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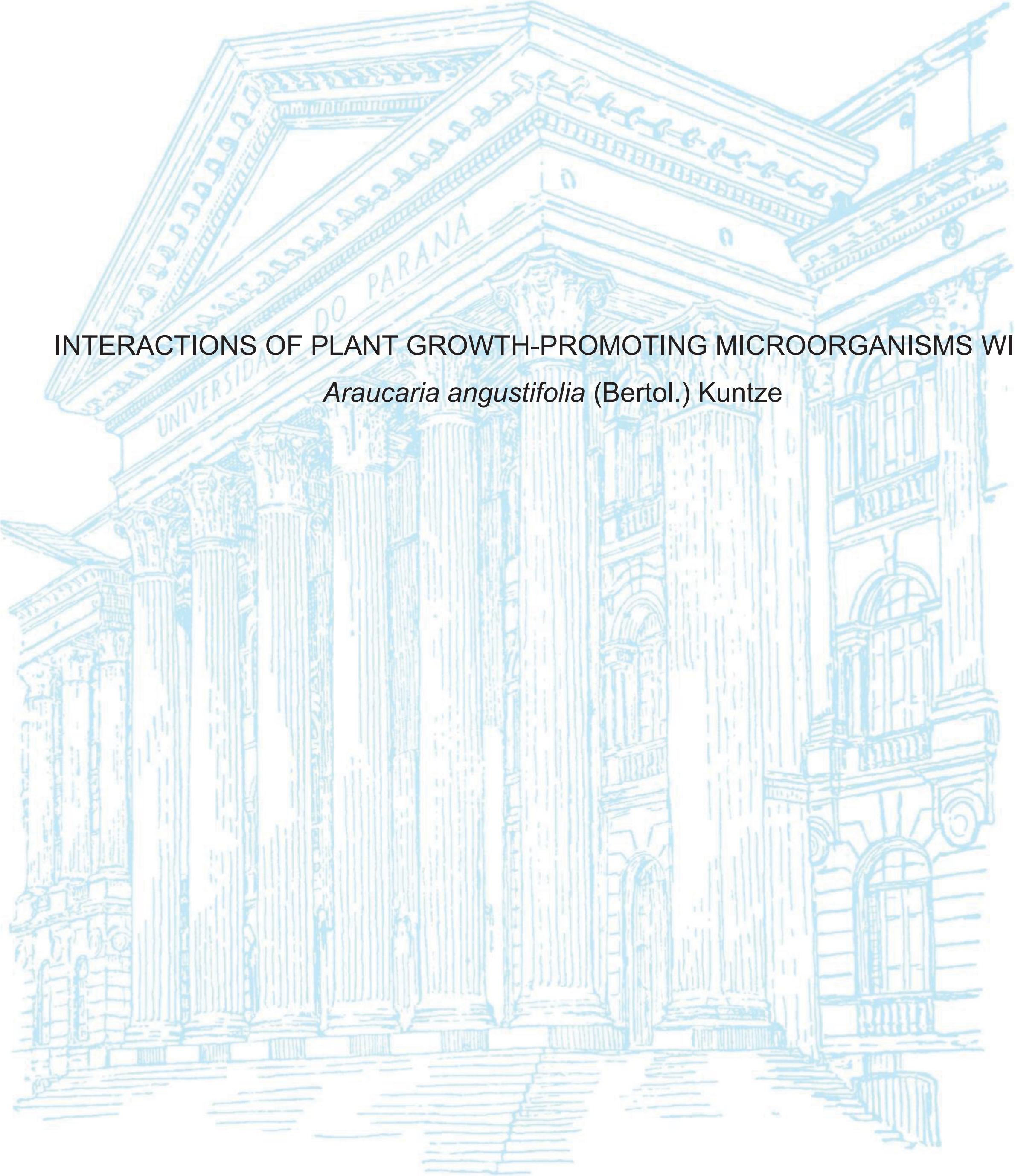

INTERACTIONS OF PLANT GROWTH-PROMOTING MICROORGANISMS WITH *Araucaria angustifolia* (Bertol.) Kuntze

CURITIBA
2026

YANKA ROCHA KONDO

INTERACTIONS OF PLANT GROWTH-PROMOTING MICROORGANISMS WITH *Araucaria angustifolia* (Bertol.) Kuntze

Tese apresentada ao curso de Pós-Graduação em Ciência do Solo, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciência do Solo, Área de Concentração Solo e Ambiente.

Orientadora: Profa. Dra. Glaciela Kaschuk

CURITIBA
2026

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP)
UNIVERSIDADE FEDERAL DO PARANÁ
SISTEMA DE BIBLIOTECAS – BIBLIOTECA DE CIÊNCIAS AGRÁRIAS

Kondo, Yanka Rocha
Interactions of plant growth-promoting microorganisms with *Araucaria angustifolia* (Bertol.) Kuntze / Yanka Rocha Kondo. – Curitiba, 2026.
1 recurso online: PDF.

