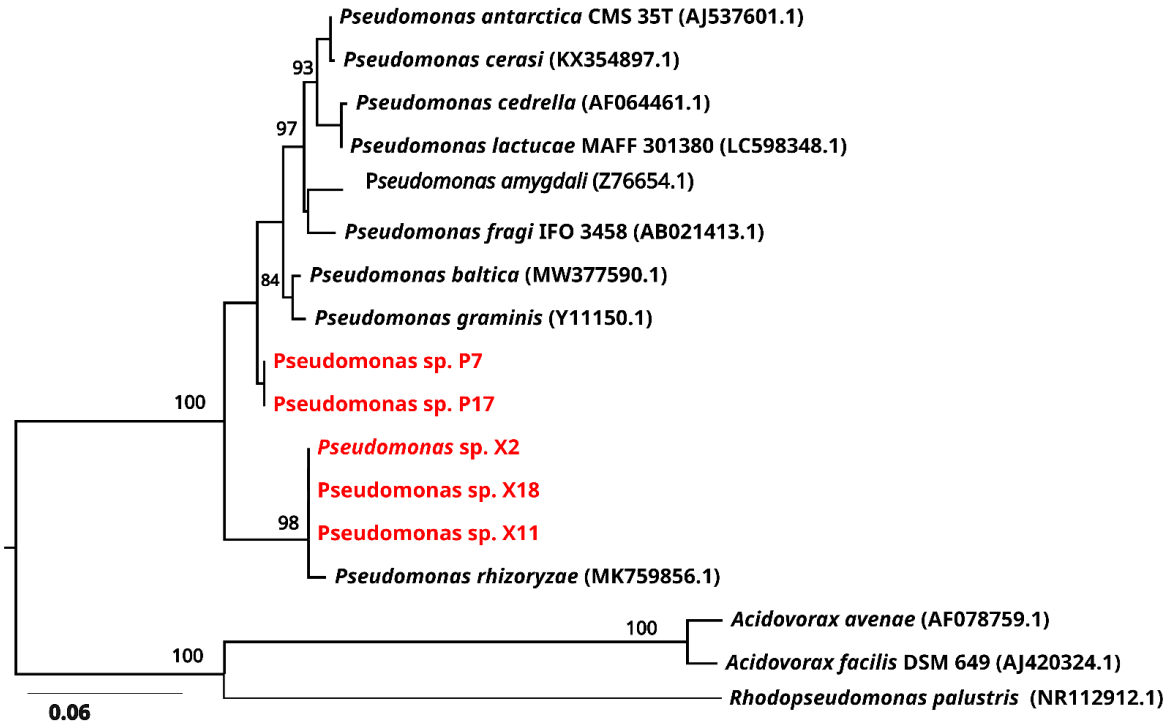


Fig. 3. The Venn diagram represents the ratio of bacterial isolates that controlled significantly the number of galls and eggs. Ten isolates significantly controlled the galls, 8 significantlly controlled the eggs and 13 bacterial isolates significantly controlled both the galls and eggs in preliminary screening.

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Pseudomonas lactucae MAFF 301380 (LC598348.1)
Pseudomonas amygdali (Z76654.1)
Pseudomonas fragi IFO 3458 (AB021413.1)
84
Pseudomonas baltica (MW377590.1)
Pseudomonas graminis (Y11150.1)
Pseudomonas sp. P7
100
Pseudomonas sp. P17
Pseudomonas sp. X2
Pseudomonas sp. X18
98
Pseudomonas sp. X11
Pseudomonas rhizoryzae (MK759856.1)
100
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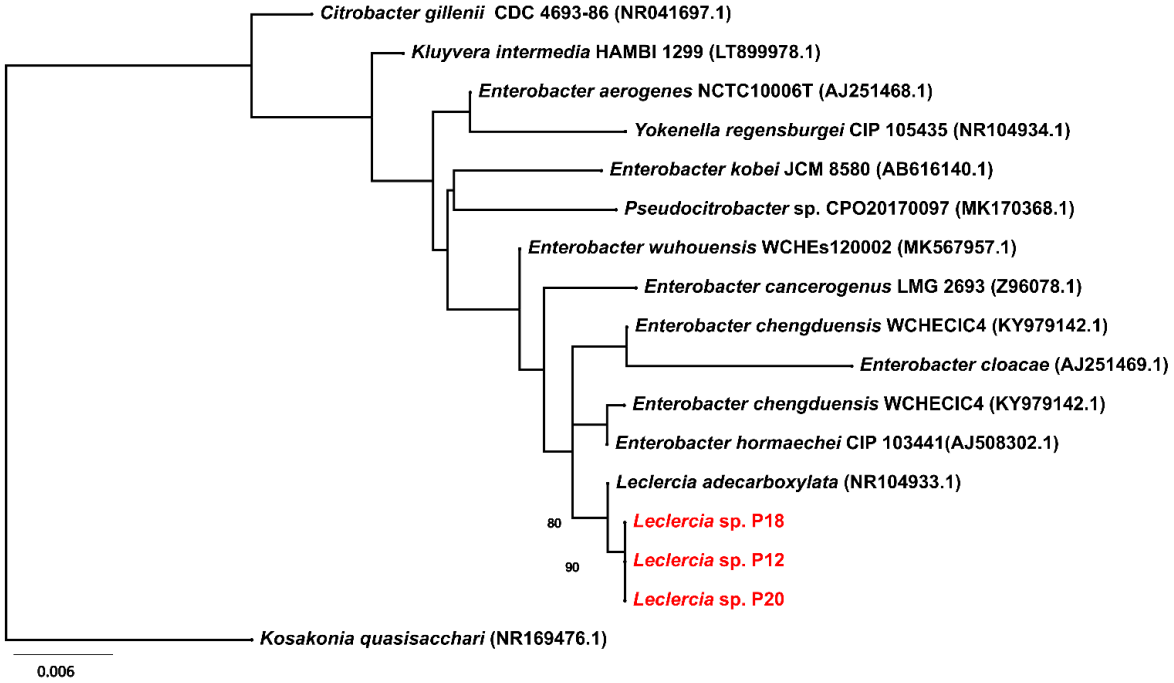
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Kluyvera intermedia HAMBI 1299 (LT899978.1)
Enterobacter aerogenes NCTC10006T (AJ251468.1)
Yokenella regensburgei CIP 105435 (NR104934.1)
Enterobacter kobei JCM 8580 (AB616140.1)
Pseudocitrobacter sp. CPO20170097 (MK170368.1)
Enterobacter wuhouensis WCHEs120002 (MK567957.1)
Enterobacter cancerogenus LMG 2693 (Z96078.1)
Enterobacter chengduensis WCHECIC4 (KY979142.1)
Enterobacter cloacae (AJ251469.1)
Enterobacter chengduensis WCHECIC4 (KY979142.1)
Enterobacter hormaechei CIP 103441(AJ508302.1)
Leclercia adecarboxylata (NR104933.1)
80
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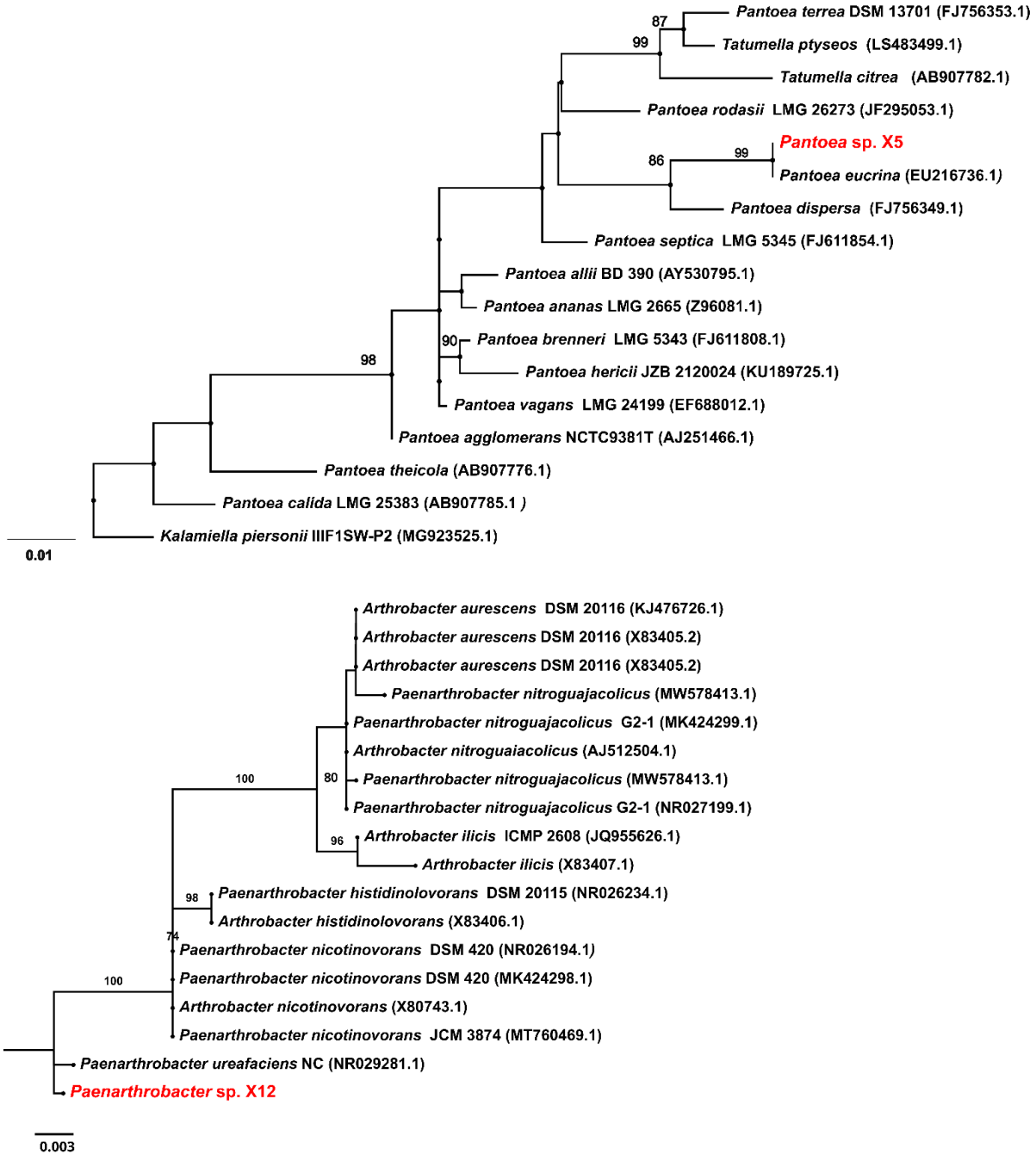
90
Leclercia sp. P12
Leclercia sp. P20
Kosakonia quasisacchari (NR169476.1)
0.006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Tatumella citrea (AB907782.1)
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Pantoea sp. X5
Pantoea eucrina (EU216736.1)
Pantoea dispersa (FJ756349.1)
Pantoea septica LMG 5345 (FJ611854.1)
Pantoea allii BD 390 (AY530795.1)
Pantoea ananas LMG 2665 (Z96081.1)
Pantoea brenneri LMG 5343 (FJ611808.1)
Pantoea hericii JZB 2120024 (KU189725.1)
Pantoea vagans LMG 24199 (EF688012.1)
Pantoea agglomerans NCTC9381T (AJ251466.1)
Pantoea theicola (AB907776.1)
Pantoea calida LMG 25383 (AB907785.1)
Kalamiella piersonii IIIF1SW-P2 (MG923525.1)
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Paenarthrobacter nicotinovorans DSM 420 (NR026194.1)
Paenarthrobacter nicotinovorans DSM 420 (MK424298.1)
Arthrobacter nicotinovorans (X80743.1)
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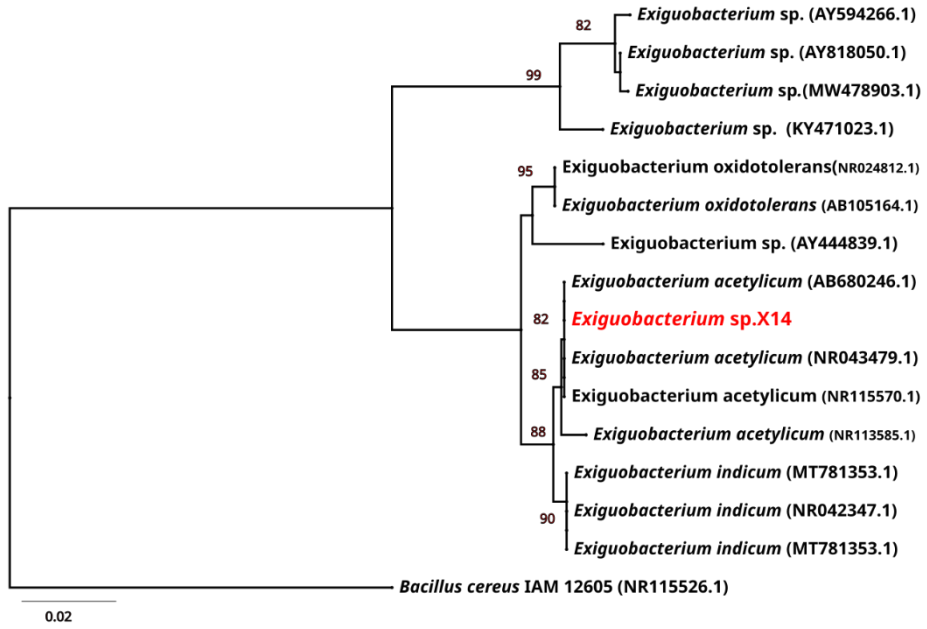

Fig. 4. Phylogenetic tree of *Bacillus, Pseudomonas, Leclercia, Pantoea* X5, *Paenarthrobacter* and *Exiguobacterium* spp inferred by analysis of 16S rRNA. The Maximum Likelihood technique and bootstrap support of 1000 replications were used to build the phylogenetic tree. The number on each node indicates the percentage of trees in. Analyses were conducted in MEGA6 Software

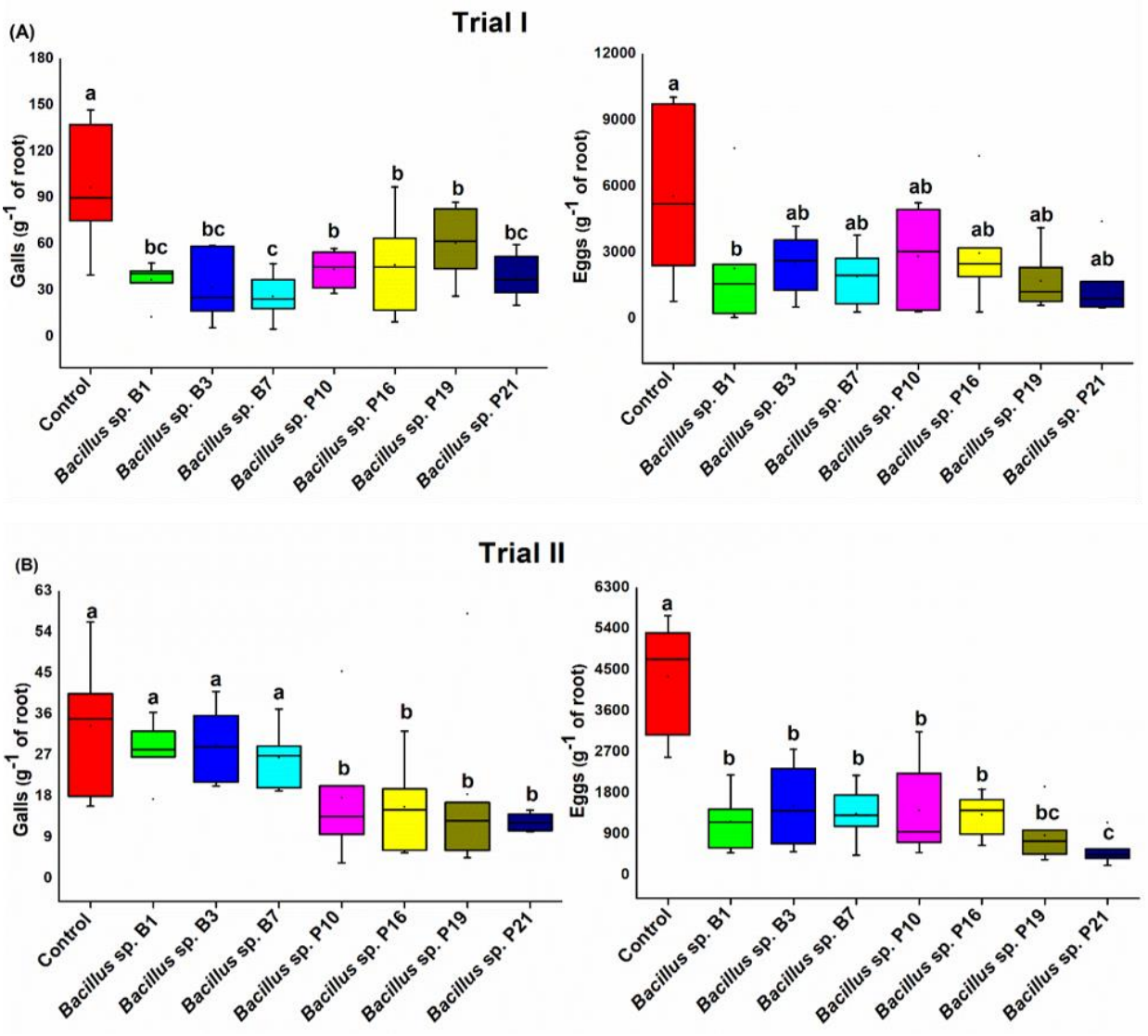

Fig 5. Biocontrol effect of *Bacillus* isolates (B1, B3, B7, P1, P16, P19, P21) on *Meloidogyne incognita* under the greenhouse environment. The number of galls/g and number of eggs/g of tomato seedlings infested with *Meloidogyne incognita* J2. Different letters indicate significant differences among treatments based on Duncan test ($p \leq 0.05$). The experiment was repeated twice (Trial1 and 2)

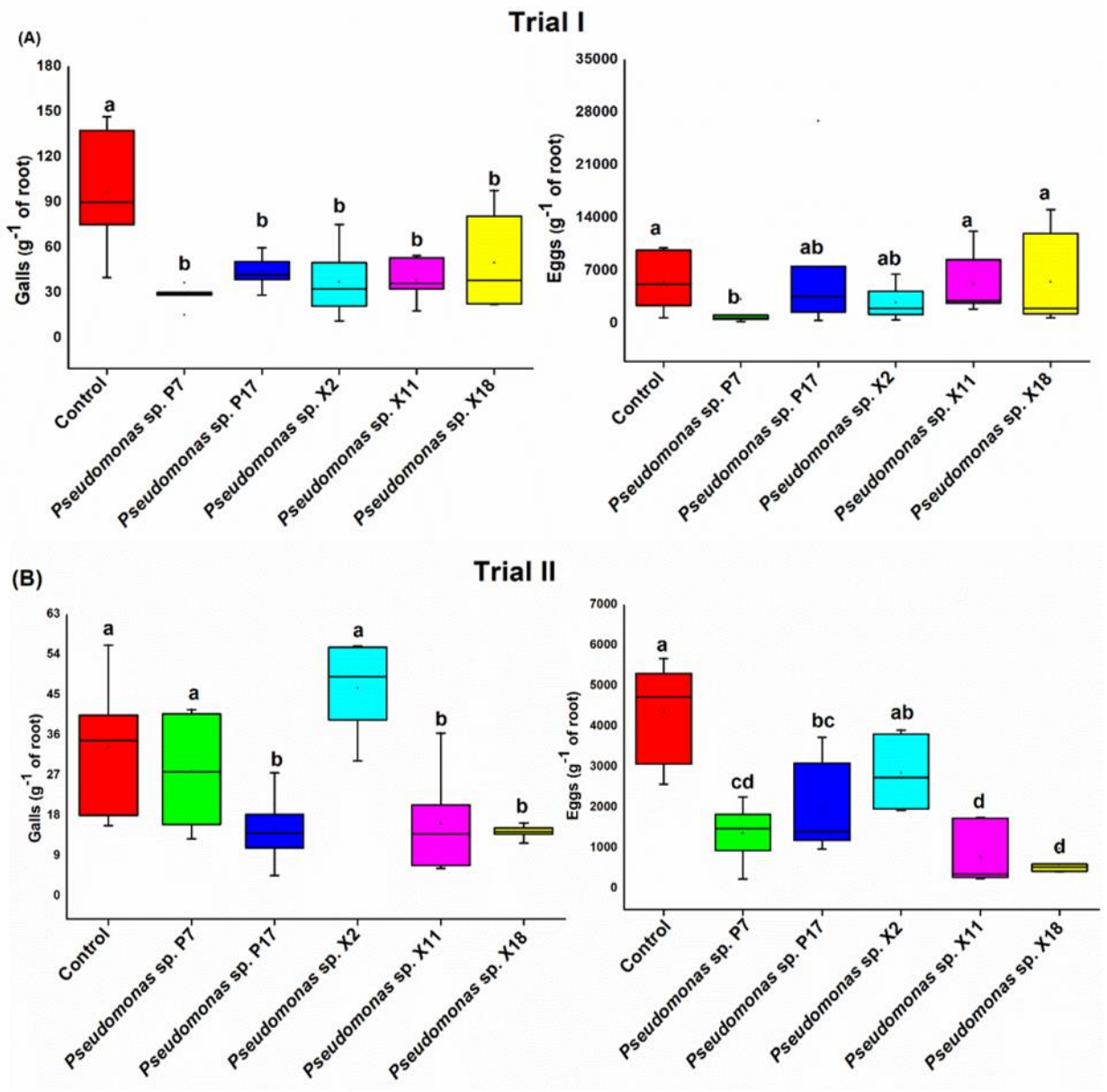

Fig 6. Biocontrol effect of *Pseudomonas* isolates (P7, P17, X2, X11, X18) on *Meloidogyne incognita* under the greenhouse environment. The number of galls/g and number of eggs/g of tomato seedlings infested with *Meloidogyne incognita* J2. Different letters indicate significant differences among treatments based on Duncan test ($p \leq 0.05$). The experiment was repeated twice (Trial1 and 2)

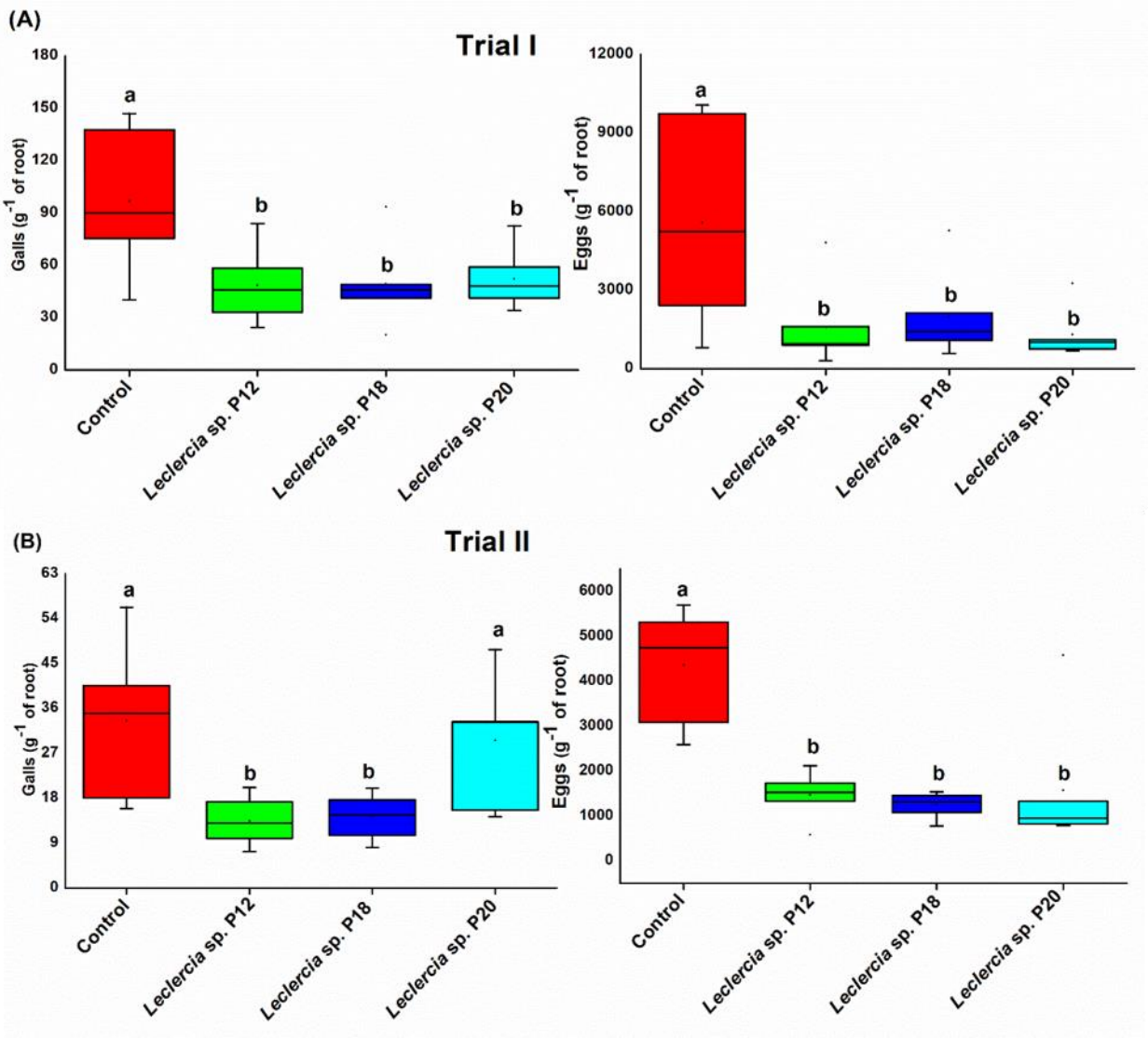

Fig 7. Biocontrol effect of *Leclercia* isolates (P12, P18, P20) on *Meloidogyne incognita* under the greenhouse environment. The number of galls/g and number of eggs/g of tomato seedlings infested with *Meloidogyne incognita* J_2. Different letters indicate significant differences among treatments based on Duncan test ($p \leq 0.05$). The experiment was repeated twice (Trial1 and 2)

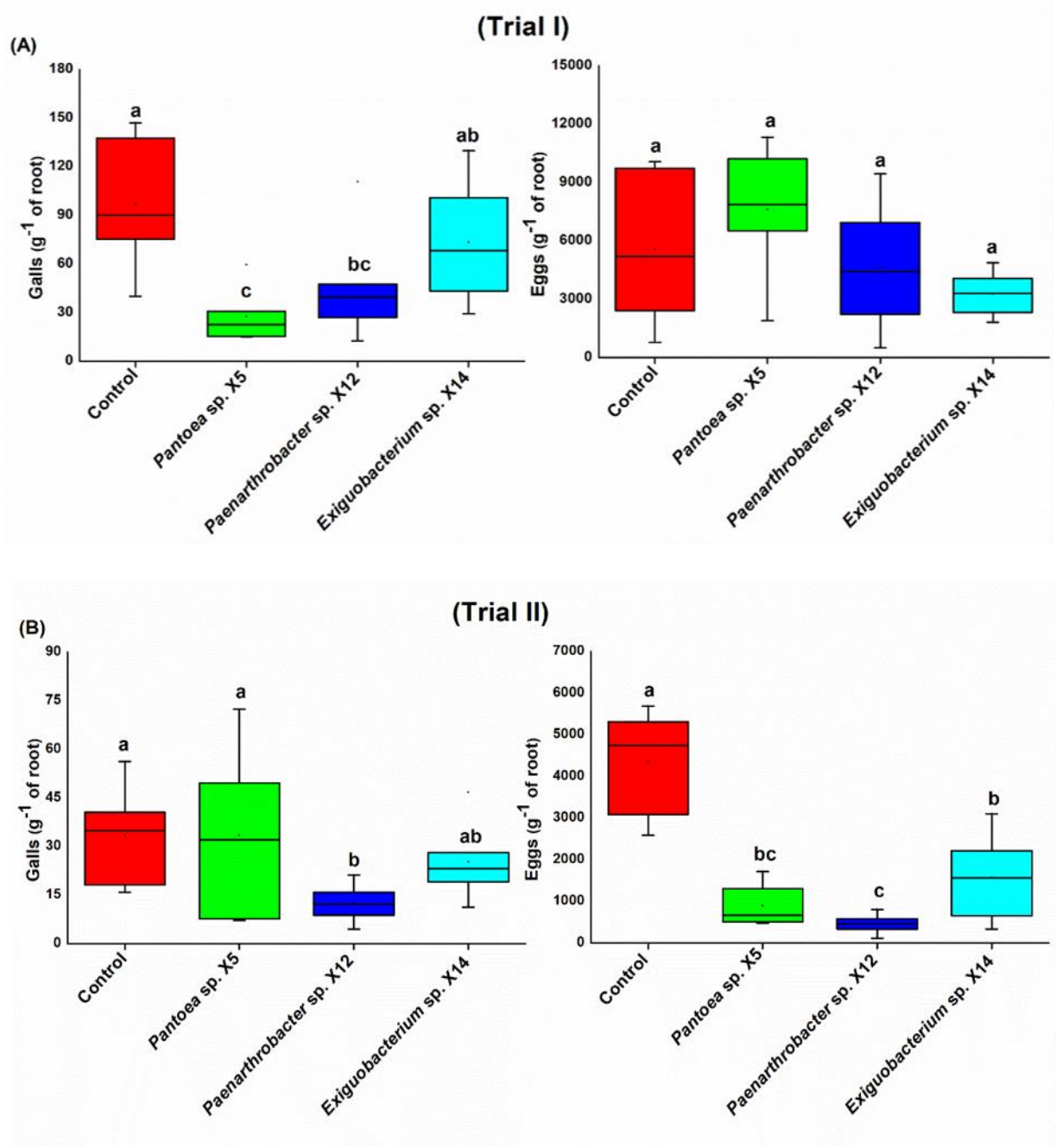

Fig. 8. Biocontrol effect of *Pantoeai* sp., *Paenarthrobacter* sp., and *Exiguobacterium* sp. (X5, X12, X14) on *Meloidogyne incognita* under the greenhouse environment. The number of galls/g and number of eggs/g of tomato seedlings infested with *Meloidogyne incognita* J_2. Different letters indicate significant differences among treatments based on Duncan test ($p \leq 0.05$). The experiment was repeated twice (Trial1 and Trial 2)

List of tables

Table 1. Physicochemical characteristics of the used suppressive soil

Soil type	Clay	Micronutrients	Unit	Macronutrients	Unit
pH	6.6	K	54.63 mg/dm³	Zn	3.1 mg/dm³
Organic matter	2.55 dag/kg	P	2.97 mg/dm³	Fe	68.2 mg/dm³
		Ca	4.61 cmolc/dm³	Mn	58.6 mg/dm³
		Mg	1.8 cmolc/dm³	Cu	3.59 mg/dm³
				B	0.16 mg/dm³
				S	0.9 mg/dm³

Table.2. Application of bio products in *M. Incognita* inoculated suppressive soil and their influence on soil microbiota activities

Treatments	Formulation	Mode of action	CFU	Dose
Suppressive soil	Microbiota			
Suppressive soil + Quart	*Bacillus subtilis;Bacillus licheniformis*	Nematicide	1×10^{11}	200ml/ha
Suppressive soil+ Bioba	*Bacillus subtilis* strain Y1336	Fungicide, bactericide	1×10^{9}	1L/ha
Suppressive soil+ Onix	*Bacillus methylotrophicus*	Nematicide	1×10^{9}	6L/ha
Suppressive soil+ Rizos	*Bacillus subtilis*	Nematicide	1×10^{9}	4L/ha

Table.3. Number of galls and eggs per gram of tomato root plant

	Soil type	Without *B. velezensis* BMH	Without *B. velezensis* BMH
Galls^{-g}	Sterilized soil	76 A a	50 B b
Eggs^{-g}	Natural soil	67 B a	58 A b
Galls^{-g}	Sterilized soil	5475 A a	3653 A b
Eggs^{-g}	Natural soil	3131 B b	4636 A a

Table. 3. The number of galls^{-1} and eggs^{-1} of root was measured at 45 days after J2 of *M. incognita* inoculation in normal and sterilized soil with and without *Bacillus* BMH. Number of eggs. The values are mean ±SD (n = 8). Upper case letters within the same column and lower case letters within the same bar indicate significant differences by the Duncan's test ($P < 0.05$)

Table.4. Root weight of tomato root plant per gram in sterilized and suppressive soil.

