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JHOICE FERREIRA BORGES

Bacillus subtilis AND *Bacillus amyloliquefaciens* DISTINCTLY SUPPRESS *Pratylenchus* spp. AND SHAPE RHIZOSPHERE MICROBIAL COMMUNITIES IN LIMA BEAN GENOTYPES

Bom Jesus – PI
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Agrárias - PPGCA, Campus Professora Cinobelina Elvas - CPCE, da Universidade Federal do Piauí - UFPI, na área de concentração Ciência do Solo, como requisito para obtenção do título de Mestre em Ciências Agrárias.

Orientador: Prof. Dr. Ademir Sérgio Ferreira de Araújo
Coorientador: Prof. Dr. Heriksen Higashi Puerari

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o amor da minha vida <3.

Dedico.

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"Somente a fé remove a desordem mental e devolve a clareza de espírito"

Dalai Lama

ABSTRACT

Bacillus species are well known for their ability to efficiently control root nematodes in plants, particularly those causing root lesions, such as *Pratylenchus* spp. However, the effectiveness of this control can vary among different *Bacillus* species. Additionally, these bacteria can influence rhizosphere microbial communities, further contributing to nematode suppression. In this study, we evaluated the effects of *B. subtilis* and *B. amyloliquefaciens*, both individually and in co-inoculation, on the suppression of *Pratylenchus* spp. and on shifts in the rhizosphere microbial communities of lima bean (*Phaseolus lunatus*) genotypes, including landraces and improved lines. Specifically, we assessed *Pratylenchus* spp. populations in soil and root samples following bacterial application. Moreover, we analyzed potential changes in the rhizosphere microbial communities of these genotypes. Our results revealed that inoculation with both *Bacillus* species reduces *Pratylenchus* populations in the roots, with *B. subtilis* showing stronger suppression effects, particularly in the landrace lima bean genotype. Co-inoculation increased microbial richness and diversity in the rhizosphere. The rhizosphere microbiome was dominated by Actinobacteria, Proteobacteria, and Firmicutes, with *B. subtilis* and *B. amyloliquefaciens* influencing the abundance of specific bacterial genera. Inoculation also altered predicted functions related to nitrogen cycling and organic matter processing, revealing genotype-specific microbial responses. This study demonstrates that both *Bacillus* species can effectively suppress *Pratylenchus* in lima bean roots and distinctly shape rhizosphere microbial communities.

Keywords: 16S rRNA, Biocontrol, Microbiome, Nematode suppression, *Phaseolus lunatus*.

RESUMO

As espécies do gênero *Bacillus* são conhecidas por controlar eficientemente nematoides em raízes de plantas, especialmente aqueles que causam lesões radiculares, como *Pratylenchus* spp. No entanto, a eficácia desse controle pode variar entre as diferentes espécies de *Bacillus*. Além disso, essas bactérias podem influenciar as comunidades microbianas da rizosfera, contribuindo ainda mais para o controle de nematoides. Neste estudo, avaliamos os efeitos de *B. subtilis* e *B. amyloliquefaciens*, tanto individualmente quanto em co-inoculação, na supressão de *Pratylenchus* spp. e nas mudanças nas comunidades microbianas da rizosfera de genótipos de feijão-fava (*Phaseolus lunatus*), incluindo variedades crioulas e melhoradas. Especificamente, avaliamos as situações de *Pratylenchus* spp. em amostras de solo e raízes após a aplicação das bactérias. Além disso, analisamos as possíveis alterações nas comunidades microbianas da rizosfera desses genótipos. Nossos resultados revelaram que a inoculação com ambas as espécies de *Bacillus* reduz as populações de *Pratylenchus* nas raízes, sendo que *B. subtilis* apresentou maiores efeitos de supressão, particularmente no genótipo crioulo de feijão-fava. A co-inoculação aumentou a riqueza e a diversidade microbiana na rizosfera. O microbioma da rizosfera foi dominado por Actinobacteria, Proteobacteria e Firmicutes, com *B. subtilis* e *B. amyloliquefaciens* influenciando a abundância de gêneros bacterianos específicos. A inoculação também alterou funções preditas relacionadas ao ciclo do nitrogênio e à manipulação de matéria orgânica, revelando respostas microbianas específicas para cada genótipo. Este estudo demonstra que ambas as espécies de *Bacillus* podem suprimir eficazmente *Pratylenchus* nas raízes do feijão-fava e moldar distintamente as comunidades microbianas da rizosfera.

Palavras-chave: 16S rRNA, Biocontrole, Microbioma, *Phaseolus lunatus*, Supressão de nematoides.

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SUMMARY

1 INTRODUCTION

Plant-parasitic nematodes are among the most significant soilborne pests, substantially reducing agricultural productivity and affecting both economically important and subsistence crops (Van Den Hoogen *et al*., 2019). These pathogens parasitize various plant organs, including roots, stems, leaves, and seeds. Roots are more severely affected by nematodes than aerial plant parts, impairing water and nutrient uptake, which leads to reduced plant growth and, in severe cases, plant death (Bernard; Egnin; Bonsi, 2017). Specifically, the genus *Pratylenchus*, commonly known as root-lesion nematodes, consists of migratory parasites that cause root lesion diseases in many host plants (Orlando *et al*., 2020).

Several strategies have been employed to control plant-parasitic nematodes, including *Pratylenchus*, such as biological control and chemical nematicides (Zasada *et al*., 2018; Sikandar *et al*., 2020;). However, commonly used nematicides are often costly, provide short-term efficacy, and pose risks to human health and the environment (Forghani; Hajihassani, 2020). Additionally, no nematicides have been approved for lima bean cultivation in Brazil. In contrast, biological control offers the advantage of being both environmentally friendly and long-lasting (Contreras-Cornejo *et al*., 2019).

Biological control of root pathogens involves the use of soil microorganisms, such as bacteria, that antagonize these pathogens. Specifically, in root nematode control, certain bacteria produce antibiotics and metabolites, disrupt nematode signaling, and compete for ecological niches (Subedi *et al*., 2020). A recent study demonstrated the effect of soil bacterial diversity on suppressing nematode infections in soybean, highlighting the crucial role of specific microbial groups in nematode deterrence (Barros *et al*., 2022).

Among well-known bacterial genera involved in biological control, *Bacillus* has shown considerable potential as a bionematicide while also promoting plant growth (Bao *et al*., 2021; Costa *et al*., 2023). For instance, *B. subtilis* produces essential antibiotics, such as iturin, which disorient *Pratylenchus* and release hormones that benefit plant growth (Radhakrishnan; Hashem; Abd-Allah, 2017; Miljaković; Marinković; Balešević-Tubić, 2020; Saxena *et al*., 2020). While *Bacillus* can directly act against parasitic nematodes, its inoculation in plants may also drive shifts in

rhizosphere microbial communities (Hashem; Tabassum; Abd-Allah, 2019). Indeed, previous studies have reported that *Bacillus* can influence plants to recruit beneficial microorganisms, potentially enhancing pathogen control and plant growth (Shi *et al*., 2022; Xiao *et al*., 2022; Nishisaka *et al*., 202;). For example, Nishisaka *et al*. (2024) evaluated *B. subtilis* inoculation in tomatoes and observed an enrichment of bacteria belonging to the phyla Verrucomicrobiota, Patescibacteria, and Nitrospirota in the rhizosphere. Similarly, Cunha *et al*. (2024) showed that *Bacillus* inoculation increased mycorrhization rates in common beans, indirectly improving plant phenotypes.

It is also well established that rhizosphere microbial recruitment varies among plant genotypes (Mendes *et al*., 2018a). Traditional genotypes, for example, recruit distinct microbial communities compared to modern cultivars, likely in response to root trait modifications resulting from plant breeding (Lund *et al*., 2022; Nunna; Balachandar, 2024). While studies have demonstrated that different plant genotypes can influence rhizosphere microbial community recruitment, little is known about the effect of *Bacillus* in steering the rhizosphere toward recruiting distinct microbial communities. This is particularly relevant, as *Bacillus*-induced changes in microbial communities may have long-term impacts on soil and plant health (Pérez-Jaramillo; Mendes; Raaijmakers, 2016; Leff *et al*., 2017; Soldan *et al*., 2021), as well as improve pathogen biocontrol.

Thus, this study evaluated the effect of two distinct *Bacillus* species (*B. subtilis* and *B. amyloliquefaciens*) on the suppression of *Pratylenchus* spp. and the modulation of rhizosphere microbial communities in lima bean (*Phaseolus lunatus* L.) landrace and line genotypes. Our hypothesis is that (i) different *Bacillus* species distinctively control *Pratylenchus* populations in lima bean roots and soil, and (ii) single and co-inoculation with different *Bacillus* species uniquely shape rhizosphere microbial communities, with these effects varying between lima bean landrace and line genotypes.