Tese (Doutorado) – Universidade Federal do Paraná, Setor de Ciências Agrárias, Programa de Pós-Graduação em Ciência do Solo.
Orientadora: Profa. Dra. Glaciela Kaschuk

1. Microrganismo. 2. Estimulante de Crescimento Vegetal. 3. Restauração florestal. 4. Araucaria Angustifolia I. Kaschuk, Glaciela. II. Universidade Federal do Paraná. Programa de Pós-Graduação em Ciência do Solo. III. Título.

Bibliotecária: Talita Nunes Silva Gonçalves CRB-9/2244

MINISTÉRIO DA EDUCAÇÃO
SETOR DE CIÊNCIAS AGRÁRIAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO CIÊNCIA DO SOLO -
40001016014P4

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CURITIBA, 20 de Fevereiro de 2026.

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À minha mãe, Adelita de Sampaio Rocha Okuda (in memoriam), que muito antes da finalização deste trabalho já me chamava de doutora. Eu te amo com cada batida do meu coração.

Dedico.

“Plants have no roots, they have mycorrhizae”

(J. L. Harley)

RESUMO

A Mata Atlântica Brasileira (MAB) é um hotspot de biodiversidade cuja restauração florestal necessita de estratégias que melhorem o desempenho inicial das mudas. Nesse sentido, microrganismos promotores de crescimento vegetal (MPCV), como fungos micorrízicos arbusculares (FMA) e bactérias promotoras de crescimento vegetal (BPCV) têm sido estudados como ferramentas biotecnológicas capazes de melhorar o estado nutricional e o vigor de espécies florestais nativas. Entre as espécies de interesse, a araucária (*Araucaria angustifolia*), que apresenta uma fitofisionomia própria dentro da MAB, nomeada Floresta de Araucárias ou Floresta Ombrófila Mista, está listada como criticamente ameaçada de extinção há mais de uma década, o que reforça a necessidade de estratégias que aumentem seu estabelecimento e sobrevivência. Portanto, esta tese foi alicerçada na hipótese de que a inoculação com MPCV melhoram o crescimento, o estado nutricional e a resiliência de mudas de espécies nativas da MAB. A pesquisa foi conduzida na literatura com dezenas de espécies florestais nativas da MAB, e experimentalmente com ênfase em *A. angustifolia*. Foram considerados diferentes grupos microbianos, condições experimentais, estresses ambientais e etapas do processo de produção e estabelecimento de mudas. O capítulo 1 consistiu em uma meta-análise com dados de 60 artigos que avaliaram os efeitos de FMA, BPCV, bactérias fixadoras de nitrogênio (BFN) e a combinação FMA+BFN em mudas nativas da MAB, considerando estágios sucessionais, grupos funcionais e condições experimentais. Os capítulos 2, 3 e 4 foram desenvolvidos com base em experimentos realizados com mudas de *A. angustifolia*, respectivamente, para: (2) avaliar o crescimento, o estado nutricional e a persistência de BPCV em diferentes substratos, utilizando técnicas de cultivo e PCR quantitativo (qPCR); (3) analisar os efeitos da inoculação com BPCV sob diferentes regimes hídricos, com avaliação de respostas morfológicas, fisiológicas e bioquímicas; (4) avaliar o efeito da inoculação com BPCV e FMA em diferentes momentos do ciclo de produção e estabelecimento das mudas em campo. A meta-análise demonstrou que a inoculação com MPCV, especialmente FMA, promove maiores benefícios em espécies pioneiras e secundárias iniciais, principalmente em condições estéreis e com fertilização fosfatada. A inoculação com BPCV melhorou o estado nutricional e o crescimento radicular de forma diferenciada em diferentes substratos, o que sugere que as BPCV utilizadas têm diferentes níveis de competitividade após a inoculação. Além disso, a qPCR mostrou-se uma ferramenta molecular eficaz para monitorar a persistência de BPCV nas raízes. A inoculação de BPCV em condições de seca afetou o acúmulo de nutrientes das mudas, o que poderia ser atribuído à maior tolerância à seca. No campo, a inoculação não afetou a altura ou a biomassa, mas as taxas de aplicação dos inoculantes ao longo do tempo influenciaram o diâmetro. Os resultados indicam que os benefícios dos MPCV dependem da adequação entre microrganismos, espécies vegetais, estágio sucessional e condições ambientais. Embora a inoculação contribua para aspectos específicos do desenvolvimento inicial e da resiliência das mudas, sua eficácia em campo requer abordagens integradas e experimentos adicionais em diferentes contextos ambientais, reforçando seu potencial, mas também suas limitações, como ferramenta para a restauração e conservação florestal.

Palavras-chave: Floresta de Araucárias. Associação planta–micróbio. *Azospirillum brasilense*. *Bacillus–Priestia–Niallia* spp. *Rhizophagus* spp.

