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Effects of *Bacillus thuringiensis* RZ2MS9 on soybean (*Glycine max*) considering volatile organic compounds, plant development in the field, and soil microbiome diversity

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To my parents

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ABSTRACT

OLIVEIRA, H. G. **Effects of *Bacillus thuringiensis* RZ2MS9 on soybean (*Glycine max*) considering volatile organic compounds, plant development in the field, and soil microbiome diversity.** 2021. 92 p. Tese (Doutorado em Ciências) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2021.

Soil microorganisms are of great importance to the sustainability of ecosystems. In agriculture, the use of plant growth-promoting rhizobacteria as bioinoculants represents an environmentally friendly alternative to improve crop yield since those bacteria are generally helpful for plant growth and protection. Among those rhizobacteria, *Bacillus thuringiensis* (*Bt*) is known as an important agent of insect control. However, it has been recently described also as a plant-growth promoter that is able to colonize plants and improve host fitness. Thus, the present research evaluated different aspects of the interaction between soybean and a plant growth-promoting rhizobacterium *Bt* RZ2MS9, through both *in vitro* and field assays. In chapter 1, a general introduction was presented. Chapter 2 discussed a field study in which soybean seeds were inoculated with *Bt* RZ2MS9 and subsequently analyzed for plant development and productivity among treatments with or without chemical treatment and inoculation. The biocontrol potential of *Bt* RZ2MS9 was evaluated by recording stink bug (*Piezodorus guildinii* and *Euchistus heros*) population density and severity of phytopathogenic fungi occurrence through a diagrammatic scale of leaf damage. We also analyzed the *in vitro* effects of the bacterial strain on the feeding habits of *P. guildinii*. The strain *Bt* RZ2MS9 was able to improve soybean plant height and slightly increase crop yield. Groups that received chemical treatment showed an increased dry mass, green stem, and leaf retention. Dry mass did not differ between inoculated and non-inoculated groups. Infestation by stink bug *P. guildinii* showed a significant difference among treatments, but that was not observed for *E. heros*. There was a significant effect of the inoculation on the severity of leaf damage caused by phytopathogenic fungi (mainly *Cercospora kikuchii*). A feeding choice study with *P. guildinii* showed preference to material without the strain. Chapter 3 presents an analysis of the volatile compounds released by *Bt* RZ2MS9, the effects of these compounds on the germination and development of soybean seeds, and on the phytopathogenic fungal mycelia growth. Volatile compounds produced by the strain had a significant effect on the soybean germination process but not on the growth of the fungi studied. Chapter 4 presents the analyzes of the effects of *Bt* RZ2MS9 inoculation on the microbiome community through metagenomics of soil samples collected before, during, and after soybean crop management applying both the strain assessed here and *Bradyrhizobium japonicum* in co-inoculation. Co-inoculation decreased plant lodging significantly but had no effect on crop productivity. The microbiome analysis showed that inoculation did not interfere in the diversity of microorganisms during the crop cycle, but treatments inoculated with *Bt* RZ2MS9 had a lower Chao1 diversity index 15 days after the crop cycle. The assessment of features from new PGPRs is of great importance to the development of new strategies to improve sustainability in crop management. Further experimentation on this strain in different crops, and field conditions, besides analysis of its metabolites and genes associated with them, may unravel new biotechnology alternatives for integrated crop management.

Keywords: Soybean. Bioinoculant. *Bacillus*. Microbiome diversity.

RESUMO

OLIVEIRA, H. G. **Efeitos de *Bacillus thuringiensis* RZ2MS9 em soja (*Glycine max*) considerando compostos orgânicos voláteis, desenvolvimento da planta no campo e diversidade do microbioma do solo** 2021. 92 p. Tese (Doutorado em Ciências) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2021.

O uso de rizobactérias promotoras de crescimento em plantas (RPCP) na agricultura como bioinoculantes configura uma alternativa para melhoria da produtividade agrícola, uma vez que tais bactérias tem demonstrado efeitos positivos no desenvolvimento de diversas culturas. Dentre tais rizobactérias, *Bacillus thuringiensis* (*Bt*) é um importante agente microbiano no controle de insetos, mas que também apresenta propriedades como promotor do crescimento de plantas. Nesta tese foi realizado um estudo de diferentes aspectos da interação entre a soja e a linhagem de RPCP *Bt* RZ2MS9, por meio de experimentos tanto in vitro quanto em condições de campo. O capítulo 1 traz uma introdução geral sobre o tema estudado nesta tese. No capítulo 2 foi apresentado um estudo sobre o desenvolvimento e a produtividade de soja inoculada com *Bt* RZ2MS9, com e sem intervenção química em condições de campo. Também foi avaliado o potencial dessa linhagem como agente de controle de percevejos praga da soja, *E. heros* e *P. guildinii*, por meio de levantamento populacional em campo e teste de preferência alimentar. A inoculação com a linhagem *Bt* RZ2MS9 aumentou a altura de plantas de soja, embora não tenha havido aumento da massa seca. Foi observado um discreto aumento de produtividade. Os grupos que receberam tratamento químico apresentaram maior massa seca em relação aos grupos não tratados. Na comparação entre grupos inoculados ou não, não houve diferença em relação à massa seca. A incidência de *P. guildinii* variou entre os tratamentos, o que não ocorreu para *E. heros*. Tratamentos inoculados apresentaram menor incidência de danos foliares causados por fungos fitopatogênicos, especialmente *Cercospora kikuchii*. Em estudo de preferência alimentar com *P. guildinii*, foi evidenciada a preferência por material não tratado com a linhagem. O capítulo 3 investiga o papel dos compostos orgânicos voláteis (COVs) emitidos por *Bt* RZ2MS9, no processo de germinação e no desenvolvimento da soja, além da avaliação de potencial para biocontrole de fungos fitopatogênicos por meio de testes in vitro. Também foi realizado um estudo inicial dos compostos produzidos, com a proposição de compostos candidatos. Houve efeito negativo na germinação de sementes expostas a COVs produzidos pelo *Bt* RZ2MS9, mas não houve inibição do crescimento dos fungos estudados. No quarto capítulo, foi estudado o efeito da linhagem *Bt* RZ2MS9 em co-inoculação com *Bradyrhizobium japonicum* sobre a cultura da soja e sobre a diversidade microbiana do solo. A co-inoculação diminuiu a incidência de tombamento das plantas, mas não apesentou efeito sobre a produtividade. A comunidade microbiana do solo não variou em diversidade devido às inoculaçãoes durante o ciclo de plantio de soja, mas a diversidade medida pelo índice Chao1 foi menor nos tratamentos inoculados com *Bt* RZ2MS9 15 dias após a colheita. A avaliação de linhagens de RPCP é de grande importância para o desenvolvimento de estratégias voltadas para uma agricultura sustentável. A continuação de estudos sobre esta linhagem em diferentes condições ambientais, bem como a análise dos metabólitos produzidos e os genes associados a eles pode trazer novas abordagem biotecnológicas para manejo integrado e sustentável de culturas.

Palavras-chave: Soja. Bioinoculante. *Bacillus*. Diversidade microbiana.

SUMMARY

1. INTRODUCTION

Soybean is a major crop worldwide due to its use as a food resource for protein and vegetable oil and also for being a feedstock source for biodiesel production (LIU et al., 2019). In countries of the Eastern World, soybean is an essential part of the diet, being consumed in various forms of foods, such as bean curd (tofu), soy milk, sprout, soy paste/sauce. It also has a vast industrial application since it may be used to produce lubricants, toner ink, cosmetics, among other uses (LEE et al., 2015).

Brazil was the largest soybean producer and exporter in the world in 2020 (EMBRAPA, 2021). According to Brazilian Company of Agriculture (EMBRAPA, 2021), soybean culture in 2019/2020 produced 124,845 million tons of grain, with an average yield of 3,379 kg/hectare. Thus, technologies that may improve the sustainable culture of this crop are of great interest to modern society.

The use of plant growth-promoting rhizobacteria (PGPR) as bioinoculants has a great potential to improve crop yields without harming the environment since those bacteria may regulate physiologic and molecular processes in plants by enhancing nutrient accessibility and plant resistance (BERGKEMPER et al., 2016; EL-ESAWI et al., 2018). This practice has been expanding worldwide with positive results for several different crops (SANTOS et al., 2019; PEREIRA et al., 2020; LANA et al., 2020).

The PGPR are a heterogeneous group of soil bacteria that improve soil properties, plant growth, and environmental conditions living in the rhizosphere or associated with plant roots (RATZ et al., 2017). These bacteria can perform several biological functions and affect plant growth in two different ways, directly or indirectly (JIANG et al., 2019). Directly occurs through PGPR providing the plant either with compounds biosynthesized by the bacteria, like hormones, or improving nutrient solubilization in the soil, like inorganic or organically bound phosphates, and therefore promoting plant growth (BENEDUZI et al., 2012). The indirect promotion of plant growth occurs when PGPR are able to decrease or prevent the harmful effects of one or more plant pathogens or pests and thus increase crop yield (MIRANSARI, 2014). Some PGPRs that are typically found in the rhizosphere are *Bacillus, Pseudomonas, Pantaoe agglomerans, Acinetobacter,* and *Paenibacillus*, showing different levels of influence on plant growth by improving nutrient acquisition, biocontrol activities, and developing resistance in plants (CHAUDHARY et al., 2021).

Recently, several studies have shown the significant role of volatile organic compounds (VOCs) emitted by plant rhizobacteria in plant growth promotion (RATH et al., 2018; JIANG et al., 2019). Even though the first study of VOCs produced by PGPR and the effects on plant growth is from 1969 (COOK; STALL, 1969), this field has become popular only after a study from 2003 discovered the promotion of growth in *Arabidopsis* by bacterial VOCs (RYU et al., 2003). In another study, a strain of *Bacillus megaterium*, named XTBG-34, showed the ability to increase the fresh weight of *Arabidopsis* by the emission of volatile compounds (ZOU et al., 2010). Another study on the genera *Bacillus* by Gutiérrez-Luna (2010) found that VOCs emitted by *Bacillus* sp. can change root architecture, directly affecting nutrient uptake by the plant, thus promoting plant growth (GUTIÉRREZ-LUNA et al., 2010).

Bacteria from the genus *Bacillus* are abundant in soil, being all Gram-positive, rod-shaped, and capable of forming endospores. Several structurally diverse secondary metabolites can be produced by members of this genus, with a wide variety of antibiotic activity (SUMI et al., 2014). Bacterial antagonists inhibit plant pathogens by excretion of antifungal metabolites, like antibiotics, toxins, and bio-surfactants (KAI et al., 2007), and thus *Bacillus* species are studied for their potential use as bioinoculants that both improve yields and help in phytopathogenic fungi control (CHAURASIA et al., 2005; TOYOTA, 2015). Some *B. subtilis* strains showed less efficiency against phytopathogen species of the genus *Fusarium*, compared to other *Bacillus* species due to the mode of action exerted or the type of antifungal metabolite produced (FRANCISCO et al., 2016). Therefore, many studies have been conducted to search for more efficient *Bacillus* strains and uncover strategies to induce secondary metabolites production (SAINI, 2012; OLA et al., 2013).

A member of the genus *Bacillus* that has been extensively studied is *B. thuringiensis* (*Bt*), mainly due to its entomopathogenic properties. *Bt* is a gram-positive aerobic or facultative spore-forming bacterium with the ability to form spores and resist adverse environmental conditions (AZIZOGLU, 2019). However, studies focused on the biostimulation, or biofertilizer potential of *Bt* are scarce, even though it has been reported that *Bt* can successfully colonize endophytically a variety of plant species, like cabbage, cotton, soybean, and rice (PRAÇA et al., 2012; ARGÔLO-FILHO et al., 2014; Qi et al., 2016).

Besides that, it is well known that *Bt* has a remarkable entomopathogenic action against some insect orders due to the production of Insecticidal Crystal Proteins (ICPs) during sporulation (HANNAY et al., 1955; BONIS et al., 2021). The crystals are coded by cry genes that produce their typical composition of δ-endotoxins that are part of a large family of Cry/Cyt

toxins currently classified according to their sequence similarity (HOFTE et al., 1989; CRICKMORE et al., 1998). These toxins act in the midgut of insect larvae, creating pores in the epithelial membrane that cause gut paralysis, systemic diffusion of bacterial spores, and ultimately killing insects from starvation or septicemia (ZHANG et al., 2017).

The first application of *Bt* as a commercial biopesticide dates from 1938, in France, with the product Sporine, which was applied for Lepidoptera control (SANCHIS, 2011). Further investigation on the *Bt* insecticidal activity and advances in molecular biology allowed the insertion of the *Bt* cry genes into the genome of several crop species, with these genetically engineered plants becoming resistant to the attack of various insect pests (OLIVEIRA et al., 2021).

This thesis further investigates the interaction of the PGPR *Bacillus thuringiensis* (*Bt*) RZ2MS9 and soybean (*Glycine max*). The strain was first isolated from the rhizosphere of the guarana tree (*Paullinia cupana* var. *sorbilis)*, an Amazon crop, and it has shown the ability to promote maize and soybean growth in screening tests performed to select PGPR microorganisms. Previous *in vitro* studies with this bacterium have shown plant growth-promoting features, such as the production of siderophores and plant hormones, phosphate solubilization, and also nitrogen fixation ability (BATISTA et al., 2018). The assays performed with *Bt* RZ2MS9 inoculation in soybean and maize (*Zea mays*) showed that it was able to improve plant height and dry mass of both plant species (BATISTA et al., 2018). A *draft* of its genome was sequenced by Batista et al. (2016), and a phylogenetic analysis placed *Bt* RZ2MS9 in the same clade of seven other bacteria, all been classified as *Bacillus thuringiensis* (BATISTA, 2017). The complete genome of this strain was obtained by Bonatelli et al. (2020), showing the presence of a single circular DNA molecule with 5,357,194 base pairs (bp), corresponding to 5,468 genes that are divided into 5,315 coding sequences and 153 non-coding sequences (42rRNAs, 106 tRNAs, and 5 non-coding RNAs (ncRNAs)). As for the classification of the strain here studied, the complete genome data was applied to a nucleotide identity analysis (NIA) along with a phylogenetic study considering more than 200 lineages belonging to the *Bacillus cereus* sensu latu, with the conclusion of the classification as *Bacillus thuringiensis* RZ2MS9 (BATISTA et al., 2021).

While PGPR can provide several positive services for the crop, it is likely that they will also influence the composition of the rhizosphere and internal microbiome in the environment (KUKLINSKY-SOBRAL et al., 2004). Soil microorganisms present quick responses to

environmental changes, with microbial communities showing changes in their activities that can be correlated to the physical and chemical dynamics of soil under the stress of heavy metal and pesticides (ASAD et al., 2017; CHAUDHARY et al., 2021). In order to better understand bacterial diversity in the environment, metagenomics is an approach that provides a global picture of the microbial community, with a comprehensive view of both cultivable and uncultivable microorganisms (CHAUDHARY et al., 2021). Considering the close interaction that can be observed between microorganisms and plants, there is great importance on the understanding of how the addition of PGPR species as bioinoculants influences the indigenous root bacterial community (KUKLINSKY-SOBRAL et al., 2004).

Metagenomics studies provide an efficient strategy to analyze community diversity and functional potential in an environment (SALAM et al., 2021). This approach is currently the most reliable to study structural and functional characteristics of the microbiome (SABALE et al., 2020) by using next-generation sequencing of the total genome in an environment and then applying extensive bioinformatics resources to reveal details of the microbial community (SALAM et al., 2021).

Thus, the present thesis shows a polyphasic and broad range investigation of different aspects of the interaction between the PGPR *B*t RZ2MS9 and soybean, considering seed germination and plant development both *in vitro* and field experiments, the potential of the strain for biocontrol of phytopathogenic fungi and soybean pests, and the impacts of the inoculation on the soil microbiome

1.1 Hypothesis

Considering the potential of *Bt* RZ2MS9 as a PGPR in the promotion of plant fitness through different pathways, the hypotheses tested were:

- The *Bt* strain RZ2MS9 benefits soybean crop development, acting alongside pesticide applications to improve plant vigor and boost plant resistance,
- Inoculation with *Bt* RZ2MS9 influences the interaction between soybean and stink bugs (*Euschistus heros* and *Piezodorus guildinii*) occurrence in the field,
- Volatile organic compounds released by *Bt* strain RZ2MS9 impact the germination process of soybean and seedling development,

- Volatile organic compounds from *Bt* RZ2MS9 have an antagonistic effect on the growth of the phytopathogenic fungi species: *Alternaria alternata, Cercospora kikuchii, Colletotrichum falcatum, Colletotrichium sublineolum, Colletotrichium truncatum, Curvularia luneta, Fusarium oxysporum, Rhizoctonia solani,* and *Sclerotinia sclerotiorum,*
- Co-inoculation of soybean with *Bt* RZ2MS9 and *Bradyrhizobium japonicum* synergistically improve crop traits,
- *Bt* RZ2MS9 interferes in the soil microorganism community when applied in the field.

1.2 Objectives

1.2.1 General Objective

Based on the hypothesis showed above, the general objective of this thesis was to investigate different aspects of the interaction between *B*t RZ2MS9 and soybean and its impacts on the environment in order to further verify the possible application of this strain as a bioinoculant that promotes plant growth and improves plant health through antagonistic effects on phytopathogenic fungi and insects that are harmful to soybean development.

1.2.2 Specific Objectives

To better understand the *Bt* RZ2MS9 inoculation effects and to answer the hypotheses proposed above, the following specific objectives were considered:

I. Examine the influence of *Bt* RZ2MS9 inoculation on the development of soybean in the field, in different chemical treatment strategies.

II. Evaluate the effect of *Bt* RZ2MS9 inoculation on soybean diseases and stink bug infestation.

III. Investigate possible effects of volatile organic compounds released by the *B*t. RZ2MS9 on the germination and initial development of soybean.

IV. Study the potential of *Bt* RZ2MS9 in plant growth-promotion as a co-inoculant with a commercial soybean bioinoculant composed of *Bradyrizhobium japonicum* and *Bradyrizhobium elkanii*.

V. Explore the inoculation impacts on the soil microbiome regarding changes in bacterial community diversity.

1.3 Structure of the thesis

This thesis is divided into one general introduction and three chapters written in the format of scientific manuscripts, each one focusing on one specific type of interaction between the *Bt* RZ2MS9 and soybean production.

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2. *Bacillus thuringiensis* RZ2MS9 inoculation effects on growth and yield of soybean (*Glycine max* (L.) Merril) and its role in biological control of stink bugs and phytopathogenic fungi at field conditions

ABSTRACT

Soybean (*Glycine max*) is an economically important food and oilseed crop, mainly due to its high protein and oil content. Soybean is also used for biodiesel production and several other industrial applications. The application of plant growth-promoting rhizobacteria can improve plant physiology, directly stimulating several plant traits or making nutrients more readily available for the plant. Rhizobacteria from the genus *Bacillus* are commonly studied for their plant growth-promoting features, with several commercial products based on species of this genus being used worldwide. Several strains of *Bacillus thuringiensis* (*Bt*) have been studied for their ability to positively interact with plants and also produce proteins that act as target-specific toxins to different agricultural pests. In this study, we have further evaluated the interaction between the strain *Bt* RZ2MS9 and soybean, considering plant development and biological control of members of the stink bug complex at field conditions with and without crop chemical management. Plants inoculated with this strain showed a higher length than non-inoculated plants, but there was no significant increase in productivity. Incidence of the stink bug *Piezodorus guildinii* was greater in the inoculated groups, but that was not observed for *Euchistus heros*. We could also observe a significant effect of the inoculation on the severity of leaf damage caused by phytopathogenic fungi (mainly *Cercospora kikuchii*). Experimentation *in vitro* showed that *P. guildinii* is repelled by the direct presence of the strain on food sources, even though there was no effect on the insect mortality rates. A broader understanding of the effects of *Bt* RZ2MS9 on plant development and on the interaction with insects may contribute to the development of new biotechnology strategies for integrated crop management.

Keywords: Soybean. Bioinoculant. *Bacillus.* Plant growth-promotion.