2 LITERATURE REVIEW

2.1 Origin and socioeconomic importance of lima bean (*Phaseolus lunatus* L.)

Lima bean (*Phaseolus lunatus* L.) is a legume belonging to the Fabaceae family (Kaplan, 2003), originating from Mesoamerica and the Andes and domesticated in the Americas (Mackie, 1943; Serrano-Serrano *et al*., 2010; Andueza-Noh *et al*., 2013; Bitocchi *et al*., 2017). The genus *Phaseolus* includes several species, among which *P. lunatus* stands out as one of the five species that underwent domestication processes (Bitocchi *et al*., 2017). It is the second most economically important species in the genus, behind only the common bean (*P. vulgaris*) (Martínez-Nieto *et al*., 2020).

Two botanical varieties have been described for this species: variety *P. lunatus var. lunatus* (domesticated) and *P. lunatus var. silvester* (wild). However, the *P. lunatus* species used alone is more appropriate, since there is no clear established distinction between both groups (Bitocchi *et al*., 2017). This crop grows under a wide range of environmental conditions and exhibits differences in seed morphology related to size, shape, and color (Silva *et al*., 2015; Sánchez-Navarro *et al*., 2019). Its pods are dehiscent and flattened, containing two to four seeds (Lustosa-Silva *et al*., 2022).

It is generally observed that wild or landrace lima bean plants tend to have an indeterminate climbing growth habit, with long flowering periods and larger pod production (Zoro Bi; Maquet; Baudoin, 2003). In contrast, domesticated lines or those undergoing breeding processes may exhibit a determinate bushy growth habit, with smaller seeds and a shorter flowering period (Santos *et al*., 2002).

Lima bean seeds, like those of other legumes, are rich in proteins and vitamins such as niacin, riboflavin, and thiamine, being a source of dietary fiber. They can be consumed fresh or dried, offering an important nutritional alternative for rural populations in northeastern Brazil (Sathe *et al*., 1984; Adebo, 2023). Their relatively low consumption in human diets compared to common beans is possibly due to the longer cooking time required and the need for specific preparation to eliminate the bitter taste associated with hydrocyanic acid (Lopes *et al*., 2010).

In Brazil's Northeast region, farmers cultivate lima beans using their own germplasm and community seed exchanges, considering them local landraces. Despite having undesirable traits such as susceptibility to pests and diseases and a long growth cycle, these genotypes are well adapted to the region and genetically diverse (Santos *et al*., 2002).

This region accounted for approximately 98.97% of the total lima bean production in Brazil in 2023, with the states of Ceará (34.23%), Paraíba (31.75%), Pernambuco (13.68%), and Rio Grande do Norte (6.51%) being the largest producers, followed by Piauí in fifth place with 6.43%, according to data IBGE (2023). Thus, to increase lima bean production and consumption in other regions of Brazil, techniques such as genetic improvement can be applied. This process involves crossing different varieties to combine desirable traits such as higher productivity and improved nutritional quality (Louwaars, 2018; Ahmar *et al*., 2020).

However, selecting more productive genotypes may reduce genetic diversity, impacting fundamental plant traits such as root morphology and exudate release, which directly influence the composition of the rhizosphere microbiome (Louwaars, 2018; Semchenko; Xue; Leigh, 2021). The microbial community in the soil and rhizosphere plays an essential role in maintaining soil fertility and nutrient cycling, contributing to processes such as nitrogen fixation, soil structure improvement, and pathogen suppression, which are crucial for lima bean development (Cardoso; Silva; Pádua, 2024).

2.2 Influence of plant genotype on the rhizosphere microbial community

The rhizosphere, the region of soil directly impacted by plant roots, hosts one of the most diverse and dynamic microbial communities associated with plants, characterized by intense microbial activity (Philippot *et al*., 2013). This space, located 1 to 3 mm around the roots, acts as an ecological niche that facilitates interactions between roots and microorganisms, it is considered one of the most dynamic interfaces on Earth (Singh *et al*., 2004; Philippot *et al*., 2013; Sasse; Martinoia; Northen, 2018).

This root-associated environment harbors complex microbiotas that play essential roles in both natural and agricultural ecosystems. Microorganisms present in the rhizosphere, such as bacteria and fungi, can play a fundamental role in biogeochemical cycles, driving nutrient and carbon cycling with a direct effect on crop productivity (Changey *et al*., 2022). In addition to these, many other functions are attributed to them, some widely known, such as biological nitrogen fixation, phosphate solubilization, phytohormone synthesis, increased water and nutrient uptake by roots (Cardoso; Andreote, 2016), plant protection against pathogens, and plant growth promotion (Mendes *et al*., 2014; Mendes *et al*., 2018a).

Some rhizobacteria, such as *Bacillus* spp., can act as biological agents that induce systemic resistance in plants against pathogen growth by producing antibiotics and siderophores (Hashem *et al*., 2019; Maheshwari; Bhutani; Suneja, 2019). Once associated with the plant rhizosphere, these rhizobacteria form a biofilm that coats the root, creating a natural barrier against root infections, competing for nutrients and occupying ecological niches, or preventing colonization by invasive pathogens such as nematodes (Chapelle *et al*., 2016; Hacquard *et al*., 2017; Mazzuchelli; Mazzuchelli; Araujo, 2020; Costa *et al*., 2023).

Roots release certain compounds, called root exudates, which have a direct effect on the microorganisms present in the rhizosphere (Pérez-Jaramillo; Mendes; Raaijmakers, 2016). These exudates are primarily sugars, amino acids, volatile and secondary metabolites, which, due to their specific composition, can selectively attract beneficial microorganisms and alter the dynamics of microbial communities in the rhizosphere (Canarini *et al*., 2019; Trivedi *et al*., 2020; Williams *et al*., 2022).

Various factors, such as plant species, genotypes, and the physical and chemical characteristics of the soil, directly influence the rhizosphere effect (Araujo *et al*., 2019; Sousa *et al*., 2020; Moroenyane *et al*., 2021). The soil serves as the primary source of microorganisms that colonize the rhizosphere, while the plant genotype contributes to the selection of these microorganisms by releasing specific exudates at the root-soil interface (Jones; Nguyen; Finlay, 2009; Mendes; Garbeva; Raaijmakers, 2013). Although soil properties have a major impact on the composition of the root microbiome (Bulgarelli et al., 2013), small changes induced by the host plant in this community can have significant effects on its adaptability and performance (Bulgarelli *et al*., 2015; Haney *et al*., 2015).

Plant-associated microbiomes exhibit remarkable similarities regardless of their environment of origin, indicating that plants can reliably select the composition of their microbiomes (Cordovez *et al*., 2019; Xu *et al*., 2018). However, plant genetic variation can impact the establishment and assembly of the microbiome in various crops (Escudero-Martinez *et al*., 2022; Meier *et al*., 2022). Studies indicate that landrace varieties tend to form more robust associations with beneficial microorganisms due to their resilience and greater genetic variability, whereas improved lines may exhibit greater specificity in interactions with certain microbial species (Pérez-Jaramillo *et al*., 2018; Tkacz *et al*., 2020).

Previous reports show that even genotypes belonging to the same species can modulate distinct microbial communities in the rhizosphere (Weinert *et al*., 2011; Bulgarelli *et al*., 2015; Walters *et al*., 2018). Investigating local varieties and modern cultivars of wheat, Rossmann *et al*. (2020) identified significant differences in the diversity of bacterial, fungal, and cercozoan communities. Additionally, He *et al*. (2024), in a study involving microbiomes from different germplasms, concluded that plant genotype has a relevant impact, especially under abiotic stress conditions. Sousa *et al*. (2020) evaluated the microbial community in the rhizosphere of four distinct lima bean genotypes under the same environmental conditions and observed that each genotype enriched specific microbial taxa in the rhizosphere.

In this context, elements such as plant species, selected genotype, and soil physical and chemical characteristics play fundamental roles in the organization and functioning of the rhizosphere microbiome (Marschner *et al*., 2001; Bulgarelli *et al*., 2015). Similarly, the composition of the microbial community can be modified through strategies such as the inoculation of biocontrol agents or the genetic improvement of plants, aiming to favor specific taxa in the rhizosphere that act as antagonists to soil-borne pathogens (Mayo *et al*., 2015; Mendes *et al*., 2018a, 2018b).

2.3 *Pratylenchus genus*

The genus *Pratylenchus*, also known as root-lesion nematodes, are intracellular endoparasites of roots, polyphagous, and exhibit migratory behavior. Their life cycle

ranges from 3 to 9 weeks, varying depending on the species and environmental conditions (Jones; Fosu-Nyarko, 2014; Francilino *et al*., 2017; Orlando *et al*., 2020). These nematodes spend a significant portion of their life cycle inside roots but can also be found on root surfaces and in adjacent soil, with a preference for sandier soils (Stirling, 2011; Freitas *et al*., 2019).

