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RHAIANA OLIVEIRA DE AVIZ

SOIL BIOLOGICAL ATTRIBUTES AND RHIZOSPHERE MICROBIAL COMMUNITY OF PRICKLY-PEAR CACTUS INOCULATED WITH *Bacillus* AND *Paenibacillus*

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Thesis submitted to the Graduate Program in Agronomy (PPGA) at the Federal University of Piauí (UFPI), in the concentration area of Soil and Water Management and the research line of Soil Microbiology and Biochemistry, as a requirement for obtaining the degree of Doctor of Agronomy.

Advisor: Prof. Dr. Ademir Sérgio Ferreira de Araújo.

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Dr. Ademir Sérgio Ferreira de Araújo – UFPI
(Orientador)

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I dedicate this thesis to my younger sister, *Rhaissa Aviz*, who has been by my side for as long as I can remember, through the good times and especially the difficult ones. Her presence, support, and constant love have been fundamental in helping me reach this point.

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Just like the soil microbiome of the semi-arid region, may you be resilient enough to face periods of stress and adapt when the environment brings you new challenges.

Rhaiana Aviz

ABSTRACT

In semiarid regions, drought-tolerant plants such as prickly pear cactus (*family Cactaceae*) are essential for sustaining food production for both humans and livestock. However, prolonged droughts intensified by climate change can compromise the growth of this plant. Some bacterial taxa, such as *Bacillus* and *Paenibacillus*, are known to colonize the rhizosphere and promote plant growth (PGPB), as well as mitigate the effects of water stress. Nevertheless, little is known about the effects of inoculation in cactus species on microbial biomass, enzymatic activity, and the rhizosphere microbiome under semiarid soil and climatic conditions. In this context, the aim of this doctoral thesis was to investigate the effects of inoculation with *Bacillus subtilis* and *Paenibacillus sp*. on two genotypes of prickly pear cactus (*Nopalea cochenillifera* Salm-Dick), 'Baiana' and 'Doce', and to evaluate their impacts on biological properties and the rhizosphere microbiome under semiarid edaphoclimatic conditions and water deficit. In Chapters I and II, a field experiment was conducted using a completely randomized design in a split-split plot arrangement (2 × 3 × 2). The main plot factor was plant growth stage (90 and 270 days after planting), the subplot factor was PGPB inoculation (non-inoculated, and inoculated with *B. subtilis* or *Paenibacillus sp*.), and the sub-subplot factor consisted of the two cactus genotypes ('Baiana' and 'Doce'). In Chapter I, microbial biomass C, N, and P, as well as enzymatic activities, were evaluated. The results showed that interactions among growth stage, PGPB taxa, and cactus genotypes significantly influenced microbial biomass and enzymatic activity in the rhizosphere, and that combining specific genotypes with *B. subtilis* can enhance phosphorus availability. In Chapter II, the diversity, composition, and enrichment of rhizosphere bacterial communities were assessed. It was concluded that the rhizosphere microbiome of prickly pear cactus was shaped primarily by plant growth stage, with key bacterial groups potentially associated with stress tolerance. Chapter III describes a greenhouse experiment arranged in a randomized block design with a 3 × 2 factorial structure. The factors included PGPB inoculation (non-inoculated, inoculated with *B. subtilis*, or inoculated with *Paenibacillus sp*.) and two water availability conditions (50% and 25% of soil field capacity). The cactus genotype used was 'Miúda' (equivalent to the 'Doce' genotype), and the structure, diversity, abundance, and co-occurrence networks of the rhizosphere bacterial community were evaluated. The results showed that water availability significantly altered bacterial community structure, with *Actinobacteriota* dominating under water deficit and *Proteobacteria* under well-watered conditions. Additionally, B. subtilis promoted cooperative microbial networks and may potentially enhance drought resilience.

Keyword: Microbial biomass; Microbiome; *Nopalea cochenillifera*; PGPB; Semiarid; Water deficit.

RESUMO

Em regiões semiáridas, plantas tolerantes à seca, como a palma forrageira (family *Cactaceae*), são essenciais para sustentar a produção de alimentos para humanos e animais. No entanto, secas prolongadas, intensificadas pelas mudanças climáticas, podem comprometer o crescimento dessa planta. Alguns táxons bacterianos, como *Bacillus* e *Paenibacillus*, são conhecidos por colonizar a rizosfera e promover o crescimento das plantas (BPCP), além de atenuar os efeitos do estresse hídrico. No entanto, pouco se sabe sobre os efeitos da inoculação em cactáceas na biomassa microbiana, na atividade enzimática e no microbioma da rizosfera de plantas sob as condições edafoclimáticas do semiárido. Nesse contexto, o objetivo dessa Tese de doutorado foi compreender os efeitos da inoculação de *Bacillus subtilis* e *Paenibacillus sp.* em dois genótipos de palma forrageira, da espécie *Nopalea cochenillifera* Salm-Dick, sendo elas a 'Baiana' e a 'Doce' nas propriedades biológicas e microbioma da rizosfera nas condições edafoclimáticas do semiárido e sob déficit hídrico. No capítulo I e II foi conduzido um experimento a campo, em um delineamento inteiramente casualizado em parcelas subsubdivididas (2×3×2). A parcela principal foi o estágio de crescimento (90 e 270 dias) após o plantio da palma, enquanto a subparcela correspondeu à inoculação com BPCP (sem inoculação e inoculação com *B. subtilis* e *Paenibacillus sp*.). A sub-subparcela foi composta por dois genótipos de palma forrageira ('Baiana' e 'Doce'). No capítulo I avaliou-se a biomassa microbiana de C, N e P, além da atividade enzimática, e concluiu-se que interação entre estágio de crescimento, táxons de BPCP e genótipos de palma influenciam significativamente a biomassa microbiana e a atividade enzimática na rizosfera, e que a combinação de genótipos específicos com *B. subtilis* pode aumentar a disponibilidade de fósforo. No capítulo II avaliou-se a diversidade, a composição e o enriquecimento das comunidades bacterianas na rizosfera, e concluiu-se que o microbioma da rizosfera da palma forrageira foi moldado principalmente pelo estágio de crescimento da planta, com grupos bacterianos-chave potencialmente associados à tolerância ao estresse. O capítulo III foi um experimento em casa de vegetação, em delineamento em blocos ao acaso, com arranjo fatorial 3 × 2. Os fatores adotados foram inoculação com BPCP (sem inoculação e inoculação com *B. subtilis* e *Paenibacillus sp*.) e duas condições de disponibilidade hídrica (50% e 25% da capacidade de campo do solo). O genótipo de palma forrageira utilizado foi a 'Miúda' (equivalente ao genótipo 'Doce'), e foram avaliados a estrutura, diversidade, abundância e redes de co-ocorrência da comunidade bacteriana da rizosfera. A partir dos resultados, concluiu-se que a disponibilidade de água alterou significativamente a estrutura da comunidade bacteriana, com *Actinobacteriota* dominando sob déficit hídrico e *Proteobacteria* sob condições bem irrigadas, além disso, *B. subtilis* promoveu redes microbianas cooperativas e pode potencialmente aumentar a resiliência à seca.

Palavras-Chave: Biomassa microbiana; Défict hídrico; BPCP; *Nopalea* cochenillifera; Microbioma; Semiárido.

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1. GENERAL INTRODUCTION

Agricultural practices in semi-arid regions can be compromised due to high temperatures, prolonged drought periods, and the salinization of soil and water (Sparacino; Argibay; Espindola, 2021). Additionally, the depletion of organic matter caused by agricultural activity occurs more rapidly in semi-arid environments compared with temperate and tropical regions (Maia *et al.*, 2019). Consequently, farmers prioritize the cultivation of species that are tolerant or resistant to these conditions, such as cowpea (Silva *et al.*, 2021), cassava (Santos *et al.*, 2020b), and cacti like mandacaru and prickly-pear cactus (Menezes *et al.*, 2022).

The cultivation of prickly-pear cactus (family *Cactaceae*) is particularly prominent, especially the species *Nopalea cochenillifera*, which has a higher nutritional value compared with other prickly-pear cactus species (Dubeux Jr *et al.*, 2021). The productivity of *Nopalea* prickly-pear cactus varies according to the cultivated variety. Under restrictive climatic conditions, the green biomass yield of the 'Baiana' genotype has been reported to reach 267 t ha^{-1} $year^{-1}$, whereas the 'Doce' genotype reached 134 t ha^{-1} $year^{-1}$ (Edivan *et al.*, 2020).

Several research efforts are currently underway to optimize management practices and improve the development and productivity of this crop, such as the adoption of supplemental irrigation (Zegbe; Servín-Palestina, 2021) and the use of different nutrient sources (Lédo *et al.*, 2021). Furthermore, recent studies have shown that plants of the Cactaceae family also establish associations with Plant Growth-Promoting Bacteria (PGPB) (Govindasamy *et al.*, 2022; Santos *et al.*, 2020a). PGPB perform several processes that support plant development, including biological nitrogen fixation, phosphate solubilization, and siderophore production, among others (Malik *et al.*, 2022).

The use of PGPB in semiarid regions has emerged as a promising strategy to increase plant resilience to abiotic stresses, especially water deficit, which is common in these areas due to prolonged drought periods (Abdelaal *et al.*, 2021). Among the most effective PGPB for stress alleviation and growth promotion, *Bacillus subtilis* and *Paenibacillus sp.* stand out for their ability to enhance plant stress tolerance through multiple mechanisms, including the production of antioxidant compounds and phytohormones (Ebrahimi-Zarandi *et al.*, 2023; Chattaraj *et al.*, 2025).

Semiarid soils harbor a diverse native bacterial community that is adapted to limiting conditions and has the potential to promote plant growth (Bonatelli *et al.*, 2021). This native community can interfere with the effectiveness of plant inoculation with specific bacteria due

to its high competitiveness. Moreover, the introduction of a new microorganism may alter the structure of the microbial community (Teijeiro; Belimov; Dodd, 2020).

Soil quality factors can also be affected by changes in the soil microbial community, including soil enzymatic activity, basal respiration, and microbial biomass (Bastida *et al.*, 2021). The introduction of PGPB into the soil can increase microbial activity within a given microbiome and, consequently, enhance soil basal respiration, as the available carbon is consumed by active microorganisms (Babur *et al.*, 2022; Uzinger *et al.*, 2020; Wang *et al.*, 2019). However, soils with low availability of organic carbon sources show reduced microbial growth (Batista *et al.*, 2022).

Plant inoculation, the microbial community, and soil quality are closely interconnected and exhibit a dynamic relationship. Therefore, a better understanding of these interactions is necessary to achieve biologically efficient soil management, especially in semiarid regions. Based on this, the hypotheses of this study were: (A) Inoculation with PGPB can modify biological attributes, increasing enzymatic activity and microbial biomass, thereby contributing to soil sustainability and conservation; (B) Inoculation with PGPB can shape the rhizosphere microbiome of the prickly-pear cactus species *Nopalea cochenillifera* Salm-Dick by recruiting other beneficial microorganisms; (C) Inoculation of prickly-pear cactus with *Bacillus subtilis* and *Paenibacillus sp.* can restructure the microbiome under water deficit; (D) Plant genotype and age may also influence the composition of the rhizosphere microbiome.

2. CHAPTER 1

LITERATURE REVIEW

2.1. Prickly-pear cactus and its importance for the semiarid region

The semiarid region is a climatic zone characterized by high solar radiation and temperature, as well as low relative humidity and annual precipitation, resulting in prolonged periods of drought. These factors directly affect agricultural activity in these areas, leading to low economic yields per unit of land (Scholes, 2020). Various agricultural strategies can be adopted to produce under these conditions, primarily focusing on water availability, such as implementing an adapted planting schedule, supplemental irrigation, and cultivating drought-resistant crops with low water requirements throughout the production cycle (Dzvene *et al.*, 2025).

In livestock production systems, one alternative for animal feed during the dry season is the use of prickly-pear cactus (*Opuntia spp.*) in animal diets (Silva *et al.*, 2024). Prickly-pear cactus is a *Cactaceae* species with high palatability and calcium content; however, it has lower dry matter and fiber content compared to other forage plants (Dubeux Jr *et al.*, 2021). When combined with other fiber sources, such as sugarcane bagasse, this forage can reduce the need for concentrated feed and lower the animals' water intake requirements (Rocha Filho *et al.*, 2021).

Among the countries that most cultivate and study this cactus are Brazil, Mexico, the United States, Tunisia, India, and Argentina. In Brazil, cultivation occurs in the northeastern region, which is characterized by the climatic conditions of the semiarid zone (Herreira-Ângulo *et al.*, 2023). The main species cultivated in northeastern Brazil belong to the genera *Opuntia* and *Nopalea*, which include individuals resistant to the cochineal insect (*Dactylopius opuntiae*), the primary pest of prickly-pear cactus (Mazzeo *et al.*, 2019).

The differences between these two genera are related to morphology and nutritional value. Plants of the genus *Opuntia* generally have larger cladodes, greater drought tolerance, and can produce even in low-fertility soils, whereas plants of the genus *Nopalea* have higher nutritional value and are rich in carbohydrates (Dubeux Jr *et al.*, 2021). In Brazil, the main spineless and cochineal-resistant prickly-pear cactus genotypes cultivated are 'Mexican Elephant Ear' (Opuntia stricta Haw), 'Doce' (*Nopalea cochenillifera* Salm-Dick, also called 'Miúda'), and 'Baiana' (*Nopalea cochenillifera* Salm-Dick, also called 'IPA Sertânia'), which

can reach yields of over 250, 134, and 267 t ha^{-1}, respectively (Sousa *et al.*, 2025; Edivan *et al.*, 2020).

2.2. Biological attributes of semiarid soils

The limiting edaphoclimatic conditions of the semiarid region can significantly affect the biological attributes of soil. These attributes, also known as "Soil Health Bioindicators," refer to parameters influenced by the activity of microorganisms present in a given soil, such as microbial biomass and enzymatic activity (Bhaduri *et al.*, 2022). Soil biological attributes are highly sensitive to the agricultural system and management practices used during cultivation. They are also strongly influenced by soil organic matter content and seasonal fluctuations (Costa *et al.*, 2024; Verma *et al.*, 2025). Among the most evaluated biological attributes are soil basal respiration, microbial biomass, enzymatic activities of β-glucosidase, urease, and phosphatase, as well as microbial relationships, such as the qCO_2 (metabolic quotient) (Semenov *et al.*, 2025).

Soil basal respiration refers to the flow of CO_2 in the soil, primarily produced by heterotrophic organisms such as the resident microbial community (Bond-Lamberty *et al.*, 2025). This parameter is mainly associated with organic matter decomposition and microbial activity, and it also serves as an important indicator of microbial stress. When soil has low organic carbon and microbial biomass carbon combined with high soil respiration, it indicates an imbalance, which can also be assessed using qCO_2, the ratio of soil respiration to microbial biomass carbon (Camilo-Cotrim *et al.*, 2025). In agricultural soils of the semiarid region, studies have shown predominance of high respiration and qCO_2 values, reflecting greater energy demand and carbon loss (Huang *et al.*, 2022; Mganga *et al.*, 2024; Oresca *et al.*, 2024).

Soil basal respiration, together with microbial biomass, is widely used to assess soil health and is therefore recommended by the FAO (Food and Agriculture Organization of the United Nations) and SMAF (Soil Management Assessment Framework) for monitoring soil quality (Semenov *et al.*, 2025). Soil microbial biomass serves as a reservoir of nutrients, such as carbon (C), nitrogen (N), and phosphorus (P), which are assimilated by microorganisms during the decomposition of organic matter (Mason-Jones *et al.*, 2022). The C, N, and P in microbial biomass are the main compartments evaluated, representing 1.2%, 2.6%, and 8.0% of the total soil C, N, and P, respectively (Fujii *et al.*, 2024). Climatic conditions influence microbial biomass, as shown by Qu *et al.* (2023), where areas experiencing prolonged drought, such as in the semiarid region, showed substantial reductions in microbial biomass C, N, and P

of approximately 22.7%, 21.2%, and 21.6%, respectively. In addition, soil enzymatic activity was also reduced under these conditions.

Soil enzymatic activity is associated with the production of enzymes by microorganisms aimed at converting C, N, and P into soluble compounds (Daunoras *et al.*, 2024). Enzyme production is influenced by the quality of organic matter and the availability of nutrients in the soil; therefore, when soil is nutrient-deficient, enzymatic activity increases as microorganisms seek to acquire resources (Zhang *et al.*, 2025). To measure enzymatic activity related to these elements, β-glucosidase (for C), urease (for N), and phosphatase (for P) are primarily quantified, although cellulases, chitinases, dehydrogenases, proteases, amidases, phytases, and others can also be measured (Upadhyay *et al.*, 2025). Quantifying enzymatic activity allows for the calculation of ecoenzymatic stoichiometry, which represents the ratio between enzymes involved in nutrient acquisition and those associated with C acquisition. This ratio helps to understand nutrient availability in the soil and the requirements of microorganisms (Kunito *et al.*, 2023).

Regarding the enzymatic activity and stoichiometry of C, N, and P in semiarid soils, a study by Medeiros *et al.* (2023) showed that enzymatic activity is influenced by soil depth and land use. Specifically, the upper layers (above 10 cm) and soils under agricultural use exhibit lower enzymatic activity. Furthermore, semiarid soils are phosphorus-limited regardless of land use.

In the literature, there are also studies that have evaluated the biological attributes of semiarid soils in areas cultivated with cacti, particularly the prickly-pear cactus species *Opuntia ficus-indica* L. (Zouitane *et al.*, 2025; Guillen-Cruz *et al.*, 2022; Bautista-Cruz *et al.*, 2017). Overall, these studies indicated that cactus cultivation exhibits characteristics similar to those of forested areas, thereby contributing to soil conservation. Areas cultivated with the prickly-pear cactus species *Nopalea cochenillifera* Salm-Dyck have also been evaluated by Camelo *et al.* (2021), who analyzed the 'IPA Sertânia' genotype. The authors observed that under monoculture conditions, these areas had lower microbial C and N contents, lower soil basal respiration, and higher qCO_2 compared to intercropped areas. This outcome was attributed to the low litter production by the prickly-pear cactus, which reduces organic matter levels and, consequently, affects the biological attributes of the soil.

2.3. The rhizosphere microbiome of cacti

Soil harbors a diverse microbial community, composed mainly of bacteria and fungi, which perform various functions such as nutrient cycling, soil conditioning, and plant growth

promotion (Iqbal *et al.*, 2025). The composition of this community forms the microbiome, whose microorganisms can either live freely in the soil or colonize the rhizosphere, where they establish beneficial interactions with plants (Banerjee; van der Heijden, 2023). These interactions can be influenced by multiple factors, including environmental conditions, agricultural management practices, and plant genotype (Han *et al.*, 2025).

Regarding genotypes as a factor shaping the rhizosphere microbiome, studies have shown differences in microbial composition in both agriculturally important crops (Tayyab *et al.*, 2022; Li *et al.*, 2024) and forest species (Onet *et al.*, 2025), demonstrating that each plant species or variety selects specific groups of microorganisms. This selection occurs primarily due to the composition of root exudates, which varies among genotypes and recruits specific microorganisms according to the plant's physiological needs. Additionally, factors such as root architecture and morphology, which also differ between plants, contribute to the variations observed in the rhizosphere microbiome (Xun *et al.*, 2024).

Given the importance of genotype as a factor shaping microbiomes, it is essential to understand the microbiome of plants adapted to limiting edaphoclimatic conditions, such as those in semiarid regions. Bonatelli *et al.* (2021) cataloged various plant growth-promoting bacteria commonly found in semiarid environments, including *Streptomyces* and *Bacillus*, which are recognized for their resistance to adverse conditions and their potential to mitigate environmental stresses. These microbial groups often form communities associated with species that thrive under water deficit, as is the case with cacti.

Several studies have investigated the microbiome and bacteria associated with different cactus species, including *Opuntia cholla* (Manríquez-Rivera *et al.*, 2025), *Echinocactus platyacanthus* (Estrada-González *et al.*, 2023), *Pereskia aculeata* Mill (Vega *et al.*, 2020), *Cereus jamacaru* (Kavamura *et al.*, 2018), *Myrtillocactus geometrizans*, *Opuntia robusta* (Fonseca-García *et al.*, 2016), and *Opuntia ficus-indica* (L.) Mill (Lyra *et al.*, 2021). Overall, these studies indicated that cacti cultivated in semiarid regions share a core group of rhizosphere-associated microorganisms, likely due to conserved prokaryotic patterns, which help ensure adaptation and survival under limiting environmental conditions (Fonseca-Garcia *et al.*, 2016; Zineb *et al.*, 2024). Karray *et al.* (2020) investigated the rhizosphere microbiomes of *Opuntia ficus-indica* across different aridity gradients and observed the enrichment of specific taxa, including *Actinobacteria, Alphaproteobacteria, Firmicutes*, and *Chloroflexi*, concluding that the rhizosphere of prickly-pear cactus harbors a bacterial community capable of tolerating and surviving under drought conditions. Thus, the association between cacti and drought-adapted microbial taxa highlights these plants as important biotechnological resources,

with the potential to produce more efficient and resilient microbial inoculants capable of improving agricultural performance in semiarid regions.