ABSTRACT

The Brazilian Atlantic Forest (BAF) is a biodiversity hotspot whose forest restoration requires strategies that improve the early performance of seedlings. In this context, plant growth-promoting microorganisms (PGPM) such as arbuscular mycorrhizal fungi (AMF) and plant growth-promoting bacteria (PGPB), have been studied as biotechnological tools capable of enhancing the nutritional status and vigor of native forest species. Among the species of interest, the araucaria (*Araucaria angustifolia*), which forms a distinct phytophysiognomy within the BAF known as Araucaria Forest of Mixed Ombrophilous Forest, has been listed as critically endangered for more than a decade, reinforcing the need for strategies that increase its establishment and survival. Therefore, this thesis was based on the hypothesis that inoculation with PGPM improve the growth, nutritional status, and resilience of seedlings of native BAF species. The research was conducted through a literature review involving dozens of native forest species from BAF and experimentally with emphasis on *A. angustifolia*. Different microbial groups, experimental conditions, environmental stresses, stages of the seedling production and establishment process were considered. Chapter 1 consisted of a meta-analysis of data from 60 articles that evaluated the effects of AMF, PGPB, nitrogen-fixing bacteria (NFB), and the AMF+NFB combination on native BAF seedlings, considering successional stages, functional groups, and experimental conditions. Chapters 2, 3, and 4 were developed based on experiments conducted with *A. angustifolia* seedlings, respectively, to: (2) evaluate growth, nutritional status, and PGPB persistence in different substrates using cultivation techniques and quantitative PCR (qPCR); (3) analyze the effects of PGPB inoculation under different water regimes, assessing morphological, physiological, and biochemical responses; and (4) evaluate the effect of PGPB and AMF inoculation at different moments of the production cycle and seedling establishment in the field. The meta-analysis demonstrated that inoculation with PGPM, especially AMF, promotes greater benefits in pioneer and early secondary species, mainly under sterile conditions and with phosphate fertilization. PGPB inoculation improved nutritional status and root growth in different ways depending on the substrate, suggesting that the PGPB strains used exhibit different levels of competitiveness after inoculation. Furthermore, qPCR proved to be an effective molecular tool for monitoring PGPB persistence in roots. PGPB inoculation under drought conditions affected nutrient accumulation in seedlings, which could be attributed to increased drought tolerance. In the field, inoculation did not affect height or biomass, but the application rates of inoculants over time influenced stem diameter. The results indicate that the benefits of PGPM depend on the compatibility among microorganisms, plant species, successional stage, and environmental conditions. Although inoculation contributes to specific aspects of early development and seedling resilience, its effectiveness in the field requires integrated approaches and additional experiments in different environmental contexts, reinforcing its potential, but also its limitations, as a tool for forest restoration and conservation.

Keywords: Araucaria Forest. Plant–microbe association. *Azospirillum brasilense*. *Bacillus–Priestia–Niallia* spp. *Rhizophagus* spp.

SUMMARY

1 GENERAL INTRODUCTION 11
1.1 OBJECTIVES 11
1.2 HYPOTHESES 12
1.3 OUTLINE OF THE THESIS 12
2 CHAPTER 1: MATCHMAKING: A META-ANALYSIS OF PLANT GROWTH-PROMOTING MICROBIAL PARTNERSHIPS DRIVING TREE SEEDLING PERFORMANCE IN THE ATLANTIC FOREST BIOME..14
2.1 RESUMO..14
2.2 ABSTRACT 14
2.3 INTRODUCTION..15
2.4 METHODS 17
2.5 RESULTS..19
2.5.1 Effects of PGPM inoculation on the biomass and nutrition of Atlantic Forest tree seedlings 20
2.5.2 Responses to functional traits, inoculant complexity, substrate conditions, and fertilization 22
2.6 DISCUSSION..29
2.6.1 Effects of PGPM inoculation on BAF tree seedlings across ecological successional groups..29
2.6.2 Biomass and nutritional responses by functional traits, inoculant complexity, substrate condition, and fertilization 30
2.7 REFERENCES..32
3 CHAPTER 2: COMPETITIVENESS OF PLANT-GROWTH PROMOTING BACTERIA INOCULATED INTO *ARAUCARIA ANGUSTIFOLIA* ASSESSED BY QPCR AMPLIFICATION..43
3.1 RESUMO..43
3.2 ABSTRACT 43
3.3 INTRODUCTION..44
3.4 MATERIALS AND METHODS..45
3.4.1 Araucaria seeds 45
3.4.2 Inoculation..45
3.4.3 Greenhouse experiments 46

3.4.4 Substrate analyses 48

3.4.5 Microbiological analyses 48

3.4.5.1 Serial dilution and plate counting of colony forming unities (CFU) 48

3.4.5.2 qPCR 49

3.4.6 Plant morphological and nutritional analyses 50

3.4.7 Statistical analyses 51

3.5 RESULTS 51

3.5.1 Microbial quantification (CFU and qPCR) 51

3.5.2 Plant growth and nutritional analyses 53

3.6 DISCUSSION 57

3.7 CONCLUSION 59

3.8 REFERENCES 60

4 CHAPTER 3: PLANT GROWTH-PROMOTING BACTERIA INOCULATION ENHANCES SHOOT NUTRIENT CONTENTS OF *ARAUCARIA ANGUSTIFOLIA* SEEDLINGS UNDER DROUGHT STRESS 67