Treatments	**Root weight (g)**	
	Bacteria -	**Bacteria +**
Sterilized soil	**6.42 Aa**	**7.86 Aa**
Suppressive soil	**6.13 Aa**	**4.78 Ba**

Table.4. Root fresh weight was measured at 45 days after J2 of *M. incognita* inoculation in normal and sterilized soil with and without *Bacillus* BMH. The values are mean ±SD (n = 8). Upper case letters within the same bar and lower case letters within the same bar indicate significant differences by the Duncan's test ($P < 0.05$)

ARTICLE 3- Investigating the mode of action of the bacterial populations obtained from a *Meloidogyne*-suppressive soil on plant pathogenic soil-borne nematode, fungi and bacteria

Abstract

Disease-suppressive soils contribute to the protection of plants from an array of soil-borne plant pathogens. The foundation of specific disease suppression in most soils affiliates commonly to soil microbial communities. Therefore, the soil microbiota of suppressive soils is considered one of the factors contributing to disease suppressiveness against soil-borne diseases. To date, a multitude of microbial taxa and genes have been documented as central players in participating disease suppressiveness of soils. Still, the dominant genera and their underlying mechanisms remain elusive for most disease-suppressive soil microbiomes. The goal of the current study was to evaluate the potential of bacteria obtained from a soil suppressive to root-knot nematode (RKN) *Meloidogyne incognita* on the direct activity towards nematodes and other soil-borne pathogens of fungal and bacterial nature. A total of 42 bacterial strains were isolated from the suppressive soil and 18 of them were identified with a high potential to control *M. incognita*. The cell-free supernatants and volatile organic compounds (VOCs) of all different genera killed more than 85% and 88% J_2 in *in vitro* experiment. Additionally, all bacterial isolates inherit nematicidal activities and reduced the hatching reduced nearly 7% M. *incognita* eggs hatching reduced nearly 7% when expose to cell-free supernatant of all bacterial strains. Moreover, all bacterial isolates inherit nematicidal activities and significantly reduced egg hatching The isolates were sequenced based on 16S rRNA. The strains significantly reduced the mycelial growth of plant pathogens *Fusarium oxysporum*, *Rhizoctonia solani* and *Ralstonia solanacearum.* These findings denote that the underlying mode of action of the selected biocontrol agent is more related to the root protection against penetration and/or downstream events in egg hatching than direct activity on the eggs and such strains may confer further protection on the release of toxic VOCs and inhibiting other soil-borne pathogens.

Keywords. Soil microbiome manipulation, soil-borne disease, biological control agent (BCA), Bio-pesticides, sustainable disease management

Introduction

The soil microbiota plays a vital role in plant health and fitness by restricting the functionality of soil-borne plant pathogens and requisitioning soil nutrients. The antagonistic microorganisms originally contribute to the establishment of disease-suppressive soil. Antagonistic microbes of suppressive soil serve as the first line of defense for plants where plant roots use rhizodeposition to enrich, nourish, and promote soil microorganisms to fend off soil-borne pathogens (Schlatter et al., 2017a).

Soil is an intricate and biodiverse belowground ecosystem ever known. A single gram soil harbor 10^9–10^{10} prokaryotic organisms (bacteria, archaea), 10^4–10^7 protists, 100–1000 m of fungal hyphae, and 10^8–10^9 viruses (Afridi et al., 2022a; Ossowicki et al., 2021; Tecon and Or, 2017; Vos et al., 2013).

The diverse and abundant microbial communities of plant rhizosphere contribute to maintain plant health and fitness (de Faria et al., 2021; Yu et al., 2020). Numerous ecosystem services, including, control of greenhouse gas emissions, carbon sequestration, erosion mitigation, pollution degradation, boosting crop nutrient uptake, reducing plant diseases, and stress resistance, are facilitated by the activities of the soil biota. (Afridi et al., 2022b; Ahkami et al., 2017; Bardgett and van der Putten, 2014; Ossowicki et al., 2021), so reducing the overuse of chemical pesticides or fertilizers. Building beneficial rhizospheric microbiomes is founded on mechanisms governing the rhizosphere microbial community assembly (Gu et al., 2020; Trivedi et al., 2020).

The primary and secondary metabolites that soil bacteria release have an impact on their environment. In this way, soil microbiota alter not only their niche but also influence their surroundings whether positively or negatively, (Pande and Kost, 2017; Schlatter et al., 2017a). The notorious disease such as Take-all, damping-off, root rot, and wilting are examples of many soil-borne diseases caused due to soil-borne pathogens in plant and significantly reduce the economic yield of important crops while also contributing to soil decline. (Lamichhane et al., 2017; Li et al., 2015; Sapkota et al., 2022). Soil-borne pathogens are one of the key factors that could reduce the productivity of agro-ecosystems, as using resistant host cultivars to tackle them is exceedingly challenging. (Jambhulkar et al., 2011).

Disease-suppressive soil (DSS) and its exploitation could be one of the most effective tools in sustainable agriculture, whose native microbial community efficiently shields host plants against pathogen invasion by activating a multitude of biocontrol mechanisms (Weller et al., 2007, 2002). Disease suppressive soil accredited the soil with stable microbial

communities and beneficial physical and chemical characteristics that improve crop health and provide protection. Because soil sterilization eliminates or lowers the soil's natural potential for disease management, this is how soil suppression against disease as a biological feature of the soil offered by its own microbiome, regarded sustainable tool in disease prevention (De Corato, 2020). The suppressive potential of a soil is determined by the abundance and functions of antagonistic microorganisms (Hadar and Papadopoulou, 2012; Schlatter et al., 2017b). Similarly, the ability of microbiota to reduce the pathogens activities and improve the soil health and functions increase the degree of suppression against disease (van Bruggen et al., 2015). For instance, the pathogens's functions can be controlled or reduced by the microbiome of disease suppressive soil and diseases through a variety of integrated mechanisms, including increased plant fitness, increased production of natural plant defenses, antibiotic production, competition with the pathogen and modification of the plant immune systems, or hyper-parasitization of the pathogen. (Liu et al., 2021; Schlatter et al., 2017b).

Despite the fact that understanding how disease-suppressive soil functions is crucial, (Kinkel et al., 2011), Some suppressive soils have already been thoroughly described. (Kyselková et al., 2009). Because soil is a sophisticated dynamic ecosystem that feeds bacteria with what are known as super-genomes in a specific habitat, comprehensive information on disease suppressive soil is still lacking (Bulgarelli et al., 2013). We can facilitate in the recruitment and utilization of particular beneficial bacteria to protect the plant from diseases by understanding how plants and microbiota interact (Liu et al., 2021). Only by studying the rhizosphere microbiome can we determine the direct and indirect modes of action that the beneficial microbiota can employ to contribute to the reduction of soil disease (Weller et al., 2007, 2002).

Contrary bacteria offer hope as potential biological pest controls for plant-parasitic nematodes, as has been consistently documented (Giannakou et al., 2004). The suppression of detrimental and pathogenic rhizosphere microorganisms has enhanced the environment for root growth as a result of the identification of many *Pseudomonas* species as plant growth-promoting rhizobacteria (PGPR). These organisms also produce siderophores that chelate iron, antibiotics, and hydrogen cyanide (Siddiqui, 2005).

In the current research, first, the suppressive soil was screened against RKN, and then the study was conducted with the hypothesis, to isolate and identify the microbes from

suppressive soil to decipher, which groups of microbes caused and validated the soil suppression against RKNs.

Materials and Methods

Study area. Soil collection site and soil sampling

The soil samples were collected from the sweet pepper-cropped (*Capsicum annuum* cv. 'Magali') cultivation system at Universidade Federal de Lavras (UFLA)-Centro de Desenvolvimento e Tranferência de Tecnologia (CDTT) (51°18′40″N, 6°12′10″E) property in Ingai, Minas Gerais, Brazil (Fig. 1). This horticultural area has been cultivated with sweet pepper (*Capsicum annuum* cv. 'Magali') continuously for since last four (4) years. using (2-3 cycles per year) applying organic practices. After proper investigation, it was revealed that this area was highly populated with *M. incognita* nematodes and the production of susceptible sweet pepper to *M. incognita* was not influenced by these invaders. The soil was sampled between harvesting and new planting from the upper layer (0-0.2 m) of the pepper cultivation system randomly selected at 10 different points (each point was distant 10m).
Each soil sample was thoroughly air dried, blended, and then placed in a plastic bag before being transported to the lab in a cool box.

Bacterial community's isolation

After the screening of suppressive soil microbiome suppressiveness, we decided to investigate the responsible candidate of root-knot nematodes *M. Incognita* disease suppression. The serial dilution technique was applied to isolate bacterial strains. Rhizosphere soil (10g) was diluted into 90 mL sterile distilled water, (Bharathi et al., 2004), by using the Drigalsky, an aliquot of 100 µL of each dilution was dispersed in Petri dishes containing nutrient agar culture media (Kado, 1970) then incubated for 24 hours at in incubator having a temperature of 28 °C. Colonies with different morphologies were collected, sub cultured and replicated to get the purified isolates. A total of fifty (42) bacterial isolates were isolated and stored in cryogenic tubes at -20 ^{0}Cfor further study.

Blood Hemolytic assay of isolates for Pathogenicity

To evaluate their hemolytic activity, the chosen bacterial isolates were cultured on human blood agar media at 37°C for 48 hours according to standard protocols. Strains were streaked onto blood agar plates that contained 40 g of a Tryptone Soya agar base and 50 ml of horse blood per liter. At 37 °C, plates were incubated for 48 hours.

The presence of clear ß), greenish-brown (α), and no zones (γ), on the plates, which, respectively, indicate complete, partial, and no hemolytic activities were evaluated according to the (Russell et al., 2006).

Compatibility of mixed strains

The bacterial strains were evaluated for the compatibility traits on NA agar by crossing two biocontrol strains (streaked at a 90° angle from one another) (Liu et al., 2018). The plates were incubated for 48 h at 28 ℃ and then assessed the inhibition zone between them. Incompatibility between strains was indicated by the presence of inhibitory zones, whereas compatibility was indicated by the absence of an inhibition zone.

In vitro antagonistic assay for biocontrol potential against Fusarium oxysporum and Rhizoctonia solani

A dual-culture assay was applied to evaluate the antagonistic activity of the eighteen selected strains against two fungi, *Fusarium oxysporum* f.sp. lycopersici CML1875 and *Rhizoctonia solani* CML 3193 followed the method of (Nandakumar et al., 2001).

Using a sterilized cork borer, five-millimeter mycelial discs were cut from the young growing edge of a seven-day-old culture of the fungus and deposited at one side of a Petri plate. The 18 bacterial isolates were incubated at 28 $\pm$2 °C for 13 days after being streaked aseptically parallel to the fungus at a distance of 15-20 mm. Each isolate was maintained in three replications. To the nearest millimeter, the difference in growth inhibition between the two cultures was quantified. Following 13 days of incubation, the percentage of the fungus that was inhibited was determined using the following formula:

Growth inhibition (%) =

Where R1 = In the absence of hostile microorganisms, the fungus's radius (mm) from the colony's center to the plate's center

R2 = Radius of the fungus (mm) from the colony's center to the antagonistic bacteria (Islam et al., 2018; Nandakumar et al., 2001)

In vitro antagonistic activity assay for biocontrol potential against Ralstonia solanacearum useing Chloroform vapour method

In vitro antagonism studies between suppressive soil-associated bacterial strains and pathogenic *R. solanacearum* strain were conducted on MBI agar media plates employing the chloroform vapour technique (Lemessa and Zeller, 2007; Ryan et al., 2004). Candidate antagonistic bacteria were spotted on MBI media and incubated for 24 h at 28 °C. After 24 h of incubation, the growing antagonists were killed by inverting Petri dishes over chloroform for 10 min. The pathogenic strain pathogenic *R. Solanacearum* was cultured in MBI broth in the incubator for 24 h and then 100µl bacterial suspension inoculated into the test tubes containing 5ml semisolid MBI media, homogenized properly and flooded into the plates possessing grown antagonists, dried and incubated further for 48 h. Measuring the inhibitory zones around antagonistic bacteria was measured to assess the biocontrol effectiveness of various bacterial strains. The study was conducted utilizing a randomized complete design and performing three replicates of each antagonistic bacterial strain.

Effect of bacterial isolates supernatants against Meloidogyne incognita J_2

The impact of all bacterial isolate's cell-free supernatants on *Meloidogyne incognita* J_2 mortality was assessed using (Cruz-Magalhães et al., 2022) methodology. Aliquots of 500 L of the supernatants from all bacterial isolates were prepared and blended with 500 L of an aqueous suspension containing 100 *M. incognita* J_2 in 2 mL microcentrifuge tubes to evaluate the effects of the cell-free supernatants on the mortality of *M. incognita*. Only 500 L of N broth medium was combined with the *M. incognita* suspension in the controls. The microcentrifuge tubes underwent a 48-hour incubation period at 28 °C. The J_2 nematode suspension from the microtubes was transferred to ELISA polypropylene microplates to analyze and quantify the number of both mobile and immobile J_2 nematodes

Toxicity of volatile compounds emitted by bacterial isolates to M. incognita J_2

A silicone layer was placed inside of 80 × 28 mm screw-top vials (Sigma-Aldrich, Bellefonte, PA, USA) to attach them to the vial and provide a full seal. These sterilized vials

contained about 25 g of dry, autoclaved sand. Three (3) ml bacterial suspension was spread over the autoclaved sands within the vials. As a control, some LB media was inoculated into the autoclaved sand. The Supelco vials were filled with sand up to halfway and put a 2 mL sterile microtube. A 500 L suspension of 100 *M. incognita* J_2 was then injected into the buried microtube using a 3 mL syringe. The vials were then sealed and maintained at 25 °C for two days without any light. Tge vials then opened, transferred the J_2 nematode suspension in the micro tubes to ELISA polypropylene microplates, and use an inverted microscope to count both the mobile and immobile J_2 of nematodes. after the J_2 nematodes had been exposed to the VOCs produced by the bacterial isolates (Gomes et al., 2020).

Evaluation of ovicidal activity in controlled conditions

The ovicidal assay was conducted to analyze the ovicidal activity following the method of (Su and Mulla, 1998). The *M. incognita* eggs were transferred into a 300 ml Erlenmeyer flask containing two (2) ml of 1% sodium aside solution (NaN3) and raised its volume up to 100 ml with tap water. The flask was stirred for 20 m on a stirring plate. The egg suspension was poured onto a 25 µm sieve and rinsed the eggs well with tap water to remove all traces of sodium azide. The egg suspension was collected into a 50 ml blue cap tube, added 2 ml of 22.5 mg /ml gentamycin sulfate solution and 2 ml of 0.75mg /ml nystatin solutions and raised the volume up to 30ml with tap water. The eggs were treated with bacterial isolates cell-free supernatants in 2 ml centrifuge tubes and kept at 28 0C for 10 days in an incubator. The hatched eggs were quantified under the microscope and used the following formulae. The experiment was repeated twice (Zhao et al., 2018).

Data analysis

All experiments were performed with six (6) replicates and in a completely randomized design. The data sets were subjected to preliminary analyses of normality (Shapiro–Wilk) and variance homogeneity (Bartlett). Analysis of variance (ANOVA) with the F-test was performed when the data followed a normal distribution. When the significance level ($P < 0.05$) occurred, using the *ExpDes.pt* package of the R statistical program (R CORE TEAM, 2022) performed the Duncan's Means Test. To use the joint analysis, the following formula was used (Pimentel-Gomes and Garcia, 2002): QM1/QM2 < 7 the test will not be significant at the 5% level. If the covariance of the errors is unknown and consistent,

the generalized least squares (GLS) estimator model was used. It is concluded that, in the case of groups of similar experiments, in which all treatments have the same number of repetitions, joint analysis can be performed if the set between the largest and smallest residual mean squares is less than 7.

Results

Isolation and preliminary screening

A total of 42 bacterial strains were isolated and purified based on using the serial dilution technique, from the root-knot nematode's suppressive soil. In a small tray experiment, all these strains were preliminarily screened against *M. incognita*. Bacterial isolates, which showed significant differences in terms of both parameters (galls, eggs) were selected and processed for further study. Among them, 18 were chosen for their controlling potential of galls g^{-1} and eggs g^{-1} of root (Supplementary Figures S1, S2). Generally, 32.30 % of the bacterial strains only reduced the galls g^{-1} while 25.80% reduced the eggs g^{-1} in plant root. Bacterial strains that show high potential for controlling both galls g^{-1} and eggs g^{-1} were observed 41.90% of the total isolates (Fig. 1) eggs while 41.90% controlled both galls and eggs.

Biological Control ability of Bacillus sp against Fusarium oxysporum and Rhizoctonia solani

Bacillus strains B1, B3, B6, P10, P16, P19, and P21's *in vitro* growth was quantified by measuring their radial growth on Petri dishes. Growth of the strains B1, B3, B7, P10, P16, P19, and P21 was 81%, 52%, 64%, 32%, 63%, 64% and 77% in dual culture with *Fusarium oxysporum* respectively (Fig. 2). The strain's inhibition growth showed significant differences (P <0.05) from one another. More effective strain B1, B7, P19 and P21 inhibited the growth of *Fusarium oxysporum* 81%, 64%, 64% and 77% respectively, while in the *Rhizoctonia solani* dual culture study, all the strains B1, B3, B6, P10, P16, P19, and P21 significantly controlled the mycelial growth of *Rhizoctonia solani*. The growth inhibition percentage was recorded 21%, 42%, 54%, 52%, 38%, 25%, 27% by each isolates respectively in dual culture bioassay study (Fig. 2).

Biological Control ability of Pseudomonas sp against Fusarium oxysporum and Rhizoctonia solani

Pseudomonas strains P7, P17, X2, X11, and X18, *in vitro* growth was quantified by measuring their radial growth on Petri dishes. Growth of the strains P7, P17, X2, X11, and X18 was 46%, 62%, 69%, 59%, and 67%, in dual culture with *Fusarium oxysporum* respectively (Fig.3). The strains's inhibition growth showed significant differences (P <0.05) from one another. More effective strain X2 and X18 inhibited the growth of *Fusarium oxysporum* 69%, and 67% respectively, while in the *Rhizoctonia solani* dual culture study, P7, P17, X2, X11, and X18 demonstrated the ability for inhibition and inhibited the *Rhizoctonia solani* mycelial growth by 34%,35%,24%,14%, and 41%, respectively (Fig. 3).

Biological Control ability of Leclercia sp against Fusarium oxysporum and Rhizoctonia solani

Leclercia strains P12, P18 and P20's *in vitro* growth was quantified by measuring their radial growth on Petri dishes. Growth of the strains P12, P18, and P20 was 61%, 63%, and 67%, in dual culture with *Fusarium oxysporum* respectively (Fig. 4). The strains's inhibition growth showed significant differences (P <0.05) from one another. More effective strain P18 and P20 inhibited the growth of *Fusarium oxysporum* 63%, and 67% respectively, while in the *Rhizoctonia solani* dual culture study, P12, P18 and P20's demonstrated the ability for inhibition and inhibited the *Rhizoctonia solani* mycelial growth by 28%, 45%, and 50%, respectively (Fig. 4).

Biological Control ability of Paenarthrobacter sp, Pantoea sp and Exiguobacterium sp against Fusarium oxysporum and Rhizoctonia solani

Paenarthrobacter sp, *Pantoea* sp and *Exiguobacterium* sp strains X5, X12 and X14's *in vitro* growth was quantified by measuring their radial growth on Petri dishes. Growth of the strains X5, X12 and X14 was 67%, 92%, and 73%, in dual culture with *Fusarium oxysporum* respectively (Fig. 5). The strains's inhibition growth showed significant differences (P <0.05) from one another. More effective strain X12 and X14 inhibited the growth of *Fusarium oxysporum* 92%, and 73%, respectively, while in the *Rhizoctonia solani* dual culture study, only X14's showed the ability and controlled the mycelial growth 14% as compared to control. (Fig. 5).

In vitro assays against Ralstonia solanacearum

A total of 18 suppressive soil-associated bacteria strains were screened against *R. solanacearum* and 5 strains had inhibitory effects, and the inhibition zone ranged from an average of 1-4 mm radius of inhibition zone. Three of the strains were from the genus *Bacillus* including *Bacillus* sp. P10, *Bacillus* sp. P12 and *Bacillus* sp. P21, while two were from the genus of *Pseudomonas Pseudomonas* sp. P7 and Pseudomonas sp. X11. Methods of in vitro test (chloroform vapour) showed significant differences, and only five antagonistic (Fig. 6)

Pathogenicity determination of bacterial isolates

The bacterial isolates (*Bacillus* sp. B1, *Bacillus* sp. B3, *Bacillus* sp. B7, *Bacillus* sp. P10, *Bacillus* sp. P10, *Bacillus* sp. P16, *Bacillus* sp.P21 *Pseudomonas* sp.P7, *Pseudomonas* sp.P17, *Pseudomonas* sp.X2, *Pseudomonas* sp.X11, *Pseudomonas* sp.X18, *Leclercia* sp. P12, *Leclercia* sp.P18, *Leclercia* sp. P20, *Pantoea* sp. X5, *Paenarthrobacter* sp. X12 *Exiguobacterium* sp. X14), Since they showed no haemolytic activity on blood agar medium during their evaluation for a human pathogenicity test, they were determined to be non-pathogenic, confirming their safety for use in further investigations.

Compatibility of mixed strains

By spreading the biocontrol strains at a 90° angle from one another on NA media, the compatibility among them was determined. After two days of culture at 28 °C, the plates were examined to see if there were any inhibition zones. The absence of inhibition zones suggested compatibility between the strains, but the presence of inhibitory zones suggested incompatibility.

The pronounced effective action of cell-free supernatants of Bacillus spp strains against Meloidogyne incognita

The bacterial strains were selected to investigate their nematicidal activity in *in vitro* assay against *M. incognita* J_2. The cell-free supernatants of bacterial isolates encountered *M. incognita* J_2, a significant *($P<0.05$)* increase in mortality rate of J_2 (%) was

observed in all bacterial isolates treatments compared with the control (Fig. 7.A). The cell-free supernatants of *Bacillus* sp. B1, *Bacillus* sp. B3, *Bacillus* sp. B7, *Bacillus* sp. P10, *Bacillus* sp. P16, *Bacillus* sp. P19 and *Bacillus* sp. P21 nearly killed 90% of *M. incognita* J_2 in relation to control treatment. The *Bacillus* sp. B1 2833%, *Bacillus* sp. B3 1833%, *Bacillus* sp. B7 2700%, *Bacillus* sp. P10 2633%, *Bacillus* sp. P16 2666%, *Bacillus* sp. P19 2566% and *Bacillus* sp. P21 2600% enhanced the mortality rate of J_2 (%) as compared to control (Fig. 7.A).

The pronounced effective action of cell-free supernatants of Pseudomonas spp strains against Meloidogyne incognita

The cell-free supernatants of five bacterial strains *Pseudomonas* sp. P7, *Pseudomonas* sp. P17, *Pseudomonas* sp. X2, *Pseudomonas* sp. X11, *Pseudomonas* sp. X18 exhibited high potential and significantly *(P<0.05)* killed the *M. incognita* J_2 as compared to the control. (Fig. 7.B). On exposure to cell-free supernatants of the selected bacterial strains to *M. incognita* J_2, the nematodes completely died and the mortality rate observed approximately 80% in all bacterial treated treatments in relation to control (Fig. 7. B). The mortality rate of J_2 (%) of *Pseudomonas* sp. P7, *Pseudomonas* sp. P17, *Pseudomonas* sp. X2, *Pseudomonas* sp. X11, *Pseudomonas* sp. X18, recorded 2733%, 2633%, 2666%, 2466%, and 2300% respectively (Fig. 8.B).

The pronounced effective action of cell-free supernatants of Leclercia spp strains against Meloidogyne incognita

The cell-free supernatants of all three potential isolates *Leclercia* sp. P12, *Leclercia* sp. P18 and *Leclercia* sp. P20 treated with the 24 h old energetic J_2 of *M. incognita* in a two ml microtube and observed their mortality after 72 h. All isolates effectively infected the *M. incognita* and significant differences *(P<0.05)* were noticed in all *M. incognita* treated with *Leclercia* sp. P12, *Leclercia* sp. P18 and *Leclercia* sp. P20, as compared to untreated control ((Fig. 7.C). The mortality rate of *Leclercia* sp. P12, *Leclercia* sp. P18 and *Leclercia* sp. P20 was 2733%, 2633% and 2666%, respectively as compared to untreated control (Fig. 7.C).

The pronounced effective action of cell-free supernatants of Paenarthrobacter, Pantoea and Exiguobacterium spp strains against Meloidogyne incognita

The cell-free supernatants of bacterial strains *Pantoea* sp. X5, *Paenarthrobacter* sp. 12 and *Exiguobacterium* sp. 14 showed significant *(P<0.05)* differences in mortality rate (%) of J_2 of *M. incognita* ((Fig. 7. D). The mortality rate (%) of J_2 is almost 90% in relation to control. On combination the cell-free supernatants with J_2, *Pantoea* sp. X5 2833%, *Paenarthrobacter* sp. 12 2700% and *Exiguobacterium* sp. 14 killed 2600% J_2 of *M. incognita* population in comparison to control (Fig. 7.D)

Toxicity and effect of Bacillus spp volatile compounds (VOCs) on Meloidogyne incognita J2s in in vitro condition

Microbial volatiles are capable to enhnace plant growth, paralyzing phytonematodes and suppress plant pathogens in *in vitro* and *in vivo* conditions. The bacterial volatile compounds (VOCs) from *Bacillus* sp, isolated from suppressive soil, showed high significance *(P<0.05)* and killed more than 90 % of *Meloidogyne incognita* J_2s in *in vitor* experiment (Fig. 8.A). Contrary, only 15% *Meloidogyne incognita* J_2s were found dead in control. Individually, the mortality rate of volatile compounds of *Bacillus* sp. B1 *w*as 1037% *Bacillus* sp. B3, 1000% *Bacillus* sp. B7 1075%, *Bacillus* sp. P10 912%, *Bacillus* sp. P16 1037%, *Bacillus* sp. P19 1025% and *Bacillus* sp. P21 875 % reported in each treatment in relation to control.

Toxicity and effect of Pseudomonas spp volatile compounds on Meloidogyne incognita J2s in in vitro condition

We determined the nematicidal effects of VOCs produced by *Pseudomonas* spp on *M. incognita* J2s in two ml microcentrifuge tubes. The movement and mortality were evaluated after 72 h and almost the rate of mortality was observed from 90-100% in all *Pseudomonas* spp treatments (Fig. 8.B). All *Pseudomonas* spp showed significant differences *(P<0.05)* in terms of killing the *M. incognita* J_2s as compared to control. The VOCs produced by *Pseudomonas* spp were highly toxic and *Pseudomonas* sp. P7, *Pseudomonas* sp. P17, *Pseudomonas* sp. X2, *Pseudomonas* sp. X11, *Pseudomonas* sp. X18, killed the *M. incognita* J2s 950%,1012%, 1075%, 1050%, and 1025, respectively, when compared to control (Fig. 8.B).

Toxicity and effect of Leclercia spp volatile compounds on Meloidogyne incognita J2s in in vitro condition

The Leclercia spp filtrate showed toxicity and killed 100% *M. incognita* J2s when exposed to volatile compounds Leclercia spp in two ml microcentrifuge tubes. In contrast, ranging from 5-20% M. incognita J2s alone in distilled water was found dead after 72 h (Fig. 8.C). The mortality rate was significant ($P<0.05$) in all treatments of the Leclercia spp in relation to control and *Leclercia* sp. P12, killed 850%, Leclercia sp. P18, 1012% and *Leclercia* sp. P20, 650% as compared to untreated control (Fig. 8.C).

Toxicity and effect of Paenarthrobacter, Pantoea and Exiguobacterium spp volatile compounds on Meloidogyne incognita J2s in in vitro condition

Three different bacteria *Paenarthrobacter* sp. X5, *Pantoea* sp. X12 and *Exiguobacterium* sp. X14 isolated from suppressive soil were screened for their nematicidal activity using their volatile compounds against *M. incognita* J_2s in two ml microcentrifuge tubes. All three different species *Paenarthrobacter* sp. X5, *Pantoea* sp. X12 and *Exiguobacterium* showed significant differences *($P<0.05$)* and the mortality rate was recorded at 100% in all *M. incognita* J_2s exposed to bacterial isolates as compared to *M. incognita* J_2s alone maintained in water (Fig. 8.D). The mortality rate of *M. incognita* J_2s treated with volatile compounds of *Paenarthrobacter* sp. X5, *Pantoea* sp. X12 and *Exiguobacterium* sp. X14, 1112% 1000%, 1037% respectively after 72 h of exposure. The control exhibited only 15% killing rate (Fig. 8.D).

The exhibition of nematicidal activity of cell-free supernatant of Bacillus spp against Meloidogyne incognita eggs

The cell-free supernatant of *Bacillus* spp was treated with *Meloidogyne incognita* eggs in two ml microcentrifuge tubes and kept for 10 days maintaining 25-28 ^{0}C in a BOD. The bacterial cell-free supernatant effect was observed under an inverted microscope after 10 days. The total number of J_2s that emerged from eggs were quantified. The cell free supernatant of all *Bacillus* spp significantly *($P<0.05$)* reduced the egg's hatching and almost ranging from 60-80% reduction observed in relation to control (Fig. 9.A). All species showed reduced egg hatching equally, and no significant difference was observed between isolates (Fig. 10.A). Individually, the eggs hatching percentage of cell-free supernatant of *Bacillus* sp. B1, which was 75% *Bacillus* sp. B3, 45% *Bacillus* sp. B7

80%, *Bacillus* sp. 50%, *Bacillus* sp. P16 60%, *Bacillus* sp. P19 75% and *Bacillus* sp. P21 80 % reported in each treatment in relation to control (Fig. 9.A).

The exhibition of nematicidal activity of cell-free supernatant of Pseudomonas spp against Meloidogyne incognita eggs

The nematicidal potentials of isolated bacterial isolates were evaluated against the eggs of *M. incognita* in *in vitro* egg hatch assay. (Fig. 9.B). The cell-free supernatant was inoculated with *M. incognita* eggs in microcentrifuge tubes and analyzed the J2s emerging from eggs and quantified after 10 days. The analysis revealed that all *Pseudomonas* spp have the potential and significantly *(P<0.05)* reduced the hatching of *M. incognita* eggs (Fig. 9.B). Overall, *Pseudomonas* spp cause a reduction from 60% to 85% as compared to control and the highest ovicidal potentials were recorded in *Pseudomonas* sp. X2, *Pseudomonas* sp. *Pseudomonas* sp. X18, 90% and 85% respectively comparatively other *Pseudomonas* spp. These results conclude that all the *Pseudomonas* sp cell-free supernatant are capable to reduced significantly *(P<0.05)* the egg hatching (Fig. 10.9B).

The exhibition of nematicidal activity of cell free supernatant of Leclercia spp against Meloidogyne incognita eggs

Cell-free supernatant from *Leclercia* spp showed high potential in reducing the eggs hatching of *M. incognita* eggs shown in (Fig. 9.C). The *M. incognita* eggs were treated with cell free supernatant of *Leclercia* spp and significant *(P<0.05)* reduction was observed after 10 days in all treatments when compared to control. The reduction in eggs hatching was from 50-70% observed in relation to control, while individually *Leclercia* sp. P12, reduced eggs hatching 50%, *Leclercia* sp. P18, 70% and *Leclercia* sp. P20, 65% as compared to untreated control (Fig. 9.C)

The exhibition of nematicidal activity of cell-free supernatant of Pantoea sp. X5, Paenarthrobacter sp. X12 Exiguobacterium sp. X14 against Meloidogyne incognita eggs

The cell-free supernatant of three different bacteria spp *Pantoea* sp. X5, *Paenarthrobacter* sp. X12 *Exiguobacterium* sp. X14 showed a significant impact (Fig. 9.D).; (P<0.05) on *M. incognita* eggs compared to the untreated control after 10 days of exposure (Fig. 10.D). The maximum reduction in eggs hatching of *M. incognita* occurred when the cell-

free supernatant of Paenarthrobacter sp. X12 treated with *M. incognita* eggs (75%) and followed by Pantoea sp. X5 (70%) relatively to alone control egg. The cell free supernatant causes a significant ($P<0.05$) reduction in all bacteria cell free supernatant in relation to untreated control (Fig. 9.D).

