ARIANE MARIA RIZZOLI MORENO

SELECTION OF XEROTOLERANT RHIZOBACTERIA TO PROMOTE EUCALYPTUS GROWTH AND MINICUTTINGS ROOTING

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

Orientador: Maurício Dutra Costa

Coorientadores: Glêison Augusto dos Santos
Marcos Rogério Tótola

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APROVADA: 20 de março de 2024.

Assentimento:

Ariane M. R. Moreno
Ariane Maria Rizzoli Moreno
Autora

Maurício Dutra Costa
Maurício Dutra Costa
Orientador

Aos meus avós Ermelinda e Afonso
(in memorian)

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“ A única forma de chegar ao impossível é acreditar que é possível “

(Lewis Carroll)

RESUMO

MORENO, Ariane Maria Rizzoli, D. Sc., Universidade Federal de Viçosa, março de 2024**. Seleção de rizobactérias xerotolerantes para promover o crescimento de eucaliptos e o enraizamento de miniestacas**. Orientador: Maurício Dutra Costa. Coorientador: Glêison Augusto dos Santos e Marcos Rogério Tótola.

Períodos longos de estiagem proporcionam graves consequências socioeconômicas e ecológicas, levando a impactos nos setores agrícola e florestal. As rizobactérias promotoras do crescimento de plantas (RPCPs) podem ajudar as plantas a enfrentar condições de déficit hídrico por meio de vários mecanismos. Assim, os objetivos deste trabalho foram: (I) determinar os mecanismos envolvidos na promoção de tolerância à seca e promoção do crescimento de plantas em bactérias isoladas de *Eucalyptus grandis*; (II) investigar o potencial dessas rizobactérias em promover tolerância à seca em clones híbridos de eucalipto [VM01 (*Eucalyptus urophylla* x *Eucalyptus camaldulensis*) e I144 (*Eucalyptus urophylla* x *Eucalyptus grandis*)] submetidos a reduções na irrigação no viveiro e, (III) avaliar se isolados produtores de AIA e que proporcionaram incrementos na biomassa radicular sob déficit hídrico promovem maior eficiência na rizogênese de miniestacas. Vinte um isolados foram caracterizados *in vitro* quanto à xerotolerância e à halotolerância. Rizobactérias foram cultivadas em meio próprio para avaliação da produção de exopolissacarídeos e biofilme a 28 °C e 37 °C. A fixação de nitrogênio foi confirmada em meio NFb e JNFb. A solubilização de fosfato foi verificada cultivando os microrganismos em meios com fosfato de cálcio. Quantificou-se a produção de AIA utilizando o reagente de Salkowiski e a produção de amônia pelo reagente de Nessler. A produção e quantificação de sideróforos foi realizada utilizando o reagente de CAS. Verificou-se a síntese das enzimas celulase e quitinase e a produção de HCN. Por fim, foi avaliado o potencial desses isolados em inibir o crescimento de fungos fitopatogênicos através do confronto direto, produção de sobrenadante e voláteis. Todas as rizobactérias apresentaram ao menos quatro mecanismos diretos de promoção de tolerância à seca. Esses isolados foram inoculados em miniestacas de VM01 e I144 aos 41 e 58 dias de idade. A umidade do substrato foi constantemente reduzida até 30 % de sua capacidade de retenção de água, no 83º dia. Ao final do 90º dia, as mudas foram avaliadas quanto às características morfológicas e condição nutricional. Algumas rizobactérias foram eficazes em melhorar as características morfológicas e as condições nutricionais das plantas, principalmente os isolados LEM 50 *Ralstonia pickettii* e LEM 14 *Ralstonia pickettii* nos clones VM01. A inoculação de rizobactérias em clones I144 proporcionaram incrementos em um número menor de variáveis. As rizobactérias que

forneceram incrementos significativos na biomassa radicular de VM01, LEM 13 *Staphylococcus kloosii* e LEM 50 *R. pickettii* e para I144 LEM 10 *Ralstonia pickettii* e LEM 41 *Burkholderia cenocepacia* foram selecionadas para o teste de enraizamento. Estes isolados foram inoculados por imersão do caule nas soluções rizobacterianas, seguidas de inoculação de reforço na interface do substrato com o caule. As plantas propagadas foram então colocadas por 35 dias em casa de enraizamento, onde foi avaliado o percentual de enraizamento. Ao final do experimento, as raízes foram submetidas a análises morfológicas utilizando-se o sistema WinRHIZO. Em miniestacas de I144, o volume radicular foi significativamente maior em tratamentos com LEM 10 *R. pickettii* and LEM 50 *R. pickettii.* Com base nos resultados apresentados, pode-se afirmar que as RPCP podem auxiliar os clones VM01 e I144 em condições de deficit hídrico. Não foram observados ganhos significativos em porcentagem de enraizamento, para ambos os clones. A eficiência dos microrganismos pode ser distinta dependendo do genótipo, do estágio de desenvolvimento das plantas e das condições abióticas de produção, como a disponibilidade de água.

Palavras-chave: Déficit hídrico; Mudanças climáticas; Microrganismos, Bactérias; Rizosfera; VM01; I144; Enraizamento; Produtividade; Viveiro.

ABSTRACT

MORENO, Ariane Maria Rizzoli, D. Sc., Universidade Federal de Viçosa, March, 2024**. Selection of xerotolerant rhizobacteria to promote eucalyptus growth and minicuttings rooting** Adviser: Maurício Dutra Costa. Co-adivisers: Glêison Augusto dos Santos and Marcos Rogério Tótola.

Long periods of drought have serious socioeconomic and ecological consequences, leading to impacts on the agricultural and forestry sectors. Plant growth-promoting rhizobacteria (PGPR) can help plants cope with water deficit conditions through several mechanisms. Thus, the objectives of this work were: (I) to determine the mechanisms involved in promoting drought tolerance and promoting plant growth from bacteria isolated from *Eucalyptus grandis*; (II) investigate the potential of these rhizobacteria to promote drought tolerance in eucalyptus hybrid clones [VM01 (*Eucalyptus urophylla* x *Eucalyptus camaldulensis*) and I144 (*Eucalyptus urophylla* x *Eucalyptus grandis*)] subjected to reductions in irrigation in seedling nurseries and, (III) evaluate whether IAA-producing isolates that provide increases in root biomass under water deficit promote greater efficiency in the rhizogenesis of minicuttings. Twenty-one isolates were characterized *in vitro* for their xerotolerance and halotolerance capacity. Rhizobacteria were cultivated in a specific medium to evaluate the production of exopolysaccharides and biofilm at 28 °C and 37 °C. Nitrogen fixation was confirmed in NFb and JNFb medium. Phosphate solubilization was verified by cultivating the microorganisms in media with calcium phosphate. The production of IAA was quantified using the Salkowiski reagent and the production of ammonia using the Nessler reagent. The production and quantification of siderophores was performed using the CAS reagent. The synthesis of cellulase and chitinase enzymes and the production of HCN were verified. Finally, the potential of these isolates to inhibit the growth of phytopathogenic fungi through direct confrontation, production of supernatants and volatiles was evaluated. All rhizobacteria presented at least four direct mechanisms for promoting drought tolerance. These isolates were inoculated into VM01 and I144 minicuttings at 41 and 58 days of age. The substrate moisture was constantly reduced to 30% of its water retention capacity on the 83rd day. At the end of the 90th day, the seedlings were evaluated for their morphological characteristics and nutritional condition. Some rhizobacteria were effective in improving the morphological characteristics and nutritional conditions of plants, mainly the LEM 50 *Ralstonia pickettii* and LEM 14 *Ralstonia pickettii* isolates in VM01 clones. The inoculation of rhizobacteria in I144 clones provided increases in

a smaller number of variables. The rhizobacteria that provided significant increases in the root biomass of VM01, LEM 13 *Staphylococcus kloosii* and LEM 50 *R. pickettii* and for I144 LEM 10 *Ralstonia pickettii* and LEM 41 *Burkholderia cenocepacia* were selected for the rooting test. These isolates were inoculated by immersing the stem in rhizobacterial solutions, followed by reinforcing inoculation at the interface between the substrate and the stem. The propagated plants were then placed for 35 days in a greenhouse rooting, where the rooting percentage was evaluated. At the end of the experiment, the roots were subjected to morphological analyzes using the WinRHIZO system. In I144 minicuttings, root volume was significantly greater in treatments with LEM 10 *R. pickettii* and LEM 50 *R. pickettii*. Based on the results presented, it can be stated that PGPR can help clones VM01 and I144 in conditions of water deficit. No significant gains in rooting percentage were observed for both clones The efficiency of microorganisms can be different depending on the genotype, the stage of plant development and abiotic production conditions, such as water availability.

Keywords: Water deficit; Climate changes; Microorganisms, Bacteria; Rhizosphere; VM01; I144; Rooting; Productivity; Nursery.

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GENERAL INTRODUCTION

In recent decades, drought occurrences have become more frequent in various regions of Brazil. Water crises are not merely historical climatic events but present and future challenges closely associated with global climate change.

The Representative Concentration Pathways (RCPs) for carbon dioxide (CO_2) emissions, specifically the medium (RCP4.5) and high (RCP8.5) trajectories, express average CO_2 concentrations corresponding to a radiative forcing of 4.5 watts per square meter and 8.5 watts per square meter, respectively, across the entire planet. These projections indicate that we can expect more frequent and intense dry days in the future, accompanied by a reduction in consecutive wet days throughout almost the entire Brazilian territory. Considering eucalyptus plantations, climate change projections indicate reductions in the mean annual increment (MAI) (m^3/ha/year) by -1 % to -6 % (RCP4.5) and -8 % to -12 % (RCP8.5). Brazil possesses the largest planted and productive areas of eucalyptus in the world, which may be susceptible to climate change impacts, resulting in uncertainties in forest production. Consequently, the development of strategies and technologies that make plants that are more tolerant to low rainfall conditions are essential.

The use of microorganisms that promote drought tolerance in plants may be an alternative to this challenge. Some bacteria have mechanisms capable of facilitating plant survival and growth under water-deficit conditions, such as the production of exopolysaccharides, biofilm formation, production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, nitrogen fixation, phosphorus solubilization, production of siderophores, volatile compounds and various phytohormones, which, either individually or collectively, stimulate plant growth.

The use of bioinputs has increased and gained notoriety, particularly in the agricultural sector, as a result of research demonstrating drought-tolerance in plants when inoculated with microorganisms. Although studies have demonstrated positive effects of using microorganisms on the nutrition and growth of eucalyptus under regular water conditions, there is still limited research on rhizobacteria and their potential to promote drought tolerance.

In this context, the objectives of this work were (I) to review the impact of drought on eucalyptus forests and explore how microorganisms can contribute to the development of more tolerant seedlings (II) to determine the mechanisms involved in promoting drought tolerance and promoting plant growth of bacteria isolated from the rhizosphere of *Eucalyptus grandis*, (III) to investigate the potential of these rhizobacteria in providing growth and drought tolerance

in hybrid eucalyptus clones [VM01(*Eucalyptus urophylla* x *Eucalyptus camaldulensis*) and I144 (*Eucalyptus urophylla* x *Eucalyptus grandis*)] subjected to constant reductions in irrigation in a seedling nursery and (IV) evaluate whether IAA-producing isolates that provided increases in root biomass under water deficit, promote greater efficiency in the rhizogenesis of minicuttings, in a greenhouse rooting.

Chapter I

IMPACT OF DROUGHT ON EUCALYPTUS FORESTS: HOW MICROORGANISMS CAN HELP IN THE DEVELOPMENT OF MORE TOLERANT SEEDLINGS: REVIEW

IMPACT OF DROUGHT ON EUCALYPTUS FORESTS: HOW MICROORGANISMS CAN HELP IN THE DEVELOPMENT OF MORE TOLERANT SEEDLINGS: REVIEW

1. Climate Change: drought

According to the Intergovernmental Panel on Climate Change (IPCC), drought is defined as "a period of abnormal dry weather long enough to cause a serious hydrological imbalance" (IPCC, 2012). It is important to highlight that drought is a complex and multifaceted phenomenon, originating from the intricate interaction of natural and anthropogenic processes. The phenomenon varies across different regional climates, making it difficult to compare over time and space (Van Loon *et al.,* 2016; Erian *et al.,* 2021). Human activities, such as deforestation and urbanization, trigger modifications in infiltration processes, evapotranspiration, surface runoff, and water storage, impacting the development of drought (Van Loon *et al.,* 2016).

Droughts can have serious socio-economic and ecological consequences, leading to disruptions in drinking water supply, hydroelectric power generation, and the agricultural and forestry sectors (Ciais *et al.,* 2005; Sheffield *et al.,* 2012; Mosley, 2015; Stahl *et al.,* 2016; Van loon *et al.,* 2016). According to the World Meteorological Organization (WMO), data from 1970 to 2019 show that droughts were responsible for the highest number of deaths worldwide (650,000 deaths). In Brazil, drought is the main cause of significant economic losses (Zhongming; Wei, 2021). The country is highly vulnerable to periods of drought, as 70 % of its energy comes from hydroelectric power plants, and agriculture relies on 32 % of the available water (Getirana, 2016; Melo *et al.,* 2016).

The first energy crisis caused by drought in Brazil was reported in 1924-1925, specifically due to the dry of the Tietê River. In 2001, Brazil faced its most severe energy crisis, surpassing all previous ones in terms of duration and geographical scope. The triggering factor was unfavorable rainfall in 1999-2000 and a dry summer in 2001, resulting in severe water scarcity (Hunt *et al.,* 2018). Similarly, from 2010 to 2013, the Northeast region experienced one of the worst droughts reported in the last 100 years, causing devastating losses in agriculture and livestock sectors (Gutiérrez *et al.,* 2014). Considering the past decade, in 2014, Brazil faced a record loss of $5 billion among South American countries (Zhongming; Wei, 2021). Over the past 60 years, most of the Brazilian territory has experienced more severe and intense droughts (Cunha *et al.,* 2019), and it is expected that in the future it will be subject to more intense and frequent dry days, with a reduction in consecutive wet days across almost the entire Brazilian

territory (Avila-Diaz *et al.,* 2020). Drought periods will cause serious problems for plant growth on more than 50% of the world's arable land by 2050 (Vurukonda *et al.,* 2016). Drought is considered the primary risk factor for eucalyptus production (Assad *et al.,* 2022).

2. Eucalyptus forests

The Brazilian forestry sector ranks first in the world in terms of planted area and productivity of eucalyptus. In 2022, eucalyptus plantations totaled 7.6 million hectares, showing an increase compared to the previous year with 7.5 million hectares (IBÁ, 2022; IBÁ, 2023). Of all the trees planted in Brazil, 76 % correspond to eucalyptus, being, therefore, the most cultivated species. The largest eucalyptus plantation areas are found in Minas Gerais (29 %), followed by Mato Grosso do Sul (15 %), São Paulo (13 %), Bahia (8 %), Rio Grande do Sul (8 %), and Paraná (6 %) (IBÁ, 2023), regions that may be affected by climate change.

Eucalyptus trees are the most widely planted trees worldwide due to their rapid growth and quality of wood (Oberschelp *et al.,* 2022). They are an important source of forest products such as cellulose, paper, panels and laminated floors, sawn timber and plywood, charcoal, firewood, and bioenergy (IBÁ, 2022). Consequently, eucalyptus plays a significant role in reducing the exploitation of tropical forests and preserving their related biodiversity (Cook *et al.,* 2016).

The productivity of eucalyptus plantations in Brazil stands out globally as the highest, resulting from investments in technology, professional training, advances in management, disease and pest control, soil fertilization, and favorable natural conditions in Brazil. Over the years, productivity has increased from 10 m³/ha/year in 1970 to 38.9 m³/ha/year in 2021 (IBÁ, 2022). However, in 2022, the forestry sector saw a drop in productivity to 32.7 m³/ha/year, the lowest result observed since 2014 (IBÁ, 2023). The main exported product from eucalyptus forests is cellulose, which increased from US$ 6.7 billion in 2021 to US$ 8.5 billion in 2022. Currently, Brazil stands out as the largest exporter of cellulose in the world market, with a difference of US$ 0.7 billion compared to the United States, the second largest exporter (IBÁ, 2023).

Eucalyptus plantations worldwide are dominated by nine species and their hybrid combinations: *E. camaldulensis*, *E. dunii*, *E. globulus*, *E. grandis*, *E. nitens*, *E. pellita*, *E. saligna*, *E. tereticornis*, and *E. urophylla* (Harwood, 2011). Each species has a specific bioclimatic niche for optimal growth (Jovanovic *et al.,* 2000). Various inter-specific hybrid combinations have been achieved through controlled pollination to obtain clones with desirable characteristics that allow better adaptation to climatic and environmental conditions.

Environmental variations such as soil class and texture, as well as climatic conditions, influence the growth of *Eucalyptus* spp., and therefore, plant performance can vary depending on the environment in which they are cultivated (Mauri *et al.,* 2014).

Hybrids of *E. grandis* and *E. urophylla* are well adapted to humid tropical and subtropical climates and are the most planted in Brazil. Furthermore, this hybrid possesses characteristics that provide greater disease resistance compared to pure species. On the other hand, the hybrid between *E. grandis* and *E. camaldulensis* exhibits drought tolerance compared to *E. grandis* and superior growth compared to *E. camaldulensis*. Due to their ability to tolerate drier climates and their superior growth performance compared to parental species, they are ideal for planting in regions with limited water availability (Harwood, 2011). However, genetic improvement through hybridization involves silvicultural aspects, intermediate selections and technological analyses of the wood, processes that are highly expensive and time-consuming. It is necessary to combine several properties, such as plant growth, physical characteristics of the wood and chemical composition, bringing together these qualities in a single individual (Paludzyszyn Filho; Santos, 2011). This process can take 10 to 20 years until new hybrid varieties are developed and ready for commercialization (Paludzyszyn Filho; Santos, 2011).

3. Impact of drought on eucalyptus forests

The effects of drought on plants are observed in all tissues and subcellular compartments, negatively impacting the quantity and quality of crop growth (Vurukonda *et al.,* 2016). High temperatures can induce physiological stress in eucalyptus plantations due to increased evapotranspiration and consequent water deficit (Elli *et al.,* 2020). Reductions in rainfall in some regions have sparked the need to seek innovations that allow plants to tolerate drought. In 2014, approximately 200 thousand hectares of planted forests were lost in the northern region of Minas Gerais (Gonçalves *et al.,* 2017).

A recent modeling study found that the regions between the states of Paraná and São Paulo will experience water reductions exceeding 300 mm/year from 2021 to 2040, limiting eucalyptus production (Assad *et al.,* 2022). Projections of future climate change indicate average annual temperature increases of 1.9 °C to 2.4 °C between 2040 and 2069 under RCP4.5 and RCP8.5, respectively, with annual rainfall reductions in the North, Northeast, Southeast, and Midwest regions. According to these projections, eucalyptus plantation productivity in Brazil will be impacted by a mean annual increment (MAI) (m^3/ha/year) decrease of -1 % to -6 % (RCP4.5) and -8 % to -12 % (RCP8.5). The influence of climate change suggests that

production may be negatively affected by drought periods, bringing concerns regarding the forest production (Elli *et al.,* 2020).

The growth of the forestry industry has led to the expansion and to search for new regions for eucalyptus plantations, including those characterized by low precipitation. Therefore, genotypes or technologies that provide greater tolerance to water deficit are essential for increasing productivity (Oliveira, *et al.,* 2021). In 2021, Brazilian forestry companies increased investments in research and development (R&D) by 17 % compared to 2020. Of these investments, 1.6 % was allocated to innovation in the forestry sector, with 0.6 % specifically invested in R&D (IBÁ, 2022). Investments in innovation and R&D often come from partnerships with technological research centers. In 2022, 31% of companies signed projects in partnerships with startups and 23 % with universities (IBÁ, 2023).

Although the percentage allocated to R&D may seem small, it demonstrates that forestry companies understand the limitations imposed, such as the need for expanding planting areas and the consequences of climate change, and they are seeking innovations to address these challenges. Therefore, the development of strategies and technologies that mitigate the effects of climate change on eucalyptus plantations, specifically reducing the impacts of water stress to which the plants may be exposed, is essential.

4. Plant Growth-Promoting Rhizobacteria (PGPR) mechanisms that aid drought tolerance

An alternative for mitigating the effects of water stress in plants is the utilization of microorganisms. In the rhizosphere, endosphere, and phyllosphere, thousands of microbial species are continuously interacting with the plant, influencing its growth and productivity. Plant Growth-Promoting Rhizobacteria (PGPR) can assist plants in facing water stress conditions through various mechanisms (Vurukonda *et al.,* 2016).

Water stress caused by the absence or low precipitation and salt concentration in the environment is one of the most common challenges that microorganisms must confront in natural habitats (Hernández-Canseco *et al.,* 2022). To withstand such conditions, xerotolerant microorganisms possess mechanisms that help them cope with limited water availability, such as modifications in membrane phospholipids to maintain fluidity, accumulation of solutes to preserve osmotic potential, and secretion of extracellular polymeric substances (EPS) (Lebre *et al.,* 2017). Many of these mechanisms are also activated under other stressful conditions, such as salt stress, enabling the survival of these microorganisms in such environments (Daniel *et*

al., 2004). The ability to survive in low water availability and saline environments is necessary, as both conditions are often interconnected, such as in semiarid regions (Bonatelli *et al.,* 2021).

In addition to possessing mechanisms that ensure their own survival in diverse environments, some microorganisms can assist plants in mitigating the effects of water stress by promoting greater drought tolerance (Kavamura *et al.,* 2013). Plant Growth Promoting Rhizobacteria can act to mitigate the effects of water stress in plants through direct and indirect mechanisms. Direct mechanisms are immediate actions carried out by microorganisms that positively affect plant growth through the acquisition of nutrients, vitamins and hormones. Indirect mechanisms are related to the actions of antagonism against phytopathogens, which, when eliminated, indirectly promote plant growth and health (Orozco – Mosqueda; Flores; Rojas-Sánchez, 2021). In the context of water deficit, we can consider that the mechanisms for the production of EPS (exopolysaccharides) and biofilm are also direct mechanisms, as they immediately benefit plants in the face of water scarcity.

Exopolysaccharides (EPS) are high-molecular-weight polymers produced and released by bacteria. In stressful situations, these molecules protect bacteria against environmental adversities, such as desiccation and/or salinity (Morcillo; Manzanera, 2021). The exopolysaccharides produced by bacteria retains water, providing a hydrated environment that dries more slowly than the surrounding environment, thus increasing the available water in the rhizosphere. As a result, plants have more time to make metabolic adjustments to cope with water deficits. Due to its viscous nature, EPS can form a protective capsule around soil particles, forming aggregates that alter various soil properties such as water infiltration, aeration, root penetration, and increased nutrient absorption by plants (Vurukonda *et al.,* 2016; Latif *et al.,* 2022). Thus, the absence of EPS can affect the drought tolerance capacity of plants (Lu *et al.,* 2018). Exopolysaccharides contributes to the formation of biofilms; therefore, the production of these compounds is crucial for cells to have the ability to aggregate and adhere to plant roots (Hori; Matsumoto, 2010).

The combination of bacterial surface components, extracellular compounds, environmental conditions, and quorum-sensing signals is fundamental for the **biofilm formation** (Costerton *et al.,* 1995). Briefly, the biofilm formation model begins when planktonic cells adhere to a surface. At this initial stage, microbial cells are loose and reversibly adhered to the surface via the cell pole or flagellum. Subsequently, these cells change their orientation to longitudinal, going from a reversible to an irreversible attachment with flagellar reduction (Sauer *et al.,* 2022). It is at this moment that the molecule called bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) comes into action. c-di-GMP restricts flagella-

mediated swimming motility and increases biofilm matrix production. On the microbial surface, there is the presence of a surface detection system, Pil-Chp, which regulates the production of c-di-GMP, increasing it with each binding event. Therefore, initial biofilm formation consists of the conversion of cells that exhibit a low concentration of c-di-GMP and have not yet encountered the surface, to cells that exhibit a high concentration of c-di-GMP and that have encountered surfaces and established an irreversible bond. With successful adhesion, microorganisms begin cell-to-cell multiplication and aggregation (Rather; Gupta; Mandal, 2021). The 3-dimensional structure of the biofilm, derived from EPS, allows the formation of channels that facilitate the exchange of water, nutrients, and signals among bacteria (Rudrappa *et al.,* 2008). Also, the biofilm acts as a protective structure, shielding against UV radiation, pH changes, osmotic stress, and desiccation (Flemming, 1993). Bacteria present in the soil mostly form biofilms when colonizing plant roots. However, this interaction is complex and involves environmental and genetic factors, exudates, cell-cell interactions, as well as plant-bacteria interactions (Bogino *et al.,* 2013). When associated with plants, the biofilm can protect roots from desiccation, salinity, pathogens, aid in nutrient uptake, soil fertility, and promote plant growth (Rudrappa *et al.,* 2008; Seneviratne *et al.,* 2011; Gupta, G. *et al.,* 2017; Naseem *et al.,* 2018; Gupta, S.; Pandey, 2019).

Certain microorganisms are capable of producing phytohormones such as **indole-3-acetic acid (IAA)**, which is the predominant auxin in plant growth and development (Vurukonda *et al.,* 2016). Over 80 % of rhizobacteria are capable of producing IAA, and in terms of quantity, it is the most produced phytohormone by them (Ullah *et al.,* 2019). AIA biosynthesis can be through the tryptophan-independent pathway or the tryptophan-dependent pathway, which represents the majority of studies. As tryptophan within the microbial cell is low, microorganisms generally use the tryptophan released by root exudates (Gamalero; Glick, 2022). By using tryptophan as a precursor, IAA biosynthesis can be carried out through five main pathways: (1) indole-3-acetonitrile (IAN) pathway, (2) indole-3-pyruvate (IPy) pathway, (3) indole-3-acetamide pathway (IAM), (4) indole-3-acetaldoxime pathway (IAOx) and (5) tryptamine pathway (TAM). Some pathways are characteristic of pathogenic microorganisms (IAN), while the IPy pathway is the main one used by many endophytic and rhizospheric plant growth-promoting microorganisms. In the IPy pathway, the aminotransferase enzyme catalyzes the reaction, transferring an amino group from L-tryptophan to alpha-ketoglutarate, resulting in the formation of indole-3-pyruvate. Then, by the action of the enzyme indole-3-pyruvate decarboxylase, indole-3-pyruvate is converted to indole-3-acetaldehyde which, through different reactions and specific oxidation enzymes, is converted to indole-3-acetic acid (IAA)

(Ahmad *et al.,* 2022). The IAA produced by bacteria can be absorbed by plant roots, contributing to the increase in the endogenous pool of IAA (Gamalero; Glick, 2022). Under stress conditions, auxins play a crucial role as they promote root elongation, the formation of root hairs and lateral roots, allowing the plant to access a larger soil area and enhance water and nutrient uptake (Zarea, 2019). Genes related to the formation of primary (OsIAA4 and OsIAA1) and lateral (OsIAA13 and OsIAA11) roots of rice were regulated through endogenous alteration of IAA synthesized by *B. altitudinis* FD48. Inoculation with the microorganism promoted increases in the number of roots, root thickness, area, volume and branching (Ambreetha *et al.,* 2018). The ability to absorb water and previously inaccessible nutrients helps the plant tolerate water stress. Furthermore, it is suggested that auxin enables greater tolerance to water stress by reducing oxidative stress, regulating chloroplast structure, and the abundance of photosynthetic components (Tognetti *et al.,* 2012; Lecube *et al.,* 2014). Drought-adapted plants exhibit increased levels of IAA; Thus, rhizobacteria that produce auxins can assist them in drought tolerance.

Some bacteria can also produce the **phytohormone abscisic acid (ABA)**, which alleviates water stress in plants (Kaushal; Wani, 2016). ABA is associated with stomatal closure under drought conditions, limiting the transpiration rate (Salvi *et al.,* 2021). Microorganisms capable of producing ABA and **gibberellic acid** (GA) appear to enhance plants' ability to cope with the effects of water stress, suggesting that GA could also contribute in some way to drought tolerance (Cohen *et al.,* 2009).

Plants subjected to stress generate **reactive oxygen species** (ROS) that, when reacting with proteins, lipids, and deoxyribonucleic acid, cause damage and impair cellular functions. The accumulation of ROS in plants is regulated by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) (Vurukonda *et al.,* 2016). However, some microorganisms can also eliminate ROS. Neotropical trees (*Cecropia pachystachya* and *Cariniana estrellensis*) inoculated with bacteria demonstrated a greater ability to cope with water deficit by increasing APX activity, thereby reducing oxidative damage (Tiepo *et al.,* 2020).

Ethylene is a gaseous hormone in plants, regulated by various physiological processes and stimuli, such as abiotic and biotic stresses (Vurukonda *et al.,* 2016). Ethylene synthesis occurs from a precursor called S-adenosylmethionine (S-AdoMet), which is converted by the enzyme 1-aminocyclopropane-1-carboxylate synthase (ACS) to 1-aminocyclopropane-1-carboxylate (ACC). **1-aminocyclopropane-1-carboxylate** is the direct precursor for ethylene synthesis, and the conversion of ACC to ethylene occurs through the action of the enzyme ACC

oxidase (ACO) (Glick, 2014). Endogenous ethylene production increases under stressful conditions such as water limitation, nutrient deficiency, and high temperatures. Consequently, increased ethylene production reduces root and shoot growth. This is due to the effects of ethylene on cell division in vegetative tissues, root elongation, and branching (Vurukonda *et al.,* 2016; Naing *et al.,* 2021). Some rhizobacteria are capable of producing an enzyme called ACC deaminase. These microorganisms, when interacting with plants, sequester the available ACC and degrade it to α-ketobutyrate and ammonia (NH_3) through ACC deaminase, using the products as a source of nitrogen and energy (Vurukonda *et al.,* 2016). As a result, ethylene levels in the plant are reduced, leading to improvements in the various effects of ethylene on plant growth. Bean plants inoculated with rhizobacteria with ACC deaminase activity showed increases in biomass and water use efficiency under water deficit. The positive effects observed appear to be related to a decrease in ACC in the roots (- 45 %) when compared to non-inoculated bean plants, which resulted in lower ethylene emission (Brunetti *et al.,* 2021). Rhizobacteria identified as *Aneurinibacillus aneurinilyticus* and *Paenibacillus* sp. were selected *in vitro* for ACC activity and inoculated as a consortium into French bean seedlings under saline stress. The isolates provided a significant decrease in ethylene levels (60 %) and promoted biomass gains throughout the plant, as well as an increase in chlorophyll content (Gupta; Pandey, 2019). Gene expression studies observed decreases in the expression of the ethylene-responsive gene (ERF-1B) in millet seeds under water stress inoculated with *Bacillus amyloliquefaciens*, with ACC deaminase activity. Inoculation with *B. amyloliquefaciens* resulted in an increase in seed germination (91.75 %), improvement in chlorophyte content and relative water content (RWC), as well as an increase in osmolyte (proline) content, APX and SOD activity (Murali *et al.,* 2021). The inoculation of PGPR with ACC deaminase activity results in positive impacts on the induction of tolerance to water stress in plants, reinforcing the potential for use in areas subject to water scarcity.

Some bacteria produce **volatile organic compounds** (VOCs) which are low molecular weight molecules (<300 Da) that can be short-chain aliphatic aldehydes, ethers, esters, alcohols, ketones, sulfur compounds and hydrocarbons (Vaishnav *et al.,* 2017). VOCs can help plants against abiotic and biotic stresses (Ali; Khan, 2021). These molecules can help plants cope with the effects of water stress. 2R, 3R-butanediol, a volatile metabolite produced by certain bacteria, acts as signaling molecules to induce stomatal closure, preventing water loss (Cho *et al.,* 2013; Vurukonda *et al.,* 2016). Additionally, VOCs appear to induce the accumulation of choline and glycine betaine, osmoprotectants that prevent dehydration in plants (Liu; Zhang, 2015). Volatile organic compounds produced by *Pseudomonas pseudoalcaligenes* such as dimethyl disulfide,

2,3-butanediol, and 2-pentylfuran were able to increase the germination rate, vigor, root length and biomass of corn plants under water stress. Furthermore, there were improvements in the relative water content (RWC), which was 29.9 % higher than that of control plants, and an increase in the proline content in the roots and shoots. There was an increase in the levels of chlorophyll a, b and carotenoids, as well as the activities of antioxidant enzymes. Volatile organic compounds produced by *P. pseudoalcaligenes* also reduced visible symptoms of drought stress, such as pale leaves, wilted and curled edges (Yasmin *et al.,* 2021).

Plant growth-promoting bacteria can provide an increase in the contents of **osmolytes**, such as proline, trehalose, choline, glycine betaine and sugars, which help maintain proper water status in cells, protecting membranes and proteins from osmotic stress (Vurukonda *et al.,* 2016). In response to water stress, microorganisms excrete osmolytes that can act synergistically with osmolytes from plants (Bouremani *et al.,* 2023) Proline is an amino acid that has a high hydration potential and accumulates in plants during periods of drought. It is capable of controlling the redox potential of cells, osmotic adjustment, protecting enzymes, proteins and membrane structures, as well as reducing acidity by neutralizing free radicals (Bouremani *et al.,* 2023; Fadiji *et al.,* 2022). Bacteria inoculated into plants subjected to water deficit caused a four-fold increase in proline content (Fadiji *et al.,* 2022). Metabolomics analyzes in sorghums [*Sorghum bicolor* (L.) Moench] treated with rhizobacteria and subjected to water stress revealed that stress-tolerant sorghums showed several metabolomic changes, including the production of the osmolytes proline, glutamic acid and choline (Carlson *et al.,* 2020). Trehalose is capable of protecting proteins and biological structures such as the lipid bilayer, preventing it from solidifying during periods of drought. Wheat plants (*Triticum aestivum* L.) inoculated with the rhizobacteria *Enterobacter bugadensis* had attenuation of the effects of water stress through multiple growth-promoting mechanisms, including increased levels of the osmolytes proline, trehalose and sugars. Gene expression analysis demonstrated altered expression in the genes that encode osmolyte synthesis (P5CS, P5CR and TPS1), suggesting that microorganisms are capable of regulating gene expression, helping plants in stress situations (Arora; Jha, 2023). Choline is a precursor to glycine betaine, an important osmoprotectant that regulates cell osmolarity, reduces reactive oxygen species levels, and protects the plasma membrane (Gowtham *et al.,* 2022). The accumulation of choline and glycine betaine provided by the inoculation of *Klebsiella variicola*, *Raoultella planticola* and *Pseudomonas fluorescens* in corn under water deficit caused an increase in relative leaf water content (RWC) and dry mass weight (SDM). The inoculation of these microorganisms resulted

in osmotic adjustment, improvement in water relations and consequently growth of corn plants (Gou *et al.,* 2015).

Good nutritional conditions are essential for plants to have good physiological performance even under stress conditions (Zhong *et al.,* 2017). Nitrogen (N) is one of the most limiting nutrients for plants, as they cannot fix atmospheric nitrogen. In conditions of water limitations, nutrient absorption by plants is even more difficult. Diazotrophic rhizobacteria play a fundamental role in the absorption of this essential nutrient for plant growth (Xu; Wang, 2023). **Nitrogen-fixing bacteria** whether symbiotic or non-symbiotic, converts atmospheric nitrogen (N_2) into plant-assimilable forms (NH_3) (Rehman *et al.,* 2020). Additionally, ammonifying and nitrifying microorganisms can provide assimilable nitrogen to plants by converting organic nitrogen compounds into inorganic forms (ammonium, NH_4^+ and nitrate, NO_3^-) increasing nutrient availability in the environment (Etesami; Adl, 2020). In studies carried out with diazotrophic rhizobacteria isolated from *Mimosa artemisianana* in the Caatinga region, it was observed that these bacteria promoted the growth of soybean under drought conditions, resulting in an increase in the dry weight of the roots and shoots (Braga *et al.,* 2023). In the context of eucalyptus cultivation, in which forest productivity is directly related to nutrient levels in the soil, diazotrophic rhizobacteria have demonstrated to be effective in promoting increases in the levels of macro-and micronutrients (N, P, K, Ca and Mg), resulting in significant gains in root and total biomass of *Eucalyptus grandis* (Martins Filho, 2021). These diazotrophic bacteria emerge as a promising strategy to reduce dependence on nitrogen fertilizers and increase biomass production (Madhaiyan *et al.,* 2021).

Another essential nutrient for plants is phosphorus, required for the biosynthesis of nucleic acids, membrane phospholipids, ATP and essential in processes such as photosynthesis and biological nitrogen fixation (Bargaz *et al.,* 2021). The majority of phosphorus (P) present in soils is insoluble, found in phosphate minerals (Glick *et al.,* 2012). The release of P from these minerals is slow and dependent on several factors related to the soil, and, therefore, not readily available to plants. The form in which P is available for absorption by plants is the orthophosphate ion (PO_4^{3-} ion). Although the total P content in the world is 400–1000 mg/kg, only a small fraction of P is available, 1.00–2.50 %, below that required by plants (Pan; Cai, 2023). Some bacteria can solubilize phosphate and are known as phosphorus solubilizing bacteria (PSB). **Phosphate-solubilizing bacteria**, which are mostly Gram-negative, can make P available through solubilization, secreting different organic acids (oxalic, citric, gluconic, succinic, α-ketoglutaric and fumaric acid) into the soil, reducing pH and thereby helping to solubilize insoluble phosphates (Glick 2012; Mendes *et al.,* 2013;). In this case, chelation of

cations (Al, Fe and Ca) linked to phosphorus occurs through hydroxyl and carboxyl groups, forming a soluble form of phosphorus, which plants can absorb (Pan; Cai, 2023). Plant growth-promoting rhizobacteria, capable of solubilizing phosphate, can help plants absorb this nutrient, especially under conditions of water deficit, providing a significant increase in biomass (Gupta; Pandey, 2019). The benefits of PSB have been observed in forest seedlings, such as *Quercus brantii*, under drought conditions. Oak seedlings inoculated with *Microbacterium* sp. and *Streptomyces* sp., and subjected to different levels of water deficit showed increases in root length and biomass, in addition to an increase in RWC (Zolfaghar *et al.,* 2020).

Potassium is one of the most important macronutrients, along with N and P, and together they form the widely used NPK chemical fertilizer in agriculture. Similar to P, the majority of K is not readily available to plants in assimilable forms, with its mineral form accounting for 90-98 % of K in the soil. Some microorganisms, such as **potassium-solubilizing bacteria (KSB)**, possess mechanisms such as the production of organic acids, EPS, siderophores, and biofilms that can convert insoluble K into a soluble form, available for plant uptake (Etesami; Adl, 2020). Potassium is an important macronutrient for plant survival under water deficit conditions. It plays fundamental roles such as osmotic adjustment, in which plants accumulate K^+ in cells to maintain osmotic pressure, and regulation of stomatal conductance, ensuring a balance between water uptake by roots and water loss through stomata during water deficit (Hassan *et al.,* 2017). Eucalyptus clones VM01 (*E. urophylla* x *E. camaldulensis*) under water deficit conditions and supplemented with K maintained aboveground biomass accumulation, increased K content in leaves and stems, water use efficiency, gas exchange, and photosynthetic efficiency (Santos *et al.,* 2021).

Indirect plant growth promotion mechanisms carried out by rhizobacteria can also help plants cope with drought. Some bacteria may possess indirect mechanisms, such as the production of siderophores, hydrogen cyanide (HCN), cellulase enzymes, and chitinase, which contribute to the biocontrol of plant pathogens and indirectly to the nutritional status of plants (Sehrawat *et al.,* 2022; Sriwati *et al.,* 2023).

Iron is generally found in the soil as ferric iron (Fe^{3+}), which is difficult for plants and bacteria to assimilate. Thus, these microorganisms developed iron chelating molecules called siderophores that enable their use. **Siderophores** are low molecular weight compounds (400 - 4000 kDa) secreted by bacteria that sequester Fe^{3+} present in the soil and reduce it to Fe^{2+} (Arora; Jha, 2019; Singh *et al.,* 2019). Four types of siderophores are known, which differ due to their structure (moieties) involved in iron chelation: catecholate, phenolate, hydroxamate and carboxylate, a mixture of several types is also commonly found. The way Fe uptake occurs

differs between Gram-positive and negative bacteria. It is known that Gram-positive bacteria do not have an external membrane and, therefore, a receptor is not necessary for the passage of siderophores. In Gram-negative bacteria, the passage of the Fe-containing siderophore through the outer membrane requires recognition by a receptor (β-barrel receptor), which, after binding, undergoes a conformational change and transports the Fe-containing siderophore to the periplasm. During this process, the TonB complex is involved, which provides energy through the proton-motive force. Transport of Fe siderophore to the cytosol is mediated by the ATP-binding cassette transporter in the inner membrane. In the cytosol, iron is generally reduced to Fe^{2+}, which has low affinity for the siderophore and is then released into the cell; however, in the periplasm the reduction of Fe can occur, and only Fe^{2+} is transported to the cytosol (Kramer; Özkaya; Kümmerli, 2020). In this way, PGPR can limit the presence of Fe in the environment, inhibiting the growth of pathogens, since Fe is essential for cell growth and metabolism. It is an efficient mechanism present in some PGPR capable of protecting plants against pathogens (Khoso *et al.,* 2024). A study of 80 rhizosphere microbiomes revealed the effectiveness of these microorganisms in suppressing the bacteria *Ralstonia solanacearum*. The secretion of siderophores by these members was capable of altering microbiome-pathogen interactions, resulting in the inhibition of pathogen growth *in vitro*, in natural soils and greenhouses, providing protection to tomato plants (Gu *et al.,* 2020). Furthermore, under stress conditions, microorganisms that secrete siderophores can also stimulate plant growth and development (Arora; Jha, 2019). *Burkholderia pyrrocinia* strain P10 under saline stress conditions demonstrated the ability to secrete siderophores. In conjunction with other growth-promoting mechanisms, siderophore secretion resulted in higher biomass in peanut seedling (Han *et al.,* 2021). Similarly, *Bacillus aryabhattai* MS3 also demonstrated siderophore production, estimated at 43 % under saline conditions. This presents a promising alternative to the use of chemical fertilizers, especially in saline soils where iron availability to plants is limited (Sultana; Alam; Karim, 2021).

Cellulase and chitinase enzymes act on the degradation of the cell walls of phytopathogenic fungi, which can be composed of cellulose or chitin, as well as on the degradation of insect exoskeletons (Subbanna *et al.,* 2018; Sriwati *et al.,* 2023). Water stress conditions leave plants more vulnerable to pathogen attacks (Gonçalves *et al.,* 2017). Therefore, indirect plant growth-promoting mechanisms are essential as they provide additional resources, enabling better energy management to cope with water deficit and greater tolerance to drought.

5. Inhibition of phytopathogens by bacteria: an essential action under water deficit conditions

Abiotic stresses such as drought influence the interaction between plant and pathogen, promoting changes in the host's physiology and increasing susceptibility to infection. In response to water stress, plants tend to accumulate osmolytes, such as proline and asparagine, with the aim of preserving cell turgidity. Although it is a strategy to face adverse conditions, the nutritional deficit resulting from drought, together with the accumulation of osmolytes, intensifies infection by the pathogen (Lawlor; Cornic 2002; Lawlor 2002). Proline and asparagine, amino acids associated with stress, have been efficiently used as a source of nutrients by pathogens, giving them advantages under water deficit conditions (Ijaz *et al.,* 2013). The presence of pathogens that interfere with water relations by colonizing xylem vessels significantly intensifies the impact of drought (Pandey *et al.,* 2015).

Ceratocystis fimbriata, first described in 1890 by Ellis and Halsted, is considered an important pathogen capable of infecting a variety of hosts, including forest species (Roux; Wingfield 2009). Wilt caused by *Ceratocystis* is one of the most prevalent and serious diseases observed in eucalyptus plantations. The fungus establishes itself in parenchymatic tissues, phloem and xylem, causing plant wilting, responsible for decreases in volumetric growth of up to 87 % and losses in cellulose production of up to 14 % (Ferreira *et al.,* 2006; Mafia *et al.,* 2013). The first observation of *C. fimbriata* in eucalyptus trees in Brazil was in 1997 in southwestern Bahia. In addition to the state of Bahia, in 2001, the disease was again reported in monoclonal plantations of *E. grandis* in Mato Grosso do Sul. In 2003 it was reported in regions of Minas Gerais and in 2004 in São Paulo, suggesting that *C. fimbriata* could be disseminated through clonal seedlings. In the years in which wilt was reported, southwestern Bahia, Mato Grosso do Sul, Minas Gerais and São Paulo faced periods of drought that were abnormal than the average for the region (Ferreira, 2006). The effects of *C. fimbriata* infection mimic plant responses to water deficit. Clones susceptible to infection with the pathogen reduced water transport by 50 %, manifesting nutritional deficiencies in the leaves. Furthermore, partial closure of the stomata was observed, reducing the assimilation of CO_2 to avoid water loss, resulting in a lower growth rate and biomass of the aerial part. Root biomass also decreased, probably due to a reduction in CO_2 assimilation and/or limitations in the transport of photoassimilates through phloem vessels, resulting in a restricted supply of carbohydrates to root cells (Da Silva, 2018).

Fungi of the species *Calonectria* sp. are also capable of infecting a diversity of agronomic and forestry crops, distributed in tropical and subtropical climates. Most reports are

of diseases in forestry crops, mainly in commercial eucalyptus nurseries. The first report of the disease in Brazil occurred in 1970, where more than 80 % of *E. grandis* leaves showed severe defoliation (Alfernas *et al.,* 2015). Infection by the fungus can occur through spores or microsclerotia present in the soil, which spread through the most basal branches, penetrating the plants through open stomata. On the leaves, whether at the margin, base or apex, the beginning of the lesions is established, which when worsening results in severe defoliation. As a result of defoliation, there may be a reduction in the photosynthetic rate and consequently a reduction in the wood volume. Furthermore, the greater light incidence facilitates the growth of other plants that may compete for nutrients (Alfenas *et al.,* 2009; Graça *et al.,* 2009). When the infection occurs in forest nurseries, during seedling production, cutting rot, damping-off, bud blight, stem canker and root rot are observed. In adult plants, aged from 6 months to 2-3 years, *Calonetria* sp. results in severe foliar diseases, shoot burning and consequently tree defoliation (Lombard *et al.,* 2010). In Brazil, 35 species of the fungus have been described, the majority of which are related to eucalyptus cultivation, 11 isolated from tissues and 10 from plantation soil (Sanchez – Gonzalez *et al.,* 2022). The expansion of production in hot and humid regions, such as the north and northeast, led to leaf burn, caused by *Calonetria* sp. (CLB), becoming the main fungal foliar disease (Alfenas *et al.,* 2015).

Plants have the ability to establish associations with different microorganisms, thus constituting their microbiome. In unfavorable scenarios, such as in situations of abiotic and biotic stress, microorganisms can be recruited, through root exudates and through other processes not yet characterized, to collaborate in the management of these challenging conditions (Liu *et al.,* 2021). In this context, microorganisms play an essential role in disease prevention, being a fundamental part of plant health and resistance. The inoculation of beneficial bacteria can have positive results on the plant's immune system. Inoculated bacteria have been shown to provide early warning for plant defense, increasing protection against pathogens (Liu *et al.,* 2020). The microbial population of the rhizosphere uses the release of antibiotics, production of organic compounds, toxins, hydrolytic enzymes (e.g., chitinase, β-1,3-glucanase) to eliminate the pathogen (Pandit *et al.,* 2022). Volatile organic compounds (VOCs), such as 3-methyl-1-butanol, phenylethyl alcohol and 2-methyl-1-butanol produced by *Pseudomonas chrororaphis* subsp. a*ureofaciens* SPS – 41 has been shown to inhibit mycelial growth and spore germination of *C. fimbriata*, the causative agent of black rot in the roots of sweet potato tubers (Zhang *et al.,* 2019). *Bacillus tequilensis* XK29 also demonstrated antifungal activity through the production of VOCs (isovaleric acid, isobutyric acid and 2-methylbutanoic acid), inhibiting the formation and germination of *C. fimbriata* spores (Xu *et*

al., 2021). In addition to the production of VOCs, some microorganisms such as *Bacillus altitudinis* are capable of inhibiting mycelial growth and the germination of *C. fimbriata* spores through the supernatant. Enabling a reduction in the severity of the disease in sweet potato roots (Zhang *et al.,* 2023). Endophytic microorganisms such as *Bacillus amiloliquefaciens* were able to inhibit at least 50 % of the growth of *Calonectria gracilis*, *in vitro*, through the metabolite diffusion test. *In vivo* tests, with minicuttings of hybrid clones of *E. urophylla* x *E. grandis* submerged in a bacterial solution and subsequently infected with *C. gracilis*, demonstrated significant reductions in the incidence and severity of the disease, when compared to the control. Furthermore, higher rooting rates and disease reductions were observed (Paz *et al.,* 2018). Different species of *Calonetria* sp. can have their growth inhibited by a variety of microorganisms. *Burkholderia cepacia* were able to lyse all *Calonectria pseudonaviculata* conidia *in vitro*. The isolates inoculated into boxwood resulted in a decrease in *C. pseudonaviculata* spores and was able to reduce the incidence of rust by 90 % (Kong; Hong, 2020). Microorganisms represent an arsenal that can present different mechanisms against plant pathogens, as well as indirectly helping to promote plant growth. Therefore, an ideal rhizobacterial isolate must be competitive in the rhizosphere, guarantee nutrient absorption, mitigate the effects of drought and promote plant growth under water deficit. Furthermore, they must present a broad spectrum of action mechanisms, such as those cited in this work, be environmentally safe, compatible with other rhizobacteria, and tolerant to abiotic stresses.