Species of the *Pratylenchus* genus generally follow a typical plant-parasitic nematode developmental cycle, beginning within the egg, where the transition to the first juvenile stage (J1) occurs. Subsequently, the second juvenile stage (J2) emerges from the egg. Unlike sedentary endoparasites, *Pratylenchus* juveniles and adults remain mobile, capable of entering and exiting root systems, and exhibit vermiform morphology, allowing them to invade host plant roots or storage organs. Adult females lay eggs both inside infested roots and in the surrounding soil, either in clusters or individually. The occurrence of males varies among species, sometimes being rare, while reproduction generally occurs through parthenogenesis. Under adverse conditions, survival and long-term persistence in the soil can be ensured by the egg stage or anhydrobiosis (Castillo; Vovlas, 2007).

Approximately 100 species belonging to the *Pratylenchus* genus have been identified, ranking third among the plant-parasitic nematodes causing the most significant damage to crops, following gall-forming (*Meloidogyne* spp.) and cyst-forming (*Heterodera* spp.) nematodes (Castillo; Vovlas, 2007; Qing *et al*., 2019; Handoo *et al*., 2020). Due to its polyphagous nature, *Pratylenchus* has a broad host range, which is facilitated by the fact that it does not need to induce the formation of specialized feeding cells, unlike *Meloidogyne* and *Heterodera* genera (Dias *et al*., 2010). According to Vieira *et al*. (2018), for example, *Pratylenchus penetrans* has an extensive host range, affecting more than 400 plant species.

Plant-parasitic nematodes are generally attracted to plant roots, especially to the elongation zone, through signals such as carbon dioxide, pH gradients, and plant allelochemicals, which influence their interaction with specific genotypes (Ali; Alborn; Stelinski, 2011; Linsell *et al*., 2014). Root penetration by this nematode occurs through its stylet, which releases enzymes responsible for degrading the cortical parenchyma cell wall, allowing the nematode to feed (Francilino *et al*., 2017).

Infection by root-lesion nematodes causes necrotic spots that expand as the nematode moves within cells, leading to cellular clutter and, consequently, spreading

to epidermal cells, forming large visible lesions on the roots (Jones; Fosu-Nyarko, 2014; Francilino *et al*., 2017). These lesions directly impact the roots' ability to absorb water and nutrients (Whish *et al*., 2014; Bernard; Egnin; Bonsi, 2017; Thompson; Clewett; Timothy, 2021).

Nematodes of the *Pratylenchus* genus are highly significant due to their negative impact on economically important crops (Back, 2009). For example, *P. brachyurus* is a parasite of major crops such as soybean, wheat, corn, rice, cotton, lima bean, sugarcane, and coffee (Dias *et al*., 2010; Grigolli; Asmus, 2014). This species is responsible for more than 30% of annual soybean yield losses (Brum *et al*., 2019; Castanheira *et al*., 2020), and it is considered one of the main phytosanitary challenges faced by this crop (Vilela *et al*., 2011).

The control of plant-parasitic nematodes, including *Pratylenchus*, can be achieved mainly through the use of chemical nematicides and bionematicides, as well as physical methods such as soil solarization and agronomic practices like crop rotation, resistant variety use, biofumigation, and green manure application (Zasada *et al*., 2018; Sikandar *et al*., 2020; Sasanelli *et al*., 2021). However, chemical nematicides have been particularly criticized due to environmental concerns and the promotion of nematode resistance (Desaeger; Wram; Zasada, 2020; Poveda; Abril-Urias; Escobar, 2020; Sasanelli *et al*., 2021).

Bionematicides use is considered a suitable approach for agriculture, and has shown promising results in controlling nematodes such as *Pratylenchus* (Saad *et al*., 2022). Costa *et al*. (2023) observed a high efficacy of the rhizobacterium *Bacillus subtilis* in suppressing *Pratylenchus* populations in both corn and lima bean roots. Additionally, previous studies have indicated an average 50% reduction in this nematode’s presence in sugarcane and common beans following *B. subtilis* application (Wepuhkhulu *et al*., 2011; Mazzuchelli; Mazzuchelli; Araujo, 2020).

2.4 Bacillus as a plant growth promoter and bionematicide agent

The *Bacillus* genus comprises a highly diverse group of bacteria belonging to the phylum Firmicutes and the class Bacilli (Fritze, 2004; Harirchi *et al*., 2022).

Morphologically, these bacteria are described as rod-shaped, Gram-positive, aerobic, or facultatively anaerobic. Their physiological capabilities and ability to form endospores allow *Bacillus* species to survive extreme environmental conditions, leading to their wide distribution across various habitats, particularly in soil. They show a preference for the rhizosphere region, where they establish a mutualistic relationship with the plant host (Etesami; Jeong; Glick, 2023).

In the soil, these bacteria perform essential functions such as organic matter decomposition (Smagacz; Martyniuk, 2023), plant growth promotion, and protection against plant pathogens (Dobrzyński *et al*., 2023). Several *Bacillus* species are known to enhance crop productivity, including *B. velezensis*, *B. pumilus*, *B. subtilis*, *B. cereus*, *B. megaterium*, and *B. amyloliquefaciens*. These bacteria employ mechanisms such as the production of phytohormones or growth regulators, including abscisic acid, auxins, cytokinins, ethylene, jasmonic acid, and gibberellins (Radhakrishnan; Lee, 2016; Asari *et al*., 2017; Park *et al*., 2017; Ambreetha *et al*., 2018; Khan *et al*., 2018; Miljaković; Marinković; Balešević-Tubić, 2020b; Dame; Rahman; Islam, 2021).

In addition to biosynthesizing plant hormones that act as bio-stimulants for plant growth, *Bacillus* can also function as biofertilizers by facilitating the absorption of specific nutrients from the environment through mechanisms such as biological nitrogen fixation (BNF) and phosphate solubilization (Borriss, 2011; Hashem; Tabassum; Abd-Allah, 2019). A recent study by Costa *et al*. (2023) demonstrated that the individual inoculation of *Bacillus subtilis* in lima bean resulted in a significant increase in the number, biomass, and size of nodules and stimulated specific nodulation. These findings suggest that the use of this rhizobacterium enhances the nitrogen-fixing capacity of lima bean efficiently.

Bacillus species are also known to secrete compounds such as exopolysaccharides, siderophores, and various extracellular enzymes, including proteases, chitinases, collagenases, lipases, and enzymatic complexes. These metabolites have the ability to inhibit pathogen development and affect nematode populations at different stages of their life cycle (Radhakrishnan; Hashem; Abd-Allah, 2017; Ayaz *et al*., 2021; Migunova; Sasanelli, 2021; Liang *et al*., 2022; Pires *et al*., 2022). In a study by Oliveira *et al*. (2019), a reduction of approximately 88% in the reproduction factor of *P. brachyurus* in soybean was observed 60 days after seed treatment with *B. subtilis*.

Furthermore, *Bacillus* produces antibiotics such as fengycins, iturins, and surfactins, which together exhibit high potential for controlling pathogenic microorganisms and plant-parasitic nematodes (Mnif; Ghribi, 2015; Hashem; Tabassum; Abd-Allah, 2019).

In general, *Bacillus* species with bionematicidal potential, such as *B. subtilis* and *B. amyloliquefaciens*, can control nematodes directly or indirectly by competing for nutrients and ecological niches, forming biofilms, promoting plant growth, and producing lytic enzymes, antibiotics, volatile compounds, and toxic metabolites. Indirect antagonism occurs through the induction of systemic resistance in plants or the release of small molecules into the rhizosphere that affect nematode feeding behavior, migration, and sex ratio (Wang *et al*., 2018; Araujo *et al*., 2021; Ayaz *et al*., 2021; Migunova; Sasanelli, 2021).

2.5 Analysis of prokaryotic soil communities through sequencing

In the past, soil was viewed as a "black box," where only its elemental composition, gas fluxes, and total microbial biomass were accessible. Its microbiota was predominantly characterized through isolation and cultivation techniques (Žifčáková *et al*., 2017). However, considering the immense microbial diversity present in soils, with estimates suggesting that one gram of soil can harbor up to 10 billion microorganisms, only a minority can be cultivated using conventional laboratory methods (Staley; Konopka, 1985; Amann; Ludwig; Schleifer, 1995; Rosselló-Mora; Amann, 2001).