2.1 Introduction

Soybean is an annual leguminous crop that was initially domesticated in East Asia and is principally cultivated in North and Latin America at the present time (TEMESGEN; ASSEFA, 2020). In Brazil, soybean production has increased even in poor soils, with low fertility and little organic matter (CORDEIRO; ECHER, 2019). This occurs mainly due to the development of new strategies in crop management, like the inoculation of plant growth-promoting rhizobacteria (PGPR) that can directly stimulate plant growth or make available nutrients for the plant (MUMTAZ et al., 2019). Recent studies on those bacteria are unraveling several strains that can benefit plant development through different mechanisms that change plant physiology, improving plant nutrition and resistance against biotic and abiotic stresses (SILVA et al., 2020).

The studies of strategies to improve soybean production also investigate the interaction between PGPR and insects that negatively affect plant development, like members of the stink bugs complex (Heteroptera: Pentatomidae). During pod development, those insects injure pods and seeds within them, impairing pod filling, besides causing a severe reduction in seed vigor and facilitating pathogen infection (GRAÇA et al., 2016). Stink bug feeding also leads to leaf retention associated with delayed maturation (DEPIERI; PANIZZI, 2011). In Brazil, the species that are most frequently associated with economic losses consists of three species: *Nezara viridula* (southern green), *Piezodorus guildinii* (redbanded), and *Euschistus heros* (neotropical brown) (SANTOS et al., 2018). Losses caused by *Euchistus heros* reach up to 30% in soybean (VIVAN; DEGRANDE, 2011). According to Schünemann et al. (2018), toxins from *B. thuringiensis* MTox 126-6 and *B. thuringiensis* MTox 3146-4 are efficient in the biological control of *E. heros.* Further screening and interaction studies are therefore necessary in order to identify adequate strains for integrated control against the stink bug complex in soybean.

Another factor that negatively impacts soybean production is the occurrence of plant diseases caused by phytopathogenic fungi (MESSA et al., 2020). Several different diseases affect soybean worldwide, and many are caused by fungi, like *Fusarium* root rot (caused by *Fusarium solani)*, pod and stem blight (*Diaporthe sojae*), Cercospora leaf blight (*Cercospora kikuchii)*, Rhizoctonia aerial blight (*Rhizoctonia solani), Sclerotinia* stem blight (*Sclerotinia sclerotiorum*), among others (BORAH; DEB, 2020).

In recent years, researchers worldwide are interested in screening effective *Bacilli* to control fungal diseases in plants since those bacteria have been described as biological control agents through the production of direct antifungal substances, like volatile organic compounds

(VOCs), non-volatile metabolites, and extracellular lytic enzymes (YOU et al., 2021). Members of the endospore-forming genus *Bacillus* possess a high potential for the biocontrol of phytopathogenic fungi (HOLLENSTEINER et al., 2017). They have advantages when compared to other biocontrol agents such as (i) a longer life-shell, (ii) the possibility of dry-product formulation, with lower contamination rates, (iii) established large-scale treatment, and (iv) a cheap and easy usage (HAAS; DÉFAGO, 2005). Members of the genus *Bacillus* are also of great interest for their insecticidal activity, with the species *Bacillus thuringiensis* (*Bt*) being extensively studied and used worldwide (SCHÄFER; LUNDSTRÖM, 2014; ZHANG et al., 2017). This bacterium produces insecticidal proteins called cry proteins that create pores in the gut membrane of the insects, leading to starvation, septicemia, and death (EMPEY et al., 2021). There has been found a number of different cry toxins, each showing specificity against a group of insects from orders Lepidoptera, Coleoptera, and Diptera (MARTÍNEZ-ZAVALA et al., 2020). The application of *Bt* toxins for the control of sucking pests has been mainly investigated using cry genes in the genetic transformation of plants (FIUZA et al., 2017), but the direct ingestion of cry protein by the pentatomid *Euschistus heros* was studied by Schünemann (2015), concluding that the strain *Bt* MTox144-9 was able to increase the mortality rate of the insect.

Besides the studies focused on the activity of *Bt* against several insect species, this bacterium shows potential as an antagonist to phytopathogenic fungi due to its production of antimicrobial molecules, such as chitinases chitin-binding proteins and quorum sensing-quenching enzymes (ROCHA et al., 2014). Hollensteiner et al. (2017) showed that *Bacillus thuringiensis* and *Bacillus weihenstephanensis* inhibit the growth of phytopathogenic *Verticillium* species. In another study, the maize pathogen *Fusarium verticilloides* had its growth negatively impacted by *Bt* serovar *kurstaki* (ROCHA et al., 2014).

The strain of *Bt* RZ2MS9 was here studied to further assess its effects on soybean crops and stink bug *Piezodorus guildinii*. Previous studies with this strain have shown plant growth-promoting features, such as the production of siderophores and plant hormones, phosphate solubilization, and also nitrogen fixation ability, with a direct improvement of soybean and maize height and dry mass (BATISTA et al., 2018). Moreover, Longatto (2020) detected cuboid and spherical cry proteins produced by *Bt* RZ2MS9, which killed *Diatraea saccharalis*, *Helicoverpa armigera*, *Agrotis ipsilon*, and *Anthonomus grandis* larvae, likewise the commercial bioinsecticide DiPel® (*Bt* serovar *kurstaki* HD1) in rearing assays.

This study aimed to investigate the effects of *Bt* RZ2MS9 on soybean development, stink bugs infestation, and foliar disease incidence in field conditions. Thus, soybean seeds were

cultivated in a field, and their performance was compared among treatments with and without chemical applications and bacterium inoculation

2.2 Material and Methods

2.2.1 Biological material

The PGPR *Bt* RZ2MS9 was previously related as a potential new bioinoculant by Batista et al. (2018). The strain was isolated from the rhizosphere of the Amazon guarana tree (*Paullinia cupana* var. *sorbilis*) and stored in 20% glycerol at -80ºC, at the Laboratory of Genetics of Microorganisms, at ESALQ/USP, Piracicaba-SP, Brazil. For the studies here presented, it was cultivated at 28ºC on Luria-Bertani (LB) medium. A field experiment was conducted to examine the effect of inoculation of this strain on soybean (*Glycine max* L. Merr 'Potencia') growth as well as its role on biological control of stink bugs and phytopathogenic fungi.

2.2.2 Experimental area characterization

The field experiment was conducted from December 2017 to April 2018 in an area of 1 hectare of the Anhumas São Paulo University Research Station, in Piracicaba-SP, (latitude 22° 50' 26" South, longitude 48° 1' 20" West), Brazil. The experiment was installed in an area previously occupied by soybean (summer). Chemical and physical characterizations of the soil in which soybean was cultivated are presented in Table 1.

Table 1. Chemical and physical properties of the soil (0-20 cm) at Anhumas Research Station, Piracicaba-SP, the location where the experiment was conducted. The analysis was performed immediately before sowing

pH	OM	P	K	Ca	Mg	H+Al	Al	SOB	CEC	SB	AS	S SO_4
$CaCl_2$	$g.dm^{-3}$	$mg.dm^{-3}$	----------$mmol_C.dm^{-3}$----------							V%	m %	$mg.dm^{-3}$
Anhumas, Piracicaba - SP: medium texture												
4.9	15	40,5	2,4	18	6,5	29,5	0	27	56,5	48	0	6

P extracted by anionic resin; OM: organic matter; H+Al: potential acidity; SOB: sum of bases; CEC: cation exchange capacity; SB: saturation of CEC by bases; AS: Al saturation

2.2.3 Bioinoculant preparation and seeds treatment

The bacterial inoculum of *Bt* RZ2MS9 was prepared in the Laboratory of Genetics of Microorganisms at ESALQ/USP, Piracicaba-SP, Brazil, and immediately transported to the experimental areas, where seeds bacterizations were performed prior to seeding. Bacterial suspensions were prepared by growing the bacterium in LB medium at 28 °C with 150 rpm agitation, and then measuring the optical density of the culture and adjusting the final cell concentration to approximately $1x10^8$ CFU ml^{-1} in saline solution. The inoculation dosage applied was 8 mL of the bioinoculant for each 1 kg of seeds, which were slurry inoculated and dried in the shade before mechanical planting.

2.2.4 Field experiment

The experiment was conducted in a strip design in order to have restricted areas of inoculant application on the field. Replications were performed within each strip, with 15 randomly chosen sample points consisting of 2 rows with 5 m each. Treatments were Control (C), with neither inoculation nor chemical application during the crop cycle, *Bt* (B), with just bacterium inoculation, Full (F), with inoculation and chemicals application and treated (T), with the application of chemicals but no inoculation (Figure 1).

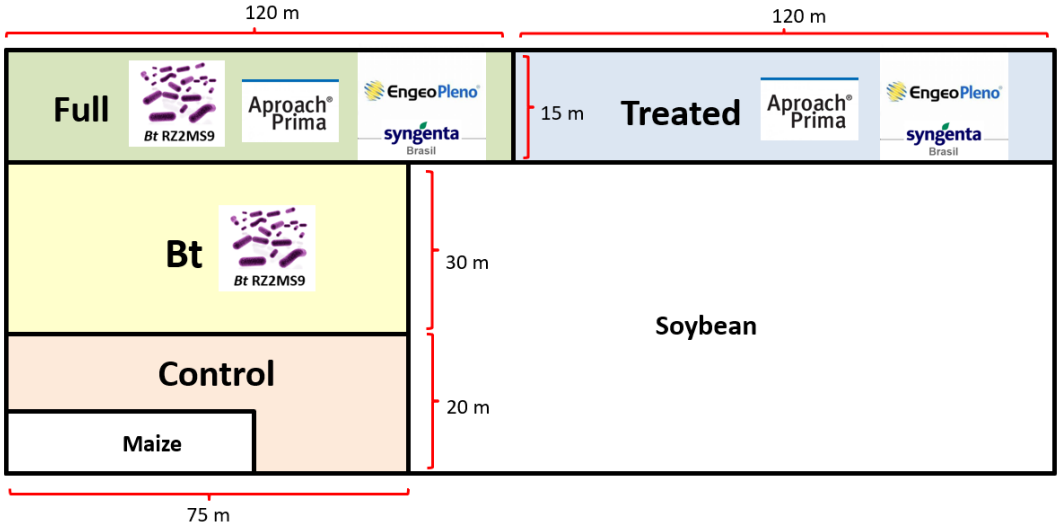

Figure 1. Field experiment design, showing areas divided into the treatments Control - no inoculation or chemical treatment; Bt – inoculation with *Bt* RZ2MS9, Full – inoculation with *Bt* RZ2MS9 and chemical treatment; and Treated – only chemical treatment

Mechanical seeding occurred on December 18, 2018, with soybean been planted at a depth of 3 cm. Each experimental strip had 40 rows wide, spaced by 45 cm and 75 m in length. Prior to seeding, fertilizer Nutrisafra® 04-20-20 was applied. The chemicals applied were the fungicide Approach®Prima (300ml.ha^{-1}) and the insecticide Belt® (70ml.ha^{-1}) in applications carried out at February 22, 2019, and March 23, 2019.

2.2.5 Soybean development parameters related to plant growth promotion and productivity

At the beginning of the flowering stage (R1), we measured plant height – 40 plants from the 15 points randomly sampled amognt the inner lines of each treatment were measured from the base of the plant (on the ground) up to the apex of the main stem using a metric table, according to Rocha et al. (2015).

At R5 stage, foliar damage by phytopathogenic fungi was evaluated through a diagrammatic scale, according to Martins et al. (2004) (Figure 2). Two days prior to harvest, green stem and leaf retention were evaluated according to Rocha et al. (2015), applying a grading system for leaf retention ranging from 0 for plants with normal senescence to 5 for plants with high leaf retention (mechanical harvest impracticable) and a grading system for green stem ranging from 1 – green stem to 3 – dry stem.

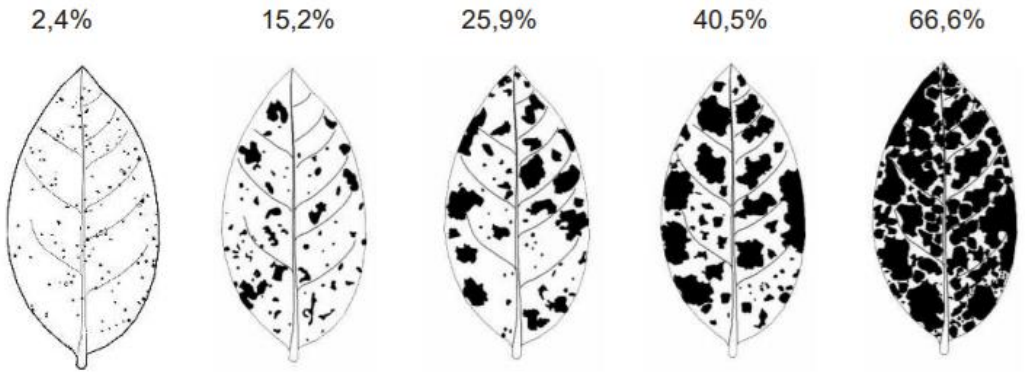

Figure 2. Diagrammatic scale for foliar damage in soybean caused by *Cercospora kikuchii* (MARTINS et al., 2004)

Soybean harvest was carried out on April 25, 2018. Each strip was harvested in the 15 sampling points consisted of 2 rows of plants with 5 m each that had been previously randomly chosen. Plants from these points were harvested completely and weighted for total grain yield

and 100 seed weight for assessment of soybean productivity. From each sampling point, three plants were separated to measure dry mass, total pod number, and weight per plant.

2.2.6 Stink bug population evaluation

The insect population density was surveyed for the occurrence of stink bugs *Euschistus heros* (Hemiptera: Pentatomidae) and *Piezodorus guildinii* (Hemiptera: Pentatomidae) – recorded weekly according to the cloth beating method for total insects per 0,5m of plants in 2 neighbouring lines (STÜRMER et al., 2012).

2.2.7 *Piezodorus guildinii* stock rearing

Adults of Piezodorus guildinii were obtained from the company Pragas.com Biological Supplies, Piracicaba, SP, Brazil, and maintained at 21 ± 5 °C, 70% r.h., and L12:D12 photoperiod. Insects were maintained in plastic containers (2,5 L, 20cm length, 20cm width, 8cm height) whose lids were cut in the center and covered with organdy ("voil") to allow adequate ventilation. The containers were lined with filter paper to absorb excrement and maintain sanitary conditions. A total of 25 insects were placed in each container and were maintained on a natural diet of green pods [P. vulgaris (L.)] (3 pods/container) and raw peanuts [Arachis hypogaea (L.)] in portions of 25 g/ cage, which were deposited in plastic Petri dishes. Hydration of the insects was provided by cotton moistened with distilled water placed in plastic Petri dishes. Containers were cleaned every 4 days to avoid fungal contamination.

2.2.8 Dual choice arena test (DCAT) for food preference of *Piezodorus guildinii* exposed to *Bt* RZ2MS9

A total of 25g of green pods were cut and then treated with 1ml of a *Bt* RZ2MS9 solution at $1x10^8$ CFU ml^{-1}, prepared as previously described in the bioinoculant preparation and seeds treatment section. Control groups consisted of the same mass of freshly cut green pods treated with saline solution. Plant materials were placed in opposite quadrants of an arena in the interior of plastic containers (2,5 L, 20cm length, 20cm width, 8cm height), as described by

Mensah-Bonsu et al. (2020), with some modifications. Three insects were placed in the center of each arena using a brush. Insects were then allowed to choose between the two food substrates freely. The experiment was performed with four replicates (Figure 3). The number of insects present on each food substrate was recorded at 15-min intervals for 3 h starting from the release time, for 5 days.

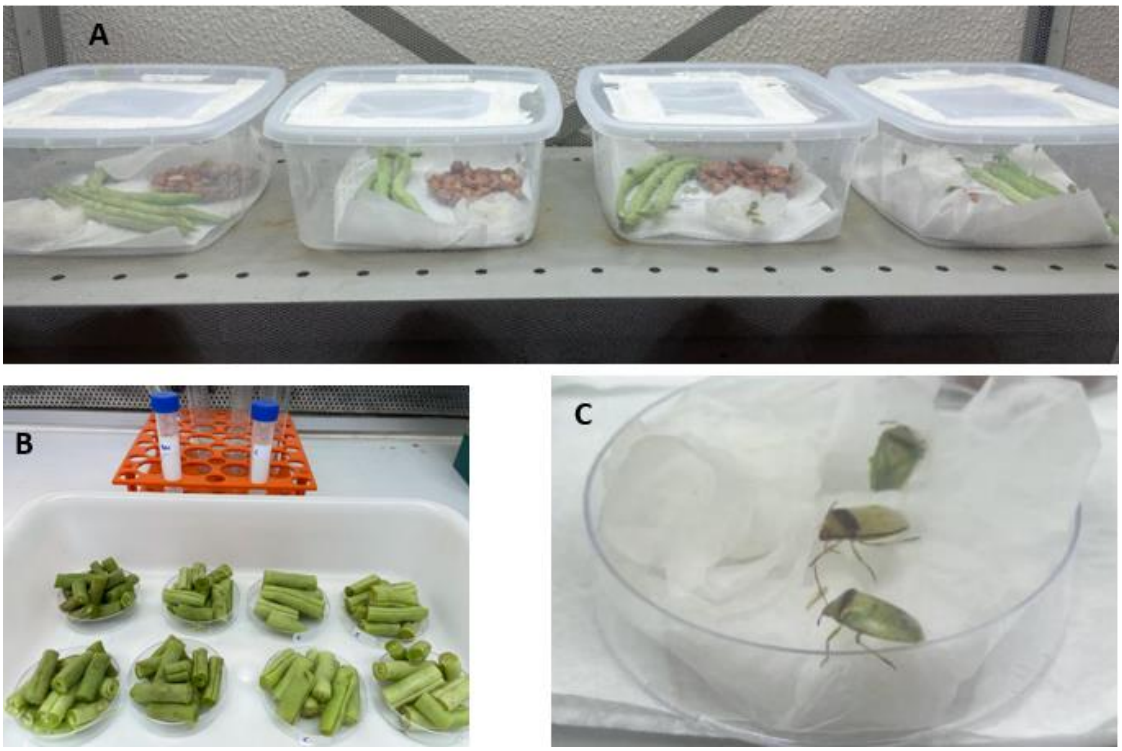

Figure 3. *Piezodorus guildinii* rearing (A), inoculum preparation for DCATs (B), and insects to be used in DCATs (C)

2.2.9 Direct effect of *Bt* RZ2MS9 on the mortality rate of *Piezodorus guildinii*

Adults of redbanded stink bug *P. guildinii* were divided into two populations and maintained in plastic containers as described in the stock rearing section. In one of the containers, food substrates were green pods treated with *Bt* RZ2MS9 solution containing approximately $1x10^{8}$ CFU ml^{-1}, while the control group container received green pods treated with saline solution, with food substrates for both control and treatment groups being replaced every 3 days. The number of dead insects was recorded 15 and 30 days after inoculation (d.a.i.) to evaluate the possible effects of the strain on the mortality of *P. guildinii* throughout the insect adult life stage.

2.2.10 Data analysis

The soybean crop field experiment was statistically evaluated by ANOVA tests for all soybean crop development and productivity parameters, with means obtained compared by the Tukey test. For the DCATs, the index of comparison is the Preference Index (PI). The value of this index for treated and control green pods was calculated as PI = (NT – NC)/(NT + NC) (Abed-Vieillard et al., 2014), where NT is the number of insects at the test green pod and NC is the number of insects at the control. PI can vary between −1 (strong aversion against inoculated green pods) and +1 (strong attraction to inoculated green pods). Analysis of the results was made using a Wilcoxon signed rank test, after checking the normality of data through a Shapiro-Wilk normality test. All analysis was performed in R software (R Core Team 2017), and the significance level adopted in all tests was 5%.

2.3 Results

2.3.1 Soybean plants development and grain production

The Control group had a lower average height than all treatment groups but greater 100 seed weight when compared to all treatments. The full group had the lowest 100 seed weight. There was no difference among groups for grain yield, but both *Bt* and Treated groups were similar and slightly more productive than the Control group. (Figure 4).