4.1 RESUMO 67

4.2 ABSTRACT 67

4.3 INTRODUCTION 68

4.4 MATERIALS AND METHODS 69

4.4.1 Soil characterization 69

4.4.2 PGPB inoculants 70

4.4.3 Soil water regimes 70

4.4.4 Experimental design 70

4.4.5 Plant growth conditions 71

4.4.6 Plant analyses 72

4.4.7 Statistical analyses 72

4.5 RESULTS 73

4.6 DISCUSSION 75

4.7 CONCLUSION 78

4.8 REFERENCES 78

5 CHAPTER 4: EDAPHOCLIMATIC CONDITIONS AFFECT THE FIELD GROWTH OF *ARAUCARIA ANGUSTIFOLIA* SEEDLINGS INOCULATED WITH PLANT GROWTH-PROMOTING MICROORGANISMS 87

5.1 RESUMO 87

5.2 ABSTRACT 87
5.3 INTRODUCTION 88
5.4 MATERIALS AND METHODS 89
5.4.1 Experimental conditions 89
5.4.2 Seeds acquisition 89
5.4.3 Soil characterization 89
5.4.4 PGPB and AM inoculants 91
5.4.5 Inoculation in the greenhouse 91
5.4.6 Seedling growth and acclimatization 91
5.4.7 Field reinoculation and planting conditions 92
5.4.8 Experimental design 92
5.4.9 Growth monitoring in the field 92
5.4.10 Weather conditions 94
5.4.11 Harvest 95
5.4.12 Growth rates 95
5.4.13 Statistical analyses 95
5.5 RESULTS 95
5.6 DISCUSSION 98
5.7 CONCLUSION 100
5.8 REFERENCES 100
6 GENERAL CONCLUSION 106
7 FINAL CONSIDERATIONS 107
8 REFERENCES 108
APPENDIX 1 – ARTICLE 1 132
APPENDIX 2 – ARTICLE 2 142
APPENDIX 3 – ARTICLE 3 143
APPENDIX 4 – ARTICLE 4 145

1 GENERAL INTRODUCTION

The Brazilian Atlantic Forest (BAF) biome provides essential ecosystem services and improves the quality of life of millions of people, particularly due to its extensive area in Brazilian territory. However, the majority of BAF is considered a biodiversity hotspot requiring strategies of conservation of native forest species and reforestation, especially in the face of climate change. In addition to traditional conservation strategies, it is also necessary to expand our understanding of the ecological potential of biological interactions.

The BAF plant species establish positive interactions with soil microorganisms, resulting in plant growth promotion and resilience against edaphoclimatic stresses. The beneficial effects of symbiosis between arbuscular mycorrhizal fungi (AMF) and a wide range of plant species are well documented in the literature. In contrast, plant–bacteria interactions, particularly those involving plant growth-promoting bacteria (PGPB), remain poorly explored in the context of seedlings of native tree species. Moreover, from a practical and economic perspective, the use of commercial inoculants originally developed for agricultural crops represents a promising strategy for forest nurseries, since the large-scale production of species-specific inoculants for each threatened native species in Brazil is unlikely to be commercially viable. Therefore, testing the efficacy of existing commercial formulations in forest species may constitute a more feasible and scalable approach.

1.1 OBJECTIVES

The four chapters of this thesis were done to:

1. Identify the most effective plant growth-promoting microorganism (PGPM) and the most suitable conditions to produce native seedlings from BAF, aiming to contribute to the production of seedlings with greater biomass and improved nutritional status for the restoration of degraded areas within the biome.
2. Evaluate the PGPB strains *Azospirillum brasilense* Ab-v5 and Ab-v6, *Bacillus pumilus* CCTB05, *B. subtilis* CCTB04, and *B. amyloliquefaciens* CCTB09, present in commercial inoculants, regarding their capacity for root colonization in *Araucaria angustifolia* seedlings, to verify their ability to establish initial association with seedlings of this tree species.

3. Determine whether inoculation with the PGPB *A. brasilense* Ab-v5 and Ab-v6, *Priestia aryabhattai* (formerly *Bacillus aryabhattai*) CBMAI1120, *B. haynesii* CCT7929, and *Niallia circulans* (formerly *B. circulans*) CCT0026, present in commercial inoculants, confers greater drought tolerance to *A. angustifolia* seedlings, to assess whether inoculation can be considered a management strategy for this species to mitigate the impacts of this abiotic stress.
4. Determine whether reinoculation with PGPB contained in commercial inoculants and AMF increases the survival rate of *A. angustifolia* seedlings planted under field conditions.

1.2 HYPOTHESES

The author of this thesis hypothesized that:

1. Native seedlings of BAF are context-dependent to PGPM inoculation, being jointly influenced by plant successional group and functional traits, microbial composition of the inocula, substrate conditions, and nutrient availability.
2. The PGPB strains *A. brasilense* Ab-v5 and Ab-v6, *B. pumilus* CCTB05, *B. subtilis* CCTB04, and *B. amyloliquefaciens* CCTB09, present in commercial inoculants, can colonize and associate with the roots of *A. angustifolia* seedlings.
3. Inoculation with the PGPB strains *A. brasilense* Ab-v5 and Ab-v6, *P. aryabhattai* CBMAI1120, *B. haynesii* CCT7929, and *N. circulans* CCT0026, present in commercial inoculants, enhances the tolerance of *A. angustifolia* seedlings to water deficit stress.
4. Reinoculation with PGPB contained in commercial inoculants and with AMF of the genus *Rhizophagus* spp. promote greater survival and vigor of *A. angustifolia* seedlings under field conditions.