Discussion

Disease suppressive soil harbors specific bacterial communities that establish disease soil suppression

Plants harbor a multitude of groups of microorganisms by performing various functions in a complex ecosystem. Microbial communities associated with plants, particularly in the below ecosystem, carry out many activities in favor of their host's growth and protection. Unfortunately, diverse groups of phytopathogens inhabit the same ecosystem and interact with host plants for food acquisition and survival. The most destructive plant pathogens among them are plant parasitic nematodes, such as root-knot nematodes (RKN) from the genus *Meloidogyne* spp. These nematodes damage a variety of plant species, including vegetables (Tapia-Vázquez et al., 2022).

Identification of potential biocontrol agents and their harnessing for plant protection against plant parasitic nematodes (PPNs) is an emerging topic in sustainable and eco-friendly disease management approaches. The advent of sustainable, environment-friendly and protective measurement implementation against PPNs diseases must be prioritized both under *in vivo* and *in vitro* circumstances across Brazil. Hence, this research was conducted to investigate and isolate the potential biocontrol agents inhabiting the suppressive soil and causing soil suppression against RKNs. Thus eighteen (18) isolates from 42 bacterial strains that exhibited biocontrol efficacy against RKNs were chosen for further analysis. 16S rRNA identification revealed 6 different genera: *Bacillus*, *Pseudomonas*, *Leclercia*, *Paenarthrobacter*, *Pantoea*, and *Exiguobacterium*. Some bacteria strains have the ability to suppress *M. incognita*, a bacterium about which there is no published data till date. Recent studies revealed that suppressive soil microbiomes foster special microbial groups that are involved in the suppression of soil-borne diseases (Chapelle et al., 2016). A large body of research has been witnessed on numerous microbe's role as biocontrol agents against RKN in greenhouse and field conditions and they have been successfully bio-controlled the RKNs employing an arsenal of mechanisms (Xiang et al., 2017). Antagonistic strains achieve range of strategies to attenuate nematodes infection intensity and significantly escalate J_2 mortality,

reduce egg hatching in a controlled environment (Wei et al., 2010).

In this investigation, a dual culture test was conducted to fully assess the antagonistic activity of the selected suppressive soil-associated bacterial strains against the *F. oxysporum and R. solani* isolate pathogenic toward tomato seedlings. *Bacillus* spp., including *Bacillus subtilis* V26 (Ben Khedher et al., 2021), *Bacillus subtilis* strains BS 10-4 and *Bacillus subtilis* strains BS 26D (Lastochkina et al., 2020), and *Bacillus subtilis* (Al-Fadhal et al., 2019), *Pseudomonas* spp. including *Pseudomonas aeruginosa* (Islam et al., 2018), *Pseudomonas fluorescens* (Al-Fadhal et al., 2019) shown efficient antagonistic action both *in vitro* and *in vivo* against a variety of soil-borne fungal infections. The *in vitro* studies conducted here revealed that *Bacillus* B1 and *Bacillus* P21, *Pseudomonas* X2 and *Pseudomonas* X18, *Leclercia* P18, *Pantoea* X5, *Paenarthrobacter* X12, and *Exiguobacterium* X14 species inhibited the *F. oxysporum* and 3193 were both.

The VOCs that soil bacteria produce have been proven to enhance plant growth, possess antimicrobial and nematicidal characteristics, and induce systemic resistance in crops (Audrain et al., 2015). Plant disease control and the stimulation of plant development are significantly aided by the volatile organic compounds (VOCs) that soil bacteria produce. Additionally, the virulence features of *R. solanacearum* were dramatically reduced by the VOCs of *P. fluorescens* WR-1 (Raza et al., 2016). These strains may therefore actively suppress the pathogen due to the production of antibiotics. Antibiotics appear to be the cause of action when an antagonist is equally effective in the presence and absence of iron, according to (Ran et al., 2005).

*F. oxysporum and R. solani*were examined, and their growth was significantly suppressed (Topalović et al., 2020). The specific microbial groups reside in the suppressive soil Microbiome which behave as antagonists against plant pathogens and parasites. An essential biocontrol mechanism known as antibiosis occurs the production of metabolites by an adversary, such as toxins, lytic enzymes, and antibiotics or VOCs that may prevent the invasion of the pathogen. In contrast to the higher initial concentration of parasites required for effective nematodes control, it has been observed that nematode suppression by a variety of rhizobacteria using alternate modes of action, such as antibiosis, can be carried out at lower microbial densities (Hussain et al., 2016). This was the case with soils that inhibited ring nematode (*M. xenoplax*) growth., when it was discovered that Pseudomonas species that produce salicylic acid significantly disrupt egg development and are responsible for preventing egg hatch (Hussain et al., 2016). While the primary mechanism of soil

suppressiveness was once thought to be the direct antagonism of microorganisms to PPN, the plant is now more recently recognized as a halobiont in interaction with its Microbiome (Hussain et al., 2016).

Multiple studies have been carried out on how the mode of action of some PGPR and they reported the bacteria produce bioactive secondary metabolites which might be the major cause of mortality of sedentary endo-parasitic nematodes *M.incognita* (Mendoza et al., 2008). (Huang et al., 2010a) claimed in their studies that bacteria produce some major nematicidal volatiles compounds such as benzeneacetaldehyde, 2-non-anone, decanal, 2-undecanone, and dimethyl disulfide, which actively controlled the *M.incognita* J_2s infection and parasitized eggs at 0.5 concentration resulting in inhibiting hatching of eggs. In addition to the mobile stages of PPN that they target in soil, microorganisms can colonize the roots and parasitize the immobile stages of end parasitic nematodes. Some *Pantoea* isolates have been used in order to combat fire blight on apple and pear trees by developing commercial biocontrol like BlightBan C9-1 and Bloomtime Biological (Johnson et al., 2000).

The researchers constantly suggest that rhizobacteria produce some metabolites including toxic compounds, antibiotics and diverse extracellular enzymes that might be contributing in the reduction of egg hatching and the intensity of J_2 mortality (Wei et al., 2014). One of the most promising nematicidal bacteria, *Bacillus firmus*, has received a lot of attention in recent years. It is capable of killing, paralyzing, and preventing PPN egg hatching. The secondary metabolites produced by *Bacillus firmus* that have all these nematicidal qualities are most likely to be responsible (Engelbrecht et al., 2018; Horak et al., 2019). The cell free supernatant of *B. subtilis* strains OKB105 treated with J_2 of *M. incognita* and induced the mortality rate when compared with the mutated strain (Xia et al., 2011). Under laboratory and greenhouse conditions, pseudomonad rhizobacteria filtrate intoxicated second-stage juveniles (J_2), increased their mortality, and successfully managed plant disease (Sharma and Sharma, 2017).

Alkaline protease (Hasp) is an extracellular enzyme that potentially degrades the cuticle proteins of nematodes and significantly kills the stage two juvenile of *Heterodera glycine* (Wang et al., 2009). Plants can harbor a variety of phytopathogenic bacteria and fungi, however *Pantoea*'s epiphytic colonization has been shown to lower the incidence of plant disease (Johnson et al., 2000). Numerous *Pantoea* strains have been shown to be ferocious environmental competitors that produce a variety of organic substances with antibiotic activity, such as pantocins, herbicolins, microcins, and Phenazines. The more recent PNP-1

natural product from P. ananatis BRT175 also inhibits E. amylovora and is probably similar to FVG, or 4-formylaminooxyvinylglycine (Trippe et al., 2013). This study reveals that tomato plants treated with such culture filtrate had better plant health when infected with the root-knot nematode, proving the effectiveness of the bacterial cell-free culture filtrate, which is responsible for the mortality of nematode second-stage juveniles (J_2). The management of culture filtrates against *M. incognita* is an effective biocontrol strategy. These bacterial isolates may be utilized as efficient biocontrol agents to manage root-knot nematodes, according to the study's findings (Huang et al., 2010b) reported that *B. pumilus* synthesize various extracellular hydrolytic enzymes or produce some nematotoxic compounds which destroy the cuticle of juvenile or nematode eggshell and consequently inhibit the hatching percentage of *M. incognita* eggs.

For instance, some bacteria produce some major nematicidal volatiles compounds such as benzeneacetaldehyde, 2-non-anone, decanal, 2-undecanone, and dimethyl disulphide, which were actively controlled the *M. incognita* J_2s and parasitized eggs at 0.5 concentration. For instance, the PGPR strain *B. megaterium* YMF 3.25 significantly reduced the hatchings of nematode eggs. Native *Bacillus thuringiensis* was used to produce nine distinct Cry protein profiles, all of which completely inhibited the development of J_2 juveniles from *M. incognita* egg masses (Ramalakshmi et al., 2020). Conclusively, eighteen bacterial strains of six different genera namely *Bacillus*, *Pseudomonas*, *Leclercia*, *Paenarthrobacter*, *Pantoea*, and *Exiguobacterium*, among the non-chemical solutions found for managing root-knot nematodes were these different genera *Bacillus*, *Pseudomonas*, *Leclercia*, *Paenarthrobacter*, *Pantoea*, and *Exiguobacterium* have shown high nematocidal and might be employed regularly in the field to manage the root-knot nematode. However, further research is needed to fully understand how these unique strains will be implemented commercially.

Conclusion

Plant-parasitic nematodes highly damage vegetables and other agronomic crops and cause unbearable loss to the agricultural economy across the world. Plant-associated microbes contribute in engineering the soil's suppression against soil-borne pathogens specifically against plant parasitic nematodes (PPN) and their contribution has been proven in both culture-dependent and culture-independent techniques. Our findings concluded that soil suppressiveness has been credited to the specific microbial communities. The isolated microbes of suppressive soil controlled *Meloidogyne incognita*, *Fusarium oxysporum*, and

Rhizoctonia solani. These beneficial microbes produced VOCs which were used to suppress *M. incognita*. Intriguingly, on direct contact, the microbes completely paralyzed the J_2 and eggs and were unable to move or hatch respectively. To understand the insights of plant microbes and microbe's nematodes interactions and functions in the rhizosphere microbiome, metagenomics studies could be harnessed in future

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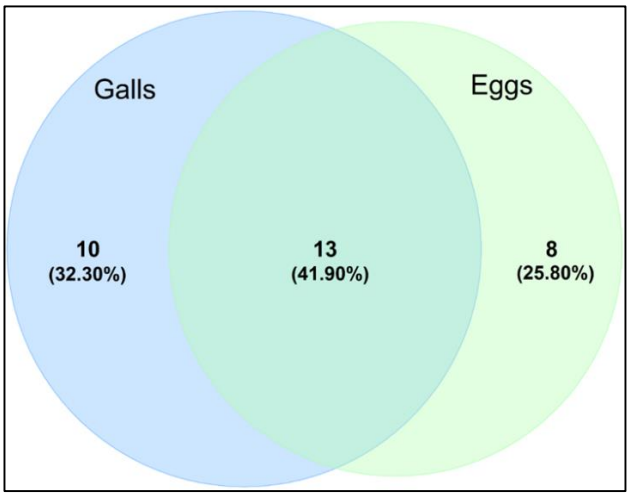

Figure1. The Venn diagram represents the control potential of the isolates based on galls and eggs reduction. 32 % bacterial isolates controlled only galls, 25.80% only controlled

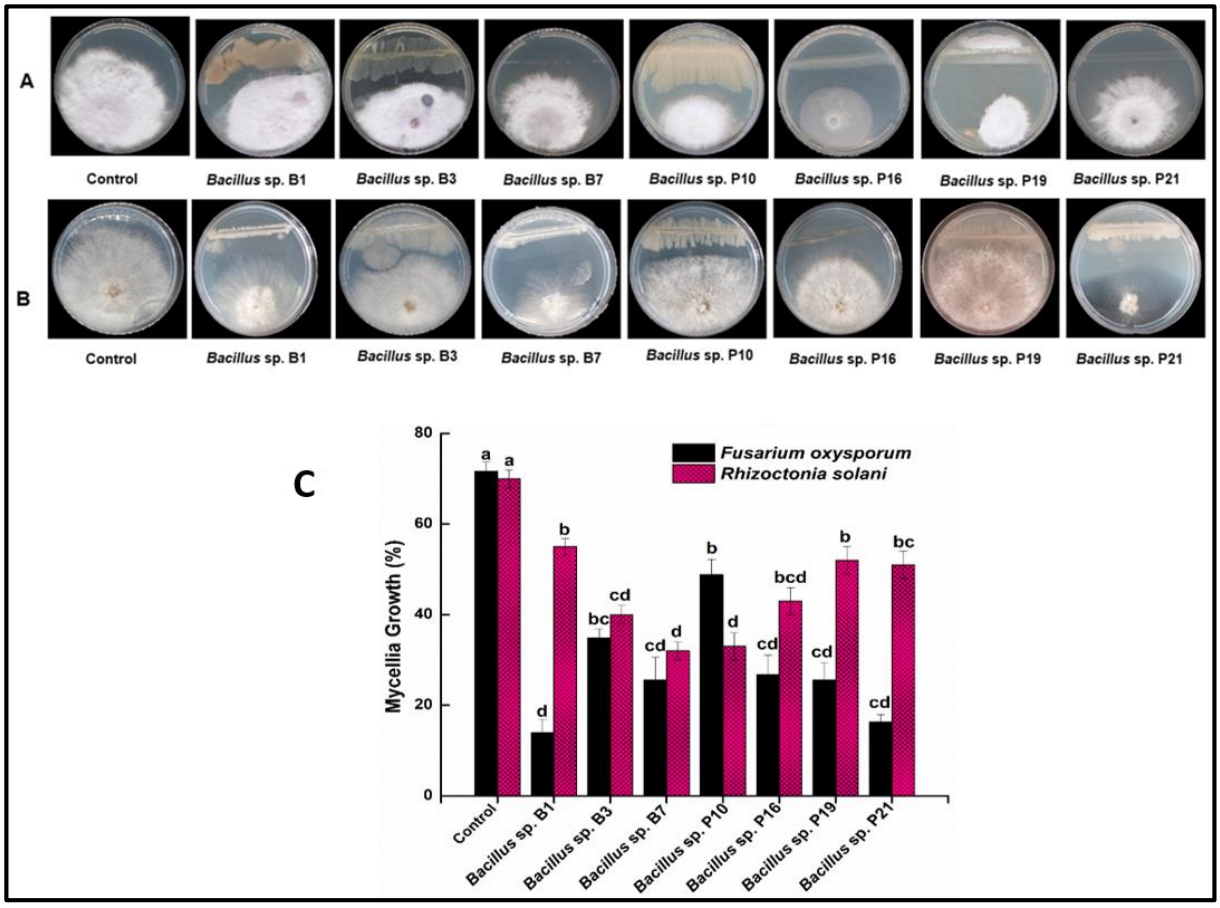

Figure.2. Biological control activity of *Bacillus* isolates (B1, B3, B7, P1, P16, P19, P21) against *Fusarium oxysporum* (A) and *Rhizoctonia solani* (B) representative photographs showing inhibition *Fusarium oxysporum* and *Rhizoctonia solani* (inoculated at the edge of petri plate) by *Bacillus* isolates. Isolates visible as straight streaking at the edge of petri plate. (C) Bar graphs showing growth inhibition of *Fusarium oxysporum* and *Rhizoctonia solani* by *Bacillus* isolates.

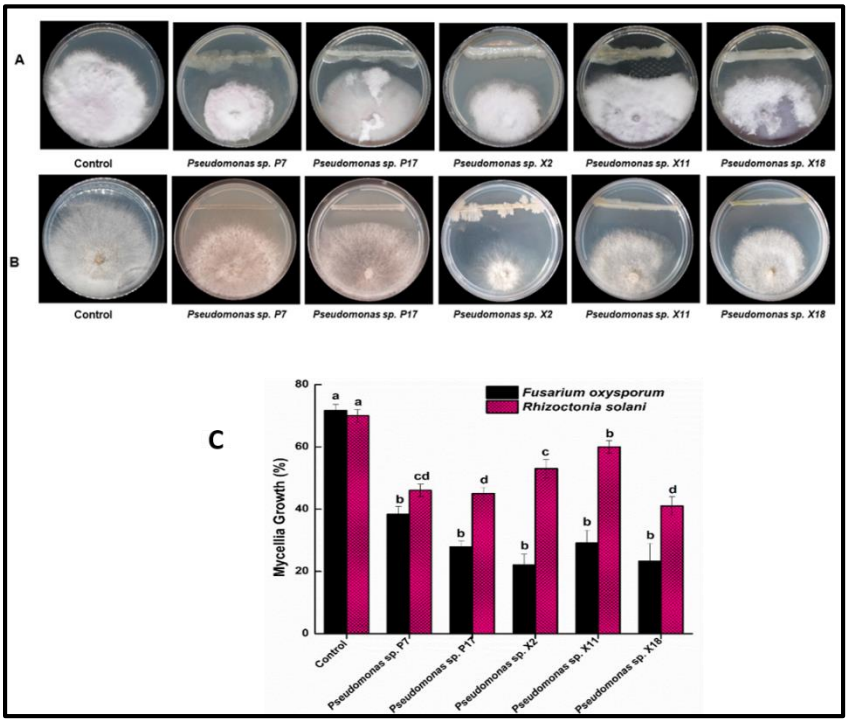

Figure.3 Biological control activity of *Pseudomonas* isolates (P7, P17, X2, X11, X18) against *Fusarium oxysporum* (A) and *Rhizoctonia solani* (B) representative photographs showing inhibition *Fusarium oxysporum* and *Rhizoctonia solani* (inoculated at the edge of petri plate) by *Pseudomonas* isolates. Isolates visible as straight streaking at the edge of petri plate. (C) Bar graphs showing growth inhibition of *Fusarium oxysporum* and *Rhizoctonia solani* by *Pseudomonas* sp.

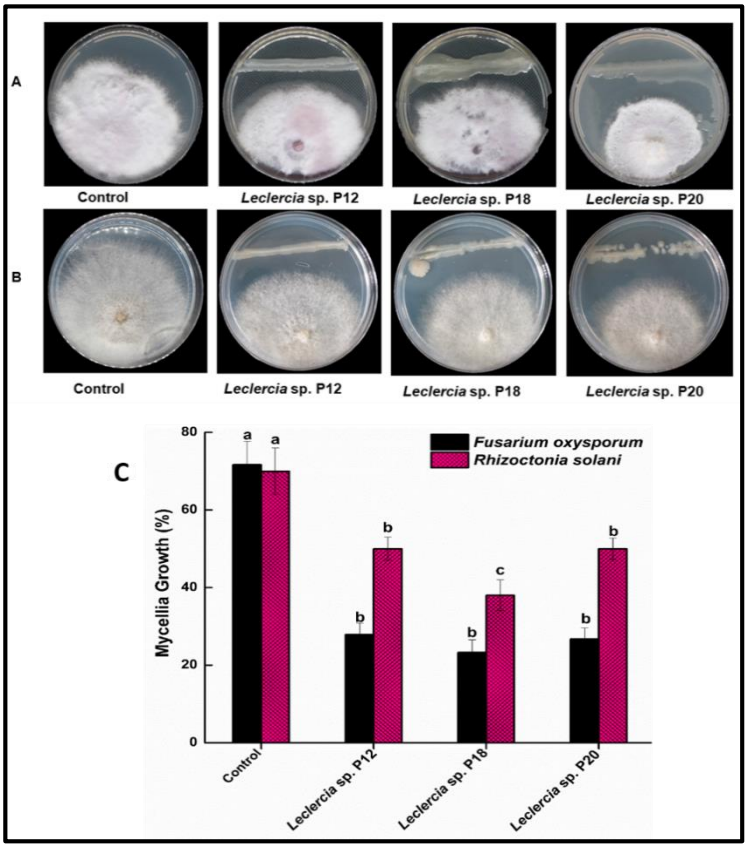

Figure.4 Biological control activity of *Leclercia* isolates (P12, P18, P20) against *Fusarium oxysporum* (A) and *Rhizoctonia solani* (B) representative photographs showing inhibition *Fusarium oxysporum* and *Rhizoctonia solani* (inoculated at the edge of petri plate) by *Leclercia* isolates. Isolates visible as straight streaking at the edge of petri plate. (C) Bar graphs showing growth inhibition of *Fusarium oxysporum* and *Rhizoctonia solani* by *Leclercia* sp.

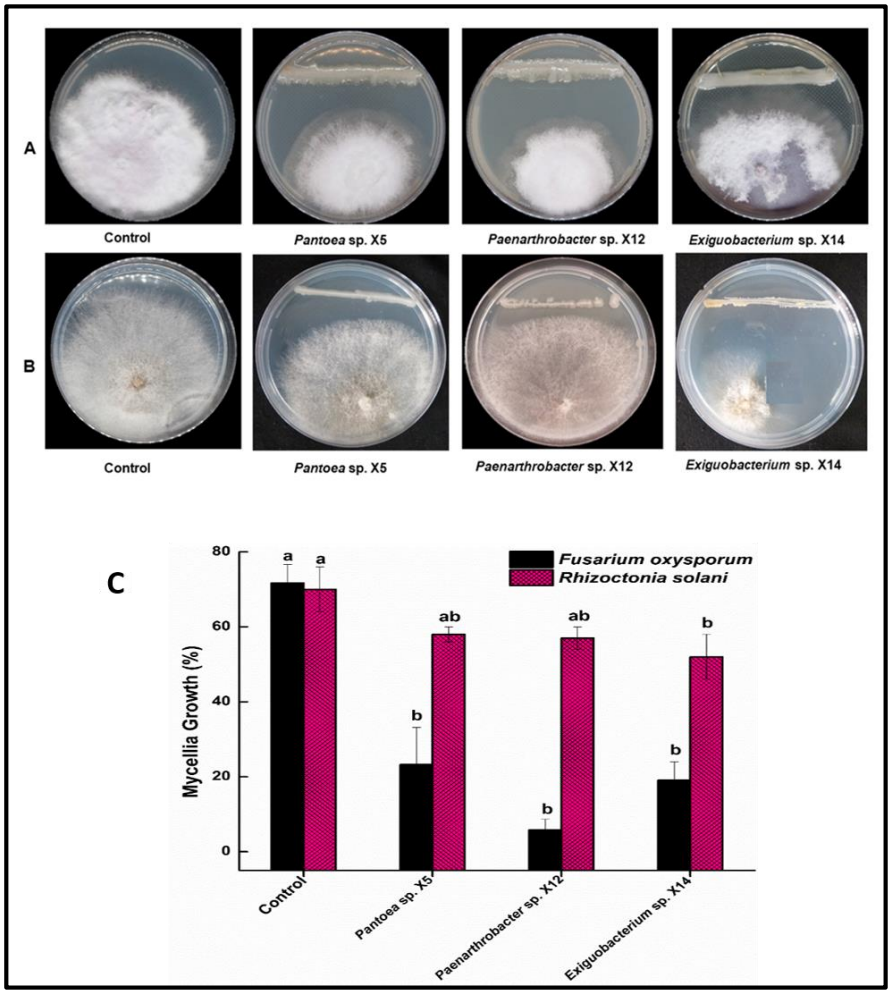

Figure.5. Biological control activity of *Pantoeai Paenarthrobacter* and *Exiguobacterium* isolates (X5, X12, X14), against *Fusarium oxysporum* (A) and *Rhizoctonia solani* (B) representative photographs showing inhibition *Fusarium oxysporum* and *Rhizoctonia solani* (inoculated at the edge of petri plate) by *Paenarthrobacter* sp, *Pantoea* sp and *Exiguobacterium* sp. Isolates visible as straight streaking at the edge of petri plate. (C) Bar graphs showing growth inhibition of *Fusarium oxysporum* and *Rhizoctonia solani* by *Paenarthrobacter* sp, *Pantoea* sp and *Exiguobacterium* sp.

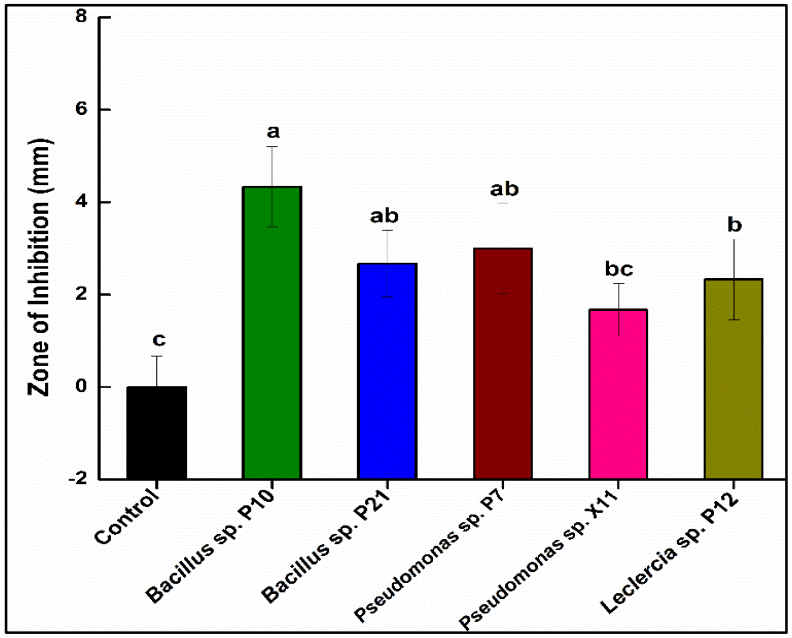

Figure.6 Biological control activity of *Bacillus* sp. 10, *Bacillus* sp. 21, *Pseudomonas* sp. P7, *Pseudomonas* sp. X11 and *Leclercia* sp. P12 against *Ralstonia solanacearum*

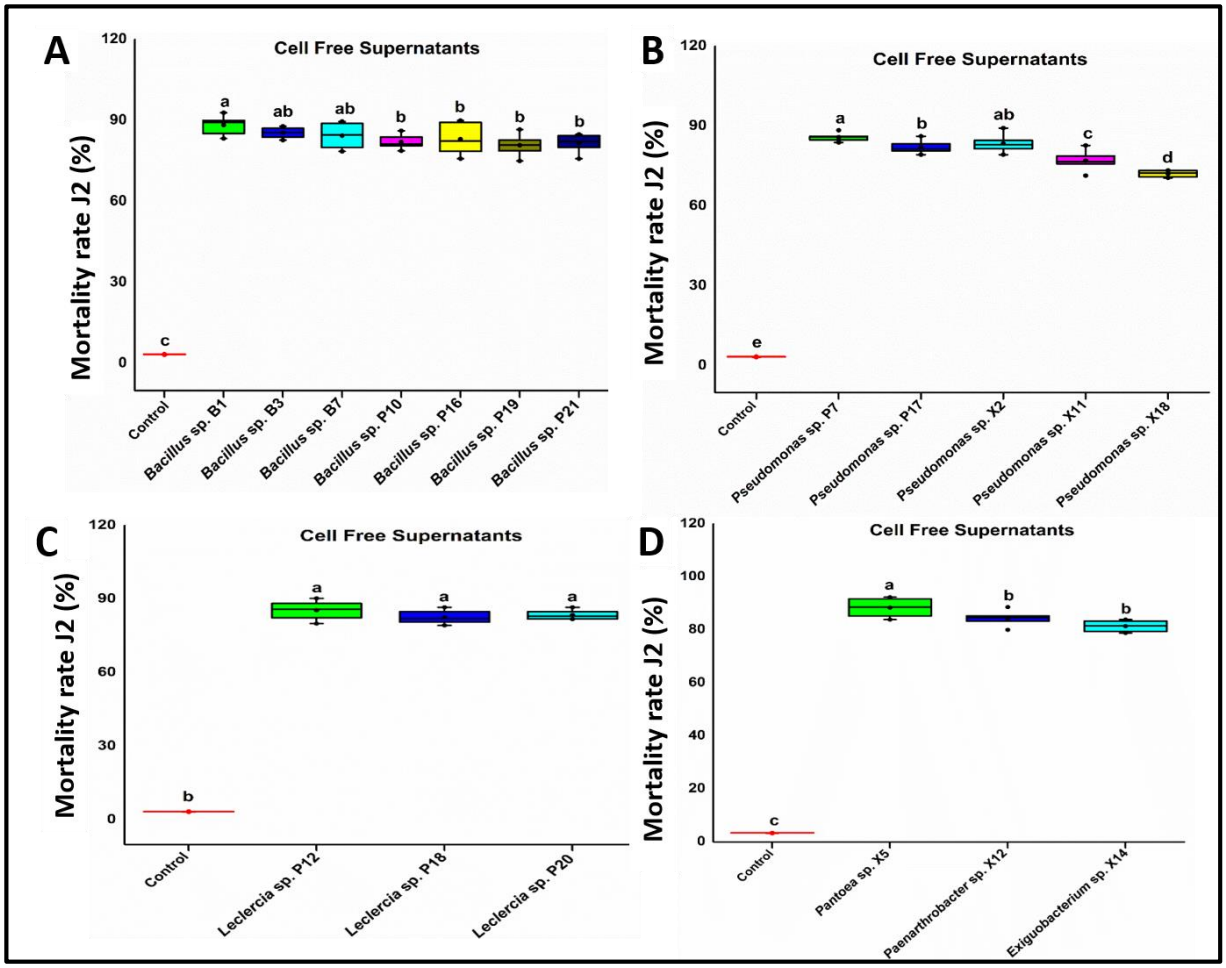

Figure. 7 The J_2 of *M. incognita* exposed to cell-free supernatants of *Bacillus* spp (B) *Pseudomonas* spp *Leclercia* spp sand *Pantoea* sp., *Paenarthrobacter* sp. and *Exiguobacterium* sp. in *in vitro* conditions. Estimated percentage of *in vitro* mortality of J_2 after 48 h of exposure to cell-free supernatant of (A) *Bacillus* spp (B) *Pseudomonas* spp (C) *Leclercia* spp sand (D) *Pantoea* sp., *Paenarthrobacter* sp. and *Exiguobacterium* sp. The mortality rate of J_2 (percentage) is represented by the data in the plot. The Duncan test ($p \leq 0.05$) was applied and different letters determine significant differences on each box among the bacterial isolates. The assay repated twice.

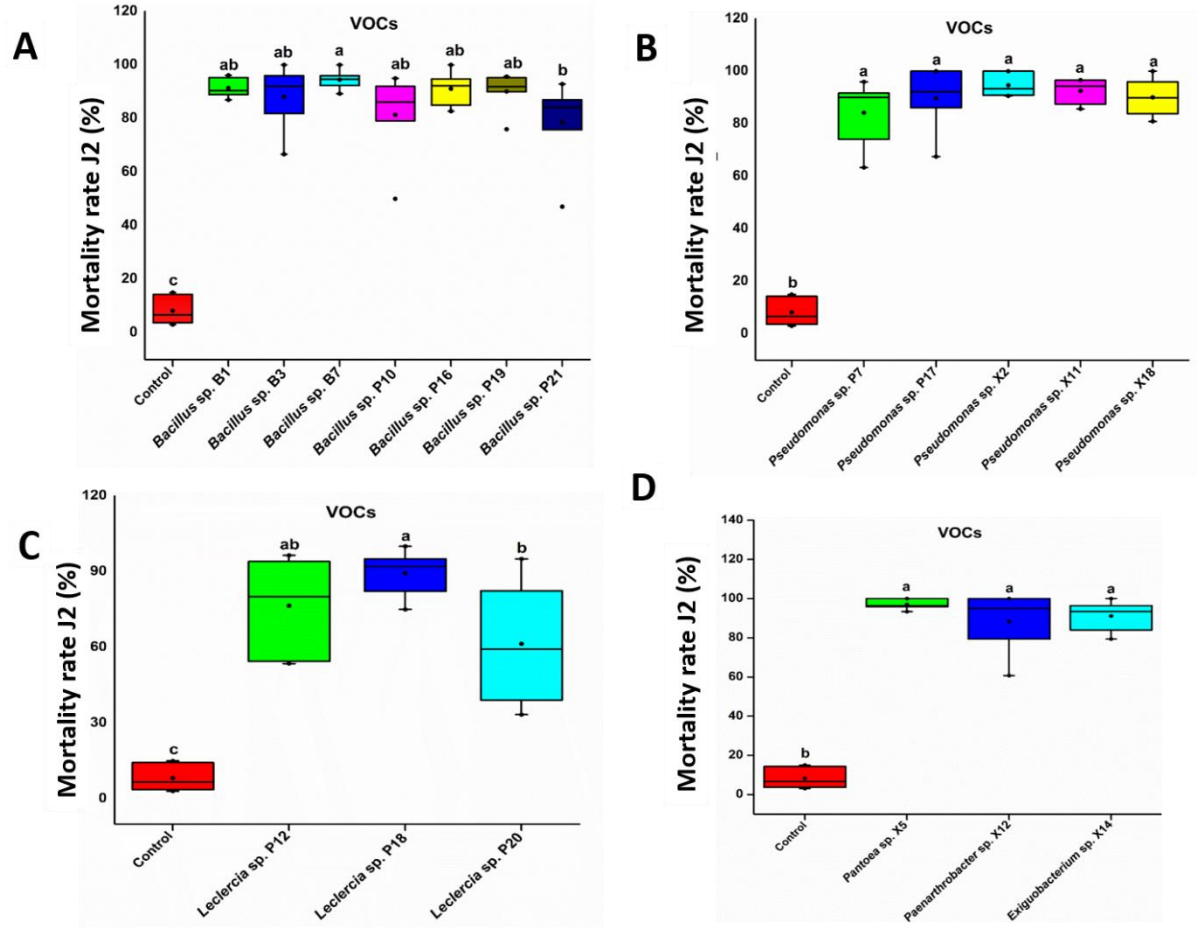

Figure. 8. The J_2 of *M. incognita* exposed to volatile organic compounds (VoCs) of *Bacillus* spp *Pseudomonas* spp *Leclercia* spp sand *Pantoea* sp., *Paenarthrobacter* sp. and *Exiguobacterium* sp. Estimated percentage of *in vitro* mortality of J_2 after 48 h of exposure to volatile organic compounds of (A) *Bacillus* spp (B) *Pseudomonas* spp (C) *Leclercia* spp sand (D) *Pantoea* sp., *Paenarthrobacter* sp. The mortality rate of J_2 (percentage) is represented by the data in the plot. The Duncan test ($p \leq 0.05$) was applied and different letters determine significant differences on each box among the bacterial isolates. The assay repated twice.