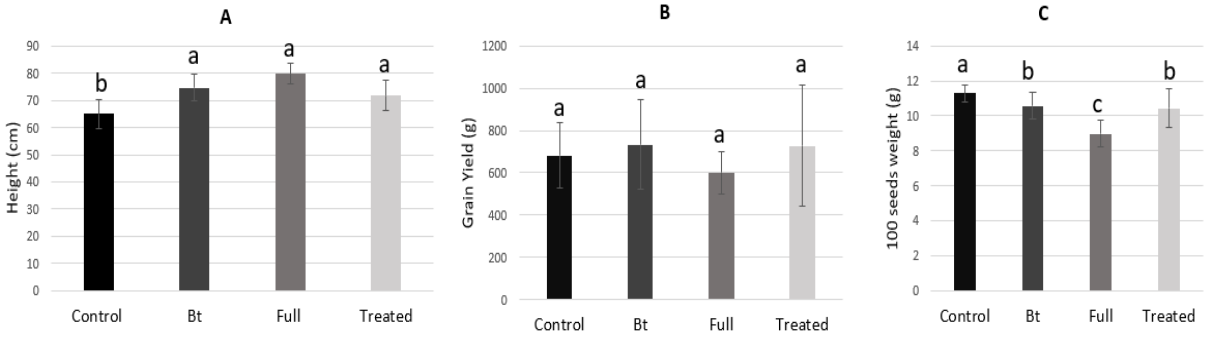

Figure 4. Average plant height (A) measured at the beginning of flowering stage (R1); average grain yield from 2 lines of plants with 5m each (B) and average 100 seeds weight (C) for each treatment: control with no chemical treatment nor inoculation (Control); inoculation with Bt RZ2MS9 (Bt); inoculation with Bt RZ2MS9 and chemical treatment (Full); and chemical treatment without inoculation (Treated). Means values (from 45 replicates for each treatment for A and C, and 15 replicates for B) followed by similar letters do no differ statistically by the Tukey test ($p < 0.05$)

Control and *Bt* showed a similar average dry mass, despite the lower average plant height from the Control group, but both differed from Full and Treated, which presented a higher dry mass than Control and *Bt.* Pod number and pod weight was similar among all groups (Figure 5).

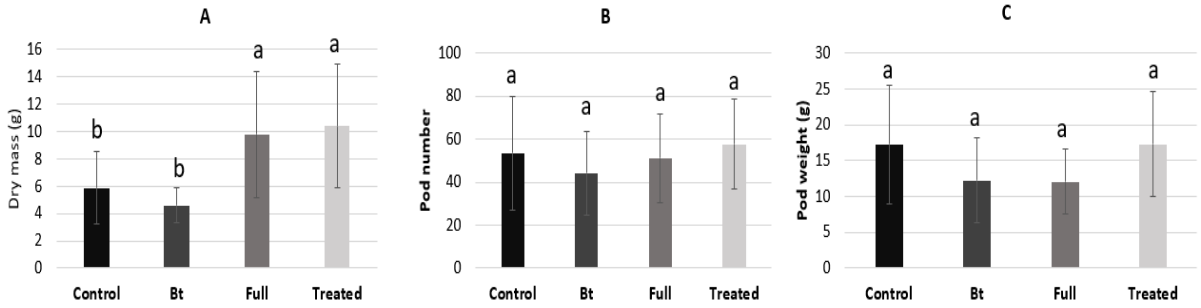

Figure 5. Average plant dry mass (A) weighed from 45 plants collect at the end of the crop cycle for each treatment; average pod number (B) and average total pod weight (C) for 45 replicates for each treatment, measured at harvest for each treatment: control with no chemical treatment nor inoculation (Control); inoculation with Bt RZ2MS9 (Bt); inoculation with Bt RZ2MS9 and chemical treatment (Full); and chemical treatment without inoculation (Treated). Means values followed by similar letters do no differ statistically by the Tukey test ($p < 0.05$).

2.3.2 Effect of inoculation on stink bug complex

There was no significant difference in the number of *E. heros* among groups, but *P. guildinii* was less present in the Treated and Control groups, differing from *Bt* and Full, which had a higher occurrence of *P. guildinii* (Table 2).

Table 2. Average number (±DV) of stink bugs *Euschistus heros* and *Piezodorus guildinii* per meter of soybean plants sampled by the cloth beating method once per week during seven weeks of crop development. Means followed by the same letter do not differ statistically by the Tukey test (p<0,05).

	Euschistus heros			
Day	Control	*Bt*	Full	*Treated*
I	0,5±0,85	0,7±0,67	0,5±0,53	0,2±0,42
II	0,4±0,52	0,9±0,88	0,5±0,53	0,2±0,42
III	0,8±0,79	0,7±0,95	0,9±0,57	0,8±0,63
IV	1,4±0,52	1,9±1,29	1,3±0,67	1,0±0,82
V	1,3±0,67	1,8±0,79	0,4±0,70	0,2±0,42
VI	0,3±0,67	0,0±0,0	0,7±0,67	0,2±0,42
VII	0,2±0,42	0,3±0,48	0,2±0,42	0,3±0,48
Mean	**0,7±0,6a**	**0,9±0,7a**	**0,6±0,5a**	**0,4±0,5a**

	Piezodorus guildinii			
Day	Control	*Bt*	Full	*Treated*
I	0,9±0,88	0,7±0,67	0,6±0,70	0,5±0,71
II	0,9±0,74	1,6±0,84	1,3±0,95	1,7±0,82
III	2±0,67	1,6±0,84	2,6±1,43	1,7±0,95
IV	3,9±0,88	4,7±2,21	5,8±1,69	4,2±2,10
V	4,8±1,62	6,2±2,35	1,7±1,77	0±0,00
VI	2,2±1,14	3,4±2,80	2,3±1,77	0,3±0,48
VII	0,8±0,79	0,8±0,92	1,7±1,34	1,1±0,74
Mean	**2,2±0,9bc**	**2,7±1,5ab**	**2,3±1,4ab**	**1,4±0,8c**

2.3.3 Effect of inoculation on plant senescence

For soybean leaf retention and green stem at the end of the crop cycle, the Control group did not differ from *Bt*, with both groups presenting a lower level of leaf retention at the end of the cycle, corresponding to normal senescence process and lower incidence of green stem at the end of the cycle. The Full group had the highest average leaf retention and also the higher incidence of green stem, with plants from these treatments presenting themselves with more green leaves and green stem at the end of the cycle. The Treated group differed from all others, being placed in between Control and *Bt*, and Full group (Figure 6).

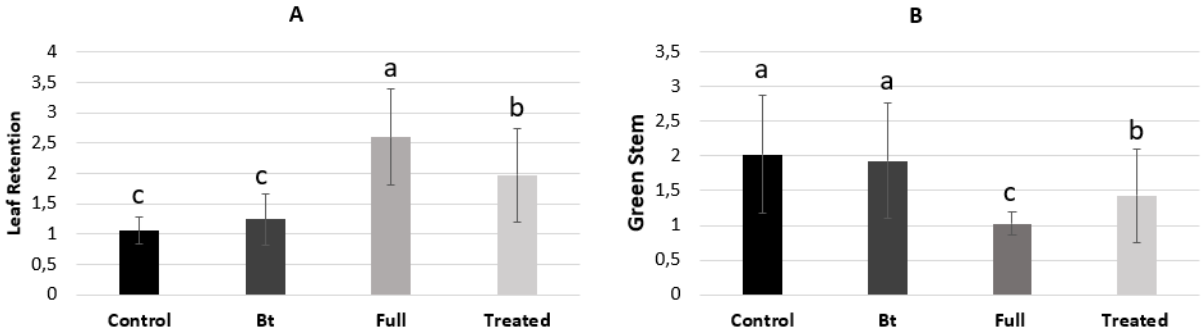

Figure 6. Soybean leaf retention (0 meaning no leaf retention and 5 total leaf retention) and green stem (1 meaning green stem and 3 dry stem) at the end of the cycle. Means followed by similar letters do no differ statistically by the Tukey test ($p < 0.05$)

2.3.4 Effect of inoculation on foliar damage due to phytopathogenic fungi

The Control group had the highest average values of foliar damage typical of *C. kikuchii* and, while the Full group had the lowest average values. *Bt* group did not differ from the Treated group (Figure 7).

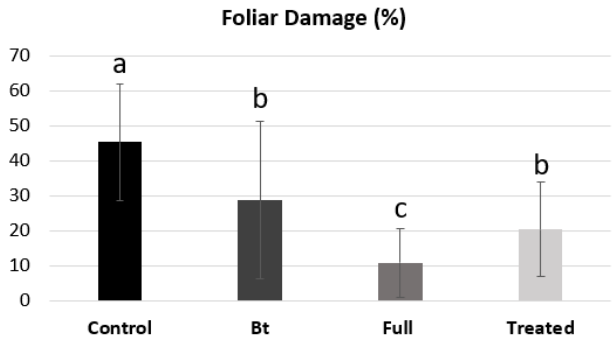

Figure 7. Average foliar damage (%) for each treatment at R5 stage. Means followed by similar letters do no differ statistically by the Tukey test ($p < 0.05$)

2.3.5 Dual choice arena test (DCAT) for food preference of *Piezodorus guildinii* exposed to *Bt* RZ2MS9

The preference index (PI) calculated for *P. guildinii* showed that insects preferred the Control group than Inoculated group, with a final mean PI of -0,59. This preference towards the Control group was statistically significant through Wilcoxon signed rank test, with a p-value of 6.797e-07 (Figure 8).

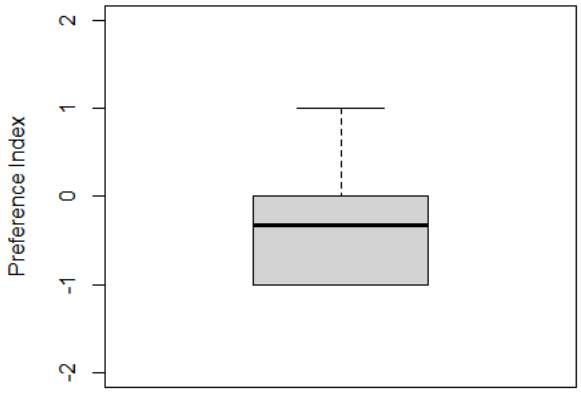

Figure 8. Mean preference index for *Piezodorus guildinii* in a dual choice arena test between green pods inoculated with *Bt* RZ2MS9 and control group (non inoculated). Negative values indicate a preference towards the Control group, and positive values indicate a preference towards the Inoculated group

2.3.6 Effect of *Bt RZ*2MS9 on the mortality rate of *Piezodorus guildinii*

The mortality rate of *P. guildinii* adults fed with green beans supplemented with RZ2MS9 (10^8 CFU/mL) was similar to the Control group, showing no statistical difference between both groups according to Tukey test (5% significance) (Figure 9).

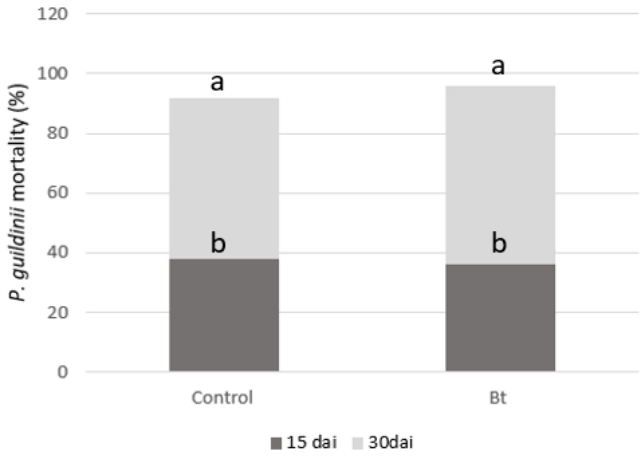

Figure 9. Effects of *Bt* RZ2MS9 on *Piezodorus guildinii* adults *in vitro* tests. Mortality was measured after 15 and 30 days after inoculation (d.a.i.). Data correspond to the mean percentage of dead adults from 5 replicates, with 10 insects each. Values with the same letter were not significantly different according to Tukey test ($p > 0.05$).

2.4 Discussion

Biological screening for plant beneficial microorganisms is crucial to the development of effective bio-fertilizers and technologies applied to more sustainable agriculture (STURZ et al., 2000). Such technological advances are of great value for our modern society, since the human population is continuously growing, and food insecurity and malnutrition is a major challenge in developing countries. Therefore, environment-friendly, low-cost technology is needed to ensure sustainable food production (SCHREINER et al., 2013).

The bacterial strain studied here, *Bt* RZ2MS9, has already been shown to cause positive results when inoculated on soybean, maize, and tomato (BATISTA et al., 2018; BATISTA et al., 2021). Here we have analyzed for the first time the potential of this strain as a biocontrol agent against phytopathogenic fungi and stink bug complex, besides having studied the role of the inoculant in crop development in relation to chemical treatment of the area.

Soybean plant height was positively influenced by inoculation since Control showed lower average height than the other groups. There was a tendency of higher height in the Full treatment, indicating that the combination of bacterial inoculation and chemical treatment may contribute to crop development, allowing the reduction of fertilizer application on the soil. Moreover, the use of bacteria consortia can be even more effective, as has been shown by Jamal et al. (2018) that studied the effect of the combined use of five bacteria, including *Bacillus amyloliquefaciens* Y1, as a biofertilizer in low bush blueberry. This study showed that the consortia improved plant growth, rhizosphere bacterial community, and enzyme production at various pH, indicating that knowledge on different strains may be applied to consortia formulations with different beneficial aspects.

The application of biofertilizers in leafy vegetable production is gaining importance because they constitute cost-effective and eco-friendly products. Different microorganisms, like *Azospirillum, Azotobacter,* and *Bacillus megaterium* have been studied in leafy vegetable production to increase the yield and reduce disease incidence (NANDISH et al., 2018).

Inoculants that increase plant height are of particular interest for leafy vegetable production, and thus further studies on the strain presented here may help to develop a useful inoculant for this type of culture.

There was no effect of inoculation on the soybean shoots dry mass in this study between Control and *Bt*, but both had lower dry mass than the groups that received chemical treatment. Since *Bt* group had a similar height to Full and Treated but lower dry mass, the perspectives for studies regarding stem elongation effect caused by *Bt* RZ2MS9 are promising.

Ku et al. (2018) investigated the effects of *Bacillus cereus* YL6 on soybean, observing higher biomass, total phosphorous content, and higher number of pods in groups treated with the bacterium. Different metabolites produced by different *Bacillus* species have shown a great diversity of action in plant growth-promoting activity. In a study with another *Bacillus* strain, *Bacillus licheniformis* DS3, Silpa et al. (2018) have found gibberellic acid production, which was highly correlated to some crop parameters, like pH, carbon, and nitrogen sources. The known effect of this hormone in plants, causing stem elongation, might explain higher heights not followed by increased dry mass in plants as was seen in this study.

Grain yield analysis showed no statistical difference between groups, indicating that inoculation did not significantly increase soybean production, but it was possible to observe a slight increase in grain yield in *Bt* group compared to Control. Furthermore, *Bt* showed results similar to Treated, indicating that it may be possible that the benefits from inoculation could allow a reduction in the use of chemicals. In a review concerning the *Bt* as a biofertilizer and biostimulator, Azizoglu (2019) affirms that there are several commercial *Bt*-based biopesticides on the market, and since *Bt* is a successful endophyte in many plants, the continuous *Bt* strain exploration may unravel strains with biofertilizer activity. Further testing of the positive impact of *Bt* RZ2MS9 on plant height observed in this study might show the potential of this strain as a useful biostimulator to plant development.

Evaluation of soybean pods showed no statistical difference among groups, neither for pod number nor for pod weight. In this experiment, chemical control was applied when the insect population was above the action threshold since we meant to evaluate a possible action of the bacterium as a biological control agent, and a bigger insect population on both Full and Treated would allow us to perform better comparisons. Since the normal action threshold is 4 stink bugs per 2 m row on a drop cloth considering grain production and 2 if we consider seed production (CORRÊA-FERREIRA; PANIZZI, 1999), the negatives effects on pod weight, grain yield, and 100 seed weight were severe. This might have masked the real effects

of *Bt* RZ2MS9 on soybean productivity, and thus new studies with chemical treatment according to action threshold may clarify the role of this strain on the culture.

The weight of 100 seeds was smaller on the Full group, which might be correlated with the increased leaf retention and green stem found on this treatment. These are signs of delayed maturation commonly associated to stink bugs attacks, just like other negative effects, like decreased number of pods, seed deformation, reduced seed size, and decreased seed production (GAZZONI, 1998; PANIZZI; SLANKY, 1985). It is possible to notice a tendency of inoculated groups to show a higher occurrence of stink bugs, even though it was not statistically significant for *E. heros*. Higher height in the Full group might have had a stronger attraction to stink bug complex, thus causing reduced grain quality.

During visits to the crop, the pods presented visual signs of damage caused by stink bug infestation. Hence, we tested the effects of *Bt* RZ2MS9 on the attractivity of *Piezodorus guildinii*, a common soybean pest from the stink bug complex in Brazil. Our results showed that green pods inoculated with a suspension of the strain had a negative effect on the food preference by the insect, but there was no effect of the strain on the mortality rates of the insects. The apparent repellent effect was also observed by Disi et al. (2018) for *Diabrotica virgifera virgifera*, the western corn rootworm, which showed a preference for the non-inoculated maize plants in comparison to plants treated with *Bacillus pumilus* strain INR-7. In a study with feeding preference of pine weevil *Hylobius abietis*, a major forest pest, the response of the insect to three strains of *Bt* showed differential eating, with just one of the bacterial strains being avoided by the insect (TUDORAN et al., 2020). The absence of effect on mortality rates indicates that proteins produced by this *Bt* strain are not effective against the stink bug tested under these experimental conditions, which might not occur with other insect species. A previous study with this strain RZ2MS9 by Longatto (2020) verified a higher mortality rate of *Diatraea saccharalis* and *Helicoverpa armigera* (both Lepidoptera). The presence of the *Bacillus* strain tested in this study on food substrate may also have this effect on other insects, which prompts further studies on the interaction between this strain and other species for a broader understanding of the potential of this strain on biocontrol strategies.

Different studies have found antagonist effects of species of the genus Bacillus against phytopathogenic fungi. Some genera, like *Bacillus*, *Paenibacillus* and *Pseudomonas* are actively being used for this purpose against plant pathogens like *Rhizoctonia solani*, *Rhizoctonia bataticola* and *Colletotrichum* (PÉREZ-MONTAÑO et al., 2014). In this study, the Full group had the lowest percentage of foliar damage, and *Bt* group showed less foliar

damage than Control. This suggests a protective effect of *Bt* RZ2MS9 inoculation, which may be used in integrated crop management to lower levels of fungicides applied on crops.

Proteins produced by *B. thuringiensis* strains may include bacteriocin, vegetative insecticidal proteins, and hydrolytic enzymes, including chitinases. The practical utility of chitinases in agriculture comes from their established direct toxicity to pathogenic fungi, nematodes, and synergism with Cry toxins (SUBBANNA et al., 2018). Two other members of the genus *Bacillus*, *B. amyloliquefaciens* QST713 (synonymous with *Bacillus subtilis* QST713) and *B. firmus* I-1582 are important strains used ad active ingredients in commercially-available biological products Serenade® and VOTiVO®, respectively. These bacteria colonize plant roots and promote plant growth and protection against pathogens. VOTiVO® is a seed treatment applied to protect against nematode infection, and Serenade® is used as a soil treatment for managing soilborne and seedling diseases (MENDIS et al., 2018).

Inoculation in soybean with the PGPR strain *Bt* RZ2MS9 has shown a positive effect on plant growth promotion, causing inoculated plants to present a higher height when compared to both chemically treated and non-treated counterparts. Effects on grain yield and grain quality were not conclusive since stink bugs might have impaired analysis. There was no significant effect of inoculation over the stink bug complex. We have seen positive results on this strain on lowering levels of foliar damage to leaves due to phytopathogenic fungi, which indicates the necessity of further experimentation on the antagonistic effects of this strain against different fungi species. Screening of PGPR strains and their metabolites may reveal promising alternatives for sustainable agriculture. Knowledge of different bacteria strains, along with further experimentation on bacterial metabolites and genes correlated, may contribute to the formulation of bio-inoculants that will benefit integrated crop management.