1.3 OUTLINE OF THE THESIS

Given the inherently slow growth of tree species, which is closely linked to their anatomical and physiological characteristics, the integrated analysis of studies evaluating the effects of inoculation with PGPM represents an important approach to support decision-making prior to species selections and nursery management. Within this framework, the first chapter of this thesis was developed to provide more specific guidance for seedling production, aiming to optimize both productivity, expressed as biomass accumulation, and plant nutritional status, particularly N and P contents, while also contributing to a reduction in costs (e.g., fertilization).

Chapter 2 was designed as a continuation of the results previously reported by Kondo et al. (2024), who identified the most effective concentrations of inoculants based on *A. brasilense* and *Bacillus* spp. for the growth of *A. angustifolia* seedlings. However, assessing morphological responses following inoculation alone is not sufficient to unequivocally demonstrate an effective plant-bacteria association. Therefore, bacterial strain identification using qPCR was employed to link the observed morphological benefits to the inoculation process, thereby confirming the interactions between *A. angustifolia* and the evaluated PGPB.

Climate change poses additional challenges to the survival and longevity of forest species, particularly when considering species that are already threatened with extinction, such as *A. angustifolia*. In this context, Chapter 3 aimed to evaluate whether interactions between PGPB and seedlings subjected to different levels of water stress could result in synergistic effects, leading to improved early plant development. The results indicate benefits associated with inoculation, especially with respect to the nutritional status of the seedlings.

Finally, chapter 4 sought to address a recurring question in studies conducted under controlled conditions: can the positive effects of inoculation with PGPM observed in greenhouse experiments be extrapolated to field conditions? To this end, seedlings were produced in a nursery, subsequently planted in the field, and their growth was monitored over time. Although no significant increase in plant growth was observed, this study remains relevant to prove that extending the benefits of inoculation are not a simple path, and many environmental factors need to be studied.

Overall, the findings presented in this thesis provide valuable insights into a specific ecological niche that remains underexplored within the field of forest engineering, with the potential to contribute meaningfully to conservation strategies for native species of the Atlantic Forest biome.

2 CHAPTER 1: MATCHMAKING: A META-ANALYSIS OF PLANT GROWTH-PROMOTING MICROBIAL PARTNERSHIPS DRIVING TREE SEEDLING PERFORMANCE IN THE ATLANTIC FOREST BIOME[1]

2.1 RESUMO

Introdução: A Mata Atlântica Brasileira (MAB) é um hotspot de biodiversidade, e a inoculação de mudas nativas com microrganismos promotores de crescimento vegetal (MPCV) pode aumentar o vigor das mudas para a restauração florestal. Objetivos: Realizamos uma meta-análise para avaliar os efeitos dos MPCV em mudas da MAB e identificamos estratégias para otimizar os resultados da restauração. Métodos: Sintetizamos dados de 60 estudos que relataram a biomassa e o conteúdo de nutrientes em mudas inoculadas com fungos micorrízicos arbusculares (FMA), bactérias promotoras de crescimento vegetal (BPCV), bactérias fixadoras de nitrogênio (BFN) ou FMA+BFN. As respostas foram comparadas entre estágios sucessionais (pioneira, secundária inicial, secundária tardia), grupos funcionais (fixadores de N_2 vs. não fixadores de N_2) e condições experimentais (complexidade do inóculo, fertilização, esterilidade do substrato). Resultados: A inoculação com FMA produziu os maiores benefícios, particularmente para espécies pioneiras e secundárias iniciais. As espécies pioneiras também apresentaram maior crescimento e teor de P. De modo geral, a inoculação com MPCV foi eficaz em espécies não fixadoras de N_2 e em espécies de estágios iniciais de sucessão ecológica, utilizando cepas únicas, em condições estéreis, combinadas com fertilização fosfatada. Conclusões: A adequação dos inoculantes de MPCV ao estágio sucessional das espécies alvo pode aprimorar as práticas de viveiro e aumentar o sucesso da restauração.

Palavras-chave: Fungos micorrízicos arbusculares (FMA). Inoculantes microbianos. Restauração florestal. Sustentabilidade ambiental. Funções de sucessão ecológica.