6. Rooting of eucalyptus minicuttings by rhizobacteria, essential development for drought-tolerant seedlings

The propagation of clonal seedlings of *Eucalyptus* sp. in forest nurseries, occurs mainly through the minicutting technique, as it provides better percentages and rooting quality, in addition to reducing the time it takes for seedlings to form. For minicutting, vegetative propagules originate from the conventional cutting technique and are established in mini clonal gardens. The minicuttings production stage consists of collecting stems (4 - 8 cm) from ministumps from the mini clonal garden (Xavier; Wendling; Da Silva, 2013). Minicuttings normally have one to three pairs of leaves and a 50 % leaf cut to avoid excess transpiration. After collection, the minicuttings are placed in tubes with substrates and taken to a climate-controlled greenhouse, with a temperature of around 27 °C and humidity above 80 %. The minicuttings remain in the greenhouse rooting for around 20 to 45 days for rooting, depending on the climatic conditions of the region, as well as the selected genotype. At the end of the rooting process, the rooted mini-cuttings are placed in a shade house for acclimatization (10

days) and subsequently taken to the patio for rustification, before being taken to the field. The seedlings will be ready to be transferred to the field, normally at 90 to 120 days old (Xavier; Wendling; Da Silva, 2013).

The rooting of minicuttings depends on intrinsic clone factors such as; youthfulness, genetics, nutritional and water conditions of the plant of origin, as well as hormonal balance (Xavier; Wendling; Da Silva, 2013). Furthermore, environmental factors during the collection procedure also impact rooting success, such as the time of collection, oxidation reactions at the base of the cuttings, quality of the substrate and the abiotic conditions of the greenhouse (temperature, light and humidity) at which minicuttings will be exposed (Xavier; Wendling; Da Silva, 2013). The formation of adventitious roots begins after cutting, with a scarring reaction, where the injury is sealed with suberin, protecting against pathogens and desiccation. Cells begin to divide, and calluses often form from parenchyma cells. Cells that became meristematic from cells adjacent to vascular tissues give rise to root primordia and consequently adventitious roots (Goulart *et al.,* 2014; Hartmann; Kester's, 2014). Therefore, it is concluded that the rhizogenesis process is highly complex and influenced by multiple factors, which can limit the productivity of forestry companies, especially when there is commercial interest in recalcitrant genotypes (Vilasboa; Da Costa; Fett-Neto, 2019).

The use of the phytohormone auxin may bring, in recalcitrant and easy-to-root genotypes, an increase in the percentage, speed and uniformity and consequently increased productivity (Xavier; Wendling; Da Silva, 2013). Minicuttings of *Eucalyptus cloeziana* clones that were submerged for 10 seconds in solutions with different dosages of indolebutyric acid (IBA) (0, 1,500, 3,000 and 6,000 mg L^{-1}), demonstrated significant effects on the rooting percentage. For clones that have greater rooting potential, lower doses of IBA were efficient, while recalcitrant clones were more responsive to higher concentrations (Almeida *et al.,* 2007). According to the literature, the main auxins used for rooting cuttings and/or minicuttings are indole-3-acetic acid (IAA), of natural origin, and the synthetic auxins indole-3-butyric acid (IBA) and naphthaleneacetic acid (ANA) (Vilasboa; Da Costa; Fett-Neto, 2019). The application of different doses of IBA (0, 500, 1000, 2000 and 3000 mg kg^{-1}) in eight commercial clones provided better results in the average rooting rate, however the dosage of IBA for each clone was different, highlighting the variability between genetic materials. Two hybrid clones of *E. urophylla* showed an increase in rooting percentage at a dose of 500 mg kg^{-1} of IBA (Higashi *et al.,* 2003). Different dosages of AIB in VM01 clones also resulted in variation in the rooting percentage. The dosage of 2,000 mg L^{-1} led to 80 % rooting, while minicuttings not

treated with IBA showed 20 % rooting. Furthermore, greater root length and biomass were observed (De Queiroz, 2014).

The use of auxin-producing PGPR to induce rhizogenesis presents itself as an alternative to the use of synthetic auxins, in addition to presenting several mechanisms for promoting plant growth that can favor the plant in a broad spectrum. Inoculation of minicuttings with rhizobacteria can be more efficient in terms of rooting percentage and root dry mass than when treated with IBA. Substrates treated with rhizobacterial isolates were able to improve the average rooting percentage by 110 % and biomass by 250 % of *E. grandis* hybrids aged 35 days. The isolates that were capable of producing IAA *in vitro* also demonstrated significant increases in the percentage of rooting and root biomass when compared to minicuttings treated with IBA (1000 µg g^{-1}) (Teixeira *et al.,* 2007). Another study evaluated the effects on the rooting of 44 minicuttings of different clones distributed throughout Brazil inoculated with rhizobacterial isolates in the substrates. The increases in rooting and biomass were variable depending on the rhizobacterial isolate and clone, with the average gain in rooting being 20.4 % and biomass 73 % (Mafia *et al.,* 2007). Different methods of microbiolization of ministrains can be used. Eucalyptus can be inoculated by spraying the aerial part, inoculating rhizobacteria directly into the substrate, immersing minicuttings in the rhizobacteria suspension, and a combination of methods. The application of bacterial isolates by spraying the aerial part on minicuttings of clone I144 proved to be efficient in promoting an increase in root fresh weight, ranging from 43.7 % to 43.8 % (Shiomi; Accordi; Sabino, 2016). Methods such as inoculation in the substrate, submersion of minicuttings and the combination of both methods, in hybrid clones of *E. grandis* x *E. urophylla*, 25 days old, were effective in causing increases in rooting, root biomass and biological control of *Cylindrocladium* spp. (Mafia *et al.,* 2009). Both microbiolization methods used were efficient in root gains, demonstrating that it is possible to obtain similar results with different ways of applying the inoculants. Efficient and quality root development is desirable in the forestry seedling production sector, and is also fundamental for the success of these seedlings in the field, especially in the face of abiotic stresses such as drought. It has been observed that the conditions provided during the production system affect the quality of the seedling and its initial performance in the field (Alves *et al.,* 2022).

7. The use of microorganisms in the agricultural and forestry sectors to mitigate the effects of drought

The use of microbial inoculants has been increasing in the agricultural and forestry sectors due to research indicating promising results in inducing tolerance to water stress. Recent

studies with maize (*Zea mays* L.) have shown that inoculation with selected bacteria led to a reduction in the effects of water stress and improvement in plant vegetative properties, with increases of 33.8 % in root length, 39.21 % in root dry mass, 83.33 % in photosynthetic rate, 41.67 % in intrinsic water use efficiency, and 16.15 % in total chlorophyll content (Mishra *et al.,* 2020). Similarly, the use of bacteria isolated from desert cacti (*Euphorbia trigonas* Mill) also conferred tolerance to water stress and enhanced growth in tomatoes (*Solanum lycopersicum* L.). After 20 days of induced drought, 86.9 % of the inoculated crops were able to recover, while the non-inoculated plants did not (Eke *et al.,* 2019). Recently, the Brazilian Agricultural Research Corporation (Embrapa) launched the Auras inoculant, composed of bacteria isolated from Caatinga cacti, capable of inducing tolerance to water stress and cellular protection in maize and soybean (*Glycine max* L.) crops (Kavamura *et al.,* 2013; Braga *et al.,* 2022).

In the forestry sector, the use of PGPR offers several benefits for seedling development. Rhizobacteria isolated from *E. grandis* have been shown to increase dry mass, macronutrient content, and nutrient use efficiency in *E. grandis* (Martins Filho, 2021). The use of *Azotobacter* spp. and *Azospirillum* sp. as biofertilizers in *E. grandis* cultivation has shown a significant effect on seedling development, increasing growth, root formation, and leaf number (Díaz *et al.,* 2009). Under drought conditions, *E. grandis* plants associated with arbuscular mycorrhizal fungi have demonstrated improved growth, increased biomass, aboveground height, root length, and relative water content (RWC). Compared to non-colonized plants, there was an increase in the activity of antioxidant enzymes such as peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT), demonstrating the ability of microorganisms to enhance drought tolerance in eucalyptus (Wang *et al.,* 2023).

However, to date, few studies have been found in the literature on the benefits of rhizobacteria in inducing drought tolerance in *Eucalyptus* spp., and none involving hybrid eucalyptus clones. So far, only one study in the forestry field has addressed the use of bacteria to induce tolerance to water stress. In this study, *E. grandis* seedlings inoculated with bacteria wrapped in an acrylic-cellulosic superabsorbent, showed a significant increase in survival after 55 days of water suspension due to rapid stomatal closure, suggesting that the inoculated plants can modify their water use strategy to avoid excessive water loss through transpiration. Additionally, the inoculated plants showed a greater capacity to maintain foliage during water stress and produce new leaves more rapidly upon resumption of watering (Chaín *et al.,* 2020). These results suggest that the use of PGPR mitigates the effects of physiological stress and

promotes adaptive physiological responses, showing promise in aiding plants under water stress.

8. Conclusions and Perspectives

The use of microorganisms holds promise for promoting the growth of several crops and mitigating the effects of drought. Inoculation with rhizobacteria, such as those isolated from Eucalyptus grandis and other species, has promoted significant increases in root dry mass, water use efficiency, and plant growth. These microorganisms help plants maintain better physiological conditions and respond more effectively to water stress, demonstrating improvements in photosynthetic rate and chlorophyll content. Despite the advances observed, research is still limited, especially for hybrid eucalyptus clones, but initial results indicate that rhizobacteria can induce adaptive physiological responses, such as efficient stomatal closure and maintenance of foliage during periods of drought. Thus, the application of rhizobacteria emerges as a valuable strategy to increase plant resistance to drought and contribute to more sustainable practices in agricultural and forestry management.

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Chapter II

IN VITRO CHARACTERIZATION OF DIRECT AND INDIRECT MECHANISMS PERFORMED BY RHIZOBACTERIA TO PROMOTE DROUGHT TOLERANCE

IN VITRO CHARACTERIZATION OF DIRECT AND INDIRECT MECHANISMS PERFORMED BY RHIZOBACTERIA TO PROMOTE DROUGHT TOLERANCE

ABSTRACT

Abiotic stress, such as drought, is likely to become a frequent agricultural problem due to global climate change. The use of microorganisms to induce tolerance to water deficit has shown promising results, as certain bacteria have mechanisms capable of facilitating the survival and growth of plants under stress conditions. **Aims** Here, we identified rhizobacteria from *Eucalyptus grandis* that present mechanisms for plant drought tolerance. **Methods** EPS production was confirmed using the modified culture medium of Guimarães *et al.* (1999). Biofilm formation was verified through cultivation in TSB – 1 % glucose, at 28 °C and 37 °C. The ability to fix nitrogen was evaluated by inoculating the isolates in NFb and JNFb medium. Phosphate solubilization was evaluated in NBRIP, Pikovskaya and NBRIPY media. The quantification of Indole-3-acetic acid (IAA) was determined by cultivation in TSB broth - 5 mM L-tryptophan and subsequent addition of Salkowski's reagent (1:1) to the supernatant. Indirect mechanisms for drought tolerance were examined. Ammonia production was confirmed by cultivation in bacteriological medium peptone (1 %) and NaCl (0.5 %) and to the supernatant (200 µL), 1 mL of Nessler's reagent was added. The production of siderophores was determined by qualitative and quantitative methods, using the CAS solution. Cellulase synthesis was verified by cultivation in CMC agar medium and chitinase synthesis was confirmed by cultivation in medium proposed by Skujins *et al.* (1965). Hydrogen cyanide production (HCN) was verified by cultivation in TSA medium - 4.4 g L^{-1} of glycine and filter papers with solution of picric acid (0.5 %) in sodium carbonate (Na_2CO_3 – 2 %) were placed on the lids of the Petri dishes. The inhibition of *Ceratocystis fimbriata* and *Calonectria variabilis* by rhizobacteria was examined by direct confrontation, supernatant and volatile organic compounds tests. The data were subjected to analysis of variance (ANOVA), and means were compared by Scott-Knott test ($p<0.05$). **Results** All rhizobacterial isolates were capable of exerting mechanisms *in vitro*, which can help plants in water deficit conditions. The isolates LEM 11 *P. validus*, LEM 01 *R. pickettii* and LEM 50 *R. pickettii* showed greater scope in the execution of the mechanisms, with LEM 11 *P. validus* presenting the best *in vitro* responses for the set of tests. **Conclusion** The confirmed mechanisms *in vitro* tests demonstrated that rhizobacteria can meet the need for technologies that help plants subject to periods of drought, enabling the maintenance of the productivity of various crops.

Keywords: Climate change; Water deficit; Bacteria; Eucalyptus.

1. INTRODUCTION

In the past six decades, a significant portion of the Brazilian territory has experienced increasingly severe and intense droughts (Cunha *et al.,* 2019). Furthermore, it is anticipated that in the future, there will be more frequent and intense dry days, accompanied by a reduction in consecutive wet days throughout almost the entire Brazilian territory (Avila-Diaz *et al.,* 2020). These periods of drought will pose significant challenges to plant growth in over 50% of the world's arable lands by 2050 (Vurukonda *et al.,* 2016). Water scarcity affects all tissues and subcellular compartments of plants, impacting the survival, quality and consequently the productivity of several economically important crops. (Vurukonda *et al.,* 2016). High temperatures, generally observed concomitantly with periods of drought, can induce physiological stress in eucalyptus plantations due to increased evapotranspiration and consequent water deficit (Elli *et al.,* 2020). In 2014, approximately 200 thousand hectares of planted forests were lost in the northern region of Minas Gerais, after a long period of drought. In addition to the effects of water stress, reductions in rainfall in some regions have made outbreaks of pests and diseases more common (Gonçalves *et al.,* 2017). Drought is considered as the primary risk factor in eucalyptus production (Assad *et al.,* 2022).

Given this scenario, it becomes crucial to identify effective strategies to mitigate the effects of water deficit on plants. In this context, microorganisms have garnered interest as allies in addressing water deficit challenges. Water stress, caused by the absence or low precipitation and the concentration of salts in the environment, represents one of the most common challenges faced by microorganisms in natural environments. In response, microorganisms have developed mechanisms to adapt to these adverse conditions (Hernández-Canseco *et al.,* 2022). Plant Growth-Promoting Rhizobacteria (PGPR) have the capacity to assist plants under water stress conditions through several direct and indirect mechanisms, including the production of exopolysaccharides (EPS), biofilm formation, synthesis of phytohormones (indole-3-acetic acid), enzymatic production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, and the release of volatile compounds. In addition to the production of siderophores and enzymes such as cellulase and chitinase that can promote the inhibition of phytopathogens. These mechanisms stimulate plant growth, protect it from biotic stresses and consequently enhance their tolerance to water deficit (Vurukonda *et al.,* 2016).

The use of microorganisms in the agricultural and forestry sector has demonstrated efficiency at mitigating the effects caused by drought on plants (Kavamura *et al.,* 2013; Chaín *et al.,* 2020; Mishra *et al.,* 2020; Braga *et al.,* 2022). Therefore, the objective of this study was

to identify rhizobacteria isolated from *Eucalyptus grandis* that present direct and indirect mechanisms for drought tolerance in plants.

2. MATERIAL AND METHODS

The experiments were carried out at the Microbial Ecology Laboratory (LEM) of the Microbiology Department at the Universidade Federal de Viçosa (UFV), Viçosa, MG, Brazil. The experiments were conducted with twenty-one rhizobacteria isolated from the rhizosphere of *E. grandis* maintained in the LEM - UFV microorganism collection. The rhizobacteria were isolated from an eight-year-old *E. grandis* plantation at the UFV Forestry sector (S 21° 01 741`; W 042° 34 258`); of plants with one-year regrowth from the district of Santo Antônio do Rio Preto, Miraí, MG (S 21° 01 638'; W042° 34 156') and eight-year-old trees, located in the same district (S 21 ° 01 698 `; W042° 33 842`) (Martins Filho, 2021).

The rhizobacteria were selected based on the results from principal component analysis (PCA) of the production variables of *E. grandis* plants, macronutrient and micronutrient contents, nutrient utilization efficiency in root dry matter and nutrient utilization efficiency in total dry matter obtained from the work de Martins Filho, 2021. The isolates that showed results in several variables, belonging to group a, b, or c, were selected for investigations in this study. Thus, 21 rhizobacteria were selected to identify the direct mechanisms of drought tolerance and indirect mechanisms to plant growth promotion, *in vitro*.

The isolates were reactivated by transferring an aliquot of 50 µL of rhizobacteria stored in an ultrafreezer at -80 °C, to tryptic soy broth (TSB). The cultures were then incubated in an orbital shaker at 150 rpm at 28 ºC for 48 h. Bacterial growth was confirmed by measuring the optical density ($O.D._{600\ nm}$) using a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific). The cultivation conditions prior to the evaluation of plant growth promotion mechanisms followed those described above.

2.1. Ability of rhizobacteria to tolerate low precipitation and high salinity environments

2.1.1. Identification of xerotolerant rhizobacteria

The identification of xerotolerant rhizobacteria was carried out by confirming colonies capable of growing on solid tryptic soy agar (TSA) supplemented with 405 g L^{-1} of sorbitol ($C_6H_{14}O_6$). TSA culture media with and without added sorbitol was prepared and sterilized in an autoclave for 15 min at 121 °C. The medium was then poured into Petri dishes with three dividers (90 x 15 mm). TSA media without sorbitol supplementation were used to confirm the viability of the isolates.

The water activity values (a_w) were determined using an aqualab 3TE equipment, calibrated with distilled water at 25 °C, with a_w = 1.000 (± 0.003). The a_w measurements corresponding to TSA-sorbitol (405 g L^{-1}) culture medium were carried out at 28 °C, and the a_w values defined after three consecutive readings. Thus, the value defined for the TSA-sorbitol medium (405 g L^{-1}) was a_w = 0.898 (± 0.003), and for the TSA medium a_w = 0.992 (± 0.003). The inoculation of microorganisms to the media occurred through the transfer of a 5 µL aliquot of suspension of each rhizobacteria. Subsequently, the Petri dishes were incubated at 28 °C in a Biochemical Oxygen Demand (B.O.D.) until colonies appeared.

2.1.2. Identification of halotolerant rhizobacteria

Rhizobacteria previously grown in TSB medium were centrifuged at 10,000 rpm for 10 minutes and resuspended in 10 mL of TSB medium making up O.D. $_{600\ nm}$ = 0.05. In order to determine the ability to tolerate different levels of salinity, the following concentrations of NaCl (w/v) were prepared: 0, 0.5, 2, 8, 12, and 15 % plus TSB and sterilized in an autoclave at 121 ° C for 15 min. Microorganisms considered halotolerant tolerate cultivation conditions containing NaCl concentrations ranging from 1 % to 25 % NaCl (w/v) (Hernández-Canseco *et al.,* 2022).

After preparing the TSB media with different concentrations of NaCl, an aliquot of 250 µL of each solution was placed in wells of a 96-well plate. Subsequently, an aliquot of 50 µL of the rhizobacteria was inoculated into each well, resulting in a final volume equal to 300 µL. The 96-well plates were incubated in a microplate reader for 72 hours at 28 °C with optical density readings every 6 hours.

2.2. Direct mechanisms for drought tolerance

2.2.1. Exopolysaccharide production

Isolates that produce exopolysaccharides were identified through cultivation in the modified culture medium of Guimarães *et al.* (1999). The culture medium used was composed of (g L^{-1}): 20 g of yeast extract, 15 g of K_2HPO_4, 0.2 g of $MgSO_4$, 0.015 g of $MnSO_4$, 0.015 g of $FeSO_4$, 0.03 of $CaCl_2$, 0.015 of NaCl, 15 g of agar, sucrose (10 % w/v), pH 7.3 and sterilized in an autoclave and poured into Petri dishes (90 x 15 mm).

Sterilized filter paper discs (5 mm Ø) were placed in triplicates on the solid-modified medium plates. On each filter paper disc, a 5 µL aliquot of the rhizobacteria was inoculated. Subsequently, the Petri dishes were incubated in B.O.D. at 28 °C for 72 h.

After the incubation time, the colony halos of each rhizobacterial isolate were measured and classified as described below (Montaldo *et al.,* 2021):

+ Low production: EPS halo ≤ 10 mm Ø
++ Moderate production: EPS halo of 10-14 mm Ø
+++ Optimal production: EPS halo ≥ 14 mm Ø

Confirmation of EPS production was verified by collecting cellular material using a subculture loop and immersion in test tubes containing 2 mL of absolute alcohol (99.5 %). The precipitation of the cell mass indicates EPS production, while the turbidity of the solution indicates negative production (Paulo, 2010).

2.2.2. Biofilm formation assay

After reactivation of the isolates, the cells were centrifuged at 10,000 rpm for 10 minutes. The formed pellets were resuspended at an O.D. $_{600\ nm}$ = 0.1 in test tubes containing 5 mL of TSB medium supplemented with 1 % (w/v) glucose. In 96-well plates with a flat bottom made of polystyrene, aliquots of 200 µL of each isolate were added, in quadruplicates. The negative control consisted only of the culture medium lacking bacterial cells, which was inoculated in sextuplicates. Before the plates were placed in incubation, the initial optical density of the isolates was measured using a microplate reader at a wavelength of 600 nm. Subsequently, the microplates were allocated to different B.O.D. with temperatures of 28 °C and 37 °C. The microplates remained under static conditions for 72 hours. After the growth period, the O.D was 600 nm, in order to confirm bacterial growth. Subsequently, the contents of the wells were discarded, and each well was washed three times with 200 µL of distilled water using a micropipette, emptying it by flicking. The 96-well plates were then inverted on absorbent paper to remove excess water. The attached bacteria were fixed by exposing the plates to a hot air circulation oven at 60 °C for 60 min. The attached biofilm was stained with 200 µL of crystal violet (1 % - 2 %) for 15 min at room temperature. After the aforementioned period, the dye was removed with the help of the micropipette, and the excess was removed by rinsing the microtiter plate under running tap water until the washings were free of stain. The microplates were placed under static conditions at room temperature until the wells dried. Biofilm formation was assessed by gently adding 200 µl of ethanol to the wells. The microplates were closed with the lid and kept at room temperature for 30 minutes. Subsequently, absorbance was measured in a microplate reader at a wavelength of 570 nm.

The negative control was defined as three standard deviations above that of the uninoculated medium (Christensen *et al.,* 1985). The quantification of the biofilm formed was carried out using the methodology proposed by Stepanović *et al.* (2007) and the intensity of biofilm formation was divided into the following categories: no biofilm producer, weak biofilm (+) producer, moderate biofilm producer (++), and strong biofilm producer (+++).

2.2.3. N fixation in NFb and JNFb media

The 21 rhizobacterial isolates were confirmed for their nitrogen-fixing capability in nitrogen-free semi-solid NFb and JNFb. The composition of NFb includes (g L^{-1}): 5 g of D-Malic acid, 5 mL of K_2HPO_4 (sol. 10 %), 2 mL of $MgSO_47H_2O$ (sol. 10 %), 1 mL of NaCl (sol. 10 %), 2 mL $CaCl_22H_2O$ (sol. 1 %), 2 mL Bromothymol Blue (0.5 % in 0.2 M KOH), 2 mL micronutrient solution*, 4 mL of Fe EDTA (sol. 1.64 %), 4.5 g of KOH, 1 mL vitamin solution**, 1.8 g of agar and pH 6.8. On the other hand, JNFb comprises (g L^{-1}): 5 g of D-Malic acid, 6 mL of K_2HPO_4 (sol. 10 %), 18 mL of KH_2PO_4 (sol. 10%) (mL), 2 mL of $MgSO_47H_2O$ (sol. 10 %), 1 mL of NaCl (sol. 10 %), 2 mL of $CaCl_22H_2O$ (sol. 1 %), 2 mL of Bromothymol Blue (0.5 % in 0.2 M KOH), 2 mL micronutrient solution, 4 mL of Fe EDTA (sol. 1.64 %), 4.5 g of KOH, 1.9 g of agar, pH 5.8) media (Döbereiner *et al.,* 1995).

Five mL of NFb and JNFb media were dispensed into 10 cm test tubes and sealed with cotton plugs. To the sterilized media, a 100 uL aliquot of the isolates was added and conditioned in a B.O.D. incubator at 28°C for 5 days. Tubes containing only NFb and JNFb media were used as negative controls. Bacteria were considered diazotrophic rhizobacteria if they formed a film on the medium surface.

*0.2 g of $Na_2MoO_42H_2O$, 0.235 g of $MnSO_4$, 0.280 g of H_3BO_3, 0.008 g of $CuSO_45H_2O$, 0.024 g of $ZnSO_47H_2O$ in 200 mL of distilled H_2O.
**10 mg of Biotin, 20 mg of Pyridoxine HCl in 100 mL of sterile distilled H_2O.

2.2.4. Phosphate solubilization

The ability of the isolates to solubilize phosphate was evaluated in different culture media. An aliquot of 5 uL of the isolates was inoculated at three equidistant points in Petri dishes (90 x 15 mm) containing NBRIP (g L^{-1}): 10 g of glucose, 2.5 g of $Ca_3(PO_4)$, 5 g of $MgCl_26H_2O$, 0.25 g of $MgSO_47H_2O$, 0.2 g of KCl, 0.1 g $(NH_4)_2SO_4$. Plates were incubated in B.O.D. at 28 °C for 72 h. After this period, the appearance of halos was observed and the phosphate solubilization index (psi) was calculated according to the formula (Nautiyal, 1999):

$$psi = (colony\ diameter + halo\ zone\ diameter) / colony\ diameter \quad (1)$$

The isolates were also evaluated for their ability to solubilize phosphate in Pikovskaya's culture media (g L^{-1}): 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of NaCl, 0.1 g of $MgSO_47H_2O$, 0.2 g of KCl, 0.002 g of $MnSO_4H_2O$, 0.002 g of $FeSO_47H_2O$, 0.5 g of yeast extract, 15 g of agar and NBRIPY (g L^{-1}): 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 0.1 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_47H_2O$, 0.2 g of KCl, 5 g of $MgCl6H_2O$ and 15 g agar. The plates were incubated under the same conditions described earlier, and halo formation was verified after 20 days.

2.2.5. Indole-3-acetic acid (IAA) production

For IAA quantification, TSB medium was prepared and sterilized in an autoclave at 115 °C for 15 min. The medium was supplemented with 5 mM L-tryptophan, which was sterilized by filtration using a 22 µm membrane filter. To initiate the experiment, 10 mL of the medium was dispensed into test tubes, followed by inoculating the isolates (O.D. $_{600\ nm}$ = 0.1). The cultures were maintained in the dark, in a shaker incubator with orbital shaking (150 rpm) at 28 °C for 72 h. After this incubation period, the samples were centrifuged at 10,000 rpm for 10 min to obtain the supernatant. Salkowski's reagent, composed of 50 mL of 35 % perchloric acid and 1 mL of 0.5 mol L^{-1} of $FeCl_3$, was used to determine the amount of IAA produced. To perform the assay, 500 µL of Salkowski's reagent was added to 500 µL of the supernatant in microtubes. The mixture was then incubated for 30 min in the dark at room temperature. Subsequently, the absorbância. was measured using a spectrophotometer at 530 nm (Hartmann *et al.,* 1983). The concentration of IAA in the culture medium (y) was determined by constructing a standard curve using commercial IAA with concentrations of 0, 2, 5, 10, 15, 30, 50, and 100 µg mL^{-1}. The equation used to determine the IAA concentration was y = 0.0066x + 0.255 (R^2 = 0.9905), where x represents the absorbance values.

2.3. Indirect mechanisms for drought tolerance

2.3.1. Ammonia (NH_3) production assay

The isolates were inoculated with an initial O.D.$_{600\ nm}$ = 0.1 in a test tube containing 10 mL of bacteriological peptone (1 % w/v) and sodium chloride (0.5 % w/v). The cultures were transferred to a shaker incubator with orbital shaking (150 rpm) at 28 °C for 48 h. To the supernatant (200 µL), 1 mL of Nessler's reagent (containing 100 g L^{-1} of HgI_2, 70 g L^{-1} of KI, and 500 mL of 32 % NaOH) was added, followed by distilled water to make a final volume of 8.5 mL (Cappuccino; Sherman, 1992).

The concentration of ammonia was estimated using a standard curve of ammonium sulfate (0, 0.1, 0.2, 0.2, 0.4, 0.6, 0.8, 1 µg mL^{-1}) with the equation: y = 1.1435x + 0.1734 (R^2 = 0.9948). Quantification was performed by measuring the absorbance at 450 nm using a spectrophotometer. The presence of a yellow-orange precipitate indicated ammonia production.

2.3.2. Siderophore production

The production of siderotrophs by the isolates was verified using the qualitative method proposed by Schwyn and Neilands (1987) modified. The first step consisted of inoculating the rhizobacterial isolates in Petri dishes (90 x 15 mm) containing nutrient agar (NB), which were incubated in B.O.D. at 28 °C until colonies appeared. After colony growth, a sterile CAS solution composed of (L^{-1}): 60.5 mg of chromeazurole (CAS), 72.9 mg of hexadecyltrimethyl ammonium bromide (HDTMA-Br), 30.2 g of Piperazine-N, N′-bis (2-ethanesulphonic acid) (PIPES), 9 g agar and 10 mL of 1 mM $FeCl_3 6H_2O$ in 10 mM HCl was added to the surface of the Petri dishes in a laminar flow hood. The plates were incubated again in B.O.D under the same conditions as above, for observation of the appearance of yellow halos, a positive indicator for siderophore production.

The quantification of siderophores was conducted based on the reactivation of rhizobacteria. An aliquot of 1 mL of each isolate was added to 2 mL microcentrifuge tubes and centrifuged at 10,000 rpm for 10 minutes. After centrifugation, 500 µl of the supernatant was added to 2 mL microcentrifuge tubes, followed by the addition of 500 µl of the CAS solution (absent agar), totaling 1 mL of reaction. The negative control consisted of 500 µl of medium lacking bacterial cells and 500 µl of the CAS solution. The reaction was carried out in the dark for 20 minutes, and subsequently the absorbance was determined at a wavelength of 630 nm.

Siderophore quantification was measured using the percent siderophore unit (psu) equation proposed by Payne, 1993:

$$psu = (Ar - As) \times 100 / Ar \quad (2)$$

Where: Ar = absorbance of reference (CAS solution and uninoculated broth); As = absorbance of sample (CAS solution and supernatant of sample).

2.3.3. Cellulase production

To verify cellulase production, the CMC agar culture medium was prepared with the following components (g L^{-1}): 2.0 g of CMC (carboxymethyl cellulose), 2.0 g of $NaNO_3$, 1.0 g of K_2HPO_4, 0.5 g of KCl, 0.5 g of $MgSO_4$, 0.2 g of peptone and 17.0 g of agar (Kasana *et al.,*

2008). The test was conducted in Petri dishes (90 x 15 mm) with 3 dividers, containing CMC agar medium. An aliquot of 5 µL of the isolates was added to each divider. The Petri dishes were sealed and incubated in a B.O.D. for 48 h at 28 °C. After the growth of the colonies, a Gram iodine solution (2 g of KI and 1 g of iodine in 300 mL of distilled water) was dripped onto them with a pasteur pipette and waited for 3 to 5 min for the colonies to grow. halo formation. A halo formation around the colonies indicated CMC degradation and therefore, cellulase production by the isolates. The cellulolytic index (CI) was calculated according to the following equation (Teather; Wood, 1982):

$$CI = \text{halo's diameter} / \text{the colony's diameter} \quad (3)$$

2.3.4. Chitinase production

The identification of chitinolytic rhizobacteria occurred from the formation of halos in culture medium containing 1% (w/v) colloidal chitin and (g L^{-1}) 1.0 g of $(NH_4)_2SO_4$, 0.2 g of KH_2PO_4, 1.6 g of K_2HPO_4, 0.2 g of $MgSO_47H_2O$, 0.1 g of NaCl, 0.01 g of $FeSO_47H_2O$, 0.02 g of $CaCl_22H_2O$ and 15 g of agar (Skujins *et al.,* 1965). An aliquot of 5 µL of the isolates was added to the Petri dishes (90 x 15mm) containing the previously described medium. The plates were sealed with plastic film and kept at B.O.D. at 28 °C for 96 h. The formation of halos by the isolates indicated the ability to degrade chitin and, therefore, chitinolytic microorganisms.

2.3.5. Hydrogen cyanide production (HCN)

A modified method based on Bakker and Schippers (1987) was employed for the identification of HCN production by the rhizobacterial isolates. The TSA medium was supplemented with 4.4 g L^{-1} glycine, sterilized and poured into Petri dishes (60 x 15 mm). Quantitative filter papers JP42 (Quanty®, with a pore size of 8 µm) were cut into 1 x 3 cm strips and sterilized by dry heat. The filter papers were soaked in a solution of picric acid (0.5 %) in sodium carbonate (Na_2CO_3 – 2 %) and placed on the lids of the Petri dishes (Bakker; Schippers, 1987). Subsequently, the reactivated rhizobacterial isolates were streaked onto TSA-glycine culture medium, and the plates were sealed with plastic film and incubated in a B.O.D. at 28°C for 5 days. The change in color of the filter papers from yellow to orange, brown, or reddish-brown indicated the production of HCN.

2.3.6. Antagonisms assays

The isolates *Calonetria variabilis* (LPF125) and *Ceratocystis fimbriata* (LPF1594) fungal pathogens of several single species or clones of *Eucalyptus* spp. were obtained from the mycology collection of the Forest Pathology Laboratory of the UFV. The fungi were grown in Petri dishes (90 x 15 mm) with potato dextrose agar (PDA) medium for 14 days in B.O. D at 26 °C to perform the tests described below. Images from the tests of antagonism by direct confrontation, supernatant and volatile organic compounds (VOC) were processed using the QUANT software with a color reduction method to 32, obtaining the area (%) of fungal growth.

2.3.6.1. Antagonism by direct confrontation

Antagonism by diffusible compounds in the culture medium was verified using the double culture technique (Morton; Stroube, 1995). The rhizobacterial isolates had their optical densities standardized to O.D. $_{600\,nm}$ = 0.6. Subsequently, using a micropipette, a 3 cm horizontal streak containing 20 µL of inoculum was made on Petri dishes (90 x 15 mm) with PDA culture medium. Mycelial discs (7 mm diameter) obtained from the margins of 14-day-old cultures of fungal pathogens were inoculated 3 cm distance from the rhizobacterial streaks. The control treatment consisted of only the fungal isolate in the culture medium. At the end, the plates were incubated in a B.O.D. with constant light at 26 ± 1 °C for 14 days.

2.3.6.2. Antagonism by supernatant

The potential of rhizobacterial supernatants to inhibit the growth of plant pathogenic fungi was evaluated using the agar well-diffusion method (Atta, 2015).

In order to obtain the supernatant, a 2 mL aliquot of the rhizobacterial isolates was centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and filtered through a 0.22-µm pore filter membrane. In Petri dishes (90 x 15 mm) with PDA medium, perforations were made with the aid of a micropipette tip to obtain wells. Using the side with the largest diameter of the tip (7 mm), 4 equidistant perforations were made in the culture media. An aliquot of 50 µL of the rhizobacterial supernatant was added to the wells and 7-mm diameter mycelial discs obtained from the edge of 14-day-old cultures of the pathogenic fungi were inoculated in the center of the plates. One of the negative controls consisted of TSB medium in the wells and the phytopathogenic fungus in the center, while the other only contained phytopathogenic fungus in the center. The plates were sealed with plastic film and placed in B.O.D. at 26 ± 1 °C, with constant light for 10 days.

2.3.6.3. Antagonism by volatile organic compounds (VOCs)

The antagonism of volatile compounds was evaluated in triplicates using a Petri dish with a divider (90 x 15 mm), allowing only the sharing of air. An aliquot of 20 µL of the rhizobacterial isolates at a $D.O._{600\ nm}$ = 0.6 was inoculated using the spread plate technique in one compartment of the dish. In the adjacent compartment, 7 mm mycelial discs obtained from the margins of 14-day-old cultures of both pathogenic fungi were inoculated in PDA medium. Controls were performed only with the inoculation of 7 mm mycelial discs in one compartment, the adjacent compartment consisted only of PDA medium. The plates were sealed first with parafilm, followed by plastic wrap, and incubated in a B.O.D with constant light, at 26 ± 1 °C for 14 days.

2.4 Statistical analyses

The experiments were conducted using a completely randomized design (CRD), with at least 3 replications. The data were subjected to analysis of variance (ANOVA), and when significant (p<0.05), the treatment means were compared by Scott-Knott procedure using the Software R and ExpDes package (Ferreira *et al.,* 2013). The construction of the graphics was carried out using GraphPad Prism 5 and the figures using InKscape.

3. RESULTS

3.1 Ability of rhizobacteria to tolerate low precipitation and high salinity environments

3.1.1. Xerotolerant rhizobacteria

The results demonstrated that ten rhizobacteria (47.6 %) exhibited growth in TSA-sorbitol media, with reduced water availability (0.898a_w). It is noteworthy that microorganisms capable of tolerar values de a_w equal to or lower than 0.90 are recognized as xerotolerant according to Grant (2004). Hence, xerotolerants isolates were: LEM 01 *Ralstonia pickettii*, LEM 05 Unrequited, LEM 07 Unrequited, LEM 11 *Paenibacillus validus*, LEM 12 *Stenotrophomonas maltophilia*, LEM 15 *Virgibacillus pantothenticus*, LEM 16 Unrequited, LEM 17 *Stenotrophomonas maltophilia*, LEM 19 *Virgibacillus pantothenticus* and LEM 50 *Ralstonia pickettii* (Figure 1). Furthermore, to assess the cellular viability (control test) all 21 aforementioned isolates were inoculated onto TSA medium with an a_w = 0.990 (28 °C). Under these specific conditions, all isolates successfully demonstrated their growth capabilities.

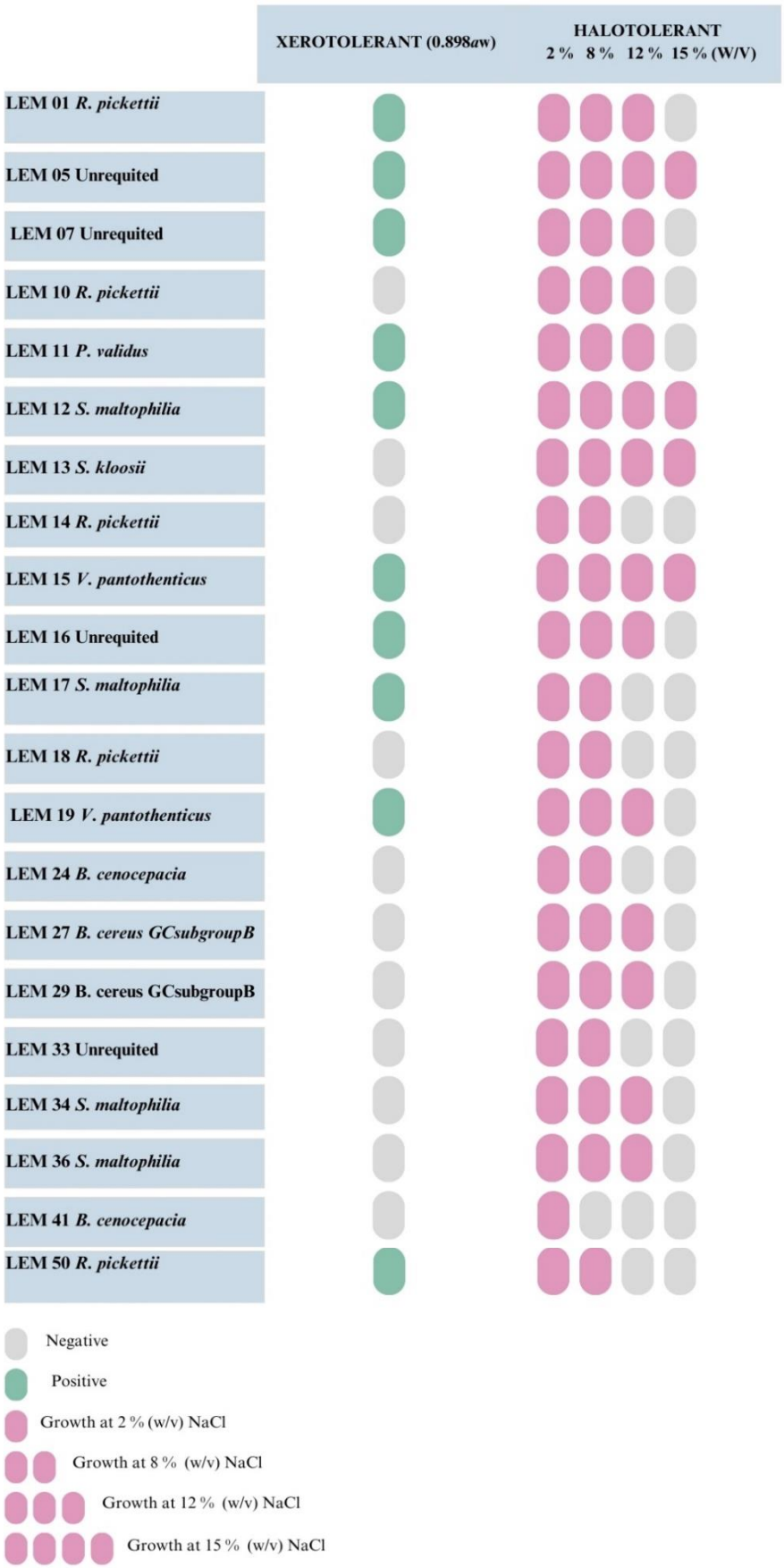

Figure 1. Identification of xerotolerant rhizobacteria capable of growing in medium with low water availability (0.898a_w) and halotolerant, capable of growing in saline conditions.

3.1.2. Halotolerant rhizobacteria

All rhizobacteria isolates were able to grow at NaCl concentration, 2 % (w/v) (Figures 1, 2 and 3) and therefore considered halotolerant isolates (Hernández-Canseco *et al.,* 2022). Six isolates showed growth at a concentration of 8 % (w/v) NaCl and ten demonstrated the ability to grow at 12 % (w/v) NaCl. Notably, four isolates displayed growth even in the presence of 15 % NaCl (w/v), the highest salt level in the test. The isolates that supported this condition were LEM 05 Unrequited, LEM 12 *S. maltophilia*, LEM 13 *Staphylococcus kloosii*, and LEM 15 *V. pantothenticus*.

Among the isolates growing at 15 % (w/v) NaCl, the LEM *05* Unrequited and LEM 15 *V. pantothenticus* isolates exhibited the highest $OD._{600\ nm}$ in 72 h, these being the $O.D._{600\ nm}$ = 0.41 and $O.D._{600\ nm}$ = 0.40, respectively (Figure 2). Isolates LEM 12 *S. maltophilia* and LEM 13 *S. kloosii* presented $O.D._{600\ nm}$ = 0.29, and $O.D._{600\ nm}$ = 0.13, respectively (Figure 2).

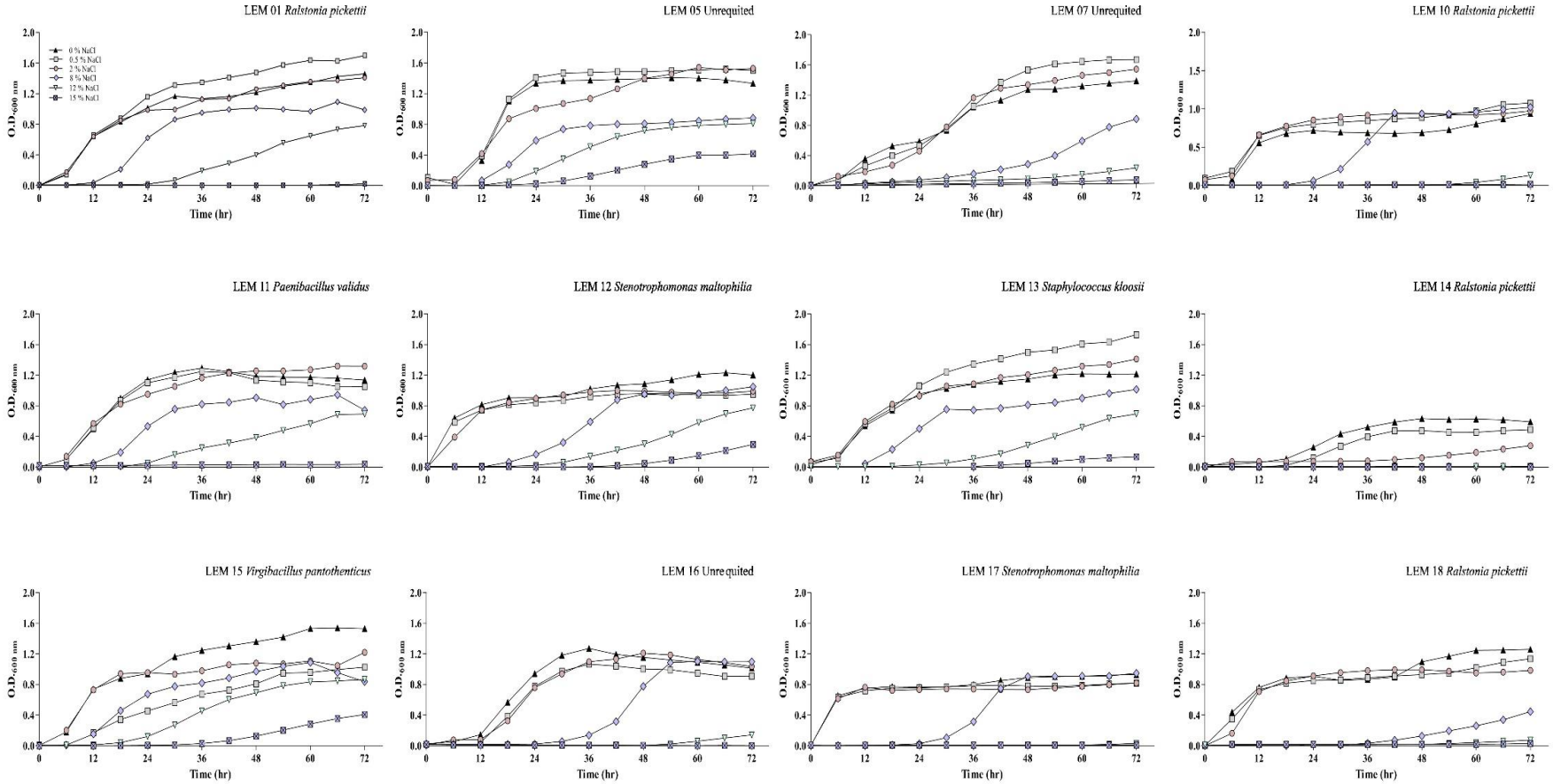

Figure 2. Growth curve of isolates LEM 01 *Ralstonia pickettii* to LEM 18 *Ralstonia pickettii* in medium containing different concentrations of NaCl, including 0, 0.5, 2, 8, 12 and 15 % (w/v).