Conflict of Interest: The authors declare that they have no conflict of interest.

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3. Volatile Organic Compounds Produced by *Bacillus thuringiensis* RZ2MS9 – identification, evaluation of effects on soybean germination, and antagonistic effects over nine species of phytopathogenic fungi

ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) are bacteria that colonize the rhizosphere of plants and may positively influence crop performance through direct or indirect mechanisms. Direct beneficial effects include solubilization of nutrients for plant absorption and the synthesis of diffusible or volatile phytoregulators, like siderophores and phytohormones, that can increase plant growth. PGPR application in agriculture can improve plant physiology, directly stimulating several crop traits and optimizing production in adverse environmental conditions. Several species of PGPR release volatile organic compounds that may directly affect plant development or have an antagonistic effect against phytopathogenic fungi. Rhizobacteria from the genus *Bacillus* are commonly studied for their plant growth-promoting features, with several commercial products based on species of this genus being used worldwide. In this study, we have performed the first analysis of the volatile organic compounds released by the strain *Bacillus thuringiensis* RZ2MS9 and assessed their effect on soybean seed germination, seedling development, and phytopathogenic fungi development. The compounds released by the strain have negatively impacted the seed germination process, but there was no effect on seedling development. An antagonistic effect was only seen for two species of phytopathogenic fungi and to a very low extent. Final identification of volatile compounds, along with a deeper understanding of the effect of culture medium on the volatile compounds released by the strain, may still show a significant interaction of this strain with plants and fungi. Evaluations of new PGPR like the one assessed in this study are of great importance to the development of new strategies on the use of active molecules for integrated crop management.

Keywords: Volatile organic compounds. Plant growth-promotion. *Bacillus. S*oybean

3.1 Introduction

Microbial and plant volatile metabolites are of great interest, with the term volatilome recently been adopted to describe the complex mixture of compounds released by those organisms (FARBO et al., 2018). These molecules are generally small, carbon-based molecules, with a low water solubility and a high vapor pressure, which causes them to be in gaseous status in normal ambient conditions (1 atm pressure and 25ºC temperature) (TILOCCA et al., 2020). Microorganism volatile organic compounds (mVOC) have long been know to influence human preference over fermented foods, like cheese, wine, beer, and yogurt (DAVIS et al., 2013). In terms of chemical composition, VOCs show heterogeneity of molecular classes, including hydrocarbons, alcohols, thioalcohols, aldehydes, ketones, thioesters, cyclohexanes, heterocyclic compounds, phenols, and benzene metabolites (TILOCCA et al., 2020). Such compounds act as important signals for interacting organisms, affecting behavior, population dynamics, and gene expression (PATEL et al., 2020).

The research field on mVOCs is currently growing due to their application possibilities in agriculture, medicine, and biotechnology, besides their newly discovered role in inter and intraspecifc communication (CAPPELLARI et al., 2020). In agriculture, these compounds have shown the ability to promote plant growth and induce systemic resistance against pathogenic organisms, hence improving the quality and vigor of crops (KAHN; BANO, 2019). Currently, the emission of mVOCs is recognized as having an important role in microorganism-plant interaction (NASEEM et al., 2018), which has stimulated research on the applications of mVOCs as a new category of fertilizers, described as ‘gaseous fertilizer’ (SHARIFI; RYU, 2018). One of the first studies on the effects of mVOCs in plants found that *Arabidopsis thaliana* exposed to mVOCs produced by *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937 showed increased leaf area, for which 2,3-butanediol and acetoin demonstrated active participation (RYU et al., 2003). In a study by Camarena-Pozo et al. (2019), bacterial volatiles emitted by a member of the prokaryotic microbiome of agave and cacti had beneficial effects in *Arabidopsis thaliana* and improved growth and development of *A. thequilana* and *A. salmiana.*

Plant growth-promoting rhizobacteria (PGPR) can promote plant growth and protect plants from pathogen infection, with the ability to produce mVOCs that show inhibitory effects on pathogens (XIE et al., 2020). A study by Jun et al. (2012) showed that VOCs produced by *Bacillus amyloliquefaciens* NJN-6 (benzothiazole phenol and 2,3,6-trimethyl-phenol) impaired the growth and spore germination of *Fusarium oxysporum* f. sp. *cubense*. Another volatile

compound, 2-ethyl-3-methylpyrazine, produced by *Bacillus megaterium*, had a broad-spectrum antimicrobial activity in a study by Munjal et al. (2016). VOCs produced by *Pseudomonas chlororaphis* subsp. *aureofaciens* SPS-41 exhibited wide-spectrum antifungal activity against several plant pathogens, with different levels of pathogen inhibition following distinct inoculation strategies, like inoculation concentration and quantity (ZHANG et al., 2019).

In another study, VOCs released by the strain *Bacillus* sp. BCT9 promote shoot and root growth on *Lactuca sativa*, which had the best yield with exposition to VOCs in a range from 30 to 60 µL of inoculum, according to culture conditions (FINCHEIRA et al., 2017). Besides that, *Bacillus* has been proven to produce nonvolatile or volatile substances which have inhibitory effects against *Trichoderma* sp. (ZHENG et al., 2013), *Colletotrichum gloeosporioides* (LEE et al., 2013), and *Penicillium* sp. (ANDERSEN, et al., 1994). Moreover, it has been shown that volatile organic compounds (VOC) released by microorganisms act as insect semiochemicals, affecting insect behavior (DAVIS et al., 2013). Some VOCs attract insects by signaling the presence of food resources or oviposition sites (RERING et al., 2018; SOBHY et al., 2019), while other VOCs have been found to repel insects (STENSMYR et al., 2012; GOELEN et al., 2020).

Studies on the chemical nature and applications of mVOCs released by rhizobacteria are of great interest for biocontrol, productivity, and health of plants, with a special interest in the biosynthetic equipment of the members of the *B. subtilis* complex for bioactive compounds (PASCAL et al., 2020). Here we investigate the mVOCs produced by a PGPR *Bacillus thuringiensis* RZ2MS9. This strain was first isolated from the rhizosphere of the guarana tree (*Paullinia cupana* var. *sorbilis)*, an Amazon crop, and it has shown the ability to promote maize and soybean growth in screening tests performed to select PGPR microorganisms (BATISTA et al., 2018). Previous *in vitro* studies with this bacterium have shown plant growth-promoting features, such as the production of siderophores and plant hormones, phosphate solubilization, and also nitrogen fixation ability (BATISTA et al., 2018). However, little is known about the mVOCs produced by this strain and their effects on plants or phytopathogenic fungi. Thus, here we have evaluated the effects of mVOCs produced by this strain on the germination of soybean and on the early development of soybean plants. We also investigated the potential of *Bt* RZ2MS9 as a biocontrol agent against phytopathogenic fungi.

3.2 Material and Methods

3.2.1 Biological material

The *Bt* RZ2MS9 was isolated from the rhizosphere of the Amazon tree guarana (*Paullinia cupana* var. *sorbilis*) and stored in 20% glycerol at -80ºC, at the Laboratory of Genetics of Microorganisms, at ESALQ/USP, Piracicaba-SP, Brazil. For the studies presented here, it was cultivated on Luria-Bertani (LB) medium (tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l) at 28 °C with 150 rpm agitation. As a double control group, the strain *Escherichia coli* DH5α was also cultivated on LB medium, at 37°C and 150 rpm agitation. Bacterial suspensions used for the experiments contained approximately $1x10^8$ CFU ml^{-1}.

The phytopathogenic fungi tested in the antagonism study were provided by the same laboratory, in which they were kept at -80ºC. The Bt RZ2MS9 strain was subjected to *in vitro* dual-culture analysis for antifungal activity evaluation against the phytopathogenic fungi indicated in Table 1. All fungi were grown on potato dextrose agar (PDA; 4.0 g L−1 potato infusion, 20 g L−1 dextrose, and 15.0 g L−1 agar, pH 5.6) at 28 °C for 3-5 days before assays.

Table 1. Phytopathogenic fungi evaluated in the present study

Fungal pathogen	Disease
Alternaria alternata	Leaf spot
Cercospora kikuchii	Cercospora leaf blight
Colletotrichum falcatum	Red root disease
Colletotrichium sublineolum	Anthracnose
Colletotrichium truncatum	Anthracnose
Curvularia lunata	Seedling blight
Fusarium oxysporum	Fusarium wilt
Rhizoctonia solani	Rhizoctonia aerial blight
Sclerotinia sclerotiorum	Sclerotinia stem blight

3.2.2 Bacterial volatiles collection and identification

Colonies of *Bt* RZ2MS9 were grown on LBA media (10 µl of inoculum at $1x10^8$ CFU ml^{-1}) for 24 hours. After that, the media with well-established colonies were transferred to glass vials with two small openings, and those were used as volatile collection chambers. For control

group, glass vials with LBA but not the bacterial inoculum were used. All chambers were left sealed for 24h to allow the accumulation of mVOCs (Figure 1). The collection was performed connecting the chambers to an automated collection system ARS (Gainesville, USA) and to a glass tube (8 cm length, 6 mm diameter) with 30mg of absorbing polymer Hysep® (80/100 mesh) to capture the compounds released by the bacteria. The air entrance in the system was generated by the ARS and kept at a constant flux of 1 L/min. After 24 hours, the collection filters were eluted with 150 µl of hexane. The eluted extracts were then kept at -30ºC until chemical analysis.

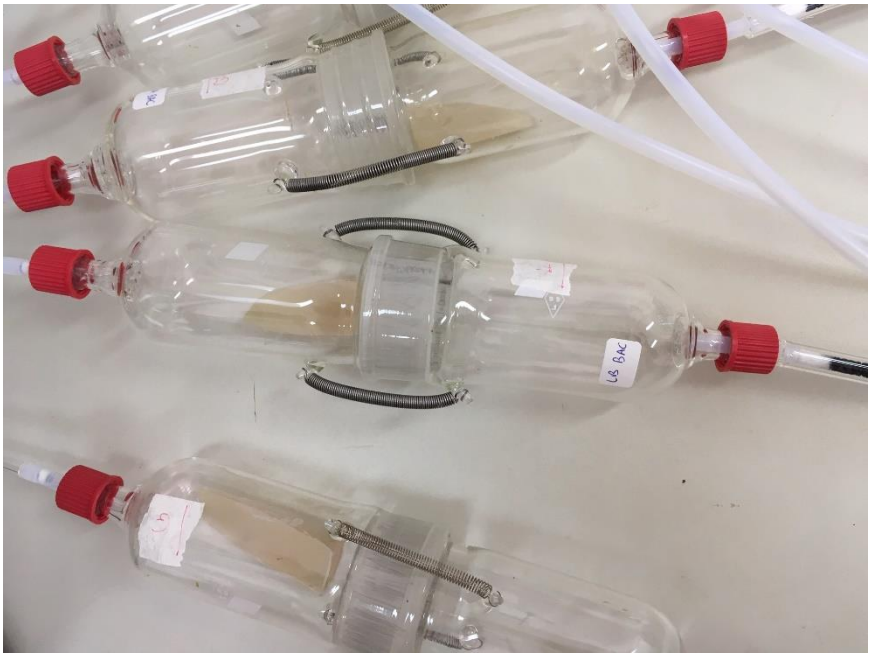

Figure 1. Collection chambers with LBA medium for mVOCs collection

For the identification process, the samples were injected into a gas chromatograph equipped with a flame ionization detector (GC–FID 2010, Shimadzu, Japan) with a capillary column HP-1 (MS 30 m x 0.25 mm x 0.25 mm, Agilent J&W, USA). A 1 µL aliquot of each sample was injected. The column temperature was kept at 35 ºC for 1 min, then increased to 150 ºC at 5 ºC min-1, and subsequently decreased at 20ºC min-1, with a final temperature of 220 ºC. Selected peaks were then analyzed in a gas chromatograph with a mass spectrometer (GC-MS, QP2010, Shimadzu, Japan), with a splitless injector, flame ionization detector, and helium as a carrier gas (24 cm/s). The detector signal was processed by MSD ChemStation software. The same temperature and times specifications used for GC-FID were applied to GC-

MS. Compounds were identified based on the Kovats Index (KI), and their mass spectra were compared with those of the NIST 2011.

3.2.3 Effect of mVOCs volatiles on the germination process

Soybean seeds were surface sterilized with 70% ethyl alcohol for 3 min, followed by soaking in 1.5% sodium hypochlorite solution for 5 min, then for another 2 min in 70% ethyl alcohol, with final washing in sterile distilled water twice for 5 min. The experiment was designed according to Fincheira et al. (2017). Seeds were then placed on sterile filter paper at one of the sides of an I-plate system, which consisted of centrally partitioned plastic Petri dishes (85 × 15 mm). Humidity for the germination consisted of 1.5 ml of sterile distilled water applied to the filter paper. On the other half of the plate, 10 µl of the liquid overnight-grown culture of *Bt* RZ2MS9 (1x 108 CFU ml-1) in LB medium were dropped in LBA medium, with no physical contact between seeds and bacterium. The Petri dishes were sealed with parafilm for 7 days to the germination process to occur under mVOCs effect undisturbed. Another group of plates was open every day to renew the internal micro-atmosphere. Parameters analyzed were root total length, root superficial area, mean root diameter, root volume, lateral root length, and axial root length. After 7 days of development, seeds with initial root development were analyzed with an Epson® scanner and then processed with the software WinRHIZO®.

3.2.4 Effect of mVOCs on soybean seedlings development

The assessment of mVOCs effects on soybean seedlings was performed according to Tahir et al. (2017). Soybean was seeded on plastic pots with soil and kept at 25 ± 5ºC, 14/10 light/dark photoperiod, and 85% humidity for seven days. Plastic pots were fixed on glass jars (80 x 140 mm) and sealed with Parafilm to avoid VOCs escaping from the jar. Bacterial strains *Bt* RZ2MS9 or *Escherichia coli* DH5α for comparison were inoculated on LBA medium in a Petri dish (35 x 12 mm) and placed at the bottom of the jar, as shown in Figure 5C. A double control group consisted of LBA medium with no inoculation. Eight cuts were made at the bottom of the plastic pots to allow mVOCs exposure. After 21 days, seedlings had their length, and dry weight measured. The experiment was designed with five replicates and repeated three times.

3.2.5 Antagonistic assay of mVOCs against fungal pathogens

This experiment was performed as described by Tahir et al. (2017). Phytopathogenic fungi tested here were culture in PDA medium until well established. A disc (0.6 cm in diameter) of the fungi mycelium was cut with a cork borer and placed upside down in PDA medium on one of the sides of an I-plate system, which consisted of centrally partitioned plastic Petri dishes (85 × 15 mm) (Figure 3). After 2 hours, 10 µl of the liquid overnight-grown culture of *Bt* RZ2MS9 (1x 108 CFU ml-1) were dropped on the other side, which contained LB medium, with no physical contact between the two microorganisms grown on either side. The Petri dishes were sealed with parafilm and incubated at 28ºC for five days. Control groups consisted of the dishes with both media, but only the pathogens inoculated them. Each experiment was performed with three replicates and repeated three times. The inhibition rate of mycelium growth was calculated according to Chen et al. (2020) by:

$$\text{Inhibition rate (\%)} = \left(1 - \frac{Dt - Dd}{Dc - Dd}\right) * 100\%$$

where *Dc* is the average diameter of the mycelial in the control plate, *Dt* is that of the mycelium on the test plate, and *Dd* is that of the inoculated mycelium discs.

3.3 Results

3.3.1 Bacterial volatiles collection and preliminary analysis

The preliminary analysis of the mVOCs released by the strain in LBA medium indicated the existence of 6 compounds, which were analyzed for candidate molecules (Figure 2).

A

	RT	Kovats Index	Compound
Peak 1	7,79	798	2 - methylpyrazine
Peak 2	8,43	868	2 - heptanone
Peak 3	10,061	911	pentanoic acid
Peak 4	10,343	919	NI
Peak 5	10,933	935	nonanal
Peak 6	28,125	1520	NI

B

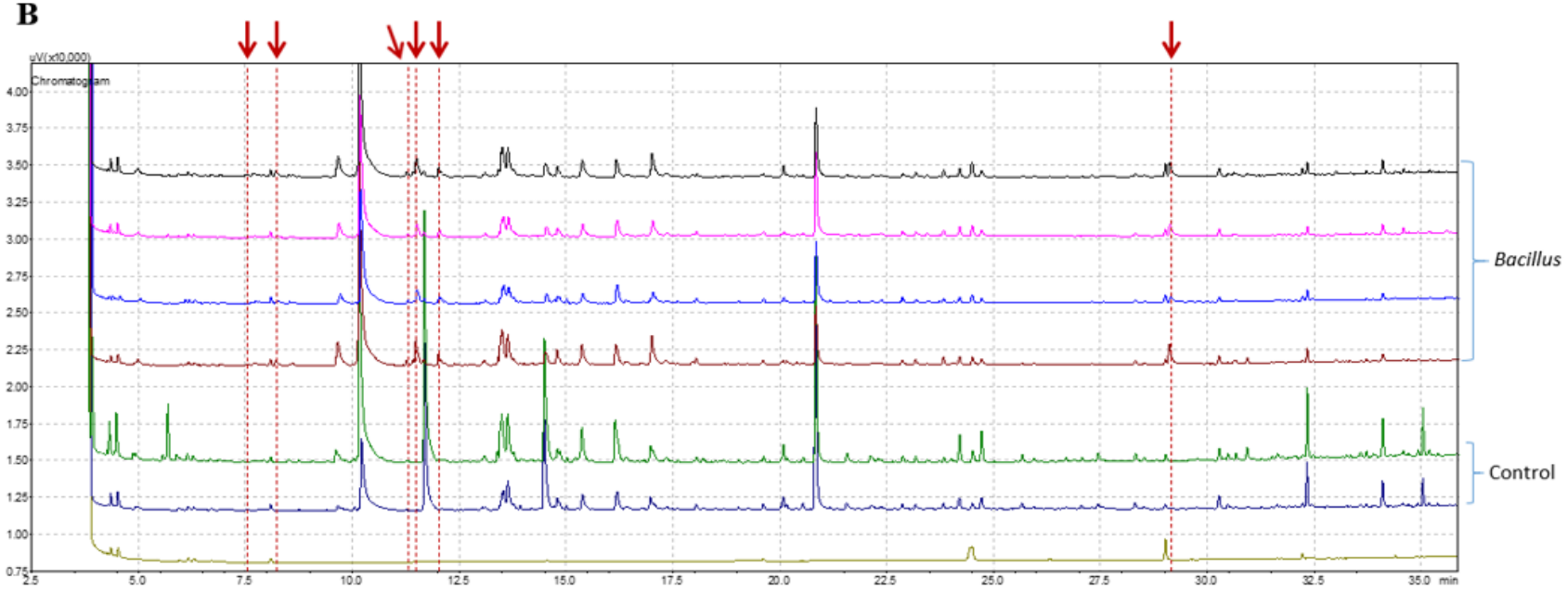

Figure 2. Candidate volatile organic compounds released by *Bt* RZ2MS9 cultivated in LBA medium (A) (RT = retention time) and volatile profile chromatogram (B).

3.3.2 Effect of mVOCs volatiles on soybean germination process

Soybean seeds presented an impaired germination process when in contact with the mVOCs released by the strain, with slow growth at the beginning of the process and then coming to a complete halt on germination (Figure 3). There was no elongation of the root, which presented a smaller length, superficial area, and volume (Figure 4).