2.2 ABSTRACT

Introduction: The Brazilian Atlantic Forest (BAF) is a biodiversity hotspot, and inoculating native seedlings with plant growth-promoting microorganisms (PGPMs) may enhance seedling vigor for forest restoration. Objectives: We performed a meta-analysis to evaluate the effects of PGPMs on BAF seedlings and identify strategies to optimize restoration outcomes. Methods: We synthesize data from 60 studies reporting biomass and nutrient content in seedlings inoculated with arbuscular mycorrhizal fungi (AMF), plant growth-promoting bacteria (PGPB), nitrogen-fixing bacteria (NFB), or

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[1] Published as Kondo, Y. R., Antunes, P. M., Siminski, A., & Kaschuk, G. (2026). Matchmaking: A meta-analysis of plant growth-promoting microbial partnerships driving tree seedling performance in the Atlantic Forest biome. *Restoration Ecology*, e70328. http://doi.org/10.1111/rec.70328

AMF+NFB. Responses were compared across successional stages (pioneer, early, late secondary), functional groups (N_2-fixing vs. non-N_2-fixing), and experimental conditions (fertilization inoculum complexity, substrate sterility). Results: AMF inoculation produced the strongest benefits, particularly for pioneer and early secondary species. Pioneers also showed enhanced growth and N content with NFB, while both AMF and PGPB increased P content. Overall, PGPM inoculation was most effective in non-N-fixing species and early successional species, using single strains, under sterile conditions, combined with P fertilization. Conclusions: Matching PGPM inoculants with the successional stage of target species can improve nursery practices and enhance restoration success.

Keywords: Arbuscular mycorrhizal fungi (AMF). Microbial inoculants. Forest restoration. Environmental sustainability. Ecological successional functions.

2.3 INTRODUCTION

The Brazilian Atlantic Forest (BAF) is one of the largest and most biodiverse biomes in the world, spanning ~1.5 million km^2 (580,000 mi^2) along Brazil's Atlantic coast and extending into Paraguay and Argentina. It harbors more than 20,000 plant species, including 8,000 endemics (Scheer & Blum, 2011; Silva et al. 2021). Despite this richness, less than 24% of the original forest remains, and only 12.4% consists of mature, well-preserved stands, highlighting the urgent need for restoration (SOS Mata Atlântica, 2024). The biome is also one of the most densely populated regions, exposing its biodiversity to habitat fragmentation, pollution, invasive species, and other pressures (Strassburg et al. 2020). These threats underscore the need for restoration strategies that are not large-scale but also tailored to specific ecological and conservation objectives (Siminski et al. 2021; Romanelli et al. 2025).

The conservation and large-scale restoration of the BAF, beyond socioeconomic and political considerations, require a multidisciplinary approach. Interventions may draw on forest ecology and forestry to improve seedling production – a fundamental step for restoration success. However, seedling production remains challenging for many BAF species, likely due to persistent knowledge gaps, particularly regarding the role of soil microorganisms and plant–microbe interactions in supporting seedling growth and establishment.

Inoculating BAF tree species with plant growth-promoting microorganisms (PGPMs) is not yet a systematic practice at any scale, whether commercial or otherwise – a potentially missed opportunity. Most studies testing PGPM inoculation on native BAF seedlings have focused on endangered species (e.g., *Araucaria angustifolia*) (Zandavalli et al. 2004; Kondo et al. 2024) or on species of medicinal and commercial value (e.g., *Ilex paraguariensis*) (Bergottini et al. 2015). However, inoculation with plant growth-promoting bacteria (PGPB) (Barcellos et al. 2021; Kondo et al. 2024), N_2-fixing bacteria (NFB) (Bergottini et al. 2015), arbuscular mycorrhizal fungi (AMF) (Zandavalli et al. 2004), or combinations of these organisms (Faria et al. 1995) many have broader applications in nurseries. By improving seedling growth, PGPM inoculation may promote environmental sustainability in many ways. For example, several studies in Brazil have demonstrated the successful use of N_2-fixing leguminous tree species in rehabilitating areas degraded by soil erosion (Macedo et al. 2008; Fiore et al. 2019), construction, and mining (Balieiro et al. 2018). These species also contribute to recovering soil organic matter and restoring biodiversity and other key ecosystem functions (Chaer et al. 2011; Gama-Rodrigues, 2020).

The literature indicates that early phases of plant succession in BAF species are more AMF dependent than later successional species (Matsumoto et al. 2005; Zangaro et al. 2012b; Zangaro et al. 2013). Moreover, Matsumoto et al. (2005) reported that the pioneer and early secondary species show greater dependence on PGPB and NFB, as reflected in positive correlations between plant growth and functional microbial communities. In this context, conducting a meta-analysis of PGPM associations across BAF successional groups is essential for determining whether the trends reported in the literature are supported by a consistent signal across studies, particularly regarding differential plant responses. Increases in seedling biomass and nutrient uptake are often regarded as advantageous traits for pioneer establishment (Poorter, 2007; Wright et al. 2010). However, the transition from nursery to restoration sites involves multiple ecological filters – including competition, herbivory, soil heterogeneity, and climate (Andivia et al. 2021) – and direct evidence linking seedling-scale inoculation effects to long-term restoration outcomes remains scarce. Accordingly, rather than repeating seedling-scale trials in isolation, a broader meta-analysis can situate PGPM effects during early establishment with the broader framework of successional trajectories.