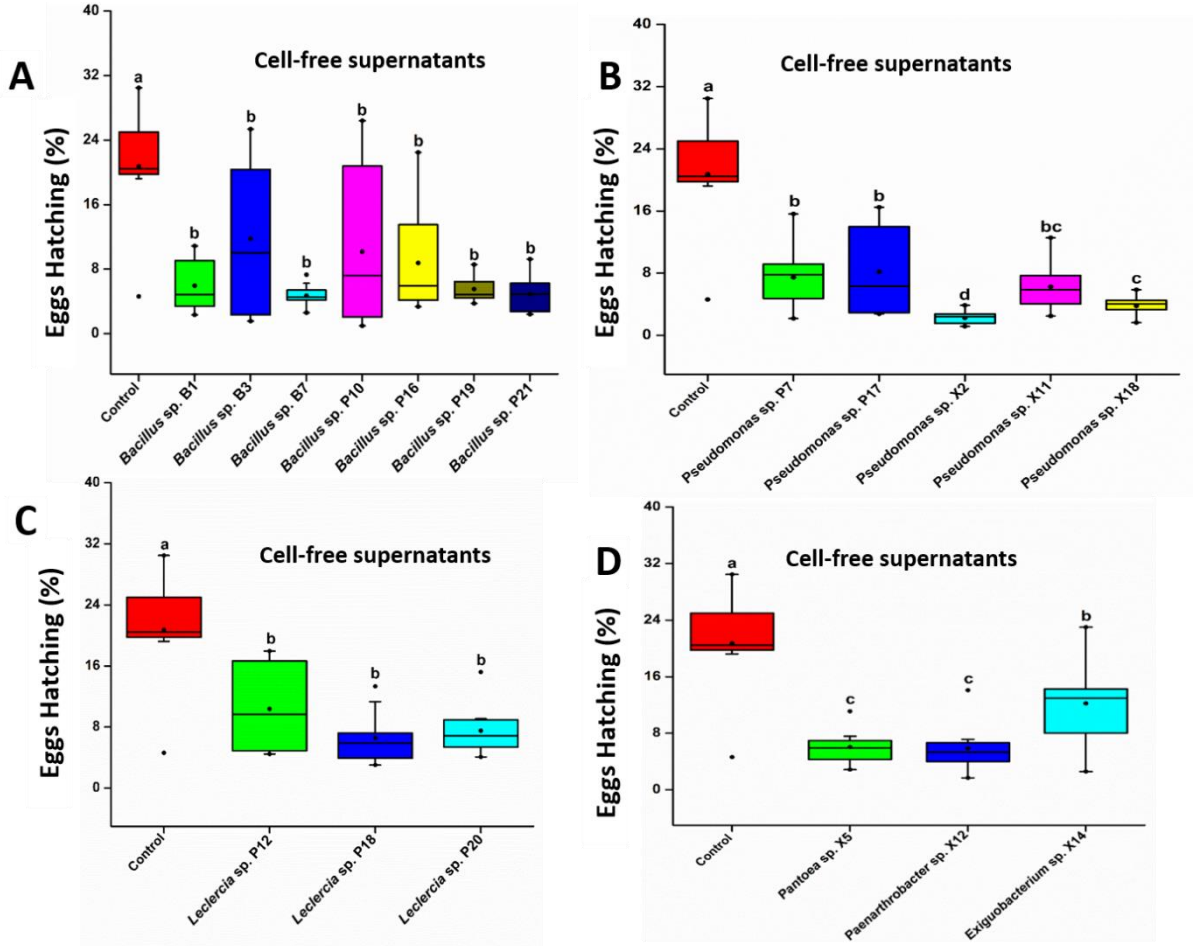

Figure. 9. The eggs inhibition of *M. incognita* exposed to cell-free supernatants of *Bacillus* spp *Pseudomonas* spp *Leclercia* spp sand *Pantoea* sp., *Paenarthrobacter* sp. and *Exiguobacterium* sp. in *in vitro* conditions. The eggs inhibition rate (percentage) of *M. incognita* is represented by the data in the plot. Estimated percentage of eggs inhibition *in vitro* after 10 days of exposure to cell-free supernatant of (A) *Bacillus* spp (B) *Pseudomonas* spp (C) *Leclercia* spp sand (D) *Pantoea* sp., *Paenarthrobacter* sp. and *Exiguobacterium* sp. The eggs inhibition rate (percentage) is represented by the data in the plot. The Duncan test ($p \leq 0.05$) was applied and different letters determine significant differences on each box among the bacterial isolates. The assay repated twice.

ARTICLE 4- The influence of bacterial and fungal-based bioproduct application on cyst nematode (*Heterodera glycines*) and rhizosphere microbiome profiling on two consecutive years

Abstract

Plant-associated beneficial microorganisms assist plant health, fitness and suppress disease resulting in the enhancement of plant growth and protection against certain plant parasites. Such strategy has been adopted for the integrated management of plant parasitic nematodes, such as soybean cyst nematodes (SCN) Heterodera *glycines.* However, little is yet know about the long term use of a biocontrol product on the nematode management and soil microbiome. The study aimed to evaluate the biocontrol efficacy of biocontrol products against soybean cyst-nematode (SCN) employing two seed or furrow treatments under field conditions. The commercially-available biological products based on *Pochonia chlamydosporua* (CEPA PC-10) (Rizotec), *Bacillus methylotrophicus* UFPEDA 20 (Onix) and *Trichoderma koningiopsis* GF362 (not commercially available) were applied as seed treatment or in-furrow upon planting. The total number of females in root, cysts, eggs, J_2 population (%) eggs/cyst and J_2 population (%) mortality rate at 30 and 60 days after sowing as well as plant yield were assessed in two consecutive years, but no significant differences were observed between control and bioproducts applied treatments. Additionally, we evaluated the diversity and community composition of bacteria, fungi and eukaryotes in the rhizosphere soil of bioproducts treated plants and the dominant phyla in bacterial, fungal and eukaryotic community were *Proteobacteria, Acidobacteria Actinobacteria*, Ascomycota, *Basidiomycota, Mortierellomycota,* and *Ascomycota, Cercozoa* respectively in both consecutive years. Overall, no significant difference was observed in bacterial, fungal, and eukaryotic community's diversity in both years of data. The co-occurrence network unearthed that bacterial, fungal and eukaryotic species formed a network structure of high complexity in all bioproducts applied treatments. Our findings suggest that the introduction of exogenous beneficial microbes into field conditions is unable to modulate overall the microbial structure but the selective recruitment of key microbial taxa, some of which is also implicated in the nematode suppressiveness.

Keywords: *Biological control agents,* soybean cyst- nematode (SCN) parasitism, Rhizosphere microbiome profiling.

Introduction

Plant-parasitic nematodes are the major notorious and destructive plant parasite, Jeopardize plant life at high risk throughout the plant life cycles. These pests cause plant damage and loses more than US$100 billion crop yields annually across the globe. They considere the destructive and lethal damaging pests of wheat, potato and soybean crops(Savary et al., 2019). The huge and most loses are caused by the root infecting small groups of nematodes such as root cysts nematodes (RCN) and root-knot nematodes (RKNs) which are also called sedentary endoparasitic nematodes.

Soybean cyst nematodes (SCN); (*Heterodera glycines*) is obligatory sedentary and biotrophic endoparasites that establish complex relation with host plants. The first action of SCN is to invade host (soybean) root tissue to start infection and following deterioration of the root metabolism. subsequently plant shows stunting of growth of aerial parts and, impact the photosynthesis, flowering stage and reduced crop yield (Stirling and West, 1991; Tian et al., 2014a)

It is widely documented that plants rely on associated microorganisms for a multitude of beneficial processes, include the ability to absorb nutrients, disease tolerance, and stress resistance. The rhizosphere, an interface between plant roots and soil, supports a dynamic microbial community with substantial microbe-microbe and plant-microbe communication, which is mediated by molecular signals from plants, especially secondary metabolites in the below-ground ecosystem.

A dynamic microbial population with significant inter- and intra-microbial communication is supported in the rhizosphere, which is the interface between plant roots and soil. Plant molecules, particularly secondary metabolites in the below-ground ecosystem, mediate this communication. (Philippot et al., 2013). Plants and bacteria have developed close relationships at this root-microbe interface. A substantial amount of photosynthates are allocated by plants as root exudates, which microbes use as resources. Microbes then contribute to plant fitness by exerting a variety of dircect and indircect mechanisams that encourage plant growth (Dutta et al., 2010; Pieterse et al., 2016). Additionally, rhizosphere also serve the plant's first line of defense against pathogen invasion (Mendes et al., 2018), It serves as the first filter for the assortment of microbes that will invade the root as endophytes (Lundberg et al., 2012). Identification of prospective targets for future disease management will be aided by knowledge of the important factors shaping the formation of the rhizosphere microbiome and the methods by which microbes and plants adapt to one another to

exogesnous microbes/microbial community intoroduction to the existing microbiota in rhizosphere microbiome. The use of soil nematicides is expensive, rarely effective, and hazardous to both the environment and human well-being (Evans et al., 2003)

Therefore, it is the need of the day to advent the ecofriendly, cost effective and highly effective strategy for the control of SCN.

Biological control is one of the most conducted and practiced management approaches which have been used since last 40 years for the control of SCN in field condition. This ecofriendly management headed many strategies in the discipline of SCN control and got attention across the world (Davies et al., 2018). Plant parasitic nematodes and particularly SCN are soil dwelling, this is why they could be controlled using soil born beneficial microbes (Huang et al., 2021). Fungi and bacteria are the most abundant owing to their fecundity soil microorganisms which have been used for as parasite and biological control agents for the control of Plant parasitic nematodes.

There are many bacteria and fungi known and reported owing biological control activities against plant parasitic nematodes such as *Burkholderia cepacia* which affect the *Meloidogyne incognita* hatching process and reduce the mobility of stage two juvenile, while the *Trichoderma* species has the potential parasite fungi (myco parasite) and demolish the infection capacity of plant parasitic nematodes (Ibrahim et al., 2020).

Both pathogenic and beneficial plant microbes interact with other soil organisms and alter their community structure, composition and functions and behavior. However, because microbial additions are frequently used against soil-borne fungi and bacteria in field condition, we are unsure of 1) how plant beneficial bacteria and fungi show antagonism against plant parasitic nematodes in field conditions 2) their influence on pre-existing microbiome (bacteria, fungi and protist communities.

The current study was conducted to evaluate the SCN inhibition and control employing three bioproducts Onix, Rizotech and GF 362 bacterial and fungal based bioproducts respectively in field condition against SCN in soybean crops. We applied two strategies seed inoculated and furrow in field conditions. Moreover, we used 16S rRNA, ITS, and 18S rRNA gene sequencing to track the effects of two well-studied plant-beneficial microbes- a bacterial biological control agent (Bacillus Methylotrophicus) and a fungal biological control agent (*Trichoderma* sp.) on the soil microbial and protist communities following the application of two strategies-seed inoculated and furrow-under field conditions.

Material and Methods

Description of Experimental Area

A field experiment was conducted at the South West Minas Gerais state of Brazil in the agriculture farm "Varjao de Minas" (18′23″S 40°02′1″W). The crop was cultivated during 2019-2020 seasoning year and the soybean plantation was carried out each year in November 2019-2020. SCN-susceptible soybean variety (AGROESTE AS 3730) for all of the soybean plots (Table 1). Seven replicates per crop sequence treatment were used in the full block design for the crop sequence treatment. A field chisel was used to plow the plots with conventional tillage before planting in the spring and fall. In this experiment, crops were fertilized to reduce the effect of soil nutrients on crop yield. Using a scythe, pre- and post-emergence weed control was carried out.

Plant material, Experimental Treatments and Design

A randomized complete block design (RCBD) with seven replications was used to set up the experiment. The seeds of susceptible soybean variety (AGROESTE AS 3730) was used in the experiment in Varjao de Minas (18º 22' 40" S 46º 01' 54" W). Plants were treated with three different biological control products employee two methodologies (seed inoculation and furrow), the data are shown in table 1. The treatment without biological products application was taken as control (T1). The seeds were treated and soaked with Rizotec ST (T2), GF362 TS (T3) and Onix TS (T6) and left overnight, mixed them thoroughly in polythene bags and were sown immediately in the field. The treatment (4 and 5) applied as a furrow methodology in each line. The experimental unit area (plot) was 7 m^2 consisting of 4 rows, and distance between each row was 0.5m and were sown with hand drill method keeping row to row distance of 0.5m. The crop was harvested after 116 days of sowing.

Soil Sampling for Soybean cysts nematodes (SCN)

A soil naturally infested with the soybean-cyst nematode *Heterodera glycines* in field condition. Each plot, which was divided into four rows and measured (7 m^2 long, 6 m wide); had six separate places (at a depth of 20 cm) from which soil samples were collected.

The soil samples were collected two times after biological control products application (Collection1; 30days Collectio2; 60days). All the samples were brought to the lab and preserved in cold chamber at 4 ^{0}C for further studies

Extraction and determination of Cysts from Soil Samples

Cysts have been extracted from the soil using a modified hand-decanting technique (Chen et al., 2001). To disintegrate soil aggregates, a 200m^3 subsample of soil being immersed in a container for 5 m and agitated with an electric drill stirrer.

A jet of water was used to suspend the soil solution, wait until settle down and then the water containing cysts was filtered via an 850 µm aperture sieve nested on top of a 250 µm aperture sieve. To recover the cysts throughout the soil sample, this process was carried out at least three times for each soil sample and each bucket of soil. Cysts containing debris and soil particles were gathered on the 250 µm -aperture sieve, and the cysts were afterwards segregated from the debris and soil using a sucrose flotation and centrifugation technique in 63% (w/v) sucrose solution (reporter and 1964, n.d.)(Jenkins, 1964).

Extraction of Eggs population and estimation from the Cysts

The soil cores have been passed through a sieve containing 4 mm holes in order to homogenize evenly. A revised sucrose suspension and centrifugation approach was employed to extract the cysts from the soil in a subsample of 200 cm^3 soil from the soil samples (Jenkins, 1964). A mechanical tool was used to release the eggs from the cysts (Faghihi and Ferris, 2000).

The Centrifuging method was followed in a 45% (w/v) sucrose solution, and allowed the eggs to be recovered from the debris, and retrieved from the top of the 25 ml aqueous suspension of sucrose. The eggs were determined by counting a subsample of the egg suspension under an inverted microscope. Under an inverted microscope, a subsample of the egg suspension was used to quantify the number of eggs.

In order to release the *H. glycines* eggs, suspensions of *H. glycines* cysts collected from soil were placed on a 100, 200, or 500 pore sieve and crushed using water and a mechanical rubber stopper. subsequently, centrifuged at 1800 rpm for 5 minutes followed by suspended in a 45% sucrose solution, centrifuged at 1600 rpm for 1 or 2 minutes. Wash well in tap water

and collect the eggs from the 500 pore sieve in water. Count the eggs density under the microscope

Extraction of adult females from Roots

Take a Beaker, make a water suspension with roots and shake the roots in water (with your hands) gently to detach the adult females from the roots and dissolve in water. Pour the water suspension into 20 and 100 sieves pour and collect the adult females from 100 sieve in water. Repeat this step as much as needed to guarantee all of cysts were extracted from the roots. Count the cysts under the microscope

Extraction of J2 from soil

We added 200 cm^3 of soil sample with the J_2 into falcon tube, calibrate their weight, shake and centrifuged them for 5m at 18000 rpm. The supernatant (water) discarded from samples and prepare the tubes for the step two. Add 45 % sucrose into falcon tubes calibrate their weight and shook them well to loosen the J_2 from the bottom. Centrifuged at 1600 rpm for 1 or 2 minutes. Recovered the supernatant (sucrose and J_2) into 500 mesh sieve, wash carefully in tap water to remove the sucrose and collect the J2 in water. The J_2 were counted under microscope.

J2 Mortality assay

To determine the mortality of J_2, the extracted J_2 (above extracted) were examined and studied for their mobility characteristics to know the efficacy of products that reduce J_2 mobility. The mortality was estimated under an inverted microscope. Percentage mortality was calculated for all treatments and controls (Hamid et al., 2017).

Soil microbial community

DNA extraction and quantification

Total DNA extraction was performed from the plant rhizosphere (control) and inoculated bioproducts plant rhizosphere using the DNeasy Power Soil DNA Isolation kit (MoBio, 12888), which has been adopted for microbial surveys. For each treatment, 0.25 g of the total soil were used for DNA extraction. DNA was quantified by using Nano-drop and stored at - 80 °C until further processing.

Amplicon library preparation, PCR analysis and high-throughput sequencing

The generation of PE sequencing reads of 16S, 18S rRNA gene and ITS2 summarized in the below subsection. The 16S V4–V5, (bacteria and archaea), 18S V4-V5 (eukarya) and ITS2 (fungal amplicon) examples are provided here. PCRs and library preparation were as described in (Comeau et al., 2017), with a change to Phusion Plus polymerase and maximum of 25 cycles for the PCRs. Pooled libraries were then sequenced on an Illumina MiSeq using a V3 chemistry kit in paired-end 2x300bp mode (, Integrated Microbiome Resource (IMR), The Langille Lab, Dalhousie University, Canada)

Sequencing data processing

QIIME 2 version 2019.765 was used to process the raw sequences (Bolyen et al., 2019) based on Microbiome Helper's operational guidelines (Comeau et al., 2017). Cutadapt was used to remove primers (Martin, 2011) and joined with the use of the QIIME 2 VSEARCH (Rognes et al., 2016) join-pairs plugin. Stitched reads were then quality filtered using the quality-filter plugin and reads were denoised using Deblur to produce amplicon sequence variants (OTUs) (Amir et al., 2017).

Statistical analyses

R version 3.5.3 was used to conduct the statistical analysis (Rproject.org). The experiment was carried out using a completely random block design**.** Community level differences in alpha- and beta-diversity were analyzed using QIIME 2 version 2019.765. For alpha-diversity, we calculated the Observed richness (number of OTUs/sample) and the Shannon diversity index. For beta-diversity, between-sample differences were assessed visually through principal coordinates analyses (PCoA) based on UniFrac distances. First, the metadata and non-rarefied feature tables were converted into a phyloseq object (version 1.29.0). Prior to differential and network analysis, taxa with a prevalence lower than 5% (i.e. taxa with a non-zero count in less than 5% of the samples) were trimmed. NetCoMi (Network Construction and Analysis for Microbiome Data) was used to construct microbial association networks (Peschel et al., 2021). First, taxa abundance data was filtered to the 150 most

abundant taxa on each sample. SparCC, a technique for inferring correlations from compositional data that assumes the true correlation network is “sparse”, was applied in the network construction step. When sparsified associations were converted into dissimilarities, the handling of negative associations was done using a "signed" method. A 0.5 threshold was employed and the fast-greedy technique was used to infer node clusters. Based on the Eigenvector centrality characteristics and taking into account the nodes with the highest centrality values, the hub node identification for each estimated network was accomplished.

Results

The major goals of the current research are to investigate the influence of exogenous biological control agents on (1) indigenous microbiota (2) microfauna and (3) their biological control efficacy against cysts nematodes in field conditions. A total of three bioproducts were applied using two strategies Rizotec (*Pochonia chlamydosporia* CEPA PC-10) ST; seed treatment, GF362 (*Trichoderma koningiopsis*) Furrow and ST; seed treatment, and Onix (*Pochonia chlamydosporia* (Pc123)) furrow and ST; seed treatment to control cyst nematodes in field conditions.

The impact of biological control based bioproducts on bacterial diversity (16Sr RNA)

The diversity of bacteria was evaluated using alpha diversity indices. Native bacterial community diversity in all treatments was lower than in control soil in the first year, while only Onix ST increased the Native bacterial community diversity in followed second year, though significant differences between the control and all treatments in the first and second years were not observed (Fig. 1). The Trichoderma-based biological product (GF362) reduced the native bacterial community followed by Bacillus-based biological product (Onix) in the first-year trial in relation to control. The second consecutive year trial also followed the same pattern and no significant differences were detected between the control and treatments, although Bacillus-based biological product (Onix) increased the bacterial community diversity when compared to the control and GF362 Fig. 1).

The impact of biological control based bioproducts on fungal and diversity (ITS2)

In both the first and second years, the average indigenous fungal community diversity

in all treatments was higher than in control soil. Despite the fact that there were no significant differences between the control and any of the treatments in the first and second years (Fig. 2). In the first-year study, the Bacillus-based biological product (Onix) improved the fungal community diversity in comparison to the control, while in the second year, both bioproducts Onix and GF362 highly increased the fungal community diversity when compared to the control (Fig. 2).

The impact of biological control based bioproducts on 18S diversity

Intrestingly the diversity of indigenous eukaryotes communities in both the first and second years in all treatments was observed higher than in control soil. Despite the fact that there were no significant differences observed between the control and any of the treatments in the first and second years (Fig. 3). In the first-year study, Trichoderma- based biological bioproduct GF362 furrow improved the indigenous eukaryotes communities followed by Onix ST while in the second year trial, the treatment Onix ST increased the indigenous eukaryotes communities followed by GF362 furrow treatment. Overall, both bioproducts improved the indigenous eukaryotes communities in both trials in relation to the control (Fig. 3)

Bacterial community composition and taxonomic distribution under different bioproducts application 1st year

The bacterial community`s taxonomy was assessed at the phylum and genus level. The total of 418 OTUs were recovered, following chimera removal and resampling. The dominated bacterial profile of *Proteobacteria, Acidobacteria, Actinobacteria, Planctomycetota, Bacteroidota, Gemmatimonadetes, Chloroflexi, Methylomirabilota, Myxococcota,* and *Nitrospirota* were observed accross all treatmnts (control, GF362, Onix). The average relative abundance of *Proteobacteria* was highest compared to other phyla but similar in all treatments (Fig. 4). The average relative abundance of *Proteobacteria* was slightly higher in GF362 (31.98%), Onix (325) than control (31.18%) (Fig. 4). The relative abundance of all observed phyla except *Proteobacteria* was similar and no variation between treatments and control was shown (Fig. 4A). For genus, the most dominated genus of family V*icinamibacteraceae Acidibacter*, *Bradyrhizobium, Sphingomonas* and *Rokubacteriales* were recorded in all treatments. The highly relative abundance genus of Vicinamibacteraceae

family were found the most prevalent in the bacterial communities in all treatments comparatively to other genera. Consequently, the relative abundance of all genus *Acidibacter*, *Bradyrhizobium*, *Sphingomonas* and *Rokubacteriales* were equally dominated in all treatments (Fig. 4B). The bioproducts showed no impact on bacterial genus composition and taxonomy when compared to control. The Venn diagram showed 253 common OTUs by various biologic control products and 98.7% of all reads were characteristic of the shared OTUs (Fig. 4C).

Bacterial community composition and taxonomic distribution under different bioproducts application 2nd year

The taxonomy of the bacterial community was evaluated at the phylum and genus levels. Following chimera removal and resampling, a total of 418 OTUs were found (Fig. 4). *Proteobacteria* predominated the bacterial composition across all treatments, followed by Acidobacteria, *Actinobacteria*, *Planctomycetota*, *Bacteroidota*, *Gemmatimonadetes*, *Chloroflexi*, *Methylomirabilota*, *Myxococcota*, and *Nitrospirota* (Fig. 4D). The control and both applied biological control products were highly dominated by *Proteobacteria* nearly 43% in comparison with other phyla, while the Onix treatement was dominated highly by *Acidobacteriota* (17%) approximately followed by control (16%) and GF362 (15%) when compared with GF362 and control treatments (Fig. 4D).

The dominated bacterial profile was *Sphingomonas, Solirubrobacter, Lysobacter, Microvirga, Massilia, Acidibacter, Ramlibacter, Vicinamibacteraceae* and *Bradyrhizobium* in all treatments

The *Sphingomonas* was recorded as the highly abunadant genus in GF362 (5%) and Onix 5%) comparatively to the control (4%) (Fig. 4E). A total of 236 common OTUs by different biological control products were displayed in the Venn diagram, and 97.6% of all reads were indicative of the shared OTUs (Fig. 4F).

Fungal community composition and taxonomic distribution under different bioproducts application 1st year

The taxonomy of the fungal community was evaluated at the phylum and genus levels. Following chimera removal and resampling, a total of 293 OTUs were found (Fig. 5A).

The taxonomic profiling of fungi revealed the dominated phyla such as *Ascomycota, Basidiomycota, Mortierellomycota, Chytridiomycota, Mucoromycota, Chlorophyta* in all treatments. Interestingly, the relative abundance of *Ascomycota,* was recorded higher in control (89.3%) than GF362 (88.2%) and Onix (58.6%). These data showed the relative abundance higher in control treatment than GF362 and Onix treatments, which shows that biological products declined the population of *Ascomycota,* in relation to control (Fig. 5A).
Figure 5B illustrates the fungal community structure in soil samples at the genus level. The major genera in the all treatments were determined and the dominated genera were *Fusarium*, *Talaromyces*, *Trichocladium*, *Purpureocillium*, Sistotrema, *Mortierella*, *Paratrimmatostroma*, *Microascus*, *Macrophomina*, *Trichoderma* (Fig. 5B). The relative abundance of *Fusarium* (16%) in control, while in GF362, and Onix (10%) and (11.4%), respectively. Hence the bioproducts reduced the community of *Fusarium* in soybean fields, many of which can be harmful to the soybean. Total of 221 common OTUs by different biological control products were displayed in the Venn diagram, and 99.2% of all reads were indicative of the shared OTUs (Fig. 5C).

Fungal community composition and taxonomic distribution under different bioproducts application 2nd year

The trial was repeated the following year, and the fungi's taxonomy was assessed at the phylum and genus levels. After chimera removal and resampling, 293 OTUs in total were obtained (Fig. 5D). The dominated phyla were *Ascomycota*, *Basidiomycota*, *Mortierellomycota, Chytridiomycota*, *Chlorophyta*, *Mucoromycota***,** *Calcarisporiellomycota*, *Cercozoa*, and *Kickxellomycota* in fungal community (Fig. 5D). The average relative abundance *Ascomycota* was recorded higher in control (90%), GF362 (89.1%) and Onix (80.1%). On the other hand, *Mortierellomycota* was highly observed in Onix treatment (11.5%) followed by control (2.5%) and GF362 (0.2%) treatment. (Fig. 5D). In the second year trial, the genus-level fungal community structure in all treatments depicted in (Fig. 5E). *Fusarium, Mortierella, Phoma, Fusicolla, Plectosphaerella, Talaromyces, Minimedusa, Articulospora, Trichocladium* and were the dominant genera in all treatments. The relative abundance of Fusarium (13%) in control, GF362, and Onix (17%) and (14%), respectively, an increase compared to the first year but not necessarily a buildup in the species virulent to soybean. Total of 184 common OTUs by different biologic control products were displayed in

the Venn diagram, and 97.6% of all reads were indicative of the shared OTUs (Fig. 5F).

Eukaryotes community composition and taxonomic distribution under different bioproducts application 1st year

The trial was repeated the following year, and the Eukaryotes's taxonomy was assessed at the phylum and genus levels. After chimera removal and resampling, 253 OTUs in total were obtained (Fig. 6A). The increased relative abundance of Phyla, *Ascomycota, Cercozoa, Phragmoplastophyta, Mucoromycota, Nematozoa, Chytridiomycota, Gracilipodida, Amoebozoa, Chlorophyta*, and *Zoopagomycota* in all treatments helped us understand the impact of biocontrol products on soil microbiome. The dominant phylum in the control, GF362, and Onix was *Ascomycota*. The average relative abundance of Ascomycota in control (82%), GF362 (78%) and in Onix (79%) observed (Fig. 6A). The relative abundance of the dominant genera were *Polymyxa, Cladosporium, Microascus, Talaromyces, Chaetomium, Magnoliophyta, Trichoderma, Cercomonas, Arachnomyces,* and *Mortierella* detected in all treatments (Fig. 6B).

The major phyla *Polymyxa,* detected *(*5%) in the control, (7%), in the GF362 and (9%), in the Onix (Fig .6A) *Total* of 157 common OTUs by different biologic control products were displayed in the Venn diagram, and 98.6% of all reads were indicative of the shared OTUs (Fig. 6C).

Eukaryotes community composition and taxonomic distribution under different bioproducts application 2nd year

The trial was repeated the following year, and the Eukaryotes's taxonomy was assessed at the phylum and genus levels. (Fig. 6D). The taxonomic analysis further revealed that the Eukaryotes's taxonomy differed markedly among the different treatments (Figure 3) It was observed that, on average, more than 40% of observed Eukaryotes's taxonomy were affiliated to ten eukaryotic phyla, including *Ascomycota, Cercozoa, Nematozoa, Mucoromycota, Apicomplexa, Amoebozoa, Gracilipodida, Annelida, Rotifera,* and *Chytridiomycota* (Fig. 6E). However, the relative abundance of these taxa varied among different treatments (Fig. 6E.). For example, the highly dominated phyla was *Ascomycota* in all treatments, which was account its relative abundance 65 %, 69% and 67% in control,

GF362 and Onix respectively.

The relative abundance of the dominant genera was *Polymyxa, Cercomonas, Tylenchida,* Mortierella, *Microascus, Trichoderm, Talaromyces, Heteromita,* and *Rhabditida* detected in all treatments (Fig. 6E). The relative abundance which was observed in all treatments was *Polymyxa* comparatively higher than other genus. The average relative abundance of *Polymyxa* 10%, 12% and 15% in control, GF362 and Onix respectively (Fig. 6E). A total of 116 common OTUs by different biologic control products were displayed in the Venn diagram, and 95% of all reads were indicative of the shared OTUs (Fig. 6F).

Beta diversity PCoA plots

The principal coordinate analysis (PCoA) was applied to assess the bacterial, eukaryotic and fungal community composition across various treatments at two consecutive years. PCoA results showed that bacterial, eukaryotic and fungal communities were dispersed and showed dispersed patterns from one another in control, GF_362, and Onix_ST treatments in the first and second year. This difference indicates that the application of biological control couldn't induce any changes in the microbial community profile in the first year. PCoA results showed that bacterial communities were showed concentrated pattern in the first year in all treatments but scattered patterns in all treatments in the second year. The variation was found between first and second year in bacterial community's composition (Fig. 7A). The PCo1 and PCo2 contributed 39.67% and 13.15% towards variations in bacterial community respectively.

PCoA results showed that eukaryotic communities were concentrated and showed clustered patterns in all treatments in first year, **(**Fig. 7B). Interestingly, in the second year all microbial Eukaryotic communities showed slightly scattered patterns and observed dispersed pattern from one another in control, GF_362, and Onix_ST treatments **(**Fig. 7B). This difference indicates that the application of biological control influenced the Eukaryotic community's composition and induced changes in the microbial community profile in the second year. The PCo1 and PCo2 contributed 38.82% and 14.74% towards variations in Eukaryotic community respectively **(**Fig. 7B).