2.4. Plant inoculation in the semiarid region and its effects on biological attributes and the rhizosphere microbiome

The practice of inoculating plants with Plant Growth-Promoting Bacteria (PGPB) in agriculture has gained attention as a sustainable strategy to ensure crop production under abiotic stresses, such as drought and salinity, and biotic stresses, through the control of pests and diseases, while also enhancing nutrient acquisition by plants (Pérez-Montaño *et al.*, 2025). These stress responses occur through mechanisms such as the production of antioxidant enzymes, ACC deaminase, antibiotic compounds, plant hormones like cytokinins and auxins, and the induction of systemic resistance. Additionally, PGPB perform biological nitrogen fixation, siderophore production, phosphate solubilization, among other functions (Fanai *et al.*, 2024). Thus, PGPB represents a promising alternative to enhance plant cultivation in semiarid regions and reduce the use of chemical inputs in agricultural systems.

Given the importance of PGPB, studies evaluating the inoculation of agriculturally important plants with strains isolated from the rhizosphere of species adapted to semiarid conditions have demonstrated the effectiveness of this practice in mitigating stress, particularly water stress (Pishchik *et al.*, 2024; Sousa *et al.*, 2023; Braga *et al.*, 2022). Sousa *et al.* (2023) tested a strain of *Bacillus aryabhattai* obtained from the rhizosphere of *Cereus jamacaru*, a cactus characteristic of the Brazilian semiarid region, and found that the inoculation alleviated both water and salt stress in maize plants.

As previously discussed, cacti play a significant role in semiarid agricultural systems and harbor a rhizosphere microbiome composed of taxa adapted to stress conditions. Despite this potential, inoculation with beneficial microorganisms in these plants remains largely unexplored. Notable studies evaluating PGPB in cacti have been conducted with *Pachycereus pringlei* (Puente; Bashan, 1993; Bashan *et al.*, 1999; Carrillo *et al.*, 2002; Carrillo-Garcia *et al.*, 2000; Bacílio *et al.*, 2006), *Mammillaria fraileana* (Lopez *et al.*, 2012; Chávez-Ambriz *et al.*, 2016), *Echinocactus platyacanthus* (Castillo-Reyes *et al.*, 2014), and *Opuntia ficus-indica* (L.) Mill (Platamone *et al.*, 2023). Overall, most of these studies focused on the potential of *Azospirillum brasilense* and *Bacillus* to promote the growth of these cacti.

In addition to promoting plant growth, inoculation can also induce changes in the biological properties of soil and in the rhizosphere microbiome of plants cultivated in semiarid regions. Regarding biological attributes, Vikram *et al.* (2025) demonstrated that inoculation has

positive effects, increasing soil enzymatic activity and microbial biomass carbon content. Despite the potential of PGPB inoculation to enhance soil biological quality, few studies have investigated these effects in semiarid environments.

Regarding inoculation as a factor shaping the microbiome, the introduction of an external microorganism into soils that already harbor a well-established native microbiome, such as those in semiarid regions, can elicit various responses. Among these, the native community may compete with and suppress the introduced organism in the rhizosphere, thereby maintaining the function and structure of the community. The microbiome may also adapt and reorganize, resulting in a new stable state; this process is referred to as resilience (Mawarda *et al.*, 2020; Kost *et al.*, 2024). Another response involves the recruitment of additional beneficial taxa, which complement the functions of the rhizosphere microbiome and contribute to plant growth. Studies investigating the effect of inoculation on the microbiome of plants cultivated in semiarid regions have highlighted this type of response (Ilahi *et al.*, 2025; Francioli *et al.*, 2025; Ollio *et al.*, 2025). In their study, Ollio *et al.* (2025) concluded that inoculation promoted a reduction in pathogenic taxa and functional enrichment through the increased abundance of the *nifH* and *nirK* genes, which are involved in nitrogen cycling.

2.5. Final considerations

Semiarid soils harbor a microbial community highly adapted to limiting edaphoclimatic conditions, such as drought and salinity. Additionally, plants adapted to these conditions engage in beneficial interactions with this community, making them a promising tool for the development and generation of new microbial inoculants. Despite this importance, there remain significant gaps in the literature regarding the use of microbial inoculants in cacti, particularly prickly-pear cactus, which is a crop of great significance in the agricultural landscape of dry regions. Furthermore, the effects of inoculation on soil biological properties are still poorly explored, and a better understanding of these effects could contribute to improved plant growth, while promoting agricultural sustainability and soil conservation. Therefore, studies addressing inoculation in cacti under semiarid conditions, along with evaluations of the microbiome and soil biological attributes, are essential to expand knowledge of these interactions and develop sustainable strategies for plant production in semiarid regions.

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3. CHAPTER 2

INOCULATION WITH *Bacillus subtilis* AND *Paenibacillus sp.* SHAPES THE RHIZOSPHERE BACTERIOME OF PRICKLY PEAR CACTUS UNDER WATER DEFICIT

ABSTRACT

In semiarid regions, drought-tolerant plants like the prickly pear cactus (*Nopalea cochenillifera* L.) are critical for sustaining food production for humans and livestock. However, prolonged droughts exacerbated by climate change can impair cactus growth. Inoculation with plant growth-promoting bacteria (PGPB), such as *Bacillus subtilis* and *Paenibacillus* sp., has emerged as a promising strategy to enhance plant resilience to water deficit. Yet, the effects of these PGPB on the prickly pear cactus rhizosphere microbiome under drought remain poorly understood. Here, we hypothesized that *B. subtilis* and *Paenibacillus sp.* inoculation would differentially reshape the rhizosphere bacterial community under water deficit. A greenhouse experiment evaluated prickly pear cactus subjected to two watering regimes (well-watered vs. water deficit) and three inoculation treatments (*B. subtilis*, *Paenibacillus* sp., and a non-inoculated control). After 150 days, rhizosphere bacterial communities were characterized via 16S rRNA gene sequencing. Water availability significantly altered bacterial community structure, with *Actinobacteriota* dominating under water deficit and Proteobacteria under well-watered conditions. Importantly, inoculation with *B. subtilis* shifted community composition and increased the relative abundance of drought-adapted taxa, while also enhancing network connectivity through positive bacterial interactions. *Paenibacillus* sp. induced distinct but less pronounced effects. These results demonstrate that water deficit and PGPB inoculation interactively restructure the rhizosphere bacteriome, with *B. subtilis* promoting cooperative microbial networks and potentially enhancing drought resilience through microbiome engineering.

Keywords: drought stress, microbial ecology, 16S rRNA, plant-microbe interaction

3.1. Introduction

In semiarid regions, cultivating drought-tolerant plant species is crucial for maintaining food security for both human and animal populations. The Brazilian semiarid region, characterized by limited annual rainfall (~500 mm), relies heavily on drought-resistant crops such as the prickly pear cactus (*Nopalea cochenillifera* L.), a key forage source for livestock (Lyra *et al.*, 2021). Physiologically, this cactus employs crassulacean acid metabolism (CAM), an adaptation that optimizes water-use efficiency by regulating transpiration and minimizing water loss critical advantage under drought conditions (Niechayev and Mayer, 2023). However, despite its inherent drought tolerance, prolonged water scarcity exacerbated by climate change can impair its growth and physiological performance. Alternative strategies to enhance plant resilience are urgently needed to address this challenge. One promising approach is the use of plant growth-promoting bacteria (PGPB), which can improve stress tolerance and support crop productivity in water-limited environments.

The use of PGPB has emerged as a promising strategy to enhance plant resilience to abiotic stresses, particularly water deficit. These beneficial microbes improve drought tolerance by enhancing water and nutrient uptake, producing stress-related metabolites, and facilitating osmotic adjustment (Abdelaal *et al.*, 2021). In semiarid regions, PGPB inoculation has demonstrated significant benefits for key crops such as cowpea, maize, sorghum, and cassava (Leite *et al.*, 2018; Aquino *et al.*, 2019; Nascimento *et al.*, 2021). Among the most effective PGPB, *Bacillus subtilis* and *Paenibacillus* sp. are notable for their ability to enhance plant stress tolerance through multiple mechanisms. For example, *B. subtilis* synthesizes auxins (Jensen *et al.*, 2024) and promotes osmoprotectant accumulation, mitigating plant oxidative stress (Etesami *et al.*, 2023). They also can modulate the growth and root architecture through the production of volatile organic compounds (Bavaresco *et al.*, 2020). A recent study reported that *B. subtilis* inoculation increased the drought tolerance index in soybeans by 51% under water deficit (Moraes *et al.*, 2025). Similarly, *Paenibacillus* sp. enhances drought resilience by producing phytohormones that stimulate root growth and exopolysaccharides that improve soil moisture retention (Chattaraj *et al.*, 2025).

Beyond their direct growth-promoting effects under water deficit, *Bacillus subtilis* and *Paenibacillus* sp. can shape rhizosphere microbial community assembly. These recruited communities play a vital role in drought mitigation by improving water acquisition and producing stress-protective compounds, collectively enhancing plant resilience (Abdelaal *et al.*, 2021). Crucially, root traits are key drivers of microbial recruitment (Solomon *et al.*, 2024), and

PGPB inoculation can modify these traits to optimize resource use efficiency and stress tolerance (Grover *et al.*, 2021).

Previous studies have demonstrated that *Bacillus subtilis* modifies root architecture and signaling pathways in plants under water deficit conditions (Jensen *et al.*, 2024; Moraes *et al.*, 2025). A notable study by Moraes *et al.* (2025) revealed that *B. subtilis* inoculation in drought-stressed soybean plants significantly altered root morphology, particularly through increased root tip formation. Such architectural modifications, induced by *B. subtilis* under water stress, likely influence rhizosphere microbiome assembly (Gifford *et al.*, 2024). Supporting this, Zhang *et al.* (2024) reported that while *B. subtilis* inoculation selectively reduced the abundance of specific soil bacterial taxa in cucumber rhizospheres, it maintained overall bacterial diversity.

While numerous studies have characterized the effects of *Bacillus subtilis* and *Paenibacillus* sp. on both plant growth promotion and rhizosphere bacterial communities, their specific impacts on the prickly pear cactus rhizosphere microbiome under water deficit conditions remain unexplored. We hypothesized that inoculation with these PGPBs will differentially modulate the rhizosphere bacteriome of *N. cochenillifera* under drought stress. To test this hypothesis, we employed 16S rRNA gene sequencing to (i) characterize drought-induced shifts in the rhizosphere bacterial community, and (ii) evaluate how *B. subtilis* and *Paenibacillus* sp. inoculation restructure these microbial assemblages under water deficit.

3.1. Material and Methods

3.2.1. Experimental design

This study was conducted in a greenhouse at the Federal University of Piauí (UFPI), Bom Jesus, Piauí, Brazil (9°04′45.6″S, 44°19′37.9″W, 277 m). Soil samples were collected from a depth of 0–20 cm in the Experimental Field located at UFPI. The soil is classified as dystrophic Oxisol and has the chemical and physical properties shown in Appendix 1. The experiment employed a randomized block design with a 3 × 2 factorial arrangement and three replicates (18 experimental units total). The treatments consisted of: *Paenibacillus* sp. (strain IPACC38), *B. subtilis (*strain IPACC29), and a non-inoculated control. The second factor involved two water availability conditions: well-watered (W+: 50% of soil field capacity, FC) and water deficit (W-: 25% FC).

3.2.2. Experimental conduction

Before planting, soil fertilization was applied according to the requirements for cactus cultivation. Bacterial inoculants were prepared by culturing each PGPB strain in 50 mL of Tryptone Soya Broth (TSB) and incubating at 120 rpm for 72 hours on an SL-180/DT shaker table. For cactus sowing, healthy cladodes of the 'Miúda' cultivar were inoculated with 1.0 mL of each bacterial strain at a concentration of 10^9 cells mL^{-1}. For the control treatment, 1.0 mL of uninoculated TSB medium was applied. Nitrogen fertilization was carried out in two applications, at 30 and 60 days after planting, using urea as the nitrogen source. Initially, all treatments were maintained at 50% soil field capacity (FC). After 40 days, plants under drought conditions were subjected to 25% FC. Daily irrigation was managed using the pot-weighing method, replacing water lost through evapotranspiration to maintain the designated moisture levels. Plants were harvested 190 days after planting, following 150 days under drought stress.

3.2.3. Plant analysis

At harvest, non-destructive biometric parameters of cladodes per plant, were measured (Appendix 2). Plants were separated into cladodes and roots to obtain the dry mass (RDM) and root length (RL). The number of cladodes (CN) was counted, and cladode area (CA) and width (CW) were determined using a digital area meter (LI-3100C, LI-COR). Then, the material was immediately frozen and lyophilized to obtain the dry mass (CDM). Rhizospheric soil samples were collected by placing the roots and adhered soil onto a 1 mm mesh sieve and washing thoroughly with a gentle stream of tap water to remove excess soil. All soil samples were stored at -80°C before DNA extraction.

3.2.4. DNA extraction and amplicon sequencing

DNA was extracted from 0.5 g of rhizospheric soil using the Power Lyzer Power Soil DNA Isolation Kit. The quality and concentration of the extracted DNA were determined by using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA). The V4 region of the 16S rRNA gene was amplified with region-specific primers (515F/806R). Each 25 µl PCR reaction contained the following: 12.25 µL of nuclease-free water (Certified Nuclease-free, Promega, Madison, WI, USA), 5.0 µL of buffer solution 5x ($MgCl_2$ 2Mm), 0.75 µL of solution of dNTP's (10 mM), 0.75 µL of each *primer* (515 F 40 µM e 806 R 10µM), 1.0 unit of Platinum Taq polymerase High Fidelity in a concentration of 0.5 µL (Invitrogen, Carlsbad, CA, USA), and 2.0 µL of template DNA. Moreover, a control reaction was performed by adding water instead of DNA. The conditions for PCR were as follows: 95°C for

3 min to denature the DNA, with 35 cycles at 98°C for 20 s, 55 °C for 20 s, and 72°C for 30 s, with a final extension of 3 min at 72°C to ensure complete elongation.

After indexing, the PCR products were cleaned up using Agencourt AMPure XP – PCR purification beads (Beckman Coulter, Brea, CA, USA), according to the manufacturer's manual, and quantified using the dsDNA BR assay Kit (Invitrogen, Carlsbad, CA, USA) on a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). Once quantified, equimolar concentrations of each library were pooled into a single tube. After quantification, the molarity of the pool was determined and diluted to 2 nM, denatured, and then diluted to a final concentration of 8.0 pM with a 20% PhiX (Illumina, San Diego, CA, USA) spike for loading into the Illumina MiSeq sequencing machine (Illumina, San Diego, CA, USA).

Sequence data were processed using QIIME 2 version 2024.5.0 (Boylen *et al.*, 2019). Firstly, the sequences were demultiplexed and quality control was carried out using DADA2 (Callahan *et al.*, 2017), using the consensus method to remove any remaining chimeric and low-quality sequences ($q < 20$). The 16S rRNA sequencing approach yielded approximately 3,394,000 reads, averaging 161,591 sequences per sample. These sequences were then rarefied to 94,000 sequences, based on the lowest sample count, and singleton and doubleton sequences were removed. The taxonomic affiliation was performed at 97% similarity using the Silva database v. 138 (Quast *et al.*, 2013), and the generated matrix was further used for statistical analyses. The sequences were submitted to the NCBI Sequence Read Archive under the identification PRJNA1257415.

3.2.5. Data analysis

To compare microbial community structures across treatments, we analyzed 16S rRNA gene sequencing data. Following sequence processing, we verified data normality and homogeneity of variance using Shapiro-Wilk and Levene's tests, respectively. Community structure was visualized via Principal Component Analysis (PCA), while rhizosphere microbiome-environment relationships were examined through Redundancy Analysis (RDA). Both PCA and RDA were employed using Canoco 5 (Biometrics, Wageningen, The Netherlands). Significant differences between treatments were tested using PERMANOVA (Anderson, 2001). Alpha diversity metrics were calculated and compared via Tukey's HSD tests implemented in PAST 4.01 (Hammer *et al.*, 2001). Differential abundance of bacterial taxa at phylum and ASV levels was assessed in STAMP v2.1.3 (Parks *et al.*, 2014) using two-sided Tukey-Kramer post-hoc tests with Benjamini-Hochberg false discovery rate correction (Benjamini; Hochberg, 1995). Microbial co-occurrence networks were constructed based on

SparCC correlation analysis (Friedman; Alm, 2012), considering only robust ($r > 0.9$) and statistically significant ($p < 0.01$) relationships, with network visualization performed in Gephi 0.10.1 (Bastian *et al.*, 2009).

3.3. Results

3.3.1. The structure, richness, and diversity of bacterial community in the rhizosphere

The structure of the bacterial community in the rhizosphere of prickly pear cactus differed significantly between well-watered and water-deficit conditions ($P = 0.0001$; Figure 1A). In terms of inoculation, we observed a slight effect on the rhizosphere bacterial community ($P = 0.03$), particularly under well-watered conditions when PGPB inoculation was applied. Furthermore, all biometric parameters of the prickly pear cactus were closely associated with the rhizosphere bacterial community under well-watered conditions (Figure 1B). Overall, bacterial richness and diversity in the rhizosphere were not significantly affected by water availability (Figure 1C), except for a reduction in bacterial diversity observed with B. subtilis inoculation under well-watered conditions.

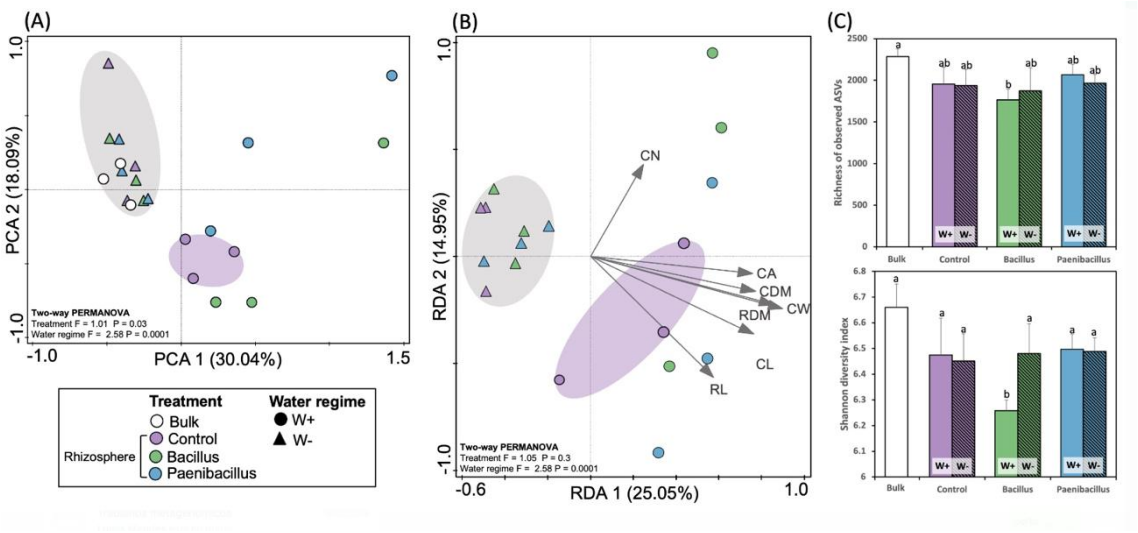

Figure 1 - Effects of water deficit and PGPB inoculation on the structure and diversity of bacterial communities in the rhizosphere of prickly pear cactus. (A) Principal Component Analysis (PCA) showing the distribution of bacterial communities of bulk soil and rhizosphere, based on Bray–Curtis dissimilarity. (B) Redundancy Analysis (RDA) illustrating the relationship between the rhizosphere bacterial community structure and plant traits (CA: cladode area; CDM: cladode dry mass; CW: cladode width; CL: cladode length; RL: root length; RDM: root dry mass; CN: cladode number) (C) Alpha diversity metrics of bacterial communities: richness (top) and Shannon diversity index (bottom). Different lowercase letters indicate statistically significant differences ($P < 0.05$, Tukey's test).