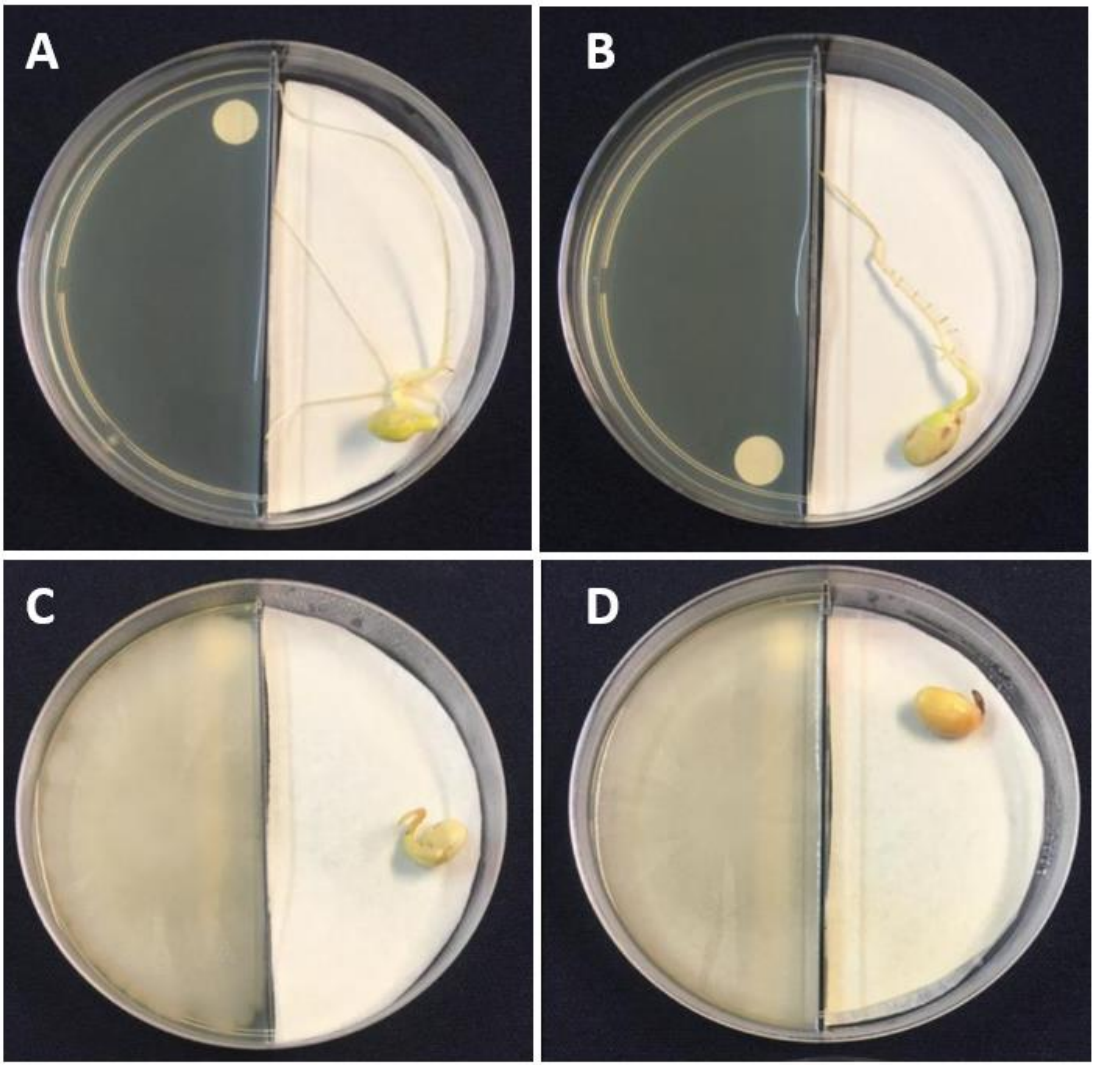

Figure 3. Effect on soybean germination process after 7 days of exposure to *Bt* RZ2MS9 mVOCs in a I-plate system test, consisting of Petri dishes with central partition. Figure shows control group open (A), control group closed (B), exposure to *Bt* RZ2MS9 mVOCs open (C), and exposure to *Bt* RZ2MS9 mVOCS closed (D).

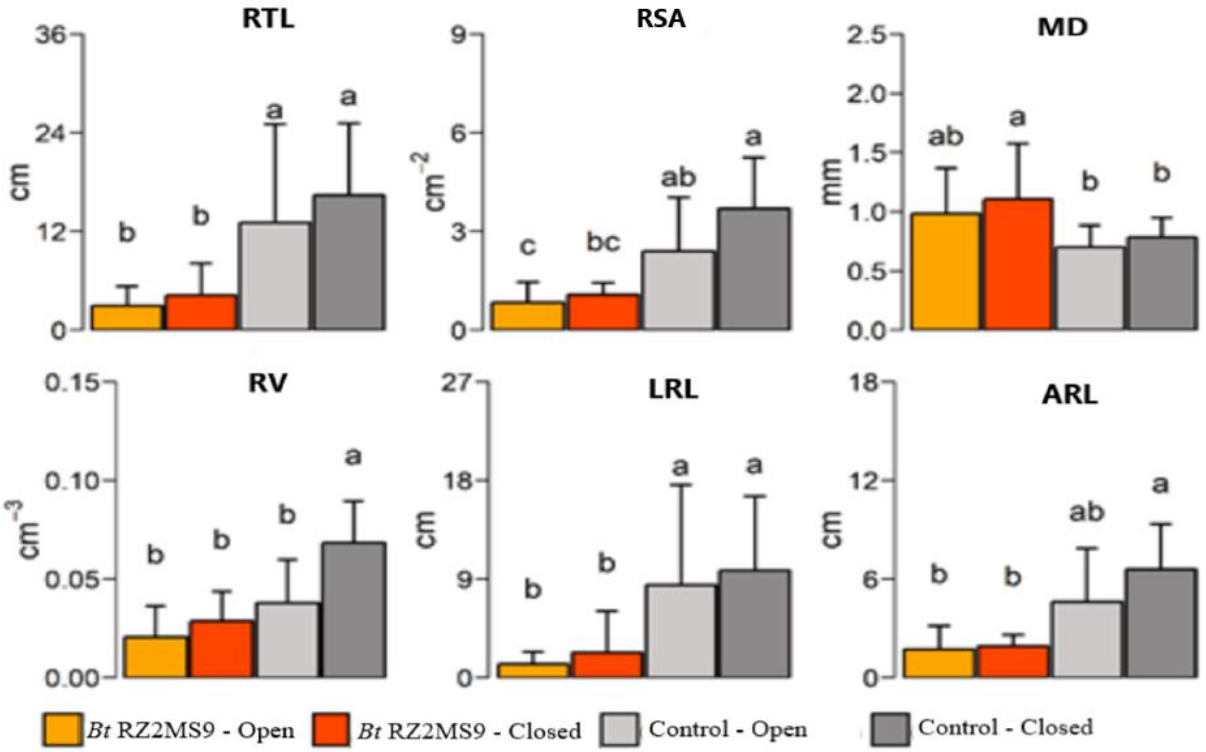

Figure 4. Effects of *Bt* RZ2MS9 on soybean germination process, in both sealed Petri dishes (closed) and regularly, opened Petri dishes (open). RTL – root total length (cm); RSA – root superficial area (cm2); MD - Mean diameter (mm); RV – root volume (cm3); LRL – lateral root length (cm); ARL – axial root length (cm). Means followed by the same letter do not differ by the Tukey test at (p<0,5).

3.3.3 Effect of mVOCs on soybean seedlings development

Plants treated with volatile compounds released by *Bt* RZ2MS9 in pot systems with strain colonies growing directly under the pots with plants did not show any influence on soybean plant development (Figure 5).

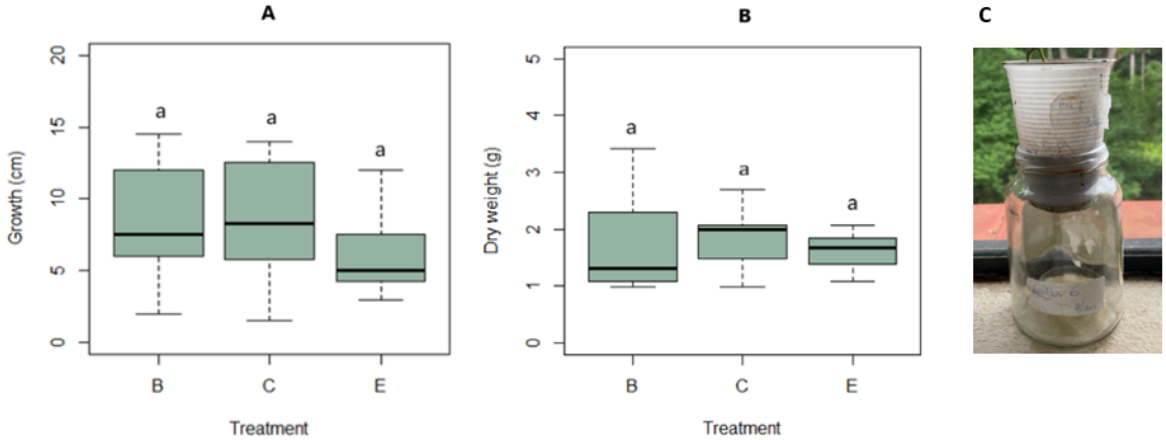

Figure 5. Effects of mVOCs from *Bt* RZ2MS9 on soybean seedlings length (A) and dry weight (B) (B = *Bt* treated group; C = control – LBA; E = *Escherichia coli* DH5α) grown on plastic pots over bacterial cultures in a sealed glass vial (C). Means followed by the same letter do not differ by the Tukey test at p<0,5.

3.3.4 Antagonistic assay of mVOCs against fungal pathogens

It was not observed strong antagonistic effects of volatiles released by *Bt* RZ2MS9 against any of the phytopathogenic fungi tested (Table 2). There was a minor inhibitory effect of *Bt* RZ2MS9 on the growth of *Colletotrichium sublineolium*, while *Colletotrichium truncatum* had its growth accelerated when in contact with the mVOCs from *Bt* RZ2MS9.

Table 2. Average inhibition rate (%) of fungal mycelial growth when cultivated in an environment with mVOCs of *Escherichia coli* DH5α and *Bt* RZ2MS9. Mean values on each line followed by the same letters do not differ statistically by the Tukey test (p<0,5).

	***E. coli* DH5α**	***Bt* RZ2MS9**
Alternaria alternata	1,9a	1,7a
Cercospora kikuchii	0,7a	1,2a
Colletotrichium sublineolium	4b	10,8a
Colletotrichium truncatum	-6,1a	-63,4b
Colletotrichum falcatum	0,8a	0a
Curvularia lunata	1,4a	0,5a
Fusarium oxysporum	0,8a	1,1a
Rhizoctonia solani	0,5a	0,8a
Sclerotinia sclerotiorum	1,2a	0,2a

3.4 Discussion

Soil microbes release volatile organic compounds that have been reported to promote plant growth (RYU et al., 2003), influence plant-insects interaction (BELL et al., 2020), and induce systemic resistance in crops (TAHIR et al., 2017), including against viral diseases (GUO et al., 2019). In this study, we have explored the potential of the plant growth-promoting rhizobacteria *Bt* RZ2MS9 as a producer of mVOCs with an influence on soybean development.

Members of the genera *Bacillus* are known PGPR of great importance as industrially relevant strains, with applications on crop productivity enhancement, disease resistance, biostimulation, and biocontrol (MÜLNER et al., 2020). One of the research fields for biotechnology strategies using PGPRs is their biochemical equipment of bioactive peptides and volatiles. Different strains of *Bt* have been proven to produce nonvolatile or volatile molecules with inhibitory effects against *Fusarium oxysporum* (MINERDI et al., 2009; YUAN et al., 2012), *Botryosphaeria berengeriana* (ZHANG, et al. 2010), *Trichoderma sp.* (ZHENG, et al., 2013) and *Colletotrichum gloeosporioides* (LEE et al., 2012). Volatile organic compounds responsible for the antifungal effect include 2,3,6-trimethylphenol, nonan-2-one, decan-2-one,

dodecan-2-one, undecan-2-one, 2-methylpyrazine, etc. and effective protection against fungi is generally not the action of one substance but a combination of many compounds (HE et al., 2020).

Studies that explored volatile-mediated impacts of bacteria on plants have been mainly performed using a divided Petri dish setup, which consists of a dish equally divided in half by a plastic border. This configuration allows volatile compounds to be exchanged between both sides, and at the same time, it prevents any diffusion of non-volatiles metabolites through the medium (BAILLY; WEISSKOPF, 2012). Moreover, a study by Kai and Piechulla (2019) indicated that Petri dishes sealed using porous tape that allowed gas exchange with the surrounding atmosphere had similar effects with *Bacillus* mVOCs on plant development as plates sealed with parafilm causing accumulation of carbon dioxide. This result indicates that this factor alone does not influence plant germination or development (KAI; PIECHULLA, 2019), which was also observed in the germination assay of this study that compared parafilm sealed plates and plates that were opened periodically.

The preliminary analysis of the volatiles released by *Bt* RZ2MS9 showed the presence of the volatile 2-heptanone, which is a methyl n-amyl ketone that has been previously reported to promote plant growth (JIANG et al., 2019), besides being toxic to both insect and plant fungal pathogens (LEE et al., 2017). Zhu et al. (2019) showed that *Bacillus nematocida* releases 2-heptanone, which has an attractive effect on a soil nematoid used as a host by the bacteria. Another candidate compound as been released by *Bt* RZ2MS9, 2 – methylpyrazine, has a potential effect of inhibiting phytopathogenic fungi. In a study by Patel et al. (2021), the strain *Pseudomonas putida* BP25 released 2-methylpyrazine, which proved to reduce disease incidence and severity caused by *Magnaporthe oryzae* in rice. Even though this study was not able to identify strong direct effects of the volatiles on the reduction of phytopathogenic fungi mycelia, new studies with different methodologies may still show this effect. Tahir et al. (2017) tested the antagonistic effect of mVOCs released by a *Bacillus* strain on tobacco plants inoculated with *Ralstonia solanacearum,* and was able to verify a protective effect of the volatiles, with decreased disease severity on the plants tested. This strategy of in-plant studies may still show induction of systemic resistance in plants by *Bt* RZ2MS9.

The third candidate compound, pentanoic acid, has previously been reported as being produced by *Bacillus altitudinis* strains BT3 and CT8 in a study that also showed the plant growth-promoting effect of both strains (KUSHWAHA, et al., 2021). That might indicate a possible similar effect of *Bt* RZ2MS9 if pentanoic acid production is confirmed.

Finally, the last compound to possibly be released by *Bt* RZ2MS9 grown in LBA is nonanal, which has been reported to exhibit antimicrobial activity against gram-positive and gram-negative bacteria in the concentration of 100 to more than 800 mg/kg (MUROI et al., 1993). This compound has been reported as a mVOC released by *Bacillus amyloliquefaciens* strains T-5 and SQR-9 in experiments showing significant inhibition of the growth and virulence traits of *Ralstonia solanacearum* (RAZA et al., 2016). In this experiment, medium MS (1.5% agar, 1.5% sucrose, and 0.4% TSA (w/v) was used, emphasizing the importance of multiple testing of growth media types to a better understanding of interaction processes.

Different studies have explored the effect of volatiles from bacteria from the genus *Bacillus* on the development of plants. VOCs from *Bacillus* sp. BCT9 showed a low toxicity effect on seeds, and ketone compounds had a stimulation effect on the germination stage of *Lactuca sativa* (FINCHEIRA et al., 2017). A study by Asari et al. (2016) found negative effects on *Arabidopsis thaliana* plant growth and plant development exposed to *Bacillus amyloliquefaciens* when the bacterial culture grew on TSA or LBA, but a change on the culture medium to MSA supplemented with root exudate proved to cause an increased total root length of the plant. In the same study, when *Bacillus* strains were grown on LBA and TSA, plant leaves more closely located to bacteria developed chlorosis (ASARI et al., 2016). In our study, the production of 2 – methylpyrazine and 2 – heptanone may contribute to the negative effect on plants since pyrazine derivatives can show herbicide properties and cause deleterious effects on the seed germination process (DOLEZAL; KRÁL'OVA, 2011). The deleterious effect was not present in the seedlings, which may have been caused by earlier stages of development being more susceptible to potentially harmful volatiles than older plants (BAILLY; WEISSKOPF, 2012).

It is known that the effects on mVOCs emission of *Bacillus* strains tend to point to greater promoting growth effects on plants when MS medium is used rather than LB medium (RYU et al., 2003; RYU et al., 2004; ZOU et al., 2010). The two media differ considerably: MS is a mineral acid medium with sucrose as a C source and low in agar concentration, while LB is slightly alkaline, with hydrolyzed proteins and higher agar concentration. Therefore, the same strain grown on both media would not surprisingly produce different volatile profiles, with differing effects on plant development (BAILLY; WEISSKOPF, 2012). A study by Blom et al. (2011) showed that the production of mVOCs depends heavily on culture condition, with the strongest effects of inhibition and stimulus occurring when bacteria were grown on LB, which was explained by the authors saying that the high cell density reached quickly on such high

nutrient medium influences the occurrence of more drastic effects. A study of forty *Bacillus* strains carried out by Goelen et al. (2020) revealed that phylogenetically closely related *Bacillus* strains emitted similar blends of mVOCs and elicited a comparable olfactory response of *A. colemani* in Y-tube olfactometer bioassays, varying between attraction and repellence. These findings emphasize the importance of screening new microorganism strains considering different culture media and further investigate volatile profiles to understand their biochemical routes of interactions.

This work is, to our knowledge, the first to show inhibitory effects of volatile compounds from a *Bt* strain on the germination process of an important crop as soybean. Hence, further studies with the strain *Bt.* RZ2MS9 in different culture conditions and with different methodologies of volatile extraction and identification may still demonstrate beneficial effects of this strain on plant development and also on biocontrol of phytopathogenic fungi.

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4. Co-inoculation with *Bacillus thuringiensis* RZ2MS9 and *Bradyrhizobium japonicum* effects on soybean (*Glycine max* (L.) Merril) development and soil microbiome diversity

ABSTRACT

Microorganisms known as plant growth-promoting rhizobacteria establish beneficial interactions with plants, providing biological services like nitrogen fixation, increased nodulation in leguminous plants, and the production of plant hormones and siderophores. This study has evaluated the effects of the co-inoculation of soybean with *Bacillus thuringiensis* (*Bt*) RZ2MS9 and Masterfix® Soja, a rhizobia commercial bioinoculant composed by *Bradyrhizobium japonicum* (SEMIA 5079) and *Bradyrhizobium elkanii* (SEMIA 5019) on soybean development and soil microbiome diversity. *Bt* RZ2MS9 and rhizobia are known to interact positively with leguminous species, promoting soybean growth. Results showed that soybean development was altered due to inoculation, with plants inoculated with only *Bt* RZ2MS9 and in co-inoculation with rhizobia showing a higher shoot length than those inoculated with only rhizobia and control group. There was no significant effect of either inoculation or co-inoculation on shoot dry mass, stem diameter, grain quality, or productivity. The evaluation of crop lodging showed that co-inoculated plants had a lower level of lodging at the end of the cycle. Soil microbiome diversity of bacteria was not altered by *Bt* RZ2MS9 inoculation or co-inoculation with this strain and rhizobia. However, after harvesting, the Chao1 index of alfa diversity showed lower bacterial diversity of treatments inoculated with *Bt* RZ2MS9 for both sole inoculation and co-inoculation with rhizobia. This study has shown that the synergistic activity of rhizobia and *Bt* RZ2MS9 on soybean development, considering the indigenous bacterial diversity increased plant growth without interfering significantly with soil diversity over time. The study of different rhizobacteria consortia for their potential synergistic positive effects as bioinoculants is a promising approach for sustainable crop management. In a scenario of increasing demand for food production along with environmental concerns, biotechnological alternatives that positively impact crop health and productivity are a promising option that may contribute to food security in more sustainable ways.

Keywords: co-inoculation, soybean, *Bacillus*, microbiome diversity

4.1 Introduction

The symbiosis formed between *Bradyrhizobium* species and soybean is considered one of the most important natural relations exploited by agricultural activities for the ability of these microorganisms to increase nitrogen fixation and grain yield with reduced dependence on inorganic nitrogen fertilizers (HUNGRIA et al.; 2015). This symbiosis ultimately provides an increase in nitrogen availability in several agroecosystems (EGAMBERDIEVA et al., 2020). *Bradyrhizobium* spp. is commonly used in biofertilizer formulations to increase nitrogen bioavailability for plants (PANZIERI et al., 2000). Thus, several studies on different *Bradyrhizobium* strains are performed worldwide, screening for new beneficial rhizobia that may be applied to agriculture in soybean crops (ULZEN et al., 2016; TEMESGEN; ASSEFA, 2020).