The fungal communities in the host rhizosphere clustered closer in the first year and second year (2019-2020). Noteworthy in the second year, all fungal communities in the control, GF_362, and Onix_ST treatments displayed slight dispersion patterns and detected more in cluster form than bacterial and eukaryotic communities **(**Fig. 7C). This distinction

shows that the application of biological control altered the profile of the fungal community in the second year and affected the composition of fungal communities, where The PCo1 and PCo2 contributed 32.03% and 14.64% towards variations in the fungal community respectively **(**Fig. 7C).

Co-occurrence Network Analysis for bacterial communities

A co-occurrence network pattern was studied to investigate how the existed microbiota interact and respond to exogenously inoculated fungal and bacterial species in two consecutive years. We applied two biological control products (fungal and bacterial) in soybean fields with aimed to control SCN and investigated the topological characteristics of the microbial communities in the plant rhizosphere compartment for both years. The network of all treatments was observed to have characteristics similar (Table 1). The investigation revealed that there were differences in the network topological metrics between the control and inoculated treatments, including the Clustering coefficient, Modularity, Positive edge, Edge density, and Natural connectivity in both first and second year. In general, clustering coefficient (average degree of connection of a node) was slightly higher in the second year, especially for 16S (where it ranged from 0.507 to 0.514 in the second year, while it ranged from 0.462 to 0.480 in the first year). The *Trichoderma* based bio product had the highest modularity values (strength of division of a network into modules), except in the second year when taking into account the 16S microbial community. The networks for control in the second year had higher edge densities (percentage of possible links between nodes) than in year 1. In general, all treatments resulted in dense networks, with taxa clustering in different ways (Fig. 8).

Co-occurrence Network Analysis for fungal communities

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Positive edge, Edge density, and Natural connectedness in both the first and second year, varied between the control and inoculated treatments. Interestingly, all the topological characteristics were reduced in second year in both *Bacillus* and *Trichoderma* applied treatments as compared to control. The Modularity was slightly higher in the first year, in both treatments as compared to control while reduced in the second year. The networks for control in the second year had higher edge densities (percentage of possible links between nodes) than in year 1. In general, all treatments resulted in dense networks, with taxa clustering in different ways (Figures.9).

Discussion

The potential of microorganisms that exhibit promising results in laboratory and greenhouse experiments to overcome obstacles and maintain their features when applied in the field is crucial for the successful deployment of bacterial inoculants to increase plant productivity(Sessitsch et al., 2019). Understanding the permanence and effectiveness of the introduced biological products and its impact on the plant-associated microbiota as well as their compatibility with farming practices are crucially important. Many Plant growth promoting microbes have been reported as successfully controlling the plant disease (Bhattacharyya and Jha, 2012; Mehmood et al., 2021; Pathania et al., 2020) including root knot nematodes (Haarith et al., 2021; Tian et al., 2014b). But it is also indisputable that many Plant growth promoting microbes have been reported unsuccessful in controlling the plant disease, when treated both in combination (consortia) or alone. For instance, *P chlororaphis* PCL1391 and *P. fluorescens* WCS365 (Bardas et al., 2009) and *P. chlororaphis* PCL1391 and *P. fluorescens* P3/pME6863, (Molina et al., 2003) as a as a consortia completely failed to perform biocontrol activities plant disease.

According to the research of the (Hubbard, 1983)*Trichoderma hamatum* for Pythium failed to control plant diseases. In this study we analyzed the effects of the biological control agent based bioproducts and chemical nematicides at different combinations (Table.1) on the control of root-knot nematodes and their impact on plant rhizosphere microbiome profile in a field trial. All the biological control products and chemical nematicide had not shown significant impact on root-knot nematodes control and did not show significant differences between control and treatments. Although PGPB-containing inoculants have been employed to plant crops for more than 120 years (Arora et al., 2017). Especially in field condition, some of the bioproducts show inconsistent efficacy in terms of control of parasites due multitudes

of biotic and abiotic factors (Mitter et al., 2021; Naamala and Smith, 2020). The inconsistent effectiveness of microbial inoculants in the field may be explained by a variety of unavoidable biotic and abiotic conditions that can put the existence of the introduced microorganism (French et al., 2021a). Additionally, the inoculants may interfere with other agricultural chemicals used on crops or may be rendered useless by native plant-associated microbiota that persists at low and ineffective levels in the soil (French et al., 2021b). Understanding the persistence and efficiency of such products thus requires testing microbial products in field conditions, evaluating their compatibility with other products, and investigating their effects on the indigenous plant microbiome. The failure of bioproducts in filed conditions extensively studied.

To establish a successful long-term interaction with the existing microbiota and host plant, and enhance their persistency and efficacy in field conditions, the following points must be considered to address. 1) Abundant and diverse soil microorganisms in the soil/plant ecosystem, distribution of microorganisms, and 2) appropriate formulations that should guard against desiccation and other detrimental circumstances for microbial cells. 3) the recipient environment's biotic and abiotic circumstances, as well as the organism's physiological activity and compatibility with the target plant 4). The colonizing microbes must be able to tolerate various environmental factors, such as pH or oxygen availability, in order to establish themselves. These factors include their ability to recognize and metabolize these chemicals. 5) the inoculant microorganisms must contend with a microbiota that is extremely diverse and plant genotype diversity (De Roy et al., 2013; French et al., 2021c; Sessitsch et al., 2019; Thilakarathna and Raizada, 2017).

We noticed that the inoculation had no influence on the number of bacteria present in the maize roots, leaves, or rhizosphere. (Estrada-Bonilla et al., (2021) used compost and phosphate-solubilizing bacteria on sugarcane in a greenhouse experiment, and they reported similar results. Inoculation transformed slightly the structure of the soil's bacterial community, the authors discovered little variation in the diversity of microorganisms in soil samples. Additionally, similar to previous research, we found that microbial diversity and structure fluctuated among niches associated with plants (Cregger et al., 2018; Dickey et al., 2020). Previous research has suggested that the presence, abundance, and activities of particular bacterial taxa are influenced by the microenvironment supplied by the plant compartment, which indicates that niche adaptation may play a significant role in filtering and recruiting various microorganisms(Compant et al., 2021; Trivedi et al., 2020). According to

ecological theory, more diverse ecosystems are more stable and, as a result, less vulnerable to invade organisms (Chen et al., 2013; Ecology and 1997, 1997). Exogenous organisms will encounter more difficulties while trying to invade the biodiverse communities (Ecology and 1997, 1997). It has been suggested that one of the main factors affecting the diversity of microorganisms in the rhizosphere is the range of organic compounds produced by plants (Curl and Truelove, 2012).

A few research has examined the long-term effects of immunization. Several months after receiving the vaccination, several of them have reported effects on the microbiome(Wang et al., 2018; Yin et al., 2013) and some reported the shift after the few days of innoculation (Johansen and Olsson, 2005). For instance, *Pseudomonas fluorescens* DR54 altered the composition of the barley-related rhizosphere microbiome up to 6 days after inoculation, but after 9 days, it recovered to its pre-inoculation state, according to research by (Johansen and Olsson, 2005). Mawarda et al., 2020 proposed a meta-analysis to determine whether microbial inoculants change the composition of the soil microbial community. Over 96% of the 26 studies that used high-throughput sequencing and found that microbial application changed the composition of the native microbial population. However, of the 78 studies that were analyzed and using profiling approaches, 82% found an impact after innoculation whereas only 18% did not find any appreciable effects. Additionally, depending on the kind of soil, inoculants may have different effects on the microbial population in the rhizosphere. However, these effects are challenging to measure in the field since the soil bacterial community composition is influenced by a variety of factors, including cropping history, agricultural management practices, and weather patterns (Costa et al., 2006; Schreiter et al., 2014).

The bacterial community in all treatments for both years was dominated by *Proteobacteria*, *Acidobacteria*, *Actinobacteria* (Figure 2), which is in line with previous research on the soil microbiome (Wang et al., 2016; Zhou et al., 2015). However, there were no discernible variations in the relative abundance of *Proteobacteria, Acidobacteria*, and *Actinobacteria* between treatments in the two consecutive years. *Ascomycota*, *Basidiomycota*, and *Mortierellomycota* dominated the fungus community in all treatments for both years (Figure 2), however in the second year, the *Ascomycota* decreased in treatment Onix_ST (Figure.) The relative abundance of Phyla, *Ascomycota*, and *Cercozoa* increased in the second year in both treatments GF_362 furrow and Onix_ST, indicating a shift in the population of eukaryotic organisms. When compared to control in the current investigation, the bacterial

diversity and diversity index values were all insignificant.

Note that in this study, we first, aimed to control the root-knot nematodes through bioproduct application in field conditions. Additionally, we also focused on how the dynamic changes in the rhizosphere microbiome happen and could affect the soil bacterial diversity, and community composition, after the bioproducts application. However, the impact of root exudates on the soil microbial population, soil sampling, and other environmental factors were not considered in the soybean plantation area. It is indisputable that biotic and abiotic factors, tree age, and rhizodeposition phenomenon will have an impact on the rhizosphere microbiome. Hence, further studies are needed to understand the influence of biotic and abiotic factors in a soybean plantation under field conditions.

Conclusion.

In summary, this research was conducted to evaluate the efficacy of biological control agent-based bioproducts under field conditions. In field conditions, we found no potential bioproduct to control root-knot nematodes. The application of bioproducts has no impact on the diversity of the microbial population of the soybean rhizosphere. The co-occurrence network, however, revealed that bacterial species established a complex network structure in the second year (2020), pointing to a more permanent microbial interaction in the rhizosphere of soybean plants. Due to the demand for inoculants whose effects are long-lasting and repeatable in the field, we think that this knowledge has the potential to improve the development of microbial products for agricultural applications.

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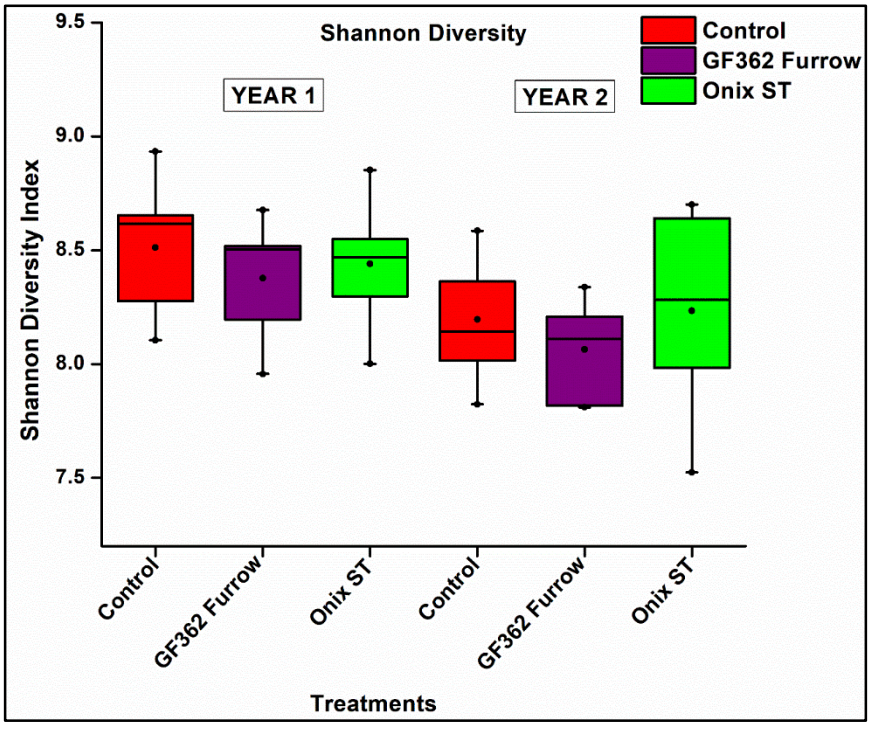

Figure.1. Alpha diversity indexes of bacterial community (16S rRNA) gene bar code for the microbiome of all seven replicates under different treatments in two consecutive years (2019 and 2020). Changes in the Shannon diversity index were observed under different applied bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2).

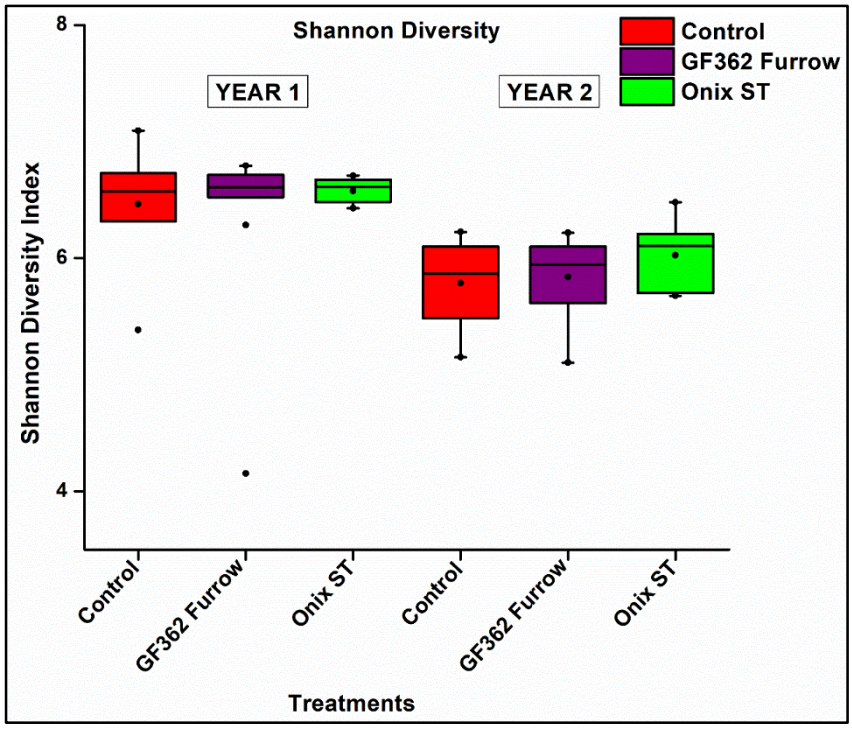

Figure.2. Alpha diversity indexes of the fungal community (ITS2) gene bar code for the microbiome of all seven replicates under different treatments in two consecutive years (2019 and 2020). Changes in the Shannon diversity index were observed under different applied bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2).

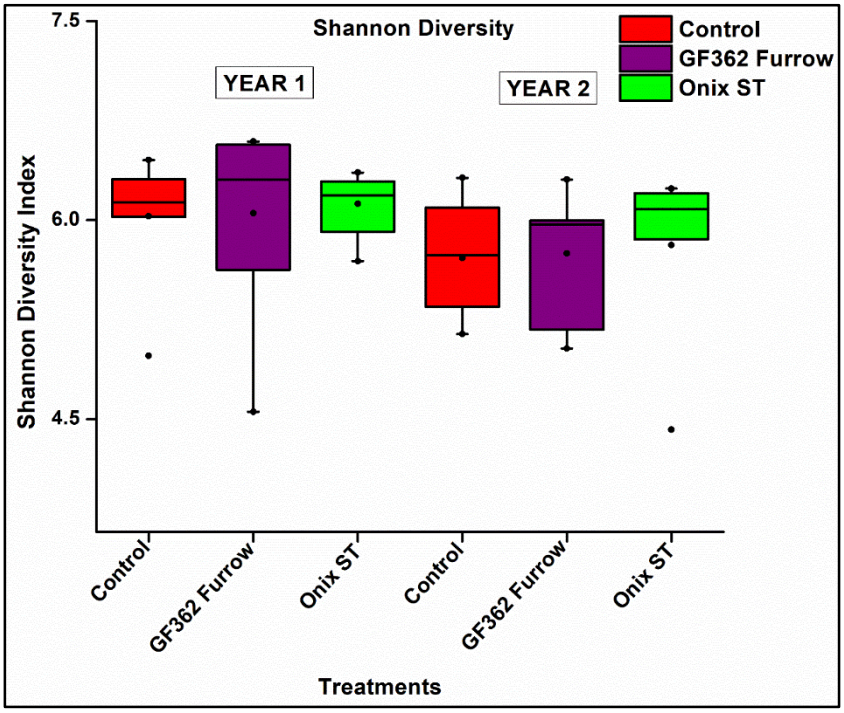

Figure.3. Alpha diversity indexes of eukaryotic community (18S) gene bar code for the microbiome of all seven replicates under different treatments in two consecutive years (2019 and 2020). Changes in the Shannon diversity index were observed under different applied bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2).

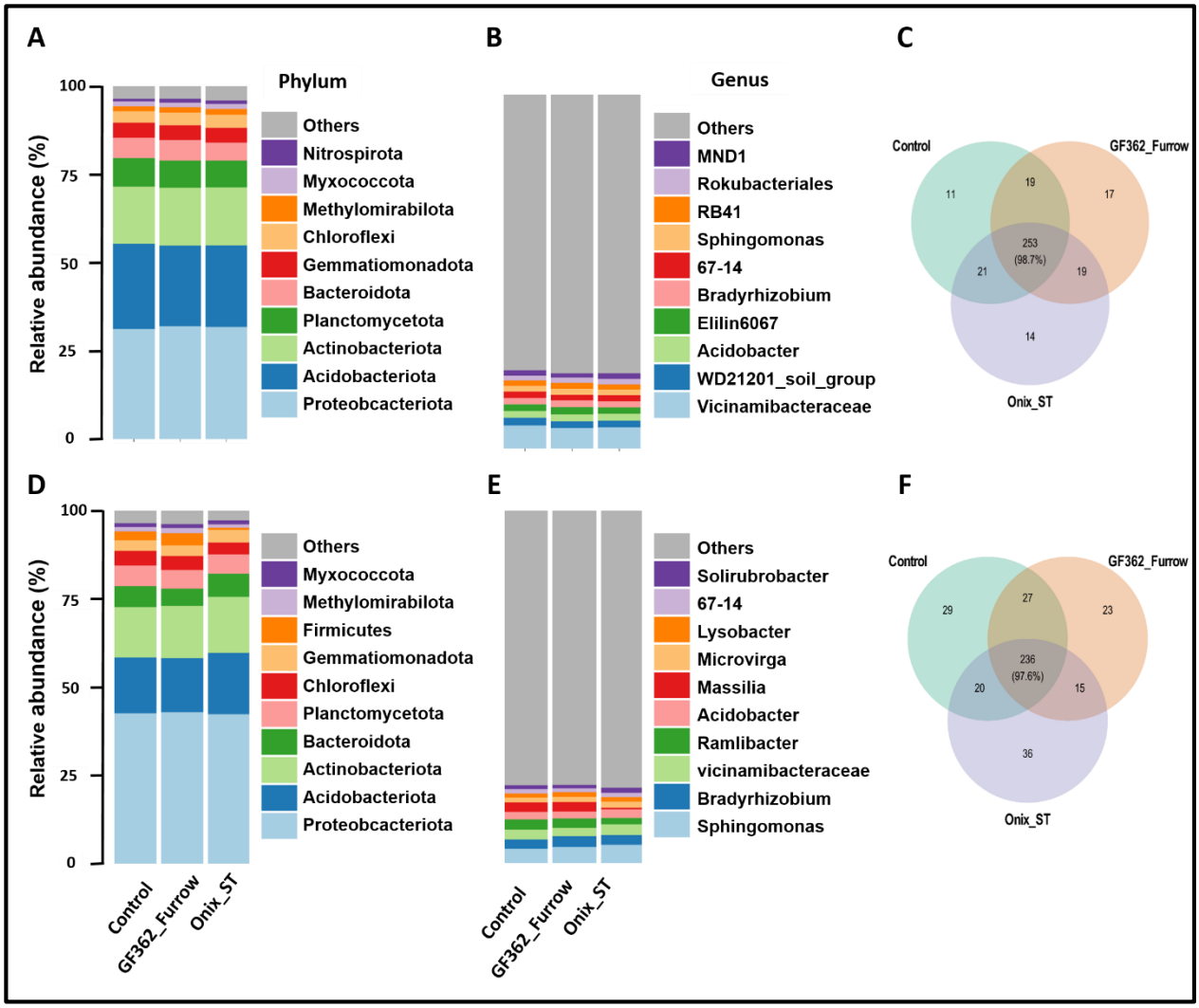

Figure 4. Distribution of bacterial communities (16S rRNA) in soybean plant rhizosphere samples treated with bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2). Different treatments resulted in changes in relative abundance and a comparison of the bacterial community (16S rRNA) over the course of two years, (A, B, C) (2019) and (D, E, F) (2020). The bioproducts were applied using two strategies, furrow and seed treatments. The figure depicts the relative abundance of bacterial communities at the Phylum and genus level. Bacterial community composition is shown in bar charts (only significant taxa greater than 1% are shown). The first 10 bacteria that are displayed at the bottom of the bars are referenced in the legend

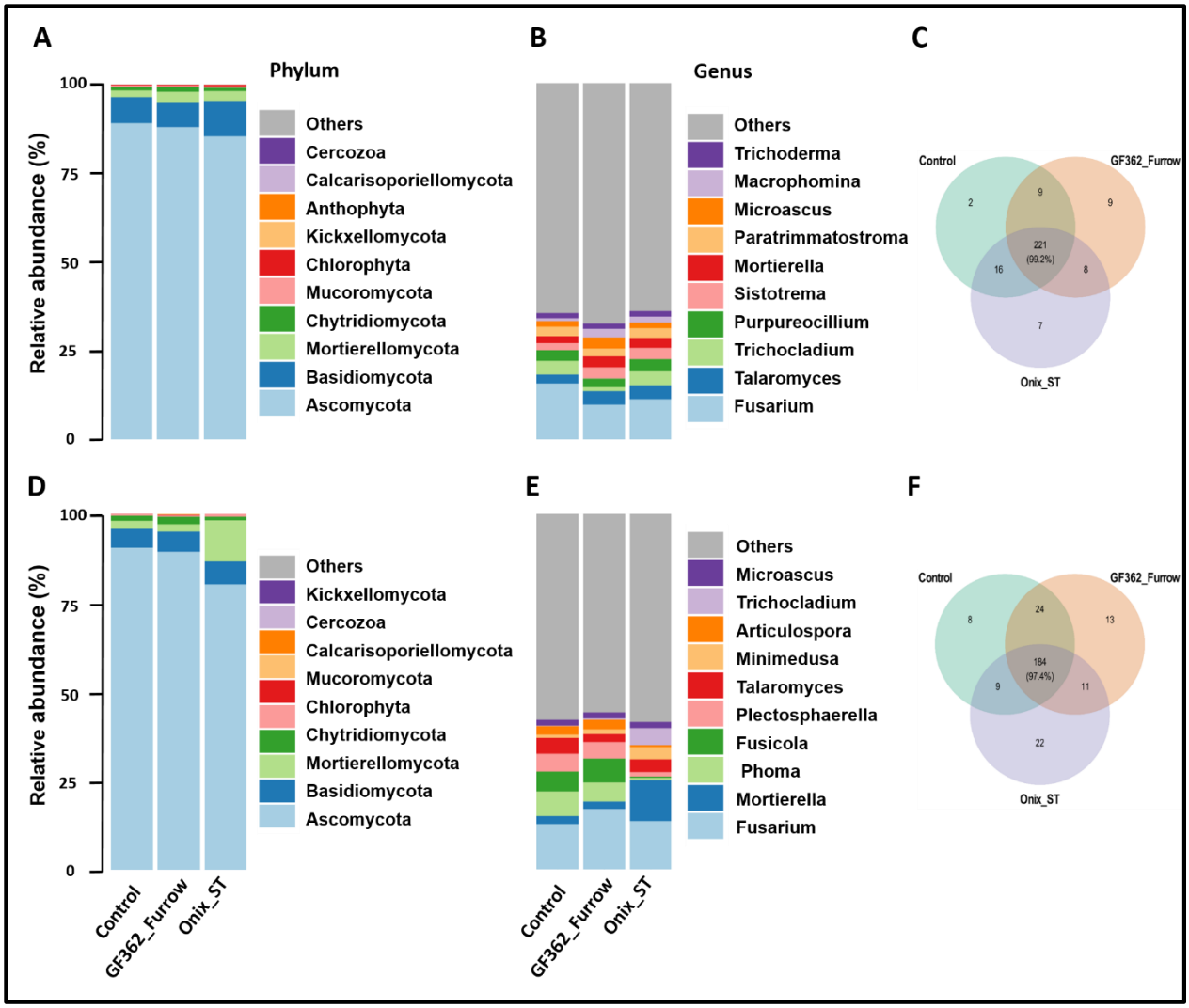

Figure 5. Distribution of fungal communities (ITS2) in soybean plant rhizosphere. Different treatments resulted in changes in relative abundance and a comparison of the fungal communities (ITS2) over the course of two years, (A, B, C) (2019) and (D, E, F) (2020). The bioproducts were applied bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2). The figure depicts the relative abundance of fungal communities at the Phylum and genus level. Fungal community's composition is shown in bar charts (only significant taxa greater than 1% are shown). The first 10 bacteria that are displayed at the bottom of the bars are referenced in the legend

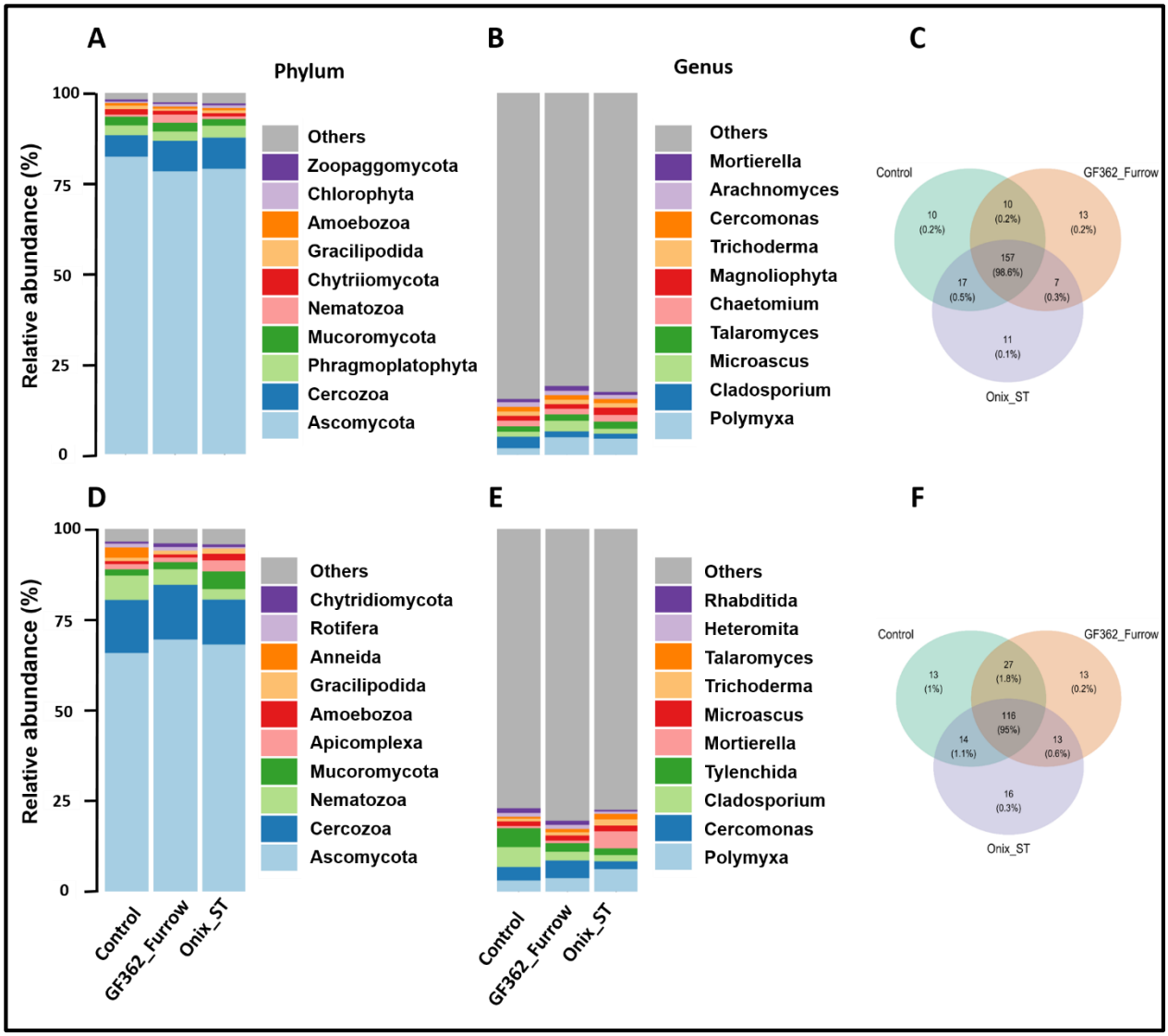

Figure 6. Distribution of eukaryotic communities (18S) in soybean plant rhizosphere samples (Control, Gf362_Furrow and Onix_St). Different treatments resulted in changes in relative abundance and a comparison of the Eukaryotic communities (18S) over the course of two years, (A, B, C) (2019) and (D, E, F) (2020). The bioproducts were applied bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2). The figure depicts the relative abundance of Eukaryotic communities at the Phylum and genus level. Eukaryotic community's composition is shown in bar charts (only significant taxa greater than 1% are shown). The first 10 bacteria that are displayed at the bottom of the bars are referenced in the legend

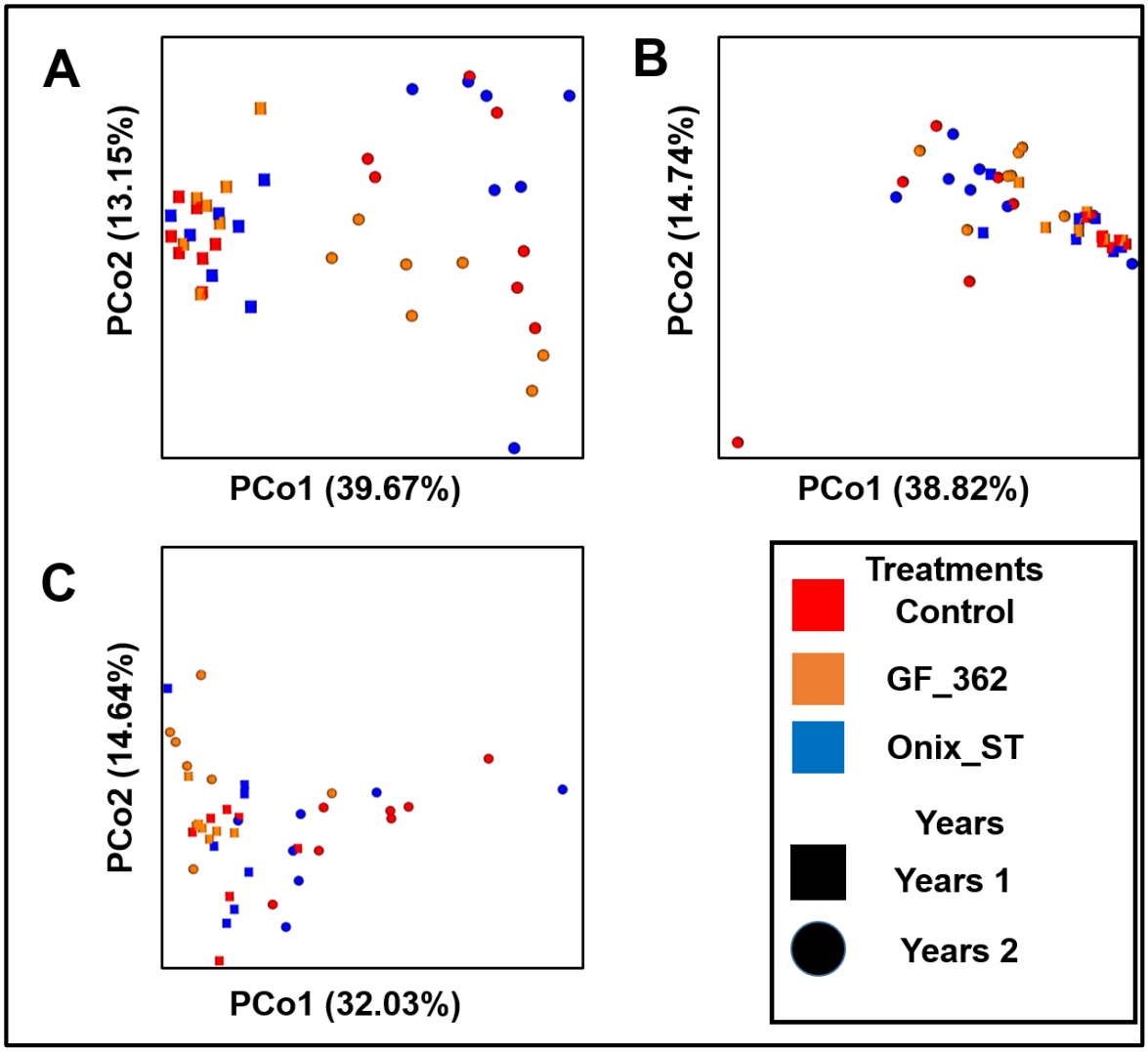

Figure. 7. Principal Component Analysis (PCoA) of Bacterial (16S r RNA), eukaryotic (18S), and fungal community (ITS2) structures and composition. The bioproducts were applied bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2). Differences in bacterial community composition. (A). PCoA of the bacterial community (16S rRNA), composition across all treatments. (B) PCoA of the eukaryotic community (18S), composition across all treatments. (C) PCoA of the fungal community (ITS2) composition across all treatments.