3.3.2. The composition of the bacterial community in the rhizosphere

The bacterial community composition was dominated by the phyla *Actinobacteriota* (approximately 50%), *Firmicutes* (20%), *Proteobacteria* (12%), *Chloroflexi* (10%), and

Acidobacteria (5%) in both bulk soil and the rhizosphere of prickly pear cactus (Figure 2A). Comparing water regimes, Actinobacteria were more abundant under water-deficit conditions, whereas *Proteobacteria* were more prevalent under well-watered conditions. Regardless of water availability, *Actinobacteriota* and *Gemmatimonadota* were enriched in the rhizosphere of prickly pear cactus inoculated with *Paenibacillus* sp., while Proteobacteria and RCP2-54 were more abundant following *B. subtilis* inoculation (Figure 2B). In contrast, *Acidobacteria* and *Cyanobacteria* dominated the rhizosphere of non-inoculated plants. When specifically comparing water conditions, *Actinobacteria* remained consistently more abundant under water deficit, whereas *Proteobacteria* were enriched in well-watered conditions (Figure 2C).

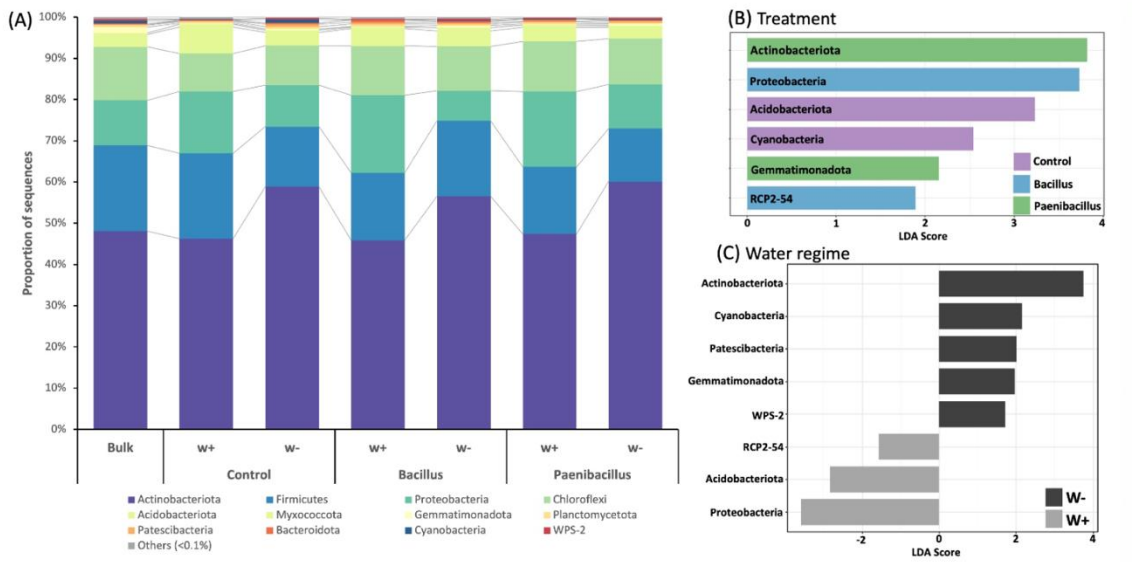

Figure 2 - Composition and differential abundance of bacterial communities in the rhizosphere soil of prickly pear cactus under different PGPB treatments and water regimes. **(A)** Relative abundance of bacterial phyla across treatments. Bars represent the mean proportion of sequences for each phylum under bulk soil, control, and rhizosphere treatments inoculated with Bacillus subtilis or *Paenibacillus sp.*, under well-watered (W+) and water-stressed (W-) conditions. **(B)** Linear discriminant analysis (LDA) showing bacterial phyla that are differentially enriched among treatments (Control, *Bacillus, Paenibacillus*). **(C)** LDA indicating bacterial phyla that are differentially enriched under different water regimes (W+ vs. W-).

3.3.2. The enrichment of specific bacterial groups in the rhizosphere

Differential analysis of the bacterial community revealed seven main clusters (Figure 3A). Bulk soil formed a distinct cluster, comprising 31 ASVs. The rhizosphere of well-watered plants clustered separately from that of plants under water-deficit conditions. Within each water regime, treatments inoculated with PGPB formed distinct clusters based on specific ASVs.

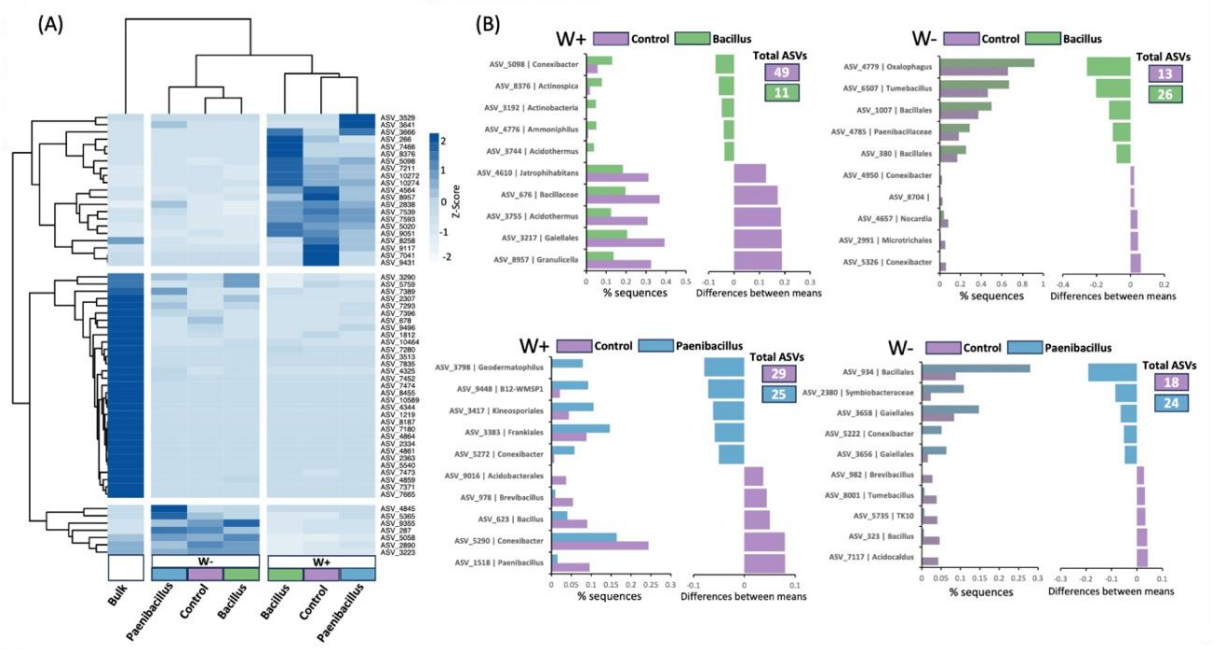

Figure 3 - Differential enrichment of bacterial ASVs in the rhizosphere of prickly pear cactus under different PGPB inoculations and water regimes. (A) Heatmap showing the Z-score normalized abundance of the significant differential ASVs across bulk soil and rhizosphere samples inoculated with Bacillus subtilis, Paenibacillus sp., or left as control, under well-watered (W+) and water-stressed (W-) conditions. (B) Differential abundance analysis of ASVs between control and inoculated samples under W+ and W- conditions. Bar plots represent the relative abundance (% sequences) and the difference between group means for the top five ASVs that were significantly enriched in each treatment ($p < 0.05$). The total number of significantly different ASVs for each comparison is shown in boxes.

To assess the enrichment of bacterial genera in the rhizosphere, we compared non-inoculated (control) plants with each PGPB-inoculated treatment under both well-watered and water-deficit conditions (Figure 3B). Under well-watered conditions, the rhizosphere of control and *B. subtilis*-inoculated plants enriched 49 and 11 ASVs, respectively. The control treatment showed a higher enrichment of *Galellales*, while *B. subtilis* inoculation primarily enriched *Conexibacter*. Under water-deficit conditions, 13 and 26 ASVs were enriched in the control and *B. subtilis*-inoculated rhizospheres, respectively. *Conexibacter* was more abundant in the control, whereas *Oxalophagus* was enriched by *B. subtilis* inoculation.

For *Paenibacillus* sp., under well-watered conditions, 29 ASVs were enriched in the control and 25 in the inoculated rhizosphere. The control enriched for *Conexibacter*, while *Paenibacillus* sp. inoculation promoted the enrichment of *Frankiales*. Under water-deficit conditions, the control and *Paenibacillus* sp.-inoculated rhizospheres enriched 18 and 24 ASVs, respectively. *Bacillus* was more abundant in the control, whereas *Bacillales* were enriched by *Paenibacillus* sp. inoculation.

3.3.3. The co-occurrence network in the rhizosphere

The analysis of bacterial interactions using co-occurrence networks revealed a lower number of nodes and edges in bulk soil (455 nodes and 2,228 edges) compared to rhizosphere samples (Figure 4). Comparing well-watered and water-deficit conditions, we observed distinct patterns in bacterial networks depending on inoculation status. In non-inoculated plants, the number of nodes was similar under both conditions (505 nodes in well-watered and 499 in water-deficit), while the number of edges increased under water-deficit conditions (4,034 edges), indicating a denser interaction network. Inoculation with *Bacillus* subtilis under well-watered conditions increased the number of nodes (530) but decreased the number of edges (3,210) compared to the water-deficit condition (496 nodes and 3,290 edges). In contrast, *Paenibacillus* sp. inoculation led to increases in both nodes and edges under well-watered conditions (521 nodes and 3,832 edges), suggesting enhanced network complexity. Overall, we observed a higher proportion of positive than negative interactions across all treatments, although the balance between them varied depending on water availability and inoculation (Appendix 3) For non-inoculated plants, the proportion of positive and negative interactions remained similar between water conditions. However, for inoculated plants, the trends diverged: *Bacillus subtilis* increased positive interactions under water deficit, whereas *Paenibacillus* sp. inoculation resulted in fewer positive interactions under the same condition.

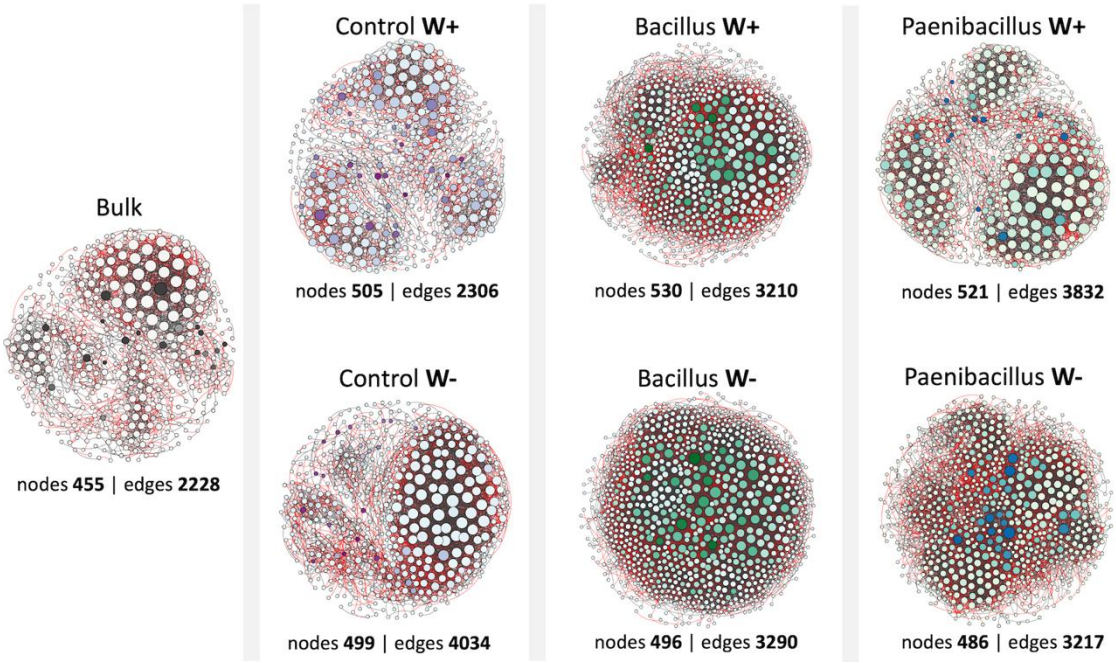

Figure 4 - Co-occurrence networks of bacterial ASVs in bulk soil and rhizosphere samples of prickly pear cactus under different PGPB inoculations and water regimes. Each network represents microbial co-occurrence patterns inferred using Spearman correlations ($\rho > 0.7$, $p < 0.01$). Nodes represent ASVs, and edges represent significant positive (gray) or negative (red) correlations. Node size reflects ASV degree of correlations, and node color intensity indicates betweenness centrality. The number of nodes (ASVs) and edges (significant associations) are shown for each condition: bulk soil, and rhizosphere

under control, *Bacillus subtilis*, and *Paenibacillus sp.* treatments, under well-watered (W+) and water-stressed (W-) conditions.

3.4. Discussion

This study evaluated the effects of *Bacillus subtilis* and *Paenibacillus* sp. inoculation on the rhizosphere bacterial community of prickly pear cactus under well-watered and water-deficit conditions. We hypothesized that each PGPB strain would distinctly shape the rhizosphere bacterial community, particularly under water-deficit stress. Our results partially support this hypothesis: while water availability significantly influenced the structure of the bacterial community, inoculation with either *B. subtilis* or *Paenibacillus* sp. had only a minor effect. These findings suggest that PGPB inoculation did not substantially alter the existing microbial structure, indicating a preservation of the native bacterial community in the rhizosphere. This result contrasts with previous studies, such as one conducted on common bean plants, which demonstrated that inoculation with different bacterial strains and a synthetic community significantly altered the rhizosphere microbiome, increased microbial diversity, and promoted more complex microbial interactions (Cunha *et al.*, 2024).

In this study, *Paenibacillus polymyxa* and *Bacillus cereus* were shown to influence nutrient dynamics, microbial assembly, and plant physiological responses, highlighting the potential of inoculants to reshape the microbial community and benefit plant health. In a study with *Azospirillum brasilense*, the inoculation significantly influenced the structure and interactions of the rhizosphere microbiome in maize, with effects dependent on the population size of the inoculant (Pedrinho *et al.*, 2024). The discrepancy between our findings and those of other studies may reflect host plant specificity, inoculant compatibility, or differences in experimental context, particularly regarding soil conditions, plant species, and the composition of the native microbiota.

In contrast, water deficit emerged as the primary factor shaping the structure of the bacterial community in the rhizosphere, consistent with previous studies examining the impact of drought on soil microbiota (Willing *et al.*, 2020; Wang *et al.*, 2022). Specifically, we observed significant differences in bacterial community structure only when comparing well-watered and water-deficit conditions. This indicates that, under semiarid conditions, water availability is a key driver of rhizosphere microbiome composition in prickly pear cactus (Lyra *et al.*, 2021; Li *et al.*, 2022).

When exploring the relationship between bacterial community structure and biometric parameters of the cactus, we found that these plant traits clustered closely with the well-watered treatments. This suggests that neither *B. subtilis* nor *Paenibacillus* sp. inoculation was sufficient

to modulate the rhizosphere microbiome in a way that could alleviate the negative effects of water deficit on plant performance. It is important to note that the response of the rhizosphere microbiome to water deficit may also depend on the intrinsic drought tolerance of the host plant.

Recent findings by Silva *et al.* (2025) demonstrate that drought-tolerant common bean cultivars exhibit a distinct and functionally enriched rhizosphere microbiome under water-limited conditions, particularly in microbial genes associated with osmotic adjustment, oxidative stress mitigation, and biofilm formation. These results suggest that drought-tolerant plants are more effective in selectively recruiting beneficial microbial functions under stress, contributing to a proactive microbial-driven resilience. In our study, the limited effect of PGPB inoculation and the strong influence of water availability on the rhizosphere microbiome of prickly pear cactus may reflect the species' inherent drought tolerance and its pre-established microbial associations in semiarid environments. Thus, the extent to which microbial inoculants reshape the rhizosphere community may depend not only on environmental factors but also on the host plant's adaptive capacity to water stress.

Despite the strong influence of water availability in shaping the structure of the rhizosphere bacterial community, both bacterial richness and diversity remained stable across treatments. This indicates that, although water conditions (i.e., well-watered and water-deficit) altered the composition of the bacterial community, the overall number of bacterial species and their within-community diversity (Delgado-Baquerizo *et al.*, 2017) were not significantly affected. Interestingly, *B. subtilis* inoculation led to a reduction in bacterial diversity under well-watered conditions, suggesting that this PGPB may play a selective role in modulating the rhizosphere microbiome by favoring specific bacterial taxa when water is not a limiting factor (Forneau *et al.*, 2024).

Although the inoculation of *B. subtilis* and *Paenibacillus* sp. did not significantly affect the overall structure of the bacterial community in the rhizosphere, both PGPBs altered its composition. Notably, we observed a higher abundance of *Actinobacteriota* under water-deficit conditions, suggesting that this phylum is particularly well-adapted to colonize the rhizosphere under drought stress (Narsing Rao *et al.*, 2022; Ebrahimi-Zarandi *et al.*, 2023). For example, Ebrahimi-Zarandi *et al.* (2023) reported an increase in *Actinobacteriota* abundance during drought, likely due to their ability to produce a wide array of secondary metabolites that enhance stress tolerance. In contrast, Proteobacteria were more abundant under well-watered conditions. This is consistent with previous findings showing that Proteobacteria are more sensitive to water deficit, as they include a high proportion of non-dormant cells that prefer moist environments (Jones; Lennon, 2010). Similarly, Fan *et al.* (2023) reported an enrichment of

Actinobacteria and a decline in *Proteobacteria* under drought, reinforcing the role of water availability in shaping dominant bacterial groups in the rhizosphere.

When comparing both PGPB, inoculation with *Paenibacillus* sp. led to an increased abundance of *Actinobacteriota* and *Gemmatimonadota* in the rhizosphere. *Paenibacillus* is well recognized for its functional traits, including biological nitrogen fixation, phosphorus solubilization, and production of plant growth-promoting metabolites (Grady *et al.*, 2016), which can shape rhizosphere microbial communities by selecting for bacteria adapted to nutrient-enriched or altered microenvironments.

Actinobacteriota are known for their metabolic versatility and production of stress-related secondary metabolites, which may enhance the resilience of the microbial community under stress conditions (Narsing Rao *et al.*, 2022). Similarly, *Gemmatimonadota* are often associated with oligotrophic conditions and may benefit from shifts in nutrient dynamics promoted by *Paenibacillus* (Wang *et al.*, 2021). These changes suggest that *Paenibacillus* inoculation may facilitate a more functionally specialized microbiome that contributes to plant nutrient acquisition and drought tolerance.

In contrast, *B. subtilis* inoculation resulted in a marked increase in *Proteobacteria* abundance in the rhizosphere. *Proteobacteria* include many copiotrophic taxa that thrive under nutrient-rich and well-aerated conditions, and several members are known for their roles in disease suppression, siderophore production, and hormone modulation (Fierer *et al.*, 2007). The increase in *Proteobacteria* following *B. subtilis* inoculation may reflect either direct microbial interactions or plant-mediated effects such as altered exudation patterns or immune signaling. Indeed, *B. subtilis* is known to enhance plant resistance to both biotic and abiotic stressors through induced systemic resistance and biofilm formation (Hashem *et al.*, 2019).

Furthermore, a recent study showed that *B. subtilis* inoculation can indirectly recruit beneficial microbial taxa, including *Proteobacteria*, reinforcing a positive feedback loop that enhances microbiome-mediated plant protection (Rigobelo *et al.*, 2024). Taken together, these findings highlight that the influence of PGPB on the rhizosphere microbiome is both taxa-specific and context-dependent, shaped by the functional capabilities of the inoculant and its interaction with plant physiology and native microbial communities.

The enrichment of specific bacterial groups revealed that *Conexibacter* and *Oxalophagus* were favored by the inoculation of *B. subtilis* under both well-watered and water-deficit conditions. Previous studies have reported *Conexibacter* as a common and abundant taxon in soils (Deng *et al.*, 2015), including those from the Brazilian semiarid region (Pacchioni *et al.*, 2014; Lacerda-Junior *et al.*, 2019). Our findings show an increase in *Conexibacter*

abundance in the rhizosphere of prickly pear cactus following *B. subtilis* inoculation, likely due to the bacterium's ability to promote nitrogen and phosphorus availability (Pedrinho *et al.*, 2020; Xia *et al.*, 2025), which may support the recruitment and enrichment of *Conexibacter* in the rhizosphere (Wu *et al.*, 2022).