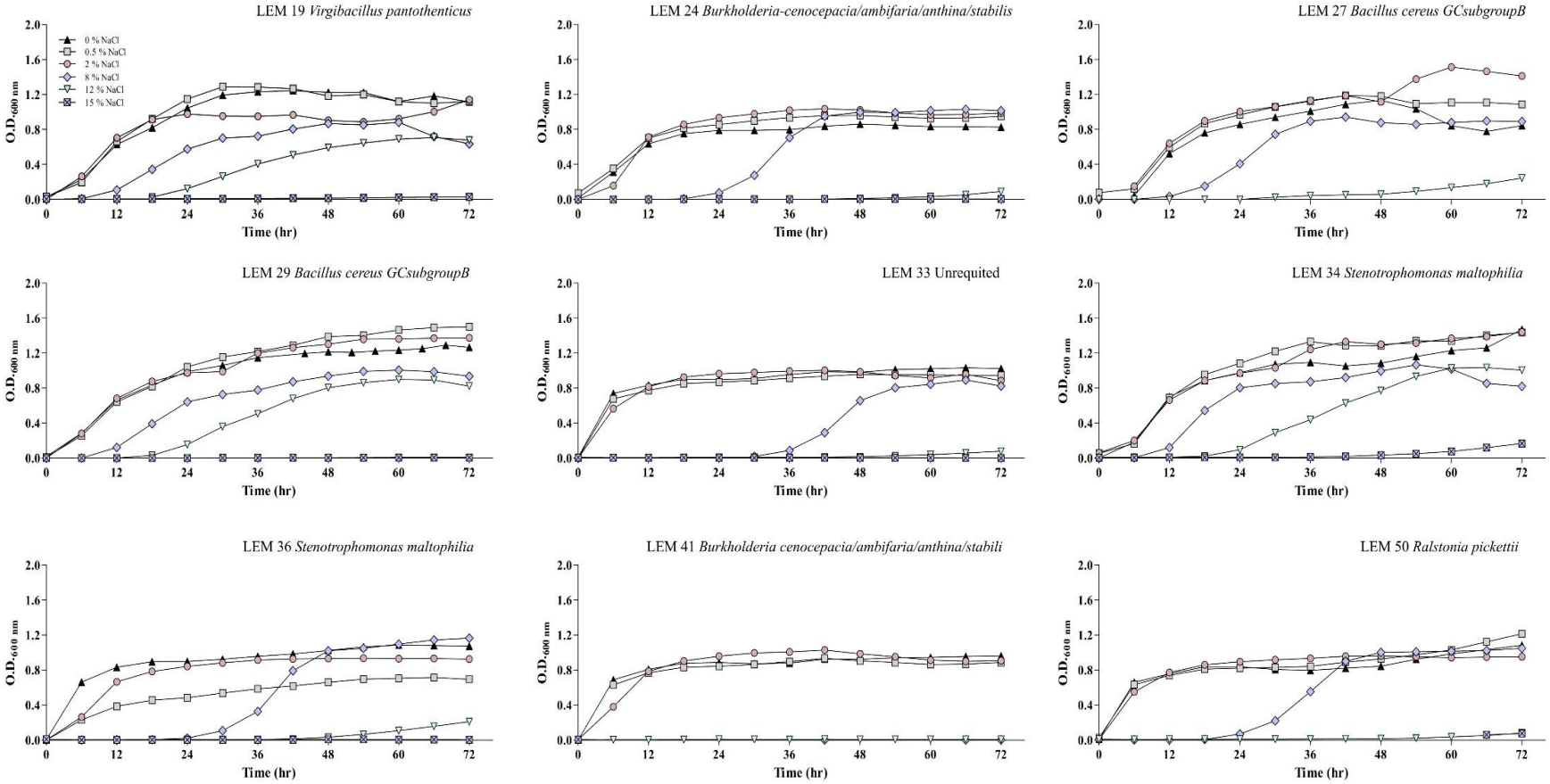

Figure 3. Growth curve of isolates LEM 19 *Virgibacillus pantothenticus* to LEM 50 *Ralstonia pickettii* in medium containing different concentrations of NaCl, including 0, 0.5, 2, 8, 12 and 15 % (w/v).

3.2. Direct mechanisms for drought tolerance

3.2.1. Exopolysaccharide (EPS) production

All 21 rhizobacteria isolates were able to produce exopolysaccharides (EPS) under the conditions evaluated (Table 1). The EPS production by these isolates was categorized into three groups according to the halo diameter: low production (≤ 10 mm), medium production (10-14 mm), and high production (>14 mm) (Montaldo *et al.,* 2021).

Seven isolates stood out in the production of EPS, as excellent producers, being LEM 07 Unrequited, LEM 17 *S. maltophilia*, LEM 19 *V. pantothenticus*, LEM 24 *Burkholderia cenocepacia*, LEM 27 *Bacillus cereus* GCsubgroupB, LEM 29 *Bacillus cereus* GCsubgroupB and LEM 33 Unrequited. Among these, the LEM 07 Unrequited showed the highest EPS production with a halo diameter of 18 mm, followed by the LEM 33 Unrequited (16.6 mm). Both isolates showed no statistical differences between them and were categorized in group a, according to the scott-knott test (p<0.05). Five EPS-producing isolates were classified into group b: LEM 17 *S. maltophilia* (15.3 mm), LEM 24 *B. cenocepacia* (15.3 mm), LEM 27 *B. cereus* GCsubgroupB (15 mm), LEM 29 *B. cereus* GCsubgroupB (15 mm), and LEM 19 *V. pantothenticus* (14.6 mm). Eleven isolates presented average EPS production, being in groups c and d, defined by scott - knott (p<0.05). Group C, comprised seven isolates: LEM 05 *Unrequited* (13.6 mm), LEM 13 *S. kloosii* (13.6 mm), LEM 11 *P. validus* (13.3 mm), LEM 12 *S. maltophilia* (13.3 mm), LEM 34 *Stenotrophomonas maltophilia* (13.3 mm), LEM 01 *R. pickettii* (13 mm), and LEM 18 *Ralstonia pickettii* (13 mm) and group d contained four isolates: LEM 10 *Ralstonia pickettii* (12.3 mm), LEM 41 *Burkholderia cenocepacia* (12.3 mm), LEM 36 *Stenotrophomonas maltophilia* (11.6 mm), and LEM 50 *R. pickettiI* (11.3 mm). Only three isolates were classified as low EPS producers, LEM 14 *Ralstonia pickettii*, LEM 15 *V. pantothenticus* and LEM 16 Unrequited (group e).

Table 1.EPS production halo (mm) by rhizobacteria.

Rhizobacteria	Exopolysaccharide (EPS) production (28 °C)		
	Low EPS production ≤ 10 mm Ø halo	Middle EPS production 10 - 14 mm Ø halo	Optimum EPS production > 14 mm Ø halo
LEM 01 *Ralstonia pickettii*		13 c	
LEM 05 Unrequited		13.6 c	
LEM 07 Unrequited			18 a
LEM 10 *Ralstonia pickettii*		12.3 d	
LEM 11 *Paenibacillus validus*		13.3 c	
LEM 12 *Stenotrophomonas maltophilia*		13.3 c	
LEM 13 *Staphylococcus kloosii*		13.6 c	
LEM 14 *Ralstonia pickettii*	8.3 e		
LEM 15 *Virgibacillus pantothenticus*	9.6 e		
LEM 16 Unrequited	9.6 e		
LEM 17 *Stenotrophomonas maltophilia*			15.3 b
LEM 18 *Ralstonia pickettii*		13 c	
LEM 19 *Virgibacillus pantothenticus*			14.6 b
LEM 24 *Burkholderia cenocepacia*			15.3 b
LEM 27 *Bacillus cereus GCsubgroupB*			15 b
LEM 29 *Bacillus cereus GCsubgroupB*			15 b
LEM 33 Unrequited			16.6 a
LEM 34 *Stenotrophomonas maltophilia*		13.3 c	
LEM 36 *Stenotrophomonas maltophilia*		11.6 d	
LEM 41 *Burkholderia cenocepacia*		12.3 d	
LEM 50 *Ralstonia pickettii*		11.3 d	

Data represent the mean of triplicates. Values followed by the same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05).

3.2.2. Biofilm formation assay

At a temperature of 28 °C, eight rhizobacterial isolates were able to form biofilm. Six isolates showed intense biofilm formation (+++), among them the isolates LEM 18 *R. pickettii* e (4.15 ± 1.21) and LEM 17 *S. maltophilia* (3.9 ± 0.13) were statistically superior. The other four isolates that exhibited intense biofilm production were LEM 27 *B. cereus* GCsubgroupB (3.51 ± 0.02), LEM 11 *P. validus* (3.48 ± 0.83), LEM 13 *S. kloosii* (3.34 ± 0.04), and LEM 14 *R. pickettii* (3.31 ± 0.06) (group b by Scott-Knott test). The isolates that showed low biofilm formation (+) were LEM 36 *S. maltophilia* (0.66 ± 0.09) and LEM 05 Unrequited (0.59 ± 0.14). Thirteen isolates were unable to form biofilms under the specified conditions (Figure 4).

At a temperature of 37 °C, five isolates that were not capable of forming biofilm at 28 °C showed this ability, they were: LEM 01 *R. pickettii*, LEM 12 *S. maltophilia*, LEM 15 *V. pantothenticus*, LEM 16 Unrequited and LEM 19 *V. pantothenticus*. These isolates, at a

temperature of 37 °C, were classified as moderate biofilm formers (++) (Figure 4). Two isolates, LEM 05 Unrequited and LEM 36 *S. maltophilia* exhibited increased biofilm formation with increasing temperature. At 28 °C they were classified as weak biofilm formers and at 37 °C they began to exhibit intense production. At a temperature of 37 °C, 13 rhizobacterial isolates produced biofilm, 9 of which were considered intense formers (+++), LEM 05 Unrequited, LEM 11 *P. validus*, LEM 13 *S. kloosii*, LEM 14 *R. pickettii*, LEM 16 Unrequited, LEM 17 *S. maltophilia*, LEM 18 *R. pickettii*, LEM 27 *B. cereus* GCsubgroupB and LEM 36 *S. maltophilia*. The LEM 13 *S. kloosii* isolate was significantly superior in biofilm formation (4.95 ± 1.20). Four isolated classified as moderate trainers (++). Eight rhizobacterial isolates were incapable of forming biofilm at both 28 °C and 37 °C. The isolates that exhibited intense biofilm production at 28 °C maintained the same characteristics at 37 °C. Results below < 0.43 were not considered biofilm forming at 28 °C and below < 0.23 at 37 °C according to the methodology proposed by Christensen *et al.* (1985).

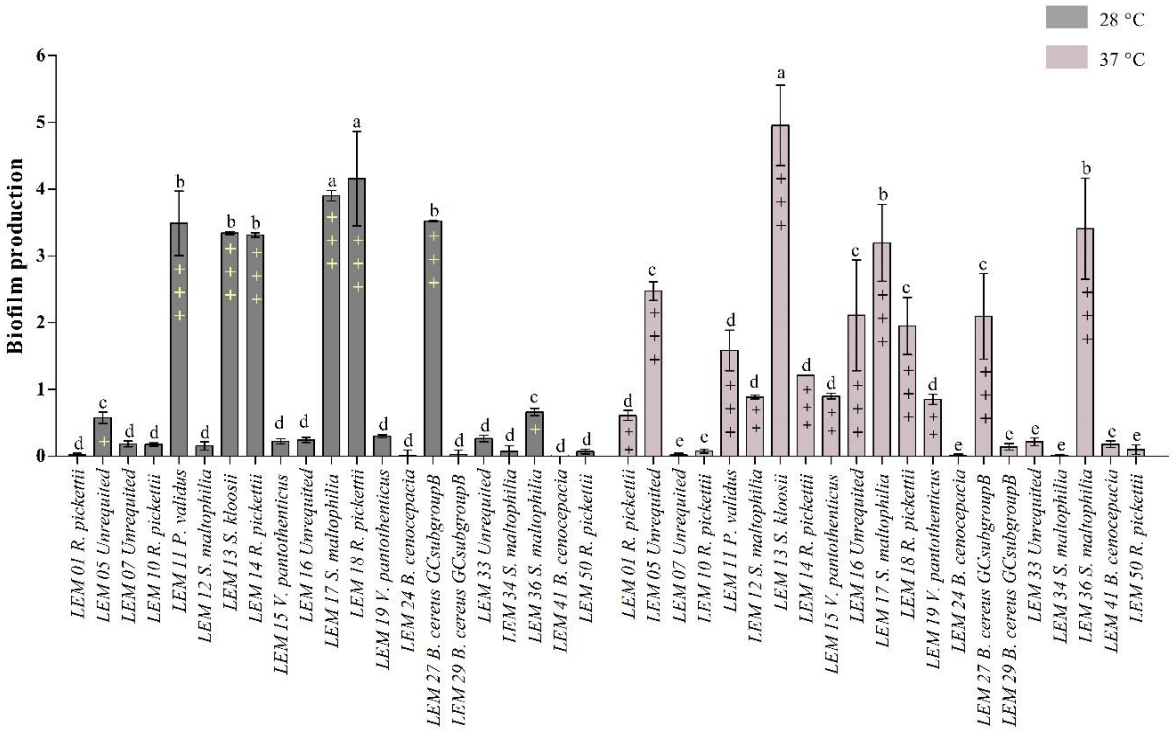

Figure 4. Biofilm production by rhizobacteria. Signals + indicate weak biofilm formation, ++ moderate biofilm formation, +++ strong biofilm formation e a ausência de sinais indica a não formação de biofilme. Data represent the mean of quadruplicates. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05).

3.2.3. N fixation in NFb and JNFb media

The ability to fix atmospheric nitrogen was confirmed in all 21 isolates cultivated in the semi-solid NFb and JNFb media (Figure 5). Twenty isolates were capable of growing and producing a film near the surface of the NFb medium, demonstrating a positive result for biological nitrogen fixation. Regarding the JNFb medium, sixteen isolates showed positive results for biological nitrogen fixation, with fifteen of them also being positive in the NFb medium. Isolates LEM 10 *R. pickettii*, LEM 11 *P. validus*, LEM 13 *S. kloosii*, LEM 14 *R. pickettii* and LEM 18 *R. pickettii* grew and exhibited nitrogen fixation only in NFb medium, while isolate LEM 41 *B. cenocepacia* demonstrated the ability to fix nitrogen only in the JNFb medium.

Figure 5. Isolates assessed for their nitrogen-fixing capability in nitrogen-free semi-solid media NFb and JNFb.

3.2.4. Phosphate solubilization

Of the twenty-one rhizobacterial isolates cultivated in the NBRIP medium, 14 (66.7 %) exhibited the ability to solubilize calcium phosphate ($Ca_3(PO_4)$) (Figure 6A). The phosphate solubilizers showed psi values ranging from 1.10 to 2.46 (Figure 6B). Isolates LEM 11 *P. validus* and LEM 36 *S. maltophilia* were statistically determined to be the most efficient, presenting psi values ranging from 2 to 2.46 (Figure 6A). Only seven isolates did not demonstrate the capability to solubilize phosphate in the NBRIP medium. However, when cultivated in different culture media such as Pikovskaya, the phosphate solubilization ability was observed in all 21 isolates (Figure 7). Therefore, for our isolates, Pikovskaya was the best medium for P solubilisation. The NBRIPY medium enabled the identification of 12 phosphate solubilizer isolates (Figure 7).

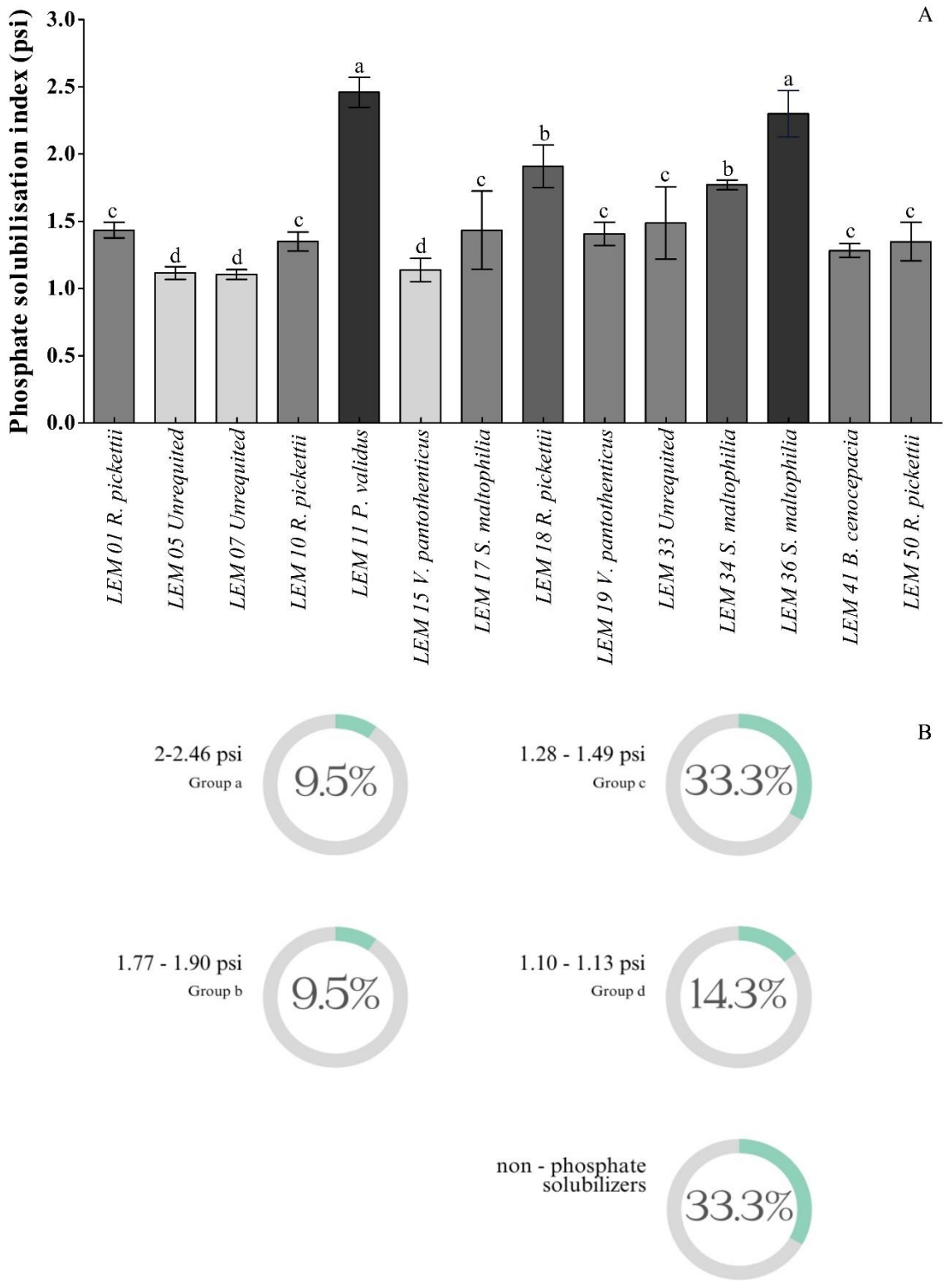

Figure 6. Phosphate solubilization by rhizobacteria in the NBRIP medium. (A) Phosphate solubilization index (psi) of rhizobacterial isolates. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05). (B) percentage of isolates with minimum and maximum psi values.

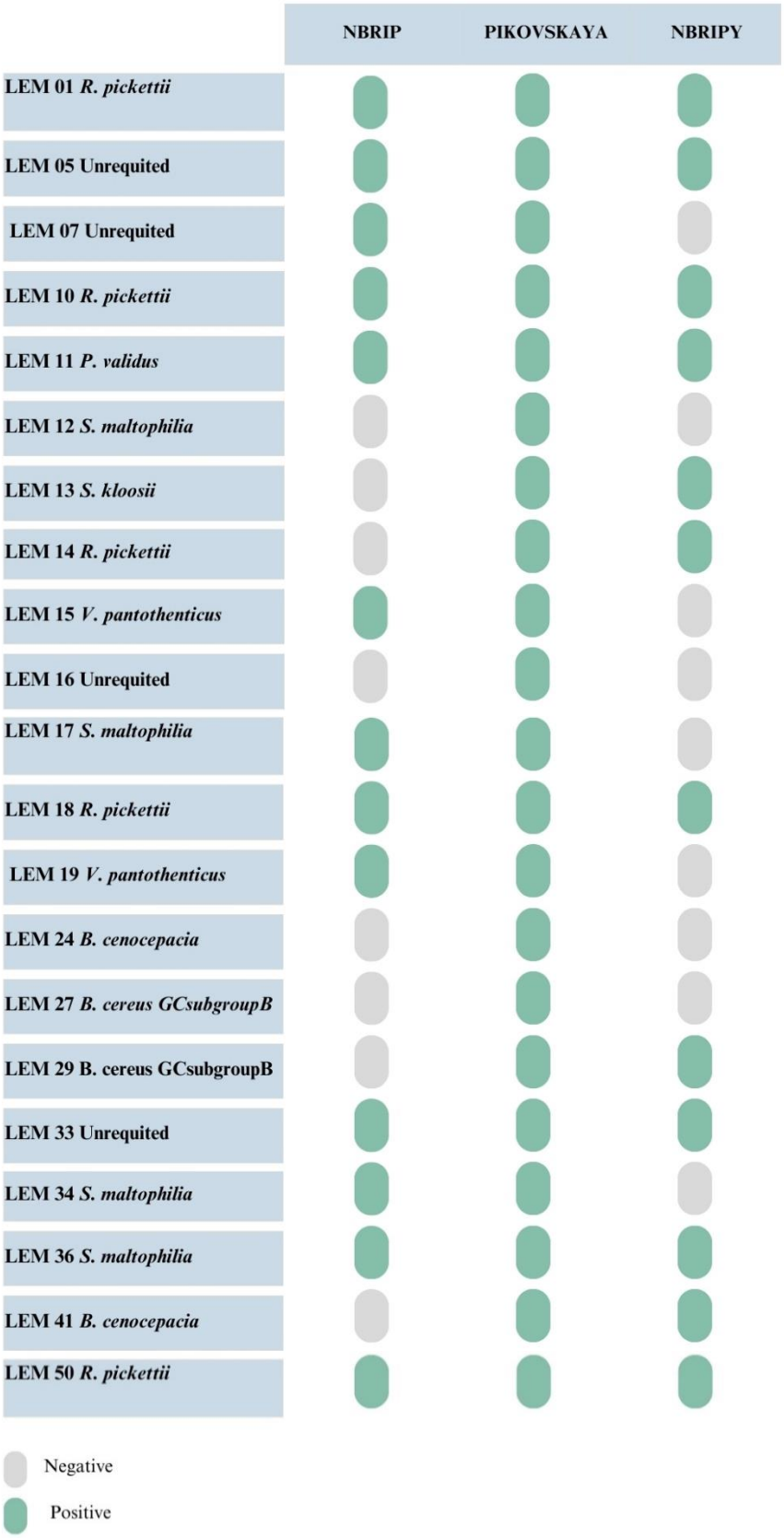

Figure 7. Phosphate solubilisation in different media, NBRIP, Pikovskaya and NBRIPY.

3.2.5. Indole-3-acetic acid (IAA) production

All 21 dizatotrophic rhizobacteria were able to produce IAA at concentrations greater than 0 μg mL^{-1} in TSB liquid medium plus 5 mM L-tryptophan (Figure 8). The LEM 14 *R. pickettii* isolate was significantly superior in IAA production, yielding 68.87 μg mL^{-1}. Subsequently, seven isolates produced substantial amounts of IAA. These isolates included LEM 01 *R. pickettii* (40.69 μg mL-1), LEM 13 *S. kloosii* (39.27 μg mL-1), LEM 07 Unrequited (36.55 μg mL-1), LEM 41 *B. cenocepacia* (36.09 μg mL^{-1}), LEM 33 Unrequited (36.09 μg mL^{-1}), LEM 24 *B. cenocepacia* (35.28 μg mL^{-1}), and LEM 34 *S. maltophilia* (35.08 μg mL^{-1}) belonging to group b by the scott-knott test ($p<0.05$). The AIA production of four isolates, namely LEM 10 *R. pickettii*, LEM 15 *V. pantothenticus*, LEM 11 *P. validus*, and LEM 17 *S. maltophilia*, ranging from 28.31 to 31.75 μg mL^{-1} (group c). Other four isolates, namely LEM 36 *S. maltophilia*, LEM 29 *B. cereus* GCsubgroupB, LEM 18 *R. pickettii*, and LEM 50 *R. pickettii*, produced IAA levels ranging from 24.37 to 25.23 μg mL^{-1} (group d). The isolates LEM 27 *B. cereus* GCsubgroupB, LEM 19 *V. pantothenticus*, and LEM 12 *S. maltophilia* demonstrated IAA concentrations ranging from 18.36 to 21.50 μg mL^{-1} (group e). Isolates LEM 16 Unrequited and LEM 05 Unrequited exhibited the lowest levels of IAA production, specifically 7.45 and 8.24 μg mL^{-1}, respectively (group f). The control treatment consisted of culture medium supplemented with 5 mM L-tryptophan, absent bacterial cells, showing no IAA production (group g).

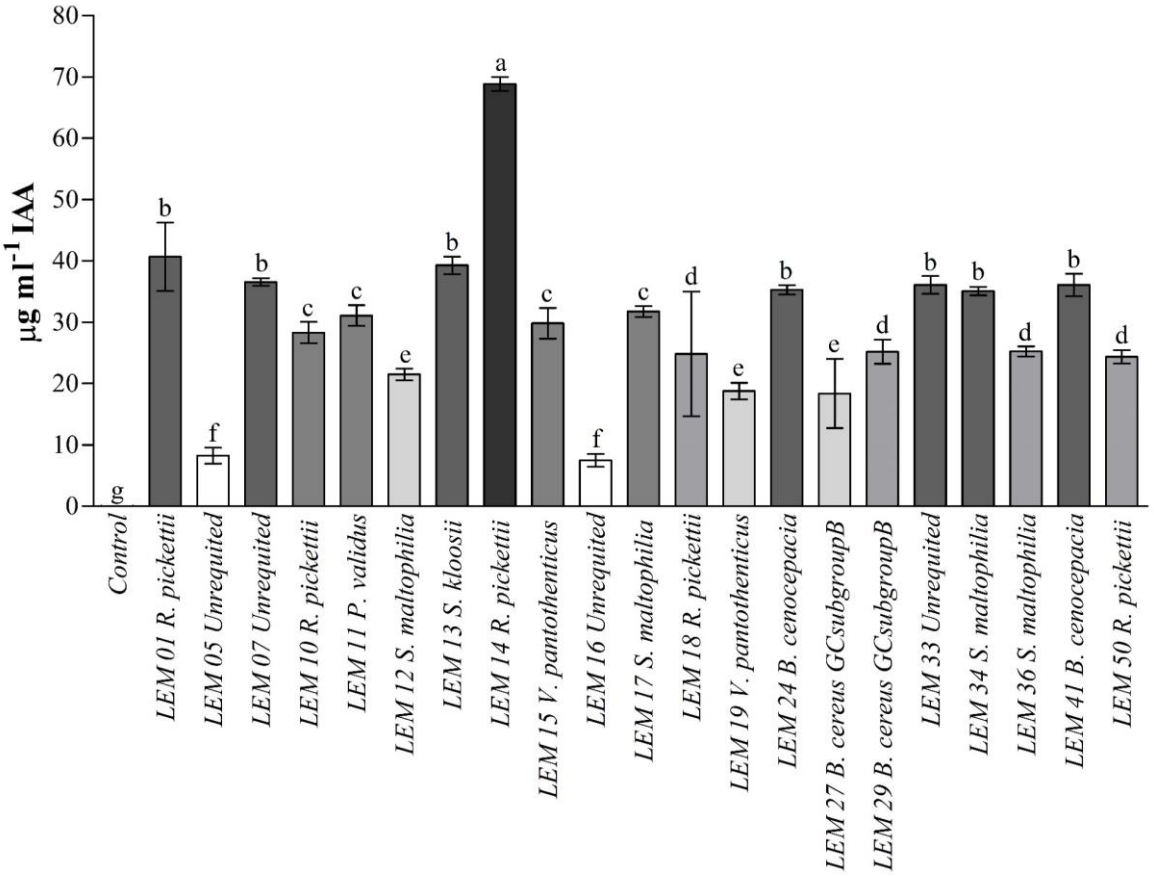

Figure 8. Production of IAA by rhizobacteria. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05).

3.3. Indirect mechanisms for drought tolerance

3.3.1. Ammonia (NH_3) production assay

The results were classified into six distinct groups, according to scott-knott (p<0.05). The highest levels of NH_3 production were observed by isolates LEM 34 *S. maltophilia* (44.37 µmol mL^{-1}), LEM 41 *B. cenocepacia* (44.30 µmol mL^{-1}), LEM 01 *R. pickettii* (42.27 µmol mL^{-1}), and LEM 36 *S. maltophilia* (39.23 µmol mL^{-1}) (Figure 9). Only one isolate was categorized into group b, the LEM 15 *V. pantothenticus*, which produced 36.08 µmol mL^{-1} of NH_3. Six isolates, namely LEM 12 *S. maltophilia*, LEM 19 *V. pantothenticus*, LEM 13 *S. kloosii*, LEM 16 Unrequited, LEM 11 *P. validus*, and LEM 07 Unrequited, were categorized into group c, with NH_3 production ranging from 29.70 to 31.82 µmol mL^{-1}. Isolates LEM 17 *S. maltophilia*, LEM 24 *B. cenocepacia*, LEM 50 *R. pickettii*, LEM 27 *B. cereus* GCsubgroupB, LEM 18 *R. pickettii*, LEM 10 *R. pickettii*, LEM 33 Unrequited, and LEM 29 *B. cereus* GCsubgroupB produced NH_3 amounts ranging from 18.38 to 24.66 µmol mL^{-1}, and were assigned to group d. The lowest NH_3 productions were observed by the isolates, LEM 05 Unrequited and LEM 14

R. pickettii, 13.85 and 12.49 μmol mL^{-1}, respectively (group e). The control treatment (C), consisted only of the culture medium without inoculum, representing a non-NH_3 producer (7.85 μmol mL^{-1}) (group f).

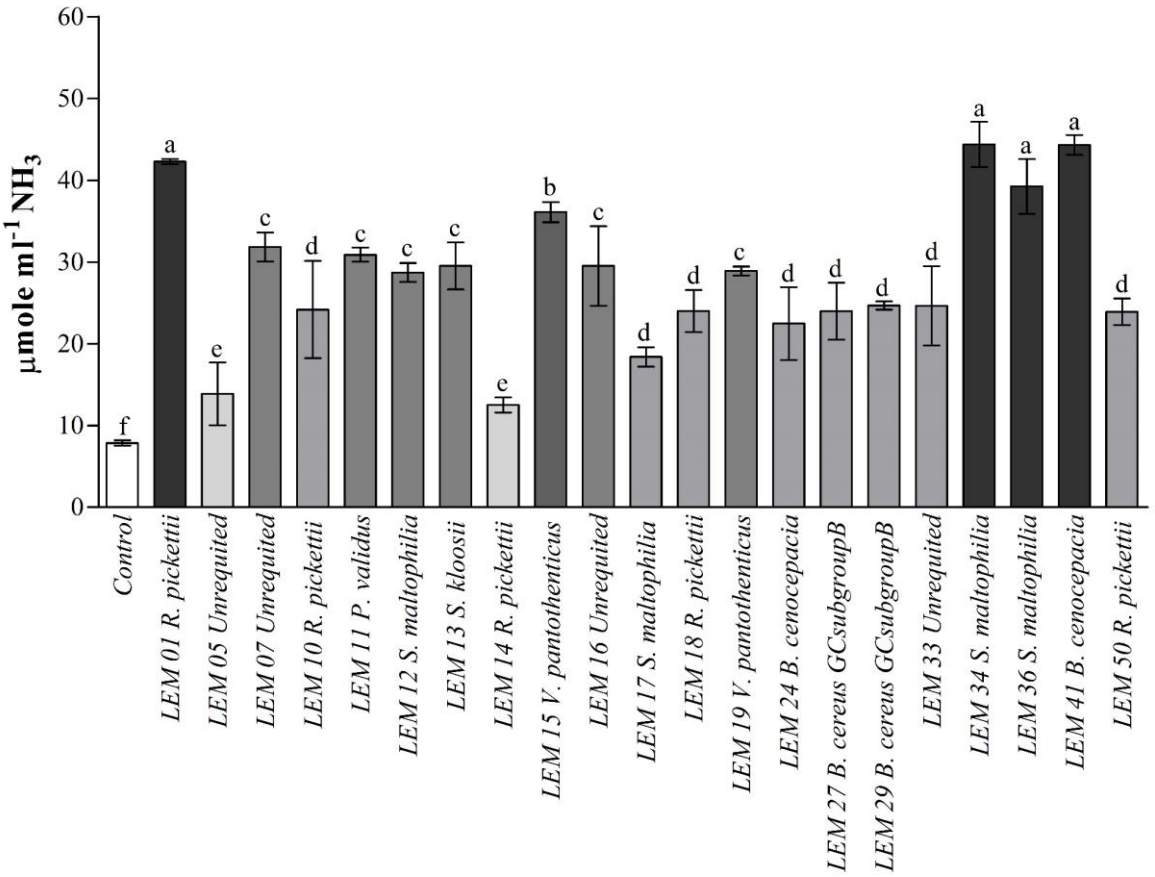

Figure 9. Production of NH_3 by rhizobacteria. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05).

3.3.2. Siderophore Production

The results obtained showed that of the 21 diazotrophic rhizobacteria, 17 presented positive results for siderophores in the qualitative assay on CAS-agar medium (Table 2). The yellowish halo surrounding the colonies on CAS-agar plates indicates siderophore production. Quantitative evaluation revealed that all 21 isolates produced siderophores, with 13 rhizobacterial isolates exhibiting significantly higher siderophore values in the range of 36.03 % - 50.92 % (psu), and eight producing siderophores in the range of 25.75 % - 34.22 % (psu) (Figure 10).

Table 2. Production of siderophore in CAS – agar medium, cellulase, chitinase and hydrogen cyanide (HCN) by rhizobacteria.

Rhizobacteria	Siderophore CAS - agar	CI	CHI	HCN
LEM 01 *Ralstonia pickettii*	+	(2.38 ± 0.15) b	-	-
LEM 05 Unrequited	+	-	-	-
LEM 07 Unrequited	+	(4.06 ± 0.20) a	(1.27 ± 0.16) b	-
LEM 10 *Ralstonia pickettii*	+	(4.19 ± 0.55) a	-	-
LEM 11 *Paenibacillus validus*	+	(3.51 ± 0.46) a	-	-
LEM 12 *Stenotrophomonas maltophilia*	-	-	(1.17 ± 0.04) b	-
LEM 13 *Staphylococcus kloosii*	+	-	-	-
LEM 14 *Ralstonia pickettii*	+	(2.57 ± 0.08) b	-	-
LEM 15 *Virgibacillus pantothenticus*	+	(2.93 ± 0.11) b	-	-
LEM 16 Unrequited	-	-	-	-
LEM 17 *Stenotrophomonas maltophilia*	-	-	-	-
LEM 18 *Ralstonia pickettii*	+	-	(1.54 ± 0.13) a	-
LEM 19 *Virgibacillus pantothenticus*	+	-	-	-
LEM 24 *Burkholderia cenocepacia*	-	-	(1.18 ± 0.08) b	-
LEM 27 *Bacillus cereus GCsubgroupB*	+	-	-	-
LEM 29 *Bacillus cereus GCsubgroupB*	+	(4.06 ± 0.46) a	(1.24 ± 0.09) b	-
LEM 33 Unrequited	+	(4.28 ± 0.64) a	(1.21 ± 0.10) b	-
LEM 34 *Stenotrophomonas maltophilia*	+	(4.24 ± 0.15) a	(1.17 ± 0.04) b	-
LEM 36 *Stenotrophomonas maltophilia*	+	-	(1.28 ± 0.05) b	-
LEM 41 *Burkholderia cenocepacia*	+	(3.93 ± 0.52) a	(1.26 ± 0.18) b	-
LEM 50 *Ralstonia pickettii*	+	(4.54 ± 0.69) a	(1.23 ± 0.08) b	-

Siderophore production; Cellulolytic Index (CI); Chitinolytic Index (CHI) e Hydrogen Cyanide (HCN). The + symbols indicate positive results, while – indicate negative results. Data represents the mean of triplicates. Values followed by the same lowercase letter belong to the same group according to the Scott-Knott test (p<0.05).

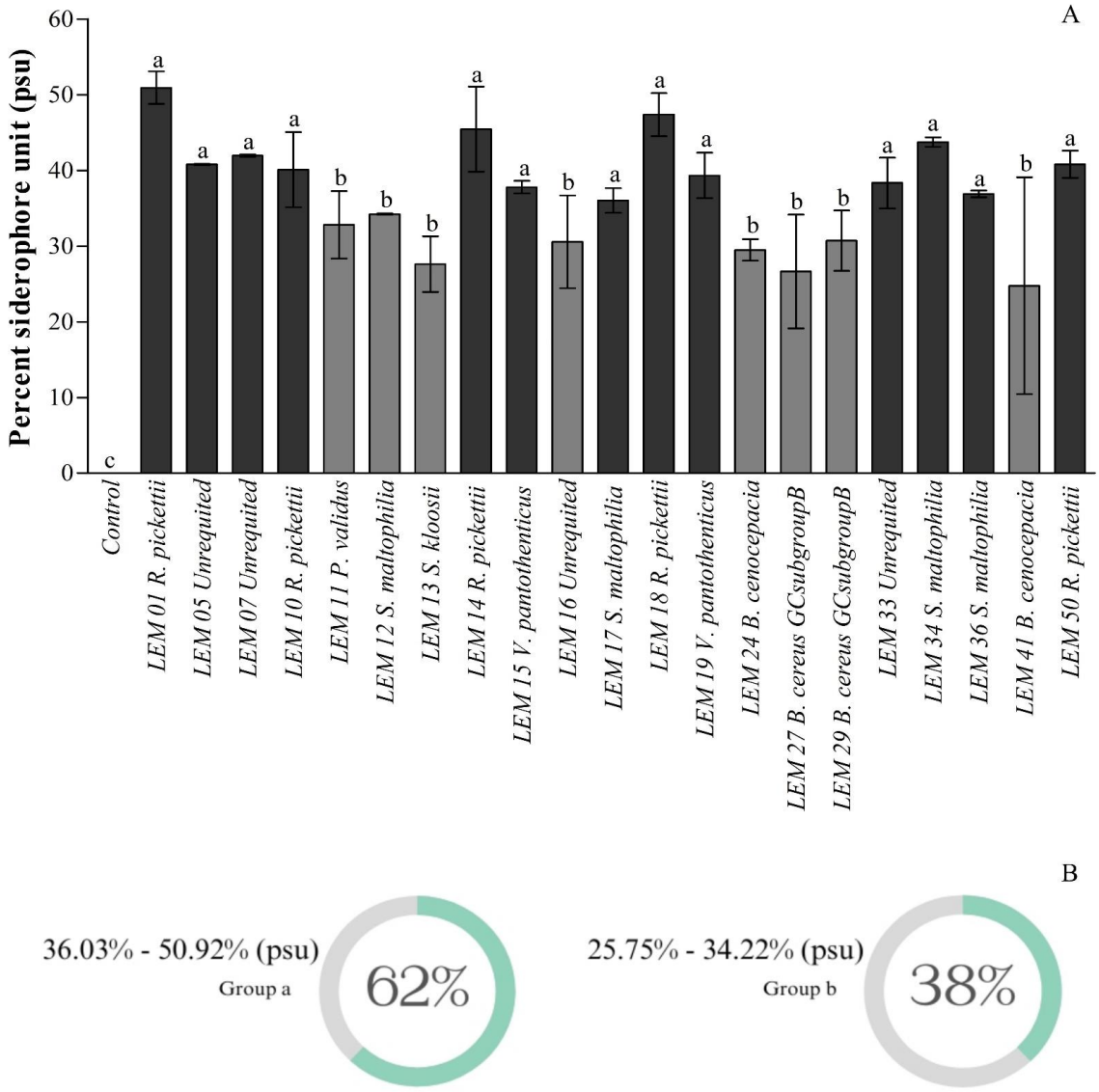

Figure 10. Siderophore production by rhizobacterial isolates. (A) Bar graph demonstrating siderophore production in percent siderophore unit (psu). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05). (B) Percentage of isolates at their respective minimum and maximum of psu.

3.3.3. Cellulase production

Under the conditions evaluated, 11 rhizobacteria exhibited a distinct halo on CMC agar medium, indicating cellulose degradation and, therefore, sintering the cellulase enzyme. Eight rhizobacteria demonstrated significantly higher cellulolytic index (CI) values and were categorized as group a based on the Scott-Knott (p<0.05). The highest IC values were observed by isolates LEM 50 *R. pickettii* (4.54 ± 0.69), followed by LEM 33 Unrequited (4.28 ± 0.64), LEM 34 *S. maltophilia* (4.28 ± 0.64), LEM 10 *R. pickettii* (4.28 ± 0.64), LEM 07 Unrequited (4.06 ± 0.20), LEM 29 *B. Cereus* GCsubgroupB (4.06 ± 0.46), LEM 41 *B. cenocepacia* (3.93 ± 0.52), and LEM 11 *P. validus* (3.51 ± 0.46) (Table 2). Only three rhizobacterial isolates

exhibited a lower CI: LEM 15 *V. pantothenticus* (2.93 ± 0.11), LEM 14 *R. pickettii* (2.57 ± 0.08), and LEM 01 *R. pickettii* (2.38 ± 0.15) (group b). Ten rhizobacterial isolates (LEM 05 Unrequited, LEM 12 *S. maltophilia*, LEM 13 *S. kloosii*, LEM 16 Unrequited, LEM *17 S. maltophilia*, LEM 18 *R. pickettii*, LEM 19 *V. pantothenticus*, LEM 24 *B. cenocepacia*, LEM 27 *B. cereus* GCsubgroupB, and LEM 36 *S. maltophilia*) were not able to synthesize the cellulase enzyme, showing no halos of cellulose degradation (Figure 11). Furthermore, the results demonstrated that isolates LEM 01 *R. pickettii*, LEM 10 *R. pickettii*, LEM 11 *P. validus*, LEM 14 *R. pickettii*, LEM 15 *V. pantothenticus* and LEM 41 *B. cenocepacia* shared the ability to exert both indirect mechanisms of plant growth promotion, siderophore production and cellulase synthesis (Figure 12).

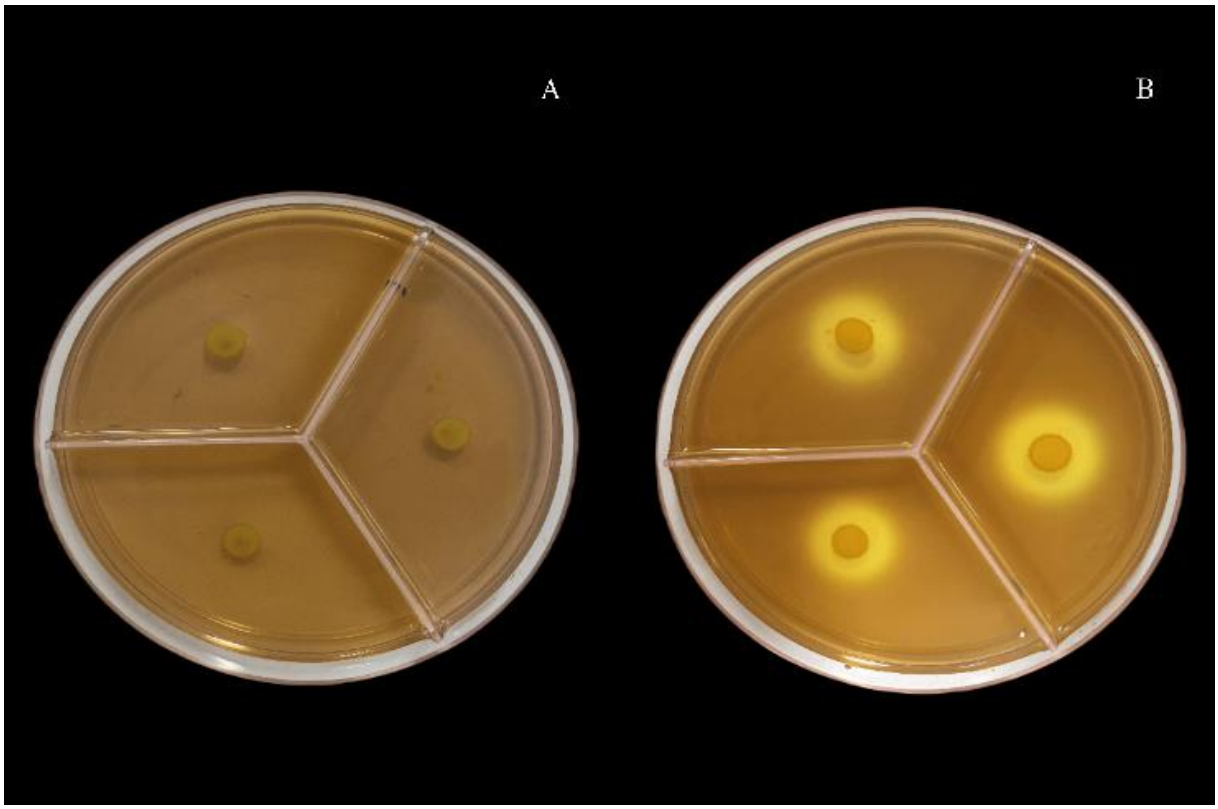

Figure 11. Cellulase production test. Isolate LEM 17 S. *maltophilia* do not produce the enzyme, and therefore is not able to degrade cellulose (A) and isolate LEM 01 *R. pickettii* produces the cellulase enzyme, and therefore forms a cellulose degradation halo (B).

3.3.4. Chitinase production

Chitin degradation was observed through the formation of halo by 10 isolates, which were considered positive for production of the chitinase enzyme (Table 2). The LEM 18 *R. pickettii* isolate presented significantly higher chitinolytic index (CHI) values (1.54 ± 0.13). The nine isolates that were also capable of degrading chitin, but presenting lower chitinolytic index values were: LEM 36 *S. maltophilia* (1.28 ± 0.05), LEM 07 Unrequited (1.27 ± 0.16), LEM 41 *B. cenocepacia* (1.26 ± 0.18), LEM 29 *B. cereus* GCsubgroupB (1.24 ± 0.09), LEM 50 *R. pickettii* (1.23 ± 0.08), LEM 33 Unrequited (1.21 ± 0.10), LEM 24 *B. cenocepacia* (1.18 ± 0.08) LEM 34 *S. maltophilia* (1.17 ± 0.04) and LEM 12 *S. maltophilia* (1.17 ± 0.04). Six rhizobacterial isolates demonstrated the ability to execute the three indirect mechanisms of plant growth promotion, LEM 07 Unrequited, LEM 29 *B. cereus* GCsubgroupB, LEM 33 Unrequited, LEM 34 *S. maltophilia*, LEM 41 *B. cenocepacia* and LEM 50 *R. pickettii* (Figure 12).