Thus, the emergence and application of molecular techniques in soil microbial ecology have paved the way for identifying previously unknown microorganisms (Whitman; Coleman; Wiebe, 1998), as they could not be easily isolated or cultivated *in vitro* due to the difficulty of replicating the same environmental conditions present in the ecosystems where these microorganisms exist (Torsvik; Øvreås, 2002). Metabarcoding analysis and the metagenomic approach are among the primary means for studying microbial DNA in soil (Nesme *et al*., 2016), as they enable the direct study of the genetic diversity of the environment (Sharon; Banfield, 2013).

Metagenomics is a systematic approach that aims to categorize and manipulate the genetic material of environmental samples through the creation of libraries and sequencing (Zeyaullah *et al*., 2009). This technique was developed to enhance traditional microbiology methods, allowing for a broader understanding of microbial diversity in a specific environment (Handelsman, 2004). It increases the chances of a more in-depth characterization of soil microbial communities without the need for prior isolation and cultivation of microorganisms, as it relies on the direct sequencing of genetic material from an environmental sample (Kunin *et al*., 2008).

Metabarcoding, on the other hand, is a technique used to identify the taxonomic composition of a community through environmental samples by amplifying and sequencing genes with specific markers, such as 16S rRNA for prokaryotes, ITS for fungi, and 18S rRNA for most eukaryotes (Coissac; Riaz; Puillandre, 2012; Ji *et al*., 2013). Advances in nucleic acid sequencing and refined analytical methods have improved the precise characterization of soil, allowing for a detailed description of microbial composition down to genus and subgenus levels (Žifčáková *et al*., 2017).

Studies on microorganisms present in the rhizosphere often use amplicon sequencing to obtain data related to the core microbiome, generating tables of Operational Taxonomic Units (OTUs) or Amplicon Sequence Variants (ASVs) (Pérez-Jaramillo *et al*., 2019). Although these analyses are widely employed and useful for estimating species, diversity, and microbial community composition, the OTU-based clustering method (Chen *et al*., 2013) has gradually been abandoned by many researchers. Currently, the most recommended approach is the use of ASVs (Callahan; Mcmurdie; Holmes, 2017).

ASVs offer greater precision and detail in taxonomic resolution, allowing for the identification of biogeographic trends, the distinction between pathogenic and non-pathogenic taxa, and the separation of strains with different ecological configurations (Callahan; Mcmurdie; Holmes, 2017). Unlike OTUs, ASVs are always identical to one another, facilitating the comparison of data from different samples and studies (Semenov, 2021). This approach is considered the most suitable for analyzing high-throughput sequencing data (Knight *et al*., 2018).

Thus, microbial composition in environmental samples can be inferred through the amplification of the 16S rRNA ribosomal gene (Alteio *et al*., 2021). This gene is considered an ideal choice for phylogenetic analyses of prokaryotes, such as soil

bacteria and archaea, due to its conservative nature throughout evolution, being present in all prokaryotes (Turnbaugh *et al*., 2009). The 16S rRNA gene is approximately 1,500 base pairs (bp) long and consists of nine variable regions (V1 to V9), interspersed with nine highly conserved regions throughout the sequence. The V3 and V4 regions are preferably used due to their ability to provide more effective resolution in differentiating between genera and, in some cases, enabling species-level identification (Jovel *et al*., 2016; Johnson *et al*., 2019).

Thus, from a soil sample and purified genetic material, millions of fragments of the 16S rRNA gene are amplified (Turnbaugh *et al*., 2009). These fragments must undergo analysis and processing through specialized bioinformatics software (Segata *et al*., 2013). Currently, thanks to next-generation sequencing (NGS) technologies (such as Illumina), with the aid of bioinformatics tools and statistical methods for DNA sequence analysis, it is possible to obtain detailed information about microbial population composition from various environmental samples (Martins *et al*., 2013).

A recent study by Lopes *et al*. (2023), which evaluated the microbial community in the rhizosphere of different *Phaseolus* genotypes through 16S rRNA sequencing, enabled the observation of distinct microbial recruitment in the rhizosphere and differences in microbial community composition among these genotypes. Beyond the composition of the rhizosphere microbial community, the comprehensive use of molecular techniques also provides insights into the genomic potential of microorganisms (Hultman *et al*., 2015; Woodcroft *et al*., 2018). For example, it is possible to describe carbon utilization throughout the entire soil microbial community (Žifčáková *et al*., 2017) or nitrogen cycling (Hesse *et al*., 2015; Mackelprang *et al*., 2018).

Therefore, this is an important tool for understanding microbial communities, from biodiversity studies and functional characteristics to the search for genes of biotechnological interest (Meneghine *et al*., 2017). These techniques are highly relevant to microbial ecology, contributing to the understanding of microorganism interactions and the investigation of potential environmental changes (Newton; Mclellan, 2015; Chávez-Romero *et al*., 2016).

3 MATERIAL AND METHODS

3.1 Experiment location

The experiment was conducted in a greenhouse at the Center for Agricultural Sciences of the Federal University of Piauí (UFPI), in Teresina, PI, Brazil (5°02'34" S, 42°47'01" W, 72 m altitude), from February to April 2024. The climate is classified as tropical (Aw), with an average annual precipitation of 1,500 mm and an average temperature of 27°C. The soil used was classified as Fluvisol, with a textural composition of 70% sand, 14% silt, and 16% clay, according to Teixeira *et al*. (2017).

3.2 Soil collection and initial quantification of *Pratylenchus* spp. population

The soil used to compose the experimental units was collected at a depth of 0-20 cm, one day before the experiment was set up, from a field area naturally infested with the *Pratylenchus* genus, with a history of lima bean cultivation. Before assembling the experiment, the soil was homogenized, and three soil samples were collected and sent to the laboratory for extraction and quantification of *Pratylenchus* spp. (root-lesion nematode), following the method described by Jenkins (1964). The initial population had an average of approximately 1,000 *Pratylenchus* individuals per pot.

3.3 Experimental procedures

Two lima bean genotypes were obtained from the UFPI *Phaseolus* Germplasm Bank: a landrace variety (UFPI-1365) and an improved line (H46-48/F8-2023). Each genotype was subjected to one of the following inoculation conditions: (a) *B. subtilis*, (b) *B. amyloliquefaciens*, or (c) co-inoculation with *B. subtilis* + *B. amyloliquefaciens*. A

control group without inoculation was also included. The experiment was conducted in a completely randomized block design with three replicates, totaling 24 experimental units.

Seeds of each lima bean genotype were sown in 9 L polyethylene pots containing 5 kg of soil. Each pot received three seeds according to the treatment. Before planting, chemical fertilization (20-80-20 kg ha^{-1} of N, P_2O_5, and K_2O, respectively) was applied, as recommended by Vieira, Vieira and Andrade (1992) for lima bean. The suspensions containing *B. subtilis* and *B. amyloliquefaciens* were prepared as follows: a) 200 µL of liquid inoculant containing the AP-3 strain at 1.0 × 10^8 CFU/mL of *B. subtilis* were mixed in 20 mL of water; b) 50 µL of the liquid inoculant NemaControl®, containing strain SIMBI BS 10 at 5.0 × 10^9 CFU/mL of *B. amyloliquefaciens*, mixed in 20 mL of water; c) a combination of 200 µL of *B. subtilis* (strain AP-3 at 1.0 × 10^8 CFU/mL) and 50 µL of *B. amyloliquefaciens* (strain SIMBI BS 10 at 5.0 × 10^9 CFU/mL) mixed in 20 mL of water.

For inoculation, 450 µL of each suspension was applied directly into the seed furrow over the lima bean seeds according to the respective treatment. This dose was divided among the three seeds. Ten days after plant emergence, thinning was performed to maintain one plant per pot. Irrigation was performed daily using the gravimetric method, based on the soil water retention capacity, maintaining soil moisture at 70% of field capacity. All plants were harvested 50 days after emergence, at the flowering stage.

3.4 Extraction and quantification of nematodes from roots and soil

Nematodes were extracted by washing the roots under running water, followed by weighing and homogenization in a blender. The homogenized material was sieved through 100 and 500 mesh screens. Nematode extraction was performed by centrifugal flotation in a sucrose-kaolin solution, following the method of Coolen and D'herde (1972). For nematode extraction from the soil, 100 cm^3 of soil near the rhizosphere was collected and subsequently mixed with 2 L of water. The resulting suspension was sieved through 100 and 400 mesh screens and clarified according to

the method described by Jenkins (1964). The resulting suspension was stored in properly labeled 50 mL Falcon tubes and kept refrigerated (10°C) until quantification. The number of nematodes in the roots and soil (final population) and the density of *Pratylenchus* spp. in lima bean roots were estimated using a Peters chamber and counted under a microscope at 40x magnification. The density of *Pratylenchus* spp. in the roots was obtained by dividing the final root population by the fresh root mass of each experimental unit. The efficiency of *Pratylenchus* reduction in both genotypes was determined by comparing the number of nematodes in the roots of *Bacillus*-treated plants with the number of nematodes in the roots of the non-inoculated control (0% *Pratylenchus* reduction efficiency).