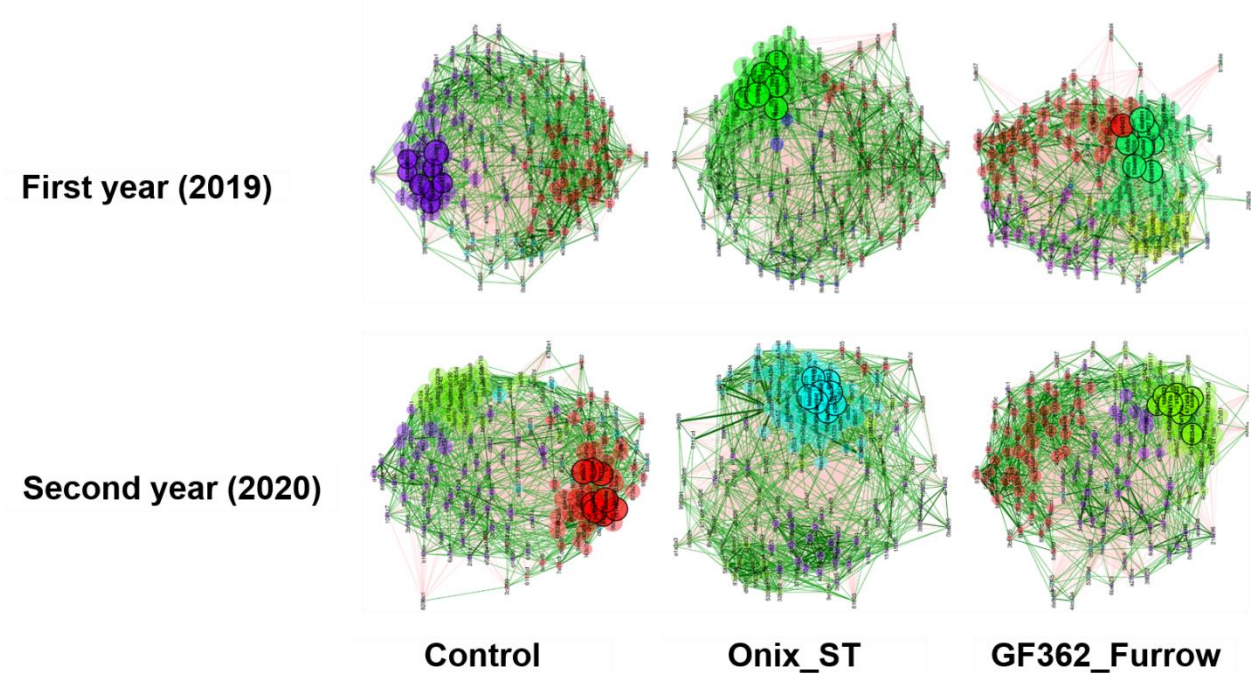

Figure 8: 16S rRNA microbial network of the rhizosphere of soybean (*Glycine max*) plants with different treatments in two consecutive years (2019 and 2020). The bioproducts were applied bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2). Nodes represent OTUS, with size reflecting the OTUs influence in the community (eigenvector centrality), and color corresponding to the cluster to which the OTUS belongs. Edges between nodes represent correlations between the nodes they connect, with edge width and shade indicating the correlation magnitude, and green and red colors indicating positive and negative correlations, respectively. See Table. 1 for full names of OTUS and corresponding taxa.

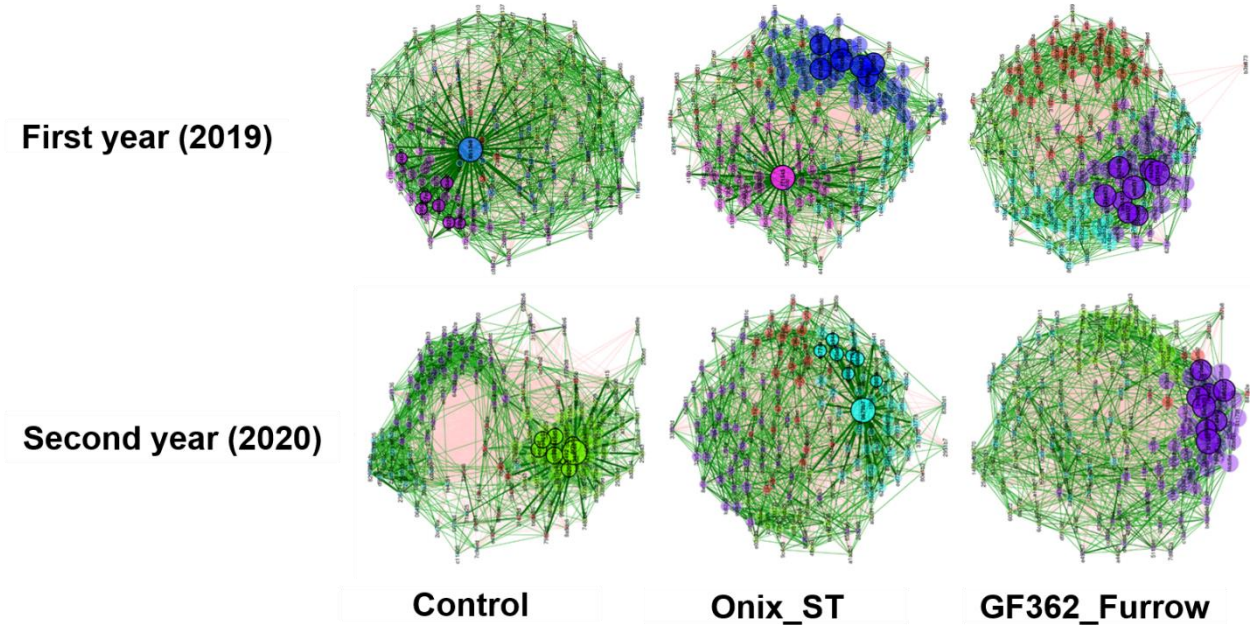

Figure 9: ITS2 microbial network of the rhizosphere of soybean (*Glycine max*) plants with different treatments in two consecutive years (2019 and 2020). The bioproducts were applied bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2). Nodes represent OTUS, with size reflecting the OTUs influence in the community (eigenvector centrality), and color corresponding to the cluster to which the OTUS belongs. Edges between nodes represent correlations between the nodes they connect, with edge width and shade indicating the correlation magnitude, and green and red colors indicating positive and negative correlations, respectively. See Table. 2 for full names of OTUS and corresponding taxa.

List of tables

Table 1: Global network properties of microbial communities (Bacterial) of soybean (*Glycine max*) plants with different treatments in two consecutive years (2020 and 2021). The bioproducts were applied bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2).

Marker	Global network property	Year 1			Year 2		
16S		**Control**	**Onix ST**	**GF362**	**Control**	**Onix ST**	**GF362**
	Components[1]	1.000	1.000	1.000	1.000	1.000	1.000
	Clustering coefficient[2]	0.462	0.480	0.466	0.514	0.549	0.507
	Modularity[3]	0.078	0.121	0.156	0.117	0.084	0.129
	Positive edge %[4]	49.838	53.670	55.456	49.690	52.132	54.206
	Edge density[5]	0.243	0.242	0.211	0.273	0.307	0.247
	Natural connectivity[6]	0.078	0.093	0.065	0.093	0.126	0.085

1 Number of components in the network
2 Average degree of connection of a node
3 Strength of division of a network into modules
4 Percentage of edges with positive estimated association of the total number of edges
5 Ratio of the number of edges and the number of possible edges.
6 Robustness measure of complex networks

Table 2: Global network properties of microbial communities (Fungal) of soybean (*Glycine max*) plants with different treatments in two consecutive years (2020 and 2021). The bioproducts were applied bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2).

Marker	Global network property	Year 1			Year 2		
ITS2		**Control**	**Onix ST**	**GF362**	**Control**	**Onix ST**	**GF362**
	Components	1.000	1.000	1.000		1.000	1.000
	Clustering coefficient	0.463	0.479	0.462		0.552	0.471
	Modularity	0.078	0.083	0.109		0.109	0.079
	Positive edge %	50.198	49.187	51.305		51.664	48.652
	Edge density	0.243	0.243	0.243		0.291	0.242
	Natural connectivity	0.081	0.075	0.072		0.120	0.072

1 Number of components in the network
2 Average degree of connection of a node
3 Strength of division of a network into modules
4 Percentage of edges with positive estimated association of the total number of edges
5 Ratio of the number of edges and the number of possible edges.
6 Robustness measure of complex networks

Supplementary Materials

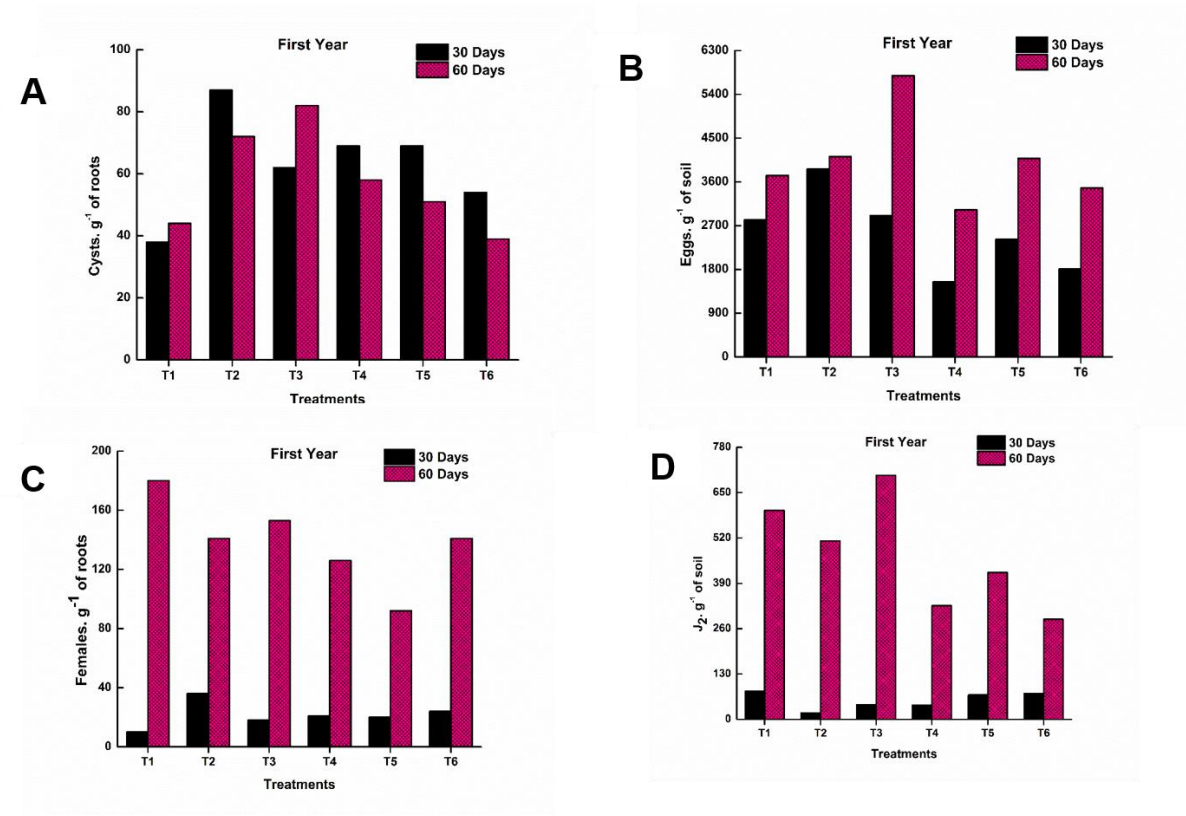

Figure 1. The effect of bioproducts on the number of (A) cysts/g of root (B), eggs/g of soil (C) female /g of root and (D) J_2 /g in soil after first (30days) and second (60days) collection in first year trial. Three bioproducts were applied *Trichoderma koningiopsis* (GF362) *Bacillus methylotrophicus* UFPEDA20 (Onix) and *Pochonia chlamydosporia* CEPA PC 10 in two consecutive years 2019 (year 1) and 2020 (year 2). The experiment was designed T1 Control, T2 Seed treatment (Rizotec ST), T3 Seed treatment (GF 362 ST), T4 Furrow treatment (GF 362), T5 Furrow treatment (Onix), T6, Seed treatment (Onix ST)

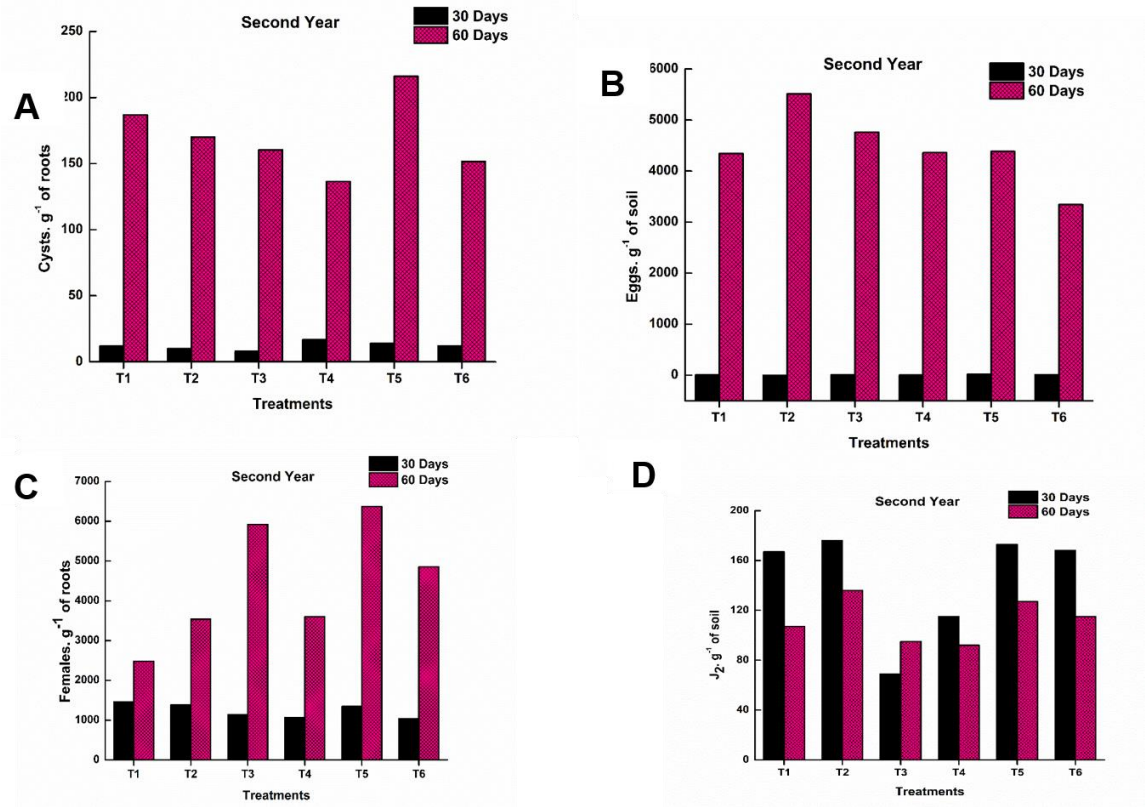

Figure 2. The effect of bioproducts on the number of (A) cysts/g of root (B), eggs/g of soil (C) female /g of root and (D) J2 /g in soil after first (30days) and second (60days) collection in second year trial. The bioproducts were applied bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2). Three bioproducts were applied *Trichoderma koningiopsis* (GF362) *Bacillus methylotrophicus* UFPEDA20 (Onix) and *Pochonia chlamydosporia* CEPA PC 10 in two consecutive years 2019 (year 1) and 2020 (year 2). The experiment was designed T1 Control, T2 Seed treatment (Rizotec ST), T3Seed treatment (GF 362 ST), T4 Furrow treatment (GF 362), T5 Furrow treatment (Onix), T6, Seed treatment (Onix ST)

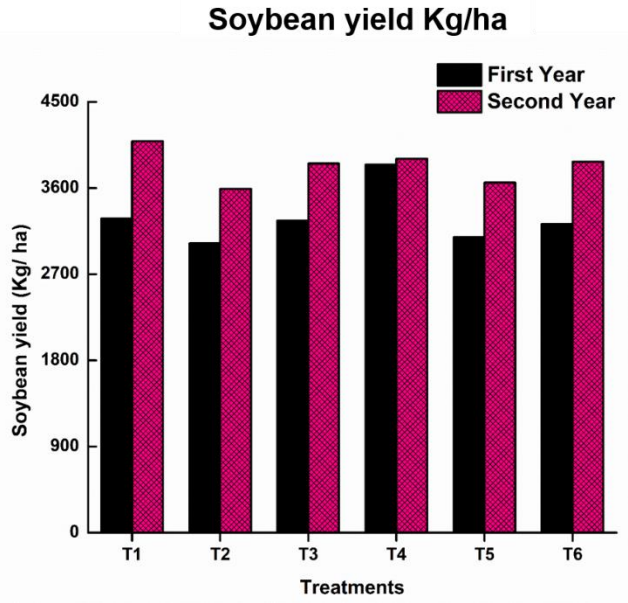

Figure 3. The effect of bioproducts on the soybean yield kg/ ha in first year and second year. The bioproducts were applied bioproducts *Trichoderma koningiopsis* (GF362) in furrow and *Bacillus methylotrophicus* UFPEDA20 (Onix) as seed treatment in two consecutive years 2019 (year 1) and 2020 (year 2). Three bioproducts were applied *Trichoderma koningiopsis* (GF362) *Bacillus methylotrophicus* UFPEDA20 (Onix) and *Pochonia chlamydosporia* CEPA PC 10 in two consecutive years 2019 (year 1) and 2020 (year 2). The experiment was designed T1 Control, T2 Seed treatment (Rizotec ST), T3 Seed treatment (GF 362 ST), T4 Furrow treatment (GF 362), T5 Furrow treatment (Onix), T6, Seed treatment (Onix ST)

ARTICLE 5- The effect of bioproducts on root-knot nematodes and rhizosphere microbiome profiling

Abstract

Plant-parasitic nematodes became notorious pest and causes massive damage to major agriculture crops worldwide. Plant beneficial microbes serve as a Biological control agent and has been exploited as antagonistic for decades against sedentary and migratory endoparasitic nematodes. However, the application of biological control products to control root-knot nematodes (RKN) in field conditions and their interaction with coffee rhizospheric microbiota remain unexplored in field conditions. The aim of this study was to analyze the effects of the biological control agent based bioproducts and chemical nematicides at different combination on root-knot nematodes and the microbial community profiling of the coffee plant rhizomicrobiome in a field trial. All the biological control products and chemical nematicide had not shown significant impact on root-knot nematodes control between control and treatments. The total number of number of galls^{-1} and eggs^{-1} and plant yield were assessed in two consecutive years, but no significant differences were observed between control and bioproducts applied treatments. Additionally, we evaluated the diversity and community composition of bacteria, fungi and eukaryotes in the rhizosphere soil of bioproducts treated plants and the dominant phyla in bacterial, fungal and community was, *Proteobacteria, Acidobacteria, Actinobacteria, Ascomycota, Mortierellomycota,* and *Ascomycota, Cercozoa* respectively in both consecutive years. Overall, no significant difference was observed in bacterial, fungal, and eukaryotic community's diversity in both years of data. The co-occurrence network unearthed that bacterial, fungal and eukaryotic species formed a complicated network structure in all bioproducts applied treatments. Our findings assist in comprehending the introduction of exogenous beneficial microbes into field conditions is unable to modulate the existing microbiota and no significant impact was exerted by them on the reshaping of the rhizosphere microbiome

Keywords**:** Plant-parasitic nematodes, Coffee, Biocontrol, Rhizosphere microbiome,

Introduction

Brazil is the largest producer of coffee (*Coffea arabica*) in the world, and Minas Gerais is the greatest producing state of coffee in Brazil (Baliza et al., 2013; Santos et al., 2018). One of the most devastating agronomic impediments to Brazil's coffee (*Coffea arabica*) production is the root-knot nematode (RKN) *Meloidogyne exigua*.

This nematode develops the distinctive rounded galls, which are primarily white to yellowish brown and then become dark brown as the root matures (Campos and Villain, 2005; Silva et al., 2010). *Meloidogyne exigua* Goeldi has infected more than 30% of Minas Gerais' coffee plantations. (Barros et al., 2014; Terra et al., 2018). Due of the perennial nature of coffee plants, this RKN causes less devastation than other species but severely decreases coffee production in heavily infected areas (Botelho et al., 2019a).

Even though there have been several attempts to control the root-knot nematode disease, no effective solutions have been established (Sharma et al., 2018). For the control of this nematode over the past couple of decades, a variety of strategies have been employed, including soil fumigants, resistant cultivars, crop rotation, soil flooding, and nematicide spraying (Sahebani and Gholamrezaee, 2021), have been utilized extensively to combat the root-knot nematode disease; But these extremely hazardous pesticides have also led to major issues with the ecosystem, human and animal health (Rajasekharan et al., 2020). The aforementioned circumstance reinforced the need for alternate approaches even more. (Montiel-Rozas et al., 2019).

Biological control will play a significant part in nematode control management in the future, due to their cost-effectiveness and environmental friendliness aspects (Zhou et al., 2021). Potentially effective solutions for controlling root knot nematodes include biological agents such as living organisms and their metabolites, anti-nematode activities by producing enzymes (chitinase, protease), volatile chemicals, and several other secondary metabolites. (Nguyen et al., 2021, 2018). Bacteria have gained increased attention in the development of commercially available biological agents to manage root-knot nematodes as a significant natural adversary of nematodes. Employing hostile microorganisms is one of the biological strategies that could be used to combat a root-knot nematode infestation (Mhatre et al., 2019), which could reduce nematode populations (Khan et al., 2016).

Microorganisms' ability to suppress root-knot nematodes through parasitism, competition for colonization sites and nutrients, or the development of antibiotics such lipopeptides and surfactin as well as other enzymes and toxins may indeed play an important

role (Lee and Kim, 2016). One of the most widely used microbial genera for biological control of pathogens and pests. *Bacillus spp. are known to produce a wide range of metabolites, antimicrobial compounds, enzymes, or toxins. These bacteria can also cause specific plant immune responses, which in turn inhibit the growth and function of cellular organisms like bacteria, fungi, insects, nematodes, and acellular organisms.* (Berini et al., 2018; Crickmore et al., n.d.; Gao et al., 2018; Goswami et al., 2016). One such biocontrol bacterium is *Bacillus subtilis*, which is currently sold as biocontrol and is supported by various studies for its significant potential in controlling a variety of diseases and nematodes infecting a wide range of host plant species (Rao et al., 2017). Additionally, field experiments were carried out to assess the efficacy of a variety of bio-nematicidal bacterial spore applications. It has been established that these lytic enzymes are detrimental to *M. javanica*. (Huang et al., 2016).

Researchers can now monitor the evolution of the plant-associated microbiome population under various conditions as well as the recruitment of bacteria to host tissues throughout time and space thanks to recent advancements in high-throughput sequencing (Dickey et al., 2020). These resources will be extremely helpful in developing bioinoculants developed with PGPB that perform well in a difficult agricultural environment, like the field, for more effective and resilient plant growth.

The objectives of this study were to: (1) to assess the potential of biological control products and nematicide to control *M. exigua*; (2) to investigate the impact of biological control products and nematicide used in combination on the yield of coffee plants grown in a commercial production system; and (3) to evaluate the effect of biological control products and nematicide on the microbiome profile of coffee plant.

Materials and Methods

Study of coffee farm and Soil and root sampling

The study was conducted in the coffee farm using two harvesting/ planation system. 1) zero coffee plantation: the coffee plants were cut after yield harvesting, 2) conventional coffee plantation: the coffee plants were remained uncut after yield harvesting. The sampling took place during the 2018–2021 season, which was a favorable season for the sampling. Soils and coffee roots were sampled in November and February of each year before and after products application from the coffee farms with susceptible *Coffea arabica* plants in the state of Minas Gerais, Brazil. The soil from the coffee plant was analyzed and found the *M. exigua*

population in the sampled soil. Gall symptoms and esterase pattern analysis allowed for the identification of *M. exigua*. The rhizosphere soil (1000 g) and 100 g of thin roots with galls collected from the coffee plants and brought to the lab for further analysis. Each soil sample was taken between 0 and 20 cm into the soil depth, beneath the aerial canopy of the plants. 100 g of roots and 1000 g of soil were taken from 10 randomly selected plants that were 5m away from each other. On each sampling date, samples were taken from the same plants. Before usage, soils were kept at 8-10°C for a maximum of three days.

Extraction of M. exigua eggs and second-stage juveniles (J2) from the coffee root and rhizospheric soil

Coffee roots containing galls were isolated from field-collected soil and chopped into 0.5 cm-long pieces for the extraction of eggs according to the described protocol of (Hussey, 1973). To separate RKN eggs, root portions were mixed for 60 seconds with 200 mL of a 0.5% bleach solution. To get rid of big particles, the soil was sieved. Using the (Botelho et al., 2019b) method, 100 g of soil was used to extract *M. exigua* J_2. The inverted optical microscope was used for the estimation of total number of eggs and J_2s per g of root and soil (density) respectively.

Soil microbial community

DNA extraction and quantification

Total DNA extraction was performed from the plant rhizosphere (control) and inoculated bioproducts plant rhizosphere using the DNeasy Power Soil DNA Isolation kit (MoBio, 12888), which has been adopted for microbial surveys. For each treatment, 0.25 g of the total soil were used for DNA extraction. DNA was quantified by using Nano-drop and stored at - 80 °C until further processing**.**

Amplicon library preparation, PCR analysis and high-throughput sequencing

The generation of PE sequencing reads of 16S, 18S rRNA gene and ITS2 summarized in the below subsection. The 16S V4–V5, (bacteria and archaea), 18S V4-V5 (eukarya) and ITS2 (fungal amplicon) examples are provided here. PCRs and library preparation were as described in (Comeau et al., 2017), with a change to Phusion Plus polymerase and maximum of 25 cycles for the PCRs. Pooled libraries were then sequenced on an Illumina MiSeq using

a V3 chemistry kit in paired-end 2x300bp mode (Integrated Microbiome Resource (IMR), The Langille Lab, Dalhousie University, Canada)

Sequencing data processing

QIIME 2 version 2019.765 was used to process the raw sequences (Bolyen et al., 2019) based on Microbiome Helper's operational guidelines (Comeau et al., 2017). Cutadapt was used to remove primers (Martin, 2011) and joined with the use of the QIIME 2 VSEARCH (Rognes et al., 2016) join-pairs plugin. Stitched reads were then quality filtered using the quality-filter plugin and reads were denoised using Deblur to produce amplicon sequence variants (OTUs) (Amir et al., 2017).

Statistical analyses

R version 3.5.3 was used to conduct the statistical analysis (Rproject.org). The experiment was carried out using a completely random block design. Community level differences in alpha- and beta-diversity were analyzed using QIIME 2 version 2019.765. For alpha-diversity, we calculated the Observed richness (number of OTUs/sample) and the Shannon diversity index. For beta-diversity, between-sample differences were assessed visually through principal coordinates analyses (PCoA) based on UniFrac distances. First, the metadata and non-rarefied feature tables were converted into a phyloseq object (version 1.29.0). Prior to differential and network analysis, taxa with a prevalence lower than 5% (i.e. taxa with a non-zero count in less than 5% of the samples) were trimmed. NetCoMi (Network Construction and Analysis for Microbiome Data) was used to construct microbial association networks (Peschel et al., 2021). First, taxa abundance data was filtered to the 150 most abundant taxa on each sample. SparCC, a technique for inferring correlations from compositional data that assumes the true correlation network is “sparse”, was applied in the network construction step. When sparsified associations were converted into dissimilarities, the handling of negative associations was done using a "signed" method. A 0.5 threshold was employed and the fast-greedy technique was used to infer node clusters. Based on the Eigenvector centrality characteristics and taking into account the nodes with the highest centrality values, the hub node identification for each estimated network was accomplished.

Results

The major goals of the current research are to investigate the influence of exogenous biological control agents on (1) indigenous microbiota (2) microfauna and (3) their biological control efficacy against cysts nematodes in field conditions. A total of three bioproducts and one chemical nematicide were applied using spray strategies: Rugby (chemical) Quality, Rizos (*Bacillus subtilis* UFPEDA 764) and Onix (*Bacillus methylotrophicus UFPEDA 20*) to control root-knot nematodes in field conditions.

Significant Bioproducts effects on microbiome diversity and plant-microbe interactions (16Sr RNA)

Native bacterial community diversity in both Rubgy and Quality + Rizos + Onix treatments were higher than in control soil except Rugby+Biologico in the first year trial, though significant differences between the control and all treatments in the first and second years were not observed (Fig. 1). The second consecutive year trial reduced the native bacterial community diversity in all treatments as compared to control and there were no significant differences were recorded between the treatments and control (Fig. 1).

Significant Bioproducts effects on microbiome diversity and plant-microbe interactions (ITS2)

Native fungal community diversity in both Rubgy and Quality + Rizos + Onix treatments were higher than in control soil except Rugby+Biologico in the first year trial, though significant differences between the control and all treatments in the first year were not observed (Fig. 2). The second consecutive year trial reduced the native bacterial community diversity in all treatments as compared to control and there were no significant differences were recorded between the treatments and control (Fig. 2).

Bioproducts effects on microbiome diversity and plant-microbe interactions (I8S)

The shannon diversity of indigenous eukaryotes communities observed high in treatments applied Rugby and Quality_Rizos_Onix in relation to control in the first year (Fig. 3) only the Rugby_Biologico treatment reduced the Shannon diversity of indigenous eukaryotes communities. Interestingly, all three treatments reduced the Shannon diversity of indigenous

eukaryotes communities as compared to control in second year trial. The significant differences were nit observed between the treatments and control in both year (Fig. 3).

Bacterial community composition and taxonomic distribution under different bioproducts application 1st year

The bacterial community`s taxonomy was assessed at the phylum and genus level. The total of 436 OTUs were recovered, following chimera removal and resampling. The dominated bacterial profile of *Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, Planctomycetota, Bacteroidota, Nitrospirota Gemmatimonadetes, Crenarchaeota* and *Myxococcota,* and were observed accross all treatmnts (Control, Quality_Rizos_Onix, Rugby, Rugby_Biologico). All observed phyla's relative abundance varied between the treatment and control groups.

The average relative abundance of *Proteobacteria* followed by *Actinobacteria,* was recorded the highest compared to other phyla in all treatments (Fig. 4). The average relative abundance of *Proteobacteria* was slightly higher in Rugby_Biologico (41%), followed by control (39%), Rugby (35%) and Quality_Rizos_Onix (33%) (Fig. 4A). For genus, the most dominated genus of *Chujaibacter, JG30−KF−AS9, Vicinamibacteraceae, Nocardioides, Streptomyces, Mycobacterium,* SC−I−84, Subgroup_13, 67−14 and Bradyrhizobium were recorded in all treatments (Fig. 4B). The highly relative abundance genus of *Chujaibacter*, *JG30−KF−AS9*, were found the most prevalent in the bacterial communities in all treatments comparatively other genus. Consequently, the relative abundance of genus *Chujaibacter* was recorded the highest in Rugby_Biologico (14%), followed by control (12%), Rugby (7%) and Quality_Rizos_Onix (7%) (Fig. 4B). The Rugby_Biologico treatment showed impact on bacterial genus composition and taxonomy when compared to control and other treatments. The Venn diagram showed 199 common OTUs by various biologic control products and 96.3% of all reads were characteristic of the shared OTUs (Fig. 4C).