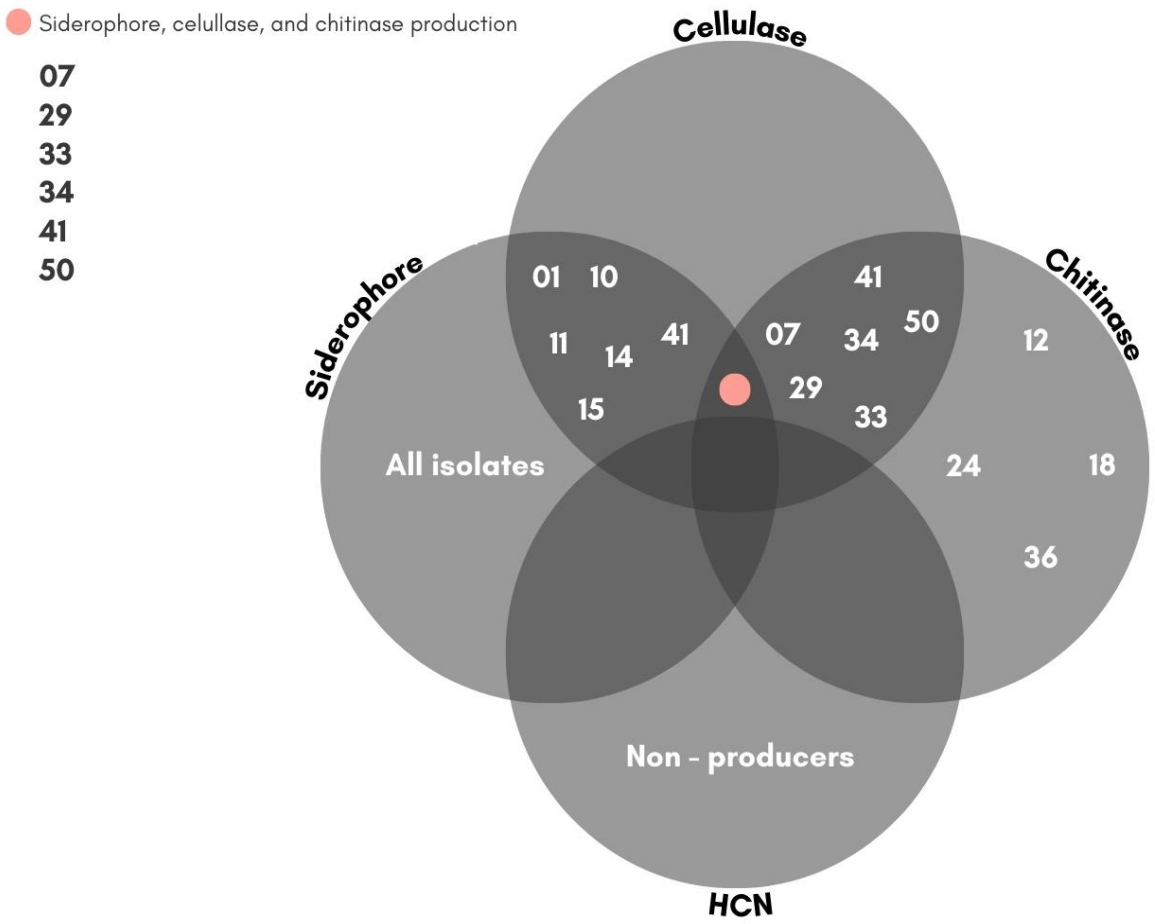

Figure 12. Venn diagram of the production of siderophores, cellulase, chitinase and HCN. Numbers represent rhizobacterial isolates and isolated intersections that exerted more than one indirect plant-promoting mechanism.

3.3.5. Hydrogen cyanide production (HCN)

HCN production was assessed using TSA culture medium supplemented with glycine (4.4 g L^{-1}). The isolates were then incubated in a B.O.D. incubator at 28°C for a duration of 5 days. HCN production was determined by observing a color change on a filter paper soaked with picric acid (0.5 %) in Na_2CO_3 (2 %), where the color transitions from yellow to brown or orange. The results revealed no color change was observed on the filter papers. Thus, it was concluded that no isolates could produce HCN under the given experimental conditions (Table 2). Figure 12 demonstrates the ability of the isolates to perform more than one indirect plant growth-promoting mechanism. Although no isolate showed HCN production, some isolates demonstrated action in more than one indirect mechanism for promoting plant growth, which may be efficient in inhibiting phytopathogens.

3.3.6. Antagonisms assays

3.3.6.1. Antagonism by direct confrontation

The antagonism test carried out using the dual culture technique showed that 17 rhizobacterial isolates were capable of inhibiting the growth of *C. fimbriata* LPF 1594 at some level (percentage of growth area). The grouping carried out by Scott-Knott provided the division of four groups (Figures 13 and 14). Five isolates were significantly superior in inhibiting *C. fimbriata* LPF 1594, namely: LEM 29 *B. cereus* GCsubgroupB, LEM 27 *B. cereus* GCsubgroupB, LEM 01 *R. pickettii*, LEM 07 Unrequited and LEM 10 *R. pickettii* and were grouped in group a (Scott – Knott). Other rhizobacterial isolates such as LEM 11 *P. validus*, LEM 12 *S. maltophilia*, LEM 13 *S. kloosii*, LEM 15 *V. pantothenticus*, LEM 33 Unrequited, LEM 34 *S. maltophilia* and LEM 36 *S. maltophilia* were also efficient in inhibition (group b), as well as isolates LEM 05 Unrequited, LEM 17 *S. maltophilia*, LEM 41 *B. cenocepacia*, LEM 14 *R. pickettii* and LEM 50 *R. pickettii*. The control treatment was categorized in group d, along with the isolates LEM 24 *B. cenocepacia*, LEM 18 *R. pickettii*, LEM 19 *V. pantotheticus* and LEM 16 Unrequited. However, there were no isolates capable of inhibiting the growth of the fungus *C. variabilis* LPF 125 (Figure 13). Statistical analyzes support the visual observation, showing no significant differences between the means of the percentage of growth area evaluated by the QUANT software (Figure 14).

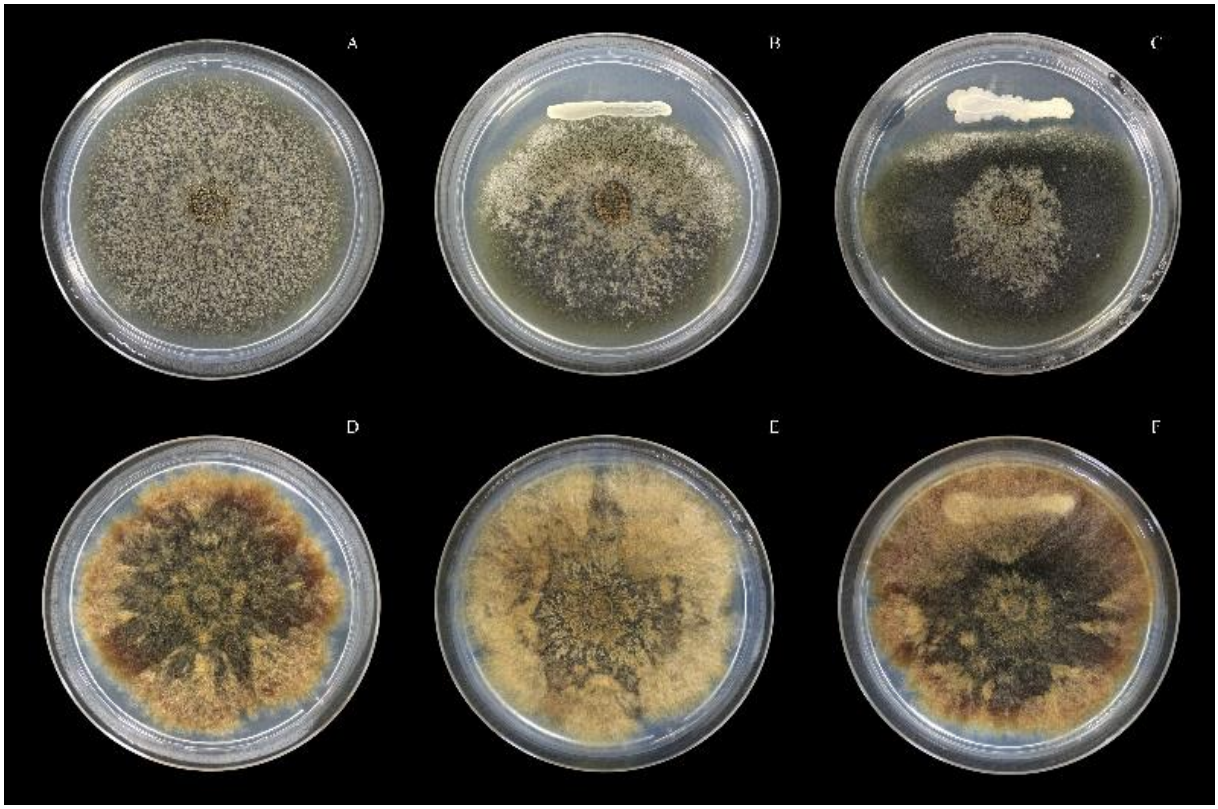

Figure 13. Dual culture antagonism test. (A) Control, only growth of the fungus *C. fimbriata* LPF 1594. (B) LEM 10 *R. pickettii* vs *C. fimbriata*. (C) LEM 29 *B. cereus* GCsubgroupB vs *C. fimbriata*. (D) Control, only growth of the fungus *C. variabilis* LPF 125. (E) LEM 10 *R. pickettii* vs *C. variabilis* (F) LEM 29 *B. cereus* GCsubgroupB vs *C. variabilis*.

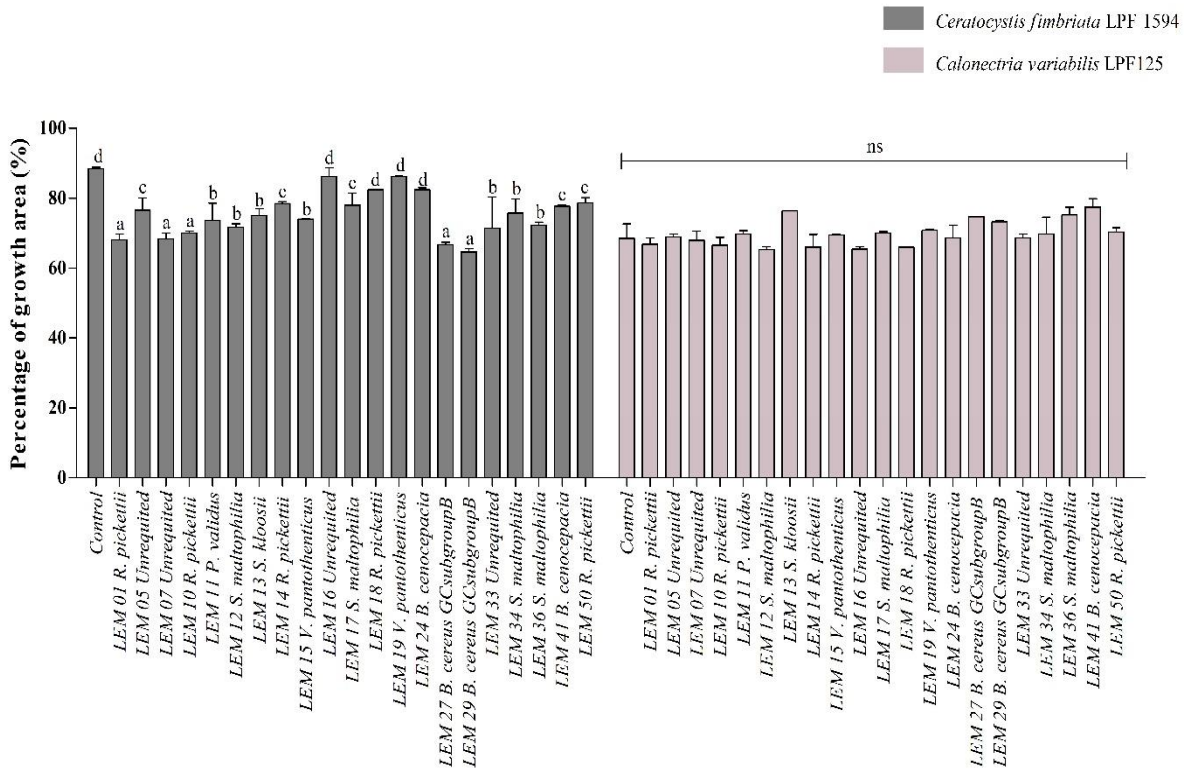

Figure 14. Bar graph demonstrating the inhibition of fungal growth of *C. fimbriata* and *C. variabilis* in dual culture. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05), ns: not significant by the analysis of variance (ANOVA).

3.3.6.2. Antagonism by supernatant

The percentage of growth area of *C. fimbriata* LPF 1594 and *C. variabilis* LPF 125 evaluated did not demonstrate significant reductions with the use of the supernatant of the 21 rhizobacterial isolates (Figures 15 and 16).

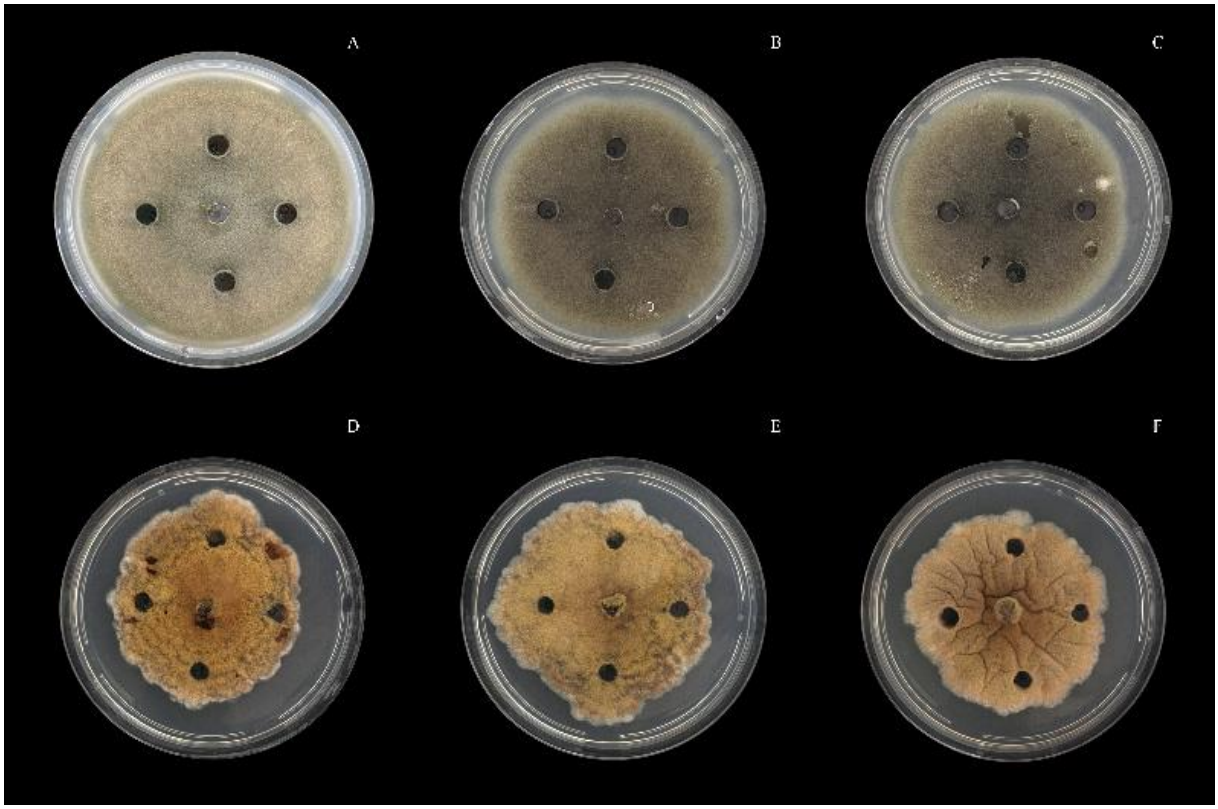

Figure 15. Antagonism test by rhizobacteria supernatant. (A) Control, only growth of the fungus *C. fimbriata* LPF 1594. (B) LEM 10 *R. pickettii* vs *C. fimbriata*. (C) LEM 29 *B. cereus* GCsubgroupB vs *C. fimbriata*. (D) Control, only growth of the fungus *C. variabilis* LPF 125. (E) LEM 10 *R. pickettii* vs C. *variabilis* (F) LEM 29 *B. cereus* GCsubgroupB vs *C. variabilis*.

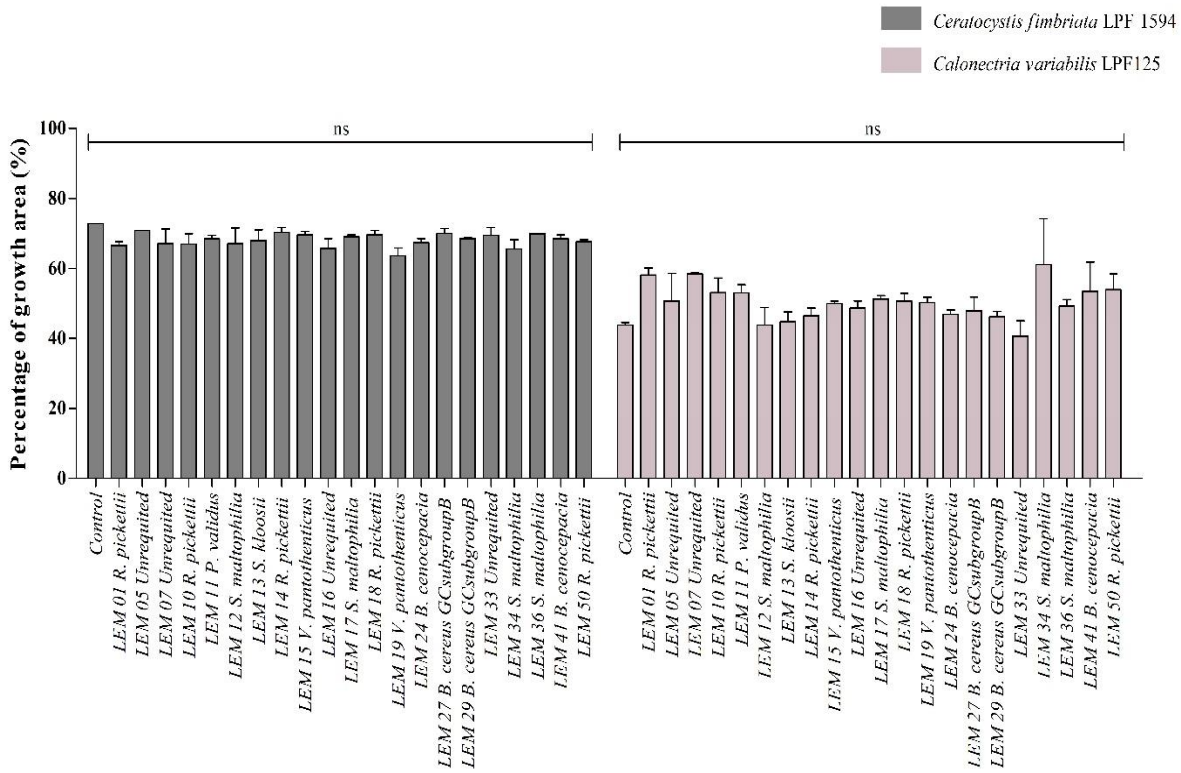

Figure 16. Bar graph demonstrating the absence of inhibition of fungal growth of *C. fimbriata* and *C. variabilis* by the rhizobacteria supernatant. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05), ns: not significant by the analysis of variance (ANOVA).

3.3.6.3. Antagonism by volatile organic compounds (VOCs)

The volatile organic compounds (VOCs) produced by the 21 rhizobacterial isolates tested were not able to reduce the fungal growth of *C. fimbriata* LPF 1594 and *C. variabilis* LPF125 evaluated (Figures 17 and 18).

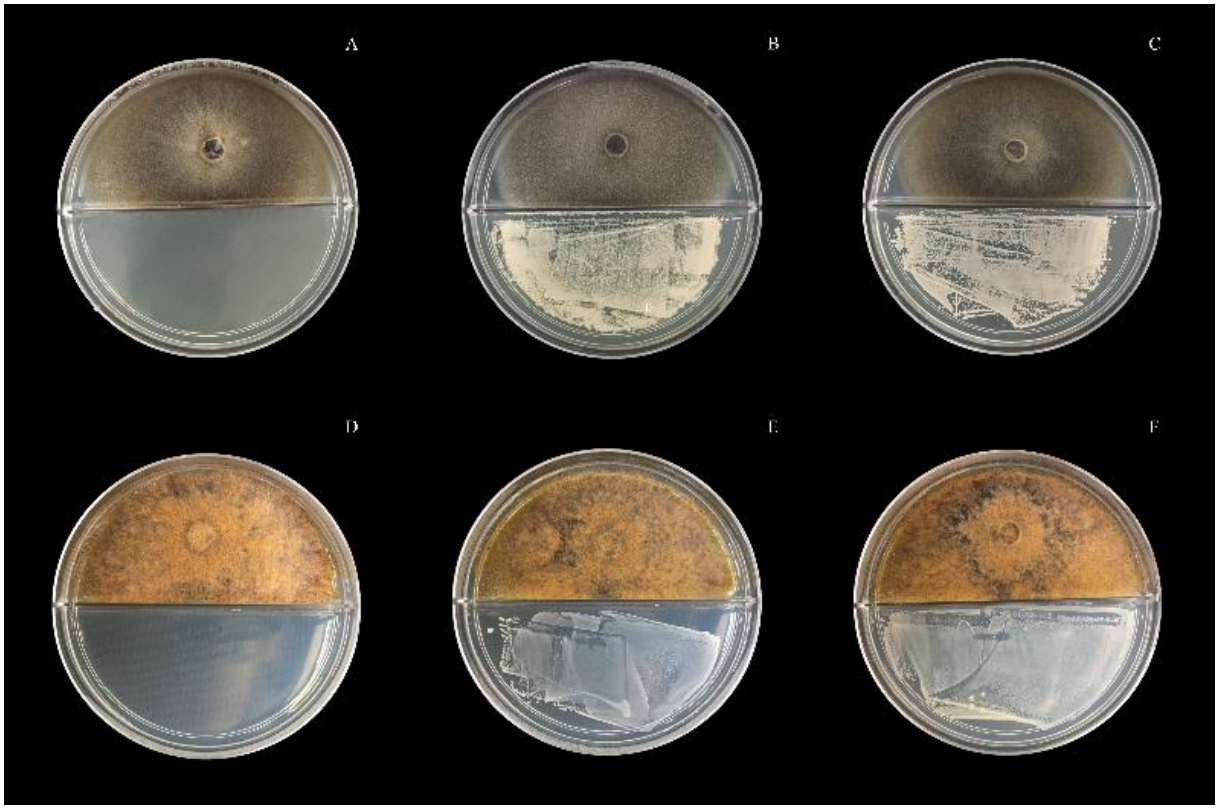

Figure 17. Antagonism test for VOCs produced by rhizobacteria. (A) Control, only growth of the fungus *C. fimbriata* LPF 1594. (B) LEM 10 *R. pickettii* vs *C. fimbriata*. (C) LEM 29 *B. cereus* GCsubgroupB vs *C. fimbriata*. (D) Control, only growth of the fungus *C. variabilis* LPF 125. (E) LEM 10 *R. pickettii* vs *C. variabilis* (F) LEM 29 *B. cereus* GCsubgroupB vs *C. variabilis*.

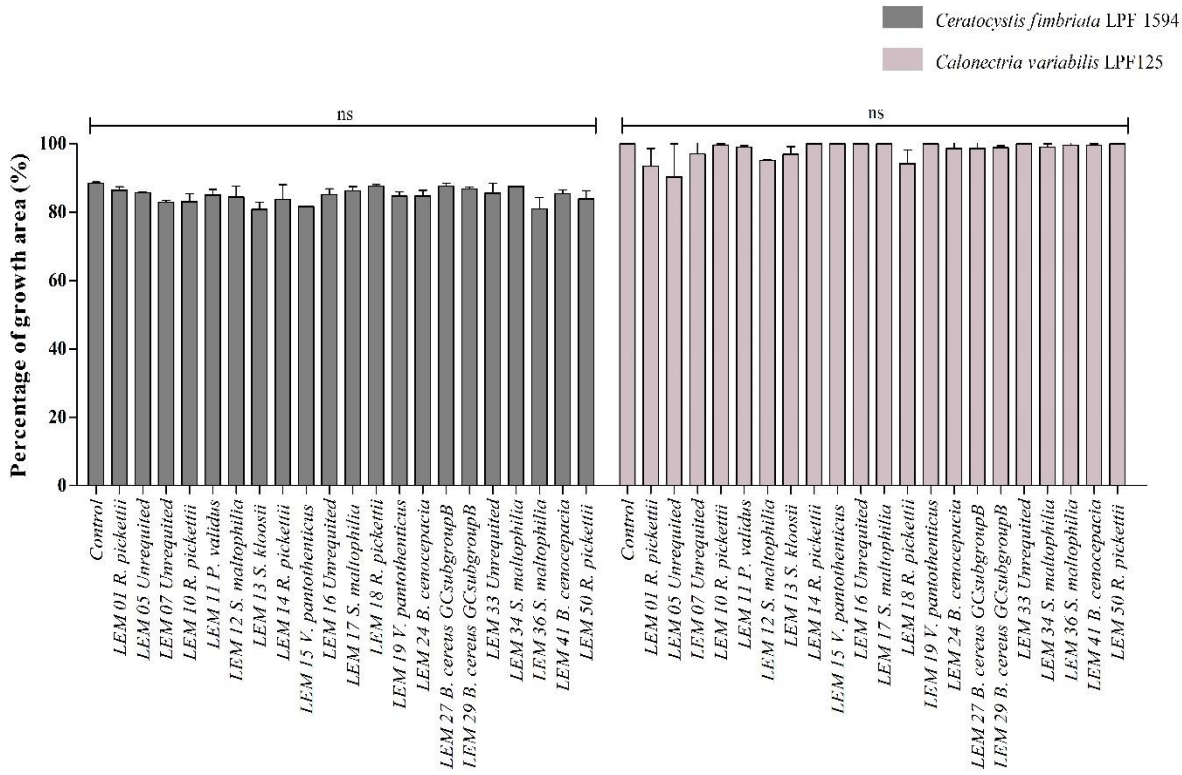

Figure 18. Bar graph demonstrating the absence of inhibition of fungal growth of *C. fimbriata* and *C. variabilis* by VOCs produced by rhizobacteria. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05), ns: not significant by the analysis of variance (ANOVA)

4. DISCUSSION

In this study, the plant growth-promoting mechanisms of 21 rhizobacteria isolated from *E. grandis* were characterized. Our results demonstrated that all rhizobacterial isolates exhibited some growth-promoting characteristics *in vitro*, which can help plants under normal conditions or under water deficit. Of the 21 rhizobacteria evaluated, 47.6 % were identified as xerotolerant, 62 % biofilm formation and all isolates (100 %) identified as halotolerant, EPS producers, diazotrophs, phosphate solubilizers and AIA producers. The four isolates LEM 01 *R. pickettii*, LEM 11 *P. validus*, LEM 17 *S. maltophilia* and LEM 19 *V. pantothenticus* presented the ability to withstand water and saline stress conditions and exert all direct mechanisms capable of assisting in tolerance to dry. However, considering the direct and indirect mechanisms, the rhizobacteria LEM 11 *P. validus*, LEM 01 *R. pickettii* and LEM 50 *R. pickettii* presented greater scope in the execution of the mechanisms, with LEM 11 *P. validus* presenting the best *in vitro* responses for the set of tests (Figure 19).

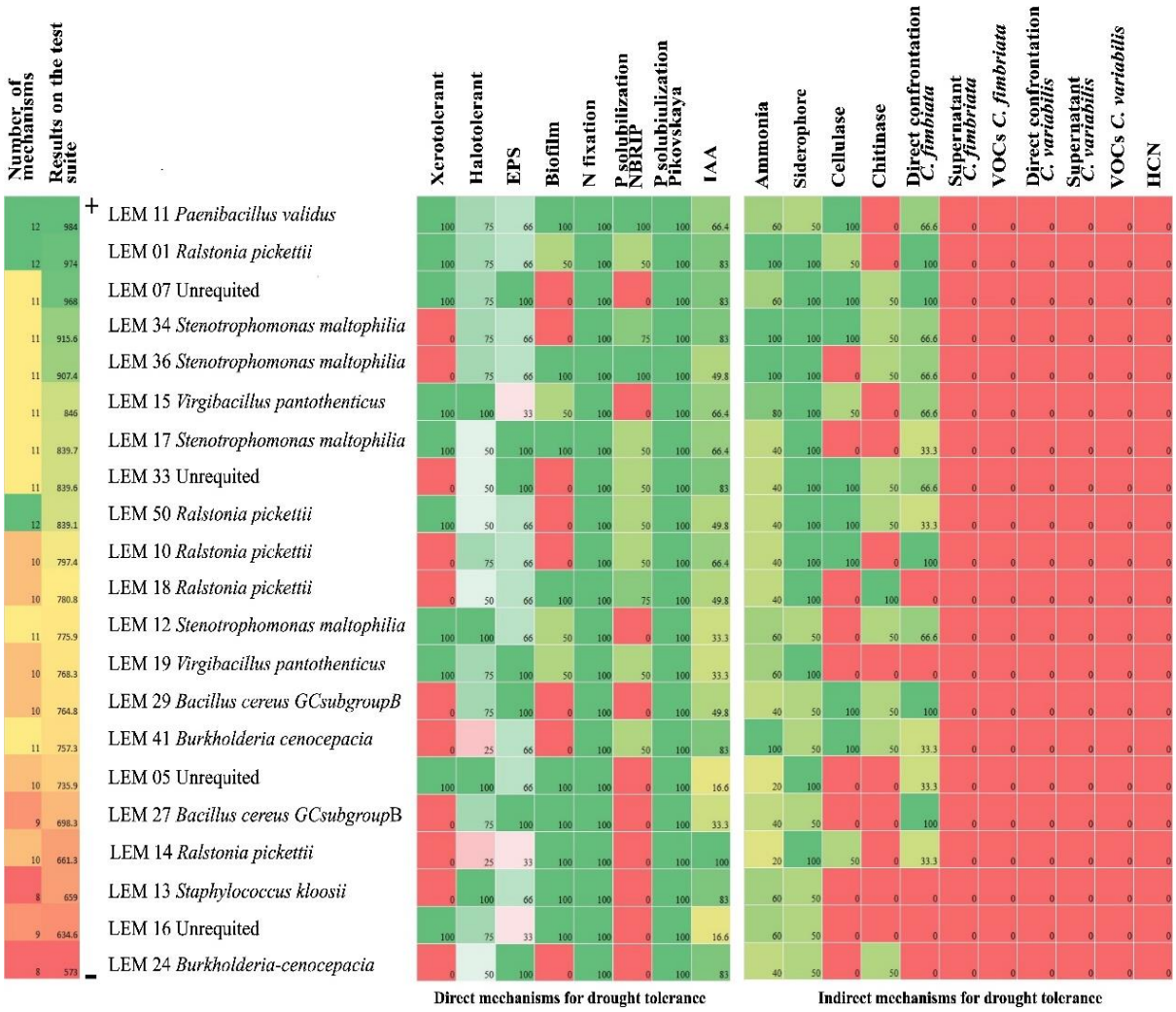

Figure 19. Heatmap of direct and indirect mechanisms for drought tolerance in plants. The ability of the microorganism to exert the mechanisms is represented by the color gradient from red to green. The strongest red indicates the absence of the mechanism (0%), while the greenest coloration indicates the full execution of the mechanism (100%).

4.1 Ability of rhizobacteria to tolerate low precipitation and high salinity environments

Of the 21 rhizobacteria evaluated, 47.6 % were identified as xerotolerant and all (100%) halotolerant. The ability to withstand conditions of low water availability and high salinity concentrations are important for the survival of these microorganisms in arid regions.

The rhizobacteria LEM 01 *R. pickettii*, LEM 05 Unrequited, LEM 11 *P. validus*, LEM 12 *S. maltophilia*, LEM 15 *V. pantothenticus*, LEM 16 Unrequited, LEM 17 *S. maltophilia* and LEM 19 *V. pantothenticus* were identified as xerotolerant and biofilm forming. LEM 05 Unrequited, LEM 11 *P. validus* and LEM 17 *S. maltophilia* were shown to be capable of forming biofilm at both temperatures of 28 °C and 37 °C. To withstand conditions of low water availability, these microorganisms likely employ physiological mechanisms such as modifications of phospholipids in the membrane to maintain fluidity, accumulation of solutes

that preserve osmotic potential, and secretion of extracellular polymeric substances (EPS) (Lebre *et al.,* 2017). Other abiotic stresses also activate many of these mechanisms, enabling xerotolerant microorganisms to survive in hypersaline environments where water availability is limited (Daniel *et al.,* 2004). This information is supported by the results obtained for halotolerance since all the isolates considered xerotolerant also grew under the highest concentrations of salinity (NaCl w/v): LEM 01 *R. pickettii* (12 %), LEM 05 Unrequited (15 %), LEM 07 Unrequited (12 %), LEM 11 *P. validus* (12 %), LEM 12 *S. maltophilia* (15 %), LEM 15 *V. pantothenticus* (15 %), LEM 16 Unrequited (12 %), LEM 17 *S. maltophilia* (8 %), LEM 19 *V. pantothenticus* (12 %), and LEM 50 *R. pickettii* (8 %). The ability of these rhizobacteria to cope with water and salt stress is necessary, given that semi-arid regions impose such conditions on microorganisms (Bonatelli *et al.,* 2021).

4.2 Direct mechanisms for drought tolerance

All 21 rhizobacteria evaluated for direct mechanisms for drought tolerance (100 %) were capable of producing EPS, fixing nitrogen, solubilizing phosphate and producing IAA. Regarding biofilm formation, this characteristic was verified by 62 % of the isolates.

Exopolysaccharide production is an essential characteristic for the formulation of inoculants as they are capable of binding soil particles, thereby altering various aspects of soil properties such as infiltration, aeration, root penetration, and surface runoff control (Latif *et al.,* 2022). Due to its hygroscopic nature, it can maintain a high water content in the rhizosphere, enabling plants to exhibit better root development, shoot growth, and overall dry biomass under water deficit conditions (Bibi *et al.,* 2021; Sati *et al.,* 2023). Furthermore, the production of EPS is essential for biofilm formation. Biofilms also enhance water retention, retaining moisture and nutrients in the rhizosphere, thereby providing plants with greater capacity to cope with water deficit conditions. They also benefit the microorganisms themselves under stressful conditions (Khan *et al.,* 2020). Hence, the ability of microorganisms to produce EPS and form biofilms is an important characteristic to assist plants in drought conditions. Biofilm formation is dynamic and complex, influenced by various biological and environmental factors such as temperature, pH, and nutrient availability, which affect cell adhesion (Alotaibi, bukhari, 2021). Our results showed that temperature was an important factor responsible for biofilm formation, isolates that did not form biofilms at 28 °C and started to form biofilm at 37 °C (LEM 01 *R. pickettii*, LEM 12 *S. maltophilia*, LEM 15 *V. pantothenticus*, LEM 16 Unrequited, and LEM 19 *V. pantothenticus*), and also contributed to an increase in biofilm quantity in isolates that already produced biofilms at 28 °C (LEM 05 Unrequited and LEM 36 S. maltophilia). The isolates

LEM 11 *P. validus*, LEM 13 *S. kloosii*, LEM 14 *R. pickettii*, LEM 17 *S. maltophilia*, LEM 18 *R. pickettii* and LEM 27 *B. cereus* GCsubgroupB maintained the same levels of biofilm formation under different temperatures. Isolates that were unable to form biofilms under the tested conditions may exhibit this mechanism under other untested laboratory or field conditions. Biofilm formation around plant roots is dependent on communication and intimate interaction between both organisms, involving polysaccharides and surface proteins. Furthermore, the interaction among bacterial populations or communities present in the extracellular polymeric matrix directly influences the adhesion capacity of bacterial cells (Danhorn, Fuqua, 2007).

All isolates cultivated in semi-solid NFb and/or JNFb medium showed the ability to fix nitrogen. Nitrogen is the most limiting nutrient for plants, as they are unable to fix atmospheric nitrogen. Therefore, diazotrophic rhizobacteria are essential for the absorption of this nutrient and consequently for plant growth (Xu; Wang, 2023). Diazotrophic rhizobacteria isolated from *Mimosa artemisianana* in the Caatinga region have been shown to promote soybean growth under drought, increasing the dry weight of roots and shoots (Braga *et al.,* 2023). Eucalyptus trees require a high availability of nutrients, with forest productivity closely related to the levels of nutrients present in the soil (Madhaiyan *et al.,* 2021). Diazotrophic rhizobacteria can promote increases in the content and nutrient use efficiency of macronutrients (N, P, K, Ca and Mg) and consequently, a significant increase in the root and total biomass of *Eucalyptus grandis* (Martins Filho, 2021). Diazotrophic bacteria are a promising strategy capable of reducing the demand for nitrogen fertilizers and increasing biomass production (Madhaiyan *et al.,* 2021). The combination of nitrogen fertilizers and the inoculation of diazotrophic bacteria are capable of increasing N absorption, height and biomass of *Eucalyptus urophylla* × *Eucalyptus grandis* clones, enabling a more sustainable alternative, promoting reductions in the use of nitrogen fertilizers (Lan *et al.,* 2023). Just like nitrogen, in the absence of microorganisms, the absorption of phosphorus (P) by plants is a limiting factor, as the majority is found in phosphate minerals, which are slowly released and dependent on several factors related to the soil (Glick *et al.,* 2012). Therefore, the ability of microorganisms to solubilize phosphates is essential for obtaining healthy and well-nourished plants, especially in conditions of water deficit. The 21 rhizobacteria tested in this work demonstrated the ability to solubilize calcium phosphate in Pikovskaya medium and 15 (71.4 %) isolates did so in NBRIP medium. Most bacteria reported to solubilize P are Gram negative and, through the secretion of organic acids such as oxalic, citric, gluconic, succinic, α-ketoglutaric and fumaric acid, solubilize P (Mendes *et al.,* 2013; Glick 2012). Our results corroborated this information, as among the 15 rhizobacteria that

solubilize P in NBRIP medium, 12 were identified as Gram negative. Plant growth-promoting rhizobacteria, capable of solubilizing phosphate, can help plants absorb this nutrient, resulting in significant increases in biomass, root length, and an increase in RWC even in water deficit conditions (Gupta; Pandey, 2019; Zolfaghar *et al.,* 2020). Regarding the ability to produce IAA, all evaluated rhizobacterial isolates were producers. It is estimated that 80 % of bacteria present in the soil, including most PGPR, have the ability to produce IAA (Kour, yadav, 2022). For the purpose of this study, the ability of isolates to produce IAA is essential since bacteria that produce IAA can assist plants in increasing root length and/or promoting lateral root formation and root hairs under water deficit conditions. This enables enhanced water and nutrient absorption, allowing plants to cope better with water deficit (Vurukonda *et al.,* 2016). Eucalyptus plants under water deficit conditions can increase o conteúdo de AIA em seus tecidos, which may be related to the adaptation process to drought (Jesus *et al.,* 2015). Thus, it is possible that inoculating IAA-producing rhizobacteria in eucalyptus plants could enhance their tolerance to water deficit conditions.

4.3 Indirect mechanisms for drought tolerance

The isolates evaluated in this study also showed the ability to execute indirect growth-promoting mechanisms. All rhizobacteria were capable of producing NH_3 and siderophore. These indirect mechanisms for promoting plant growth may also contribute to improved plant tolerance to water stress. The NH_3 production can enhance plant growth through the enzymatic assimilation pathway of NH_4^+ (ammonium), with plants utilizing it as a nitrogen source (Kojima *et al.,* 2021). Rice plants under stress conditions supplied with NH_4^+ increase the uptake of this molecule and exhibit higher contents of soluble sugars, proline, and amino acids in the leaves, as well as higher rates of photosynthesis and biomass accumulation (Cao *et al.,* 2018). Therefore, it is suggested that NH_4^+ can help alleviate water stress in plants. On the other hand, the production of siderophores by some microorganisms gives them greater competitiveness, as they are capable of sequestering ferric iron (Fe^{3+}) present in the environment and reducing it to Fe^{2+} (Arora; Jha, 2019; Singh *et al.,* 2019). Limiting the availability of Fe in the environment ends up inhibiting the growth of pathogens, which require this element for their metabolism. Secretion of siderophores in the rhizosphere can alter microbiome-pathogen interactions and result in their inhibition. In this way, microorganisms capable of producing siderophores can provide protection to different crops, including under abiotic stresses, through the suppression and/or inhibition of pathogens, in addition to stimulating plant growth and development (Arora; Jha, 2019; Gu *et al.,* 2020; Han *et al.,* 2021; Khoso *et al.,* 2024). The use of microorganisms

that have the ability to secrete siderophores and execute other growth-promoting mechanisms can be an alternative to the use of chemical fertilizers, especially in soils with low water availability (saline), where iron is little available to plants (Sultana *et al.,* 2021).

The cellulase synthesis was verified in 11 isolates (52.3 %), LEM 01 *R. pickettii*, LEM 07 Unrequited, LEM 10 *R. pickettii*, LEM 11 *P. validus*, LEM 14 *R. pickettii*, LEM 15 *V. pantothenticus*, LEM 29 *B. cereus* GCsubgroupB, LEM 33 Unrequited, LEM 34 *S. maltophilia*, LEM 41 *B. cenocepaciae*, and LEM 50 *R. pickettii*. The ability to degrade chitin was confirmed by 10 isolates (47.6 %), LEM 12 *S. maltophilia*, LEM 18 *R. pickettii*, LEM 24 *B. cenocepacia* and LEM 36 *S. maltophilia* including the ones that degraded cellulose, LEM 07 Unrequited, LEM 29 *B. cereus* GCsubgroupB, LEM 33 Unrequited, LEM 34 *S. maltophilia*, LEM 41 *B. cenocepacia* and LEM 50 *R. pickettii*. The rhizobacteria LEM 18 *R. pickettii* was the most effective in chitin degradation. Cellulase and chitinase enzymes aid the plant in promoting biocontrol against phytopathogenic fungi by degrading the cell wall, as well as the chitin exoskeleton present in arthropods (Rathore; Gupta, 2015; Bhattacharyya *et al.,* 2020). Under stress, plants experience physiological and metabolic changes that can weaken their natural defense mechanisms. This weakened state leaves them more vulnerable to opportunistic pathogens and insect pests (Gonçalves *et al.,* 2017). Thus, these indirect mechanisms for promoting plant growth are also important under water stress conditions as they provide additional resources that can assist the plant in coping with adverse conditions, enabling better energy management that favors the plant's capacity to tolerate and adapt to water deficit.

Considering the importance of PGPRs presenting indirect growth-promoting mechanisms, the *in vitro* double culture test demonstrated a significant reduction in the growth of *Ceratocystis fimbriata* LPF for 81 % of the isolates, namely: LEM 29 *B. cereus* GCsubgroupB, LEM 27 *B. cereus* GCsubgroupB, LEM 01 *R. pickettii*, LEM 07 Unrequited, LEM 10 *R. pickettii*, LEM 33 Unrequited, LEM 12 *S. maltophilia*, LEM 13 *S. kloosii*, LEM 11 *P. validus*, LEM 15 *V. pantothenticus*, LEM 34 S. *maltophilia*, LEM 05 Unrequited, LEM 41 *B. cenocepacia*, LEM 17 *S. maltophilia*, LEM 14 *R. pickettii* and LEM 50 *R. pickettii*. Microorganisms have the ability to combat pathogens through various mechanisms, such as the secretion of antibiotics, volatile compounds, toxins and hydrolytic enzymes (e.g., chitinase, β-1,3-glucanase) (Liu *et al.,* 2020; Pandit *et al.,* 2022). The inhibition of phytopathogenic fungi, *Fusarium solani* and *C. fimbriata*, was also observed through antagonism tests (dual culture) with the bacteria *Bacillus amyloliquefaciens* (Cui-Juan *et al.,* 2020). The potential for antagonism by the supernatant was also reported with the bacteria *Bacillus altitudinis*, which provided *in vitro* reductions in the growth and germination of *C. fimbriata* spores (Zhang *et al.,*

2023). The results found in both studies demonstrated that the antagonism behavior in the laboratory was consistent in *in vivo* tests, providing reductions in the severity of the disease in sweet potato roots (Cui-Juan *et al.,* 2020; Zhang *et al.,* 2023). Although the *in vitro* results demonstrate the potential of these microorganisms to execute different growth promotion mechanisms, it is essential to carry out *in vivo* tests, in this case, with eucalyptus plants. In the environment, microorganisms form complex and multifaceted interactions with plants. Interactions are subordinated to different relationships between the microorganisms themselves, environmental conditions and poorly understood molecular mechanisms, which are not simulated in *in vitro* situations, meaning that microorganisms may respond in different ways to what is observed in the laboratory (Trivedi *et al.,* 2020).

5. CONCLUSION

All rhizobacterial isolates were capable of exerting mechanisms *in vitro*, which can help plants in conditions of water deficit, however, some demonstrated a greater range in the execution of the mechanisms. Nine isolates were able to tolerate conditions of low water activity and all isolates were identified as halotolerant, suggesting that they can withstand such abiotic stress conditions in the field. The isolates that presented a greater range of direct and indirect mechanisms for drought tolerance in plants were also identified as xero and halotolerant: LEM 11 *P. validus*, LEM 01 *R. pickettii* and LEM 50 *R. pickettii*. Furthermore, isolates LEM *01 R. pickettii* and LEM 11 *P. validus* and LEM 50 *R. pickettii* exhibited indirect mechanisms such as siderophore production, cellulase and chitinase synthesis. Such mechanisms confirmed in *in vitro* tests demonstrate that rhizobacteria can help plants to withstand water deficit conditions, and promote the inhibition of pathogens, such as *C. fimbriata*. The use of these microorganisms can meet the need for technologies that help plants subject to periods of drought, enabling the maintenance of the productivity of various crops. However, the effectiveness of inoculating these isolates in plants depends on the interaction between plants and microorganisms, soil type, and environmental conditions. Therefore, it is important to evaluate these rhizobacteria directly in the soil-plant system to determine their effectiveness in promoting growth and drought tolerance in eucalyptus clones.

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SUPPLEMENTARY MATERIAL

Table S1. Gram staining and species of diazotrophic bacterial isolates obtained from the rhizosphere of *E. grandis.*

Isolados	**Gram**	**Espécie**
LEM 01	-	*Ralstonia pickettii (Burkholderia, Pseudomonas pickettii)*
LEM 05*	-	Unrequited
LEM 07*	-	Unrequited
LEM 10	-	*Ralstonia pickettii (Burkholderia, Pseudomonaspickettii)*
LEM 11	+	*Paenibacillus validus (Bacillusgordonae)*
LEM 12	-	*Stenotrophomonas maltophilia (Xanthomonas, Pseudomonas)*
LEM 13	+	*Staphylococcus kloosii*
LEM 14	-	*Ralstonia pickettii (Burkholderia, Pseudomonaspickettii)*
LEM 15	+	*Virgibacillus pantothenticus (Bacillus)*
LEM 16*	-	Unrequited
LEM 17	-	*Stenotrophomonas maltophilia (Xanthomonas, Pseudomonas)*
LEM 18	-	*Ralstonia pickettii (Burkholderia, Pseudomonaspickettii)*
LEM 19	+	*Virgibacillus pantothenticus (Bacillus)*
LEM 24	-	*Burkholderia-cenocepacia/ambifaria/anthina/stabilis*
LEM 27	+	*Bacillus cereus GCsubgroupB*
LEM 29	+	*Bacillus cereus GCsubgroupB*
LEM 33*	-	Unrequited
LEM 34	-	*Stenotrophomonas maltophilia (Xanthomonas, Pseudomonas)*
LEM 36	-	*Stenotrophomonas maltophilia (Xanthomonas, Pseudomonas)*
LEM 41	-	*Burkholderia cenocepacia/ambifaria/anthina/stabili*
LEM 50	-	*Ralstonia pickettii (Burkholderia, Pseudomonaspickettii)*

*Unidentified isolates by the FAME system that may represent new species. The listed rhizobacteria were isolated by Martins Filho, 2021.