Growing evidence indicates that other soil beneficial bacteria can positively affect rhizobia performance (KORIR et al., 2017). Soybean inoculation with different rhizobacteria strains (mainly species from the genera *Azospirillum, Bacillus* and *Pseudomonas*) in consortium with rhizobia have been reported to promote plant growth and yield of the crop, besides increasing seed germination, nodulation, and consequent N-fixation (SHARMA; KUMAWAT, 2011; SOLOMON et al., 2012; RECHIATU et al., 2015; ULZEN et al., 2016; AUNG et al., 2013; KRAVCHENKO et al., 2013). Other ways in which these plant growth-promoting rhizobacteria (PGPR) can directly facilitate plant growth are the production of siderophores (which can facilitate nutrient absorption by the plant), synthesis of phytohormones as auxins, cytokinins, and gibberellins (with direct impact on plant physiology at different stages of plant growth) and solubilization of nutrient minerals (MASCIARELLI et al., 2014). Thus, knowledge of the mechanism involved in the PGPR interaction with plants is of great interest to science since different strains show different effects on plant physiology and also vary in their symbiotic effectiveness with different cultivars (TEMESGEN; ASSEFA, 2020).

There is a complex and rich diversity of species in the soil microbiome, and the interactions among them play an essential role in plant health and productivity, and so there is increasing interest in research on beneficial PGPR strains and their diversity in soil for successful inoculation techniques (PHILIPPOT et al., 2013; JIMÉNEZ et al., 2020). Moreover, recent studies of two or more PGPRs in co-inoculation have shown improved crop morphology and physiological construction triggered by the combined action of different PGPRs (MOLINA et al., 2017; ROJAS-SOLÍS et al., 2018; BARBOSA et al., 2021).

Wasule et al. (2007) evaluated the co-inoculation of the rhizobium species *Bradyrhizobium japonicum* and *Pseudomonas striata*, reporting that there was a significant improvement in soybean growth and grain yield compared to the sole application of *B. japonicum.* Sibponkrung et al. (2020) observed that the co-inoculation of the PGPR *Bacillus* spp. with *B. japonicum* into soybean resulted in enhanced nodulation and N2 fixation due to the formation of larger nodules.

In a review made by Zeffa et al. (2020) on the results obtained from co-inoculation studies from 1987 to 2018, the authors show that co-inoculation protocols resulted in a significant increase in nodule number (11.40%), nodule biomass (6.47%), root biomass (12.84%), and shoot biomass (6.53%). However, these studies did not show an increase in shoot nitrogen content and grain yield. Positive effects observed on plant development were more evident when the PGPR genera used as co-inoculant were *Azospirillum*, *Bacillus*, and *Pseudomonas.* This review points out that the effects of co-inoculation on plant development might be more important in overcoming nutritional limitations than in increasing productivity in optimal conditions (ZEFFA et al., 2020).

Besides assessing the effect on plants, research on PGPR also evaluates their impact on indigenous soil microbiome structure (MARTÍNEZ-HIDALGO et al., 2019). Some studies measured the non-target effects of PGPR on the indigenous bacterial community and demonstrated contrasting results, with significant effects being generally rare and spatially limited (JIMÉNEZ et al., 2020). So far, we have limited knowledge on the mechanisms by which PGPRs affect indigenous bacterial diversity in the rhizosphere (WIN et al., 2020). A recently developed approach for understanding the interactions among microbial taxa in the rhizosphere is using next-generation sequencing, such as Illumina-based techniques, to study microbial taxa in a given environment, especially those with low-abundance species changes (UROZ et al., 2013). In a study with this approach, Jo et al. (2020) reported an increase in plant growth and soil microbiome diversity in a study with inoculation of *Bt* KNU-07 on pepper plants.

The PGPR *Bt* RZ2MS9 has shown positive effects in promoting plant growth in previous studies (BATISTA, 2017; BATISTA et al., 2018; LONGATTO, 2020). The study presented here is the first to evaluate the effects of the co-inoculation of *Bt* RZ2MS9 and rhizobia on soybean development and the diversity of bacteria on the soil microbiome. In the current scenario of climate insecurity, the improved plant performance observed in the co-inoculation studies is of particular interest for being aligned with modern demands of agricultural, economic, social, and environmental sustainability (CHAPARRO et al., 2012).

4.2 Material and Methods

4.2.1 Biological material

The PGPR *Bt* RZ2MS9 was first isolated from the rhizosphere of the Amazon tree guarana (*Paullinia cupana* var. *sorbilis*) (BATISTA et al., 2018). It is stored in 20% glycerol at -80ºC, at the Laboratory of Genetics of Microorganisms, at ESALQ/USP, Piracicaba-SP, Brazil. Cultures of this microorganism were made on Luria-Bertani (LB) medium (tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l) at 28 °C with 150 rpm agitation.

We applied the commercial peat bioinoculant Masterfix® Soja for the co-inoculation study, which contains the rhizobia *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* (SEMIA 5079 e SEMIA 5019, respectively). Seed treatment was performed according to the instructions provided by the manufacturer. Finally, the field study was conducted with the soybean cultivar Potencia BMX.

4.2.2 Experimental area characterization

The field experiment was conducted from December 2018 to April 2019 in an area of 1 hectare of the Anhumas São Paulo University Research Station, in Piracicaba-SP, (latitude 22° 50' 26" South, longitude 48° 1' 20" West), Brazil. The experiment was installed in an area previously occupied by soybean (summer). Chemical and physical characterizations of the soil in which soybean was cultivated are presented in Table 1.

Table 1. Chemical and physical properties of the soil (0-20 cm) at Anhumas Research Station, Piracicaba-SP, the location where the experiment was conducted. The analysis was performed immediately before sowing.

pH	OM	P	K	Ca	Mg	H+Al	Al	SOB	CEC	SB	AS	S SO_4
$CaCl_2$	$g.dm^{-3}$	$mg.dm^{-3}$	----------$mmol_C.dm^{-3}$----------							V%	m %	$mg.dm^{-3}$
Anhumas, Piracicaba - SP: medium texture												
4.9	15	40,5	2,4	18	6,5	29,5	0	27	56,5	48	0	6

P extracted by anionic resin; OM: organic matter; H+Al: potential acidity; SOB: sum of bases; CEC: cation exchange capacity; SB: saturation of CEC by bases; AS: Al saturation

4.2.3 Bioinoculant preparation and seeds treatment

The bacterial inoculum of *Bt* RZ2MS9 was prepared in the Laboratory of Genetics of Microorganisms at ESALQ/USP, Piracicaba-SP, Brazil, and immediately transported to the experimental areas where seeds bacterization was performed before seeding. The inoculum consisted of bacterial suspension in saline solution at approximately $1x10^8$ CFU ml^{-1}, which was prepared by previously growing the bacterium in LB medium at 28 °C with 150 rpm agitation and then measuring the optical density of the culture and adjusting the concentration. The inoculum dosage applied was 8 mL of the bio-inoculant for each 1 kg of seeds, which were dried in the shade before mechanical planting.

The inoculation with the commercial rhizobia product Masterfix® Soja was performed according to the manufacturer's instructions, diluting the peat product in saline solution to the final concentration of $1x10^8$ CFU ml^{-1} and directly applying the inoculant in the seeds. The material was also dried in the shade before seeding, which occurred two hours after seed treatment for all inoculations tested.

4.2.4 Experimental field design

The experiment was conducted in a strip design to have restricted areas of inoculant application in the field since the inoculants here tested are all rhizobacteria and could easily spread among treatments in case of a smaller plots design. Replications were performed within each strip, with 20 sampling points being marked in the strips considering a 5 m border for both sides of treatments. The treatments were Control (no inoculation), *Bt* (*Bt* RZ2MS9 inoculation), *Bt*_rhizobia (co-inoculation of *Bt* RZ2MS9 and rhizobia) and Rhizobia (only rhizobia inoculation) (Figure 1).

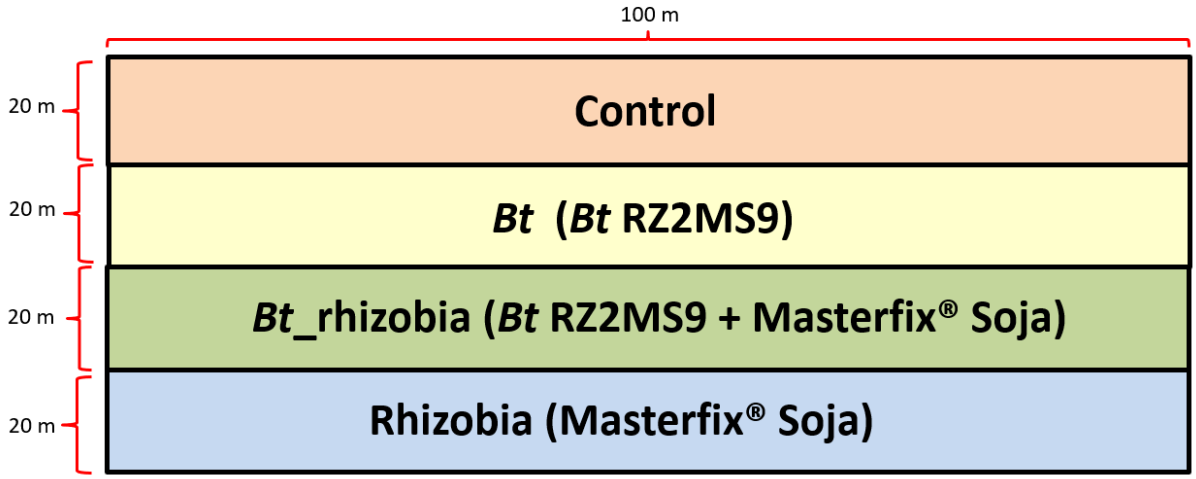

Figure 1. Field experiment design, showing areas divided into the treatments: Control (no inoculation), *Bt* (*Bt* RZ2MS9 inoculation), *Bt*_rhizobia (co-inoculation of *Bt* RZ2MS9 and rhizobia), and Rhizobia (only rhizobia inoculation).

4.2.5 Sowing and conducting of the experiment

Mechanical seeding occurred on November 28th, 2018, with soybean seeds planted at a depth of 3 cm along the experimental strips (40 rows wide, spaced by 45 cm, and 100 m in length). Prior to seeding, fertilizer Nutrisafra® 04-20-20 was applied. All treatments received the same crop treatment, which was performed with applications of the fungicide Approach®Prima (300 ml.ha^{-1}) and the insecticide Belt® (70 ml.ha^{-1}).

4.2.6 Evaluation of the response variables related to the plant growth promotion and productivity

At the beginning of the flowering stage (R1 – 45 days after sowing), we measured the plant height of 5 plants per sampling point, totalizing 100 plants per treatment measured from the base of the plant (on the ground) up to the apex of the main stem using a metric table, according to Rocha et al. (2015). Crop lodging was assessed for each sampling point based on the average erectness of the main stem of plants at R8 (full maturity), according to Antwi-Boasiako (2017). The rating system applies a scale from 1 to 5, with 1 = all plants erect, 2 = 25% of plants lodged, 3 = 50% of the plants lodged, 4 = 75% plants lodged and 5 = all plants lodged.

Soybean harvest was carried out on April 3rd, 2019. Each harvesting strip had previously been marked along with the 20 sampling points, and they consisted of 2 rows of plants with 5 m each, which were evaluated for total grain yield and 100 seed weight. Five plants from each sampling point were kept for measurements of dry mass, stem diameter, pod number, and seeds per pod for production estimates.

4.2.7 Soybean seeds oil and protein content

The percentual of oil and protein content in soybean seeds was determined through near-infrared (NIR) spectroscopy, which is a quick strategy to determine seed composition without the destruction of the sample (JIANG, 2020). This analysis was performed at the Laboratory of Applied Biotechnology for Plant Breeding at Universidade Estadual Paulista, Jaboticabal – SP, Brazil. Data gathering was performed with clean whole soybean seeds from each treatment, divided into 20 biological replicates and 3 technical replicates in Bruker® FT-NIR TANGO spectroscopy equipment.

4.2.8 Soil sampling, DNA extraction, library construction and data processing

Soil sample for metagenomic analysis was collect at 20 sampling points within each treatment strip, at each time considered (Before – before sowing; CropR1 – during crop development at R1 stage; After – 21 days after total harvesting of soybean plants), respecting a 5 m border at each side of the strips. For each sampling point, 20 cm of soil was collected with the help of a soil probe. The material was immediately transported to the Laboratory of Genetics of Microorganisms at ESALQ/USP, Piracicaba-SP, Brazil, and stored at -80ºC until DNA extraction.

Material collected was separated for DNA extraction as follows: for the time Before, the 20 soil samples for each treatment were grouped in 1 composite sample, composed of 5 g of soil obtained after mixing the soil from the 20 original samples. DNA extractions of samples from time CropR1 and After were performed in composite samples that grouped the 20 samples from each treatment in 4 composite samples, each one with 5g of soil weighed from the mix of 5 sampling points.

Total DNA extraction was performed using the DNeasy PowerSoil® Kit (Qiagen). The quality of DNA extracted was assessed using agarose gel electrophoresis, and the quantification was performed using a NanoDrop One and a fluorometer Qubit 4 with the kit DNA High Sensitivity (ThermoFisher). Samples were then stored at 4º C. For library construction, samples were sequenced with the DNA Preparation kit Nextera Flex, and then their concentrations were checked using a QuBit 4, with the DNA High Sensitivity kit. Fragment sizes were assessed with the Bioanalyser DNA (Agilent Technologies), applying the kit DNA HS 2100 (Agilent Technologies). Whole metagenomic DNAs from samples were then sequenced with an Illumina NextSeq 500/550 High Output Kit platform for 300 base pairs readings (2 x 151), which may generate 400 million single reads.

For raw sequence quality analysis, we used FastQC v0.11.9 (ANDREWS, 2020) for raw read and MultiQC v.1.9 (EWELS et al., 2016) to generate an integrated report. Sequences were then trimmed using HTStream (v.1.3.2) for deduplication, and removal of adapter sequences and low-quality reads, followed by removal of short sequences (fewer than 30 bp) also with HTSream (v.1.3.2) and quality checked again with FastQC v0.11.9 and MultiqQC v.1.9. The unique sequence set was classified into operational taxonomic units (OTU) at 97% identity. Obtained reads were mapped to the soybean reference genome (Glycine_max_v4.00) using Bowtie2 (v.2.4.2) (LANGMEAD; SALZBERG, 2012). Non-host reads were retrieved in fastq format from the alignment files using the SAMtools (v.1.11) (LI et al., 2009) and BEDtools (v.2.27.0) (QUINLAN; HALL, 2010) software. Taxonomic analysis of trimmed reads was performed using Kraken2 (WOOD; SALZBERG, 2014) using NCBI RefSeq complete genomes database.

4.2.10 Data analysis

Field experiment data were statistically evaluated with ANOVA, followed by Tukey tests to compare means obtained for each treatment. All analysis was performed in R software (R Core Team 2017), and the significance level adopted in all tests was 5%.

Soil microbiome diversity analyzes were performed with Phyloseq package v.1.34.0 in R software (v.4.0.1). Alfa-diversity indexes (Shannon index, and Chao index) were tested for normal distributions with the Shapiro-Wilk test and for variance homogeneity with the Levene test. Chao1 index reflects species richness in samples without considering the abundance of each species. Shannon index reflects the species richness and evenness of the community

(QIAO et al., 2017). Indexes were then compared among treatments and time periods with Fisher's Least Significant Difference (LSD) test. All p-values (corrected) were calculated at 95% confidence intervals, and differences were considered significant when $p < 0.05$. Beta diversity among bacteria was determined using NMDS (Non-Metric Multi-Dimensional Scaling) analysis based on Bray–Curtis distances matrix between the soil samples. Taxonomic summary bar charts were created to determine the OTU abundance ratio (%) at the phylum level using ggplot package version 3.3.3 in R software (v.4.0.1).

4.3 Results

4.3.1 Co-inoculation of soybean with *Bt* RZ2MS9 and *Bradyrhizobium japonicum*

The results from plant height showed that Control group was substantially smaller than the other groups, while *B*t (*Bt* RZ2MS9) presented the highest values for plant height. *Bt*_rhizobia group (co-inoculated) and Rhizobia (inoculation with Masterfix® Soja) had an intermediate height between Control and *Bt* (Figure 2A). There was no strong statistical difference in stem diameter among groups (Figure 2B), and no difference was observed in shoot dry mass (Figure 2C). Co-inoculation with the two rhizobacteria provided protection against lodging, with group *Bt*_rhizobia presenting lower levels of lodging at the end of the crop cycle (Figure 2D).

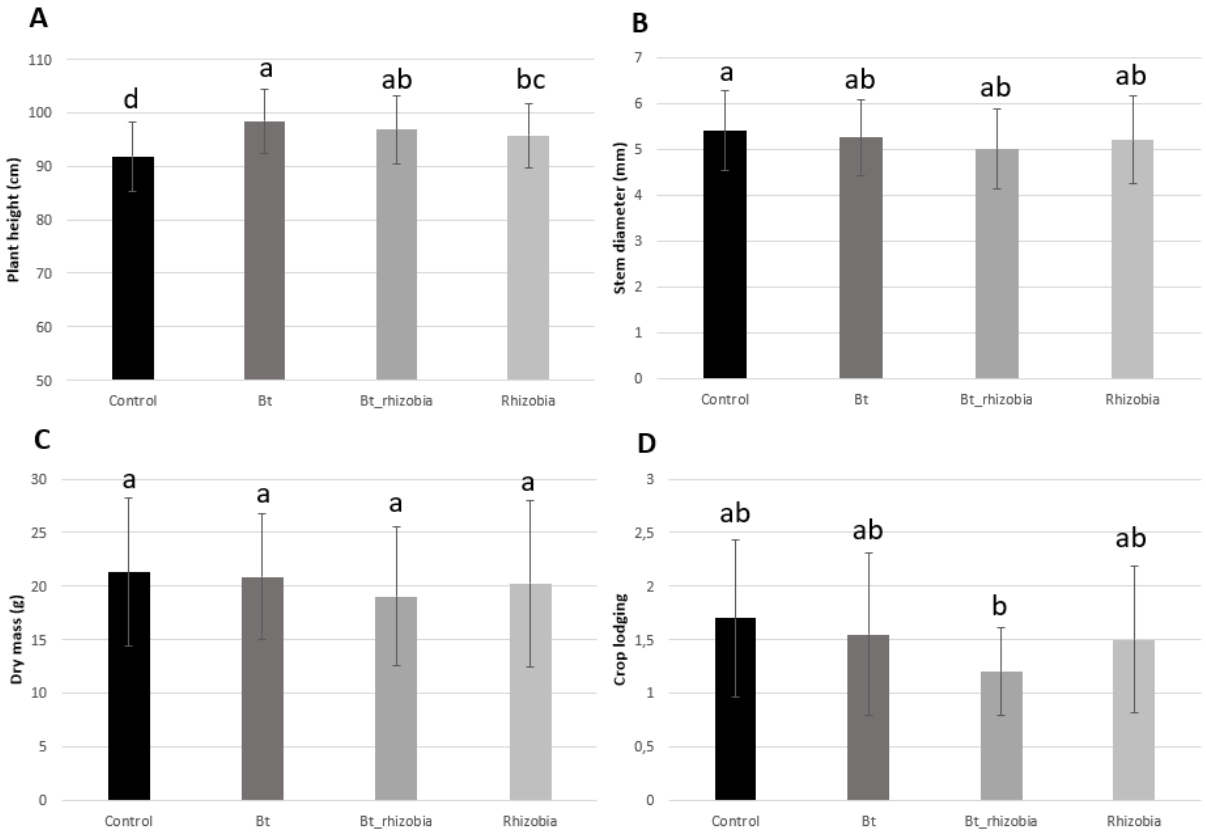

Figure 2. Average plant height (A) measured at the beginning of the flowering stage, stem diameter at harvest (B), dry mass at harvest (C), and crop lodging (D) for each treatment: Control (no inoculation), *Bt* (*Bt* RZ2MS9 inoculation), *Bt*_rhizobia (co-inoculation of *Bt* RZ2MS9 and rhizobia) and Rhizobia (only rhizobia inoculation). Different lowercase letters above the bars indicate statistical differences by the Tukey test ($p < 0.05$) between mean values (5 plants from each of the 20 sampling points, totalizing 100 replicates for each treatment).