3.5 Rhizosphere soil collection

Rhizosphere soil was collected from each pot, totaling 24 samples. Soil adhering to the roots was carefully removed and transferred to sterile Eppendorf tubes, which were immediately frozen at -80°C upon arrival at the laboratory until further processing for DNA extraction and analysis.

3.6 DNA extraction and sequencing

DNA was extracted from 0.5 g (total wet weight) of soil using the PowerLyzer PowerSoil DNA Isolation Kit (MoBIO), following the manufacturer's instructions. The quality and concentration of the extracted DNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA). The V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the region-specific primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3'). The PCR products were then purified and quantified. After quantification, the DNA pool was sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA). The 16S rRNA sequencing data were

processed using QIIME 2 (version 2024.5). First, sequences were demultiplexed, and quality control was performed with DADA2 (Bolyen *et al*., 2019), applying a consensus method to remove chimeric and low-quality sequences ($q < 20$). The 16S rRNA sequencing approach generated approximately 3,939,000 reads, with an average of 164,106 sequences per sample. These sequences were then rarefied to 140,100 sequences, based on the lowest sample count, and singleton and doubleton sequences were removed. The representative sequence of each ASV (Amplicon Sequence Variant) was taxonomically classified with 97% similarity using the SILVA database, version 138 (Quast *et al*., 2012), and the resulting matrix was used for statistical analyses. The 16S rRNA sequencing data are available in the NCBI SRA under the identification PRJNA1181910.

3.7 Data analysis

First, data were tested for normal distribution and variance homogeneity using the Shapiro-Wilk and Levene tests, respectively, in RStudio 4.4.0. The efficiency of *Pratylenchus* reduction compared to the control by *Bacillus* treatments in both lima bean genotypes (landrace and line) was calculated using Excel (version 2412). Significant differences between isolated factors (Bionematicides and Varieties) concerning the number of nematodes in the root and soil, nematode density in the root, and root mass were verified using Tukey test ($p < 0.05$) in RStudio 4.4.0. The microbial community structure was analyzed using Principal Component Analysis (PCA) to assess the rhizosphere microbial community structure of both lima bean genotypes under different treatments, using Canoco 5 software (Biometrics, Wageningen, Netherlands). After filtering, the ASV table at the phylum and genus levels was used to evaluate the bacterial community composition.

For composition, Shannon diversity and richness indices were calculated based on the taxonomic matrix at the ASV level and compared using Tukey HSD test ($p < 0.05$) in PAST, version 4.1. The differential abundance of bacterial groups at the genus level was compared using the Statistical Analysis of Metagenomic Profile (STAMP) software (Parks; Beiko, 2010), with Welch's t-test and Benjamini-Hochberg correction

(Benjamini; Hochberg, 1995). To evaluate correlations between different bacterial genera and environmental variables, Spearman's correlation test ($p < 0.05$) was performed. Finally, a functional profile analysis was conducted using the FAPROTAX 1.2.5 database (Louca *et al*., 2019), with comparisons made using Tukey-Kramer post-hoc test ($p < 0.05$), followed by Benjamini-Hochberg correction for FDR.

4 RESULTS

Since no significant interaction was observed between the inoculation treatments of *B. subtilis* and *B. amyloliquefaciens* and the lima bean genotypes, these two factors were evaluated separately for each variable related to *Pratylenchus* suppression (Figure 1).

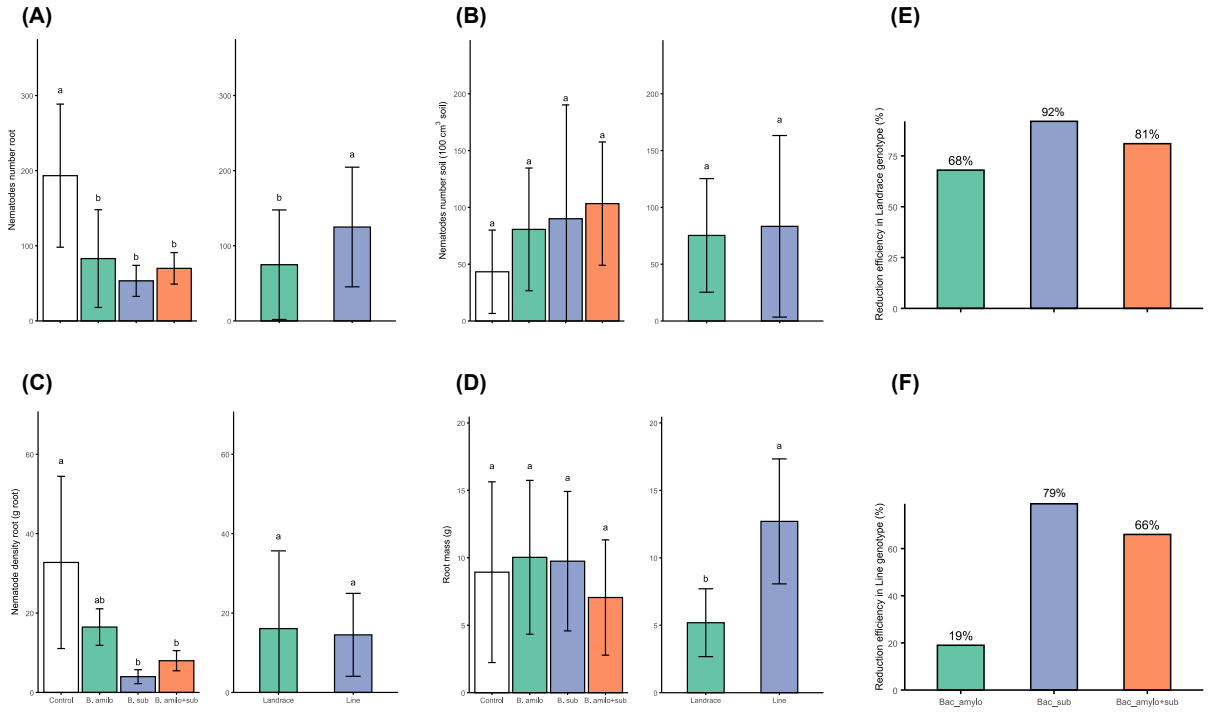

Figure 1 - Comparison within each factor (Bionematicides and Varieties) for the variables (A) Number of nematodes in the root and (B) in the soil, (C) nematode density in the root, (D) root mass, (E) efficiency in reducing *Pratylenchus* spp. in the roots of the landrace genotype, and (F) in the line genotype subjected to inoculation and co-inoculation treatments with different *Bacillus* species.

Statistical results indicated that inoculation with *B. subtilis* and *B. amyloliquefaciens*, both individually and in co-inoculation, positively reduced the *Pratylenchus* population in lima bean roots, as measured by the number of nematodes in the roots, with a greater reduction observed in the landrace genotype (Figure 1A). Additionally, inoculation with *B. subtilis* and its co-inoculation with *B. amyloliquefaciens* decreased *Pratylenchus* density in lima bean roots compared to the non-inoculated control (Figure 1C). In contrast, no differences were observed in nematode counts in

the soil (Figure 1B) or root biomass (Figure 1D) among the tested factors. Notably, when comparing the efficiency of *Pratylenchus* population reduction in roots between lima bean genotypes, inoculation with *B. subtilis* demonstrated the highest efficiency, particularly in the landrace variety (92% reduction) (Figure 1E). In contrast, *B. amyloliquefaciens* exhibited lower suppression, especially in the line genotype (19% reduction) (Figure 1F).

The PCA analysis compared the structure of the prokaryotic community in the rhizosphere of lima bean genotypes in response to inoculation with *B. subtilis* and *B. amyloliquefaciens*, both individually and in co-inoculation. Overall, no significant differences were observed in the prokaryotic community structure between lima bean genotypes and *Bacillus* species (Figure 2A).