Chapter III

PROMOTING THE GROWTH OF EUCALYPTUS CLONES IN A FOREST NURSERY UNDER WATER DEFICIT CONDITIONS THROUGH RHIZOBACTERIAL INOCULATION

PROMOTING THE GROWTH OF EUCALYPTUS CLONES IN A FOREST NURSERY UNDER WATER DEFICIT CONDITIONS THROUGH RHIZOBACTERIAL INOCULATION

ABSTRACT

Water deficits are closely linked to global climate change and are projected to become a major limiting factor for agriculture in the future. Over the past 60 years, a significant portion of Brazilian territory has experienced severe and intense droughts, and it is anticipated that the country will face even more intense and frequent dry periods in the future. One potential solution to address this issue is the utilization of microorganisms. Certain bacteria possess mechanisms that facilitate plant growth and survival under stressful conditions. **Aims** In this study, the efficacy of 21 rhizobacteria isolated from the rhizosphere of *Eucalyptus grandis* was examined in promoting growth in the hybrid eucalyptus clones VM01 (*E. urophylla* x *E. camaldulensis*) and I144 (*E. urophylla* x *E. grandis*), which were subjected to consecutive reductions in the watering regime during seedling production. **Methods** The rhizobacteria were inoculated into VM01 and I144 mini cuttings at 41 and 58 days of age. The substrate's moisture content was maintained at 70 % of its water-holding capacity for the initial 68 days, after which it was reduced to 50 %, and on the 83rd day, further reduced to 30 %. At the end of the 90th day, the seedlings were evaluated for parameters such as leaf and branch numbers, collar diameter, height, root dry mass (RDM), shoot dry mass (SDM), and total dry mass (TDM). The macronutrients nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S), as well as the micronutrient boron (B), were also assessed for each eucalyptus clone. The experiment was conducted using a completely randomized design (CRD), data regarding morphological characteristics were subjected to analysis of variance (ANOVA) (p<0.05), and the treatment means were compared by Scott-Knott. Nutritional variables were subjected to principal component analysis (PCA). **Results** Certain rhizobacteria were effective in increasing morphological parameters and nutrient amount, as well as nutrient absorption efficiency, use efficiency and ttranslocation efficiency. Notably, isolates LEM 50 *R. pickettii* and LEM 14 *R. pickettii* exhibited higher efficiency in enhancing biomass, macro and micronutrient content, and efficiency of nutrient utilization and translocation in VM01 clones. Although rhizobacteria showed improvements in nutrient levels for I144 clones, it was observed that they were more efficient in VM01 clones. Increases in absorption efficiency were provided mainly by the isolates LEM 41 *Burkholderia cenocepacia* e LEM 36 *Stenotrophomonas maltophilia*. **Conclusion** These findings demonstrate that rhizobacteria have the potential to

enhance eucalyptus growth under water-restricted conditions; however, the effectiveness vary among different hybrid clones.

Keywords: Water scarcity; Bacteria; VM01; I144; Climate change.

1. INTRODUCTION

Eucalyptus trees are the most widely cultivated trees in the world due to their fast growth and quality of wood (Oberschelp *et al.,* 2022). They are an important source of forest products such as cellulose, paper, laminated panels and floors, sawn timber and plywood, charcoal, firewood, and bioenergy. The Brazilian forestry sector holds first place globally in terms of eucalyptus planted area, and the productivity of its plantations is also the highest in the world. Approximately 75 % of the trees planted in Brazil are eucalyptus (IBÁ, 2022). However, the effects of climate change, can have a negative impact on production, bringing uncertainties to the forestry sector. Future climate change projections indicate average annual temperature increases between 1.9 °C and 2.4 °C between the years 2040 and 2069, with annual rainfall reductions in the North, Northeast, Southeast, and Midwest regions. According to these projections, the mean annual increment (MAI) (m^3/ha/year) of eucalyptus plantations in Brazil will suffer an average decrease of -1 % to -12 % (Elli *et al.,* 2020). In 2014, the forestry sector was impacted by the loss of 200 thousand hectares after a long period of drought in the northern region of Minas Gerais. Furthermore, eucalyptus planted in regions subject to intense periods of water deficit are more prone to outbreaks of pests and diseases (Gonçalves *et al.,* 2017). The main risk factor for eucalyptus production is drought (Assad *et al.,* 2022). In this context, one of the biggest challenges is the adaptation of genotypes to climate change, the development of which can take years (Gonçalves *et al.,* 2017). Therefore, the development of more agile technologies that provide greater tolerance to water scarcity are indispensable for increasing productivity (Oliveira *et al.,* 2021).

One alternative to promote drought tolerance and mitigate the effects of water stress in plants, is the use of microorganisms. Plant Growth-Promoting Rhizobacteria (PGPR) have the ability to assist plants under water deficit conditions through various mechanisms, whether direct or indirect. Among these protective mechanisms, the production of exopolysaccharides, biofilm formation, synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, nitrogen fixation, phosphate solubilization, volatile compounds production, phytohormones production (indole-3-acetic acid), and indirect mechanisms such as the production of siderophores and cellulase and chitinase enzymes. The joint action of these mechanisms is capable of providing better plant nutrition, growth, protection against pathogens and tolerance to environmental stresses (Vurukonda *et al.,* 2016).

Currently, the use of microbial inoculants has been increasing in the agricultural and forestry sectors as a result of research showing promising results in inducing drought tolerance (Kavamura *et al.,* 2013; Eke *et al.,* 2019; Chaín *et al.,* 2020; Mishra *et al.,* 2020; Braga *et al.,*

2022). Eucalyptus trees inoculated with bacteria can significantly increase survival rates, maintain foliage better during water stress, and produce new leaves more rapidly after irrigation resumes (Chaín *et al.,* 2020). These results suggest that the use of PGPR alleviates physiological stress effects and promotes adaptive physiological responses, showing promise in assisting plants under water stress (Mishra *et al.,* 2020). Thus, the aim of this work was to evaluate the capacity and identify rhizobacteria isolated from *Eucalyptus grandis* that are capable of promoting drought tolerance and growth in hybrid eucalyptus clones [VM01 (*Eucalyptus urophylla* x *Eucalyptus camaldulensis*) and I144 (*Eucalyptus urophylla* x *Eucalyptus grandis*)], subjected to reduced water regimes in a nursery forestry.

2. MATERIAL AND METHODS

The experiment was conducted at the Forest Nursery of the Forest Engineering Department (DEF) (20°46'30.6"S 42°52'37.1"W) and the Microbial Ecology Laboratory (LEM) of the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil. Twenty-one diazotrophic rhizobacteria isolated from the rhizosphere of *Eucalyptus grandis* maintained in the LEM - UFV microorganism bank were utilized. The rhizobacterial isolates were obtained from the UFV Forestry sector, from eight-year-old plants (S 21° 01 741'; W 042° 34 258'), from the district of Santo Antônio do Rio Preto, Miraí, MG, with one-year regrowth (S 21° 01 638'; W042° 34 156') and in the same district, from eight-year-old trees (S 21° 01 698 '; W042° 33 842') (Martins Filho, 2021). The isolates were used to inoculate eucalyptus seedlings and evaluate their capacity to induce water stress tolerance in hybrid eucalyptus clones.

2.1 Preparation of rhizobacteria inoculants

Rhizobacteria strains stored in the ultrafreezer (- 80 °C) were reactivated by transferring them into 250 mL erlenmeyer flasks containing 50 mL of sterile tryptic soy broth (TSB) media. The flasks were placed on an orbital shaker and incubated at 150 rpm for 48 h at 28 ± 2 °C. The growth of bacteria was determined by measuring the optical density ($O.D._{600\ nm}$) using a spectrophotometer (Thermo Scientific Genesys 10S UV-Vis) and polyethylene cuvettes with an optical path length of 10 mm. An aliquot of culture cells was collected and centrifuged at 10,000 rpm for 5 min at room temperature. The pellet was washed once with 0.85 % NaCl and suspended in falcon tubes containing 30 mL of 0.85 % NaCl sterile at an $O.D._{600\ nm} = 0.6$.

2.2 Preparation of eucalyptus seedlings

Two commercial hybrid clones of eucalyptus were used: VM01 (*E. urophylla* x *E. camaldulensis*), which is tolerant to water deficit and therefore more suitable for planting in dry regions, and I144 (*E. urophylla* x *E. grandis*), which is the most planted in Brazil.

The clonal propagation of eucalypt was carried out at the forest nursery (DEF-UFV) using the mini-cutting technique (Assis, 2005). Vegetative propagules measuring between 8 and 10 cm in length with 2 pairs of leaves, where the leaf area was reduced by 50 %, were placed in plastic tubes with a volume of 55 cm^3 (Figure 1). The tubes were filled with commercial substrate Carolina Soil, class XVI (composed by peat, vermiculite and limestone pH of 5.5 and electric conductivity of 0.7 mS/cm), and fertilized with 6.0 kg m^{-3} of simple superphosphate and 3.0 kg m^{-3} of Osmocote®. The propagated plants were then placed in a greenhouse rooting with a humidity level of 80 % and nebulizers (NETAFIM®) providing a flow rate of 7 L h^{-1}. The temperature in the greenhouse was maintained at 35 °C to promote rooting, and the plants were kept in this environment for 40 days.

2.3 Experiment assembly

After 40 days, rooted clones of VM01 and I144 with similar heights were selected for the experiment. Prior to transferring the selected clones to the shade house, rhizobacteria inoculations were conducted. Seedlings of eucalyptus hybrid clones VM01 and I144, at 41 days of age were inoculated, at the interface of the stem with the substrate, with 1 mL of rhizobacteria at an $O.D._{600\ nm}$ = 0.6 and maintained for 11 days in a shade house for acclimatization period. No rhizobacteria solution was added to the control group, 1 mL of 0.85 % NaCl was added. A total of 22 treatments were performed for each clone, with 21 inoculated with rhizobacteria and 1 control treatment, each consisting of ten seedlings (Figure 1).

After the acclimatization period, the clones were moved to the nursery courtyard for growth and hardening of the seedlings for seven days. They were irrigated five times daily for five minutes each, with a flow rate of 330 L h^{-1}. On the last day, another inoculation of rhizobacteria was carried out on the same procedure as described, on the 58-day-old eucalyptus seedlings. Subsequently, the seedlings were transferred to an area protected from rain at the forest nursery (DEF-UFV) to initiate the irrigation reduction treatment.

To standardize irrigation, the water holding capacity (WHC) of commercial substrate Carolina Soil (Class XVI) was determined in the laboratory. The seedlings were irrigated at 70 % of the WHC for the first ten days. Afterwards, the irrigation was reduced to 50 % for 14 days

and 30 % for 6 days. The soil moisture content was monitored by weighing a sample of the plastic tubes (Novais; Neves; Barros, 1991).

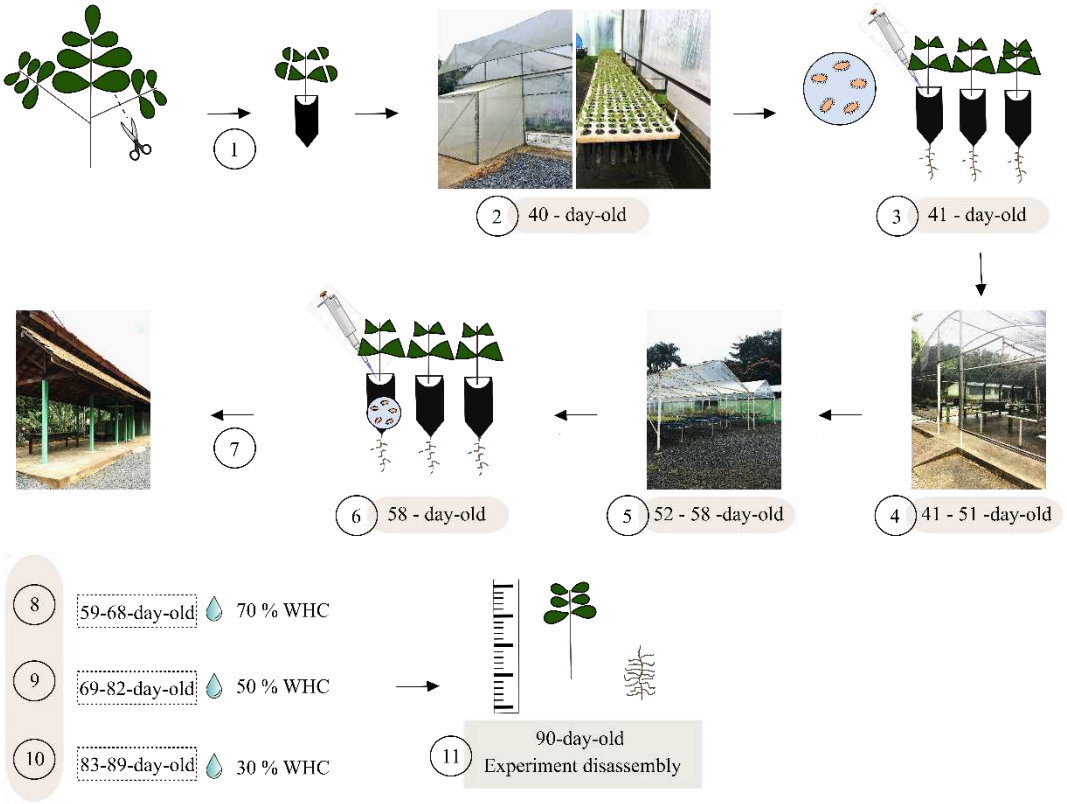

Figure 1. Scheme of the rhizobacteria inoculation methodology and conduct of the experiment with irrigation reductions in hybrid clones VM01 and I144. (1) Mini cuttings 8-10 cm long and with two pairs of leaves, with a leaf reduction of 50 %, are produced from mini stumps and transferred to tubes with substrate; (2) minicuttings are kept in a greenhouse for 40 days; (3) at the exit from the greenhouse, 41-day-old rooted clones are inoculated with rhizobacteria and 0.85 % saline solution (control); (4) the clones are transferred to a shade house and kept for eleven days; (5) after the shade house period, they are transferred to the rustification yard and remain for seven days; (6) on the 58th day, a booster inoculation is carried out; (7) on the 59th day, the clones are taken to a covered patio and (8) watering reductions begin at 70% WHC; (9) from the 69th to the 82nd day, watering is reduced to 50% WHC; (10) from the 83rd to the 89th day, watering is reduced to 30 % WHC; (11) the experiment is disassembled with clones that are 90 days old and are collected to carry out the analyzes described.

2.4 Morphological and nutritional evaluation

The experiment was harvested at 90° days. Measurements were taken for plant height (cm), leaf number per plant and branches, and collar diameter (mm). The plants were carefully removed from the plastic tubes and separated into shoots and roots. Samples of roots were washed in running water and placed with shoot samples in an oven with forced air circulation at 70 °C until reaching a constant weight. Samples were weighed on a precision scale of three

houses. The variables analyzed were root dry mass (RDM), shoot dry mass (SDM), and total dry mass (TDM).

Additionally, macronutrient chemical composition, N, P, K, Ca, Mg, and S, and micronutrient chemical composition, B, in the plant tissues were analyzed. The Kjedahl method was used to determine the N contents. The remaining macronutrients and B were subjected to nitroperchloric digestion and quantified using an inductively coupled plasma optical emission spectrometer (ICP –OES; Perkin Elmer Model Optima 8300 DV).

The total nutrient concentration (1), root nutrient amount (2), shoot nutrient amount (3), total nutrient amount (4), was determined. The nutrient absorption efficiency (NAE) (5) was determined using the equation proposed by Swiader *et al.* (1994), nutrient translocation efficiency (NTE) (6) was determined using the equation proposed by Li *et al.* (1991) and nutrient use efficiency (NUE) (7), was determined using the equation proposed by Siddiqi and Glass (1981) (Eq.7).

$$\text{Total nutrient concentration (dag kg}^{-1}\text{)} = \frac{\text{Total nutrient amount}}{\text{Plant total dry mass}} \times 100 \qquad (1)$$

$$\text{Root nutrient amount (mg plant}^{-1}\text{)} = \frac{\text{(Root concentration nutrient x root dry mass)}}{100} \times 1000 \qquad (2)$$

$$\text{Shoot nutrient amount (mg plant}^{-1}\text{)} = \frac{\text{(Shoot concentration nutrient x shoot dry mass)}}{100} \times 1000 \qquad (3)$$

$$\text{Total nutrient amount (mg plant}^{-1}) = \text{Root nutrient amount} + \text{Shoot nutrient amount} \quad (4)$$

$$\text{NAE (mg g}^{-1}) = \frac{\text{Total nutrient amount}}{\text{Root dry mass}} \quad (5)$$

$$\text{NTE (\%)} = \frac{\text{Shoot nutrient amount}}{\text{Total nutrient amount}} \times 100 \quad (6)$$

$$\text{NUE (g}^2\text{ mg}^{-1}) = \frac{(\text{Total dry mass})^2}{\text{Total nutrient amount}} \quad (7)$$

2.5 Statistical analyses

The experiment was conducted using a completely randomized design (CRD), consisting of 22 treatments with 10 experimental units in each. The data relating to morphological characteristics were subjected to analysis of variance (ANOVA), and when significant (p<0.05), the treatment means were compared using the Scott-Knott procedure using the Software R and ExpDes package (Ferreira *et al.,* 2013). Nutritional variables were subjected to principal component analysis (PCA) to condense all variables into a smaller set of dimensions. Reductions in linear combinations led to the generation of scores that were used to define principal components 1 and 2. Principal component analysis was performed with the aid of PAST software. The construction of the graphics was carried out using GraphPad Prism 5 and the figures were created in InKscape.

3. RESULTS

3.1 Morphological characteristics of eucalyptus clones under water reductions

3.1.1 Leaf number, branching, collar diameter, and height of VM01 clone

The hybrid clones of VM01, upon inoculation with some rhizobacteria, exhibited higher numbers of leaves, branches, collar diameter, and height compared to non-inoculated plants (Table 1) after the 90-day experiment. The analyzed data reveal that rhizobacteria LEM 50

Ralstonia pickettii demonstrated notable performance, leading to significant increases in all measured when compared to the averages of both inoculated and non-inoculated clones. Specifically, isolate LEM 50 *R. pickettii* resulted in a 102.27 % increase in the number of leaves, a 389.33 % increase in the number of branches, an 11.11 % increase in collar diameter, and a 19.32 % increase in height. The LEM 14 *Ralstonia pickettii* demonstrated significant increases in three of the four variables evaluated, number of leaves (105.45 %), branches (220 %), and collar diameter (9.66 %).

In terms of branch numbers, the rhizobacteria LEM 50 *R. pickettii* (389.33 %), LEM 13 *Staphylococcus kloosii* (333.33 %), LEM 27 *Bacillus cereus* gcsubgroupb (300 %), and LEM 14 *R. pickettii* (220 %) promoted increases significantly higher than the other isolates. Regarding the collar diameter, seven isolates, LEM 24 *Burkholderia cenocepacia*, LEM 50 *R. pickettii*, LEM 12 *Stenotrophomonas maltophilia*, LEM 11 *Paenibacillus validus*, LEM 14 *R. pickettii*, LEM 01 *Ralstonia pickettii*, and LEM 16 Unrequited, demonstrated significantly better performance than the control, with improvements ranging from 6.28 % to 18.84 %. When considering the height of the clones, the LEM 50 *R. pickettii* rhizobacterium induced a significant increase of 19.32 %, corresponding to a 2.72 cm increase in relation to the mean height of the control, surpassing the performance of other isolates. Height gains were also significant with the inoculation of LEM 11 *P. validus* (11.08 %) and LEM 16 Unrequited (5.54 %) isolates. The remaining rhizobacterial isolates were not able to promote a significant increase compared to the control.

3.1.2 Root, shoot and total dry mass of VM01 clone under water reductions

The RDM, SDM, and TDM of both inoculated and non-inoculated VM01 and I144 hybrid clones were evaluated after 90 days of experimentation. Certain rhizobacterial isolates demonstrated the ability to enhance growth in the inoculated VM01 clones, influencing both the root system and aerial part.

The obtained results revealed significant increases in RDM for four isolates, namely LEM 01 *R. pickettii,* LEM 11 *P. validus*, LEM 13 *S. kloosii*, and LEM 50 *R. pickettii*, and, in comparison to the average values observed in the non-inoculated clone (0.176 g). Among the aforementioned rhizobacteria, plants with LEM 13 *S. kloosii* exhibited the most pronounced enhancement in root growth (0.243 g), followed by LEM 50 *R. pickettii* (0.231 g), LEM 01 *R. pickettii* (0.228 g), and LEM 11 *P. validus* (0.218 g) (Figure 2A).

In terms of SDM, seven isolates elicited notable and significant increases compared to the non-inoculated clone (0.490 g). Notably, LEM 50 *R. pickettii* isolate exhibited the most

prominent effect among all treatments, resulting in a remarkable 66.5 % increase (0.816 g) compared to the non-inoculated plants. Furthermore, the remaining six isolates (LEM 27 *B. cereus GCsubgroupbB*, LEM 11 *P. validus*, LEM 24 *B. cenocepacia*, LEM 16 Unrequited, LEM 13 *S. kloosii*, and LEM 14 *R. pickettii*) induced increases in SDM ranging from 0.612 to 0.715 g (Figure 2B).

In relation to the TDM, a significant increase was observed in seven rhizobacterial isolates compared to the control group (0.688 g) (Figure 2C). These isolates were specifically identified as LEM 50 *R. pickettii*, LEM 13 *S. kloosii*, LEM 14 *R. pickettii*, LEM 24 *B. cenocepacia*, LEM 16 Unrequited, LEM 11 *P. validus*, and LEM 07 Unrequited. Among these isolates, LEM 50 *R. pickettii* statistically provided the greatest increase in total biomass, demonstrating the most prominent effect, resulting in a TDM of 1.06 g, representing a 54.07 % increase relative to the control.

Table 1. Morphological characteristics of hybrid clone VM01 (*E. urophylla* x *E. camaldulensis*) inoculated with rhizobacteria under constant water reduction

Rhizobacteria	Leave number	Increase (%)	Branch number	Increase (%)	Collar diameter (mm)	Increase (%)	Height (cm)	Increase (%)
LEM 01 *Ralstonia pickettii*	14.20[c]	29.09	1.00[c]	33.33	2.24[a]	8.21	12.79[c]	-
LEM 05 Unrequited	13.25[c]	20.45	0.80[c]	6.67	2.01[b]	-	14.38[c]	2.13
LEM 07 Unrequited	15.37[c]	39.73	0.62[c]	-	1.94[b]	-	13.88[c]	-
LEM 10 *Ralstonia pickettii*	12.80[c]	16.36	0.50[c]	-	1.97[b]	-	12.35[c]	-
LEM 11 *Paenibacillus validus*	14.57[c]	32.45	0.37[c]	-	2.43[a]	17.39	15.64[b]	11.08
LEM 12 *Stenotrophomonas maltophilia*	11.22[c]	2.00	0.25[c]	-	2.30[a]	11.11	13.67[c]	-
LEM 13 *Staphylococcus kloosii*	13.00[c]	18.18	3.25[a]	333.33	2.13[b]	2.90	13.00[c]	-
LEM 14 *Ralstonia pickettii*	22.60[a]	105.45	2.40[a]	220.00	2.27[a]	9.66	13.19[c]	-
LEM 15 *Virgibacillus pantothenticus*	14.44[c]	31.27	0.87[c]	16.00	2.13[b]	2.90	13.42[c]	-
LEM 16 Unrequited	10.71[c]	-	0.33[c]	-	2.20[a]	6.28	14.86[b]	5.54
LEM 17 *Stenotrophomonas maltophilia*	12.42[c]	12.91	0.56[c]	-	2.10[b]	1.45	13.06[c]	-
LEM 18 *Ralstonia pickettii*	14.00[c]	27.27	0.37[c]	-	1.99[b]	-	13.17[c]	-
LEM 19 *Virgibacillus pantothenticus*	10.62[c]	-	0.00[c]	-	2.10[b]	1.45	13.94[c]	-
LEM 24 *Burkholderia cenocepacia*	15.33[c]	39.36	1.60[b]	113.33	2.46[a]	18.84	13.58[c]	-
LEM 27 *Bacillus cereus GCsubgroupB*	17.00[b]	54.54	3.00[a]	300.00	2.03[b]	-	13.56[c]	-
LEM 29 *Bacillus cereus GCsubgroupB*	13.37[c]	21.54	0.70[c]	-	1.93[b]	-	12.50[c]	-
LEM 33 Unrequited	18.14[b]	64.91	1.78[b]	137.33	2.00[b]	-	13.17[c]	-
LEM 34 *Stenotrophomonas maltophilia*	15.12[c]	37.45	1.43[b]	90.67	2.01[b]	-	13.31[c]	-
LEM 36 *Stenotrophomonas maltophilia*	14.56[c]	32.36	1.00[c]	33.33	2.08[b]	0.48	12.93[c]	-
LEM 41 *Burkholderia cenocepacia*	13.29[c]	20.82	0.87[c]	16.00	2.13[b]	2.90	13.29[c]	-
LEM 50 *Ralstonia pickettii*	22.25[a]	102.27	3.67[a]	389.33	2.30[a]	11.11	16.80[a]	19.32
Control	11.00[c]	-	0.75[c]	-	2.07[b]	-	14.08[c]	-

Data represent the mean. Values followed by the same lowercase letter belong to the same group according to the Scott-Knott test (p<0.05).

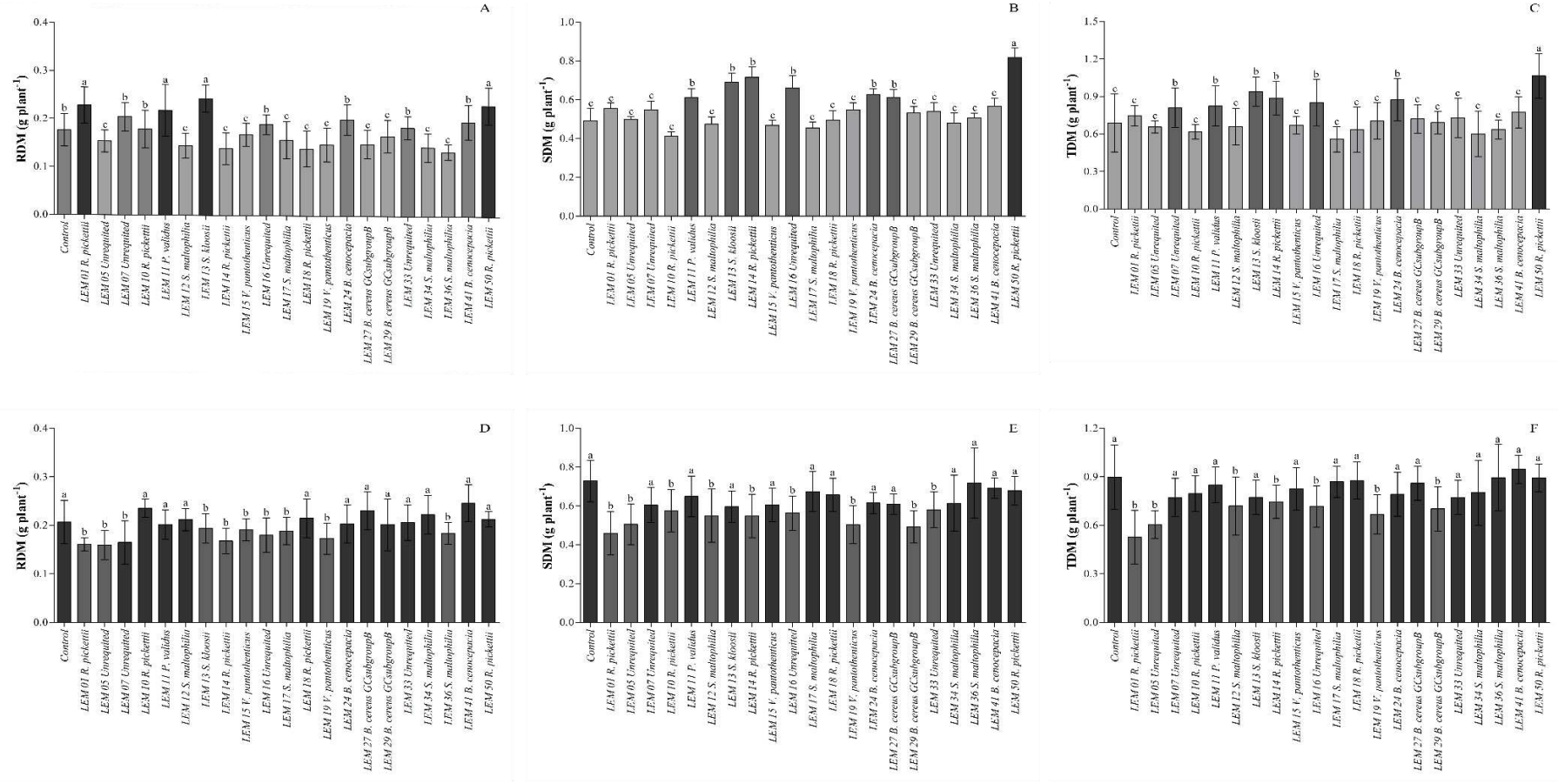

Figure 2. Effect of irrigation reduction on biomass of hybrid clones VM01 and I144, inoculated and non-inoculated with rhizobacteria. VM01: root dry mass (RDM) (A), shoot dry mass (SDM) (B), and total dry mass (TDM) (C). I144: root dry mass (RDM) (D), shoot dry mass (SDM) (E), and total dry mass (TDM) (F). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05).

3.1.3 Leaf number, branching, collar diameter, and height of I144 clone

In relation to clone I144, the isolates demonstrated significant gains only in the number of leaves and branches, and it was not possible to observe a significant increase in the stem diameter and height (Table 2). The results concerning the number of leaves indicated that 11 isolates resulted in significant increases compared to the control, varying in increases from 19.83 % to 53.12 %, these being the isolates, LEM 05 Unrequited, LEM 07 Unrequited, LEM 11 *P. validus*, LEM 12 *S. maltophilia*, LEM 15 *V. pantothenticus*, LEM 16 Unrequited, LEM 17 *S. maltophilia*, LEM 24 *B. cenocepacia*, LEM 33 Unrequited, LEM 41 *B. cenocepacia*, and LEM 50 *R. pickettii*. Notably, isolate LEM 33 Unrequited exhibited the highest increase of 53.12 % in the number of leaves, followed by LEM 16 Unrequited with a similar increase of 52.50 %. In terms of the number of branches, 13 isolates demonstrated significant increases compared to the control, ranging from 16.82 % to 77.57 %. Among them, isolate LEM 41 *B. cenocepacia* resulted in the largest increase, 77.57 %, followed by isolate LEM 12 *S. maltophilia* with a 71.49 % increase.

3.1.4 Root, shoot and total dry mass of I144 clone under water reductions

Clone I144, which was subjected to identical conditions and treatments as clone VM01, did not exhibit significant increases in RDM, SDM, or TDM (Figures 2D, E, and F). For this clone, some rhizobacterial isolates demonstrated significant reductions in dry mass. The rhizobacteria LEM 05 Unrequited, LEM 14 *R. pickettii*, LEM 16 Unrequited and LEM 19 *V. pantothenticus* were responsible for significant reductions in the three morphological characteristics, RDM, SDM and TDM. Although it was possible to observe that the rhizobacteria LEM 50 *R. pickettii* and LEM 13 *S. kloosii* showed significant increases in the biomass of clone VM01 (RDM, SDM and TDM), they did not demonstrate increases in clone I144. However, these isolates also did not promote significant reductions in biomass.

Table 2. Morphological characteristics of hybrid clone I144 (*E. urophylla x E. grandis*) inoculated with rhizobacteria under constant water reduction**.**

Rhizobacteria	Leave number	Increase (%)	Branch number	Increase (%)	Collar diameter (mm)	Increase (%)	Height (cm)	Increase (%)
LEM 01 *Ralstonia pickettii*	13.25[b]	-	1.63[b]	-	2.07[b]	-	11.60[a]	-
LEM 05 Unrequited	20.20[a]	26.25	2.50[a]	16.82	2.12[b]	-	13.39[a]	-
LEM 07 Unrequited	22.12[a]	38.25	2.57[a]	20.09	2.17[a]	-	12.92[a]	-
LEM 10 *Ralstonia pickettii*	17.43[b]	8.94	1.75[b]	-	2.14[b]	-	13.25[a]	-
LEM 11 *Paenibacillus validus*	20.40[a]	27.50	2.83[a]	32.24	2.07[a]	-	12.56[a]	-
LEM 12 *Stenotrophomonas maltophilia*	20.75[a]	29.69	3.67[a]	71.49	2.13[a]	-	12.17[a]	-
LEM 13 *Staphylococcus kloosii*	18.00[b]	12.50	2.14[b]	-	2.16[b]	-	13.64[a]	1.03
LEM 14 *Ralstonia pickettii*	16.20[b]	1.25	2.67[a]	24.77	2.24[b]	-	11.64[a]	-
LEM 15 *Virgibacillus pantothenticus*	19.25[a]	20.31	3.14[a]	46.73	2.33[a]	-	13.06[a]	-
LEM 16 Unrequited	24.40[a]	52.50	2.83[a]	32.25	2.48[a]	1.22	12.44[a]	-
LEM 17 *Stenotrophomonas maltophilia*	19.60[a]	22.50	2.67[a]	24.77	2.20[b]	-	12.81[a]	-
LEM 18 *Ralstonia pickettii*	16.87[b]	5.44	1.43[b]	-	2.28[a]	-	13.56[a]	0.44
LEM 19 *Virgibacillus pantothenticus*	17.29[b]	8.06	2.60[a]	21.49	2.20[b]	-	13.25[a]	-
LEM 24 *Burkholderia cenocepacia*	19.17[a]	19.81	3.20[a]	49.53	2.24[b]	-	13.36[a]	-
LEM 27 *Bacillus cereus GCsubgroupB*	13.87[b]	-	1.71[b]	-	2.20[b]	-	14.07[a]	4.22
LEM 29 *Bacillus cereus GCsubgroupB*	13.40[b]	-	1.80[b]	-	2.13[b]	-	12.97[a]	-
LEM 33 Unrequited	24.50[a]	53.12	3.50[a]	63.55	2.43[a]	-	12.56[a]	-
LEM 34 *Stenotrophomonas maltophilia*	14.60[b]	-	1.50[b]	-	2.12[b]	-	13.08[a]	-
LEM 36 *Stenotrophomonas maltophilia*	18.00[b]	12.50	1.83[b]	-	2.30[a]	-	12.37[a]	-
LEM 41 *Burkholderia cenocepacia*	22.25[a]	39.06	3.80[a]	77.57	1.85[b]	-	14.75[a]	9.26
LEM 50 *Ralstonia pickettii*	20.00[a]	25.00	2.86[a]	33.64	2.20[b]	-	12.93[a]	-
Control	16.00[b]	-	2.14[b]	-	2.45[a]	-	13.50[a]	-

Data represent the mean. Values followed by the same lowercase letter belong to the same group according to the Scott-Knott test ($p<0.05$). Control represents non-inoculated plants.

3.2 Principal component analysis: effect of rhizobacteria inoculation on eucalyptus nutrition under water reductions

3.2.1 VM01 clone

The reductions in nutritional variables were carried out through principal component (PC) analysis, where PC1 and PC2 explained 86.17 % of the total data variance (Figure 3). The response variables MSR, MSPA, MST, the total amount of N, P, K, Ca, B, the utilization efficiencies of N, S, B, P and Ca were separated in the first axis (PC1), which explained 57.08 % of total variability of data with positive eigenvectors. The second axis (PC2) explained 29.09 % of the total variation and was influenced by the translocation efficiencies of N, K, Ca, Mg, S and total N content, with eigenvectors also positive. Principal component analysis (PCA) revealed distinct behaviors among the isolates. The rhizobacteria LEM 50 *R. pickettii* stood out among the others for presenting the highest value in PC1, presenting high values of total amount of N (12,998 mg plant $^{-1}$), P (3,471 mg plant $^{-1}$) K (16,044 mg plant $^{-1}$), Ca (8,034 mg plant $^{-1}$), and B (451,756 mg plant $^{-1}$). Regarding utilization efficiencies, this isolate caused the highest values of N (0.103 g^2 mg-$^{-1}$), P (0.382 g^2 mg^{-1}) S (1.175 g^2 mg^{-1}), B (0.0029 g^2 mg^{-1}) and Ca (0.165 g^2 mg-$^{-1}$). LEM 14 *R. pickettii* also presented one of the highest values for PC1, providing increases in the total amount of N (14,109 mg plant $^{-1}$), P (3,039 mg plant $^{-1}$), K (17,139 mg plant $^{-1}$), Ca (7,302 mg plant $^{-1}$), and B (364,412 mg plant $^{-1}$), and the utilization efficiencies N (0.073 g^2 mg^{-1}), P (0.338 g^2 mg^{-1}), S (0.942 g^2 mg^{-1}), B (0.002 g^2 mg $^{-1}$), and Ca (0.140 g^2 mg^{-1}).

Other isolates such as LEM 7 Unrequited, LEM 13 *S. kloosii*, LEM 16 Unrequited, LEM 24 *B. cenocepacia*, LEM 27 *B. cereus GCsubgroupB*, LEM 29 *B. cereus GCsubgroupB* and LEM 41 *B. cenocepacia* also demonstrate positive positioning in PC1, providing a positive influence on related variables. The isolates that clustered close to the origin tend to result in intermediate values for the variables in PC1 and PC2.

The control was positioned negatively to the first and second axis and, therefore, did not influence PC1 and PC2, resulting in low values of total amount of N (6,702 mg plant $^{-1}$), P (1,809 mg plant $^{-1}$), K (7,895 mg plant $^{-1}$), Ca (4,416 mg plant $^{-1}$) and B (262,729 mg plant $^{-1}$) and utilization efficiencies of N (0.046 g^2 mg^{-1}), S (0.523 g2 mg-1), B (0.001 g^2 mg^{-1}), P (0.172 g^2 mg^{-1}) and Ca (0.070 g^2 mg^{-1}). And in relation to PC2, it demonstrated N translocation efficiency values of 86.88 %, K of 83.47 %, Ca of 77.11 %, Mg of 63.38 % and total N concentration of 1214.191 dag kg $^{-1}$. Similar behavior to the control was observed by isolates LEM 01 *R. pickettii*, LEM 10 *R. pickettii*, LEM 15 *V. pantothenticus*, LEM 17 *S. maltophilia*

and LEM 33 Unrequited, which do not exert influence on PC1 and PC2, and, therefore, do not confer gains in promoting growth and nutritional status of eucalyptus under water deficit.

In relation to PC2, rhizobacteria that presented positive values in this axis, demonstrate a tendency to promote higher values of translocation efficiency of N, K, Ca, Mg and S, as well as total N content, since these variables presented positive eigenvectors. The rhizobacteria LEM 14 *R. pickettii* demonstrated the highest value in the second axis, positively influencing the associated variables. Regarding this component, LEM 14 *R. pickettii* stood out with the highest positive value, demonstrating a superior influence on the translocation efficiency of N (87.56 %), K (91.56 %), Ca (85.41 %), Mg (83.30 %) and total concentration of N (1397.992 dag kg $^{-1}$). Isolate LEM 13 *S. kloosii* demonstrated the lowest negative value in PC2, and, therefore, tended to promote the lowest translocation efficiency values for N (77.07 %), K (74.57 %), Ca (71.97 %), Mg (51.00 %) and total N concentration (1091,712 dag kg $^{-1}$). Other rhizobacteria also demonstrated a positive correlation with PC2: LEM 34 *S. maltophilia*, LEM 05 Unrequited, LEM 36 *S. maltophilia*, LEM 18 *R. pickettii* and LEM 12 *S. maltophilia*. Furthermore, LEM 14 *R. pickettii* and LEM 16, LEM 27 *B. cereus GCsubgroupB* and LEM 19 *V. pantothenticus*, provided a positive influence on both axes (PC1 and PC2).

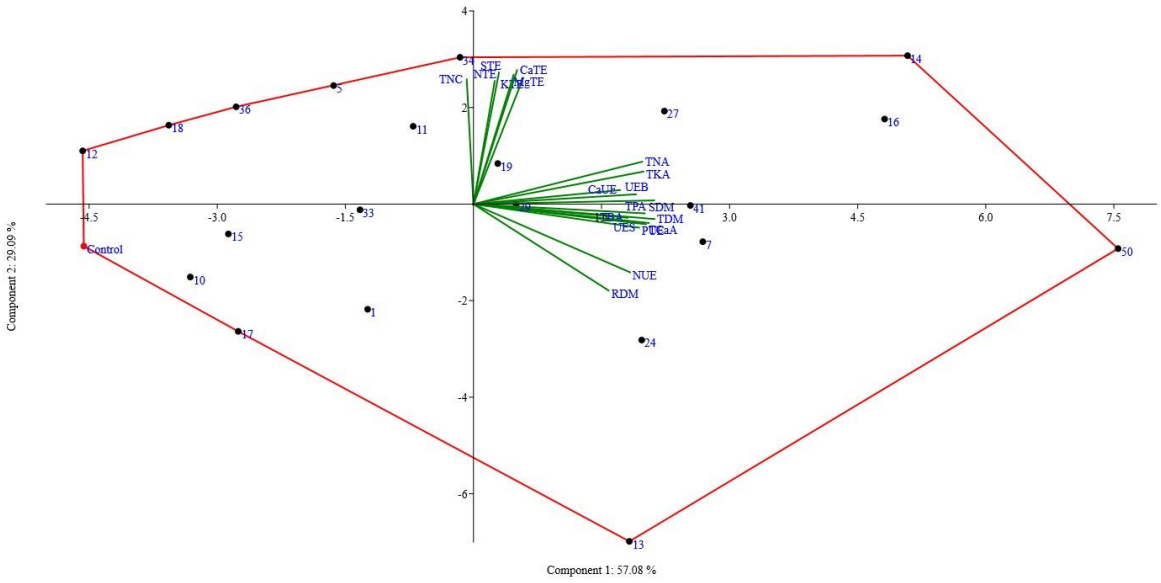

Figure 3. Principal component analysis of nutritional variables of clone VM01 inoculated with rhizobacteria under water deficit. Principal component 1 and 2 explained 86.17% of the total data variance. Treatments that presented positive eigenvectors for PC1 and PC2 influence the variables presented.

3.2.2 I144 Clone

In relation to clone I144, the reductions in nutritional variables through PCA explained 85.19 %, considering both axes (Figure 4). The response variables MSR, MSPA, MST and the utilization efficiencies of N, P, K, Ca, Mg, S and B were separated into PC1, which explained 55.73 % of the total variability of the data with positive eigenvectors. The variables of P, K, Ca, S absorption efficiency and K translocation efficiency influenced PC2, which explained 29.46 % of the variation and also demonstrated positive eigenvectors. Principal component analysis for clone I144 variables also revealed different behaviors between the isolates. The rhizobacteria LEM 41 *B. cenocepacia* and LEM 36 *S. maltophilia* presented the highest positive values for both axes, PC1 and PC2, and, therefore, are the isolates that most influence the associated variables. However, in relation to PC1 these isolates were close to the control treatment. In relation to PC2, LEM 41 *B. cenocepacia* and LEM 36 *S. maltophilia* showed P absorption efficiency of 10,730 mg g^{-1} and 13,987 mg g^{-1}, K 62,664 mg g^{-1} and 73,561 mg g^{-1}, Ca 35,168 mg g^{-1} and 37,568 mg g^{-1} and S of 6,527 mg g^{-1} and 7,877 mg g^{-1} and K translocation efficiency of 85.71 % and 86.02 %, respectively. The rhizobacteria LEM 50 *R. pickettii* was positioned close to the control treatment, in relation to PC1, and also demonstrated the ability to influence PC2 a little.

The rhizobacteria LEM 18 *R. pickettii*, LEM 34 *S. maltophilia* and LEM 12 *S. maltophilia* were positioned in the same lower right quadrant as the control, demonstrating similar behaviors for PC1 and PC2. The isolates closest to the origin demonstrate intermediate values for the variables associated with PC1 and PC2, these being: LEM 10 *R. pickettii*, LEM 11 *P. validus*, LEM 16 Unrequited and LEM 27 *B. cereus GCsubgroupB*. The isolates in the upper and lower left quadrants were not able to influence PC1, resulting in lower nutritional gains than those observed in the control treatment.

In relation to the isolates that influenced only PC2, isolates LEM 33 Unrequited, LEM 17 *S. maltophilia* and LEM 05 Unrequited presented the highest values, demonstrating P absorption efficiencies of 14,021 mg g^{-1}, 17,021 mg g^{-1} and 14,275 mg g^{-1}, K of 75,417 mg g^{-1}, 72,596 mg g^{-1} and 72,374 mg g^{-1}, Ca of 40,267 mg g^{-1}, 38,092 mg g^{-1} and 39,255 mg g^{-1}, and S of 7,100 mg g^{-1}, 7,461 mg g^{-1} and 7,271 mg g^{-1} and the translocation efficiency of K 88.00 %, 84.71 % and 85.60 %, respectively. Isolates in the lower left and right quadrants could not demonstrate effects on PC2.

Some isolates, such as the rhizobacteria LEM 01 *R. pickettii*, LEM 07 Unrequited, LEM 19 *V. pantothenticus* and LEM 29 *B. cereus GCsubgroupB*, were unable to influence both PC1 and PC2, demonstrating the absence of increases in these related variables, and even allowing

lower results than those observed in the control treatment. In this way, it was possible to perceive, according to PCA, that the inoculation of rhizobacteria was different from the control treatment, mainly in relation to PC2, demonstrating that the isolates LEM 36 *S. maltophilia*, LEM 33 Unrequited, LEM 17 *S. maltophilia*, LEM 05 Unrequited and LEM 41 *B. cenocepacia* are the ones that most positively influence this axis. For this component, the control treatment demonstrated P absorption efficiency of 11,076 mg g-1, K of 47,251 mg g^{-1}, Ca of 31,553 mg g^{-1} and S of 5.2125 mg g^{-1} and K translocation efficiency of 79.82 %.

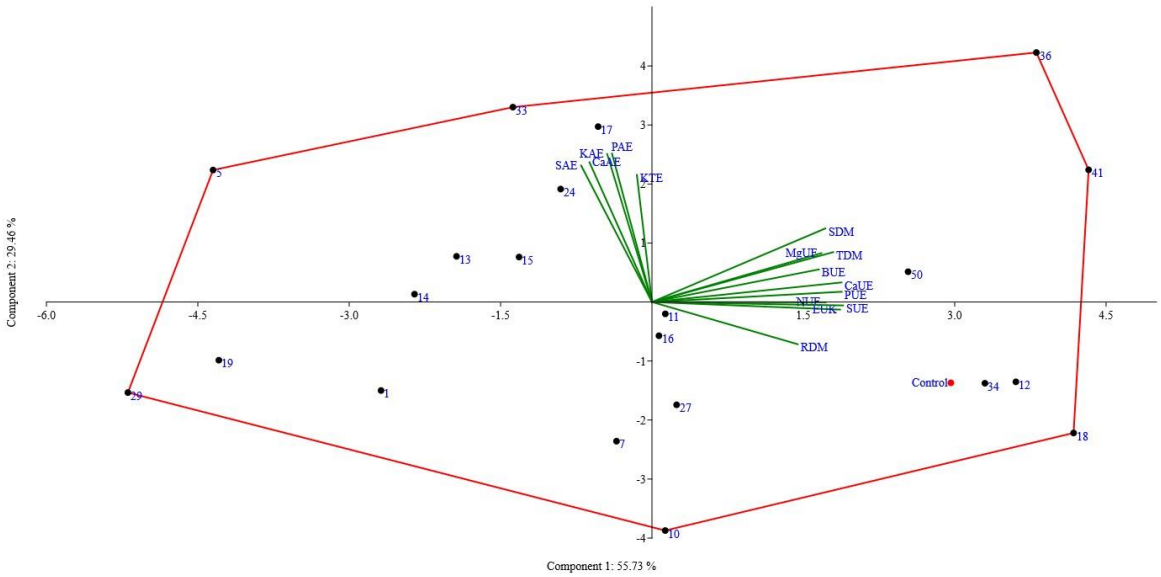

Figure 4. Principal component analysis of nutritional variables of clone I144 inoculated with rhizobacteria under water deficit. Principal component 1 and 2 explained 85.19% of the total data variance. Treatments that presented positive eigenvectors for PC1 and PC2 influence the variables presented.