In contrast, the inoculation with *Paenibacillus* sp. enriched members of the orders *Frankiales* and *Bacillales* under well-watered and water-deficit conditions, respectively. Under well-watered conditions, *Paenibacillus* may enhance phosphorus solubilization, enriching the rhizosphere with this nutrient and favoring the proliferation of *Frankiales*, a copiotrophic bacterial group involved in phosphorus cycling (Cunha *et al.*, 2023; Kang *et al.*, 2023). Under water-deficit conditions, the enrichment of Bacillales, a group within the phylum Firmicutes, suggests its adaptive capacity to drought, possibly through the production of exopolysaccharides and endospores (Díaz-Cornejo *et al.*, 2023). Supporting this, Paliwoda *et al.* (2022) reported the enrichment of Bacillales in the rhizosphere of strawberry plants exposed to water-deficit conditions.

Co-occurrence network analysis is a powerful tool for exploring microbial interactions and understanding how environmental factors, such as water availability, influence microbial community structure (Yang *et al.*, 2024; de Vries *et al.*, 2018). In this study, we demonstrated how both water deficit and PGPB inoculation shape bacterial co-occurrence patterns in the rhizosphere of prickly pear cactus. Notably, bulk soil displayed lower network complexity, evidenced by a reduced number of nodes and edges, compared to rhizosphere networks (Fu *et al.*, 2024). This lower complexity can be attributed to the absence of the rhizosphere effect, which is recognized as a hotspot for microbial activity and interactions due to root exudates and plant-driven nutrient fluxes (Sun *et al.*, 2023).

In non-inoculated plants, the number of nodes remained similar under both water conditions, suggesting the presence of a stable core bacterial community (Cai *et al.*, 2025). However, the increase in edges under water-deficit conditions indicates intensified microbial interactions, possibly driven by enhanced root activity and exudation aimed at recruiting beneficial microbes to support plant resilience (George *et al.*, 2024). In contrast, PGPB inoculation led to distinct network responses, highlighting the differential impact of microbial inoculants on rhizosphere community assembly under varying water regimes.

Under well-watered conditions, *B. subtilis* inoculation led to an increase in the number of nodes but a reduction in edges, indicating lower network complexity (Cai *et al.*, 2025). This pattern suggests that *B. subtilis* promotes the recruitment of a more specific and specialized bacterial community in the rhizosphere under favorable conditions. In contrast, under water-

deficit conditions, the number of nodes decreased while the number of edges increased, reflecting a shift toward a more interactive bacterial community. This indicates that *B. subtilis* may help plants cope with drought by enhancing cooperative interactions among rhizosphere bacteria, contributing to stress adaptation. Notably, *B. subtilis* inoculation also increased the proportion of positive interactions under water-deficit conditions, suggesting a more functionally cohesive and supportive microbial network (Pantigoso *et al.*, 2022; Ren *et al.*, 2025). For example, Ren *et al.* (2025) demonstrated that *B. subtilis* inoculation enhanced plant drought tolerance in cotton by stimulating beneficial microbial associations in the rhizosphere, increasing the frequency of positive bacterial interactions, and facilitating the recruitment of microbial taxa that aid in stress mitigation.

Distinctly, inoculation with *Paenibacillus* sp. increased both the number of nodes and edges under well-watered conditions, indicating that this PGPB promotes bacterial recruitment and enhances microbial network complexity when water is not limiting. This suggests that *Paenibacillus* sp. fosters a more connected and interactive bacterial community in the rhizosphere of cactus under favorable conditions. However, under water deficit, we observed a marked reduction in positive interactions, implying a disruption in microbial connectivity and cooperation, likely due to stress-induced shifts in rhizosphere dynamics or reduced functional compatibility among microbial taxa. Interestingly, our findings highlight a contrasting pattern between the two PGPB strains: while *Bacillus subtilis* enhanced positive microbial interactions under water deficit, *Paenibacillus* sp. appeared to weaken them. These opposing effects emphasize that the influence of PGPB on microbial network structure and functionality is highly context-dependent, varying with both the microbial inoculant and the prevailing environmental conditions.

In summary, our results demonstrate that *B. subtilis* and *Paenibacillus* sp. differentially modulate the structure and interactions of the rhizosphere bacterial community depending on water availability. Under well-watered conditions, *Paenibacillus* sp. promoted greater network complexity and microbial connectivity, suggesting its role in enhancing a diverse and functionally interactive community when environmental conditions are favorable. In contrast, *B. subtilis* appeared to recruit a more specialized bacterial community with lower connectivity, possibly reflecting selective enrichment of beneficial taxa. Under water deficit, *B. subtilis* stood out by increasing positive microbial interactions, suggesting that it facilitates a cooperative and stress-resilient microbial network capable of supporting plant tolerance to drought. Meanwhile, *Paenibacillus* sp. failed to maintain such interactions under stress, indicating limited efficacy in sustaining beneficial microbial relationships during water scarcity. These findings highlight

the importance of selecting context-appropriate PGPB strains to modulate rhizosphere microbial communities for improved plant performance under varying environmental conditions.

3.5. Conclusions

This study demonstrated that water deficit is a primary driver of bacterial community dynamics in the rhizosphere of prickly pear cactus. In contrast, inoculation with plant growth-promoting bacteria (PGPB), specifically *Bacillus subtilis* and *Paenibacillus* sp., had a limited effect on overall community structure but did influence bacterial composition and the enrichment of specific taxa. Notably, *B. subtilis* enhanced positive microbial interactions under water-deficit conditions, suggesting its potential to support drought mitigation in prickly pear cactus. Future research should investigate a broader range of PGPB species and evaluate their long-term effects under field conditions, particularly in semiarid environments, to validate the practical application of this strategy for sustainable agriculture.

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4. CHAPTER 3

MICROBIAL BIOMASS AND ENZYMATIC ACTIVITY IN THE RHIZOSPHERE OF PRICKLY-PEAR CACTUS GENOTYPES INOCULATED WITH *Bacillus subtilis* AND *Paenibacillus sp.*

ABSTRACT

Some bacterial taxa, such as *Bacillus* and *Paenibacillus*, are known to colonize the rhizosphere and promote plant growth. However, little is known about their effect on microbial biomass and enzymatic activity in the rhizosphere of plants under semi-arid conditions. This field study assessed the effects of *B. subtilis* and *Paenibacillus sp.* in the rhizosphere of two prickly-pear cactus genotypes on microbial biomass of C, N, and P, and on enzymatic activity during early and late growth stages. The analysis of variance showed that microbial biomass and enzymatic activity were significantly influenced by interactions among PGPB taxa, cactus genotypes, and growth stage. Specifically, PGPB inoculation increased microbial biomass P, β-glucosidase, and acid phosphatase, while microbial biomass of C and N was more affected by cactus genotypes. At the early growth stage (90 days), the highest values of microbial biomass C, P, and acid phosphatase were observed, whereas N biomass was higher at the later stage (270 days). *B. subtilis* increased microbial biomass P in the 'Doce' genotype and acid phosphatase in 'Baiana,' while *Paenibacillus sp.* increased β-glucosidase in 'Baiana.' Multivariate analysis showed distinct clusters of cactus genotypes and PGPB taxa, suggesting distinct microbial biomass dynamics. This study highlights that the interaction between growth stage, PGPB taxa, and cactus genotypes significantly affects microbial biomass and enzymatic activity in the rhizosphere, and that combining specific genotypes with *B. subtilis* can enhance phosphorus availability, benefiting growth in arid, nutrient-poor soils.

Keywords: Microbial phosphorus, *Nopalea cochenillifera L.*, PGPB, Semiarid.

4.1. Introduction

The Brazilian semiarid region covers approximately 1 million km² and is characterized by high annual temperatures (~30°C) and limited rainfall (~600 mm), concentrated within a four-month period (Silva *et al.,* 2023). In response to these challenging conditions, smallholders prioritize resilient crops, particularly drought-tolerant species such as the prickly-pear cactus (*Nopalea cochenillifera* L.) (Lyra *et al.,* 2021). This cactus species performs crassulacean acid metabolism (CAM) photosynthesis, which is a physiological process adapted to minimize water loss through regulated transpiration (Niechayev *et al.,* 2023). Additionally, prickly-pear cactus contains high levels of essential nutrients, including ascorbic acid, vitamin E, carotenoids, and fiber (Sipango *et al.,* 2023). These physiological and biochemical characteristics make prickly-pear cactus valuable in semiarid regions, both for conserving the plant and providing water and nutrients to animals (Alam-Eldein *et al.,* 2021).

Despite its importance in semiarid agriculture, prickly-pear cactus often has low productivity and quality, mainly due to a lack of inputs such as chemical fertilizers (Ferreira *et al.,* 2022), primarily because of their high cost (Hendricks *et al.,* 2019). However, more sustainable economic and ecological alternatives, such as plant growth-promoting bacteria (PGPB), can be employed to enhance plant growth through natural biological processes (Poria *et al.,* 2022). PGPB support plant development by producing hormones and antibiotics, fixing atmospheric nitrogen, and solubilizing phosphates (Di Benedetto *et al.,* 2017; Zhang *et al.,* 2023). In semiarid regions, studies have reported the benefits of PGPB to important crops such as cowpea, maize, sorghum, and cassava (Leite *et al.,* 2018; Aquino *et al.*, 2019; Nascimento *et al.*, 2021).

While several studies have reported the role of PGPB in promoting plant growth, less is known about their potential effects on soil microbial biomass and enzymatic activity, particularly within the rhizosphere of semiarid plant species. Particularly to semiarid regions, understanding the influence of PGPB on microbial biomass and enzymatic activity is essential, as these biological parameters play a role in increasing soil organic matter and nutrient availability (Mazzuchelli *et al.,* 2020). Thus, increased SOM contributes to conservate soil moisture, while higher nutrient availability supports the plant growth. A key impact of PGPB on the rhizosphere is the stimulation of root exudation, which provides carbon and nitrogen sources to support microbial biomass (Silva *et al.,* 2021). For instance, Silva *et al.* (2021) demonstrated that PGPB inoculation in sorghum and maize positively influenced soil microbial

biomass C. However, studies have also shown that PGPB effects can vary depending on plant genotypes and their developmental stages (Haldar; Sengupta, 2015; Navarro-Noya *et al.,* 2022).

Although previous studies have highlighted the positive effects of PGPB inoculation on the growth of plant species in semiarid regions, some gaps remain unanswered. It is unclear how the inoculation of different PGPB strains in prickly-pear cactus genotypes might impact microbial biomass and enzymatic activity within the rhizosphere. Here, we hypothesized that: a) PGPB inoculation in different prickly-pear cactus genotypes would distinctly influence microbial biomass and enzymatic activity in the rhizosphere; and b) different stages of plant growth would affect microbial biomass and enzymatic activity responses. To test these hypotheses, we inoculated *Bacillus subtilis* and *Paenibacillus* sp. in two genotypes of prickly-pear cactus ('baiana' and 'doce') and assessed the effects on microbial biomass C and N and enzymatic activity in the rhizospheric soil.

4.2. Material And Methods

4.2.1. Study area

This field-study with prickly-pear cactus was conducted at the Campus Prof. Cinobelina Elvas from Federal University of Piauí, located in Bom Jesus, Piauí State (9° 4' 30" S, 44° 21' 26" W). The predominant soil is classified as Yellow Latosol which presents 17% clay, 5% silt, and 78% sand. Soil chemical properties were assessed according to Teixeira *et al.* (2017) at the beginning of the study (Appendix 1). During the study, the climatic conditions were recorded (Figure 5).

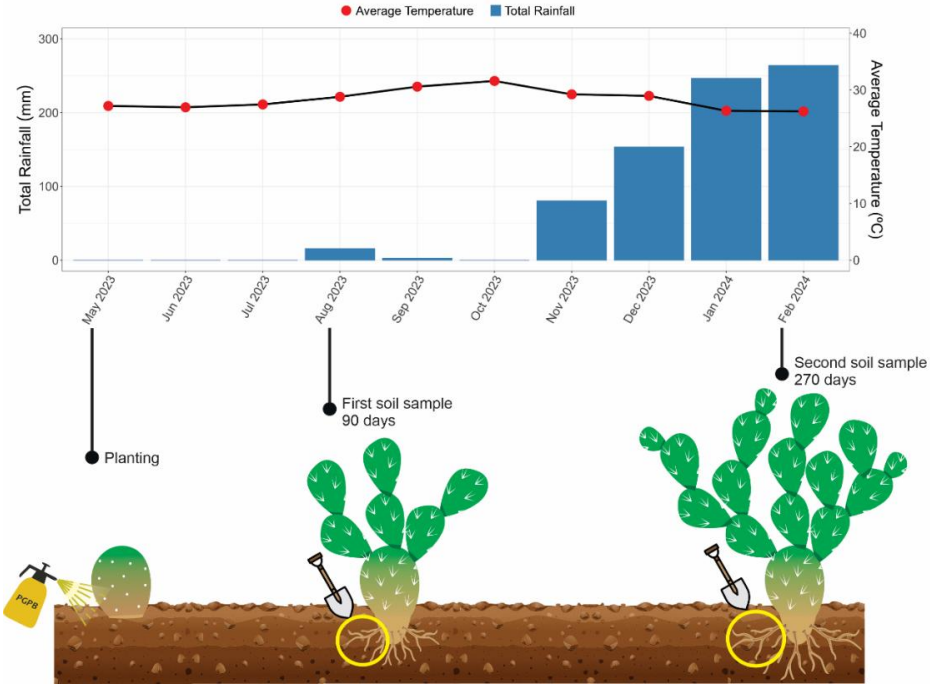

Figure 5 - Climatic conditions at planting and soil collections.

4.2.2. Experimental design and planting

The study was conducted in a completely randomized design under split-split plots (2x3x2). The main plot was growth stage (90 and 270 days) after cactus planting, while the subplot was PGPB inoculation (non-inoculation and inoculation with *B. subtilis* and *Paenibacillus sp*). The sub-subplot was two genotypes of prickly-pear cactus ('baiana' and 'doce'). The strains of both *B. subtilis* and *Paenibacillus sp* were IPA-CC29 and IPA-CC38, respectively. These strains were used in previous experiments (Aquino *et al.* , 2019), and their growth-promoting characteristics include the production of Indole-3-acetic acid (IPA-CC29: 1.41 mg L^{-1}; IPA-CC38: 1.13 mg L^{-1}) and Acetylene-reducing activity (IPA-CC29: 5.03 nmol C_2H_4 h^{-1}; IPA-CC38: 7.10 nmol C_2H_4 h^{-1}).

To obtain the PGPB inoculants, *B. subtilis* and *Paenibacillus sp* were grown in Erlenmeyer flasks containing 50 mL of liquid medium (Tryptone Soy Broth) and incubated in a rotatory shaker (200 rpm; 31 °C) for 72 hours. Bacterial growth was assessed by optical density (absorbance) at a wavelength of 540 nm using a spectrophotometer and was approximately 10^8 CFU (colony-forming unit) mL^{-1}. The planting was done using one cladode per hole, with a spacing of 0.75 x 0.50 m. After planting, the inoculation of *B. subtilis* and *Paenibacillus sp* was performed by spraying the liquid inoculant diluted in water at a 5:1 ratio directly in the root area. (Figure 5).

4.2.3. Soil sampling and analysis

Rhizospheric soil samples were collected by removing soils adhered to the roots, using a spade while preserving the plant structure as much as possible. After rhizospheric soil sampling, all samples were sieved through a 2 mm mesh sieve, and stored at 6 °C.

Soil microbial biomass C, N, and P were assessed by irradiation-extraction, as described by Islam and Weil (1998), Joergensen and Brookes (1990), and Brookes *et al.* (1982). Briefly, 10 g of soil from each sample was weighed in duplicate, with one sample being irradiated and the other non-irradiated. The irradiated samples were subjected to a microwave oven in five cycles of 30 seconds each. An extracting solution was then added to both irradiated and non-irradiated soil samples, which were shaken for 30 minutes at 150 rpm. After shaking, the samples were filtered. Potassium sulfate (K_2SO_4) was used as the extracting solution for microbial biomass C and N, while sodium bicarbonate ($NaHCO_3$) was used for P.

To measure the biomass C, potassium dichromate ($K_2Cr_2O_7$) and sulfuric acid (H_2SO_4) were added to the samples. The solution was heated, then cooled, and diluted with distilled water. The residual dichromate was measured by titration with an ammonium ferrous sulfate solution ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$) in concentrated sulfuric acid (H_2SO_4), using ferroin ($[Fe(C_{12}H_8N_2)_3]SO_4$) as an indicator.

To measure biomass N, the samples were transferred to test tubes, and citric acid ($C_6H_8O_7$) and ninhydrin ($C_9H_6O_4$) were added. The samples were then incubated in a water bath at 100°C for 25 minutes. After cooling, an ethanol:water solution (C_2H_6O:H_2O) in a 1:1 ratio was added, and the absorbance was measured with a spectrophotometer at 570 nm.

To measure microbial P, HCl (10 mol L^{-1}) was slowly added to the samples, which were then left to stand for 1 hour. After this, the samples were filtered, and reagent A (containing ammonium molybdate - $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, potassium antimony tartrate - $C_8H_4K_2O_{12}Sb_2 \cdot 3H_2O$, and sulfuric acid - H_2SO_4), reagent B (containing ascorbic acid - $C_6H_8O_6$ and sulfuric acid - H_2SO_4), and distilled water were added. The samples were left to stand for 30 minutes, after which the absorbance was measured with a spectrophotometer at 882 nm.

The quantification of microbial biomass C, N, and P was determined by the difference between irradiated and non-irradiated soils. The extracted C, N, and P were converted into microbial C, N, and P using correction factors Kc = 0.38, 0.48, and 0.40, respectively.

The determination of β-glucosidase (Eivazi; Tabatabai, 1988) and acid phosphatase (Tabatabai; Bremner, 1970) activities was carried out by measuring p-nitrophenol, released when 0.5 g of soil was incubated for 1 hour at 37°C with buffered solutions containing the

substrates p-nitrophenyl-β-D-glucoside and p-nitrophenyl phosphate disodium. The p-nitrophenol released by the reaction with the enzymes was extracted from the soil by filtration after the addition of $CaCl_2$ and NaOH solutions. Readings were taken using a spectrophotometer at a wavelength of 400 nm for both enzymes.

The methodology used for the determination of urease enzyme in soil consisted of measuring ammonia release after incubating the soil in urea solution for 2 hours at 37°C (Kandeler; Gerber, 1988). Two grams of soil were weighed and added to the urea solution, followed by borate buffer with pH 10. The samples were then incubated in a water bath for 2 hours at 37°C. After the incubation period, potassium chloride (KCl) was added, followed by filtration. After filtering the material, H_2O, salicylate/NaOH, and dichloroisocyanurate (0.1%) were added, and the mixture was incubated for 30 minutes. The intensity of the color was measured with a spectrophotometer at 690 nm.

4.2.4. Statistical analysis

The data were subjected to the Shapiro-Wilk normality test and analysis of variance (ANOVA). When the treatments differed significantly based on the F test, the means were compared using Tukey's test at a probability level of 5%. The analysis was done in RStudio using the AgroR and ggplot2 packages. To reduce the dimensionality of the data and understand which factors have influence on the biological variables, a principal component analysis (PCA) was performed using a correlation matrix with the R software (stats, factoextra, and vegan packages), version 4.1.3 (R Core Team, 2021).

4.3. Results

4.3.1. The interactions between growth stage, PGPB and genotypes of prickly-pear cactus on microbial biomass and enzymatic activity in the rhizosphere

ANOVA results showed that PGPB species, prickly-pear cactus genotypes, growth stage, and their interactions significantly affected microbial biomass and enzymatic activity in the rhizosphere (Appendix 4). PGPB inoculation specifically impacted microbial biomass P, β-glucosidase, and acid phosphatase, while cactus genotypes influenced microbial biomass C and N. Growth stage also significantly affected microbial biomass C and N, as well as acid phosphatase. Interactions between PGPB species and cactus genotypes caused notable changes in microbial biomass C, N, β-glucosidase, and acid phosphatase, while three-way interactions

among PGPB species, cactus genotypes, and growth stage impacted microbial biomass C, N, P, and acid phosphatase. The highest values of microbial biomass C, P, and acid phosphatase were observed at 90 days, while microbial biomass N was higher at 270 days, with β-glucosidase and urease activities showing no significant changes across these periods (Figure 6).