In addition to successional stage, several other factors can modulate seedling responses to PGPMs. Species capable of supporting biological N_2 fixation may interact differently with microbial inoculants due to complementary roles in nutrient acquisition (Vitousek et al. 2013). The composition of the inoculum – whether single or mixed – can also influence inoculation outcomes, as microbial compatibility may lead to additive, synergistic or antagonistic interactions (Savastano & Bais, 2024). Similarly, substrate sterilization, by reducing native microbiota, may either amplify the effects of introduced strains (Li et al. 2019) or suppress inoculation responses (Li et al. 2023). Finally, nutrient availability, particularly of N and P, can regulate plant–microbe symbioses: high nutrient levels tend to suppress mutualistic benefits, whereas moderate nutrient-poor conditions often enhance them (Tharanath et al. 2024).

In BAF restoration, species selection across successional stages is key: pioneers provide initial cover, whereas later-successional species support biodiversity and long-term stability (Rodrigues et al. 2009). PGPM inoculants can improve seedling performance under stressful or nutrient-poor conditions (Sanhueza et al. 2024), thereby facilitating the establishment of key tree species and contributing to the restoration of degraded ecosystems.

We conducted a meta-analysis to guide the use of PGPMs in cultivating native BAF seedlings for improved conservation and restoration outcomes. The guiding hypothesis of our research were:

1. Seedlings responses to PGPM inoculation differ among ecological successional groups.
2. The ability to N_2-fixation influences seedling responses to PGPM inoculation.
3. Single PGPM inocula produce stronger seedling responses than those containing multiple PGPM.
4. Substrate sterilization alters seedlings responses to PGPM inoculation.
5. Nitrogen and P availability influence seedling responses to PGPM inoculation.

2.4 METHODS

We conducted a literature search in Web of Science and Google Scholar using the following keywords: “arbuscular mycorrhizal fungi” or “AMF”, “inoculation” or “co-inoculation”, “plant growth-promoting bacteria” or “PGPB”, and “rhizobium” or “rhizobia” or “nitrogen fixing bacteria” or “BNF”. The search included all publications available in each database from its inception through 2024. We then identified the studies that included BAF tree species. Species identity was verified through the virtual herbarium available on the Flora do Brasil website (REFLORA; Flora e Funga do Brasil, 2024), the official Brazilian repository for species classification. Scientific names were updated as necessary based on the taxonomic revisions available in REFLORA. Furthermore, we classified the species into four ecological successional groups: pioneer, early secondary, late secondary, and climax (Budowski, 1965). Pioneer species establish in open areas or canopy gaps and depend on high light intensity. Early secondary species colonize sites such as small or older gaps near pioneer vegetation. Late secondary species are shade-tolerant during their juvenile stage and form seedling banks in the understory; however, they experience high mortality in the first years and typically produce small- to medium-sized seeds with low viability. Climax species grow slowly, do not tolerate high light in their adult stage, and are well represented in natural regeneration. They usually produce large seeds with short viability. For leguminous species, the capacity for N_2 fixation via nodule formation was confirmed individually by reviewing publications that directly measured N_2 fixation.

To be included in the meta-analysis, experiments had to meet the following criteria: (i) evaluate the effects of inoculation with AMF, PGPB, NFB, or a combination of AMF and NFB on seedlings of native BAF tree species; (ii) include at least one uninoculated control treatment; (iii) be conducted in greenhouse conditions; and (iv) report $n \geq 3$ for each response variable (biomass and nutrition). We focused exclusively on greenhouse experiments because they provide a controlled environment for assessing the early-stages of symbiosis between seedlings and microbial inoculants. The many factors influencing plant growth and microbial interactions under field conditions, make it difficult to isolate the effects of microbial inoculations.

We excluded studies in which soil samples were used as inoculants without specification of the microorganism(s) present. Experiments in which seedlings were grown in trays as pseudo-replicates (i.e., not in independent containers such as pots or tubes) were also excluded. In addition, we excluded studies that did not differentiate seedlings of BAF tree species from one another (e.g., when multiple species were evaluated only at the successional group level). These steps resulted in the inclusion of 60 studies in the meta-analysis (Supplementary Table S1).

We obtained the mean, standard deviation, and number of replicates for inoculated and control treatments for the following variables: dry biomass (shoot, root, or total) and nutritional data (N and P concentration or content in shoot or root). When standard deviations were not reported, they were calculated from the coefficient of variation or the standard error. If neither was provided, we estimated the coefficient of variation from the reported means and used it to derive standard deviation values.