4. DISCUSSION

4.1 Morphological characteristics of eucalyptus clones under water reductions

Among the isolates evaluated in this study, the LEM 50 *R. pickettii* was the only one that significantly promoted increases in all four assessed parameters: number of leaves, branching, stem diameter, and height of the VM01 hybrid clone. The LEM 14 *R. pickettii* rhizobacterium increased three of these parameters, excluding height. Root biomass was also higher in the VM01 clone when inoculated with LEM 01 *R. pickettii* (29.54 %), LEM 11 *P. validus* (23.86 %), LEM 13 *S. Kloosii* (38.06 %), and LEM 50 *R. pickettii* (31.25 %). Root development has been observed in various studies of plants inoculated with microorganisms, including eucalyptus species such as *E. grandis* (Teixeira *et al.,* 2007; Eke *et al.,* 2019). Some rhizobacteria have the ability to produce IAA, which induces root growth and/or increased

formation of lateral and adventitious roots (Vurukonda *et al.,* 2016). In situations of water stress, this characteristic can be advantageous as it allows the plant to explore a larger soil volume in search of water and nutrients, enabling a better absorption (Enebe; Babalola, 2018). In addition to the increased root biomass, several isolates also demonstrated significant increases in shoot and total biomass, with the LEM 50 *R. pickettii* isolate resulting in the highest increases compared to the non-inoculated clone, 66.5 %, and 54.07 %, respectively. Inoculating bacteria in water-stressed *E. grandis* can be beneficial as it promotes the formation of new leaves, increased biomass, height, and stem diameter (Chaín *et al.,* 2020). Non-inoculated VM01 eucalyptus plants under water deficit showed poorer development in various evaluated parameters, including the number of leaves, stem diameter, height, root biomass, shoot biomass, and total biomass when compared to some inoculated plants. Drought affects the plant, which can respond to the disturbance by reducing leaf size, stem elongation, root proliferation, as well as root and total plant biomass (Farooq *et al.,* 2009; Maseda, Fernández, 2016). In relation to clone I144 which was subjected to identical conditions and treatments as clone VM01, it was not possible to observe significant increases in RDM, SDM, or TDM. The absence of increases in biomass may be related to little or no influence of isolates on variables of PC1 (nutrient use efficiency). Different genotypes, such as VM01 and I144, can recruit different microorganisms, which can cause different results (Dastogeer et al., 2020).

4.2 Effect of rhizobacteria inoculation on eucalyptus nutrition under water reductions

When inoculated with rhizobacteria, the VM01 and I144 clones showed alterations in the macronutrients N, P, K, Ca, Mg, S, and the micronutrient B. These changes varied according to the eucalyptus genotype and the specificity of the isolates. Microorganisms can enhance the plant's nutritional through fixation, mineralization, and solubilization, supporting the nutritional data found in this study (Chandra *et al.,* 2021). In relation to the VM01 clone, isolates, LEM 14 *R. pickettii*, and LEM 50 *R. pickettii*, enabled the greatest increases in the total amount and/or utilization efficiency of all evaluated nutrients. However, for the I144 clone, rhizobacteria demonstrate a greater influence on P, K, Ca, S absorption efficiency and K translocation efficiency. The observed results demonstrate that rhizobacteria can help eucalyptus trees under water scarcity conditions. Plants under water stress present low nutrient absorption and nutrient utilization efficiency due to decreased transpiration rate (Farooq *et al.,* 2009). The increase in total N amount in clone VM01, can be attributed to the nitrogen-fixing capability of these bacteria, making it assimilable by the plants. Nitrogen can alleviate the effects of water stress by enhancing root vigor, increasing the production of osmoprotectants and antioxidant enzyme

activity, thereby reducing lipid peroxidation and preventing damage to the cell membrane (Zhou; Oosterhuis, 2012; Iqbal *et al.,* 2020). Some rhizobacteria have been shown to provide higher total P amount and it can be attributed to the ability of some rhizobacteria to convert insoluble phosphorus into soluble forms, thereby increasing its absorption and utilization by the plant (YU *et al.,* 2022). The increase in P can be advantageous in water deficit situations as it promotes root growth, allowing for more efficient water absorption and nutrients (Singh; Sale, 1998). The tendency in rhizobacteria to provide an increase in the macronutrient K was observed for both clones. The increase in potassium by some isolates can be explained by the mechanism of potassium solubilization by certain rhizobacteria. The potassium increase can be beneficial for the plant, as K is directly related to osmotic regulation under water stress conditions, leading to increased solute accumulation, reduced osmotic potential, and maintenance of turgor pressure (Hassan *et al.,* 2017). Potassium is primarily responsible for stomatal regulation and improved water use efficiency, allowing eucalyptus clones to perform vital functions with reduced water availability (Santos *et al.,* 2021). Therefore, increasing potassium amount in hybrid eucalyptus clones can be seen as an effective strategy to enhance their tolerance to water deficit conditions.

In addition to the macronutrient K, the micronutrient B is directly related to mechanisms that alleviate water stress in plants. However, water deficit leads to a reduction in its uptake by plants (Ahanger *et al.,* 2016). According to the results obtained, some rhizobacteria can increase the total amount and utilization efficiency of B. The inoculation of these microorganisms can be advantageous for plants under water deficit conditions, as boron promotes increased photosynthetic rate, membrane integrity, synthesis of indole-3 -acetic acid (IAA), and activity of antioxidant enzymes (Ahanger *et al.,* 2016). In Eucalyptus plants, it has been observed that boron mitigates the effects of drought by reducing stomatal conductance. *E. grandis* plants inoculated with bacteria under water deficit showed an early response of reduced stomatal conductance, occurring 7 days earlier compared to non-inoculated plants (Chaín *et al.,* 2020). In summary, boron is capable of enhancing plant adaptation to drought by stimulating early stress response mechanisms. However, it should be noted that the mitigation of drought effects by boron varies among genotypes (Pita-Barbosa *et al.,* 2016; Aydin *et al.,* 2019). As briefly mentioned, there seems to be a relationship between boron and IAA. All rhizobacteria studied in this work demonstrated the capacity to produce IAA *in vitro*, with LEM 14 *R. pickettii* exhibiting the highest production. Interestingly, LEM 14 *R. pickettii* also increased the total boron content, and the efficiency of use. It is hypothesized that boron fertilization leads to a decrease in IAA oxidase activity, thus increasing IAA content (Ayvaz *et al.,* 2012). Therefore,

it is suggested that there may be a possible, albeit indirect, influence between IAA-producing rhizobacteria and increased boron uptake by plants. However, this relationship needs to be explored and investigated in future studies.

Rhizobacteria have also been shown to have a positive influence on total Ca amount, Ca, S absorption efficiency, Ca, Mg, S utilization efficiency and Ca, Mg and S translocation efficiency. Water-soluble mineral nutrients such as calcium, magnesium, and sulfur also play important roles in mitigating the effects of water stress in plants, including osmoregulation, protein synthesis, and increased activity of antioxidant enzymes, among others (Gowtham *et al.,* 2022).

Our results indicate that rhizobacteria were more efficient in mitigating the effects of water limitation and promoting growth in clones that have greater drought tolerance, such as VM01, compared to the clone, I144 (Oliveira, 2021). During water deficit conditions, plants generally exhibit limited resource allocation and must allocate the available energy to different physiological processes. Since the plant's interaction with rhizobacteria requires energy investment, under stress conditions, the I144 clone may prefer to redirect part of its energy to respond to adverse conditions, which can affect its ability to interact with rhizobacteria. Therefore, the interaction between different genotypes and associated rhizobacteria under water deficit conditions, as well as their impact on the holobiont, needs to be explored and investigated in future studies. A deeper understanding of the interactions between eucalyptus gentotypes and rhizobacteria can provide high-quality seedlings that are tolerant to abiotic stresses

5. CONCLUSION

The results obtained in this study demonstrate that some rhizobacteria were effective in increasing nutrient content, absorption, utilization and translocation efficiency in inoculated clones compared to non-inoculated ones. The inoculation of rhizobacteria provided greater efficiency in clone VM01, as the isolates influenced the PC1 and PC2 variables, while in I144 clones, the isolates influenced PC2 only differently than the control treatment. Among the isolates studied, LEM 50 *R. pickettii* and LEM 14 *R. pickettii* showed higher efficiency in increasing biomass, macro, and micronutrient content, utilization efficiency and nutrient translocation in VM01 clones. In relation to clone I144, the LEM 36 *S. maltophilia* and LEM 41 *B. cenocepacia* isolates showed a high influence on PC2, efficiency of nutrient absorption and K translocation. However, for PC1, a proximity to the control treatment and the isolates was observed, demonstrating in lack of gains for these related variables. Based on the presented

results, it can be stated that plant growth-promoting rhizobacteria can increase nutrient amount, absorption efficiency, utilization efficiency and translocation efficiency in plants helping to mitigate the effects of water stress. However, it is important to highlight that the responses vary with the eucalyptus genotype.

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SUPPLEMENTARY MATERIAL

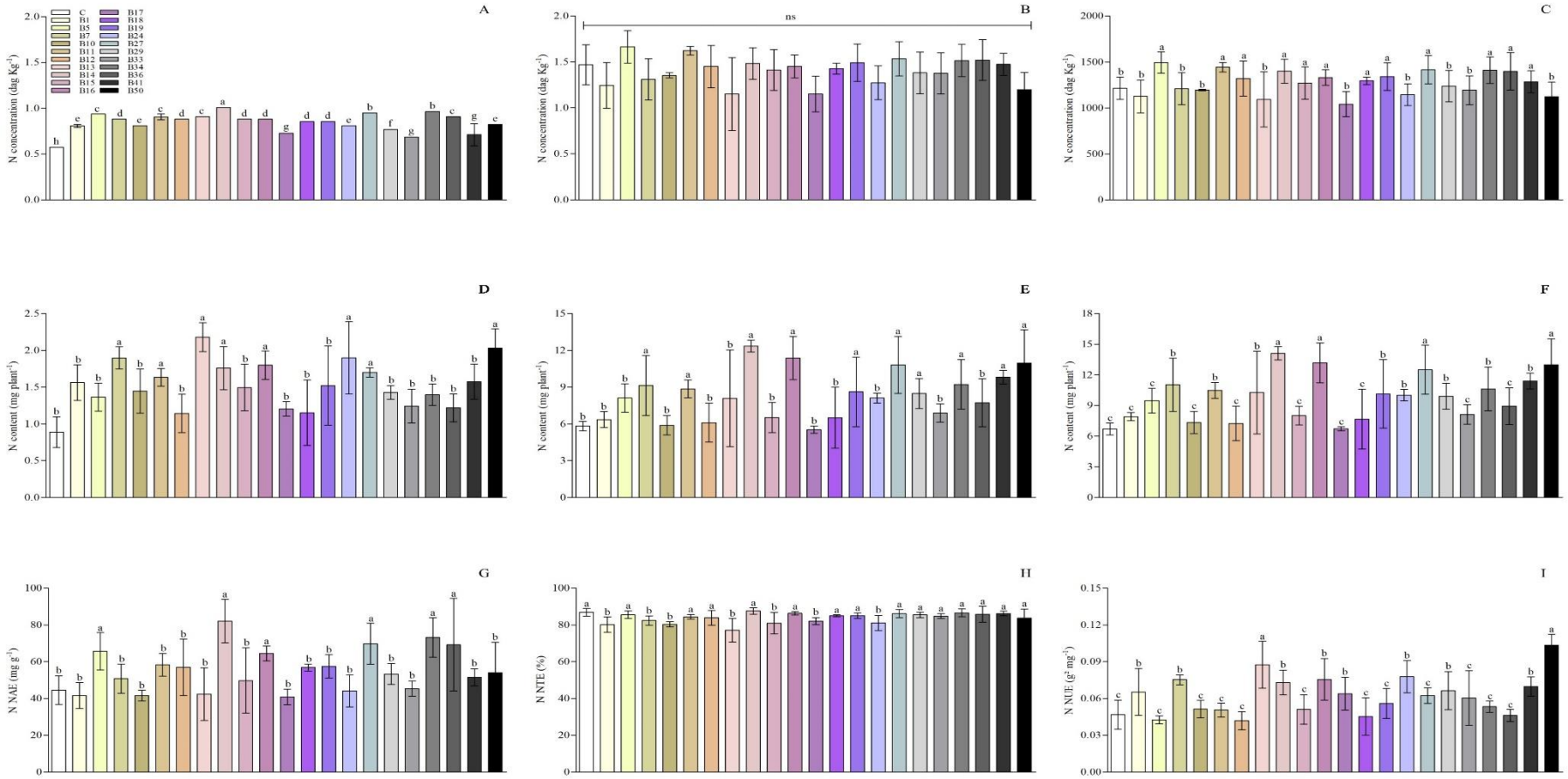

Figure S1. Nitrogen (N) in VM01 clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05), ns: not significant by the analysis of variance (ANOVA).

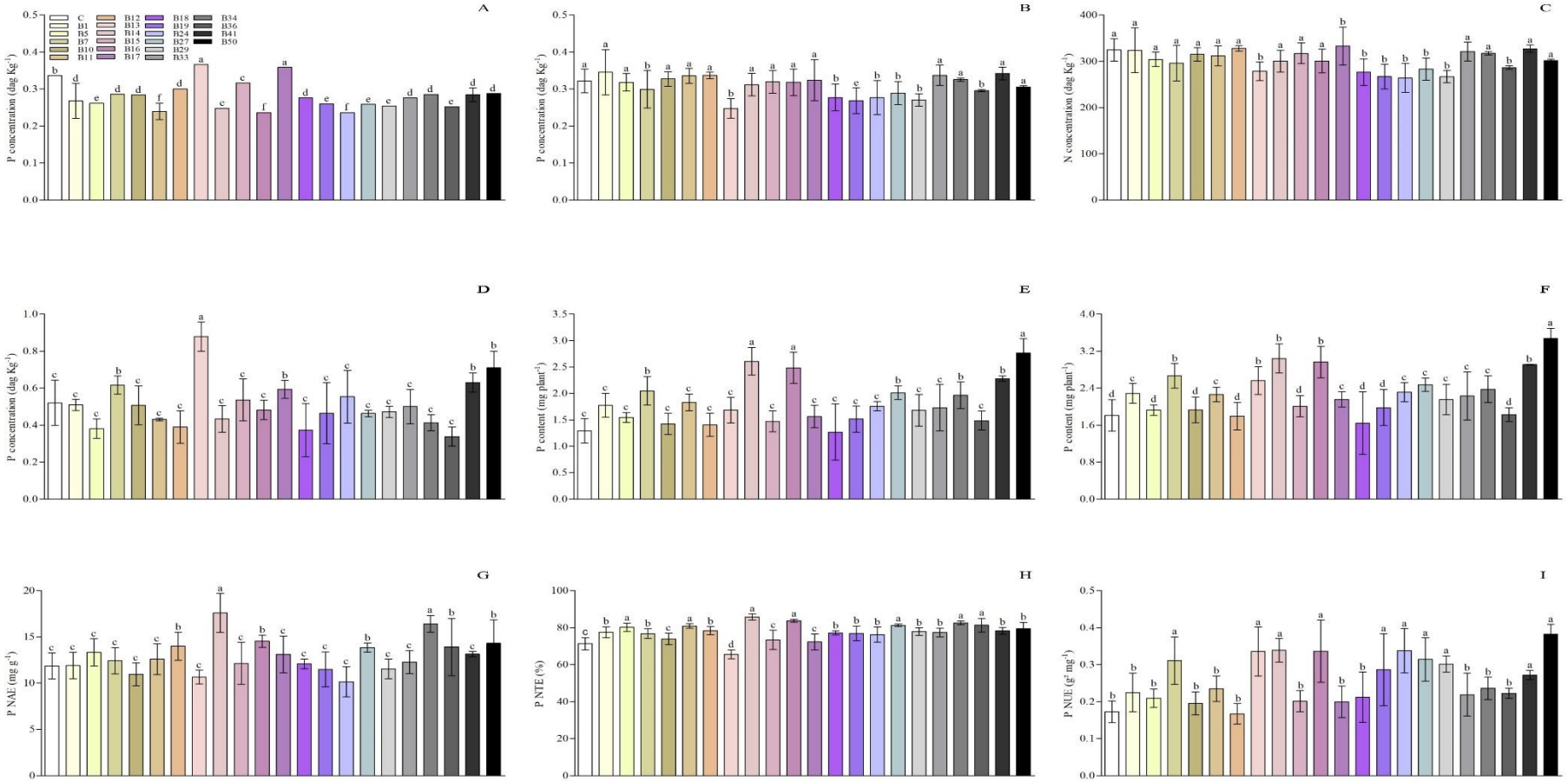

Figure S2. Phosphate (P) in VM01 clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05).

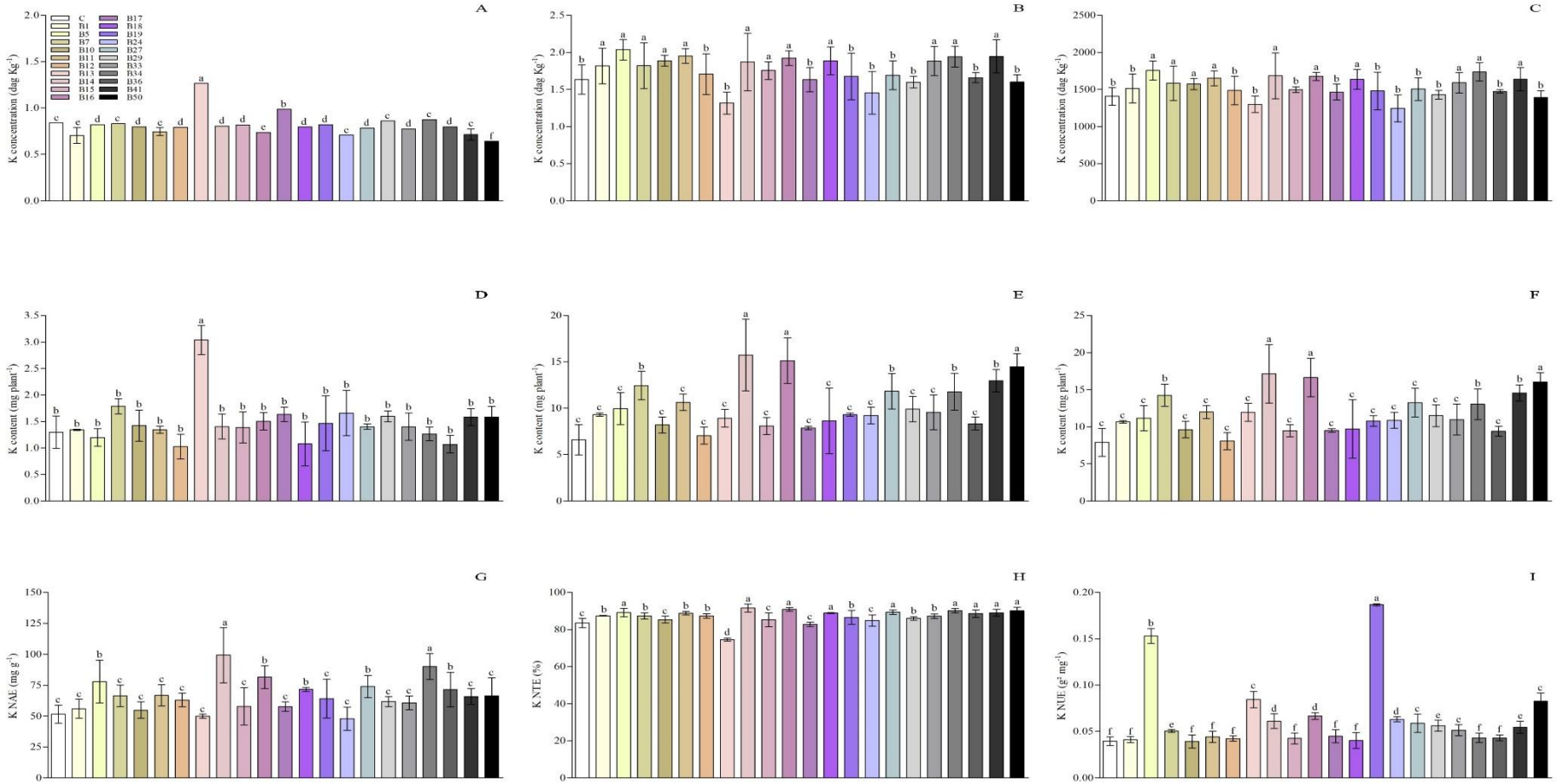

Figure S3. Potassium (K) in VM01 clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05).

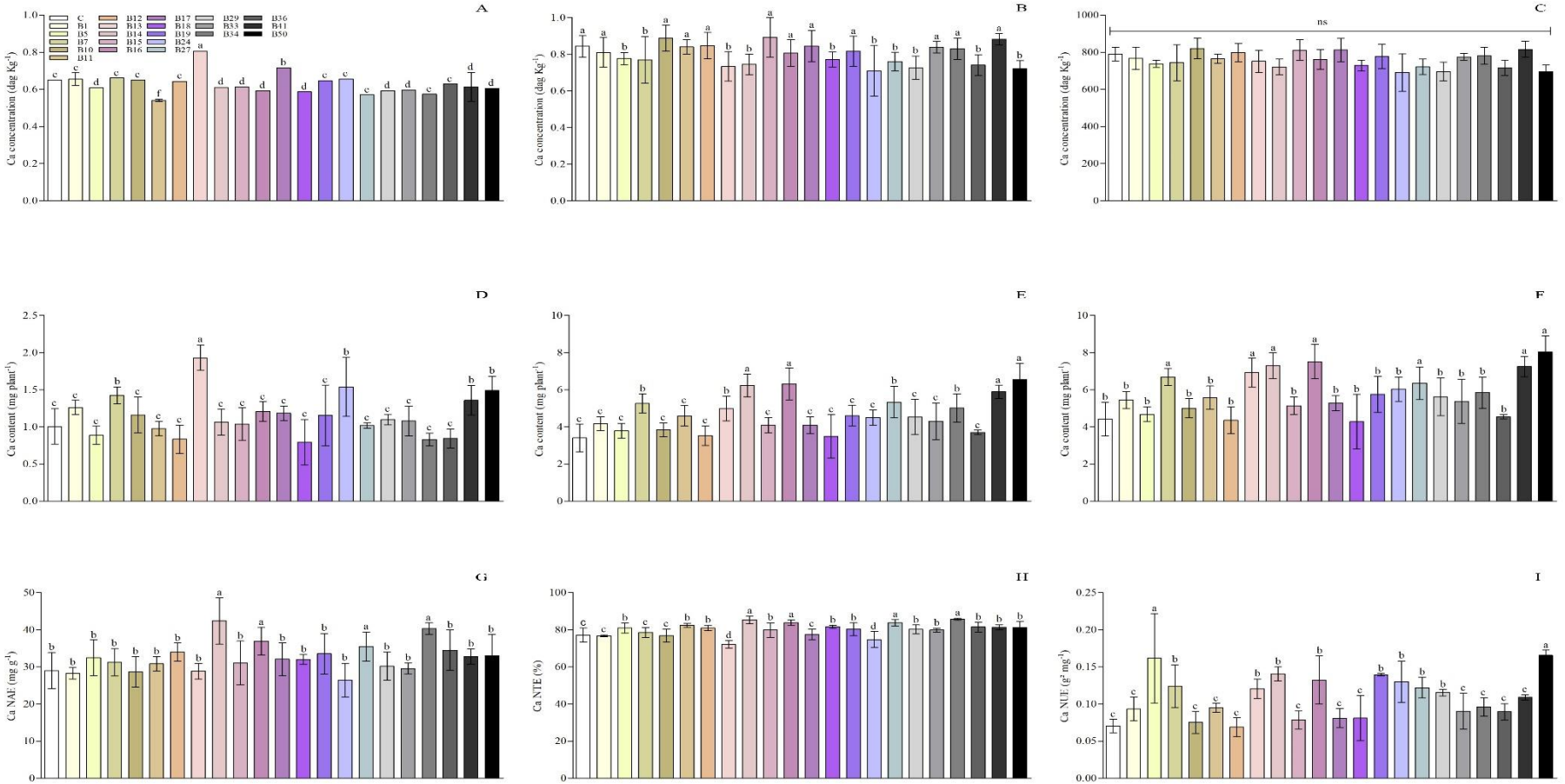

Figure S4. Calcium (Ca) in VM01 clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05), ns: not significant by the analysis of variance (ANOVA).

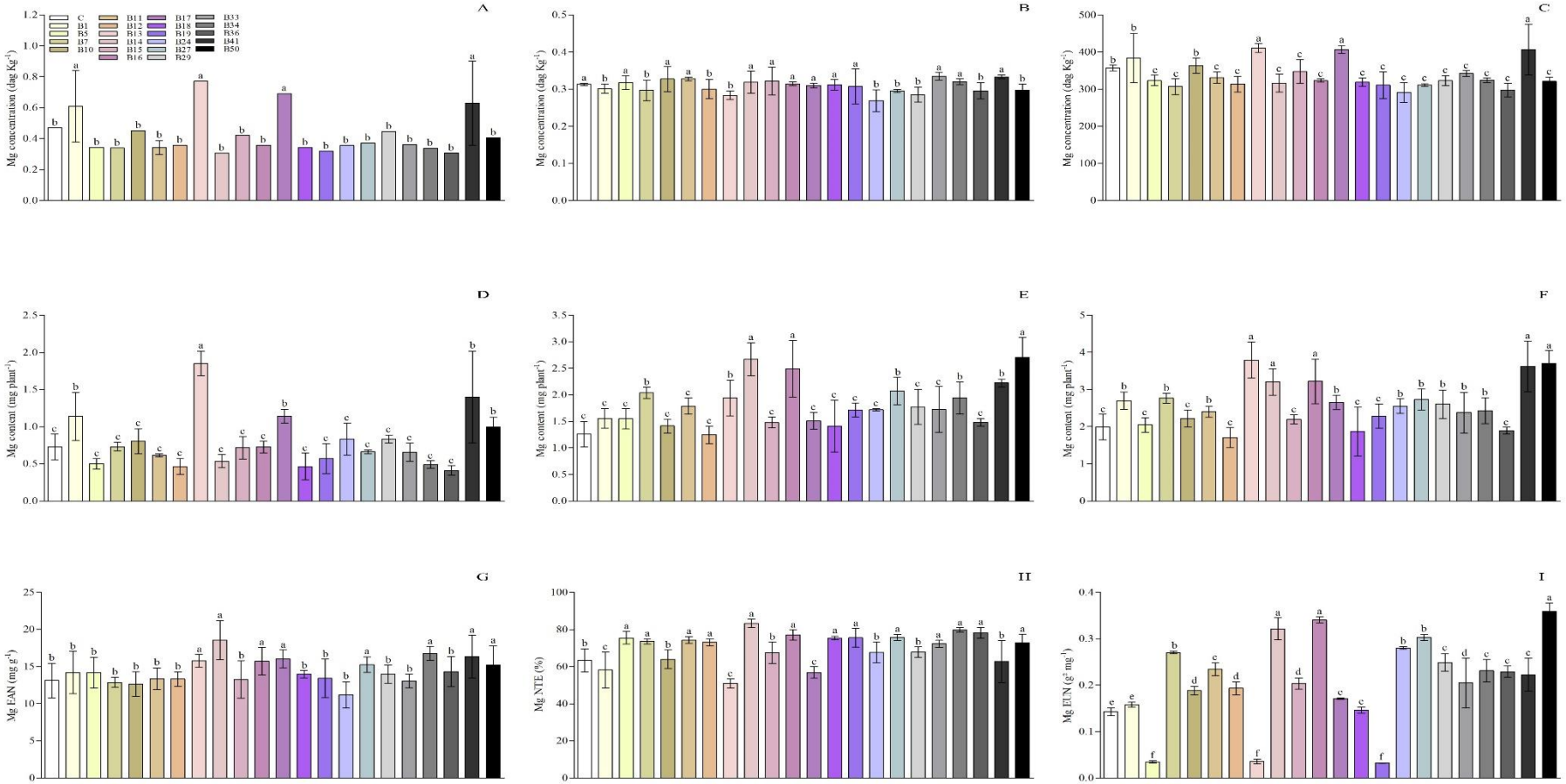

Figure S5. Magnesium (Mg) in VM01 clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05) or Scott-Knott test (p<0.10) for B.

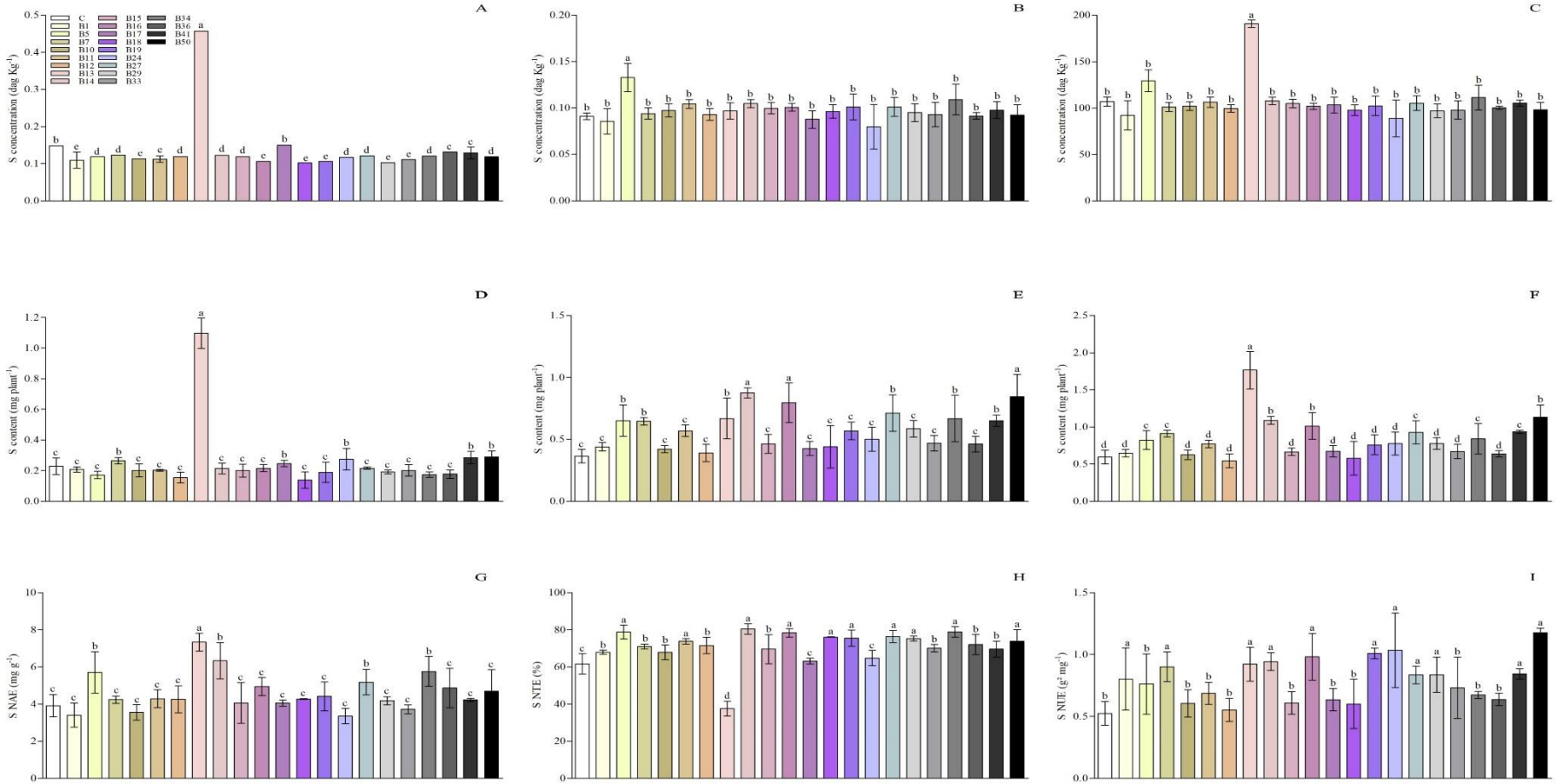

Figure S6. Sulfur (S) in VM01 clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05) or Scott-Knott test (p<0.10) for B.

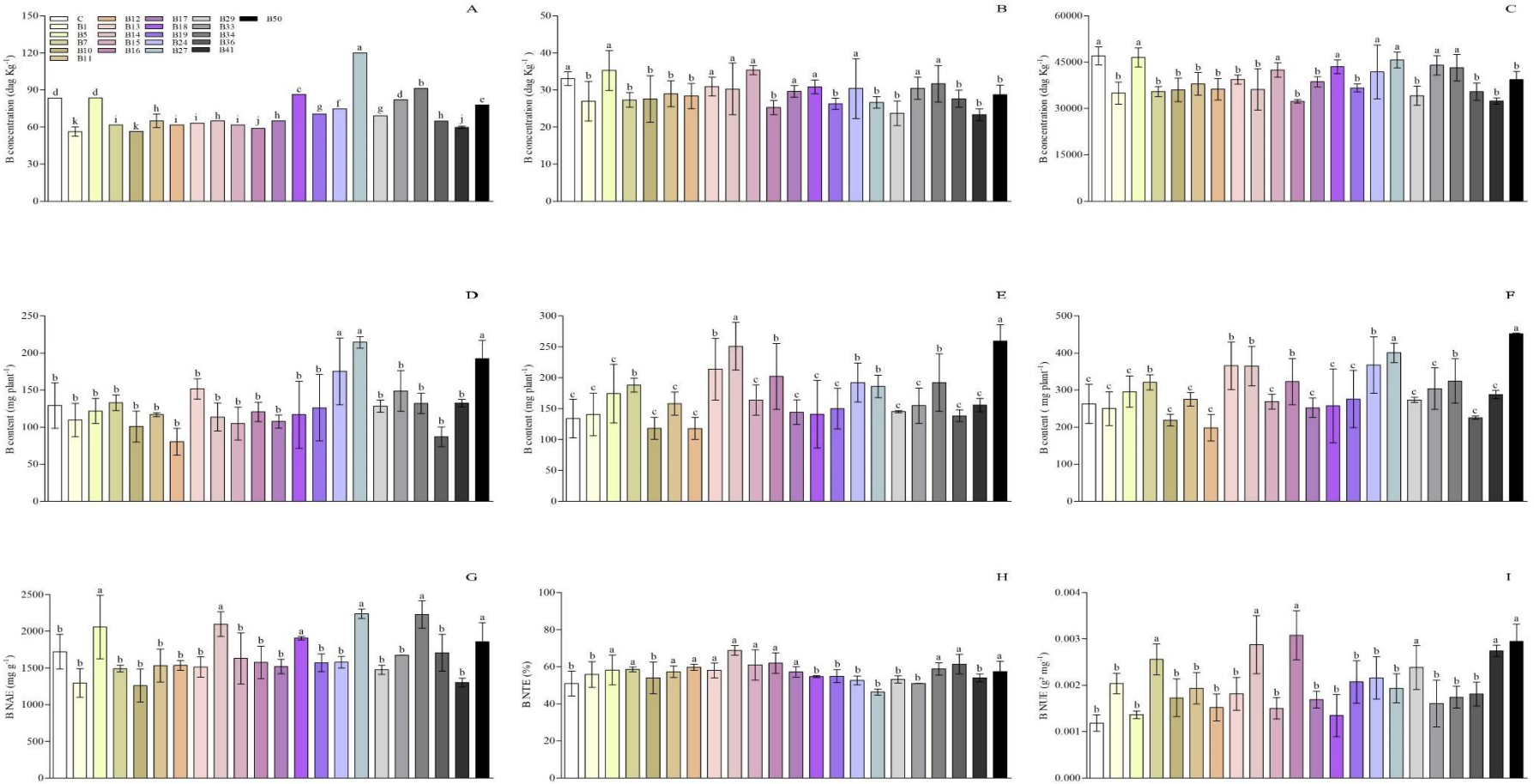

Figure S7. Boron (B) in VM01 clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05) or Scott-Knott test (p<0.10) for B.

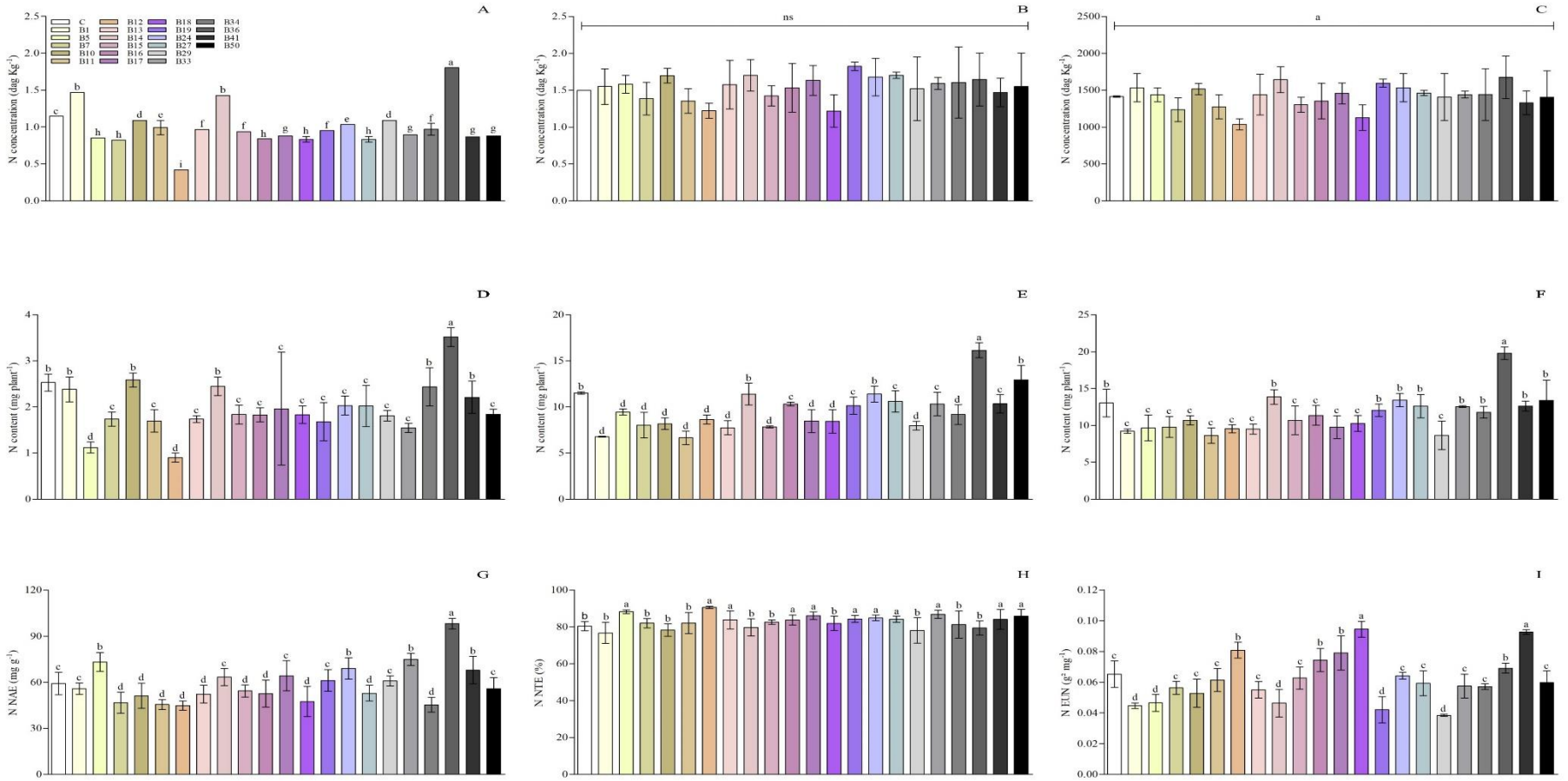

Figure S8. Nitrogen (N) in I144 eucalyptus clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05), ns: not significant by the analysis of variance (ANOVA).

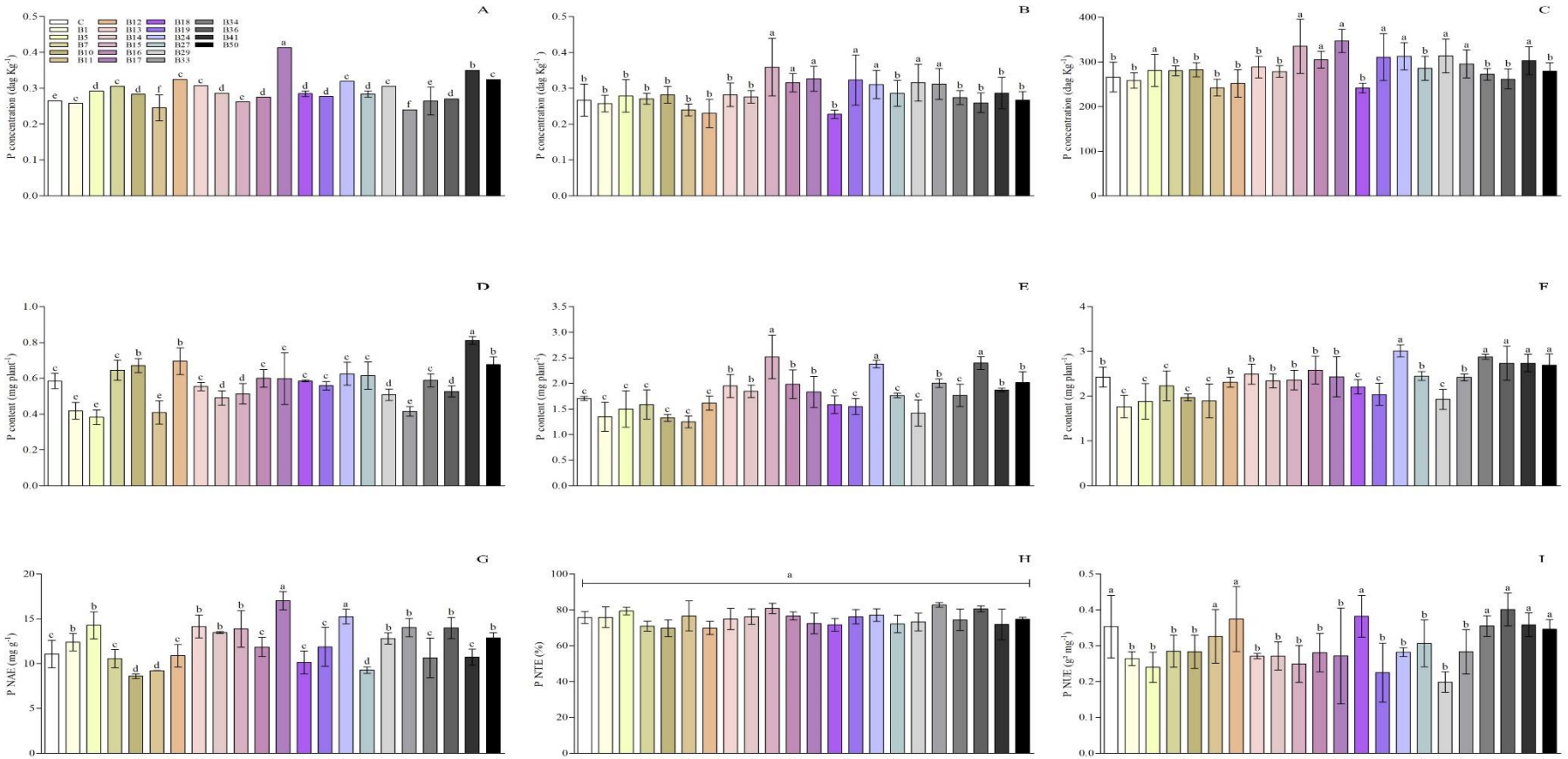

Figure S9. Phosphate (P) in I144 eucalyptus clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05).

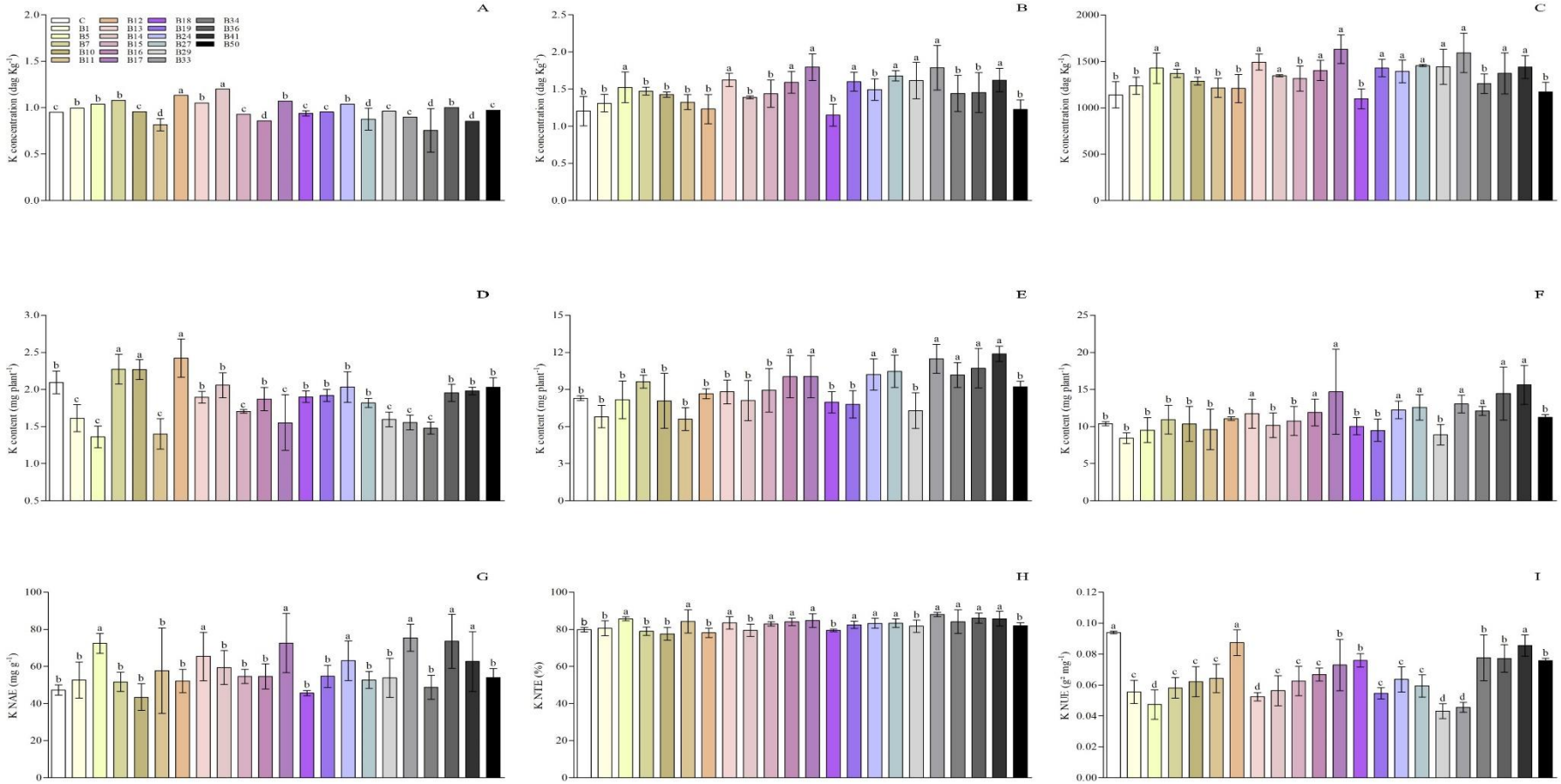

Figure S10. Potassium (K) in I144 eucalyptus clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05).