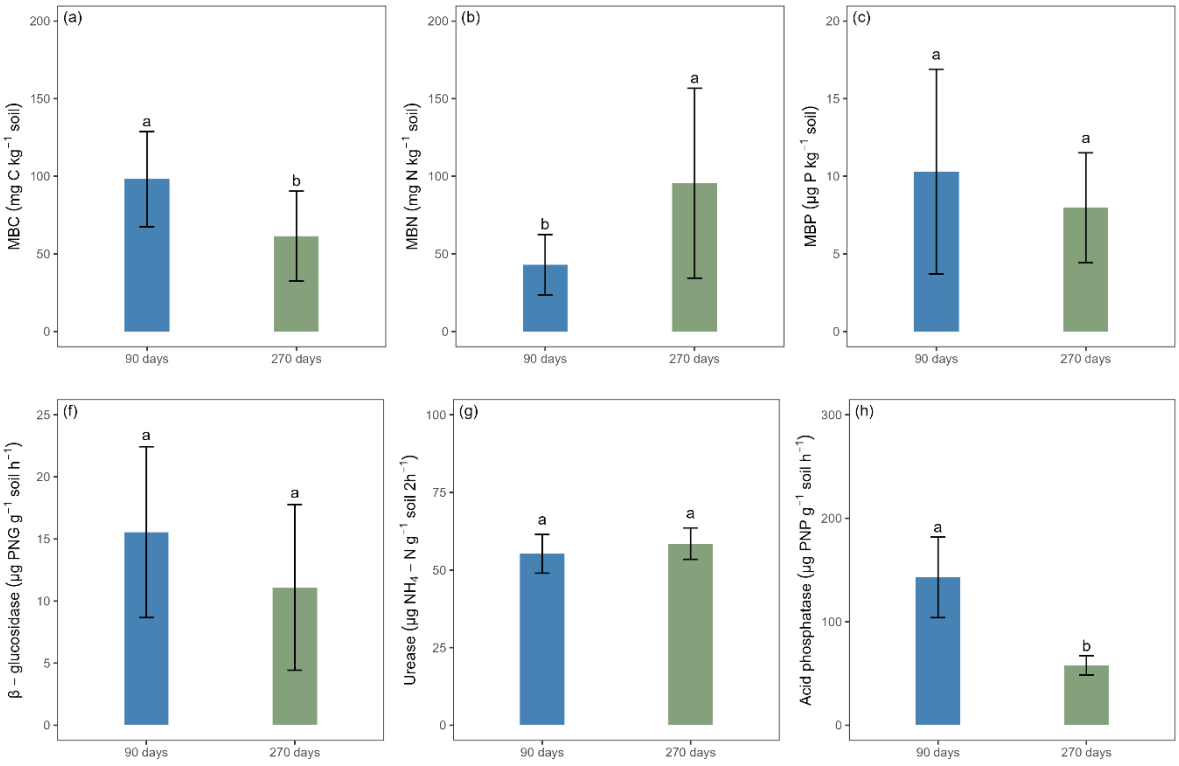

Figure 6 - Microbial biomass, microbial ratios, and enzymatic activity at 90 and 270 days of growth of prickly-pear cactus. Different lowercase letters indicate significant differences based on Tukey's HSD test ($p < 0.05$).

4.3.2. Microbial biomass and enzymatic activity in response to PGPB and genotypes of prickly-pear cactus

At 90 days, non-inoculated plants and those inoculated with *B. subtilis* showed higher microbial biomass C in the rhizosphere of genotype 'Doce' compared to 'Baiana' (Figure 7A). By 270 days, microbial biomass C remained higher in the non-inoculated rhizosphere of genotype 'Doce' relative to 'Baiana' (Figure 7B). In general, PGPB inoculation did not affect microbial biomass N in the rhizosphere at 90 days compared to non-inoculated plants (Figure 7C), although the non-inoculated genotype 'Baiana' had higher microbial biomass N than 'Doce'. At 270 days, *B. subtilis* inoculation increased microbial biomass N in genotype 'Doce' (Figure 7D). The microbial biomass P in the rhizosphere increased, at 90 days, in both genotypes with PGPB inoculation compared to non-inoculated plants (Figure 7E). By 270 days, PGPB

inoculation further increased microbial biomass P in the genotype 'Doce', while 'Baiana' showed higher microbial biomass P specifically with *B. subtilis* inoculation (Figure 7F).

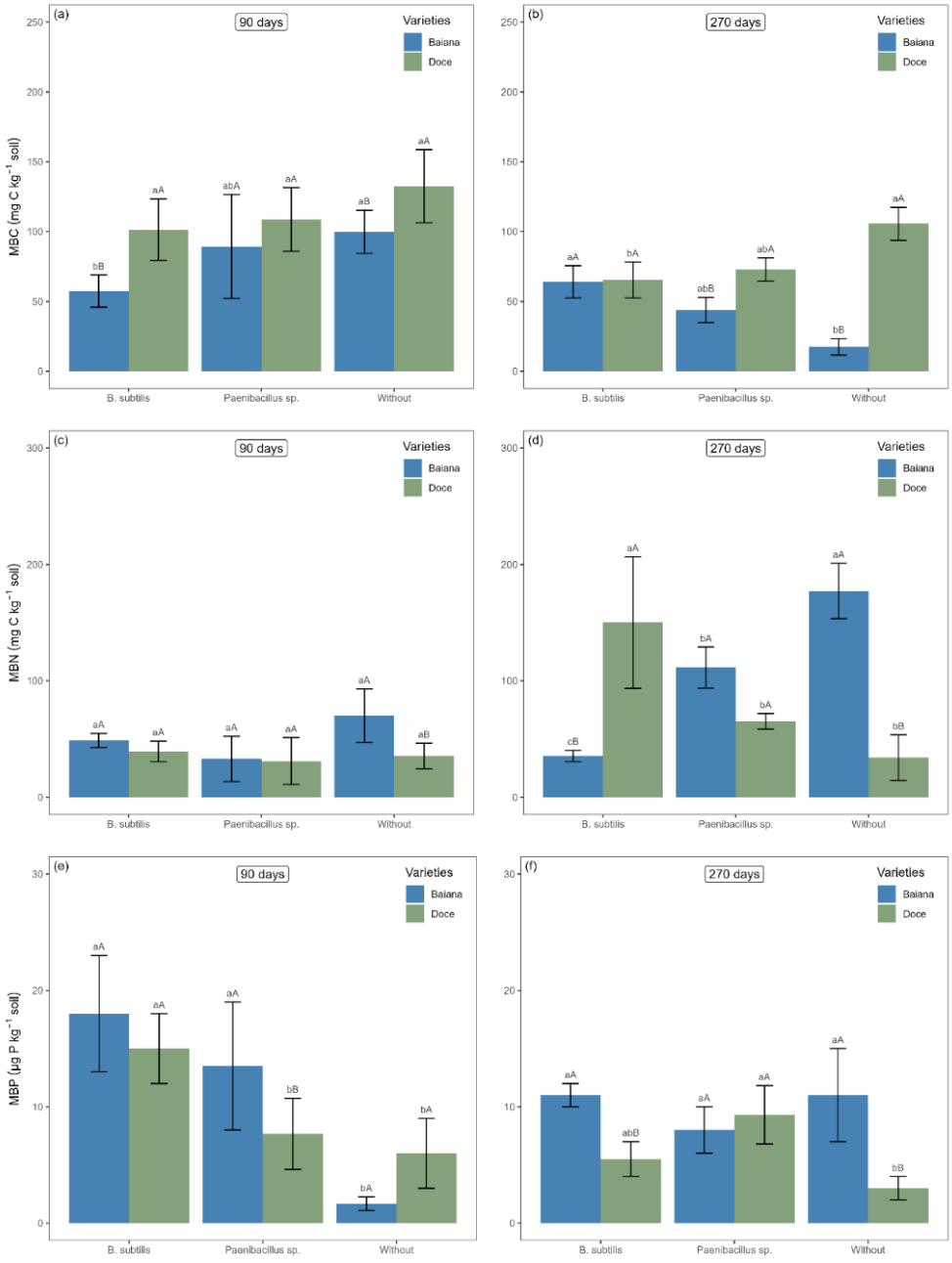

Figure 7 - Carbon (MBC), Nitrogen (MBN), and Phosphorus (MBP) of microbial biomass in the rhizosphere of 'Baiana' and 'Doce' prickly-pear cactus, including both non-inoculated and inoculated with Bacillus and Paenibacillus, at 90 and 270 days of plant growth. Lowercase letters distinguish inoculants within varieties, while uppercase letters distinguish varieties within inoculants, based on Tukey's HSD test ($p < 0.05$).

The inoculation of *B. subtilis* increased the activity of β-glucosidase in the rhizosphere of genotype 'Doce' at 90 days, while *Paenibacillus sp.* enhanced this activity in 'Baiana' (Figure 8A). At 270 days, PGPB inoculation significantly increased the activity of β-glucosidase in both genotypes (Figure 8B). The activity of urease activity in the rhizosphere was unaffected

by both PGPB and genotype treatments (Figures 8C, 8D). At 90 days, the activity of acid phosphatase activity increased in the rhizosphere of 'Baiana' with *Paenibacillus* sp. inoculation (Figure 8E), while at 270 days, acid phosphatase showed no significant differences between PGPB and genotype treatments (Figure 8F).

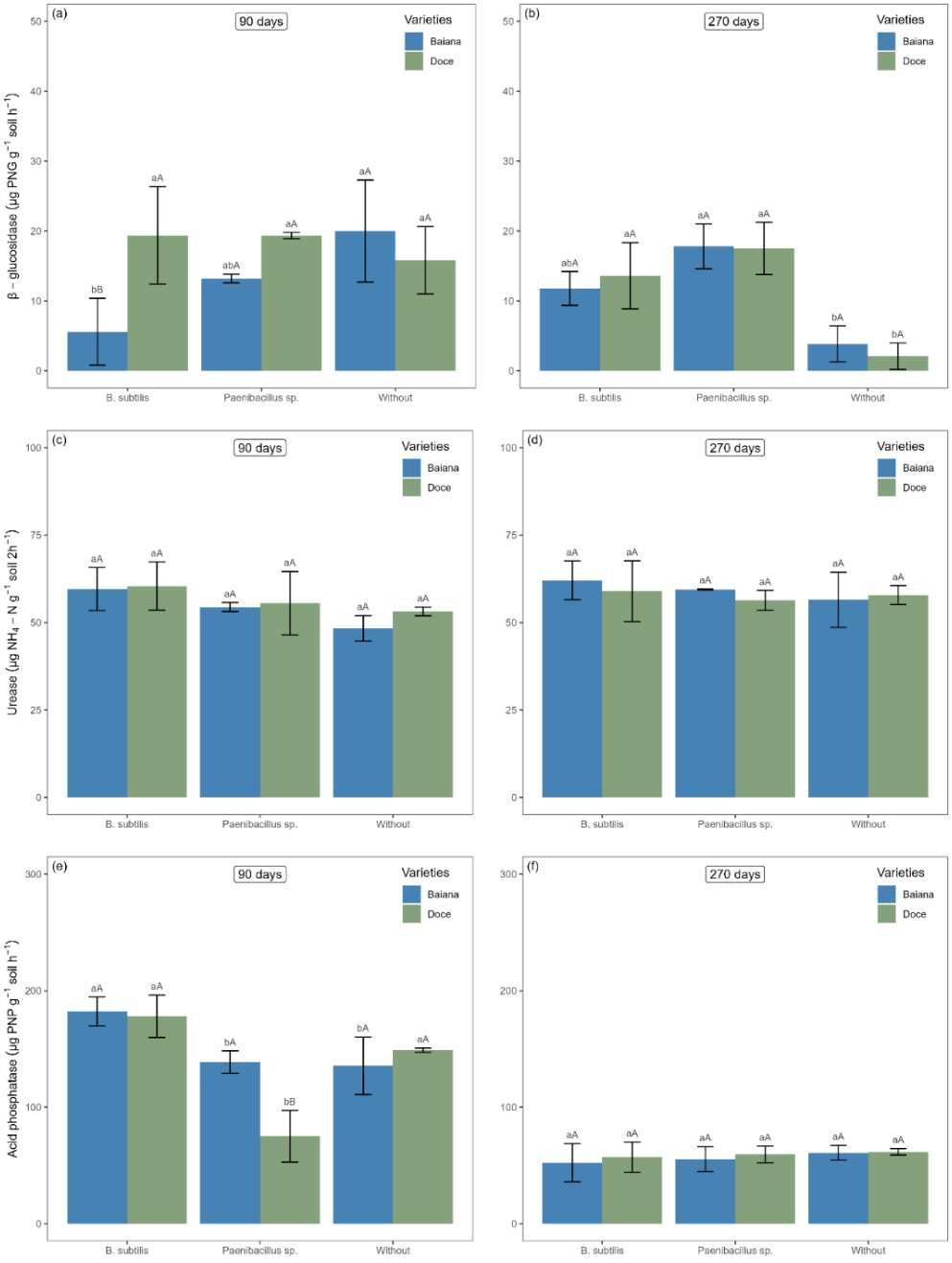

Figure 8 - The enzymatic activity of β-glucosidase, urease, and acid phosphatase in the rhizosphere of the 'Baiana' and 'Doce' prickly-pear cactus, both non-inoculated and inoculated with *Bacillus* and *Paenibacillus*, at 90 and 270 days of plant growth. Lowercase letters indicate differences among inoculants within the varieties, while uppercase letters indicate differences among varieties within the inoculants, based on the Tukey HSD test ($p < 0.05$)

4.3.3. Multivariate analysis of microbial biomass and enzymatic activity in responses to PGPB and genotypes prickly-pear cactus.

Principal component analysis (PCA) explained 68.6% and 58.3% of the total variance at 90 and 270 days, respectively (Figure 9). At 90 days, *B. subtilis* inoculation in the genotype 'Baiana' formed a distinct cluster, influenced by acid phosphatase. *B. subtilis* inoculation in the genotype 'Doce' and *Paenibacillus sp.* inoculation in the genotype 'Baiana' clustered together, driven by microbial biomass P and urease. The non-inoculated genotype 'Doce' and the one inoculated with *Paenibacillus sp.* formed a cluster, influenced by microbial biomass C and β-glucosidase, while the non-inoculated genotype 'Baiana' was influenced only by microbial biomass C.

At 270 days, the non-inoculated genotype 'Doce' formed an independent cluster, driven by microbial biomass C. In contrast, the non-inoculated genotype 'Baiana' and the one inoculated with *Paenibacillus sp.* clustered together, influenced by β-glucosidase and microbial biomass N and P. Microbial biomass C and N also influenced the genotype 'Baiana' inoculated with *Paenibacillus sp.*. *Bacillus subtilis* inoculation in the genotype 'Baiana' formed a cluster influenced by acid phosphatase, while the genotype 'Doce' inoculated with *Bacillus subtilis* was influenced by urease.

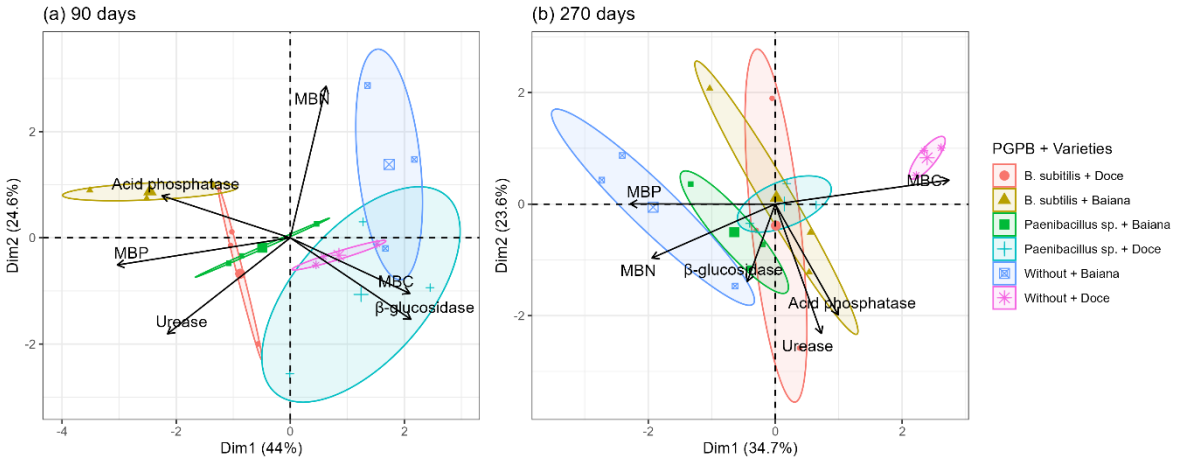

Figure 9 - The principal component analysis (PCA) of the combination of prickly-pear cactus varieties, both inoculated and non-inoculated, assessed their effects on microbial biomass (MBC, MBN, MBP), and enzymatic activity (β-glucosidase, urease, and acid phosphatase) at 90 and 270 days of plant growth. PCA a = Permanova < 0.05 and R^2: 0.634, PCA b = Permanova < 0.05 and R^2: 0.800.

4.4. Discussion

This study is the first to evaluate the effects of well-known plant growth-promoting bacteria (PGPB) on prickly-pear cactus genotypes in semiarid regions, specifically assessing their impact on microbial biomass and enzymatic activity in the rhizosphere. Both hypotheses were confirmed, showing that microbial biomass and enzymatic activity responses varied with the inoculated PGPB (*B. subtilis* and *Paenibacillus* sp.) and prickly-pear cactus genotypes ('Doce' and 'Baiana').

4.4.1. The growth stage influences microbial biomass and enzymatic activity in response to PGPB inoculation in prickly-pear cactus.

Regarding growth stage effects (90 and 270 days), our study suggests a rhizospheric influence on biological parameters, with differences observed between early (90 days; more active rhizosphere) and later (270 days; less active) stages in microbial biomass and activity (Lu *et al.,* 2018). Our results showed higher microbial biomass C and P and acid phosphatase activity at early growth stage, while microbial biomass N increased at later plant maturity. Root exudation, the primary driver of microbial communities in the rhizosphere, provides a carbon source that enhances compatibility between microorganisms and plants (Bukhat, *et al.,* 2020). Known to vary by plant growth stage (Vives-Peris *et al.,* 2020), root exudation during early growth provides abundant C sources (Ma *et al.,* 2022; Santangeli *eet al.,*2024), leading to increased microbial biomass C.

For microbial biomass P and acid phosphatase activity, higher phosphorus demand at early stages explains the observed increases in these parameters (Chodak *et al.,* 2021). Additionally, lower soil moisture at 90 days influenced root traits in cactus species (Snyman *et al.,* 2006), potentially impacting the rhizosphere and associated microbial biomass. The results showed higher microbial biomass N at 270 days which may mean that the later growth stage, reduced plant N demand allowed microbes to outcompete roots for nitrogen, boosting microbial biomass (Kuzyakov; Xu, 2013). Higher soil moisture at 270 days also facilitated organic matter decomposition, releasing nitrogen compounds for microbial use and further increasing microbial biomass N (Monaci *et al.,* 2022). β-glucosidase activity remained stable across both growth stages, suggesting a consistent rate of organic carbon decomposition (Cañizares *et al.,* 2011).

4.4.2. The interaction between PGPB and genotypes of prickly-pear cactus

There are currently no specific studies comparing the effects of PGPB species on microbial biomass and enzymatic activity across different plant genotypes, particularly in semiarid regions. Nevertheless, our results suggest a genotypic effect on microbial biomass and enzymatic responses to various PGPB species. Previous studies have reported a genotypic effect associated with PGPB on microbial communities in the rhizosphere (Salamone *et al.,* 2012; Ferrarezi *et al.,* 2022). Consequently, specific plant genotypes may optimize interactions with certain PGPB taxa, influencing the rhizosphere and its associated microbial populations (Shi *et al.,* 2024).

Interestingly, *B. subtilis* inoculation had distinct effects on microbial biomass C, N, and P in the rhizosphere of each genotype, particularly in the genotype 'Doce'. This enhancement in microbial C, N, and P may be attributed to the ability of *B. subtilis* in efficiently colonizing roots, forming biofilms, and producing exudates (Hashem *et al.,* 2019), creating favorable environments for soil microbes. Particularly, *B. subtilis* facilitates phosphorus solubilization, contributing to increased microbial biomass P (Gomez-Ramirez; Uribe-Velez, 2021). Additionally, both *B. subtilis* and *Paenibacillus* sp. increased β-glucosidase and acid phosphatase activities in the rhizosphere. These PGPB species stimulate plant roots to release organic acids and enzymes involved in the decomposition of organic compounds, such as cellulose and organic phosphate, thereby making C and P more available to plants (Rekha *et al.,* 2018; Garcia-Sanchez *et al.,* 2023).