Bacterial community composition and taxonomic distribution under different bioproducts application 2nd year

The taxonomy of the bacterial community was evaluated at the phylum and genus levels. Following chimera removal and resampling, a total of 436 OTUs were found (Fig. 4D). *Proteobacteria* predominated the bacterial composition across all treatments, followed by *Acidobacteria, Actinobacteria, Chloroflexi, Planctomycetota, Bacteroidota,*

Gemmatimonadota, *Crenarchaeota*, WPS−2 and *Myxococcota* (Fig. 4D). All treatments were slightly dominated by *Proteobacteria* as compared to control but the highest relative abundance of *Proteobacteria* (39%) was found in Quality_Rizos_Onix in relation to control and all treatments. Following the same pattern, the phyla *Acidobacteriota* was dominated in all tretaments Quality_Rizos_Onix (37%), Rugby (29%) and Rugby_Biologico (32%), as compared to control (29%), (Fig. 4D). The dominated bacterial genus profile was *Subgroup_2, Chujaibacter Subgroup_13, G30−KF−AS9, Acidibacter, Acidothermus, Burkholderia−Caballeronia−Paraburkholderia, SC−I−84* and *Nevskia* in all treatmnts. The *Subgroup_2* was recorded as the highly abundant genus in Quality_Rizos_Onix (9%), Rugby (10%) and Rugby_Biologico (13%), as compared to control (5%), (Fig. 4E). Total of 174 common OTUs by different biologic control products were displayed in the Venn diagram, and 94.5% of all reads were indicative of the shared OTUs (Fig. 4F).

Fungal community composition and taxonomic distribution under different bioproducts application 1st year

The taxonomy of the fungal community was evaluated at the phylum and genus levels. Following chimera removal and resampling, a total of 219 OTUs were found (Fig. 5).

The taxonomic profiling of fungi revealed the dominated phyla such as *Ascomycota, Mortierellomycota, Basidiomycota, Mucoromycota, Chytridiomycota, Chlorophyta* in all treatments. Interestingly, the relative abundance of *Ascomycota,* was recorded was higher in Quality_Rizos_Onix (96%) than Rugby and Rugby_Biologico. The relative abundance of *Ascomycota* was observed less in Rugby (90%) and Rugby_Biologico (90%) treatments. The relative abundance of *Mortierellomycota* found higher in Rugby_Biologico (9%) than all treatments and control (Figure. 5A). Figure 5B illustrates the fungal community structure in soil samples at the genus level. The major genera in the all treatments were determined and the dominated genera were *Fusarium, Mortierella, Trichoderma., Trichocladium, Cladorrhinum, Ilyonectria, Purpureocillium, Sarocladium, Metarhizium, Acremonium* and *Mycochlamys* (Fig. 5B) The relative abundance of Fusarium (39%) in control, while in Quality_Rizos_Onix (35%), Rugby (34%), and Rugby_Biologico (41%) respectively. It demonstrates that control has a higher *Fusarium* genre than treatments, except Rugby_Biologico, hence the bioproducts controlled the population of *Fusarium* in coffee fields (Fig. 5B). Total of 123 common OTUs by different biologic control products were displayed in the Venn diagram, and 97.3% of all reads were indicative of the shared OTUs

(Fig. 5C).

Fungal community composition and taxonomic distribution under different bioproducts application 2nd year

The trial was repeated the following year, and the fungi's taxonomy was assessed at the phylum and genus levels. After chimera removal and resampling, 219 OTUs in total were obtained (Fig. 5C). The dominated phyla were *Ascomycota*, *Mortierellomycota*, *Mucoromycota***,** *Basidiomycota*, *Chytridiomycota*, *Chlorophyta*, Glomeromycota, *Kickxellomycota, Calcarisporiellomycota*, and *Cercozoa*, in fungal community (Fig. 5C). The average relative abundanace *Ascomycota* was recorded higher in control (66%), Rugby (73%), Quality_Rizos_Onix (50%). and Rugby_Biologico (81%). Figure 5D in the second year trial, the genus-level fungal community structure in all treatments depicted in Figure. *Mortierella, Fusarium, Penicillium, Trichoderma, Polyschema, Gongronella, Fusidium, Purpureocillium, Campylospora*, and *Conlarium* were the dominant genera in all treatments. The relative abundance of Fusarium (23%) in control, Rugby (23%), Quality_Rizos_Onix (40%). and Rugby_Biologico (16%) (Fig. 5D) respectively, shows that control has a less *Fusarium* genre than treatments. Total of 87 common OTUs by different biologic control products were displayed in the Venn diagram, and 94.5% of all reads were indicative of the shared OTUs (Fig. 5E).

Eukaryotes community composition and taxonomic distribution under different bioproducts application 1st year

The trial was repeated the following year, and the Eukaryotes's taxonomy was assessed at the phylum and genus levels. After chimera removal and resampling, 233 OTUs in total were obtained (Fig. 6A). The dominent relative abundance of Phyla, *Ascomycota, Cercozoa, Mucoromycota, Ciliophora, Gracilipodida, Amoebozoa, Nematozoa, Rotifera, Phragmoplastophyta,* and *Vertebrata,* in all treatments. The dominant phylum in the control, GF362, and Onix was *Ascomycota*. The average relative abundance of *Ascomycota* in control (86%), Quality_Rizos_Onix (79%), Rugby (83%) and in Rugby_Biologico (83%) observed (Fig. 6A). The results show that biological based control products and chemicals unable to influence the phyla profile. (Fig. 6A). The relative abundance of the dominant genera were *Trichoderma, Sordariomycetes, Chaetomium, Mortierella, Sarocladium, Heteromita, Absidia, Pleosporales, Rhabditida*, and *Aspergillus* detected in all treatments (Fig. 6B). The major

genera *Trichoderma,* detected *(*9%) in the Rugby_Biologico followed by control (5%), quality_Rizos_Onix (4%) and Rugby (3%) (Fig. 6B). Total of 120 common OTUs by different biologic control products were displayed in the Venn diagram, and 97.3% of all reads were indicative of the shared OTUs (Fig. 6C).

Eukaryotes community composition and taxonomic distribution under different bioproducts application 2nd year

The trial was repeated the following year, and the Eukaryotes's taxonomy was assessed at the phylum and genus levels (Fig. 6).

After chimera removal and resampling, 233 OTUs in total were obtained. The taxonomic analysis further revealed that the Eukaryote's taxonomy differed markedly among the different treatments (Figure 6) It was observed that, on average, more than 40% of observed Eukaryotes's taxonomy were affiliated to ten eukaryotic phyla, including *Cercozoa, Ascomycota, Amoebozoa, Ciliophora, Rotifera, Mucoromycota, Nematozoa, Gracilipodida, Chytridiomycota and* LKM15 (Fig. 6D). However, the relative abundance of these taxa varied among different treatments (Fig. 6D). For example, the highly dominated phyla was *Cercozoa,* followed by *Ascomycota* and *Amoebozoa,* in all treatements, which wsas account its relative abundance 29 %, 32%, 31% and 28% in control Quality_Rizos_Onix, Rugby and Rugby_Biologico respectively. *Ascomycota* and *Amoebozoa* are the second highly abundant phyla observed in all treatments (Fig. 6D). The relative abundance of the dominant genera were *Tracheleuglypha, Adinetida, Euamoebida,* Cercomonadidae, *Dictyamoeba, Leptomyxida, Copromyxa, Arcellinida, Glissomonadida* and *Gymnophrys* detected in all treatments (Fig. 6E). The relative abundance which was observed in all treatments was *Tracheleuglypha* comparatively higher than other genus. The average relative abundance of *Polymyxa* 7%, 12% and 8% and 5% in in control Quality_Rizos_Onix, Rugby and iRugby_Biologico respectively (Fig. 6D). Total of 145 common OTUs by different biologic control products were displayed in the Venn diagram, and 98.1% of all reads were indicative of the shared OTUs (Fig. 6F).

Beta diversity PCoA plots

The principal coordinate analysis (PCoA) was applied to assess the bacterial, eukaryotic and fungal community composition across various treatments at two consecutive

years.

PCoA results showed that bacterial, eukaryotic and fungal communities were dispersed and showed dispersed patterns from one another control, Rugby_Biologico, Rugby and Quality_Rizos_Onix treatments in the first and second year. This difference indicates that the application of biological control couldn't induce any changes in the microbial community profile in the first year. PCoA results showed that bacterial communities were dispersed and showed scattered patterns in all treatments in both first and second year analyses (Fig. 7). The PCo1 and PCo2 contributed 42.87% and 21.23% towards variations in bacterial community respectively. PCoA results showed that eukaryotic communities were concentrated and showed clustered patterns in in all treatments in first year, (Figur.7 A). Interestingly, in the second year all microbial Eukaryotic communities showed scattered patterns and observed dispersed pattern from one another in control, Rugby_Biologico, Rugby and Quality_Rizos_Onix treatments (Figur.7. A). This difference indicates that the application of biological control influenced the Eukaryotic community's composition and induced changes in the microbial community profile in the second year. The PCo1 and PCo2 contributed 44.40% and 18.99% towards variations in Eukaryotic community respectively (Fig.7 B).

The fungal communities in the host rhizosphere clustered closer in the first year (2019). It's interesting to note that in the second year, all fungal communities in the control, Rugby_Biologico, Rugby, and Quality_Rizos_Onix treatments displayed dispersion patterns and detected scattered patterns from one another. (Fig.7 C.) This distinction shows that the application of biological control altered the profile of the fungal community in the second year and affected the composition of fungal communities, where The PCo1 and PCo2 contributed 52.57% and 15.79% towards variations in the fungal community respectively.

Co-occurrence Network Analysis 16S

To further understand how the preexisting microbiota, interact and respond to exogenously introduced fungal, bacterial species and chemical nematicides over the course of two years, a co-occurrence network model was investigated. we applied three biological control products (fungal and bacterial) and one chemical nematicides at different combinations in coffee fields with aimed to control Root-knot nematode (*Meloidogyne exigua*) and investigated the topological characteristics of the microbial communities in the plant rhizosphere compartment for both years. The network of all treatments was observed and having different characteristics (Table 1). The analysis showed that the network

topological measures, such as the Clustering coefficient, Modularity, Positive edge, Edge density, and Natural connectivity in both the first and second year, varied between the control and inoculated treatments. The control had the fewest and same number of components in both consecutive years (2019-2020), whereas other treatments had networks with varying and higher number of components than control. The clustering coefficient (average degree of connection of a node) was recorded the highest in treatment (Quality_Rizos_Onix) (0.550) in the second year, as compared to control and other treatments and lowest in lowest in the treatment **(**Rugby_Biologico) (0.09) in the first year. The modularity values (strength of division of a network into modules) was observed the highest and varied in all treatments in relation to control (Table.1) when taking into account the 16S microbial community. The networks for control in the both first and second year had higher edge densities (percentage of possible links between nodes) comparatively all treatments. In general, all treatments resulted in dense networks, with taxa clustering in different ways (Fig 8 and Table. 1).

Co-occurrence Network Analysis ITS2

A co-occurrence network pattern was studied to investigate how the existed microbiota interact and respond to exogenously inoculated fungal and bacterial species and chemical nematicides in two consecutive years. we applied three biological control products (fungal and bacterial) and one chemical nematicides at different combinations in coffee fields with aimed to control Root-knot nematode (*Meloidogyne exigua*) and investigated the topological characteristics of the microbial communities in the plant rhizosphere compartment for both years. The network of all treatments was observed and have different characteristics (Table 2). The network of all treatments was observed and having different characteristics (Table 2). The analysis showed that the network topological measures, such as the Clustering coefficient, Modularity, Positive edge, Edge density, and Natural connectivity in both the first and second year, varied between the control and inoculated treatments. The control had the fewest and same number of components in both consecutive years (2019-2020), whereas other treatments had networks with varying and higher number of components than control. The clustering coefficient (average degree of connection of a node) was recorded the highest in treatment (Chemical) (0.689) in the first year, as compared to control and other treatments and lowest in all other treatments than control. The modularity values (strength of division of a network into modules) was observed the highest in (Rugby_Biologico) (0.575) and (Quality_Rizos_Onix) (0.604) varied in all treatments in relation to control (Table.1) when

taking into account the 16S microbial community. The networks for control in the both first and second year had higher edge densities (percentage of possible links between nodes) comparatively all treatments. In general, all treatments resulted in dense networks, with taxa clustering in different ways (Fig. 9 and Table. 2).

Discussion

The potential of microorganisms that exhibit promising results in laboratory and greenhouse experiments to overcome obstacles and maintain their features when applied in the field is crucial for the successful deployment of bacterial inoculants to increase plant productivity(Sessitsch et al., 2019). Understanding the permanence and effectiveness of the introduced biological products and its impact on the plant-associated microbiota as well as their compatibility with farming practices are crucially important. Many Plant growth promoting microbes have been reported as successfully controlling the plant disease (Bhattacharyya and Jha, 2012; Mehmood et al., 2021; Pathania et al., 2020) including root knot nematodes (Antil et al., 2022; Cetintas et al., 2018). But it is also indisputable that many Plant growth promoting microbes have been reported unsuccessful in controlling the plant disease, when treated both in combination (consortia) or alone. For instance, *P chlororaphis* PCL1391 and *P. fluorescens* WCS365 (Bardas et al., 2009) and *P. chlororaphis* PCL1391 and *P. fluorescens* P3/pME6863, (Molina et al., 2003) as a as a consortia completely failed to perform biocontrol activities plants disease. According to the research of the (Hubbard, 1983)*Trichoderma hamatum* for Pythium failed to control plant diseases.

In this study we analyzed the effects of the biological control agent based bioproducts and chemical nematicides at different combination (Table.1) on the controlled of root-knot nematodes and their impact on plant rhizosphere microbiome profile in a field trial. All the biological control products and chemical nematicide had not shown significant impact on root-knot nematodes control and didn't show significant differences between control and treatments. Although PGPB-containing inoculants have been employed to plant crops for more than 120 years (Arora et al., 2017). Especially in field condition, some of the bioproducts show inconsistent efficacy in terms of control of parasites due multitudes of biotic and abiotic factors (Mitter et al., 2021; Naamala and Smith, 2020). The inconsistent effectiveness of microbial inoculants in the field may be explained by a variety of unavoidable biotic and abiotic conditions that can put the existence of the introduced microorganism (French et al., 2021b). Additionally, the inoculants may interfere with other agricultural chemicals used on crops or may be rendered useless by native plant-associated

microbiota that persists at low and ineffective levels in the soil (French et al., 2021a).

Understanding the persistence and efficiency of such products thus requires testing microbial products in field conditions, evaluating their compatibility with other products, and investigating their effects on the indigenous plant microbiome. The failure of bioproducts in filed conditions extensively studied. To establish a successful long-term interaction with the existing microbiota and host plant, and enhance their persistency and efficacy in field conditions, the following points must be considered to address. 1) Abundant and diverse soil microorganisms in the soil/plant ecosystem, distribution of microorganisms, and 2) appropriate formulations that should guard against desiccation and other detrimental circumstances for microbial cells. 3) the recipient environment's biotic and abiotic circumstances, as well as the organism's physiological activity and compatibility with the target plant 4). The colonizing microbes must be able to tolerate various environmental factors, such as pH or oxygen availability, in order to establish themselves. These factors include their ability to recognize and metabolize these chemicals. 5) The inoculant microorganisms must contend with a microbiota that is extremely diverse and plant genotype diversity (De Roy et al., 2013; French et al., 2021b; Sessitsch et al., 2019; Thilakarathna and Raizada, 2017).

The success of inoculations with rhizobacteria that promote plant growth depends critically on the efficiency of root colonization and rhizosphere competence. We assessed at the abundance, diversity, and community structure of the bacterial, fungal, and eukaryotic communities related to the rhizosphere when applied bioproducts and nematicide at different concentration in the rhizosphere of coffee plants in field conditions. Application of various bioproducts and nematicides at different concentration had no impact on the soil's microbial communities' structure and composition. (Chowdhury et al., 2013a) reported in their study that on application of *B. amyloliquefaciens* FZB42 strain into the soil did not significantly alter the microbial populations in the lettuce rhizosphere. A multitude of reasons favor this circumstances but one of them might be that exogenously applied biocontrol agent unable to show durable impact on rhizosphere microbiota. Various research studies investigated that the plant microbiota go through a transient modification after the application if biological control agents into the soil(Buddrus-Schiemann et al., 2010; Chen et al., 2013; Chowdhury et al., 2013a; Scherwinski et al., 2007; Yin et al., 2013).

A research by (Matos et al., 2005) found a negative correlation between *Pseudomonas aeruginosa* invisibility and rhizosphere community richness. This research may indicate that

native microorganisms, as opposed to 2P24 or CPF10, were more adapted to the environmental conditions existing in the cucumber rhizosphere (Girlanda et al., 2001).

Sampling time might be one of the significant factors which impact on the bacterial community and density, because the seasonal changes, and the soil sampling right after the products application or giving a long gap could lead different results. According to the (Chowdhury et al., 2013b; Correa et al., 2009), the biological control agents *B. amyloliquefaciens* FZB42 and *B. amyloliquefaciens* BNM122 was not significantly affected the rhizospheric microbial communities. These inconsistencies could be explained by variations in the types and strains of bacteria utilized, inoculum concentration, applications to soil directly, etc. The need of identifying the conditions under which each experiment is carried out before drawing conclusions is crucially important. We sampled rhizospheric soil for microbial profiling after 90 days of products application, the observation of no impact of microbial community's richness and densities might be attributed to the sampling timing.

Previous studies with lettuce exposed to inoculants like *Serratia plymuthica* 3Re4-18, *P. trivialis* 3Re2-7, *P. fluorescens* L13-6-12, and *P. jessenii* RU47 only had a minor and transitory impact on the native rhizosphere community, but they did demonstrate field site-specific and seasonal changes (Adesina et al., 2009; Correa et al., 2009; Grosch et al., 2012).

plant roots have a significant impact on the rhizosphere via rhizodeposition and offering favorable ecological niches for the growth and microbial activities (Bais et al., 2006). According to one theory, bacterial populations in the rhizosphere oscillate in a wave-like pattern along root axes. Accordingly, bacterial communities momentarily benefit from the nutrients released by younger roots and wave-like fluctuations in bacterial cell numbers. These phenomena can be explained by starvation-induced cell death and lysis followed by cell divisions in remaining and thus viable populations as promoted by the release of nutrients from dead and decaying cells (Semenov et al., 1999).

Conclusion

In conclusion, the biological control agents based bioproducts and chemical nematicide investigated in filed conditions in this study. We did not observe any promising bioproduct and chemical nematicide to biocontrol root knot nematodes in filed conditions. The diversity of the microbial community in the soil of the coffee rhizosphere are unaltered by the application of bioproducts. However, the co-occurrence network showed that in the second year (2020), bacterial species established a complex network structure, indicating a

more persistent microbial interaction in the rhizosphere of coffee plants. We believe that this information has the potential to enhance the development of microbial products for agricultural application, taking into account the demand for inoculants whose effects are enduring and repeatable in the field.

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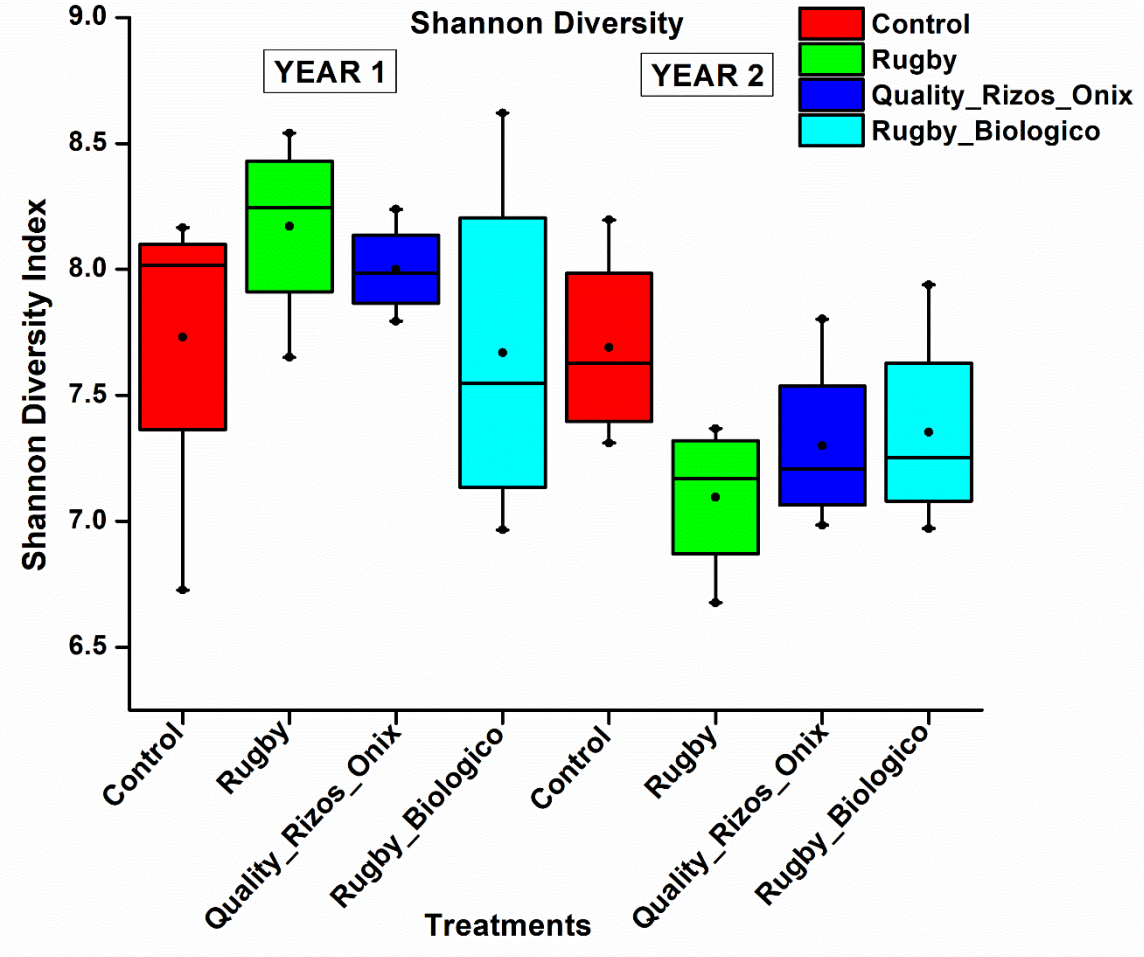

Figure.1. Alpha diversity indexes of bacterial community (16S rRNA) gene bar code for the microbiome of all four replicates under different treatments in two consecutive years (2019 and 2020). Changes in the Shannon diversity index were observed under different applied product combinations: BIOLOGICO (three different products applied simulaneaously *Trichoderma asperellum* URM5911 (Quality), *Bacillus subtilis* UFPEDA 764 (Rizos) and *B. methylotrophicus* UFPEDA20 (Onix), the chemical nematicide cadusaphos (Rugby) or a tandem application of BIOLOGICO and RUGBY in two consecutive years 2019-2020.

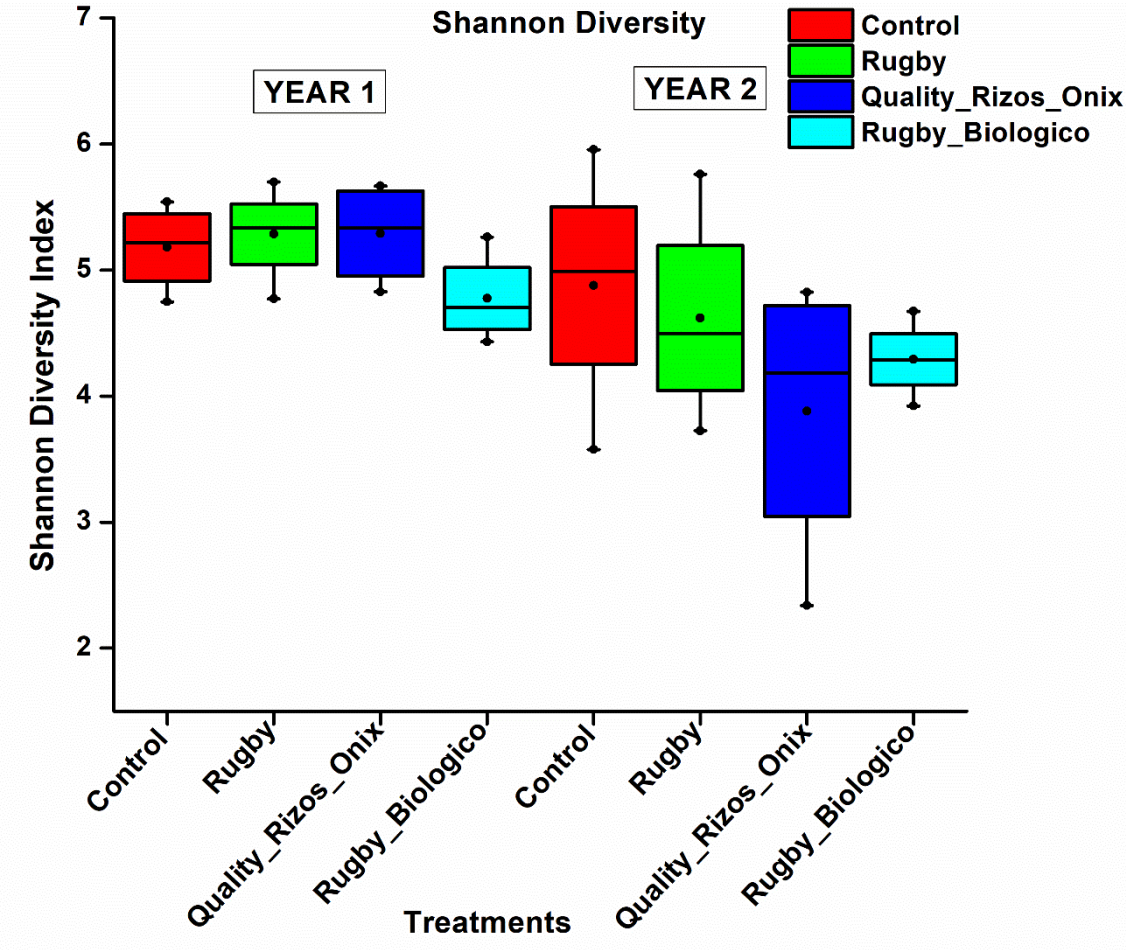

Figure.2. Alpha diversity indexes of the fungal community (ITS2) gene bar code for the microbiome of all four replicates under different treatments in two consecutive years (2019 and 2020). Changes in the Shannon diversity index were observed under different applied product combinations: BIOLOGICO (three different products applied simulaneaously *Trichoderma asperellum* URM5911 (Quality), *Bacillus subtilis* UFPEDA 764 (Rizos) and *B. methylotrophicus* UFPEDA20 (Onix), the chemical nematicide cadusaphos (Rugby) or a tandem application of BIOLOGICO and RUGBY in two consecutive years 2019-2020.

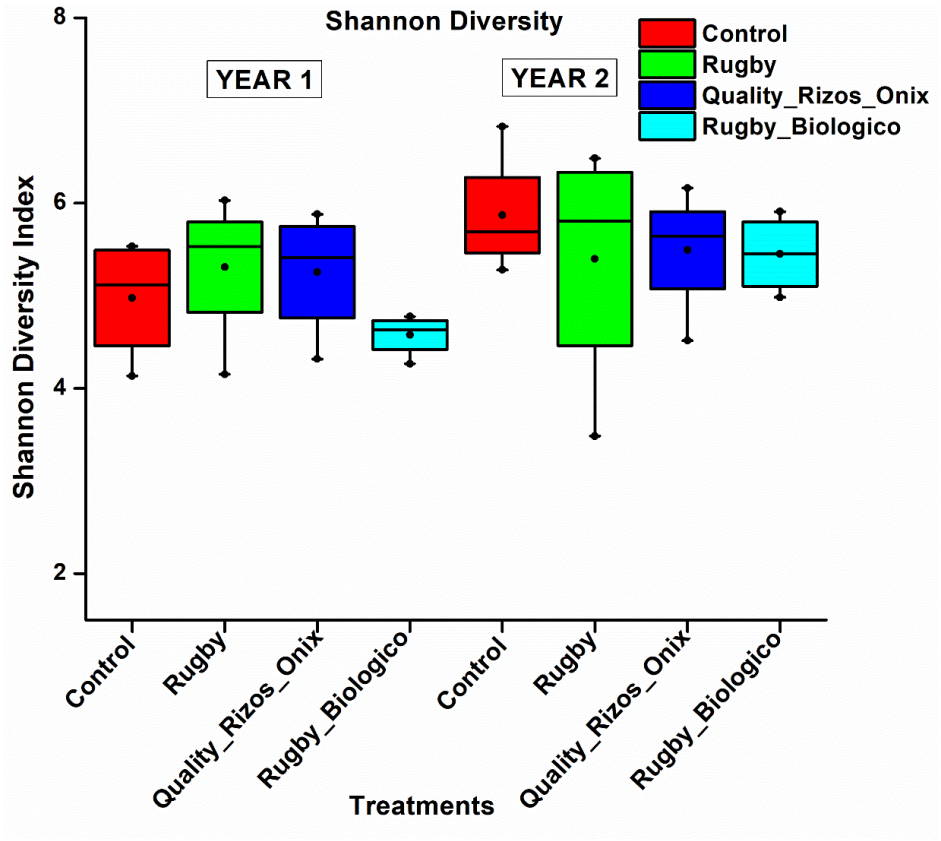

Figure.3. Alpha diversity indexes of eukaryotic community (18S) gene bar code for the microbiome of all seven replicates under different treatments in two consecutive years (2019 and 2020). Changes in the Shannon diversity index were observed under different applied product combinations: BIOLOGICO (three different products applied simulaneaously *Trichoderma asperellum* URM5911 (Quality), *Bacillus subtilis* UFPEDA 764 (Rizos) and *B. methylotrophicus* UFPEDA20 (Onix), the chemical nematicide cadusaphos (Rugby) or a tandem application of BIOLOGICO and RUGBY in two consecutive years 2019-2020.