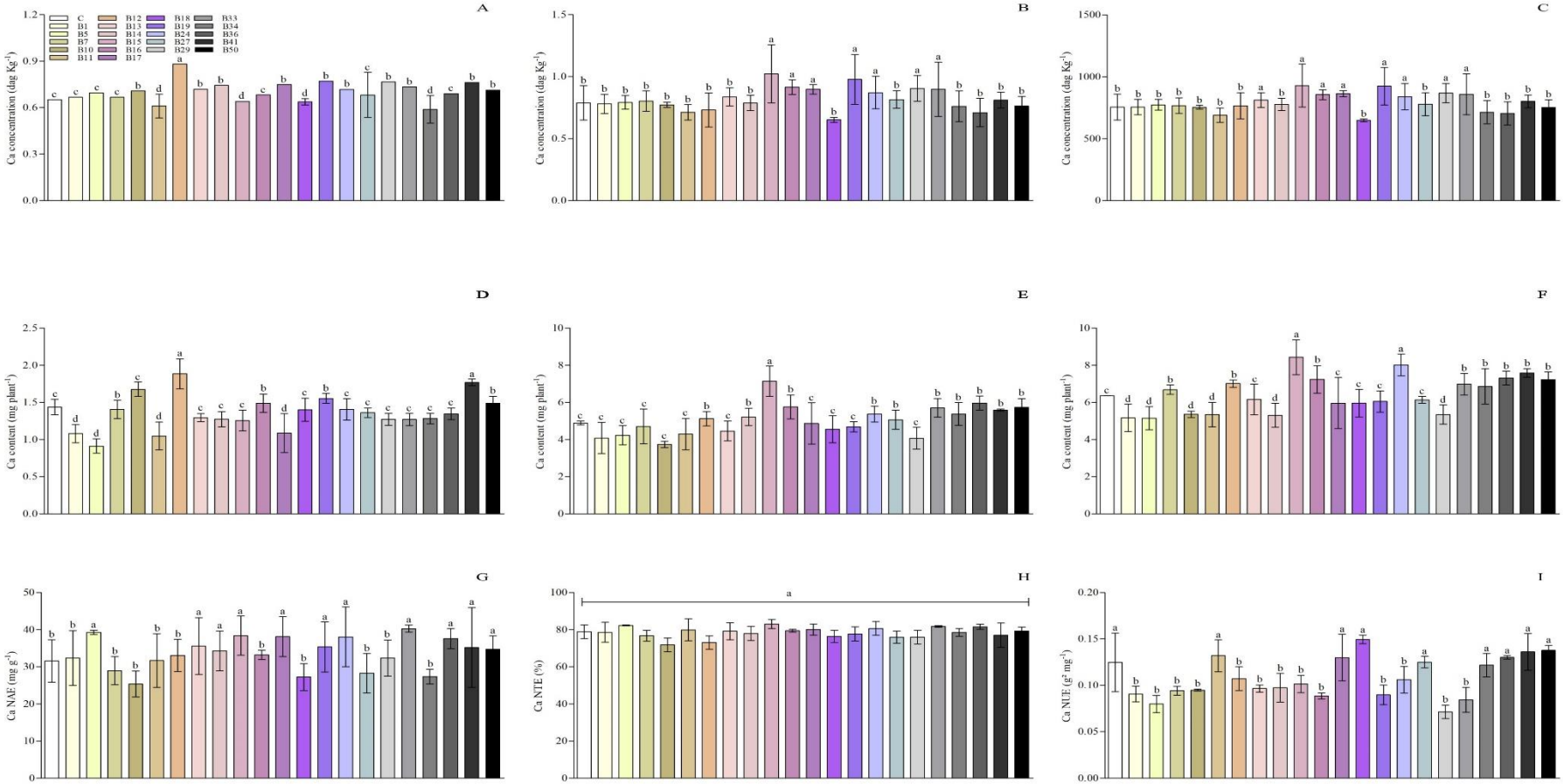

Figure S11. Calcium (Ca) in I144 eucalyptus clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05).

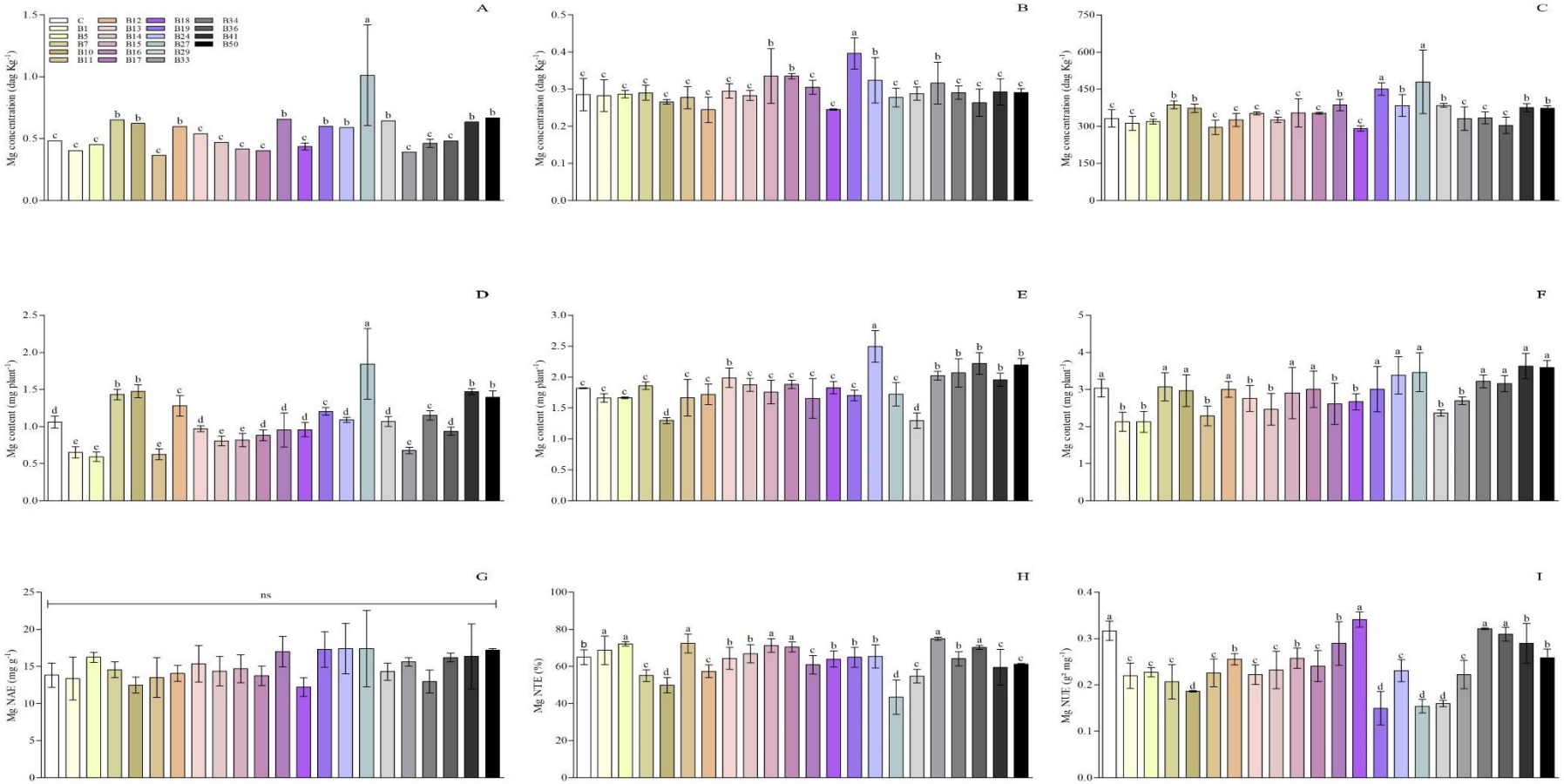

Figure S12. Magnesium (Mg) in I144 eucalyptus clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05), ns: not significant by the analysis of variance (ANOVA).

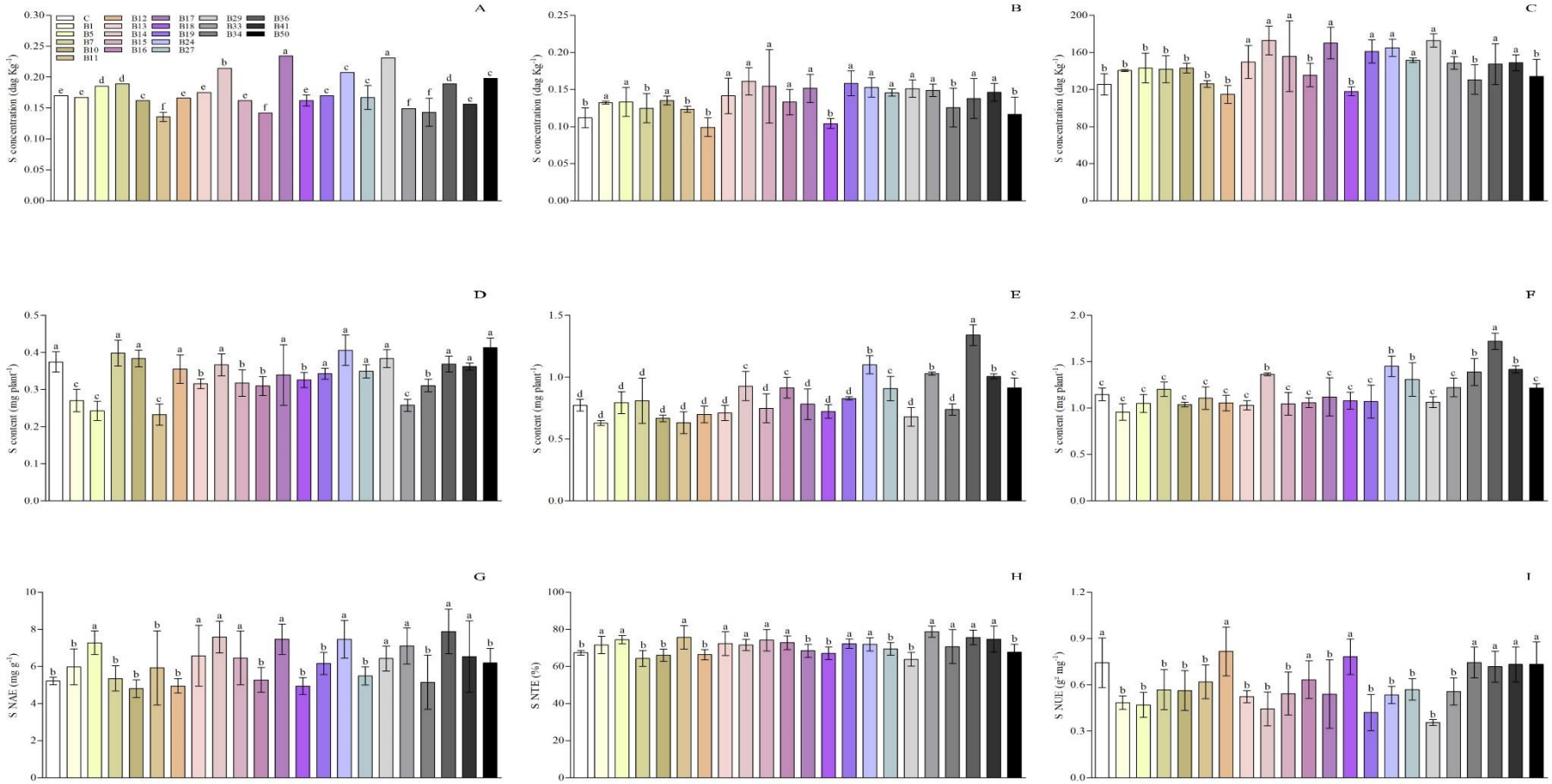

Figure S13. Sulfur (S) in I144 eucalyptus clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05) or Scott-Knott test (p<0.10) for B.

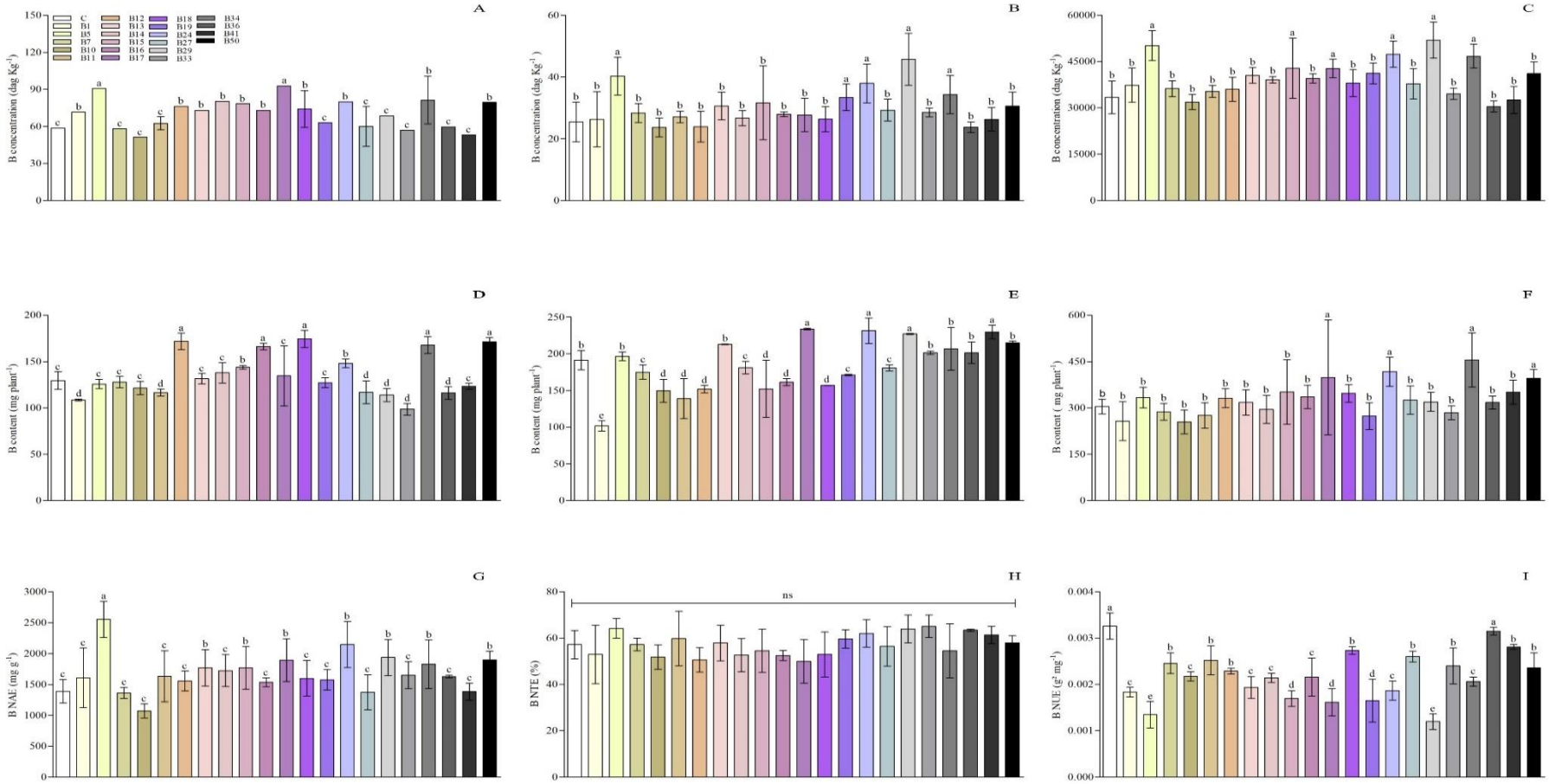

Figure S14. Boron (B) in I144 eucalyptus clones, inoculated and non-inoculated with rhizobacteria under constant irrigation reduction. Root concentration (A), shoot concentration (B), total concentration (C), root content (D), shoot content (E), total content (F), nutrient absorption efficiency (NAE) (G), nutrient translocation efficiency (NTE) (H), and nutrient use efficiency (NUE). The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.05), ns: not significant by the analysis of variance (ANOVA)

Chapter IV

POTENTIAL OF RHIZOBACTERIA IN IMPROVING ROOTING OF MINICUTTINGS OF CLONES VM01 (*Eucalyptus urophylla* x *Eucalyptus camaldulensis*) and I144 (*Eucalyptus urophylla* x *Eucalyptus grandis*) *IN GREENHOUSE ROOTING*

POTENTIAL OF RHIZOBACTERIA IN IMPROVING ROOTING OF MINICUTTINGS OF CLONES* VM01 (*Eucalyptus urophylla* x *Eucalyptus camaldulensis*) and I144 (*Eucalyptus urophylla* x *Eucalyptus grandis*) *IN GREENHOUSE ROOTING

ABSTRACT

Eucalyptus is the most planted tree in the world, mainly due to its rapid growth and wood quality. The propagation of clonal seedlings occurs through the minicutting technique. Root development is influenced by several intrinsic factors and biotic and abiotic conditions to which minicuttings are exposed. Therefore, understanding the rhizogenesis process is complex, but fundamental to increasing productivity. Auxin-producing PGPR can result in higher rooting percentages and seedling quality. **Aims** Here, we identified whether IAA-producing rhizobacteria, which promoted significant gains in root dry matter and nutrition in clones VM01 and I144 under water deficit, also provide greater efficiency in the rhizogenesis of minicuttings. **Methods** Minicuttings of clones VM01 and I144 were inoculated with rhizobacteria LEM 10 *Ralstonia pickettii*, LEM 13 *Staphylococcus kloosii*, LEM 41 *Burkholderia cenocepacia*, LEM 50 *Ralstonia pickettii* and consortium. The control treatment consisted of saline solution (0.85 % NaCl). The propagated plants were then placed for 35 days in a greenhouse rooting, where the rooting percentage and height were constantly evaluated. At the end of the experiment, the roots were separated from the shoots and preserved in 25 % alcohol for morphological evaluations using the WinRHIZO system. The experiment was conducted in randomized blocks (DBC), the data were subjected by the ANOVA test (p<0.10) and means were compared by Scott-Knott test. **Results** The rhizobacteria LEM 10 *R. pickettii* provided significant reductions in rooting percentage and greater callus formation in minicuttings of clone VM01. Furthermore, LEM 10 *R. pickettii* and LEM 41 *B. cenocepacia* demonstrated significantly greater proportions of medium and thick roots, resulting in a greater average root diameter, per LEM 10 *R. pickettii*. In minicuttings of I144, the root volume was significantly higher in treatments with LEM 10 *R. pickettii* and LEM 50 *R. pickettii*. It was observed that 35-day-old minicuttings form a higher proportion of very fine roots (Ø <0.5 mm). No significant gains were observed in terms of rooting percentage in both clones. **Conclusion** Thin roots allow greater absorption of water and nutrients, while the superior root volume provides greater capacity to explore the soil for resources. Efficient and quality root development is desirable in the seedling production process and fundamental for their success in the field. However, the efficiency of inoculation of microorganisms can be different depending on the genotype, stage of plant development and abiotic conditions.

Keywords: Roots; Rhizogenesis; Productivity; Forest nursery; Microorganisms.

1. INTRODUCTION

Brazil has the largest eucalyptus planted area in the world, 7.6 million hectares (2023). Eucalyptus plantations correspond to 76 % of all trees planted in the country, and are therefore the most cultivated species (IBÁ, 2023). The rapid growth and quality of the wood make these trees the most planted species in the world (Oberschelp *et al.,* 2022). They are an important source of raw material for a variety of forest products, such as cellulose, paper, panels and laminated floors, sawn timber and plywood, charcoal, firewood, and bioenergy (IBÁ, 2022). Consequently, planting eucalyptus plays a significant role in reducing the exploitation of tropical forests and preserving their related biodiversity (Cook *et al.,* 2016). Brazil stands out worldwide in the productivity of planted forests, due to investments in the sector that involve the development of more productive genotypes, technologies, professional training, disease management and pest control, soil fertilization, and Brazilian environmental conditions, which favor the success of plantations (IBÁ, 2022).

The production system for eucalyptus seedlings in forest nurseries occurs mainly through the minicutting technique, which provides better percentages and rooting quality, in addition to reducing the time it takes for seedlings to form. In minicutting, stems from ministumps are collected, staked in tubes and transferred to a greenhouse, where they remain for around 20 to 45 days for rooting, depending on the climatic conditions of the region, as well as the selected genotype (Xavier; Wendling; Da Silva, 2013). The success of root development is dependent on clone intrinsic factors such as; youthfulness, genetics, nutritional and water conditions of the plant of origin, as well as hormonal balance (Xavier; Wendling; Da Silva, 2013). In addition to these mentioned, the collection time, the chosen area of the ministump for the formation of the minicuttings, the oxidation reactions at the base of the cuttings, quality of the substrate and the abiotic conditions of the greenhouse (temperature, light and humidity) to which the minicuttings will be exposed also impact the success of rooting, (Xavier; Wendling; Da Silva, 2013). Therefore, rhizogenesis is a highly complex process and influenced by multiple factors that are not yet well understood, which can limit the productivity of forestry companies, especially when there is commercial interest in recalcitrant genotypes (Vilasboa; Da Costa; Fett-Neto, 2019).

The use of auxin-producing PGPR to induce rhizogenesis presents itself as an alternative to the use of synthetic auxins, in addition to presenting several mechanisms for promoting plant growth that can favor the plant in a broad spectrum (Shiomi; Accordi; Sabino, 2016). Inoculation of minicuttings with rhizobacteria can result in higher rooting percentages, root dry mass, better nutritional status and protection against pathogens, resulting in high quality

seedlings (Mafia *et al.,* 2007; Teixeira *et al.,* 2007). Efficient and quality root development is desirable in the forestry seedling production sector, and is also fundamental for the success of these seedlings in the field, especially in the face of abiotic stresses such as drought. It has been observed that the conditions provided during the production system affect the quality of the seedling and its initial performance in the field (Alves *et al.,* 2022). Therefore, the objective of this work was to evaluate whether IAA-producing rhizobacteria, which promoted significant gains in root dry matter and nutrition in clones VM01 and I144 under water deficit, also provide greater efficiency in the rhizogenesis of minicuttings.

2. MATERIAL AND METHODS

The experiment was conducted at the Forest Nursery of the Forest Engineering Department (DEF) (20°46'30.6"S 42°52'37.1"W) and the Microbial Ecology Laboratory (LEM) of the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil. In this study, four rhizobacteria isolated from the rhizosphere of *Eucalyptus grandis* maintained in the LEM - UFV microorganism bank were utilized. The rhizobacterial isolates were obtained from the UFV Forestry sector, from eight-year-old plants (S 21° 01 741'; W 042° 34 258'), from the district of Santo Antônio do Rio Preto, Miraí, MG, with one-year regrowth (S 21° 01 638'; W042° 34 156') and in the same district, from eight-year-old trees (S 21° 01 698 '; W042° 33 842') (Martins Filho, 2021). The isolates selected for the rooting experiment were chosen based on the results obtained from IAA production, described in chapter II and mainly from the root dry mass (RDM) of the VM01 (*Eucalyptus urophylla* x *Eucalyptus camaldulensis*) and I144 (*Eucalyptus urophylla* x *Eucalyptus grandis*) clones, as described in Chapter III. Two isolates that produced the highest RDM were selected for clone VM01 (LEM 13 *S. kloosii* and LEM 50 *R. pickettii*), and two for clone I144 (LEM 10 *R. pickettii* and LEM 41 *B. cenocepacia*).

2.1 Compatibility between rizobacterial isolates

The isolates LEM 10 *R. pickettii*, LEM 13 *S. kloosii*, LEM 41 *B. cenocepacia*, and LEM 50 *R. pickettii* were assessed *in vitro* for their compatibility capabilities using the spread plate method, adapted from Molina-Romero *et al.* (2017). The isolates were cultured in TSB broth at 28 °C, with constant agitation at 150 rpm for 48 hours. After growth, a 20 µL aliquot of bacterial suspension from a specific isolate was spread on a Petri dish (90 x 15 mm) containing TSA medium. Subsequently, an aliquot of 20 µL of the remaining isolates was dripped onto the previously inoculated TSA plate. All four isolates were subjected to surface spreading, altering

their arrangement in the test. The plates were incubated at 28 °C for 96 hours to observe inhibition halos.

2.2 Rhizobacterial inoculants

The impact of rhizobacteria on the rooting and biomass of the VM01 and I144 clones was assessed through six distinct treatments. Formulations were prepared, including individual isolates (LEM 10 *R. pickettii*, LEM 13 *S. kloosii*, LEM 41 *B. cenocepacia*, and LEM *50 R. pickettii*), as well as a rhizobacterial consortium comprising all four isolates together. The control treatment consisted solely of saline solution (0.85 % NaCl) without bacterial cells. Isolates LEM 10 *R. pickettii*, LEM 13 *S. kloosii*, LEM 41 *B. cenocepacia*, and LEM *50 R. pickettii* were cultured in TSB medium at 28 °C, with constant agitation at 150 rpm for 48 hours. After growth, the bacterial suspension of each isolate was transferred to sterile 50 mL Falcon tubes and centrifuged at 10, 000 rpm for 10 minutes. The pellet of each isolate was resuspended in new sterile 50 mL Falcon tubes containing saline solution (0.85 % NaCl) to achieve an optical density ($O.D._{600\ nm}$) of 0.6. The preparation of the rhizobacteria consortium followed the same cultivation and centrifugation steps. Each rhizobacterial isolate was adjusted to an $O.D._{600\ nm}$ of 0.6, and their respective pellets were resuspended in saline solution (0.85 % NaCl) in the same 50 mL Falcon tube.

2.3 Assembling the Rooting Experiment

Two commercial hybrid clones of eucalyptus were utilized: VM01 (*E. urophyl*la x *E. camaldulensis*) and I144 (*E. urophylla* x *E. grandis*). Clonal propagation of these clones was conducted at the forest nursery (DEF-UFV) using the mini-cutting technique (Assis, 2005). Each treatment consisted of 30 plants, and therefore, 360 mini-cuttings measuring between 5 and 8 cm in height with 2 pairs of leaves, with a 50 % reduction in leaf area, were prepared.

Before placing the mini-cuttings in the tubes, the five treatments containing rhizobacteria, as well as the control treatment, were inoculated by immersing on the stem of the clones. Thus, each mini-cutting was submerged in the bacterial solution for 10 seconds before being placed in plastic tubes with a volume of 55 cm^3. The tubes containing commercial substrate Carolina Soil, class XVI (composed by peat, vermiculite and limestone pH of 5.5 and electric conductivity of 0.7 mS/cm) and fertilized with 6.0 kg m^{-3} of simple superphosphate and 3.0 kg m^{-3} of Osmocote®. After the cutting, 1 mL of the same rhizobacteria used in the immersion was inoculated into the substrate near the minicutting's stem. The control treatment consisted only of saline solution (0.85% NaCl) and was conducted as description above. The

propagated plants were then placed for 35 days in a greenhouse rooting (20°46'30.6"S 42°52'37.1"W) constructed with an antechamber with aphid-proof screen and external shading screen (50 %), and an irrigation system with fogger nozzles (NETAFIM®) providing a flow rate of 7.5 L h^{-1} (Figure 1). Temperature and humidity variations were recorded by Datalogger sensors, which registered average temperatures of 26.7 °C and humidity of 90.5 % (Figure 2A and 2B).

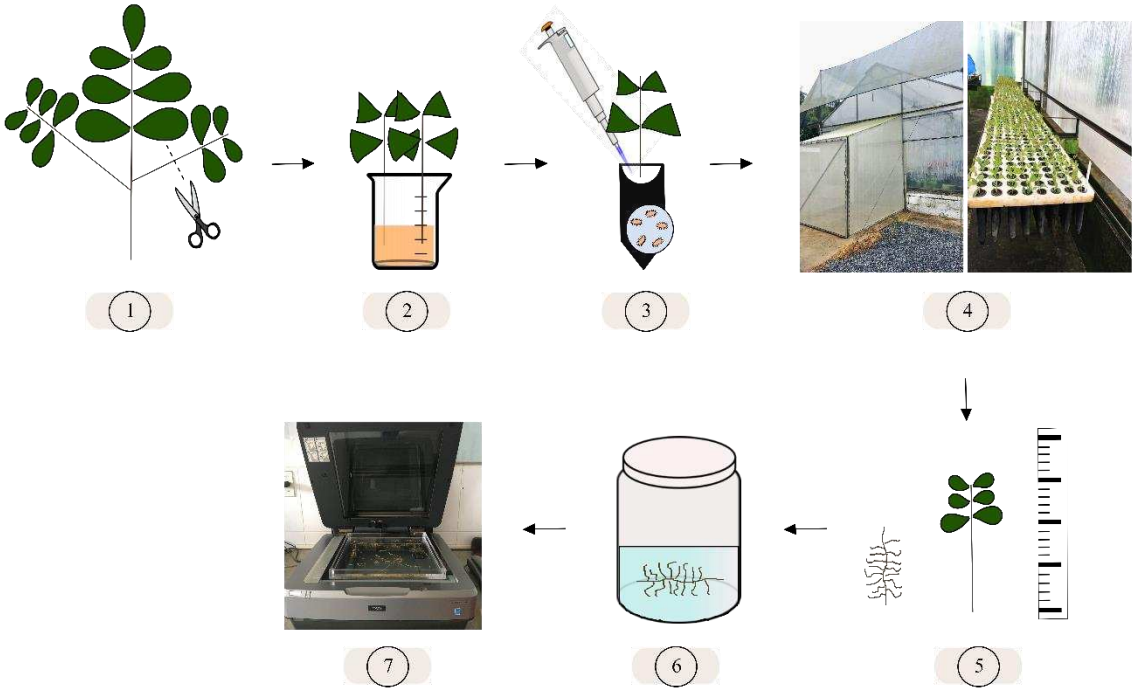

Figure 1. Scheme of the methodology for inoculating rhizobacteria in hybrid clones VM01 and I144 for rooting induction. (1) Minicuttings of clone VM01 and I144 between 5 - 8 cm tall with two pairs of halved leaves are produced from ministumps. (2) Minicuttings are submerged in rhizobacteria solutions, making up six different treatments: LEM 10 *R. pickettii*, LEM 13 *S. kloosii*, LEM 41 *B. cenocepacia*, LEM 50 *R. pickettii* and Control (NaCl 0.85 %). (3) Reinforcement inoculation is carried out by adding 1 mL of rhizobacterial and Control solutions at the interface of the stem with the substrate. (4) The mini stumps are taken to a greenhouse rooting and kept for 35 days, under frequent rooting and height assessments. (5) At the end of the experiment, the area and the root are separated and measurements of root length, height and callus observations are carried out. (6) The roots are transferred to pots with 25% alcohol. (7) Root morphological characterization is performed using the WinRHIZO system with a professional Epson XL 10000 scanner.

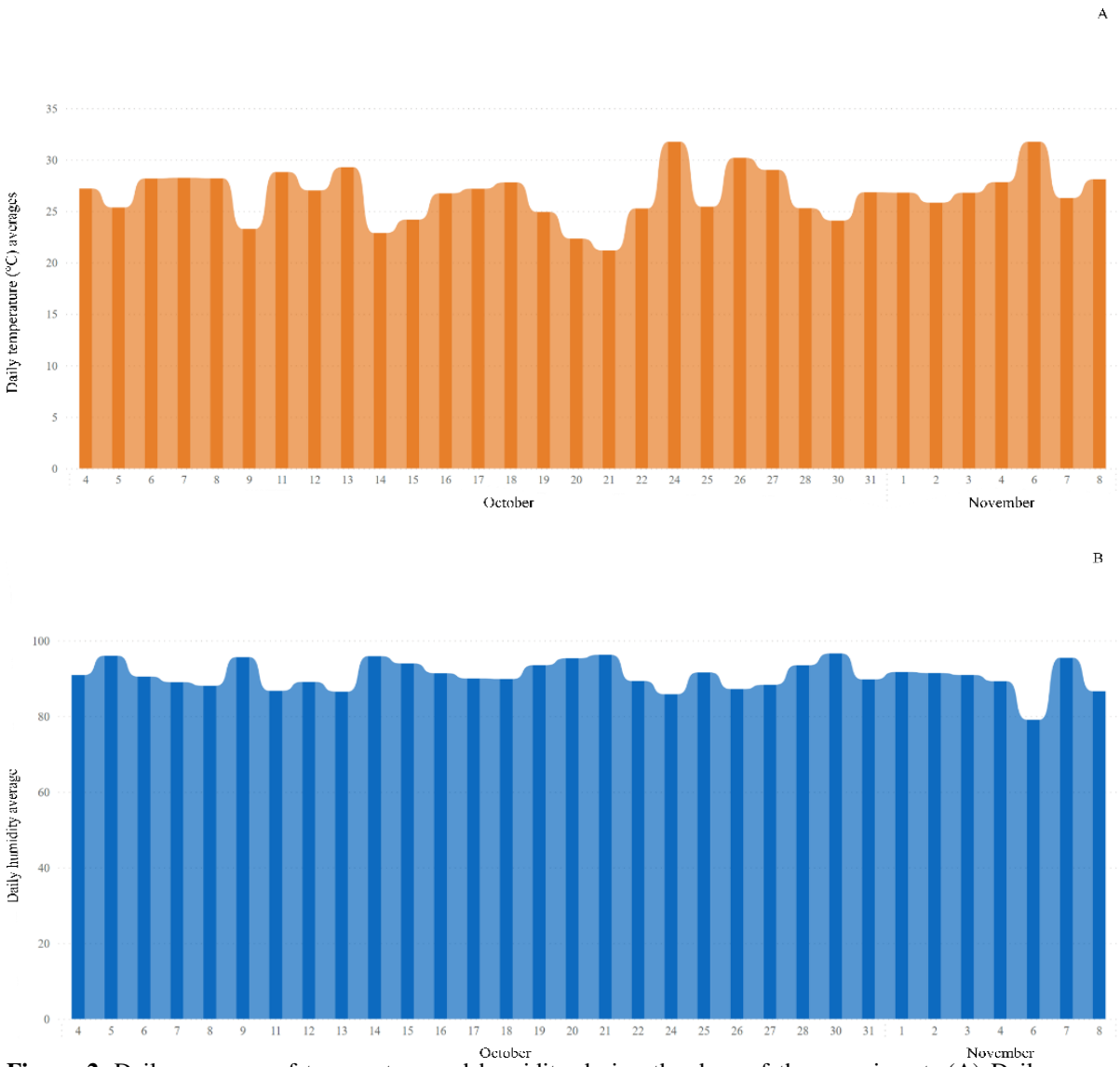

Figure 2. Daily averages of temperature and humidity during the days of the experiment. (A) Daily average temperatures, (B) daily average humidity.

2.4 Determination of rooting time in a greenhouse rooting

During the 35 days in the greenhouse rooting, the root development of the minicuttings at ages 7, 14, 19, 21, 26, 28, 30, 33 and 35 was evaluated by visualizing the opening at the lower end of the tubes. During the experiment, heights were evaluated in minicuttings aged 14, 21, 28 and 35 days.

2.5 Determination of root variables

At the end of the experiment, the aerial part of the root was separated and washed in running water to remove the substrate. Subsequently, the stems, the height of the aerial part and the root length were measured and the presence of calluses and rooted roots was verified, but

not visible in tubes. The aerial part of the plants was placed in a paper bag and subjected to an oven (70 °C) until constant weight to determine the shoot dry mass (SDM). The roots were transferred to plastic pots containing 0.25 % alcohol and stored in a refrigerator for subsequent morphological assessments using the WinRHIZO Pro 2009 system with an Epson XL 10000 professional scanner equipped with an additional light unit (TPU). The characteristics analyzed by the WinRHIZO system were: length (cm), root volume (cm^3), projected area (cm^2), surface area (cm^2), average diameter (mm) and the distribution of root length by diameter class, which was transformed into a percentage for interpretation purposes. Subsequently, the roots were stored in a paper bag and dried in an oven at 70 °C until constant weight to determine root dry mass (RDM).

2.6 Statistical analysis

The experiment was conducted using a randomized block design (DBC), with 6 replications. Each block was composed of 5 experimental units for each treatment and clone, resulting in a total of 30 plants per treatment. The data was subjected to analysis of variance (ANOVA), and when significant (p<0.10), the treatment means were compared using the Scott-Knott procedure using the Software R and ExpDes package (Ferreira *et al.,* 2013). The construction of the graphs was carried out using GraphPad Prism 5, Power BI and the R software, ggplot2 package. The illustrations were edited using InKscape.

3. RESULTS

3.1 Compatibility test between rhizobacterial isolates

The isolates selected for the consortium formulation (LEM 10 *R. pickettii*, LEM 13 *S. kloosii*, LEM 41 *B. cenocepacia*, and LEM *50 R. pickettii*) did not show incompatibility with each other in *in vitro* tests, making it possible to use and the development of products that contain the four rhizobacteria in the same solution (Figure 3). Isolates that are incompatible with each other show an inhibition halo.

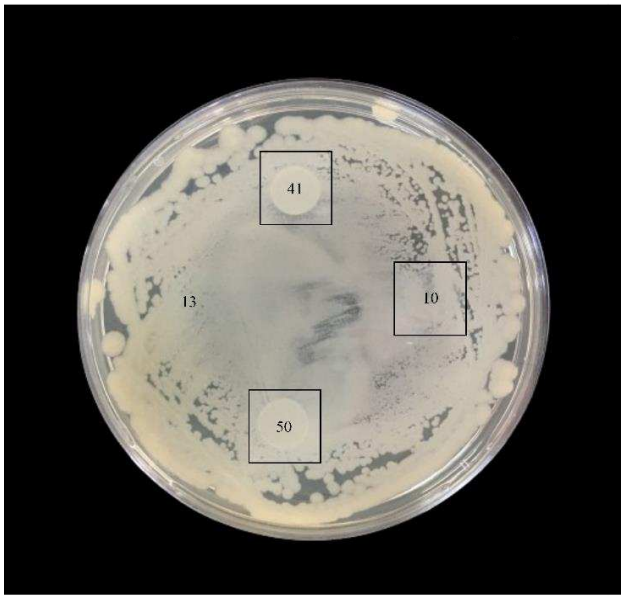

Figure 3. Compatibility test between rhizobacterial isolates. Rhizobacteria defined for the rooting test are compatible with each other.

3.2 VM01 Clone

3.2.1 Percentage and rooting efficiency of minicuttings

Minicuttings of VM01 clones had rooting in tubes after 14 days in a greenhouse rooting, for all treatments (Figure 4). At 14 days of age, LEM 50 *R. pickettii* promoted rooting of 11.67 %, Control treatment of 10.83 %, LEM 13 *S. kloosii* of 10 %, LEM 10 *R. pickettii*, LEM 41 *B. cenocepacia* and the consortium of 3.33 %. Most treatments provided rooting up to 33 days of age, with the exception of minicuttings treated with LEM 13 *S. kloosii* and LEM 41 *B. cenocepacia* which demonstrated rooting up to 35 days. On 21 day the treatments resulted in rooting of 61.67 % LEM 50 *R. pickettii*, 39.72 % control, 36.39 % LEM 13 *S. kloosii*, 33.33 % LEM 41 *B. cenocepacia*, 24.72 % Consortium and 15.83 % LEM 10 *R. pickettii*, no there are statistical differences. Minicuttings aged 26 and 30 days demonstrated statistical differences regarding rooting percentage, being grouped into a, b and c according to Scott – Knott (p<0.10). At 26 days of age, the minicuttings with a statistically higher rooting percentage were those treated with LEM 50 *R. pickettii* (80 %) and control (66.11 %). The rhizobacteria LEM 13 *S. kloosii*, consortium and LEM 41 *B. cenocepacia* resulted in a rooting percentage of 49.44 %, 47.22 % and 46.39 % respectively. The LEM 10 *R. pickettii* isolate provided a significantly lower percentage of rooted minicuttings, 18.52 %. At 33 days of age, no statistical differences were observed in the percentage of rooting between the 5 treatments: LEM 50 *R. pickettii* (87.5 %), control (74.44 %), LEM 13 *S. kloosii* (62.5 %), LEM 41 *B cenocepacia* (62.22 %),

consortium (60.28 %). Only minicuttings inoculated with LEM 10 *R. pickettii* (27.39 %) showed a significantly lower rooting percentage than the others.

At the end of 35 days, visible rooting was obtained in the tube of 87.50 % of minicuttings when treated with LEM 50 *R. pickettii*, 74.44 % of minicuttings in the control treatment, 66.39 % of minicuttings in the treatment with LEM 41 *B. cenocepacia*, 62.50 % of minicuttings with LEM 13 *S. kloosii*, 60.28 % of minicuttings in the consortium and 27.39 % of minicuttings with LEM 10 *R. pickettii*. Treatment with the rhizobacteria LEM 10 *R. pickettii* statistically provided the lowest number of rooted clones. Callus formation was observed in all treatments, however minicuttings treated with LEM 10 *R. pickettii* resulted in a higher percentage of callus, 55.83 %, while LEM 50 *R. pickettii* resulted in lower formation, 8.33 % (Figure 5A). The main factor in the minicuttings not rooting was due to the formation of calluses and not due to slow development or length not visible in the tube (Figure 6).

Total rooting, considering visible and non-visible rooting in the tube, is observed in figure 5B. A rooting rate of 91.67 % was obtained in the treatment with LEM 50 *R. pickettii*, 82.78 % Control, 70 % in the consortium, 66.83 % LEM 41 *B. cenocepacia* and 65.83 % LEM 13 *S. kloosii*. Minicuttings inoculated with LEM 10 *R. pickettii* statistically showed the lowest percentage of rooted seedlings, 28.33 %.

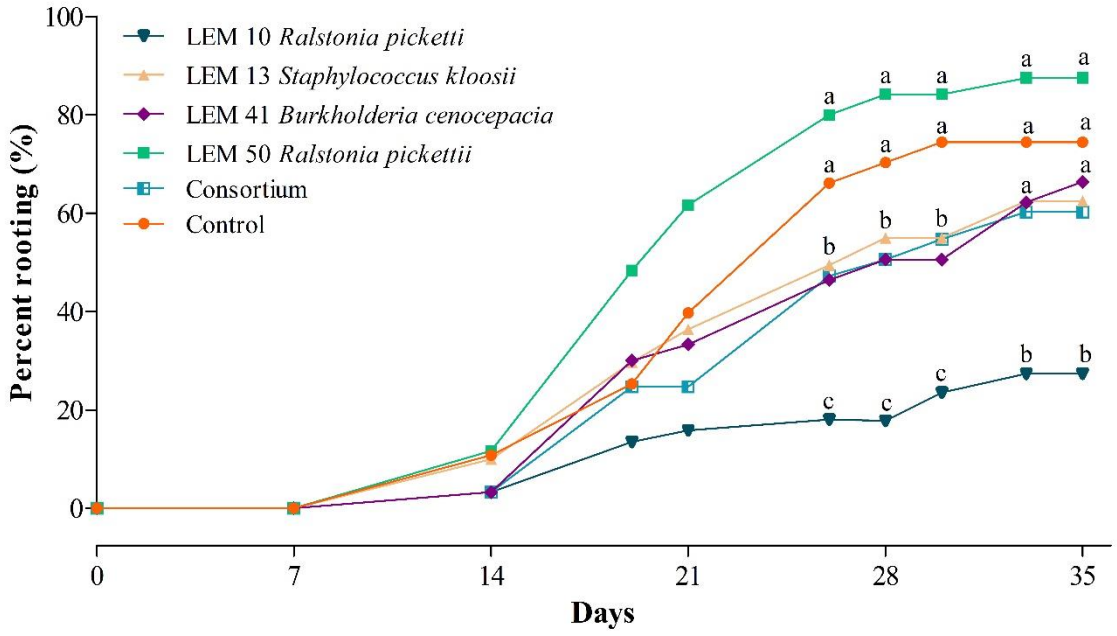

Figure 4. Percentage of rooting of VM01 clone minicuttings inoculated with different treatments over 35 days. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10).

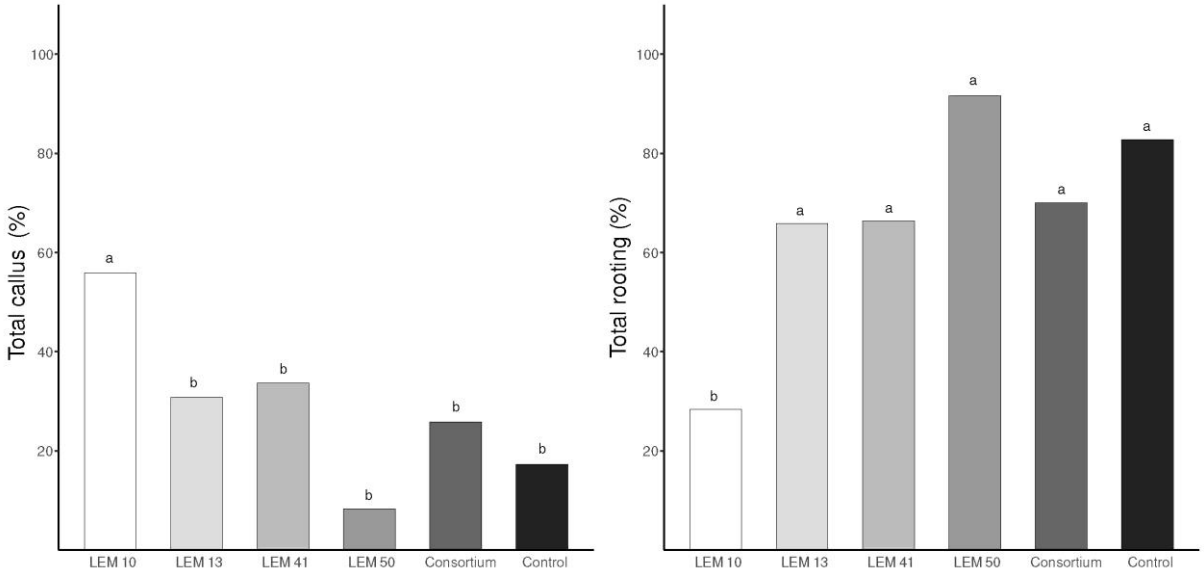

Figure 5. (A) Percentage of calluses formed in VM01 minicuttings. (B) Total rooting percentage of VM01 minicuttings, visible in the tube and not visible in the tube. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10).

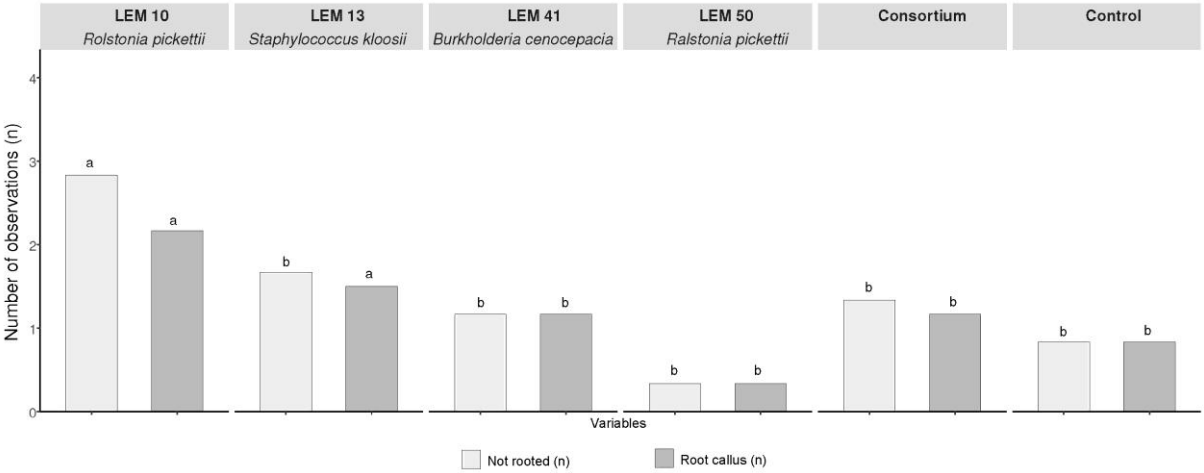

Figure 6. Average per block of unrooted VM01 clone minicuttings with callus formation in the different treatments. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10).

3.2.2 Morphological characteristics of the root system

3.2.2.1 Length (cm), diameter (mm) and distribution of root length by diameter class

Minicuttings treated with LEM 13 *S. kloosii* had a root length of 557.96 cm, LEM 50 *R. pickettii* resulted in 518.83 cm, Control 437.15 cm, Consortium 410.27 cm, LEM 41 *B. cenocepacia* 409.38 cm and LEM 10 *R. pickettii* 289.74 cm, with no statistical differences between them (Figure 7A). Although LEM 10 *R. pickettii* and LEM 41 *B. cenocepacia* had a shorter length, the average root diameter was statistically greater, 0.35 mm and 0.29 mm respectively, compared to the other treatments (Figure 7B).

The distribution of root length by diameter class showed that the majority of roots formed in 45-day-old minicuttings are fine roots (Ø <2.0mm) (Figure 8B). The majority of these roots correspond to a diameter smaller than 0.5 mm, considered very thin roots. The treatments LEM 50 *R. pickettii* (85.57 %), LEM 13 *S. kloosii* (84.69 %), Control (84.08 %) and Consortium (83.67 %) statistically promoted a higher proportion of very fine roots in relation to the treatments LEM 41 *B. cenocepacia* (75.32 %) and LEM 10 *R. pickettii* (73.22 %). The proportion of roots with a diameter of 0.5-2.0 mm was statistically higher in minicuttings treated with LEM 10 *R. pickettii* and LEM 41 *B. cenocepacia*. The proportion of medium and thick roots (> 2.0 mm) was statistically higher in minicuttings inoculated with LEM 10 *R. pickettii*.