4.4.3. Relationship between PGPB and genotypes of prickly-pear cactus and biological parameters in the rhizosphere

The results clearly demonstrated how *B. subtilis* and *Paenibacillus* sp. influenced microbial biomass and enzymatic activity in the rhizosphere of genotypes 'Baiana' and 'Doce', highlighting shifts in microbial parameters over time. At the early growth stage, *B. subtilis* associated with the genotype 'Baiana' increased phosphatase activity, enhancing phosphorus dynamics (Lemanowicz, 2018). Additionally, the association between *B. subtilis* and the genotype 'Doce' genotype led to increased microbial biomass P. Thus, the inoculation of *B. subtilis* in both genotypes can stimulate biological processes related to P cycling, increasing phosphorus availability in the rhizosphere of prickly-pear cactus (Jensen *et al.,* 2024). These findings suggest that *Bacillus* is an important PGPB for enhancing microbial biomass and activity in the rhizosphere of prickly-pear cactus. A previous study by Kavamura *et al.* (2013) identified *Bacillus* as the primary PGPB taxa in the rhizosphere of cactus.

Furthermore, the inoculation of *Paenibacillus* sp. in the genotype 'Baiana' also stimulated microbial biomass P, indicating a specific effect in this genotype.

At the later growth stage, the inoculations of *Paenibacillus* sp. in the genotype 'Baiana' and *B. subtilis* in 'Doce' were associated with β-glucosidase activity and microbial biomass P. This finding reinforces the potential of both PGPB, particularly *B. subtilis*, to enhance phosphorus availability (Jensen *et al.,* 2024) and influence carbon cycling (Duan *et al.,* 2020). Interestingly, the rhizospheres of non-inoculated genotype 'Doce' (at 90 days) and 'Baiana' (at 270 days) were primarily driven by microbial biomass N, suggesting that these genotypes may rely on nitrogen immobilized in microbial biomass.

4.4.4. Implications for inoculating *B. subtilis* and *Paenibacillus sp.* in genotypes of prickly-pear cactus

This study indicated that the inoculation of *B. subtilis* and *Paenibacillus* sp. impacts microbial biomass C and P and their associated enzymes, while microbial biomass N and its related enzyme appear unaffected by both PGPB. Although both *B. subtilis* and *Paenibacillus* sp. are known to be involved in nitrogen cycling (He *et al.,* 2023) primarily in nitrogen fixation (Liu *et al.,* 2019), our results suggest that, in the rhizosphere of prickly-pear cactus, these bacteria primarily contribute to organic matter breakdown and phosphorus solubilization rather than nitrogen mineralization and fixation. Consequently, prickly-pear cactus plants may depend on native soil microorganisms for nitrogen cycling.

4.5. Conclusions

The interactions among growth stage, PGPB taxa, and genotypes significantly shape microbial biomass and enzymatic activity in the rhizosphere of prickly-pear cactus. These dynamics suggest that specific combinations of prickly-pear cactus genotypes with PGPB taxa. Particularly, *B. subtilis* has been proved to be broadly effective for both genotypes of prickly-pear cactus, particularly in microbial biomass and enzymes associated to phosphorus. This mean that carefully pairing prickly-pear cactus genotypes with specific PGPB taxa, mainly *B. subtilis*, can enhance microbial biomass and enzymatic activity in the rhizosphere. Markedly, this can lead to improved P availability to support cactus growth, mainly in nutrient-poor arid soils.

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5. CHAPTER 4

GROWTH STAGE AS THE MAIN DRIVE OVER GENOTYPE AND PLANT GROWTH-PROMOTING BACTERIA INOCULATION OF BACTERIAL COMMUNITIES IN THE RHIZOSPHERE OF PRICKLY-PEAR CACTUS

ABSTRACT

Despite its strategic importance for food security in semiarid regions, no studies have assessed the rhizosphere microbiome of the prickly-pear cactus (*Nopalea cochenillifera)* and how its growth stage, bacterial inoculation, and genotypes may shape the rhizosphere. Thus, the aim of this study was to assess diversity, composition, and enrichment of bacterial communities in the rhizosphere of the prickly-pear cactus genotypes 'Baiana' and 'Doce', both with and without inoculation with *Bacillus subtilis* and *Paenibacillus sp.*, at 90 and 270 days of growth. In this study we cultivated samples of rhizospheric soil, extracted DNA, and assessed the bacterial communities by 16S rRNA amplicon sequencing by Illumina platform. The bacterial communities in the rhizosphere were significantly driven by plant growth stage and showed high diversity, while both inoculation and genotype showed lower effects. Key bacterial phyla typical of semiarid regions were predominant, including *Actinobacteriota, Proteobacteria*, and *Firmicutes*. Enrichment patterns of bacterial genera shifted with plant growth, with *Streptomyces* and *Bradyrhizobium* predominating at 90 days, and *Bacillus* and *Gaiella* at 270 days. PGPB inoculation further shaped specific taxa, while non-inoculated plants showed distinct enrichment over time. This study showed that the bacterial communities in the rhizosphere of *N. cochenillifera* were primarily shaped by plant growth stage, with key bacterial groups potentially associated with stress tolerance which provide important insights into soil microbial ecology in semiarid regions.

Keywords: Prickly-Pear Cactus, *Bacillus*, *Paenibacillus*, Microbial Enrichment, Resilience.

5.1. Introduction

In semi-arid regions, cactus cultivation represents a valuable strategy for supporting animal production during drought periods. This plant species presents high adaptability to semi-arid regions and contains high nutritional value, which is important to support animal production (Araújo Júnior *et al.*, 2023). Particularly, the prickly-pear cactus (*Nopalea cochenillifera*) has been used as forage in semiarid regions, mainly due to its nutritional value compared to other prickly-pear species (Dubeux Jr *et al.*, 2021). On the other hand, the productivity of *N. cochenillifera* varies significantly across genotypes, reflecting both genetic potential and adaptive traits. In addition, each genotype has specific morphological traits, such as root growth, which may influence microbial communities associated with the rhizosphere (Xie *et al.*, 2023).

The rhizosphere is the narrow soil zone surrounding plant roots, where root traits drive microbial communities (Yang *et al.*, 2024). Thus, assessing the rhizosphere microbiome of cactus is essential to understand how plant traits interact with soil microorganisms and shape beneficial associations. Previous studies have already assessed the microbial communities associated with different cactus species, such as *Opuntia cholla* (Manríquez-Rivera *et al.*, 2025), *Echinocactus platyacanthus* (Estrada-González *et al.*, 2023), *Pereskia aculeata* (Vega *et al.*, 2020), *Cereus jamacaru* (Kavamura *et al.*, 2018), *Myrtillocactus geometrizans*, *Opuntia robusta* (Fonseca-García *et al.*, 2016), and *Opuntia ficus-indica* (Lyra *et al.*, 2021). These studies showed the ecological importance of the rhizosphere microbiome and its potential role in stress adaptation, nutrient acquisition, and plant performance.

However, the rhizosphere microbiome of *N. cochenillifera* remains poorly understood, particularly when comparing genotypes and growth periods. It is known that rhizosphere–microbes interaction is strongly shaped by host-related factors, including genotype and growth stage (Han *et al.*, 2025). These differences in growth stage and genotypes have been shown to influence root exudation patterns which play a central role in selecting and recruiting rhizosphere microorganisms (Araújo *et al.*, 2025). For instance, Han *et al.* (2025) found that distinct soybean genotypes shaped rhizosphere microbial communities, with shifts across developmental stages. Beyond these factors, the inoculation of plant growth-promoting bacteria (PGPB) can modulate rhizosphere communities (Vuolo *et al.*, 2022; Cheng and Ma, 2025). For instance, Cheng and Ma (2025) inoculated PGPB in maize and found that inoculation enhanced beneficial bacteria and microbial interactions in the rhizosphere. These PGPB directly promote

plant growth, while indirectly altering microbial assembly through competition with native taxa and enrichment of beneficial microorganisms (Kampouris *et al.*, 2025).

In this context, we propose two hypotheses for this study: a) the bacterial communities in the rhizosphere of *N. cochenillifera* differ significantly comparing genotypes and growth period; and b) the inoculation of PGPB shapes the composition and interactions of the bacterial communities in the rhizosphere of *N. cochenillifera***.** Therefore, the aim of this study was to investigate the bacterial communities in the rhizosphere of *N. cochenillifera* (genotypes 'Baiana' and 'Doce') under inoculation with *Bacillus subtilis* and *Paenibacillus* sp. The rhizosphere was evaluated at two growth stages, i.e., early (90 days) and late (270 days) stages, to assess how microbial diversity, community composition, and enrichment patterns are influenced by genotype, plant growth stage, and PGPB inoculation.

5.2. Materials And Methods

5.2.1. Study area

This field-study with prickly-pear cactus was conducted at the Campus Prof. Cinobelina Elvas from Federal University of Piauí, located in Bom Jesus, Piauí State (9° 4' 30" S, 44° 21' 26" W). The predominant soil is classified as Yellow Latosol which presents 17% clay, 5% silt, and 78% sand. Soil chemical properties were assessed by Teixeira *et al.* (2017) at the beginning of the study (Appendix 1). During the study, the climatic conditions were recorded (Figure 5).

5.2.2. Experimental design and planting

The experiment was carried out in a completely randomized design, arranged in a split-split plot scheme (2 × 3 × 2), with three replications. The main plot was growth stage (90 and 270 days) after cactus planting, while the subplot was PGPB inoculation (non-inoculation and inoculation with *B. subtilis* and *Paenibacillus sp*). The sub-subplot comprised two genotypes of prickly-pear cactus, *Nopalea cochenillifera* ('Baiana' and 'Doce'). The strains of both *B. subtilis* and *Paenibacillus sp* were IPA-CC29 and IPA-CC38, respectively. These strains were used in previous experiments (Aquino *et al.*, 2019), and their growth-promoting characteristics include the production of indole-3-acetic acid (IPA-CC29: 1.41 mg L^{-1}; IPA-CC38: 1.13 mg L^{-1}) and acetylene-reducing activity (IPA-CC29: 5.03 nmol C_2H_4 h^{-1}; IPA-CC38: 7.10 nmol C_2H_4 h^{-1}).

To obtain the PGPB inoculants, *B. subtilis* and *Paenibacillus sp* were grown in Erlenmeyer flasks containing 50 mL of liquid medium (Tryptone Soy Broth) and incubated in a rotatory

shaker (200 rpm; 31 °C) for 72 hours. Bacterial growth was assessed by optical density (absorbance) at a wavelength of 540 nm using a spectrophotometer and was approximately 10^8 CFU (colony-forming unit) mL^{-1}. The planting was done using one cladode per hole, with a spacing of 0.75 x 0.50 m. After planting, the inoculation of *B. subtilis* and *Paenibacillus sp* was performed by spraying the liquid inoculant diluted in water at a 5:1 ratio directly in the root area (Figure 5).

5.2.3. Soil sampling, DNA extraction and sequencing

The sampling was conducted in the same prickly-pear cactus cultivation area at two different stages, at 90 and 270 days of plant development. Rhizospheric soil samples were obtained by using a spade to remove the roots from the soil and collected with a stainless-steel spatula, while preserving the plant structure as much as possible. All equipment used for sampling was previously sterilized with 70% alcohol. Bulk soil samples were also collected from the interplot corridor at depths of 0–10 cm.

After the collection of rhizospheric soil samples, all samples were stored at −20 °C in a freezer and subsequently sent to the Soil Microbiology Laboratory of the Sugarcane Breeding Program at the Federal University of Piauí (PMGCA-UFPI/RIDESA), where microbial DNA was extracted.

Microbial DNA was extracted from rhizosphere and bulk soil samples using 0.5 g of each sample with the PowerLyzer PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), following the manufacturer's protocol. DNA quality and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The V4 hypervariable region of the 16S rRNA gene was amplified using region-specific primers 515F and 806R (Caporaso *et al.*, 2011). Each 25 µL PCR reaction contained 12.25 µL of nuclease-free water (Certified Nuclease-Free, Promega, Madison, WI, USA), 5.0 µL of 5× reaction buffer ($MgCl_2$ 2 mM), 0.75 µL of dNTP mix (10 mM), 0.75 µL of each primer (515F at 40 µM and 806R at 10 µM), 0.5 µL (1 U) of Platinum Taq High Fidelity polymerase (Invitrogen, Carlsbad, CA, USA), and 2.0 µL of template DNA. A negative control (no DNA template) was included using nuclease-free water.

PCR conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 98 °C for 20 s, annealing at 55 °C for 20 s, and extension at 72 °C for 30 s. A final extension step was carried out at 72 °C for 3 min. Following amplification, PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) as per the manufacturer's instructions. DNA concentrations were quantified with the Qubit 2.0

fluorometer (Invitrogen, Carlsbad, CA, USA) using the dsDNA BR Assay Kit (Invitrogen). Equimolar amounts of each sample were pooled, and the pooled library was diluted to 2 nM, denatured, and further diluted to a final concentration of 8.0 pM with a 20% PhiX spike-in (Illumina, San Diego, CA, USA) for sequencing on the Illumina MiSeq platform.

5.2.3. Statistical analysis

Raw sequence data were processed using QIIME 2 version 2023.7.0. Sequences were demultiplexed, and quality filtering was performed with DADA2 (Callahan *et al.,* 2017), using the consensus method to remove chimeric and low-quality reads. Taxonomic assignment was performed at 100% similarity against the SILVA reference database (version 138) (Quast *et al.*, 2013), resulting in an ASV table. The generated table contained 10,855,758 (ten million, eight hundred fifty-five thousand, seven hundred fifty-eight) sequences; however, after rarefaction (based on the lowest sample size), the total number of sequences was reduced to 4,173,156 (four million, one hundred seventy-three thousand, one hundred fifty-six).

The generated table was loaded into the Microeco package (version 1.13.0) of the RStudio software, version 4.4.1 (R Core Team, 2021), after which beta diversity, alpha diversity, microbial community composition, enrichment, and hierarchical abundance were analyzed. Figures were also generated in RStudio using the ggplot package (version 3.5.1).

Beta diversity was assessed through the structure of the microbial community, examined using Principal Component Analysis (PCA). Additionally, Permanova was conducted to evaluate differences among the factors. Alpha diversity was assessed using observed ASV values and the Shannon diversity index. Means were compared using the Wilcoxon test with a significance level of 0.05. To illustrate the microbial community composition at the phylum level, a Venn diagram was generated, and mean values were compared using analysis of variance (ANOVA) followed by Duncan's test, with a significance level of 0.05. Enrichment was evaluated using Linear Discriminant Analysis Effect Size (LEfSe) to identify differentially abundant taxa at the genus level that characterize and distinguish microbial communities across the studied factors (Segata *et al.*, 2011). Hierarchical taxonomic abundance analysis was performed to determine which taxa at the class level predominated within each phylum in the community for each studied factor.

5.3. Results

PCA analysis showed that the two sampling periods (90 and 270 days) clustered into distinct bacterial groups (PERMANOVA < 0.05) (Figure 10A). At 90 days, neither PGPB nor

genotype significantly affected the bacterial communities (PERMANOVA > 0.05), although bulk soil clustered separately from rhizosphere samples (Figure 10B). At 270 days, only PGPB inoculation had a significant effect (PERMANOVA < 0.005), but group separation in the PCA was partial (R^2 = 25%) (Figure 10C). Bacterial richness and diversity were significantly higher at 90 days than at 270 days ($p < 0.001$; Figure 3). No significant differences were observed within each period for PGPB or genotype ($p > 0.05$).

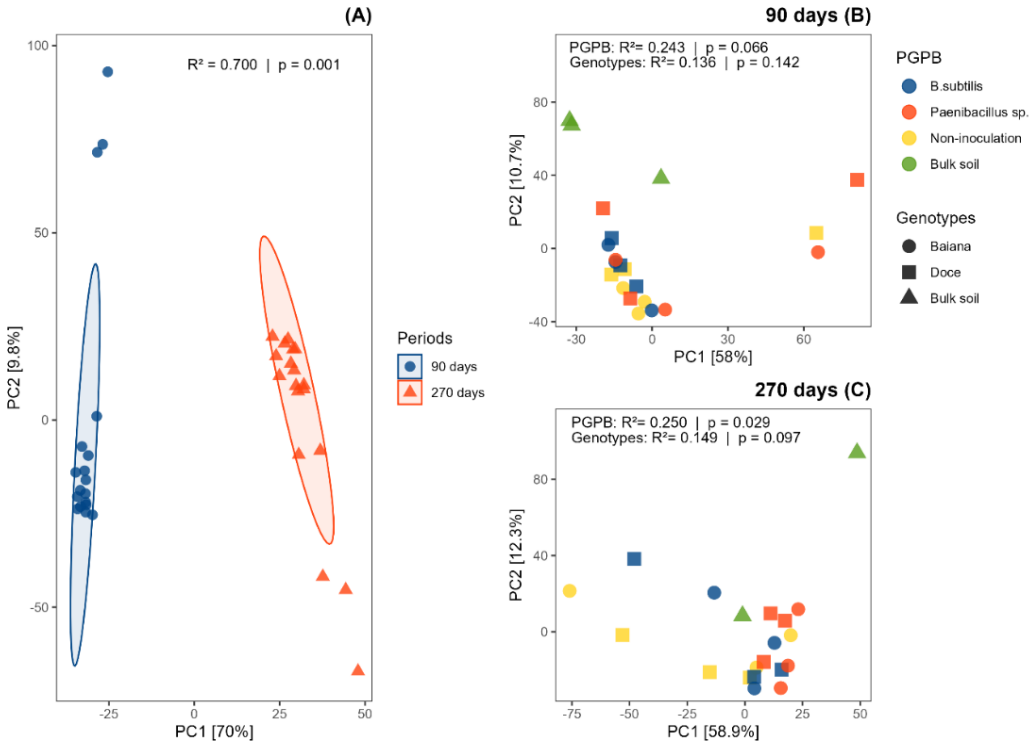

Figure 10 - Beta diversity of rhizosphere microbial communities of prickly-pear cactus genotypes 'Baiana' and 'Doce', inoculated and non-inoculated with *Bacillus* and *Paenibacillus* at 90 and 270 days of growth.

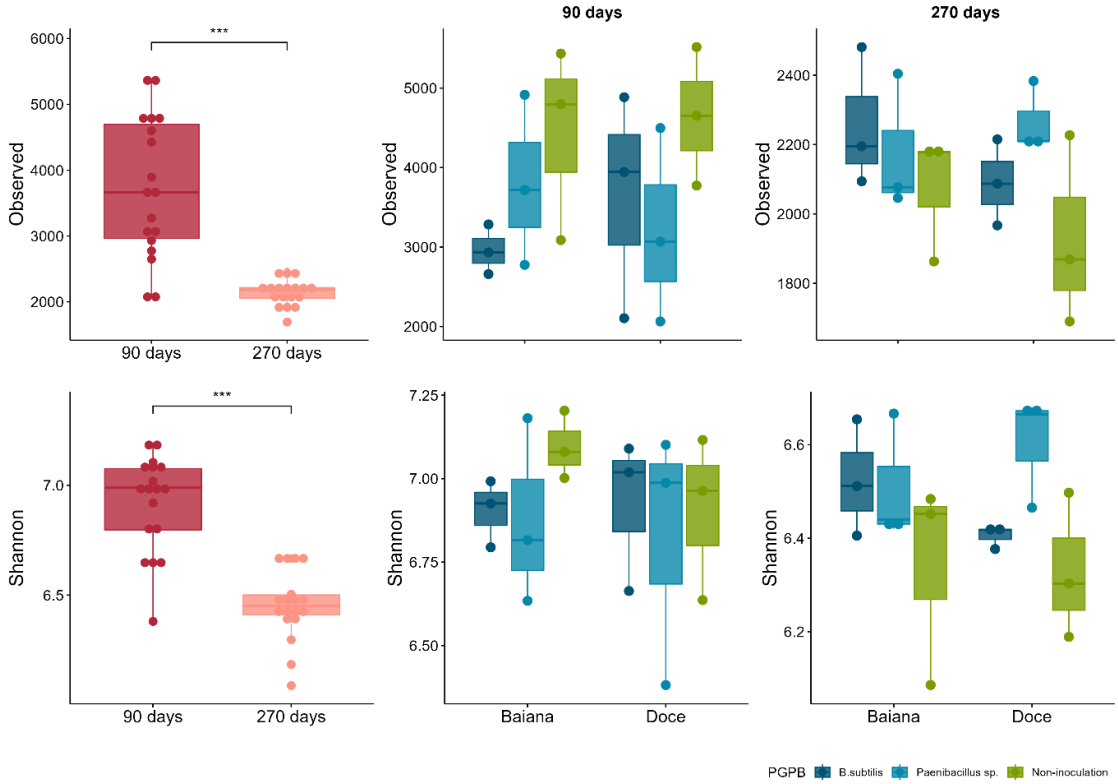

Figure 11 - Alpha diversity of rhizosphere microbial communities of prickly-pear cactus genotypes 'Baiana' and 'Doce', inoculated and non-inoculated with *Bacillus* and *Paenibacillus* at 90 and 270 days of growth, médias comparadas pelo teste de Wilcoxon ($p < 0.05$)

The five dominant bacterial phyla in the rhizosphere were *Actinobacteriota, Proteobacteria, Firmicutes, Chloroflexi,* and *Acidobacteriota* (Figure 4; Appendix 5). Period-dependent differences were detected ($p < 0.05$): *Proteobacteria, Acidobacteriota, Planctomycetota, Crenarchaeota, Myxococota, Verrucomicrobiota*, and *Gemmatimonadota* were more abundant at 90 days, while *Actinobacteriota* and *Chloroflexi* predominated at 270 days. Firmicutes showed no significant change. PGPB inoculation and genotype did not significantly influence phylum-level composition within periods ($p > 0.05$).