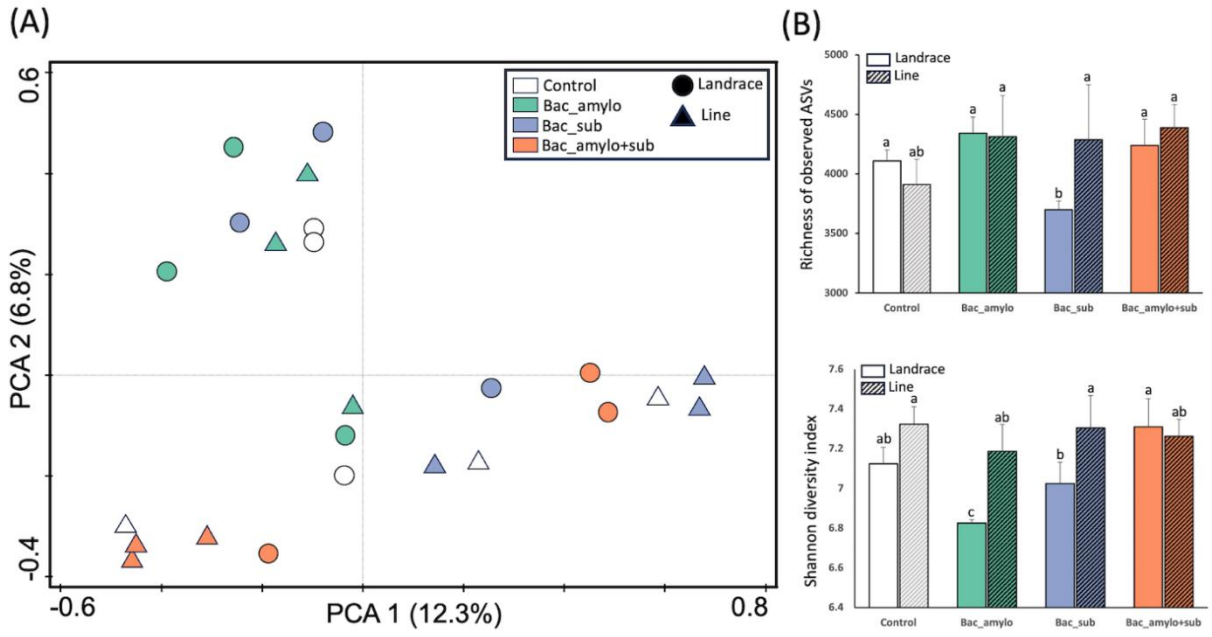

Figure 2 – Structure of bacterial communities in the rhizosphere of two lima bean (*Phaseolus lunatus*) genotypes inoculated with different *Bacillus* species, based on the 16S rRNA gene. (A) Principal Component Analysis (PCA). (B) Observed ASV richness and Shannon diversity index of microbial communities. Error bars represent the standard deviation of three independent replicates. Different lowercase letters indicate significant differences between treatments, based on Tukey's HSD test ($P < 0.05$).

However, microbial richness and diversity differed among treatments. Although co-inoculation (*B. subtilis* + *B. amyloliquefaciens*) did not promote significant differences in microbial richness and diversity in the rhizosphere between lima bean

genotypes, inoculation with *B. subtilis* reduced microbial richness and diversity in the rhizosphere of the landrace lima bean compared to the line variety. In contrast, inoculation with *B. amyloliquefaciens* led to a reduction in diversity in the landrace genotype compared to the line (Figure 2B).

The three most abundant bacterial phyla identified in the rhizosphere of the lima bean genotypes were Actinobacteria (28.3% of the total sequences), Proteobacteria (27.3%), and Firmicutes (17.5%) (Figure 3A).

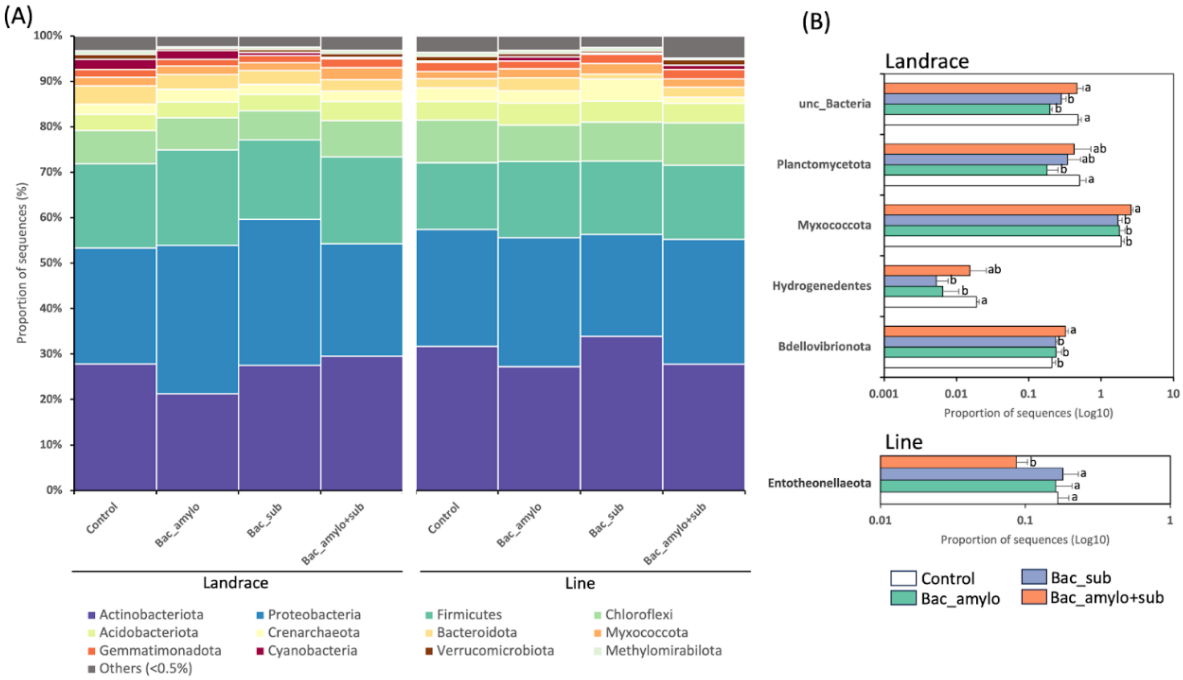

Figure 3 – (A) Microbial community composition at the phylum level in the rizosphere of lima bean (*Phaseolus lunatus*) genotypes inoculated with different *Bacillus* species, based on the 16S rRNA gene. (B) Bar graph comparing the most abundant microbial phyla between treatments within each genotype. Error bars represent the standard deviation of three independent replicates. Different lowercase letters indicate significant differences between treatments, based on the Tukey HSD test ($P < 0.05$).

We compared the most abundant microbial phyla between treatments in each genotype. In the rizosphere of the lima bean landrace, co-inoculation with *B. subtilis* + *B. amyloliquefaciens* resulted in a higher relative abundance of Myxococcota and Bdellovibrionota. On the other hand, this co-inoculation reduced the relative abundance of Entotheonellaeota in the rizosphere of the lima bean line genotype (Figure 3B).

The differential analysis of the prokaryotic community revealed a total of 96 genera with distinct abundances, grouped into five main clusters (Figure 4A). Clusters V (28 genera) and II (25 genera) contained the largest number of differentiated genera, while clusters I, III, and IV contained 14, 18, and 11 differentiated genera, respectively. In the rizosphere of the lima bean landrace, inoculation with *B. amyloliquefaciens* enriched genera grouped in cluster IV, while inoculation with *B. subtilis* showed less distinction between clusters, with genera distributed between clusters I and III. Co-inoculation with *B. subtilis* + *B. amyloliquefaciens* favored genera from cluster I and, to a lesser extent, from cluster II. In the rizosphere of the lima bean line genotype, *B. amyloliquefaciens* enriched genera in cluster III, while a clear separation was observed between genera from clusters II and V, which were enriched by *B. subtilis* and co-inoculation, respectively.