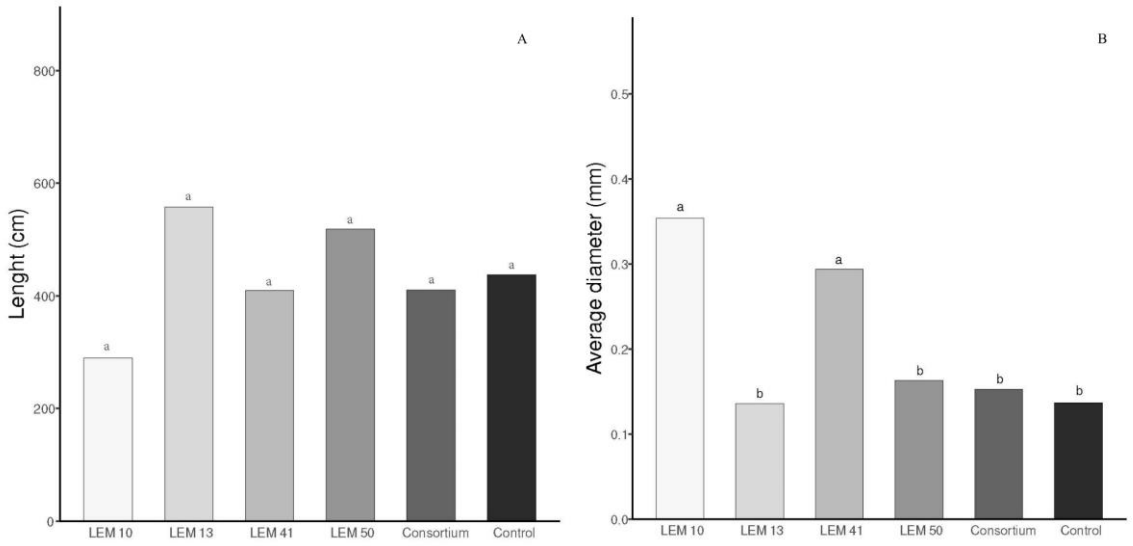

Figure 7. (A) Average length observed in minicuttings of clone VM01. (B) Average root diameter observed in VM01 minicuttings inoculated with the different treatments. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10).

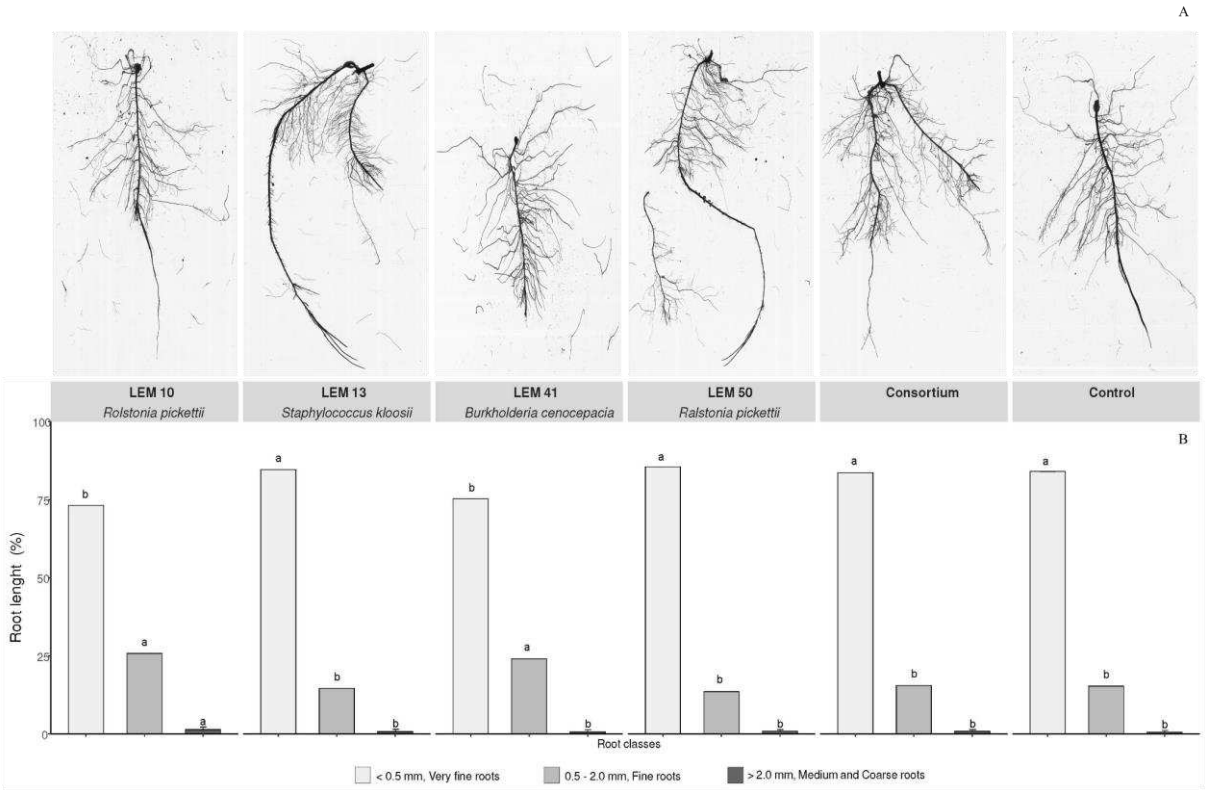

Figure 8. (A) images of the root system of VM01 at 35 days of age, obtained through the scanner, corresponding to the rhizobacterial isolates tested in this work. (B) Percentage of root length distribution by diameter class. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10).

3.2.2.2. Projected area (cm^2), surface area (cm^2) and root volume (cm^3)

The minicuttings did not demonstrate statistical differences between treatments in terms of projected root area, which varied from 11.66 to 17.73 cm^2 in horizontal distribution. In relation to the surface area, it was also not possible to observe statistical differences attributed to the different treatments, which varied from 36.63 to 55.70 cm^2, possibly reflecting the absorption of resources equally. As with the previous variables, the root volume of the minicuttings was not significantly influenced by the treatments, varying from 0.38 to 0.47 cm^3, which can demonstrate a similar capacity to explore the soil.

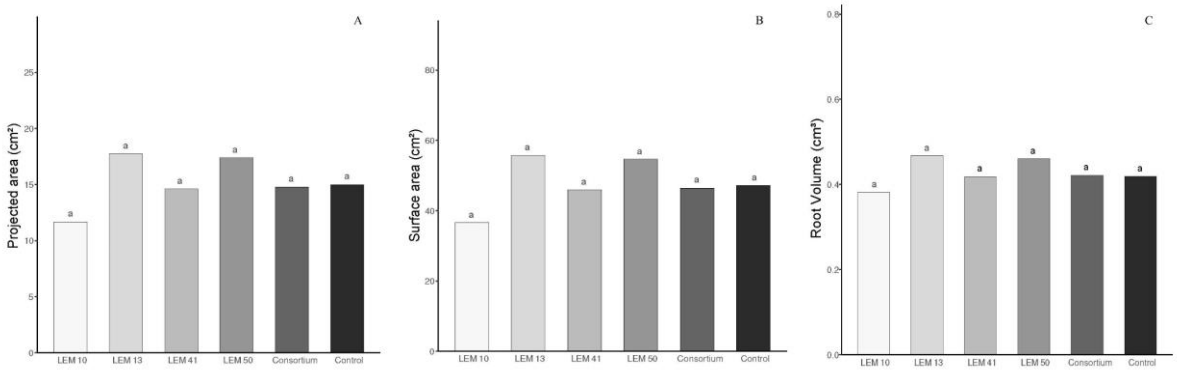

Figure 9. (A) Projected area of minicuttings of clone VM01. (B) Root surface area of minicuttings from clone VM01. (C) Root volume of VM01 clone minicuttings inoculated with different rhizobacteria. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10).

3.2.2.3 Average root dry mass

In relation to the average root dry mass, no significant increases were observed with the rhizobacteria treatments. LEM 50 *R. pickettii* provided root dry mass of 0.069 g, LEM 13 *S. kloosii* of 0.063 g, LEM 10 *R. pickettii* of 0.054 g, Control of 0.0534 g, Consortium of 0.052 and LEM 41 *B. cenocepacia* of 0.050 (Figure 10).

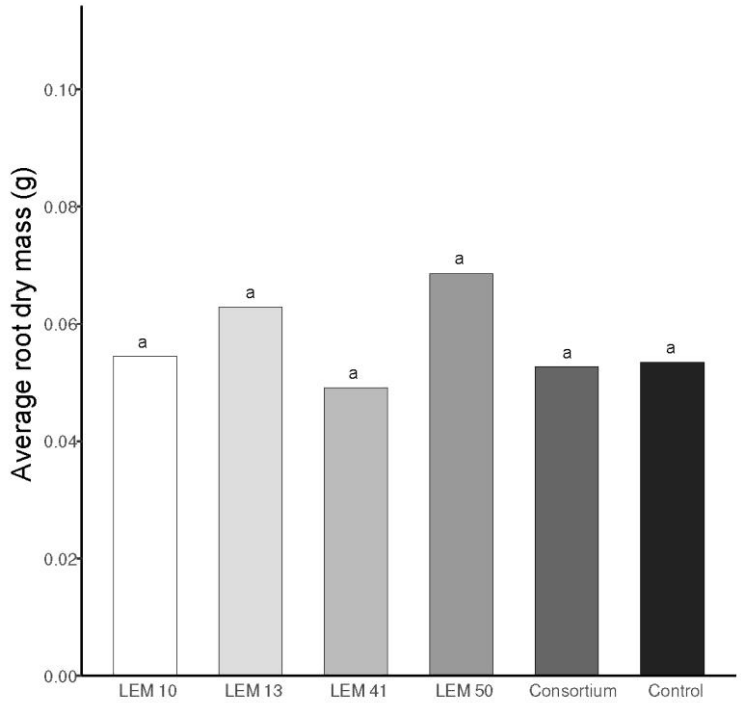

Figure 10. Average root dry mass of VM01 clone minicuttings inoculated with the different treatments. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10).

3.2.3 Development of the aerial part during the 35 days in the greenhouse rooting

The minicuttings subjected to different treatments did not result in statistical differences in aerial part biomass. Rhizobacteria LEM 50 *R. pickettii* provided minicuttings with 0.37 g, LEM 13 *S. kloosii* 0.33 g, control 0.32 g, LEM 41 *B. cenocepacia* 0.28 g, consortium 0.28 g and LEM 10 *R. pickettii* 0.19 g. In a greenhouse rooting, it was not possible to observe significant increases between treatments in terms of height evaluated in minicuttings aged 14, 21, 28 and 35 days.

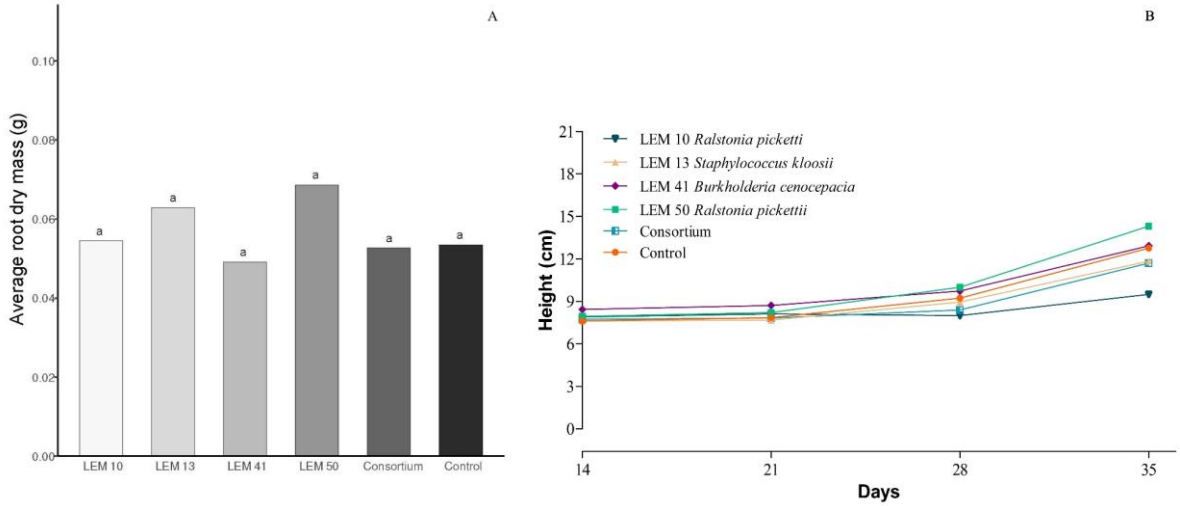

Figure 11. (A) Average dry mass of the aerial part of minicuttings from clone VM01. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10). (B) Average height of the VM01 clone minicuttings inoculated with the different isolates, over 35 days in the greenhouse rooting. Means belong to the same group according to the Scott-Knott test (p<0.10).

3.3 I144 Clone

3.3.1 Percentage and rooting efficiency of minicuttings

Root development in I144 clones was visible in minicuttings from 14 days of age, for all treatments. Eucalyptus inoculated with LEM 50 *R. pickettii* started with a rooting of 13.05 % of seedlings LEM 41 *B. cenocepacia* 7.5 %, Consortium 6.67 %, Control 4.17 % and LEM 10 *R. pickettii* and LEM 13 *S. kloosii* provided the same percentage of seedlings rooted, 3.33 %. Rooting was observed in minicuttings up to 35 days old. At 26 days LEM 50 *R. pickettii* allowed 73.33 % rooting, LEM 10 *R. pickettii* 65.55 %, LEM 41 *B. cenocepacia* 62.00 %, Consortium 58.05 %, LEM 13 *S. kloosii*, 53.88 % and Control treatment 50.55 %. Minicuttings, 28 to 30 days old, had root development ranging from 53.89 to 76.66 % and 59.44 to 77.78 % respectively. At 33 days of age, the Consortium treatment was able to cause rooting in 85.83 %, LEM 50 *R. pickettii* 84.16 %, LEM 10 *R. pickettii*, 81.11 %, Control 75.83 %, LEM *41 B. cenocepacia* 73.60 % and LEM 13 *S. kloosii* 71.11 %. The final percentage of rooting seen at

the exit from the greenhouse remained the same as observed at 33 days for the Consortium, LEM 50 *R. pickettii* and LEM 10 *R. pickettii* treatments. On the other hand, minicuttings from the control treatment, LEM 41 *B. cenocepacia* and LEM 13 *S. kloosii* had rooting rates at 35 days of 75.83, 76.93 and 79.45% respectively (Figure 12). Callus formation was observed in five treatments, with the exception of the control, however there were no statistical differences. Of the total number of seedlings in the experiment, callus formation was observed in 17.22 % in the treatment with LEM 13 *S. kloosii*, 10.83 % Consortium, 6.67 % LEM 41 *B. cenocepacia*, 4.17 % LEM 50 *R. pickettii*, 3.33 % LEM 10 *R. pickettii* (Figure 13A). In relation to the plants that did not root, as well as in the VM01 clone, the main factor was due to the formation of callus, with the exception of the control treatment (Figure 14). Regarding total rooting, including rooting not visible in the tube, a percentage of 96.67 % of rooted minicuttings was obtained in the control treatment, 93.33 % with LEM 10 *R. pickettii*, 92.50 % with LEM 50 *R. pickettii*, 89.17 % in the treatment Consortium, 79.44 % LEM 13 *S. kloosii* and 70 % LEM 41 *B. cenocepacia*, absent statistical differences (Figure 13B).

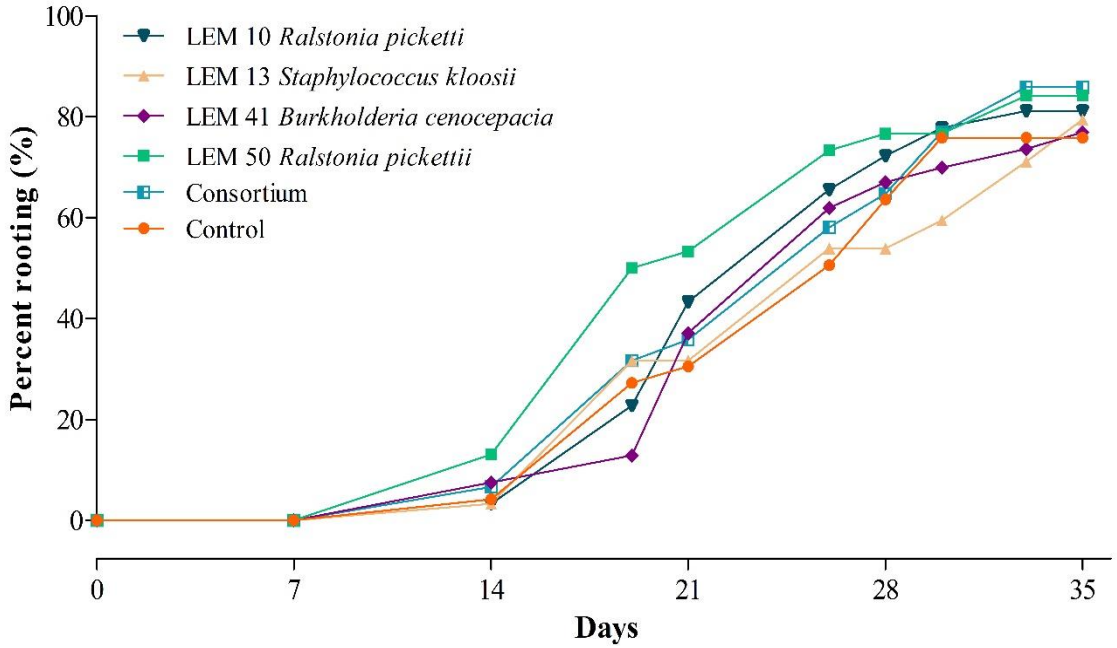

Figure 12. Percentage of rooting of clone I144 minicuttings inoculated with different treatments over 35 days. Means belong to the same group according to the Scott-Knott test (p<0.10).

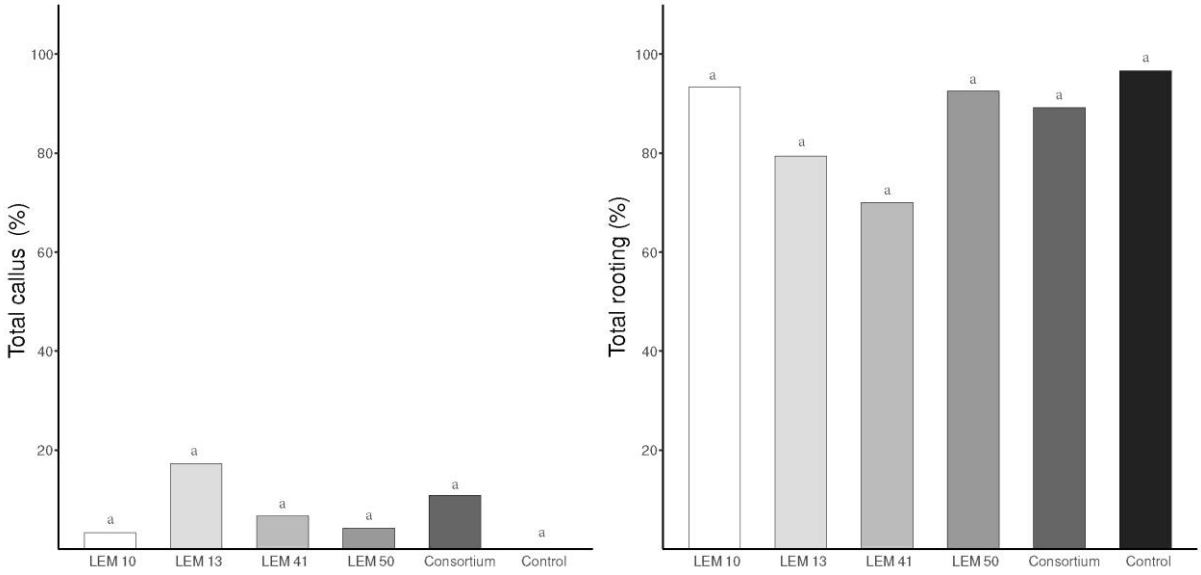

Figure 13. (A) Percentage of calluses formed in I144 minicuttings. (B) Total rooting percentage of I144 minicuttings, visible in the tube and not visible in the tube. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10).

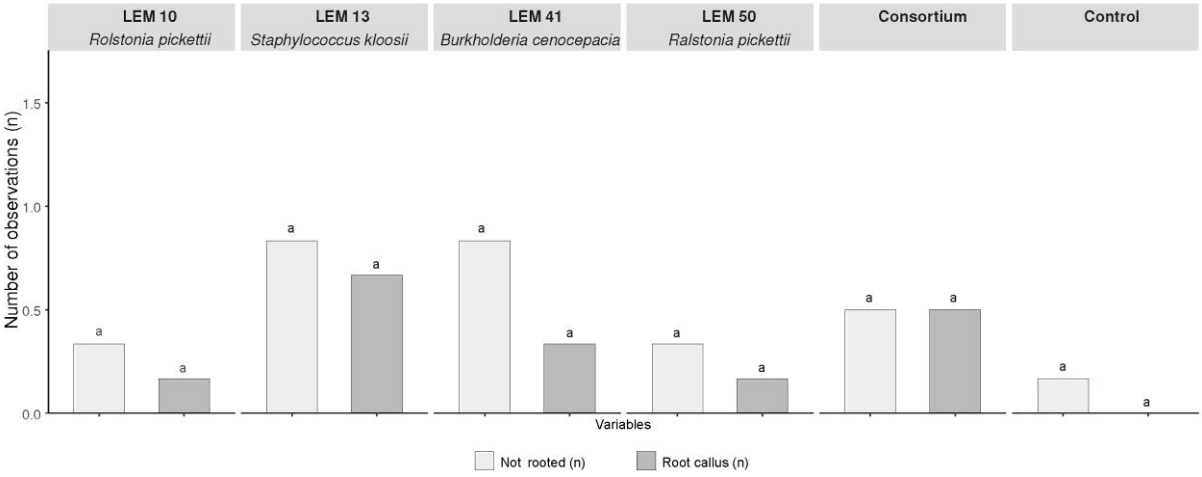

Figure 14. Average per block of clone I144 minicuttings not rooted and with callus formation in the different treatments. The same lowercase letter belongs to the same group according to the Scott-Knott test *(p*<0.10).

3.3.2 Morphological characteristics of the root system

3.3.2.1 Length (cm), diameter (mm) and distribution of root length by diameter class

It was not possible to observe statistical differences between the treatments, in relation to the variables analyzed. The root length provided by LEM 50 *R. pickettii* was 724.38 cm, LEM 13 *S. kloosii* 665.44 cm, LEM 10 *R. pickettii* 645.50 cm, LEM 41 *B. cenocepacia* 542.16 cm, Consortium 529.08 cm and Control 465.02 cm (Figure 15A). In relation to the average diameter, the LEM 50 *R. pickettii* treatments resulted in roots measuring 0.198 mm, LEM 10 *R. pickettii* 0.164 mm, LEM 13 *S. kloosii* 0.144 mm, Consortium 0.115 mm and Control 0.105 mm (Figure 15B). The roots of the minicuttings evaluated at 35 days are mainly formed by fine

roots (Ø < 2.0mm) (Figure 16B). The results showed that 85.60 to 88.72 % of the root length is up to 0.5 mm in diameter, roots considered very thin. Fine roots (0.5 - 2.0 mm) represented 10.90 to 13.97 % of the root length. Medium and thick roots represent a very small percentage of the length, ranging from 0.045 to 0.058 %.

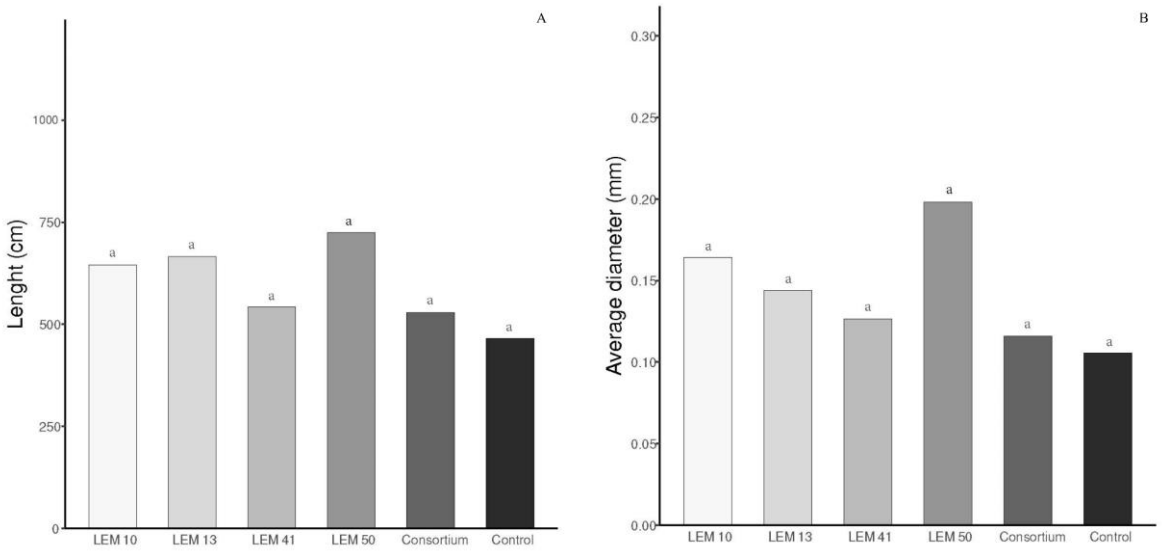

Figure 15. (A) Average length observed in minicuttings of clone I144. (B) Average root diameter observed in I144 minicuttings inoculated with the different treatments. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10).

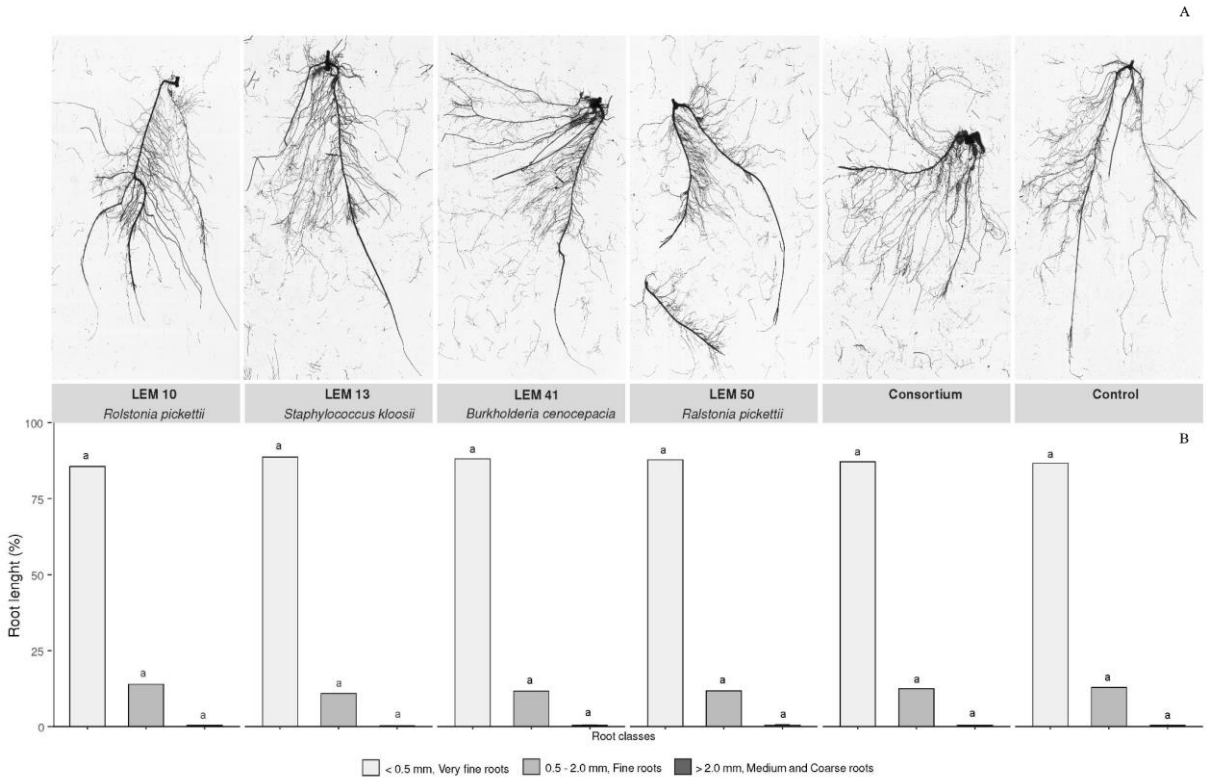

Figure 16. (A) images of the root system of 35-day-old I144, obtained through the scanner, corresponding to the rhizobacterial isolates tested in this work. (B) Percentage of root length distribution by diameter class. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10).

3.3.2.2. Projected area (cm^2), surface area (cm^2) and root volume (cm^3)

The minicuttings did not demonstrate statistical differences between treatments in terms of projected root area. The root area observed in the treatment with LEM 10 *R. pickettii* was 20.96 cm2, in the treatment with LEM 50 *R. pickettii* it was 20.60 cm^2, LEM 13 *S. kloosii* 18.89 cm2, Consortium 16.23 cm^2, LEM 41 *B. cenocepacia* 16.02 cm^2 and Control 14.48 cm^2 (Figure 17A). In relation to surface area, it was also not possible to observe statistical differences attributed to the different treatments, which varied from 45.49 to 65.85 cm^2, and reflected equally in resource absorption (Figure 17B). On the other hand, the root volume was significantly higher in minicuttings treated with LEM 10 *R. pickettii* 0.544 cm^3 and LEM 50 *R. pickettii* 0.470 cm^3, demonstrating that these plants may have a greater capacity to exploit the soil for resources (Figure 17C). The LEM 13 *S. kloosii*, Consortium, LEM 41 *B. cenocepacia* and Control treatments did not result in statistical differences for this variable, providing a variation in root volume from 0.358 to 0.423 cm^3.

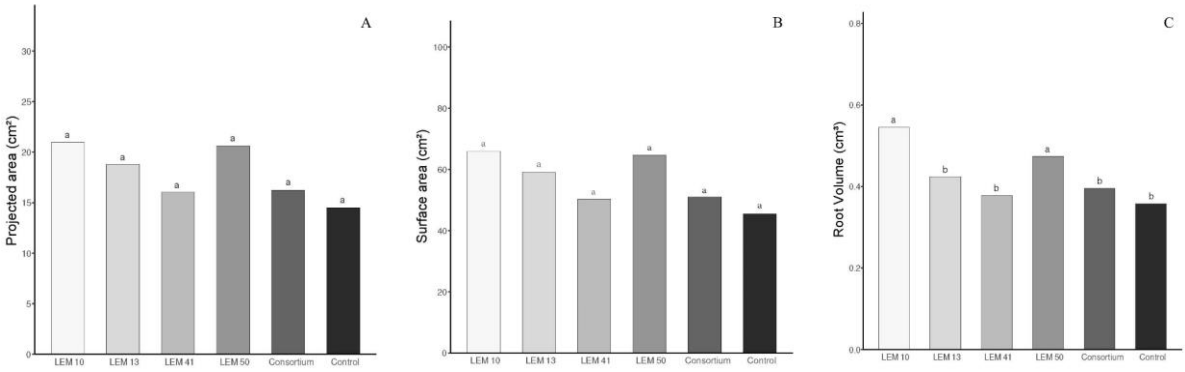

Figure 17. (A) Projected area of minicuttings of clone I144. (B) Root surface area of minicuttings from clone I144. (C) Root volume of clone I144 minicuttings inoculated with different rhizobacteria. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10).

3.3.2.3 Average root dry mass

The root dry mass of the plants did not differ statistically between treatments, varying from 0.045 to 0.065 g. The rhizobacteria provided minicuttings with an average dry mass of: LEM 10 *R. pickettii* 0.065 g, LEM 50 *R. pickettii* 0.064 g, LEM 13 *S. kloosii* 0.058 g, Consortium 0.046 g, Control 0.045 g and LEM 41 *B. cenocepacia* 0.045 g, no making it possible to obtain statistical differences between treatments.

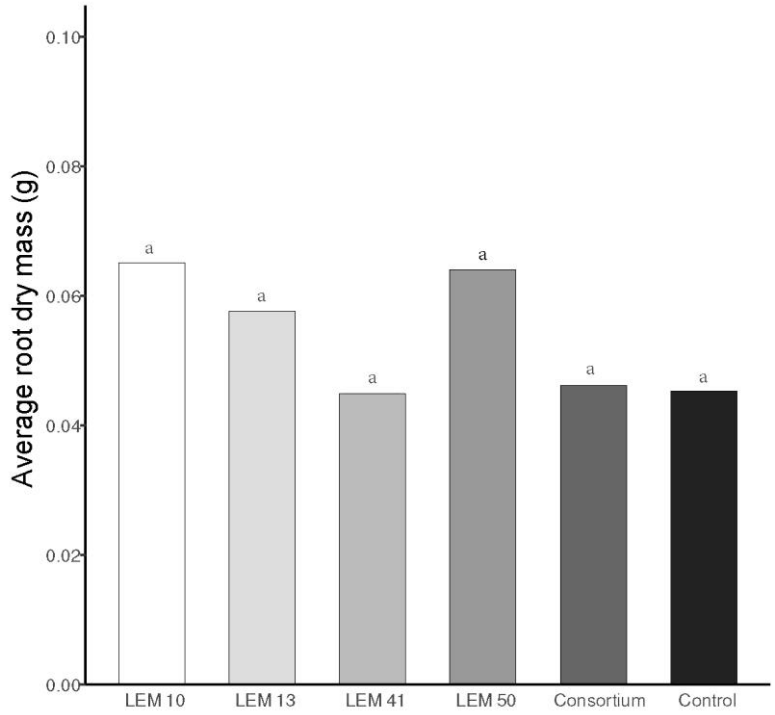

Figure 18. Average root dry mass of clone I144 minicuttings inoculated with the different treatments. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10).

3.3.3 Development of the aerial part during the 35 days in the greenhouse rooting

The minicuttings subjected to the different treatments did not result in statistical differences in the biomass of the aerial part. The observed average weight of the minicuttings in the following treatments were: LEM 10 *R. pickettii* 0.065 g, LEM 50 *R. pickettii* 0.064 g, Control 0.045 g, LEM 13 *S. kloosii* 0.057 g, Consortium 0.046 g and LEM 41 *B. cenocepacia* 0.045 g (Figure 19A). Assessments regarding plant heights also did not demonstrate statistical differences between treatments with 14, 21, 28 and 35 days old minicuttings (Figure 19B).

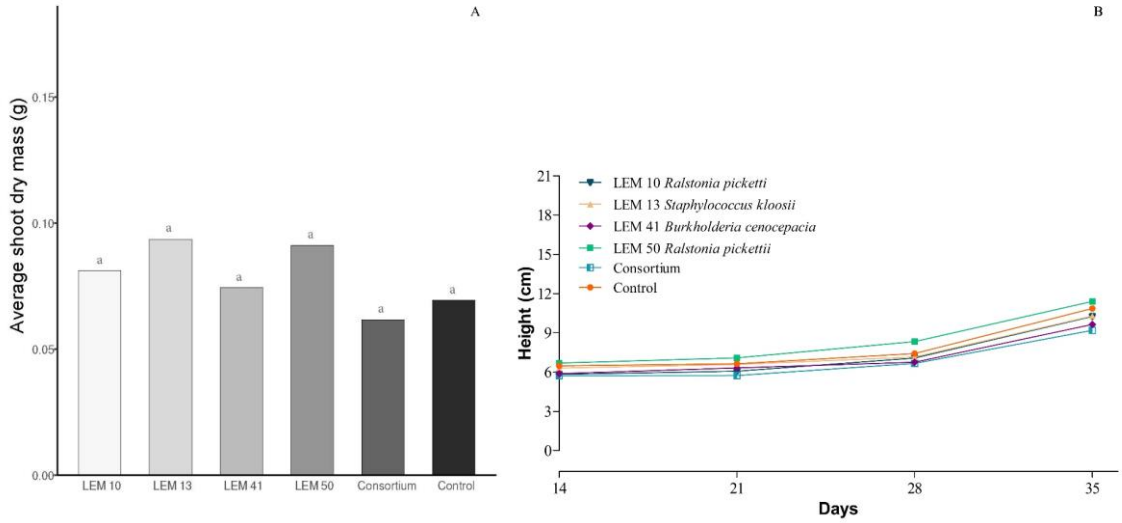

Figure 19. (A) Average dry mass of the aerial part of minicuttings of clone I144. The same lowercase letter belongs to the same group according to the Scott-Knott test (p<0.10). (B) Average height of the I144 clone minicuttings inoculated with the different isolates, over 35 days in the greenhouse rooting. Means belong to the same group according to the Scott-Knott test (p<0.10).

4. DISCUSSION

4.1 Rooting efficiency of VM01 and I144 clones

Both clones VM01 and I144 had rooting observed after 14 days in the greenhouse, with root development occurring, in most treatments, until 33 days. VM01 minicuttings inoculated with LEM 13 *S. kloosii* and LEM 41 *B. cenocepacia*, and I144 minicuttings inoculated with LEM 13 *S. kloosii*, LEM 50 *R. pickettii* and Control group showed root development up to 35 days of age.

At the end of 35 days, the percentages of total rooted VM01 minicuttings ranged from 65.83 to 91.67 %, with no statistical differences between the treatments and the Control group, with the exception of minicuttings inoculated with LEM 10 *R. pickettii*, which caused significant lower rooting percentages (28.83 %). Regarding the rooting of I144 minicuttings, it was not possible to observe statistical differences between treatments over the 35 days, resulting

in rooting of 70 to 96.67 %. The greenhouse rooting is the place where the roots develop, which have a variable development period according to the genetic material and the environmental conditions of the structure (Ferreira *et al.,* 2004). The rooting process can vary from 15 to 30 days or more (Xavier; Wendling; Da Silva, 2013). Azevedo *et al.* (2022) observed a maximum rooting percentage of 60.6 % for the VM01 clone at 33 days of age and 88.4 % for the AEC 0144 clone at 34 days of age. The rooting of minicuttings is dependent on intrinsic factors of the clone such as; youthfulness, genetics, nutritional and water conditions of the plant of origin, as well as hormonal balance (Xavier; Wendling; Da Silva, 2013). Furthermore, environmental factors during the collection procedure also impact rooting success, such as the time of collection, oxidation reactions at the base of the minicuttings, quality of the substrate and the abiotic conditions of the greenhouse (temperature, light and humidity) at which minicuttings will be exposed (Xavier; Wendling; Da Silva, 2013). After the rhizogenesis period, there is no increase in the rooting rate regardless of whether they remain in the greenhouse rooting or not (Ferreira *et al.,* 2004).

The use of phytohormone and inoculation with auxin-producing rhizobacteria seem to promote a higher rate and percentage of minicuttings rooted in a greenhouse rooting (Xavier; Wendling; Da Silva, 2013). Our results, however, demonstrated that the increases caused by LEM 50 *R. pickettii* in minicuttings of clone VM01 and Consortium in minicuttings of clone I144 were not significant. Inoculation with LEM 10 *R. pickettii* in minicuttings of the VM01 clone, statistically promoted the lowest percentages of rooted minicuttings, as well as the highest percentages of callus formation (55.83 %). In all treatments, it was observed that minicuttings that did not root were frequently observed with callus formation. Auxins are an important hormone for root development, but by themselves they do not promote rhizogenesis, requiring the involvement of other hormones and rooting cofactors (oxygenated terpenoids, phenolic compounds and isochlorogenic acid) in the process (Shiomi; Accordi; Sabino, 2016). Furthermore, it is essential that a high auxin/cytokinin ratio occurs for root development. These phytohormones have an antagonistic relationship, while auxin promotes the development of lateral roots, cytonicin inhibits them and stimulates the development of the aerial part. The highest levels of cytokines are reported at the initial stages of leaf development, a high concentration of cytokinin in relation to auxin, provides the development of the shoot (Sosnowski; Truba; Vasileva, 2023). Callus formation is also related to the auxin/cytokinin hormonal balance. Generally, a similar proportion between these phytohormones promotes the formation of calluses (Skoog, 1957). Another possible explanation for non-rooting may be related to high concentrations of auxin. Deleterious rhizobacteria can produce high

concentrations of IAA and consequently inhibit root growth (Barazani; Friedman, 1999; Kang *et al.,* 2020). In minicuttings of clone I144, no significant reductions in the percentage of rooted minicuttings were observed, mainly related to treatment with LEM 10 *R. pickettii*. The effects caused by the inoculation of LEM 10 *R. pickettii* in I144 minicuttings were opposite to that observed in the VM01 clone. While rhizobacteria can promote growth in some plants, they can induce deleterious effects in others, inhibiting their development and growth (Kang *et al.,* 2020).

4.2 Morphological characteristics of the root system

The root length and the average diameter of minicuttings of clones VM01 and I144 were not influenced by rhizobacteria inoculation. The distribution of root length by diameter class demonstrated that minicuttings up to 35 days' old have a higher proportion of fine roots (Ø <2.0mm), at least 90 %. The rhizobacteria LEM 10 *R. pickettii* and LEM 41 *B. cenocepacia* inoculated into minicuttings of clone VM01, significantly provided the lowest proportions of very fine roots (Ø <0.5mm), 73.22 and 75.32 %, respectively. In relation to fine roots with diameters of 0.5 to 2.0 mm, the isolates LEM10 *R. pickettii* and LEM 41 *B. cenocepacia* demonstrated significantly the highest proportions, these being 25.80 and 24.06 %, respectively. The higher proportion of fine roots is also observed in eucalyptus trees planted between 7 and 9 years of age, which demonstrate that 88 % of the length is formed by roots smaller than 2 mm in diameter (Grant *et al.,* 2012). The greater proportion of fine roots in eucalyptus was confirmed by several authors, who observed 70 to 95 % of the total root length (Nambiar, 1990; Falkiner *et al.,* 2006; Wang *et al.,* 2020). The root system has plasticity, which allows these plants to adapt to various adverse conditions such as drought, low nutrient availability and different types of soil (Pierik; Testerink, 2014). Different soil characteristics can influence the proportion of fine roots (Grant *et al.,* 2012). In relation to the formation of medium and thick roots (Ø >2.0mm), LEM 10 *R. pickettii* caused statistically higher proportions than the other treatments. Demonstrating that although the largest proportion is of fine roots, there is a significant distribution in the formation of these roots.

The average root diameter was significantly greater in Clone VM01 minicuttings inoculated with LEM 10 R. *pickettii* and LEM 41 *B. cenocepacia*, 0.354 and 0.293, respectively. Eucalyptus roots can be divided into two categories: supporting roots, which are mostly formed by thicker roots, and absorption roots, represented by thin roots (<2–3 mm in diameter). Because they have a low degree of suberization, the fine roots are highly permeable to water and nutrients (Gonçalves; Mello, 2015).

Regarding the projected area, surface area and root volume of the VM01 clone minicuttings, it was not possible to observe statistical differences. However, the root volume of I144 minicuttings inoculated with LEM 10 *R. pickettii* and LEM 50 *R. pickettii* were statistically higher than the other treatments, 0.548 and 0.474 cm^3, respectively. A higher root volume results in a greater ability to exploit the soil for resources such as nutrients and water, giving plants a competitive advantage (Herrero *et al.,* 2014). Kulmann *et al.* (2020) demonstrated that 90-day-old *E. grandis* clones that had greater root length, area and volume had a superior absorption efficiency that resulted in high biomass (Kulmann *et al.,* 2021). Efficient and quality root development is desirable in the forestry seedling production sector, and is also fundamental for the success of these seedlings in the field.

4.3 Rhizobacteria in root development of clones VM01 and I144

VM01 minicuttings inoculated with the four isolates defined from the results obtained in chapter III, generally did not demonstrate significant increases in root variables compared to the control treatment. The opposite was observed in chapter III, which under water deficit conditions, the isolates LEM 13 *S. kloosii* and LEM 50 *R. pickettii* promoted significant increases in root, shoot and total biomass and significant gains in relation to the nutritional content of the plants. Furthermore, differences were observed in minicuttings of VM01 and I144 inoculated with LEM 10 *R. pickettii*. In minicuttings from VM01, significant reductions in rooting percentage, increase in callus formation and proportion of medium and thick roots were observed. However, in I144 minicuttings, similar effects were not observed with the inoculation of LEM 10 *R. pickettii*. This result demonstrates that the same isolates can exert different behaviors and provide different results when associated with different genotypes and environmental conditions. Different genotypes demonstrate an influence on the recruitment of different microorganisms, reflecting a characteristic microbiome capable of modulating plant fitness (Dastogeer *et al.,* 2020). The interaction between plants and microorganisms requires essential conditions that are interpreted through two signals, (1) the production of a specific signal and (2) the interpretation of the specific signal that generates a behavioral response (Upadhyay *et al.,* 2022). These signals can be through the release of exudates, quorum sensing, electron transfer mechanism, variation in the consumption of plant resources, morphological characteristics of plants, such as tissue permeability and wettability, physicochemical properties, cuticle chemistry and even even the inherent immunity of the plant to the inoculated microorganisms (Singh; Sale, 1998; Tashiro *et al.,* 2013; Keswani *et al.,* 2020; Santoyo, 2022). The greater the phylogenetic distance between plants, the greater the variations in the associated

microbiome (Santoyo, 2022). Inferring that the signals are variable depending on the biotic and abiotic conditions, the eucalyptus genotype, the stage of development of the plants, and the different conditions found in the greenhouse rooting, these factors probably corroborated different results between the clones and what was previously observed from the inoculation in VM01 under water deficit. Furthermore, the interaction between plants and microorganisms can vary in terms of duration. Plants can associate with microorganisms for a short period and stop interacting after a certain time or associate for long periods, remaining together throughout the plant's life cycle. This association that remains for long periods is formed by the core microbiome, whose understanding can provide greater efficiency in the development and use of bioinoculants under different environmental conditions (Santoyo, 2020).

Under stress conditions, plants can play an important role in recruiting microorganisms to help cope with adverse conditions, ensuring greater nutrient absorption, physiological activities and survival, as observed in chapter III. However, in the greenhouse, the water, temperature and nutritional conditions were optimal for rooting the minicuttings, which may have enabled the plants to conserve energy for root development alone, resulting in the absence of significant increases in rooting and biomass. The positive effects of bacteria observed under water deficit conditions may not have been as evident in the greenhouse rooting, as the plants were subjected to an environment conducive to their development. However, this work did not evaluate the nutritional conditions of these minicuttings, which may present differences between treatments, resulting in high quality forest seedlings. Considering the results observed in this work, it is concluded that it is essential to study core microorganisms associated with different eucalyptus genotypes and their stages of development. This research can clarify which microorganisms are relevant and which have lasting associations with certain genotypes at certain ages. Furthermore, it is interesting to evaluate whether the benefits observed during the seedling process will extend to field planting, where the plants will be subject to adverse conditions, such as water deficit.

5. CONCLUSION

The results demonstrated that there was no significant increase in the percentage of rooting provided by the inoculation of LEM 10 *R. pickettii*, LEM 13 *S. kloosii*, LEM 41 *B. cenocepacia*, LEM 50 *R. pickettii* and Consortium in 35-day-old minicuttings of the clones VM01 and I144. In minicuttings of the VM01 clone, significant reductions in the percentage of rooting and superior callus formation were observed in eucalyptus trees inoculated with LEM 10 *R. pickettii*. Furthermore, LEM 10 *R. pickettii* and LEM 41 *B. cenocepacia* caused a lower

proportion of fine roots. A greater proportion of medium and thick roots was observed by the inoculation of LEM 10 *R. pickettii*, which, similarly to LEM 41 *B. cenocepacia*, demonstrated a higher average diameter than the other treatments. In I144 minicuttings, root volume was significantly higher when treated with LEM 10 *R. pickettii* and LEM 50 *R. pickettii*. Efficient and quality root development is desirable in the seedling production process and essential for their success in the field. However, the efficiency of microorganism inoculation can be different depending on the genotype, stage of plant development and abiotic conditions.

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