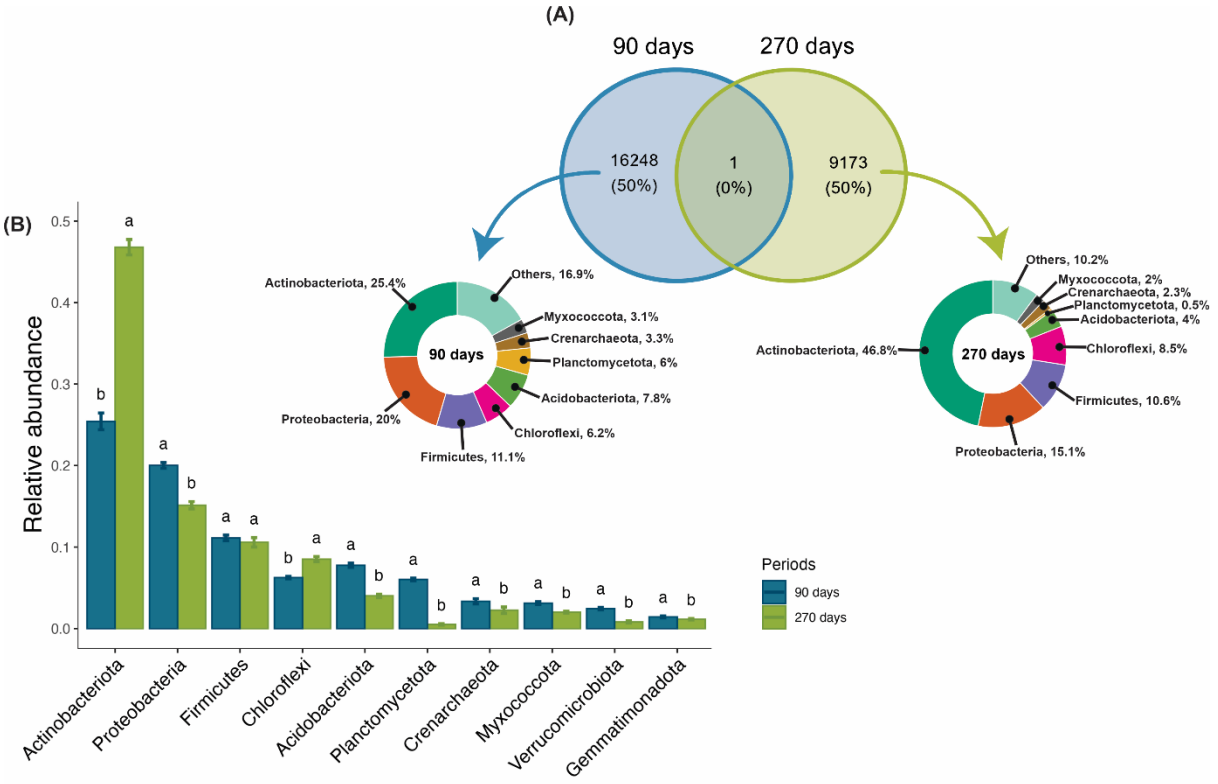

Figure 12 - Composition of the rhizosphere microbial community of prickly-pear cactus at 90 and 270 days of growth and comparison among the predominant phyla using Duncan's test ($p < 0.05$).

At the genus level, enrichment patterns also varied over time (Figure 5A). At 90 days, *Streptomyces*, *Sphingomonas*, *Mycobacterium*, *Microvirga*, and *Bradyrhizobium* predominated; at 270 days, the most enriched genera were 67-14, *Bacillus*, *Gaiella*, Glitter-*GS-136*, and *Ammoniphilus*. PGPB inoculation influenced specific taxa (Figures 5B and 5C). In *B. subtilis*-inoculated plants, *Nitrososphaeraceae*, *Candidatus_Udaeobacter*, *SC-I-84, C0119*, and *WD2101_soil_group* were enriched at 90 days, while only *Mycobacterium* was enriched at 270 days. In *Paenibacillus* sp.-inoculated plants, enrichment occurred for *Chloroplast* at 90 days and for *JG30-KF-CM66*, *Iamia*, *Rubellimicrobium*, *OLB14*, and *Ellin6067* at 270 days. In non-inoculated plants, *Hyphomicrobium* was enriched at 90 days, whereas *Sinomonas*, *Burkholderia–Caballeronia–Paraburkholderia*, and *WWH38* predominated at 270 days.

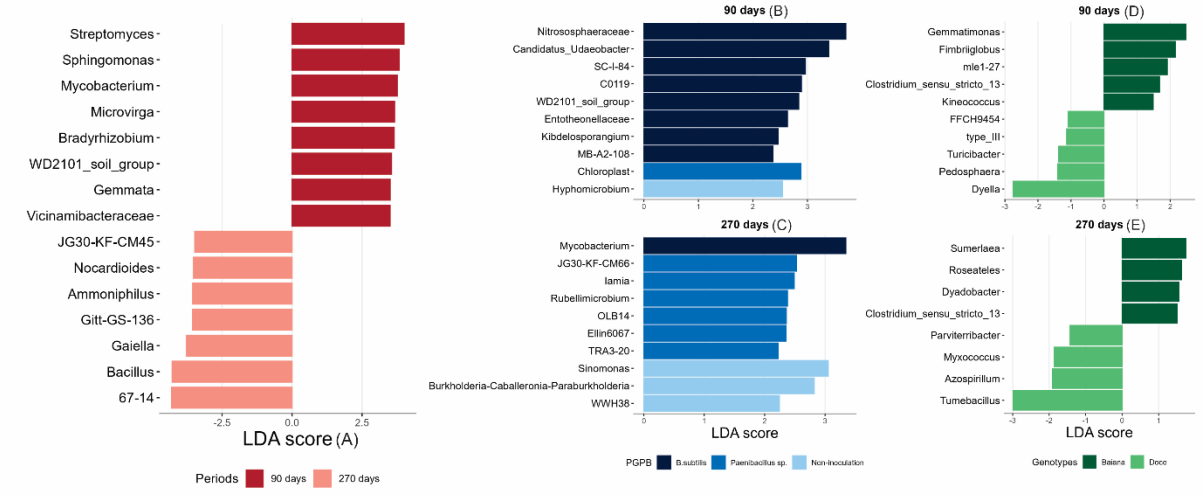

Figure 13 - Genus-level enrichment of bacteria in the rhizosphere of prickly-pear cactus genotypes 'Baiana' and 'Doce', inoculated and non-inoculated with *B. subtilis* and *Paenibacillus* sp., at 90 and 270 days of growth

Genotype-specific enrichment was observed (Figures 5D and 5E). In the 'Baiana' genotype, the genera *Gemmatimonas*, *Fimbriiglobus*, *mle1-27, Clostridium_sensu_stricto_13*, and *Kineococcus* were enriched at 90 days, whereas *Sumerlaea*, *Roseateles*, *Dyadobacter*, and *Clostridium_sensu_stricto_13* were enriched at 270 days. In the 'Doce' genotype, *Dyella* was the most enriched genus at 90 days and *Tumebacillus, Azospirillum* and *Myxococcus* at 270 days.

Class-level hierarchical analysis showed the most abundant taxa within the dominant phyla (Figure 6). *Alphaproteobacteria* and *Gammaproteobacteria* dominated *Proteobacteria*, with *Alphaproteobacteria* more abundant. *Actinobacteriota* was primarily composed of *Acidimicrobiia* and *Actinobacteria*, with the latter predominating. *Bacilli* dominated *Firmicutes; Acidobacteriae* dominated *Acidobacteriota*, and *Chloroflexia* was most abundant in *Chloroflexi*.

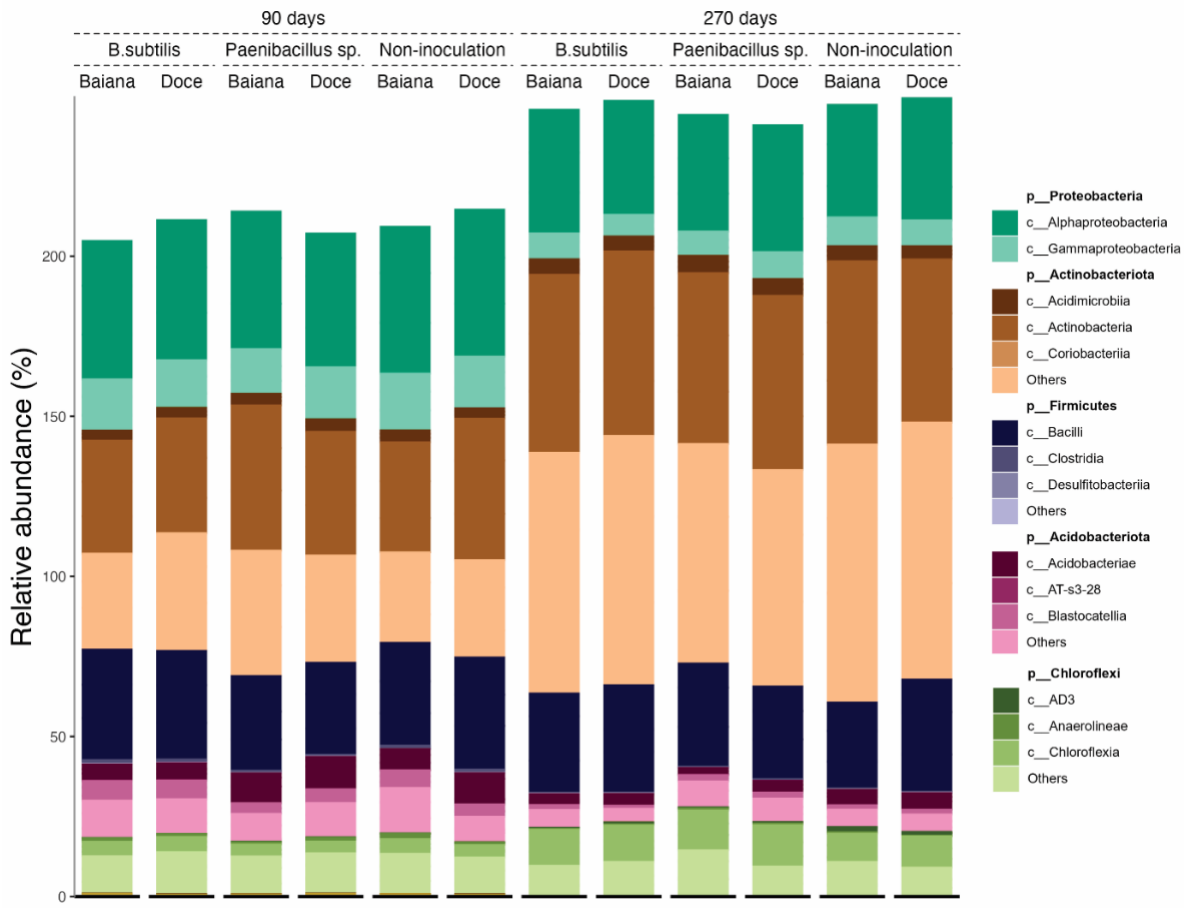

Figure 14 - Hierarchical abundance at the phylum and class levels in the rhizosphere of prickly-pear cactus genotypes 'Baiana' and 'Doce', inoculated and non-inoculated with *B. subtilis* and *Paenibacillus* sp., at 90 and 270 days of growth.

5.4. Discussion

5.4.1. Growth stage prevails over genotype and inoculation in shaping the bacterial communities in the rhizosphere of N. cochenillifera.

This is the first study assessing the bacterial communities in the rhizosphere of two prickly-pear cactus genotypes belonging to *N. cochenillifera*, which were inoculated with PGPBs and assessed at early (90 days) and late (270 days) stage of plant growth under field conditions in the semi-arid region. Our results partly confirmed the proposed hypotheses showing that the bacterial communities in the rhizosphere of *N. cochenillifera* were not significantly shaped by genotype at the community level, although specific genera were genotype-enriched, indicating a limited genotypic effect. In contrast, PGPB inoculation influenced bacterial composition only at 270 days, supporting a stage-dependent effect on certain taxa. Thus, we observed a temporal effect on microbiome structure, while genotype and inoculation drove specific microbial groups.

The results showed that the growth period significantly drives the rhizosphere microbiome in prickly-pear cactus. This means that the rhizosphere environment changes as the plant develops with changes in microbial colonization. Thus, in early stages of plant growth, the root exudation is more diverse and abundant (Liu *et al.*, 2024), providing a variety of carbon sources that favor specific microbial groups. On the other hand, during later plant stages, i.e., mature plants, the root exudation is more stable, as roots become more lignified (Hafner *et al.*, 2025), which favor other specific microbial groups. These results confirm that plant growth stage is a dominant driver of microbiome structure (Pantigoso *et al.*, 2020; Lopes *et al.*, 2023).

Previous studies with *N. cochenillifera* genotypes ('Doce' and 'Baiana') reported different levels of primary and secondary metabolites in cladodes, which varied according to seasonality and phenological stage (Alves *et al.*, 2017; Pessoa *et al.*, 2024). These variations may influence root deposition, which accounts for approximately 5–21% of photosynthetic products (Upadhyay *et al.*, 2022), potentially explaining the differences in the bacterial communities in the rhizosphere observed in our study. Indeed, in cacti (*Cereus jamacaru*), Kavamura *et al.* (2013, 2018) found distinct microbiome structures when comparing two different growth periods.

Regarding the inoculation of PGPB and *N. cochenillifera* genotypes, our results showed only subtle effects on bacterial community structure, with no changes in richness, diversity, or composition, indicating that the native soil microbiome studied can be resistant and resilient. This means that when an external organism is introduced, soils which harbor a well-established native microbiome may compete with and suppress the introduced organism, thereby maintaining community function and structure (resistance), or it may adapt and reorganize to establish a new stable state (resilience) (Kost *et al.*, 2024). Indeed, cactus species cultivated in semi-arid regions generally share a core group of adapted rhizosphere-associated microorganisms (Fonseca-García *et al.*, 2016; Zineb *et al.*, 2024).

We consistently observed high diversity in the rhizosphere, although, due to selective recruitment, it is naturally lower than in the adjacent soil. Our results differ from patterns reported in arid and semi-arid regions, which are characterized by low microbial diversity (Pechlivanis *et al.*, 2024), indicating that the bacterial community is not only highly adapted to semi-arid conditions but also resilient to seasonal fluctuations.

5.4.2. Recruitment of stress-mitigating bacterial groups in cactus rhizospheres

The analysis of the bacterial composition in the rhizosphere of prickly-pear cactus showed predominance of key phyla typical of semi-arid soils, i.e., *Actinobacteriota,*

Proteobacteria, Firmicutes, Chloroflexi, and *Acidobacteriota* (Zhu *et al.*, 2024) which varied according to plant growth stage. Interestingly, the phylum *Actinobacteriota*, known to predominate in dry soils, also thrived under wetter conditions, highlighting its capacity to adapt to diverse environmental conditions.

Studies conducted in semi-arid soils (Pan *et al.*, 2024; Liu *et al.*, 2025) and in cactus rhizosphere (De La Torre-Hernández *et al.*, 2020; Zineb *et al.*, 2024) have reported taxonomic profiles similar to those observed in our study. For instance, Karray *et al.* (2020) investigated the rhizosphere and endosphere microbiomes of *Opuntia ficus-indica* across different aridity gradients and found enrichment of certain taxa, including *Actinobacteria, Alphaproteobacteria, Firmicutes*, and *Chloroflexi*.

During the 270-day sampling, which coincided with the highest recorded precipitation, the abundance of *Actinobacteria* nearly doubled compared to the drier period (90 days). This pattern contrasts with previous reports indicating that *Actinobacteriota* predominates in soils under dry conditions (Leal *et al.*, 2024; Rodríguez *et al.*, 2024). A possible explanation is that precipitation stimulates soil mineralization processes, allowing *Actinobacteria* to adopt a copiotrophic lifestyle under such conditions (Zhang *et al.*, 2019; Zhang *et al.*, 2024).

Enrichment of genera reported as potential PGPB was observed when analyzing the two growth stages separately (*Streptomyces*, *Sphingomonas*, *Microvirga*, *Bradyrhizobium*, and *Bacillus*), the non-inoculated rhizosphere (*Hyphomicrobium*, *Burkholderia–Caballeronia–Paraburkholderia*), and both genotypes (*Clostridium*, *Dyella*, and *Azospirillum*). These findings indicate that the bacterial community is capable of tolerating and surviving under both favorable and limiting conditions, while can perform key metabolic functions that support soil and plant health. For instance, *Streptomyces* and *Bacillus* are commonly associated with plants cultivated in semi-arid regions, and due to their resilience to adverse environmental conditions, they possess high potential to mitigate plant stress (Bonatelli *et al.*, 2021). The PGPB taxa identified in our study were also predominant in the microbiomes of other cacti, such as *Echinocactus platyacanthus* (De La Torre-Hernández *et al.*, 2020).

5.4.3. Insights, limitations, and practical implications for assessing cactus rhizosphere

The findings of this study provided valuable insights into the bacterial communities in the rhizosphere of prickly-pear cactus (*N. cochenillifera*) cultivated in the semiarid region. Our results showed that plant growth stages were shown to strongly influence the bacterial community structure, which was composed of key taxa typical of semiarid environments. Despite the significance of these findings, our study showed several limitations. These include

the scarcity of recent data on PGPB inoculation and root exudation in cacti associated with the challenge of obtaining responses across different growth stages due to the long cycle of prickly-pear cactus (approximately two years). In addition, the absence of more in-depth functional analyses that could allow mapping of metabolic groups in the rhizosphere and more directly confirm the growth-promoting potential of the bacterial community.

Despite these limitations, several relevant practical implications can be obtained in this study. The inoculation with both PGPBs did not cause disturbances in the microbiome over time, which is crucial for plant health as it represents a positive and beneficial plant–PGPB interaction (Berg *et al.*, 2021). Another important aspect is that soil in semi-arid regions serves as a reservoir of native PGPBs capable of thriving under drought conditions, which could provide potential inoculants for plant species from semiarid regions.

5.5. Conclusions

The bacterial communities in the rhizosphere of the prickly-pear cactus genotypes ‘Doce’ and ‘Baiana’, both non-inoculated and inoculated with *B. subtilis* and *Paenibacillus* sp., was significantly shaped by plant growth stage. Key bacterial taxa associated with semiarid regions were predominant in the rhizosphere, many of which are known to contribute to abiotic stress tolerance and plant growth promotion. These findings provide fundamental insights into the microbial ecology of *Nopalea cochenillifera* and highlight the importance of studying the microbiome of cacti cultivated in semiarid regions. Nevertheless, further studies are required to better understand ecological interactions in the rhizosphere through network analysis, to identify functional groups and the composition of root exudates in cacti, and to determine how these factors correlate with the recruitment of specific taxa.