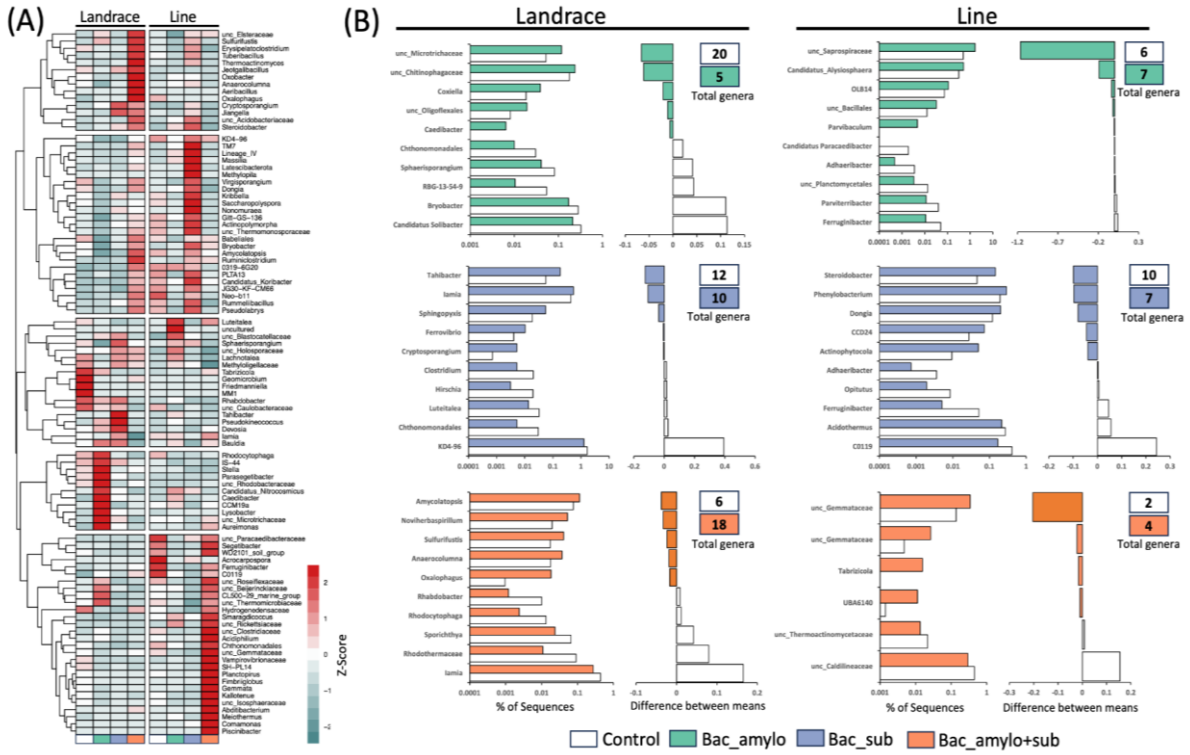

Figure 4 – Microbial community composition in the rizosphere of different lima bean (*Phaseolus lunatus*) genotypes, based on the 16S rRNA gene. (A) Heatmap showing the differential abundance of genera across all treatments. (B) Pairwise comparison between each treatment and the control within each genotype. In all graphs, only genera that differed significantly are shown, based on the Welch's t-test with Benjamini-Hochberg correction ($P < 0.05$).

We then performed a pairwise comparison between each treatment and the control, revealing that different microbial genera were enriched depending on the treatment and lima bean genotype (Figure 4B). In the rizosphere of the lima bean landrace, *B. amyloliquefaciens* enriched *Coxiella* and *Caedibacter*, while *B. subtilis* enriched *Tahibacter* and *Sphingopyxis*. Co-inoculation with *B. subtilis* + *B. amyloliquefaciens* enriched *Amycolatopsis* and *Noviherbaspirillum*. In the rizosphere of the lima bean line genotype, *B. amyloliquefaciens* enriched *Candidatus_Alysiosphaera* and *Parvibaculum*. Inoculation with *B. subtilis* enriched *Steroidobacter* and *Phenylobacterium*, while co-inoculation enriched *Tabrizicola*.

In general, more positive than negative correlations were observed between bacterial and archaeal groups and variables related to nematode suppression. For nematode density and number in the roots, a negative correlation was found with *Aureimonas* and a positive correlation with *Babeliales*. Additionally, the number of nematodes in the roots correlated negatively with *Caedibacter* and *unc_Microtrichaceae*, while showing a positive correlation with *Amycolatopsis* and *Candidatus_Koribacter*. In the soil, the number of nematodes correlated negatively with Luteitalea and positively with *Cryptosporangium*, *unc_Blastocatellaceae*, and *Steroidobacter* (Figure 5A).

For functional prediction, we evaluated functions related to the nitrogen cycle (Figure 5B). No significant differences were observed in the predicted functions associated with nitrogen fixation, nitrification, and assimilatory nitrate reduction in both lima bean genotypes in response to inoculation. However, predicted functions related to denitrification, organic matter degradation, and dissimilatory nitrate reduction differed between the lima bean genotypes. In the rizosphere of the lima bean landrace, inoculation with *B. amyloliquefaciens* reduced sequences related to denitrification and dissimilatory nitrate reduction, while increasing those associated with organic matter degradation. In contrast, in the rizosphere of the lima bean line variety, inoculation with *B. subtilis* increased sequences related to denitrification and dissimilatory nitrate reduction, while reducing those associated with organic matter degradation.

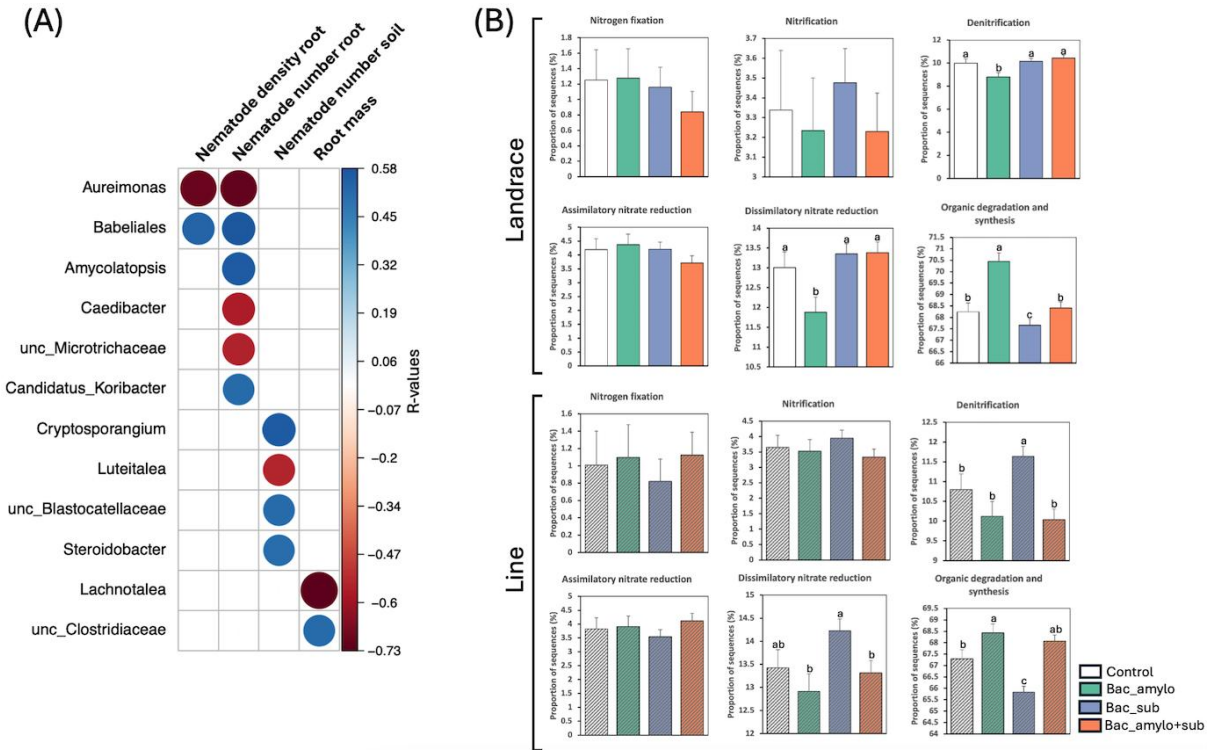

Figure 5 – (A) Heatmap of Spearman's correlation coefficients between different bacterial and archaeal genera and variables related to nematode suppression. Only significant correlations ($P < 0.05$) are shown. The color scale and circle size are proportional to the correlation weight. Positive correlations are shown in blue, while negative correlations are in red. (B) Bar graph showing the differential abundance of predicted functions related to the nitrogen cycle in the landrace and line genotypes. The classification is based on functional predictions from the FAPROTAX database. Different letters indicate significant differences ($P < 0.05$) based on the Tukey-Kramer post-hoc test followed by Benjamini-Hochberg correction for FDR.

5 DISCUSSION

In recent years, the use of biological control agents, such as species of *Bacillus*, has gained attention as an ecological alternative to chemical nematicides in the management of plant-parasitic nematodes (Engelbrecht *et al*., 2018). These nematodes are significant soil-borne pathogens that cause severe root damage in crops, leading to reduced plant growth and productivity. Species of *Bacillus*, particularly *B. subtilis* and *B. amyloliquefaciens*, have demonstrated efficacy in suppressing nematode populations through various mechanisms, including antibiotic production and competition for ecological niches (Xia *et al*., 2011; Jamal *et al*., 2017; Engelbrecht *et al*., 2018). However, the potential of *Bacillus* inoculation to alter microbial communities in the rizosphere and its interaction with different plant genotypes is still poorly understood.