On April 3rd (123 days after sowing), at the end of the crop cycle, soybean was harvested, and total grain yield from 20 sampling points was weighed, showing a slightly higher grain yield for *Bt* in comparison with Control and *Bt*_rhizobia. Group Rhizobia had a lower grain yield (Figure 3A). The average weight of 100 seeds from each treatment showed that groups without rhizobia (Control and *Bt*) had lower seed weight than groups inoculated with rhizobia (*Bt*_rhizobia and Rhizobia) (Figure 3B).

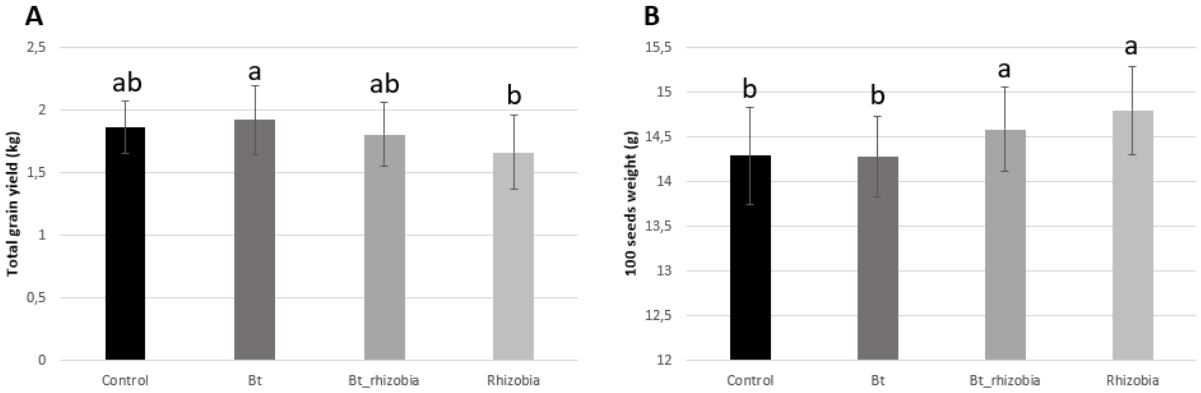

Figure 3. Average total grain yield (A) harvested from 2 rows of 5m each of soybean plants, and 100 seeds weight (B) for each treatment: Control (no inoculation), *Bt* (*Bt* RZ2MS9 inoculation), *Bt*_rhizobia (co-inoculation of *Bt* RZ2MS9 and rhizobia) and Rhizobia (only rhizobia inoculation). Different lowercase letters above the bars indicate statistical differences by the Tukey test ($p < 0.05$) between mean values (20 harvesting points for total grain yield and 3 replicates for each harvest point for 100 seeds weight).

Analysis of pod number per plant showed Control had fewer pods per plant, and all other treatments were similar (Figure 4A). Average total grain yield was used to estimate productivity in kg.ha^{-1}, showing that inoculation with *Bt* RZ2MS9 did not differ from Control or *Bt*_rhizobia in productivity, although these three groups had higher productivity than sole Rhizobia inoculation (Figure 4B).

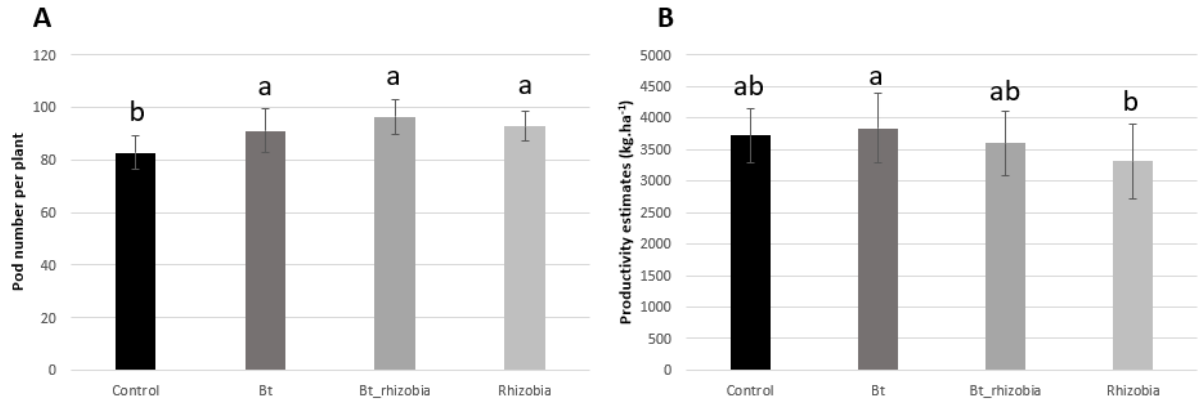

Figure 4. Average pod number per plant (A) and productivity estimates (B) for each treatment: Control (no inoculation), *Bt* (*Bt* RZ2MS9 inoculation), *Bt*_rhizobia (co-inoculation of *Bt* RZ2MS9 and rhizobia), and Rhizobia (only rhizobia inoculation). Different lowercase letters above the bars indicate statistical differences by the Tukey test ($p < 0.05$) between mean values (5 plants from each harvesting point for pod number and estimates for average total grain yield for each treatment).

4.3.2 Soybean seeds oil and protein content

No effect of inoculations was observed on oil and protein content from the seeds, with all treatments presenting very similar results (Figure 5).

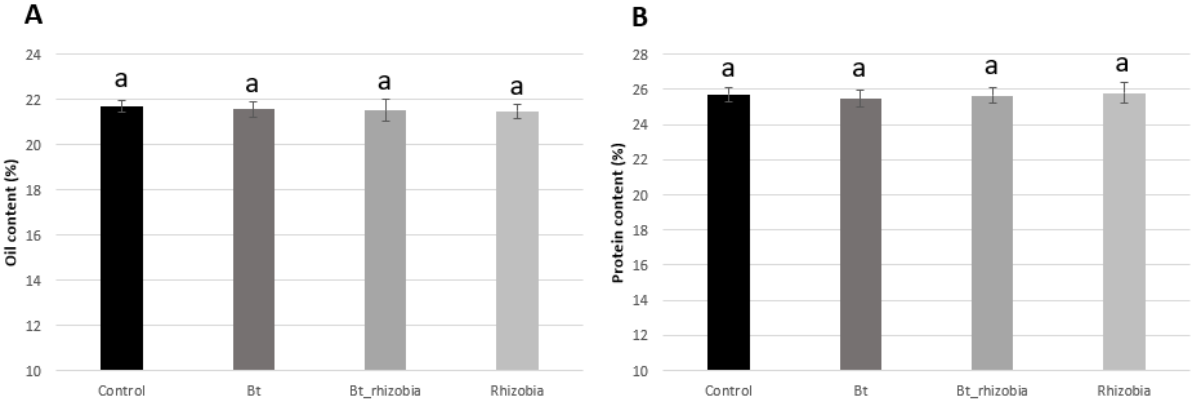

Figure 5. Average oil content (A) and protein content (B) of seed harvested at each treatment: Control (no inoculation), *Bt* (*Bt* RZ2MS9 inoculation), *Bt*_rhizobia (co-inoculation of *Bt* RZ2MS9 and rhizobia) and Rhizobia (only rhizobia inoculation). Different lowercase letters above the bars indicate statistical differences by the Tukey test ($p < 0.05$) between mean values (20 biological replicates and 3 technical replicates for each treatment).

4.3.3 Soil microbiome diversity analysis

To investigate the hypothesis that inoculations interfere with the microbial community structure, alpha diversity (within each sample) was analyzed based on the Chao1 and Shannon diversity indexes (Table 2). Alpha diversity analysis showed that Chao1 index did not differ among the three different times compared ($p > 0,05$), nor during treatments during crop cycle (CropR1), but it was lower for the *Bt* (*Bt* RZ2MS9) and *Bt*_rhizobia (co-inoculated group) after crop cycle (After) ($p = 0.02973$) (Figure 6).

Table 2. Average Chao and Shannon diversity indices of bacterial community for tested treatments - Control, *Bt* (*Bt* RZ2MS9), *Bt*_rhizobia (co-inoculation with *Bt* RZ2MS9 and rhizobia), and Rhizobia, at the times Before sowing, Crop at R1, and After (21 days after harvest). Means followed by the same letter do not differ by the LSD test ($p < 0.05$). Lowercase letters compare all treatments over time; uppercase letters compare treatments within the same time period.

Treatment	Chao	Shannon
Before	8767,936 ^{a}	7,154627^{aA}
Control_CropR1	8896,073 ^{aA}	7,172326 ^{aA}
Bt_CropR1	9037,952 ^{aA}	7,151549 ^{aA}
Bt_rhizobia_CropR1	8898,077 ^{aA}	7,166659 ^{aA}
Rhizobia_CropR1	8863,468 ^{aA}	7,147447 ^{aA}
Control_After	9097,947 ^{aAB}	7,185322 ^{aA}
Bt_After	8974,196 ^{aBC}	7,165308 ^{aA}
Bt_rhizobia_After	8930,44 ^{aC}	7,168684 ^{aA}
Rhizobia_After	9145,398 ^{aA}	7,168137 ^{aA}

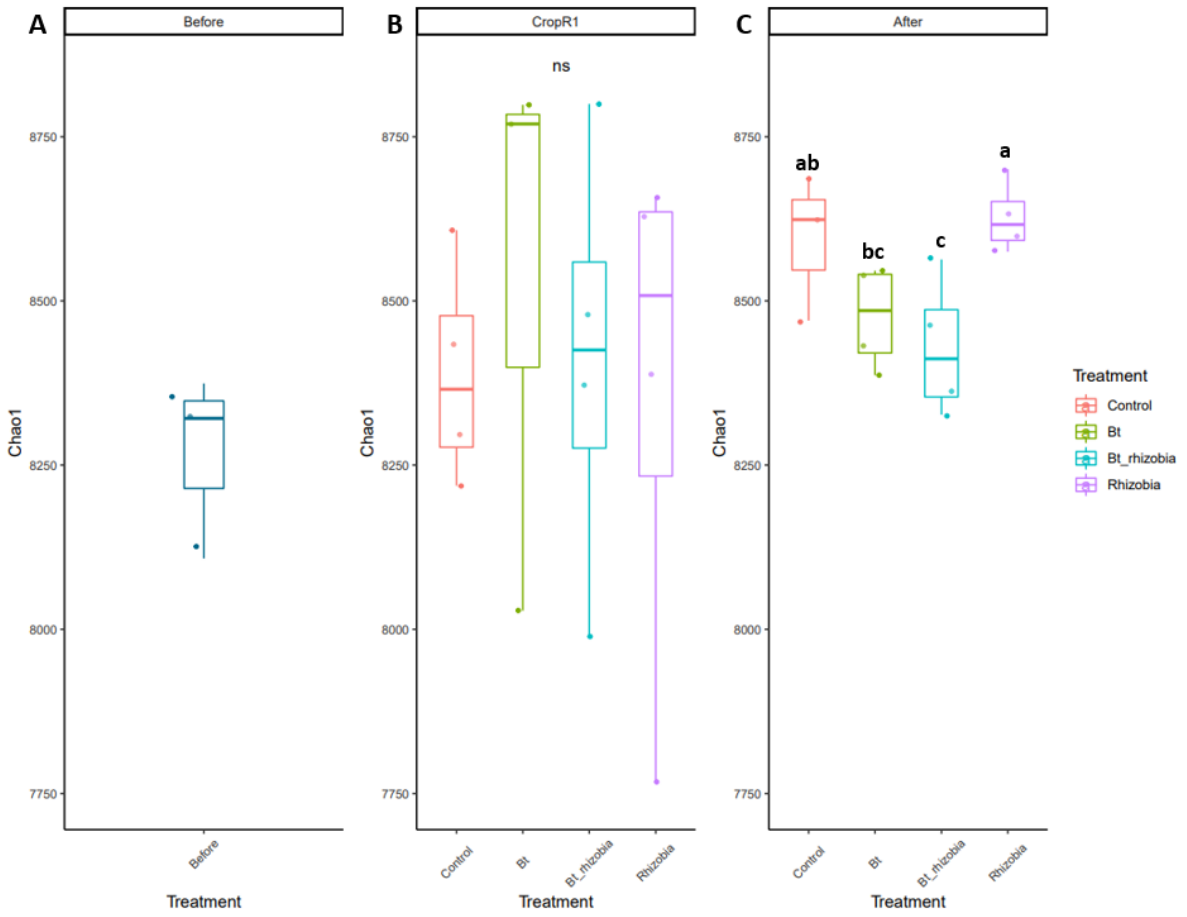

Figure 6. Alpha diversity of the soil bacterial community according to the Chao1 index before crop cycle (A), at R1 stage (B) and 21 days after crop cycle (C), for the treatments: Control, *Bt* (*Bt* RZ2MS9), *Bt*_rhizobia (co-inoculation with *Bt* RZ2MS9 and rhizobia), and Rhizobia. (NS = not significative). Boxplots with different letters above the boxes denote means that are significantly different ($p < 0.05$).

Shannon index for alpha diversity analysis showed no significant difference among time periods or among treatments within each period ($p > 0,05$) (Figure 7).

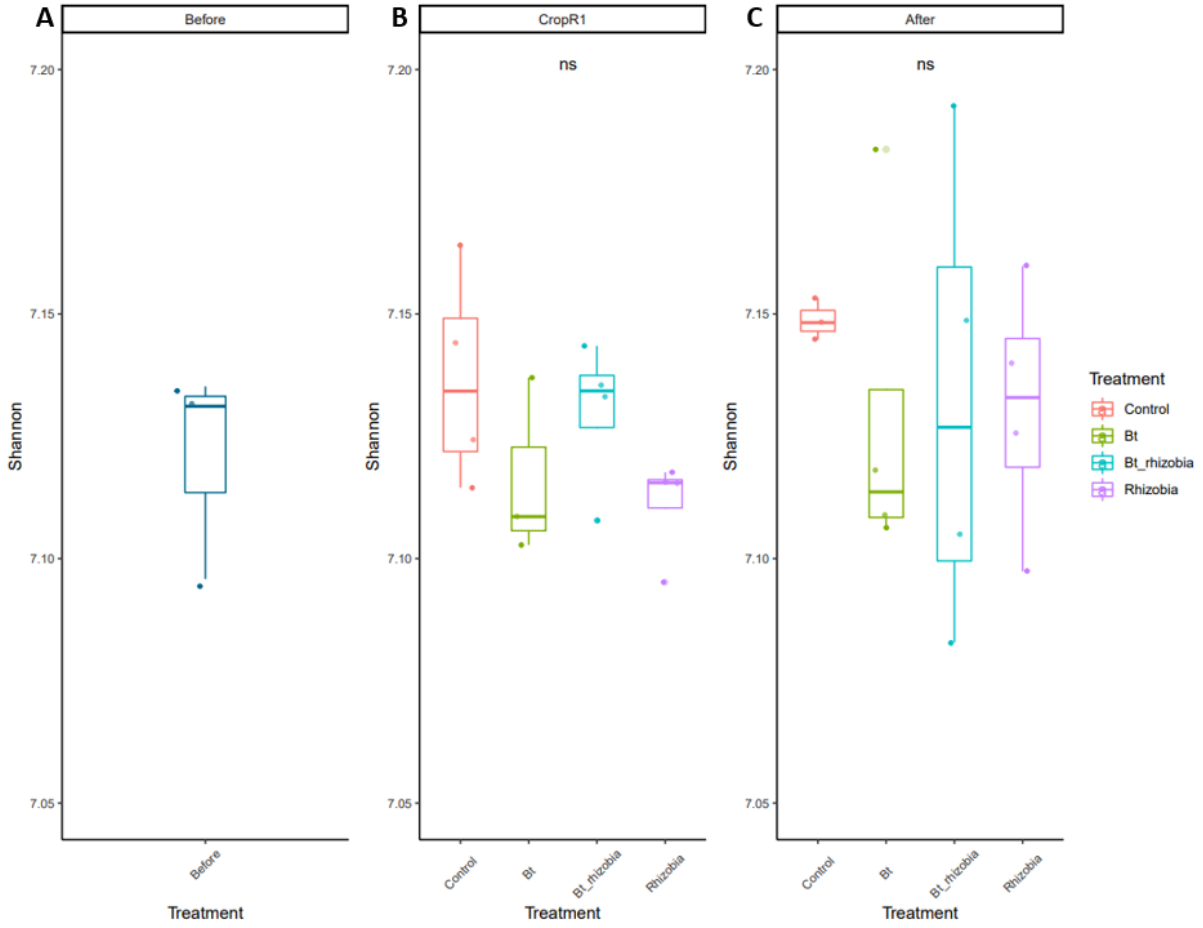

Figure 7. Alpha diversity of the soil bacterial community according to the Shannon index before crop cycle (A), at R1 stage (B) and 21 days after crop cycle (C), for the treatments: Control, *Bt* (*Bt* RZ2MS9), *Bt*_rhizobia (co-inoculation with *Bt* RZ2MS9 and rhizobia), and Rhizobia. (NS = not significative; $p < 0.05$).

To assess beta diversity, differences between inoculation treatments and time were compared through non-metric multidimensional scaling ordination, which allowed for visualization of complex datasets in two dimensions. In interpreting these plots, dots that are closer together represent samples with more similar bacterial profiles (Figure 8).

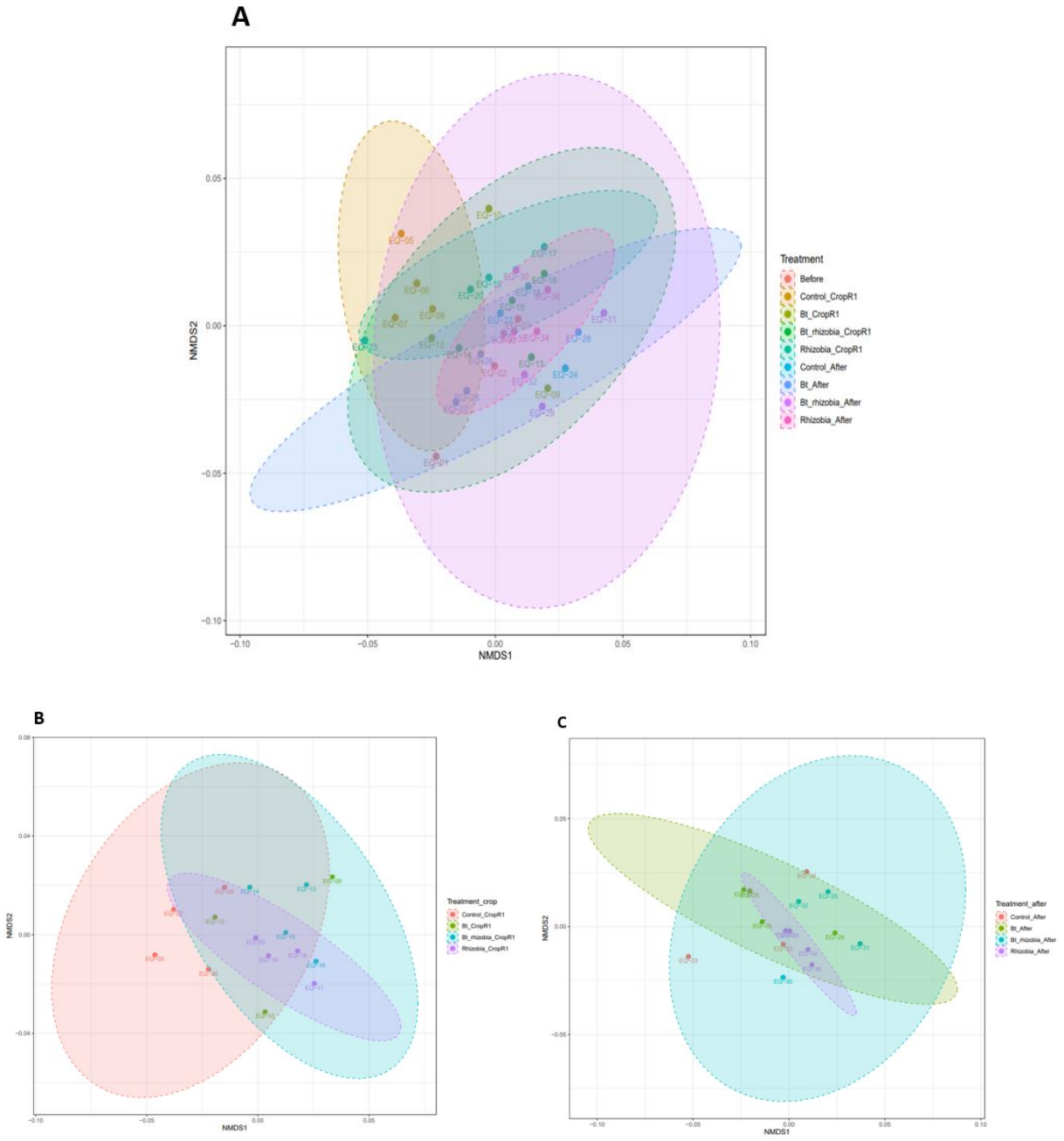

Figure 8. Comparison of soil bacterial diversity among inoculation treatments and times of the soybean crop cycle by beta diversity analysis. Data is shown with non-metric multidimensional scaling (NMDS) plots, grouping samples in ellipses. Images consider all samples from the 3 times (Before, Crop at R1, and 21 days after harvest) and treatments (A), only samples from the crop at R1 stage – CropR1 (B), and only samples from 21 days after harvest – After (C). Treatments are Control, *Bt* (*Bt* RZ2MS9), *Bt*_rhizobia (co-inoculation with *Bt* RZ2MS9 and rhizobia), and Rhizobia.