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6. FINAL CONSIDERATIONS

Overall, the results of the studies developed in this doctoral thesis demonstrate that the growth stage of prickly-pear cactus is a key factor influencing the dynamics of soil biological attributes and the structure of the bacterial community associated with its rhizosphere. The interaction between prickly-pear cactus genotypes and inoculation with plant growth-promoting bacteria also revealed distinct responses, indicating that the effect of inoculation depends on the specific combination of microorganism and plant genotype. Among the evaluated combinations, inoculation with *Bacillus subtilis* associated with the 'Doce' genotype stood out by promoting more pronounced responses, particularly with respect to microbial phosphorus.

In addition, water stress conditions were shown to act as an important driver of microbial selection, favoring bacterial taxa that contribute to stress alleviation in plants. Under these conditions, inoculation with *B. subtilis* increased the complexity of rhizosphere microbial interaction networks, indicating greater system resilience under adverse conditions.

Thus, the results indicate that, in semiarid regions and under water deficit scenarios, the adoption of the 'Doce' genotype combined with inoculation with *Bacillus subtilis* may represent a promising strategy for safer and more sustainable management of forage cactus cultivation.

APPENDICES

Appendix 1 - Soil chemical and physical properties

pH	H +Al	Al	Ca	Mg	K	TEB	CEC	P	K
H_2O	---------- $cmol_c$ dm^{-3} ----------							----- mg dm^{-3} ------	
6.6	1.48	0.0	3.45	0.87	0.25	4.57	6.06	17.4	98

Micronutrientes				BS	AS	O.M.	Clay	Silt	Sand
Cu	Mn	Fe	Zn						
---------- mg dm^{-3} ----------				------ % -----		---------- g kg^{-1} ----------			
0.46	30.10	84.7	2.44	75.5	0.0	10.54	172	54	774

TEB = Total Exchangeable Bases; Cation Exchange Capacity (CEC) at pH 7.0;
BS = Percent base saturation; AS = Aluminum saturation; O.M.: Organic matter.

Appendix 2 - Biometric parameters of cladodes obtained at 190 days after planting, following 150 days under drought stress.

Parameter	Control W+	Control W-	*Bacillus* W+	*Bacillus* W-	*Paenibacillus* W+	*Paenibacillus* W-
CA	536	458	471	436	548	423
CDM	103	77	90	78	97	79
CW	12	8	13	9	12	9
CL	21	19	22	19	21	20
RL	52	38	60	40	48	33
RDM	8	3	9	4	7	3
CN	5	4	5	5	6	4

W+: well-watered; W-: water-deficit; CA: cladode area; CDM: cladode dry mass; CW: cladode width; CL: cladode length; RL: root length; RDM: root dry mass; CN: cladode number

Appendix 3 - Topological properties of the bulk soil and rhizosphere microbiome networks as inferred by SparCC and calculated by Gephi.

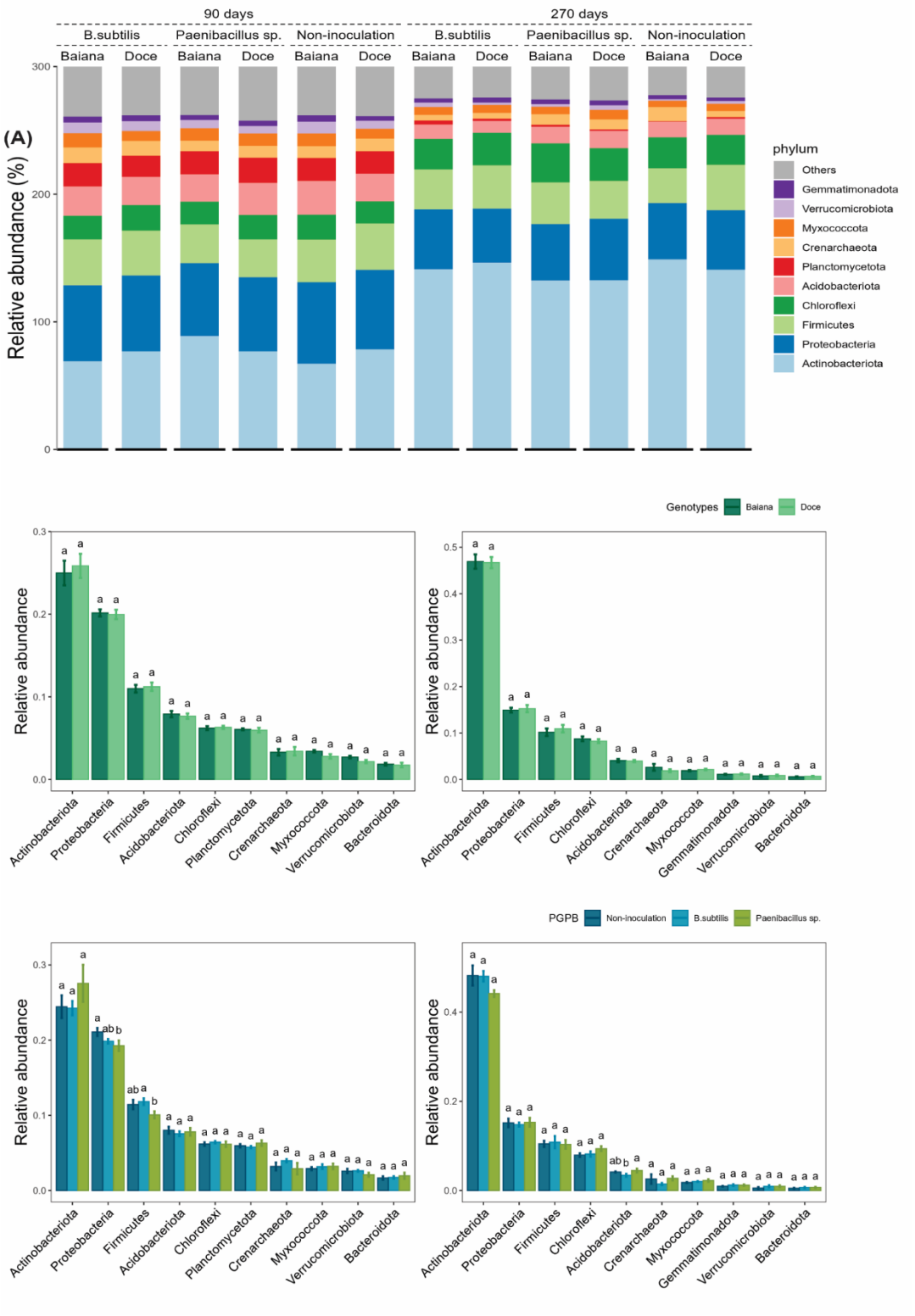
Network properties	Bulk	Control W+	Control W-	*Bacillus* W+	*Bacillus* W-	*Paenibacillus* W+	*Paenibacillus* W-
Number of nodes[a]	455	505	499	530	496	521	486
Number of edges[b]	2228	2306	4034	3210	3290	3832	3217
Positive edges[c]	1522 (68%)	1425 (62%)	2623 (65%)	2285 (71%)	2681 (81%)	2794 (73%)	2075 (65%)
Negative edges[d]	706 (32%)	881 (38%)	1411 (35%)	925 (29%)	609 (19%)	1038 (27%)	1142 (35%)
Modularity[e]	1.337	2.136	1.27	1.22	1.00	1.19	1.31
Number of communities[f]	82	68	75	87	84	82	69
Network diameter[g]	28	18	32	9	8	29	8
Average path length[h]	8.80	7.91	9.09	3.26	2.93	7.85	3.26
Average degree[i]	9.79	9.13	16.16	18.28	22.65	14.71	18.03
Average clustering coeffic.[j]	0.377	0.345	0.412	0.381	0.350	0.416	0.369

[a]Microbial taxon (at genus level) with at least one significant ($P < 0.05$) and strong (Spearman > 0.7 or < -0.7) correlation;
[b]Number of connections/correlations obtained by Spearman analysis;
[c]Positive correlation (> 0.7 with $P < 0.05$);
[d]Negative correlation (< -0.7 with $P < 0.05$);
[e]The capability of the nodes to form highly connected communities, that is, a structure with high density of between nodes connections (inferred by Gephi);
[f]A community is defined as a group of nodes densely connected internally (Gephi);
[g]The longest distance between nodes in the network, measured in number of edges (Gephi);
[h]Average network distance between all pair of nodes or the average length off all edges in the network (Gephi);
[i]The average number of connections per node in the network, that is, the node connectivity (Gephi);
[j]How nodes are embedded in their neighborhood and the degree to which they tend to cluster together (Gephi).

Appendix 4 - Results of the analysis of variance (ANOVA) of the data obtained in the experiment.

	DF	MBC	MBN	MBP	β-glucosidase	Urease	Acid Phosphatase
Days	1	0.044*	0.002**	0.100ns	0.133ns	0.063ns	0.003 **
PGPB	2	0.125ns	0.080ns	0.001**	0.009**	0.197ns	0.004**
Varieties	1	1.432e-05**	0.022*	0.016*	0.082ns	0.840ns	0.079ns
PGPB x Varieties	2	0.016*	2.835e-05**	0.588ns	0.023*	0.446ns	0.004812 **
Days x PGPB x Varieties	2	0.007**	1.436e-04**	0.006**	0.135ns	0.995ns	0.0032**

NS = non-significative; * = Significative 0.05; ** = Significative 0.01; DF = Degrees of freedom.



Appendix 5 - Microbial community composition in the rhizosphere of prickly pear cactus genotypes 'Baiana' and 'Doce', inoculated and non-inoculated with *Bacillus* and *Paenibacillus* at 90 and 270 days of growth, e comparação entre os Filos predominantes pelo teste de Duncan ($p < 0.05$)

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RESEARCH ARTICLE

Inoculation with *Bacillus subtilis* and *Paenibacillus* sp. shapes the rhizosphere bacteriome of prickly pear cactus under water deficit

Rhaiana Oliveira de Aviz · Janaira Rocha Campos · Marcos Renan Lima Leite · Sandra Mara Barbosa Rocha · Maria Helena Ferreira Duarte · Pâmalla Graziely Carvalho Moraes · Gonçalves Albino Dauala · Elaine Martins da Costa · Rafael de Souza Miranda · Cacio Luiz Boechat · Arthur Prudêncio de Araujo Pereira · Erika Valente de Medeiros · Lucas William Mendes · Ademir Sérgio Ferreira Araujo

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Abstract

Background and aims Prickly pear cactus (*Nopalea cochenillifera* L.) plays a central role in sustaining forage production in semiarid environments, yet prolonged drought associated with climate change increasingly constrains its performance. Plant growth-promoting bacteria (PGPB), including *Bacillus subtilis* and *Paenibacillus* spp., are recognized for enhancing plant tolerance to water limitation, but their influence on the rhizosphere microbiome of cactus under drought conditions remains unclear. We tested whether inoculation with these bacteria shapes rhizosphere bacterial community composition and interaction patterns under water deficit.

Methods A greenhouse experiment was conducted using a factorial design combining two water regimes (well-watered and water-deficit) with three inoculation treatments (*B. subtilis*, *Paenibacillus* sp., and a non-inoculated control). After 150 days of water stress exposure, rhizosphere bacterial communities were assessed using 16S rRNA gene amplicon sequencing.

Results Water availability was the dominant driver of rhizosphere bacterial community structure, with drought favoring Actinobacteriota and well-watered conditions enriching Proteobacteria. Inoculation with *B. subtilis* modified community composition and increased the prevalence of drought-associated taxa, while also promoting a higher proportion of positive microbial interactions under water deficit. In contrast, *Paenibacillus* sp. exerted weaker and more variable effects. Despite microbial shifts, inoculation did not significantly affect plant biomass or root traits.

Conclusions Rhizosphere bacterial communities of prickly pear cactus are primarily structured by water availability, whereas PGPB inoculation induces

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R. O. de Aviz · J. R. Campos · M. R. L. Leite · S. M. B. Rocha · A. S. F. Araujo (✉)
Agricultural Science Center, Federal University of Piauí, Teresina, PI 64049-550, Brazil
e-mail: ademir@ufpi.edu.br

M. H. F. Duarte · P. G. C. Moraes · G. A. Dauala · E. M. da Costa · R. de Souza Miranda · C. L. Boechat
Campus Profa Cinobelina, Federal University of Piauí, Bom Jesus, PI, Brazil

A. P. Pereira
Department of Soil Science, Federal University of Ceará, Fortaleza, CE, Brazil

E. V. de Medeiros
Federal University of Agreste Pernambucano, Garanhuns, PE, Brazil

L. W. Mendes
Center for Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, SP, Brazil

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Appendix 6 - Published article: Inoculation with *Bacillus subtilis* and *Paenibacillus sp.* shapes the rhizosphere bacteriome of prickly pear cactus under water deficit.

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Microbial biomass and enzymatic activity in the rhizosphere of prickly-pear cactus genotypes inoculated with *Bacillus subtilis* and *Paenibacillus Sp*

Rhaiana Oliveira de Aviz[1], Janaira Rocha Campos[1], Davila Esmelinda Oliveira Silva[1], Larissa Macelle de Paulo Barbosa[1], Romário Martins Costa[1], Jhoice Ferreira Borges[1], Marcos Renan Lima Leite[1], Sandra Mara Barbosa Rocha[1], Pâmalla Graziely Carvalho Morais[2], Gonçalves Albino Dauala[2], Arthur Prudencio de Araujo Pereira[3], Erika Valente de Medeiros[4] & Ademir Sergio Ferreira Araujo[1]✉

Some bacterial taxa, such as *Bacillus* and *Paenibacillus*, are known to colonize the rhizosphere and promote plant growth. However, little is known about their effect on microbial biomass and enzymatic activity in the rhizosphere of plants under semi-arid conditions. This field study assessed the effects of *B. subtilis* and *Paenibacillus* sp. in the rhizosphere of two prickly-pear cactus genotypes on microbial biomass of C, N, and P, and on enzymatic activity during early and late growth stages. The analysis of variance showed that microbial biomass and enzymatic activity were significantly influenced by the interaction between PGPB taxa (*B. subtilis* and *Paenibacillus* sp.), prickly-pear cactus genotypes ('Baiana' and 'Doce'), and plant growth stage (90 and 270 days). Specifically, PGPB inoculation increased microbial biomass P, β-glucosidase, and acid phosphatase, while microbial biomass of C and N were primarily driven by differences between cactus genotypes 'Baiana' and 'Doce'. At the early growth stage (90 days), the highest values of microbial biomass C, P, and acid phosphatase were observed, whereas N biomass was higher at the later stage (270 days). *B. subtilis* increased microbial biomass P in the 'Doce' genotype and acid phosphatase in 'Baiana,' while *Paenibacillus* sp. increased β-glucosidase in 'Baiana.' The combination of the 'Doce' genotype with *B. subtilis* enhanced phosphorus availability, suggesting that specific plant–microbe interactions may benefit nutrient acquisition in arid, nutrient-poor soils; however, further research is needed to confirm whether this effect extends to other genotypes.

Keywords Microbial phosphorus, *Nopalea Cochenillifera* L., PGPB, Semiarid

The Brazilian semiarid region covers approximately 1 million km², with high temperatures (~ 30 °C) and limited rainfall (~ 600 mm/year), concentrated over four months[1]. In response to these challenging environmental conditions, smallholders rely on resilient crops, such as the prickly-pear cactus (*Nopalea cochenillifera* L.), a drought-tolerant species, showing the crassulacean acid metabolism (CAM), that conserves water through regulated transpiration[2,3]. Additionally, prickly-pear cactus contains high levels of essential nutrients, including ascorbic acid, vitamin E, carotenoids, and fiber[4]. These physiological and biochemical characteristics make prickly-pear cactus valuable in semiarid regions, both for conserving the plant and providing water and nutrients to animals[5]. Particularly, the prickly-pear cactus genotypes 'Baiana' and 'Doce' are among the most widely cultivated in the Brazilian semiarid region due to their strong adaptation to harsh environmental conditions (Oliveira et al., 2010)[6].

[1]Soil Microbial Ecology Group, Universidade Federal do Piauí, Teresina, PI, Brazil. [2]Postgraduate Program in Agricultural Sciences, Federal University of Piauí, Bom Jesus, PI, Brazil. [3]Departamento de Ciencias do Solo, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil. [4]Ecology Group, Universidade Federal do Piauí, Teresina, Garanhuns, Pernambuco, PI, Brazil. ✉email: asfaruaj@yahoo.com.br

Appendix 7 - Published article: Microbial biomass and enzymatic activity in the rhizosphere of prickly-pear cactus genotypes inoculated with *Bacillus subtilis* and *Paenibacillus sp.*

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Growth stage as the main drive over genotype and plant growth-promoting bacteria on bacterial communities in the rhizosphere of prickly-pear cactus

Rhaiana Oliveira de Aviz [a], Janaira Rocha Campos [a], Davila Esmelinda Oliveira Silva [a], Mayanna Karlla Lima Costa [a], Marcos Renan Lima Leite [b], Sandra Mara Barbosa Rocha [a], Pâmalla Graziely Carvalho Morais [c], Gonçalves Albino Dauala [c], Maria Helena Ferreira Duarte [c], Cacio Luiz Boechat [c], Rafael de Souza Miranda [c], Elaine Martins da Costa [c], Arthur Prudêncio de Araujo Pereira [d], Erika Valente de Medeiros [e], Lucas William Mendes [f], Ademir Sergio Ferreira Araujo [a,*]

[a] Soil Microbial Ecology Group, Federal University of Piauí, Teresina, PI, Brazil
[b] IFPI/CAVAL, Federal Institute of Piauí, Valença, PI, Brazil
[c] Postgraduate Program in Agricultural Sciences, Federal University of Piauí, Bom Jesus, PI, Brazil
[d] Soil Science Department, Federal University of Ceara, Fortaleza, CE, Brazil
[e] Semi-Arid (Bio)Technologies Group, Federal University of the Agreste of Pernambuco, Garanhuns, PE, Brazil
[f] Center for Nuclear Energy in Agriculture, University of Sao Paulo, Piracicaba, SP, Brazil

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ABSTRACT

Despite its strategic importance for food security in semiarid regions, no studies have assessed the rhizosphere microbiome of the prickly-pear cactus (*Nopalea cochenillifera)* and how its growth stage, bacterial inoculation, and genotypes may shape the rhizosphere. Thus, the aim of this study was to assess diversity, composition, and enrichment of bacterial communities in the rhizosphere of the prickly-pear cactus genotypes 'Baiana' and 'Doce', both with and without inoculation with *Bacillus subtilis* and *Paenibacillus* sp., at 90 and 270 days of growth. In this study we cultivated samples rhizospheric soil, extracted DNA, and assessed the bacterial communities by 16S rRNA amplicon sequencing by Illumina platform. The bacterial communities in the rhizosphere were significantly driven by plant growth stage and showed high diversity, while both inoculation and genotype showed lower effects. Key bacterial phyla typical of semiarid regions were predominant, including Actinobacteriota, Proteobacteria, and Firmicutes. Enrichment patterns of bacterial genera shifted with plant growth, with *Streptomyces* and *Bradyrhizobium* predominating at 90 days, and *Bacillus* and *Gaiella* at 270 days. PGPB inoculation further shaped specific taxa, while non-inoculated plants showed distinct enrichment over time. This study showed that the bacterial communities in the rhizosphere of *N. cochenillifera* was primarily shaped by plant growth stage, with key bacterial groups potentially associated to stress tolerance which provide important insights into soil microbial ecology in semiarid regions.

1. Introduction

In semi-arid regions, cactus cultivation represents a valuable strategy for supporting animal production during drought periods. This plant species presents high adaptability to semi-arid regions and contains high nutritional value, which is important to support animal production (Araújo Júnior et al., 2023). Particularly, the prickly-pear cactus (*Nopalea cochenillifera*) has been used as forage in semiarid regions, mainly due to its nutritional value compared to other prickly-pear species (Dubeux Jr et al., 2021). On the other hand, the productivity of *N. cochenillifera* can vary significantly across genotypes, reflecting both genetic potential and adaptive traits (Alves et al., 2016). In addition, each genotype has specific morphological traits, such as root growth, which may influence microbial communities associated with the rhizosphere (Xie et al., 2023).

The rhizosphere is the narrow soil zone surrounding plant roots,

* Corresponding author.
E-mail address: ademir@ufpi.edu.br (A.S.F. Araujo).

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Appendix 8 - Published article: Growth stage as the main drive over genotype and plant growth-promoting bacteria inoculation of bacterial communities in the rhizosphere of prickly-pear cactus