In this study, we aimed to evaluate the effects of inoculation with *B. subtilis* and *B. amyloliquefaciens* on *Pratylenchus* spp. populations and microbial communities in the rizosphere of two distinct lima bean genotypes. Consistent with our first hypothesis, the results showed that both *Bacillus* species, both individually and in co-inoculation, distinctly affected the suppression of *Pratylenchus* in the roots of lima bean. This suggests that different bacterial species exert varied effects on *Pratylenchus* and its infection process (Nuaima, 2022). Specifically, in the lima bean landrace genotype, *Pratylenchus* suppression was more efficient. *Bacillus* rhizobacteria are known for their nematode-suppressive properties, through mechanisms that disrupt the orientation and migration of *Pratylenchus* towards the roots, thus confirming their role in controlling root-lesion nematodes (Hashem; Tabassum; Abd-Allah, 2019). Additionally, species of this genus, such as *B. subtilis*, produce antibiotics and biofilms in the rizosphere that enhance plant defense against nematodes (Mazzuchelli; Mazzuchelli; Araujo, 2020). Interestingly, control of *Pratylenchus* by these bacteria was more effective in the lima bean landrace genotype than in the improved variety.

The differences observed between the lima bean landrace and line genotypes in their response to *Bacillus* species for *Pratylenchus* control may be attributed to genetic changes during plant breeding (Walters *et al*., 2018; Lund *et al*., 2022). Landrace genotypes typically maintain greater genetic diversity, which may enhance

their ability to adapt and respond to beneficial microbial interactions (Lopes *et al*., 2015). In contrast, improved varieties, which are selected for specific traits, may exhibit reduced genetic diversity, limiting their microbial response capacity (Abdul Aziz; Masmoudi, 2024). A recent study by Rossmann *et al*. (2020) demonstrated reduced multitrophic interactions in the rizosphere microbiomes of modern wheat cultivars compared to landrace genotypes. We observed a similar genotype effect in our study, with a more pronounced response to *B. amyloliquefaciens*, which showed lower suppression efficiency in the lima bean line variety. However, *B. amyloliquefaciens* is known to be effective in controlling *Meloidogyne* (Moreira *et al*., 2025), although it may be less efficient against *Pratylenchus* in lima bean roots.

Our second hypothesis was partially confirmed, as the results showed that both *Bacillus* species (in single inoculations and co-inoculation) did not significantly alter the microbial community structure in the rizosphere. This suggests that *Bacillus* species effectively controlled *Pratylenchus* without compromising the stability of the microbial community in the rizosphere, which is essential for soil resilience (Jiang *et al*., 2020). However, *B. subtilis* and *B. amyloliquefaciens* promoted an increase in microbial richness and diversity in the rizosphere of the lima bean line genotype. A recent study by Cunha *et al*. (2024) showed that inoculation with bacterial strains, including *Bacillus* spp., increased prokaryotic diversity in the rizosphere of common bean, suggesting that inoculation may improve functional resilience and positively impact plant health. This increase in richness and diversity may indicate that plants, during breeding, release more complex root exudates, recruiting a broader microbial community (Pacheco-Moreno *et al*., 2023). Co-inoculation did not alter richness and diversity regardless of genotype, suggesting a beneficial interaction between *B. subtilis* and *B. amyloliquefaciens* in recruiting diverse microorganisms in the rizosphere.

The microbial composition in the rizosphere varied between lima bean genotypes in response to *Bacillus* inoculation, highlighting the role of genotype in modulating the rizosphere microbiome (Mendes *et al*., 2018a; Rossmann *et al*., 2020; He *et al*., 2024). The most abundant bacterial phyla were Actinobacteria, Proteobacteria, and Firmicutes, which are known for promoting plant growth and were observed similarly in other studies on *Phaseolus* genotypes (Bao *et al*., 2021; Costa *et al*., 2023). Co-inoculation with *B. subtilis* and *B. amyloliquefaciens* increased the abundance of phyla associated with predation, such as Myxococcota and

Bdellovibrionota, in the rizosphere of the landrace genotype, suggesting greater pathogen control (Bonfiglio *et al*., 2020; Ye *et al*., 2020). In contrast, co-inoculation reduced the abundance of Entotheonellaeota in the rizosphere of the improved variety, possibly due to competition with bacteria producing various bioactive compounds (Andrić; Meyer; Ongena, 2020).

The differential abundance analysis revealed distinct groupings of genera influenced by inoculation with *B. subtilis* and *B. amyloliquefaciens*, both individually and in co-inoculation, along with the effect of lima bean genotype. In the rizosphere of the landrace genotype, *B. amyloliquefaciens* enriched specific microbial groups, while *B. subtilis* and co-inoculation enriched broader microbial communities. This suggests that *B. amyloliquefaciens* affects a more specialized subset of microorganisms, while *B. subtilis* sustains a more diverse community (Qin *et al*., 2017; Sui *et al*., 2022). In the improved variety, each treatment recruited distinct microbial genera, indicating that plant breeding may influence how specific *Bacillus* strains shape rizosphere community recruitment (Sousa *et al*., 2020). These findings support the idea that microbial interactions in the rizosphere are genotype-specific, with co-inoculation potentially offering synergistic benefits for microbial diversity (Sokolova *et al*., 2023).

The enrichment of specific microbial genera in the rizosphere supports the hypothesis that *Bacillus* can selectively shape rizosphere communities (Hashem; Tabassum; Abd-Allah, 2019; Cunha *et al*., 2024). For example, *B. subtilis* enriched genera such as *Sphingopyxis* and *Phenylobacterium*, which are associated with organic matter decomposition and nutrient cycling, potentially benefiting plant growth (Kielak *et al*., 2016; Sharma *et al*., 2021). Additionally, the enrichment of *Amycolatopsis* and *Noviherbaspirillum*, known as plant-growth-promoting rhizobacteria, suggests that *Bacillus*-based treatments may improve soil quality and plant resilience in agricultural systems (Gao *et al*., 2020; Syiemiong *et al*., 2022). The selective enrichment observed in this study highlights the potential of *Bacillus* as a promising approach to enhance rizosphere resilience and microbial diversity in agricultural soils. Thus, using *Bacillus* as an inoculant can influence plant health and growth both directly and indirectly. Directly, the inoculant exerts its effect through the production of specific molecules, such as volatile organic compounds, that influence root growth and architecture (Bavaresco *et al*., 2020). Indirectly, inoculation affects other microbiome groups that provide beneficial support to plants (Pedrinho *et al*., 2024).

The correlations observed between bacterial and archaeal genera and nematode suppression variables highlight the importance of functional interactions in the rizosphere. Negative correlations between microbial genera such as *Aureimonas*, *Caedibacter*, *unc_Microtrichaceae*, and *Luteitalea* with nematode numbers and density suggest their potential roles in biocontrol. *Aureimonas*, known for promoting plant resilience, may suppress pathogens through mechanisms such as exopolysaccharide synthesis, protein secretion, and biofilm formation (Becker *et al*., 2022). Although little is known about *Caedibacter*, *unc_Microtrichaceae*, and *Luteitalea* in the context of nematode control, they may significantly contribute to shaping the microbial composition of the rizosphere and ecological balance.

Finally, putative functions related to the nitrogen cycle were predicted, and we evaluated the effect of inoculation on specific functions. While *Bacillus* species, particularly *B. subtilis*, are known for their nitrogen-fixing potential, our study found that the predominant functions in the community were more related to denitrification, organic matter degradation, and dissimilatory nitrate reduction. In the rizosphere of the lima bean landrace genotype, *B. amyloliquefaciens* reduced the potential for denitrification and nitrate reduction activities, while increasing organic matter degradation, potentially improving nitrogen availability for plants (Hui *et al*., 2018). In contrast, in the rizosphere of the improved genotype, *B. subtilis* increased the potential for denitrification and nitrate reduction but reduced organic matter degradation, possibly favoring microorganisms that use nitrate as an electron acceptor (Kuypers; Marchant; Kartal, 2018). The presence of these functions across genotypes suggests that inoculations with *B. amyloliquefaciens* and *B. subtilis* may help sustain essential nitrogen cycle processes, regardless of genotypic differences.

6 CONCLUSION

This study demonstrated that the inoculation of *B. subtilis* and *B. amyloliquefaciens* in lima bean is effective in suppressing *Pratylenchus* spp. in the roots, with *B. subtilis* showing particularly strong effects on the roots of the landrace genotype. These inoculations distinctly shape the richness, diversity, and microbial composition in the rizosphere of each genotype. Additionally, the enrichment of specific bacterial genera and changes in functional profiles related to the nitrogen cycle and organic matter degradation highlight genotype-specific responses to *Bacillus* inoculation. The findings emphasize how different *Bacillus* species and inoculation strategies can not only reduce nematode infestations but also influence microbial diversity in the rizosphere in a genotype-dependent manner, shedding light on the complex interaction between biocontrol agents and plant-associated microorganisms. This study provides valuable insights into how *Bacillus* spp. can be strategically used to control nematodes and support microbial stability in agricultural soils.

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