Relative abundance of bacterial taxa at the phylum level for all treatments showed high homogeneity, including when considering the time variable (Before, CropR1 and After). All bacterial OTUs were further assigned into 37 phyla, 75 classes, 174 orders, 403 families and 1513 genera, including some unclassified groups. The main phyla were Proteobacteria (48,7%),

Actinobacteria (44,4%), Planctomycetes (1,7%) Firmicutes (1,2%), Acidobacteria (0,95%) and Bacteroidetes (0,91%) (Figure 9).

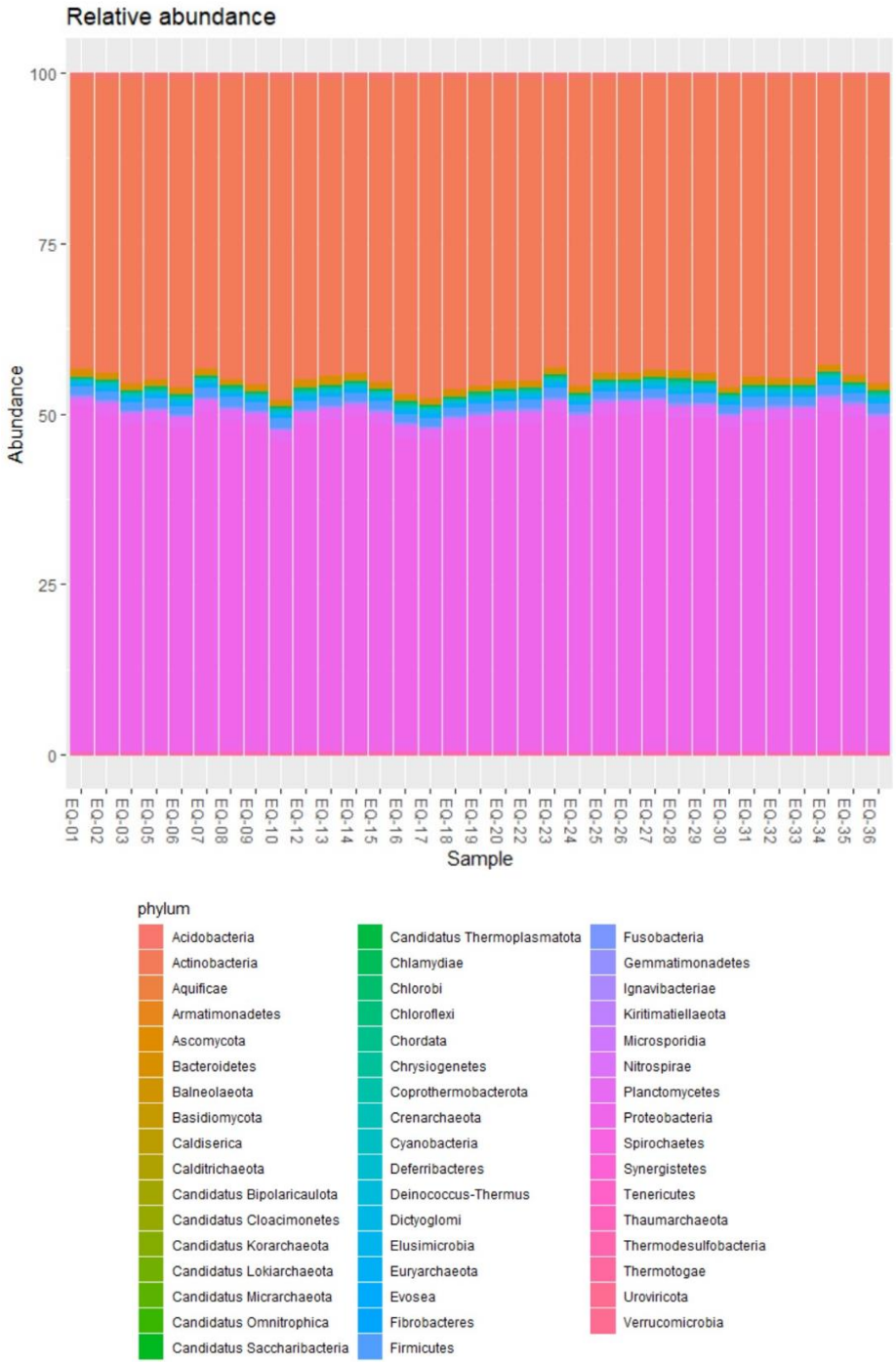

Figure 9. Relative abundance of bacterial taxa at the phylum level. Samples from EQ-01 to EQ-03 correspond to soil samples collected before sowing, EQ-05 to EQ-20 are from the R1 stage of soybean cycle, with 3-4 repetitions for each treatment, and EQ-21 to EQ-36 are from 21 days after harvest, with 3-4 repetitions for each treatment (Control, *Bt* (*Bt* RZ2MS9), *Bt*_rhizobia (co-inoculation with *Bt* RZ2MS9 and rhizobia), and Rhizobia).

4.4 Discussion

4.4.1 Effects of co-inoculation on soybean development

Low-cost agricultural technologies are currently being extensively studied worldwide in search of new strategies to help increase food offer under sustainable production models (SÁ et al., 2017). Microorganisms have notorious participation in this field, having received vast attention from researchers and farmers during the last decades when the application of bioinoculants in agriculture has become a common practice (FUKAMI et al., 2018; BELLABARBA et al., 2019; SANTOS et al., 2019).

Soybean (*Glycine max* (L.) Merr.) is one of the crops that most apply inoculants worldwide, using mainly a range of bacteria belonging to the genus *Bradyrhizobium* (SANTOS et al., 2019). Dubey (1996) and Wasule et al. (2007) showed that co-inoculation of rhizobia in consortium with other PGPR significantly improved soybean growth and grain yield compared to the sole application of rhizobia.

The bacterial strain studied here, *Bt* RZ2MS9, has already been shown to cause positive results when inoculated on soybean and maize (BATISTA, 2017; BATISTA et al., 2018; LONGATTO, 2020). Considering the potential of this strain as a bioinoculant, this study presents the first evaluation of the co-inoculation of rhizobia and the *Bt* RZ2MS9 and its effects on soybean and on the soil bacterial diversity in a field experiment.

In PGPR, the mechanisms related to the plant growth-promoting effect involve biological processes like indole acetic acid (IAA) production, phosphate solubilization and urease activities, with a direct impact on the nutrient and water uptake by the plant (KHAN et al., 2019). A previous study with *Bt* RZ2MS9 showed that this strain is able to produce IAA in the presence of L-tryptophan (BATISTA et al., 2021), which may be attributed to the nature of the strain to utilize L-tryptophan as a physiological precursor (SPAEGEN et al., 2007). Several strains of *B. thuringiensis* have been used to promote plant growth, and the findings of this study are consistent with those reports (VIDAL-QUIST et al., 2013; TAGELE et al., 2019; VILJOEN et al., 2019; JO et al., 2020).

In congruence with previous studies with this strain (BATISTA, 2017; BATISTA et al., 2018; LONGATTO, 2020), the inoculation with *Bt* RZ2MS9 increased plant growth. The average shoot length of treatments inoculated and co-inoculated with this strain were greater than control and the group inoculated with only rhizobia, but there was no significant effect on

shoot dry mass, stem diameter, or productivity. However, a tendency to a higher productivity in groups inoculated with *Bt* RZ2MS9 was seen in this study, which has been reported previously (BATISTA, 2017; BATISTA et al., 2018). Likewise, the study of Atieno et al. (2012) observed an increase in average yields of soybean co-inoculated with *Bacillus subtilis* and *B. japonicum,* but with no statistical significance.

Similar results were reported by Elkoca et al. (2010) in soybean, with no significant increase in yield from co-inoculants containing *B. japonicum* and *B. subtilis.* Even though rhizobacteria from the genus *Bacillus* are commonly seen to interact positively with plants, different species and strains within the same species may have an effect on other aspects of plant growth, such as the production of phytohormones, improvement of drought resistance, and pathogen suppression, that may not be directly correlated to expressive increments in grain yield in field conditions where those variables where not targeted in experimental design (ELKOCA et al., 2010; TSIGIE et al., 2012). Experimentation with different plant species and different environmental conditions may show different plant growth-promoting features.

In a study with another strain of *Bt* (A5-BRSC), Bandopadhyay (2020) tested the inoculation of this strain on the development of okra, with a significant increase in seed germination, shoot height, root length, leaf diameter, vigor index, fruit weight, seed weight, and total fresh weight as well as dry weight of inoculated plants in comparison to the non-inoculated control group.

Hungria et al. (2013) observed an increase of 420 $kg.ha^{-1}$ (16,1%) in soybean production co-inoculated with *Bt japonicum* and *Azospirillum brasilense* compared to control treatment inoculated only with *B. japonicum.* However, Zuffo et al. (2016) reported no significant differences in productivity in soybean co-inoculated with *B. japonicum* and *A. brasilense,* and the control group only inoculated with the former bacterium. A study with *Bacillus subtilis* in co-inoculation with *B. japonicum* in soybean by Atieno et al. (2012) showed increased soybean nodulation and biomass traits. Thus, what is not clear is the impact of co-inoculation on soybean grain yield (ZEFFA et al., 2020).

The co-inoculation in this study showed a possible protecting effect against crop lodging, with a significant reduction in crop lodging occurring only in the co-inoculated group and not on the single inoculation treatments. This protective effect may be caused by the increased bioavailability of potassium provided by the bacteria applied. According to Zaki et al. (2012), species from the soil microbiome can solubilize potassium in the environment and make it more available to plants, leading to plant growth. The role of potassium on plant physiology includes maintaining turgor, reducing water loss and wilting, and reducing lodging

(MEENA et al., 2015). In a study with inoculation of *Bacillus cereus* on potato, Ali et al. (2021) observed increased potassium solubilization and intake by the plant and increased plant height, branch number, and shoot dry weight. Further investigation of the effects of *Bt* RZ2MS9 on potassium solubilization may provide information about this possible role as a biofertilizer when co-inoculated with *B. japonicum.*

The sole inoculation with *Bt* RZ2MS9 did not cause an increase in 100 seed weight, but all treatments inoculated with rhizobia, including the co-inoculated group, had a higher 100 seed weight than Control and *Bt*. There was an increase in pod number in all inoculated groups: *Bt*, *Bt*_rhizobia (Co-inoculated) and Rhizobia (only rhizobia) compared to Control. s may interfere with the soil nutrient availability to the plant, and thus, with the grain production, which has shown difference according to the type of formulation used to inoculate the crop (MAITRA et al., 2021). The strain *Bt* RZ2MS9 was applied on the seeds in the form of a bacterial suspension in saline solution, which can be tested in other formulations to assess a possible stronger effect on pod number, 100 seed weight, and overall soybean productivity.

Protein and oil content on soybean seeds in this study did not vary among groups. However, Sheteiwy et al. (2021) tested the effect of co-inoculation of *Bacillus amyloliquefaciens* and mycorrhiza on soybean under drought stress and they could observe an increase of protein and oil content in seeds from inoculated plants cultivated under drought stress in relation to the control group. Yasmin et al. (2020) observed the same results of increased oil and protein content when testing the co-inoculation effects of *Pseudomonas pseudoalcaligenes* and *Bacillus subtilis* in soybean under salinity stress. Therefore, the inoculation of both bacteria tested in this study under the same conditions of salinity and irrigation may not have shown a potential protective effect of these rhizobacteria against drought and salinity stresses, which can be assessed with different experimental conditions.

Besides that, Barbosa et al. (2021) showed that other variables, like soybean growth habit, climate, soil texture, and management system, affect co-inoculation results, and thus they should be considered in determining the inoculation strategy to be applied. Thus, further experimentation considering different experimental conditions or plant species can reveal other potential benefits from the use of *Bt* RZ2MS9 in co-inoculation strategies.

4.4.2 Effects of co-inoculation on microbiome diversity

Based on the positive interactions of PGPR species and plant hosts, the agriculture industry is increasingly interested in the application of these rhizobacteria to improve crop production (NUZZO et al., 2020). However, two challenges to this approach are the limited number of microorganisms of known beneficial characteristics that can be cultivated under laboratory conditions (STEWART, 2012), and the formulation of synthetic microbial consortia (NUZZO et al., 2020). Particular attention must be given to avoid predation or amensalism phenomena within the microbial consortia in favor of survival and increased abundance after deployment to field soils (GROßKOPF; SOYER, 2014). Other factors affecting the efficacy of soil microbial inoculants include competition of inoculated microorganisms with the native soil microbiota, inoculum shelf life, plant genotype interactions, soil properties, and other agricultural practices (KAMINSKY et al., 2019).

In this study, inoculation with *Bt* RZ2MS9 and rhizobia showed little interference on the native soil microbiome. For bacterial diversity, the Proteobacteria and Actinobacteria were predominant in all soil samples. Both phyla are commonly related to plant growth promotion since they are able to promote degradation of aminocyclopropane carboxylate and act on the suppression of root diseases (JORQUERA et al., 2012; ZHANG et al., 2019). Analysis of percentual abundance and beta diversity over time do not show clear impacts of either sole inoculations or co-inoculation on bacterial soil diversity.

As for species richness assessed through alfa diversity, inoculation with *Bt* RZ2MS9 caused a decrease in Chao1 index in the two treatments from the After group inoculated with this strain (*Bt* and *Bt*_rhizobia), with the lowest Chao1 index occurring for the co-inoculated group *Bt*_rhizobia. This was also reported by Lebrun et al. (2021), in their study with inoculation of *Bacillus* sp. on As- and Pb-contaminated technosol. The authors noted that direct inoculation of *Bacillus* sp. caused a significant decrease in richness, which could be explained by competition between the inoculated isolate and the microorganisms already present in the soil. That could have led to the disappearance of some species less abundant or less competitive (LEBRUN et al., 2021).

Nevertheless, the reduction in Chao1 index observed here was significant only among treatments from After group, and not among the three times measured, which indicates that there was not an overall reduction in species richness with time. Bai et al. (2020) also found a small influence of inoculation with *Pseudomonas fluorescens* CLW17 and *Bacillus*

cereus CLY07 on rhizosphere microbial diversity over time, with both inoculants promoting plant growth. Considering that beta diversity analysis in this study also did not show an impact of inoculations over time, the application of *Bt* RZ2MS9 and rhizobia inocula seems to be safe for environmental application. Further experimentation, however, considering a longer time period and different environmental conditions, are necessary for a final conclusion.

Changes in soil bacterial community structure due to the inoculation of a *B. thuringiensis* strain were reported by Jo et al. (2020), and such effect also occurred after six weeks of inoculation, which is consistent with the findings reported here. A similar time interval was seen by Ke et al. (2019) in a study with inoculation of *Pseudomonas stutzeri* A150, in which the changes in indigenous soil bacterial community structure were seen after 2 months of inoculation. In this study, soil sampling for the diversity analysis during crop development occurred 45 days after sowing the inoculated seeds, and sampling after harvest occurred 144 days after inoculation. This emphasizes the importance of future analysis on the impacts over time of *Bt* RZ2MS9 inoculation on soil bacteria diversity.

Chen et al. (2020) tracked soil bacterial community responses to long-term monocropping of four different peanut varieties and proposed that there is a general loss in bacterial diversity and abundance over time, with long monocropping periods having a stronger influence on bacterial communities than peanut variety and growth stage. That information should be taken into consideration when analyzing the environmental implications of the use of bioinoculants, since they might contribute or mitigate such effects according to their interaction with indigenous soil microbiota.

In this first study on the co-inoculation of the PGPR strain *Bt* RZ2MS9 and rhizobia, it was seen that bacterial consortia caused an increase in soybean growth without disrupting soil bacterial diversity over time. This positive synergistic effect among rhizobacteria is a promising conclusion to be further investigated under different conditions to help plan new sustainable crop management strategies.

A broader understanding of PGPR interaction with both host plants and soil microbiome may allow for the validation of crop-specific inoculants tailored to specific soil or climatic environments (NUZZO et al., 2020). Accessory mechanisms of *Bt* RZ2MS9 to improve crop development under stress may be seen with future experimental conditions that will contribute to a better understanding of the dynamics of rhizobacteria consortia in the soil, adding layers of crop improvement to specific environmental conditions.

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5. Final Considerations

The inoculation with *Bacillus thuringiensis* strain RZ2MS9 was able to improve soybean height in two different field experiments. There was no significant increase in productivity, even though both field experiments showed a tendency to a higher grain yield when soybean seeds were inoculated with *Bt* RZ2MS9. Further testing with different experimental conditions focused on crop development under stressful conditions regarding salinity, pH, soil contaminants, and draught, might reveal specific positive effects of this strain on different plant species.

The role of *Bt* RZ2MS9 as a biocontrol agent against stink bugs has a promising perspective for future tests with foliar application of the strain against *Piezodorus guildinii*, since the experiment of dual choice arena showed that treated green pods repelled the insect. It is also worthy of new investigations the indirect effect of inoculation of seeds on the attraction of stink bugs by the plants seen in Chapter 2. Analysis of the volatile organic compounds released by inoculated plants and their effect on stink bugs attractivity might explain the field results of higher incidence of *P. guildinii* on *Bt* RZ2MS9 inoculated groups.

Testing of the mVOCs produced by *Bt* RZ2MS9 grown on different growth media might show different compounds that RZ2MS9 may be able to produce, with possible biotechnological applications. This approach could also further investigate the negative effects on the soybean germination shown in Chapter 3 caused by the mVOCs produced by the strain grown on LBA. Tests with commercial patterns of the candidate compounds could confirm the identification here proposed, and then enable a broader comprehension of the effect of these compounds on plant-bacterium interaction.

The methodology applied in Chapter 3 to investigate possible antagonistic effects of *Bt* RZ2MS9 on the development of phytopathogenic fungi could not identify such activity. Other methodologies, however, should be tested to evaluate other possibilities of use of this strain as a biocontrol agent, including different growth media and also the direct diffusion of metabolites on the media besides mVOCs effects.

Co-inoculation of *Bt* RZ2MS9 and rhizobia showed increased height in plants inoculated with the former bacterium, and a lower occurrence of plant lodging. Further experimentation focused on lodging in inoculated plants could promote a better understanding of this effect and thus evaluate a possible application of this co-inoculation on crop improvement. In terms of productivity, as mentioned above, co-inoculation in different environmental conditions could also be tested to show possible protective effects of the *Bt*

RZ2MS9 when applied in co-inoculation. This approach shows promising future possibilities since this thesis has shown for the first time that there was no negative effect of the co-inoculation tested on the soil microbiome diversity over time, and thus it seems that *Bt* RZ2MS9 is safe for environmental application. Further testing considering a longer period should also be considered to corroborate the results found in this study.