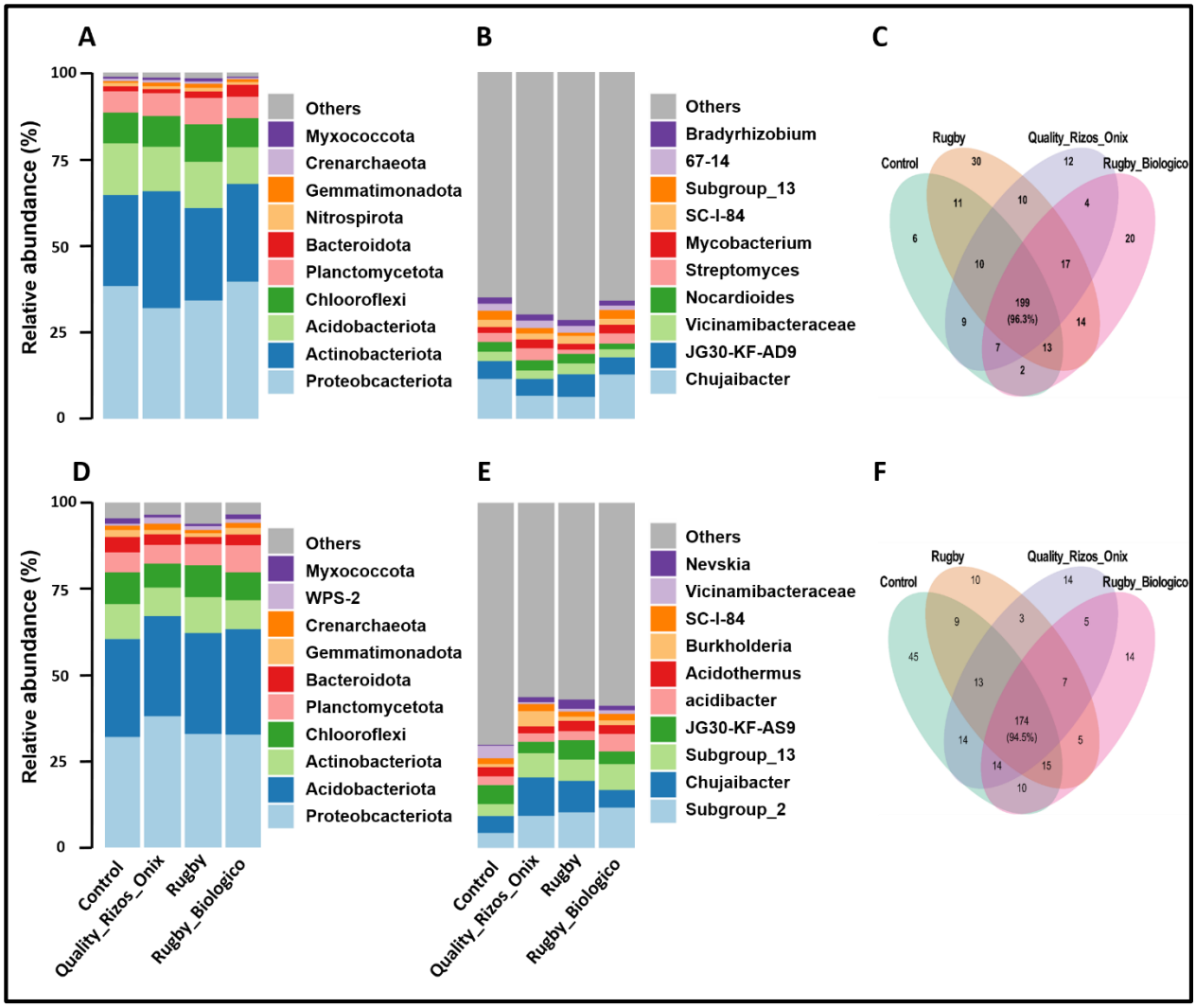

Figure 4. Distribution of bacterial community (16S rRNA) in coffee plant rhizosphere samples. Different product combination treatments were applied: BIOLOGICO (three different products applied simulaneaously *Trichoderma asperellum* URM5911 (Quality), *Bacillus subtilis* UFPEDA 764 (Rizos) and *B. methylotrophicus* UFPEDA20 (Onix), the chemical nematicide cadusaphos (Rugby) or a tandem application of BIOLOGICO and RUGBY in two consecutive years 2019-2020. The treatments resulted in changes in relative abundance and a comparison of the bacterial community (16S rRNA) over the course of two years, (A, B, C) (2019) and (D, E, F) (2020). The figure depicts the relative abundance of bacterial community (16S rRNA) at the Phylum and genus level. Bacterial community (16S rRNA) composition is shown in bar charts (only significant taxa greater than 1% are shown). The first 10 bacterial (phyla and genera) that are displayed at the bottom of the bars are referenced in the legend.

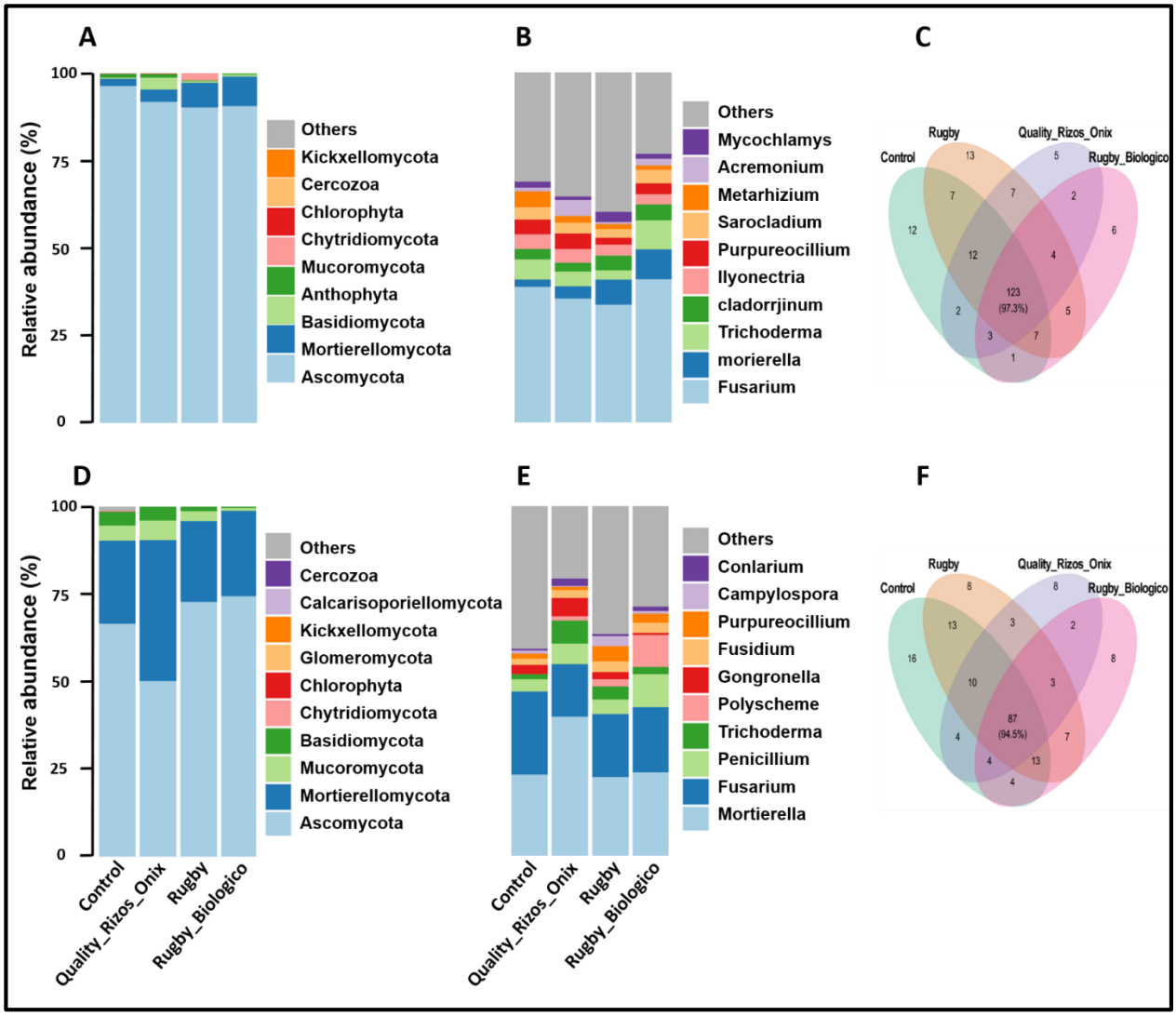

Figure 5. Distribution of fungal community (ITS2) in coffee plant rhizosphere samples. Different product combination treatments were applied: BIOLOGICO (three different products applied simulaneaously *Trichoderma asperellum* URM5911 (Quality), *Bacillus subtilis* UFPEDA 764 (Rizos) and *B. methylotrophicus* UFPEDA20 (Onix), the chemical nematicide cadusaphos (Rugby) or a tandem application of BIOLOGICO and RUGBY in two consecutive years 2019-2020. The treatments resulted in changes in relative abundance and a comparison of the fungal community (ITS2) over the course of two years, (A, B, C) (2019) and (D, E, F) (2020). The figure depicts the relative abundance of fungal community (ITS2) at the Phylum and genus level. Fungal community composition is shown in bar charts (only significant taxa greater than 1% are shown). The first 10 fungal (phyla and genera) that are displayed at the bottom of the bars are referenced in the legend.

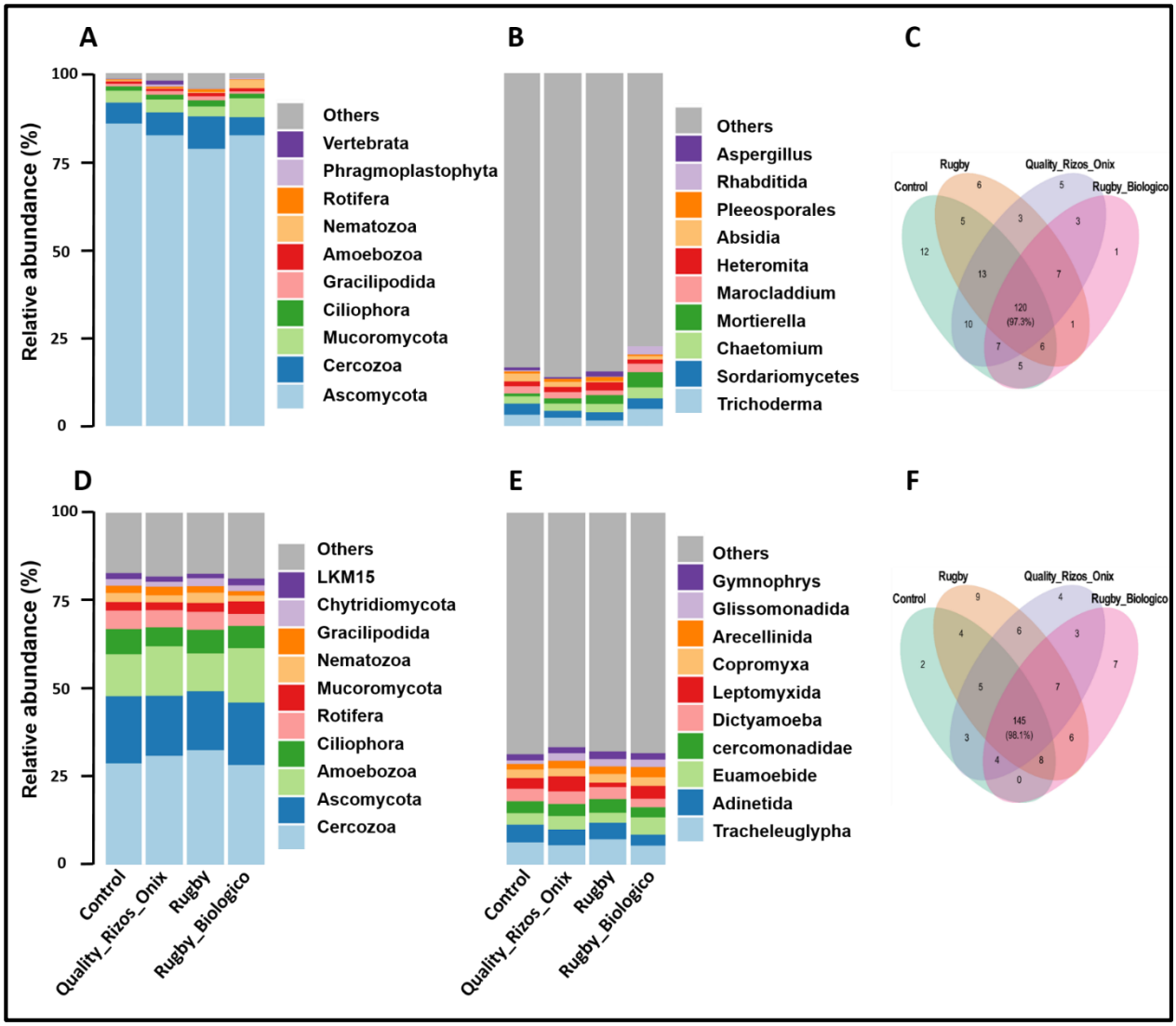

Figure 6. Distribution of eukaryotic community (18S) in coffee plant rhizosphere. Different product combination treatments were applied: BIOLOGICO (three different products applied simulaneaously *Trichoderma asperellum* URM5911 (Quality), *Bacillus subtilis* UFPEDA 764 (Rizos) and *B. methylotrophicus* UFPEDA20 (Onix), the chemical nematicide cadusaphos (Rugby) or a tandem application of BIOLOGICO and RUGBY in two consecutive years 2019-2020. The treatments resulted in changes in relative abundance and a comparison of the eukaryotic community (18S) over the course of two years, (A, B, C) (2019) and (D, E, F) (2020). The figure depicts the relative abundance of eukaryotic community (18S) at the Phylum and genus level. eukaryotic community composition is shown in bar charts (only significant taxa greater than 1% are shown). The first 10 eukaryotic (phyla and genera) that are displayed at the bottom of the bars are referenced in the legend.

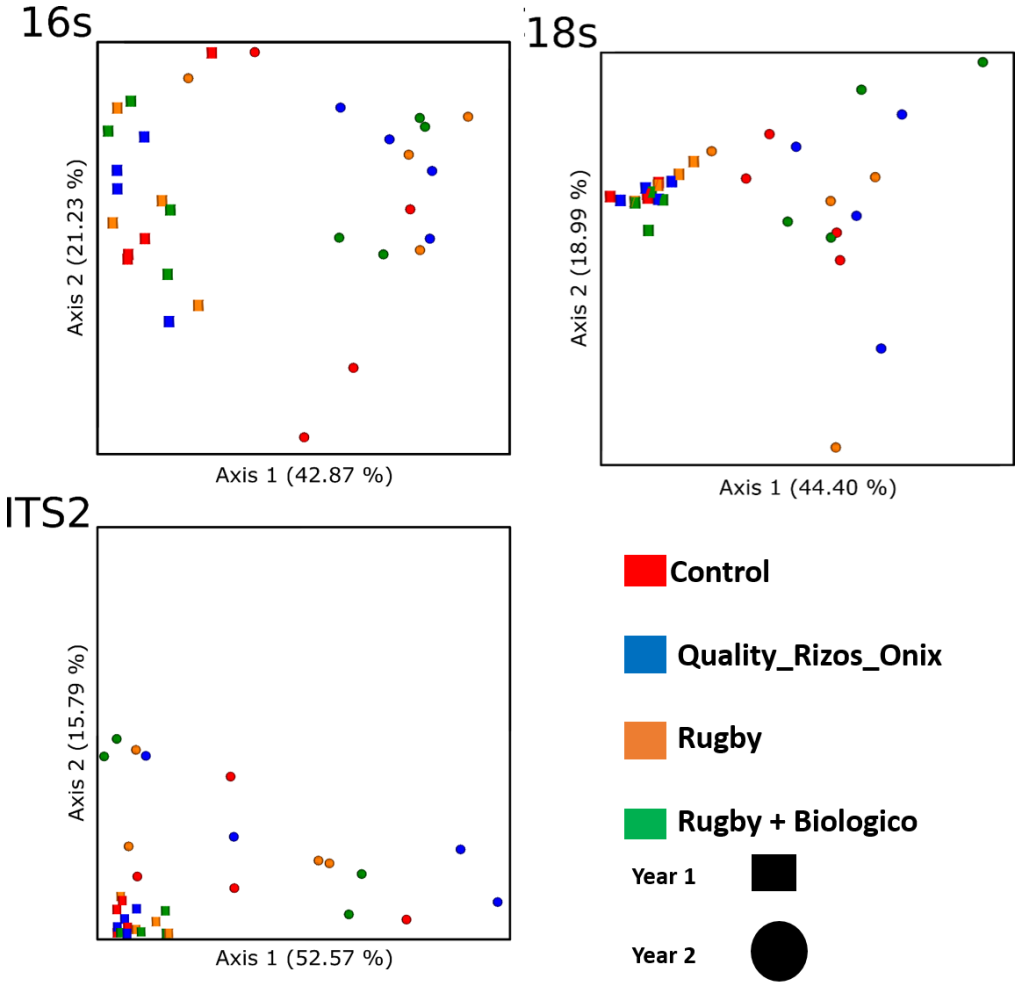

Figure. 7. Principal component Analysis (PCoA) of Bacterial, eukaryotic and fungal community structures and composition. Different product combination treatments were applied: BIOLOGICO (three different products applied simulaneaously *Trichoderma asperellum* URM5911 (Quality), *Bacillus subtilis* UFPEDA 764 (Rizos) and *B. methylotrophicus* UFPEDA20 (Onix), the chemical nematicide cadusaphos (Rugby) or a tandem application of BIOLOGICO and RUGBY in two consecutive years 2019-2020. Differences in bacterial community composition. (A). PCoA of the bacterial community 16S rRNA composition across all treatments (B) PCoA of the eukaryotic community (18S) composition across all treatments (C) PCoA of the fungal community (ITS) composition across all treatments.

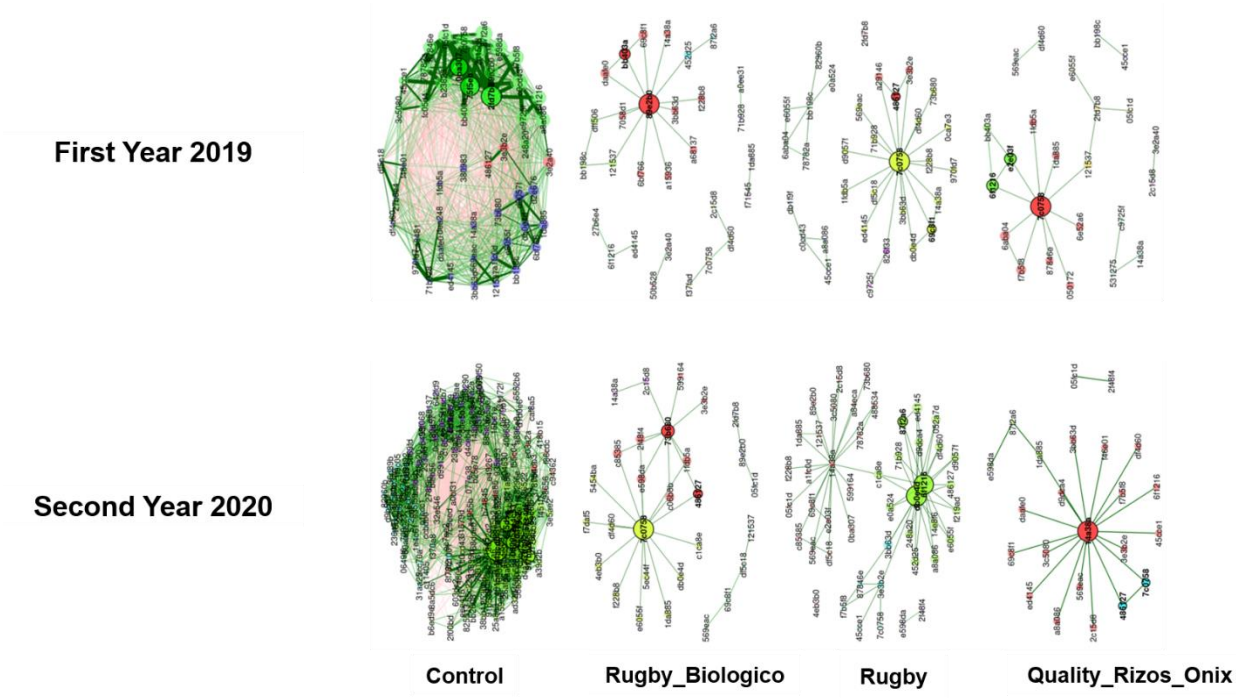

Figure 9: ITS2 microbial network of the rhizosphere of coffee (*Coffea arabica*) plants with different treatments in two consecutive years (2019 and 2020). Different product combination treatments were applied: BIOLOGICO (three different products applied simulaneaously *Trichoderma asperellum* URM5911 (Quality), *Bacillus subtilis* UFPEDA 764 (Rizos) and *B. methylotrophicus* UFPEDA20 (Onix), the chemical nematicide cadusaphos (Rugby) or a tandem application of BIOLOGICO and RUGBY in two consecutive years 2019-2020. Nodes represent OTUS, with size reflecting the OTUs influence in the community (eigenvector centrality), and color corresponding to the cluster to which the OTUS belongs. Edges between nodes represent correlations between the nodes they connect, with edge width and shade indicating the correlation magnitude, and green and red colors indicating positive and negative correlations, respectively. See Table. 1 for full names of OTUS and corresponding taxa.

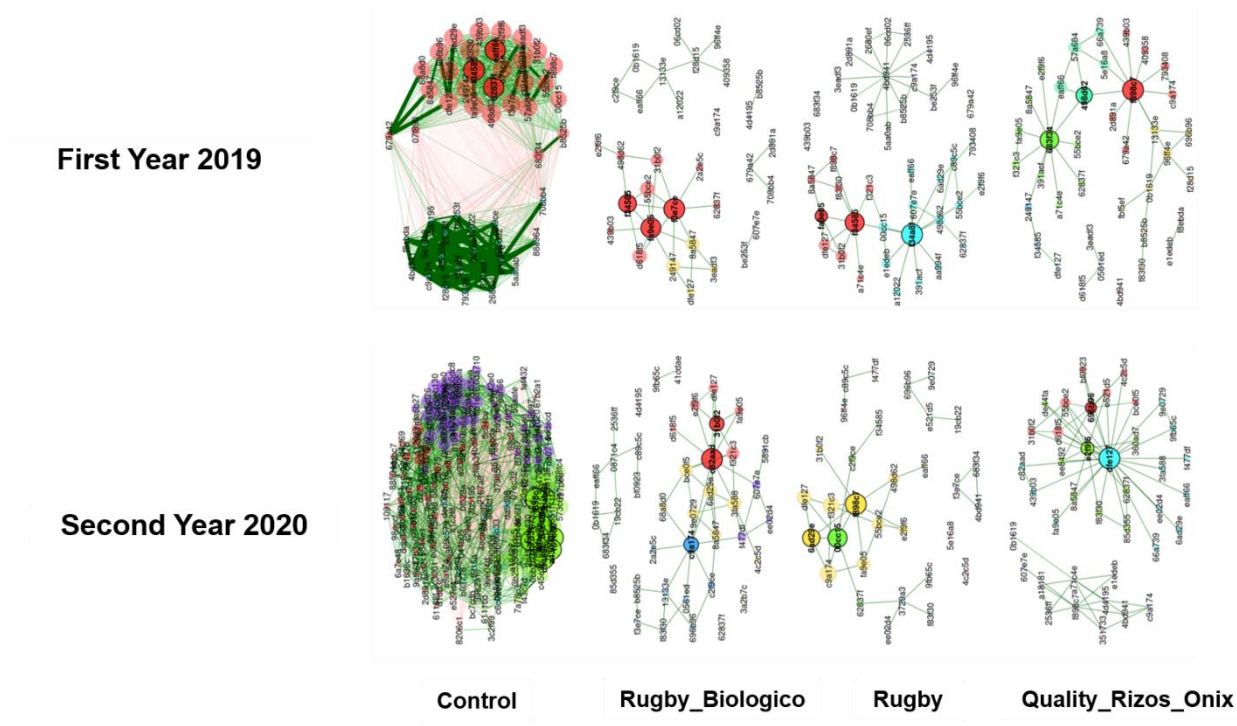

Figure 8: 16S microbial network of the rhizosphere of coffee (*Coffea arabica*) plants with different treatments in two consecutive years (2019 and 2020). Different product combination treatments were applied: BIOLOGICO (three different products applied simulaneaously *Trichoderma asperellum* URM5911 (Quality), *Bacillus subtilis* UFPEDA 764 (Rizos) and *B. methylotrophicus* UFPEDA20 (Onix), the chemical nematicide cadusaphos (Rugby) or a tandem application of BIOLOGICO and RUGBY in two consecutive years 2019-2020. Nodes represent OTUS, with size reflecting the OTUs influence in the community (eigenvector centrality), and color corresponding to the cluster to which the OTUS belongs. Edges between nodes represent correlations between the nodes they connect, with edge width and shade indicating the correlation magnitude, and green and red colors indicating positive and negative correlations, respectively. See Table. 2 for full names of OTUS and corresponding taxa.

Table 1: Global network properties of microbial communities (Bacterial) of the rhizosphere of coffee (*Coffea arabica*) plants with different treatments in two consecutive years (2019 and 2020). Different product combination treatments were applied: BIOLOGICO (three different products applied simulaneaously *Trichoderma asperellum* URM5911 (Quality), *Bacillus subtilis* UFPEDA 764 (Rizos) and *B. methylotrophicus* UFPEDA20 (Onix), the chemical nematicide cadusaphos (Rugby) or a tandem application of BIOLOGICO and RUGBY in two consecutive years 2019-2020.

Marker	**Marker Global network properties**	**Year 1**				**Year 2**			
16S		Control	Rugby_Biologico	Rugby	Quality_Rizos_Onix	Control	Rugby_Biologico	Rugby	Quality_Rizos_Onix
	Components[1]	1.00	21.00	11.00	15.00	1.00	11.00	23.00	12.00
	Clustering coefficient[2]	0.46	0.09	0.34	0.24	0.51	0.21	0.20	0.55
	Modularity[3]	0.07	0.51	0.58	0.59	0.11	0.52	0.54	0.43
	Positive edge %[4]	49.83	37.75	39.20	31.81	49.69	32.59	54.68	48.92
	Edge density[5]	0.24	0.03	0.04	0.03	0.27	0.05	0.03	0.05
	Natural connectivity[6]	0.07	0.04	0.05	0.04	0.09	0.05	0.04	0.08

Table 2: Global network properties of microbial communities (Fungal) of the rhizosphere of coffee (*Coffea arabica*) plants with different treatments in two consecutive years (2019 and 2020). Different product combination treatments were applied: BIOLOGICO (three different products applied simulaneaously *Trichoderma asperellum* URM5911 (Quality), *Bacillus subtilis* UFPEDA 764 (Rizos) and *B. methylotrophicus* UFPEDA20 (Onix), the chemical nematicide cadusaphos (Rugby) or a tandem application of BIOLOGICO and RUGBY in two consecutive years 2019-2020.

Marker	**Marker Global network properties**	**Year 1**				**Year 2**			
ITS2		Control	Rugby_Biologico	Rugby	Quality_Rizos_Onix	Control	Rugby_Biologico	Rugby	Quality_Rizos_Onix
	Components[1]	1.00	37.00	21.00	31.00	1.00	24.00	5.00	29.00
	Clustering coefficient[2]	0.46	0.24	0.68	0.48	0.55	0.00	0.36	0.40
	Modularity[3]	0.10	0.57	0.54	0.60	0.10	0.44	0.52	0.27
	Positive edge %[4]	50.16	34.66	38.04	40.00	51.66	41.33	43.94	44.89
	Edge density[5]	0.24	0.02	0.02	0.01	0.29	0.02	0.05	0.01
	Natural connectivity[6]	0.08	0.03	0.04	0.03	0.12	0.04	0.07	0.04

Supplementary materials

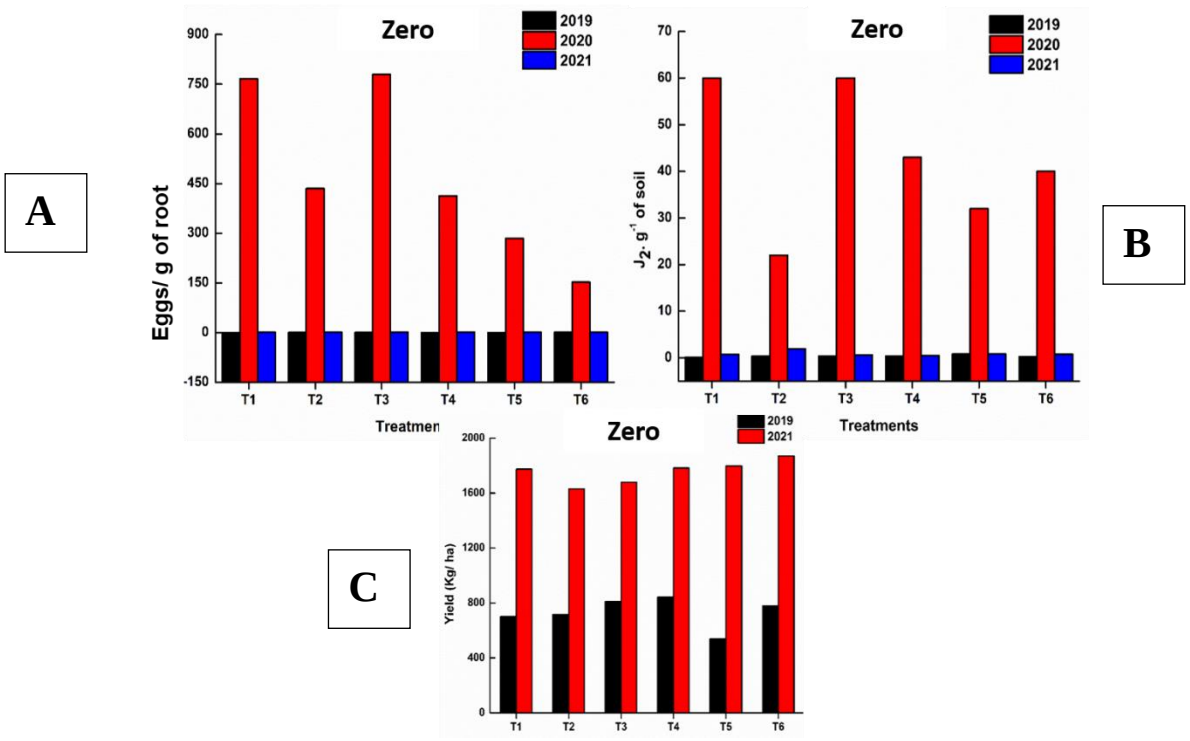

Figure 1. The effect of bioproducts on (A) the number of eggs/g of root (B), J2 /g in soil and (C) yield kg/ ha in first year (2019) second year (2020 and third year (2021) year trials. Three bioproducts were applied Quality, Rizos and Onix in three consecutive years 2019 (year 1) and 2020 (year 2) and 2021 (year 3). The experiment was designed T1 (Control), T2 (Rugby), T3 (Quality + Rizos + Onix), T4 (Quality + Rizos + Onix), T5 (Quality + Rizos + Onix), T6 (Rugby)

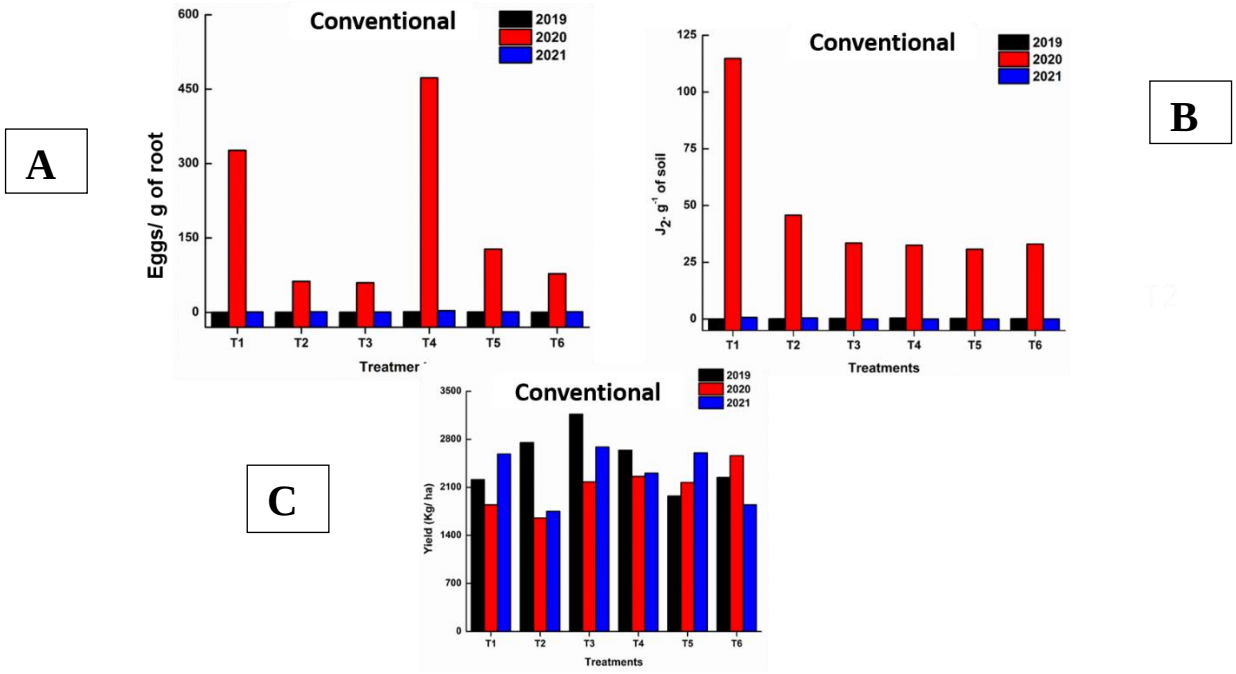

Figure 2. The effect of bioproducts on (A) the number of eggs/g of root (B), J2 /g in soil and (C) yield kg/ ha in first year (2019) second year (2020 and third year (2021) year trials. Three bioproducts were applied Quality, Rizos and Onix in three consecutive years 2019 (year 1) and 2020 (year 2) and 2021 (year 3). The experiment was designed T1 (Control), T2 (Rugby), T3 (Quality + Rizos + Onix), T4 (Quality + Rizos + Onix), T5 (Quality + Rizos + Onix), T6 (Rugby)