All analyses were performed in R, version 4.0.3 (R Development Core Team, 2020), using the metafor package (Viechtbauer, 2010). The effect of inoculation was estimated using the log response ratio (*lnRR*), calculated for each comparison between the inoculated treatment (T) and the uninoculated control (C) as:

$$lnRR = \ln\left(\frac{\bar{x}T}{\bar{x}C}\right)$$

where: $\bar{x}T$ = mean of the value of the variable in treatments (T) inoculated with AMF, PGPB, NFB, or a combination of AMF and NFB; $\bar{x}C$ = mean of the value of the variable in uninoculated control (C). The variance associated with *lnRR* was calculated following Lajeunesse (2009):

$$Var(lnRR) = \frac{SDT^2}{nT \cdot \bar{x}T^2} + \frac{SDC^2}{nC \cdot \bar{x}C^2}$$

where: SDT, SDC = standard deviations of treatments T and C, respectively; nT, nC = number of replicates in treatments T and C, respectively. Calculations were performed using the following predictor groups:

1) **Ecological group:** pioneer, early secondary, late secondary, and climax.
2) **Functional group:** N_2-fixing leguminous trees, leguminous trees with unknown N_2-fixing ability, and non-leguminous, non-N_2-fixing trees.
3) **Inoculum complexity:** single PGPM inoculum (one microorganism/strain) and multiple PGPM inoculum (several different species or strains).
4) **Substrate condition:** sterilized substrate and non-sterilized substrate.
5) **Fertilization:** fertilization with N and P (two levels: fertilized or non-fertilized) and general fertilization (four levels: N only, P only, N + P, or non-fertilized).

2.5 RESULTS

2.5.1 Effects of PGPM inoculation on the biomass and nutrition of Atlantic Forest tree seedlings

AMF inoculation produced the strongest positive responses in plant biomass and P content across all four BAF ecological groups, although some significant increases were also observed with other PGPMs. Seedlings from pioneer and early secondary stages were the most responsive to inoculation (Table 1). In pioneer species, AMF and NFB increased biomass, NFB increased N content, and AMF increased P content. In early secondary species, AMF increased biomass, N content, and P content, while PGPB increased both N and P contents. The combination AMF + NFB also enhanced biomass in pioneer species, whereas inoculation with PGPB, NFB, and AMF + NFB significantly reduced biomass in early and late secondary species.

TABLE 1 – LOG RESPONSES OF PGMP INOCULATION ON BIOMASS, N, AND P OF ATLANTIC FOREST TREE SEEDLINGS ACROSS SUCCESSIONAL GROUPS.

PGPM	**Ecological Group**	**Plant Biomass**				**Plant N**				**Plant P**			
		n	***lnRR***	***95% C.I.***	***p-value***	***n***	***lnRR***	***95% C.I.***	***p-value***	***n***	***lnRR***	***95% C.I.***	***p-value***
AMF	PI	375	0.688	0.588; 0.788	<0.0001	116	-0.114	-0.169; -0.060	<0.0001	211	0.235	0.162; 0.309	<0.0001
	ES	431	0.646	0.549; 0.744	<0.0001	107	0.155	0.033; 0.277	0.0128	302	0.424	0.332; 0.516	<0.0001
	LS	253	0.321	0.243; 0.399	<0.0001	75	0.041	-0.023; 0.105	0.2058	145	0.237	0.156; 0.317	<0.0001
	CL	57	0.177	0.079; 0.276	0.0004	16	-0.062	-0.131; 0.008	0.0810	41	0.293	0.192; 0.395	<0.0001
PGPB	PI	4	0.206	-0.044; 0.457	0.1067	20	-0.001	-0.423; 0.422	0.9981	NA	NA	NA	NA
	ES	220	-0.199	-0.228; -0.170	<0.0001	44	0.373	0.297; 0.448	<0.0001	24	0.408	0.309; 0.508	<0.0001
	LS	14	-0.023	-0.125; 0.080	0.6665	43	0.090	-0.022; 0.202	0.1142	3	0.337	0.332; 0.341	<0.0001
NFB	PI	186	0.205	0.040; 0.371	0.0151	146	0.365	0.151; 0.579	0.0008	12	0.224	-0.018; 0.465	0.0698
	ES	376	-0.149	-0.196; -0.102	<0.0001	205	0.105	0.025; 0.185	0.0104	45	-0.122	-0.294; 0.050	0.1654
	LS	49	-0.082	-0.235; 0.072	0.2980	18	-0.158	-0.302; -0.014	0.0310	4	0.021	-0.474; 0.515	0.9342
AMF + NFB	PI	42	0.478	0.042; 0.915	0.0317	26	-0.040	-0.135; 0.056	0.4164	26	-0.176	-0.394; 0.043	0.1148
	ES	87	-0.428	-0.698; -0.158	0.0019	41	0.041	-0.045; 0.127	0.3452	45	-0.310	-0.632; 0.011	0.0580
	LS	18	-0.460	-1.092; 0.172	0.1539	6	0.025	-0.291; 0.341	0.8770	6	-0.250	-0.944; 0.444	0.4809

Abbreviations: PGPM = plant growth-promoting microorganisms; AMF = arbuscular mycorrhizal fungi; PGPB = plant growth-promoting bacteria; NFB = nitrogen-fixing bacteria; PI = pioneer; ES = early secondary; LS = late secondary; CL = climax; NA = data not available; *n* = number of observations; *lr* = log response ratio, calculated as the value of the inoculated treatment divided by the non-inoculated control; 95% *C.I.* = confidence interval at *p*<0.05.

Interpretation: Negative *lr* values indicate reductions due to inoculation, positive values indicate increases, and effects are significant when *lr* and both *C.I.* limits share the same sign.

SOURCE: The author (2025).

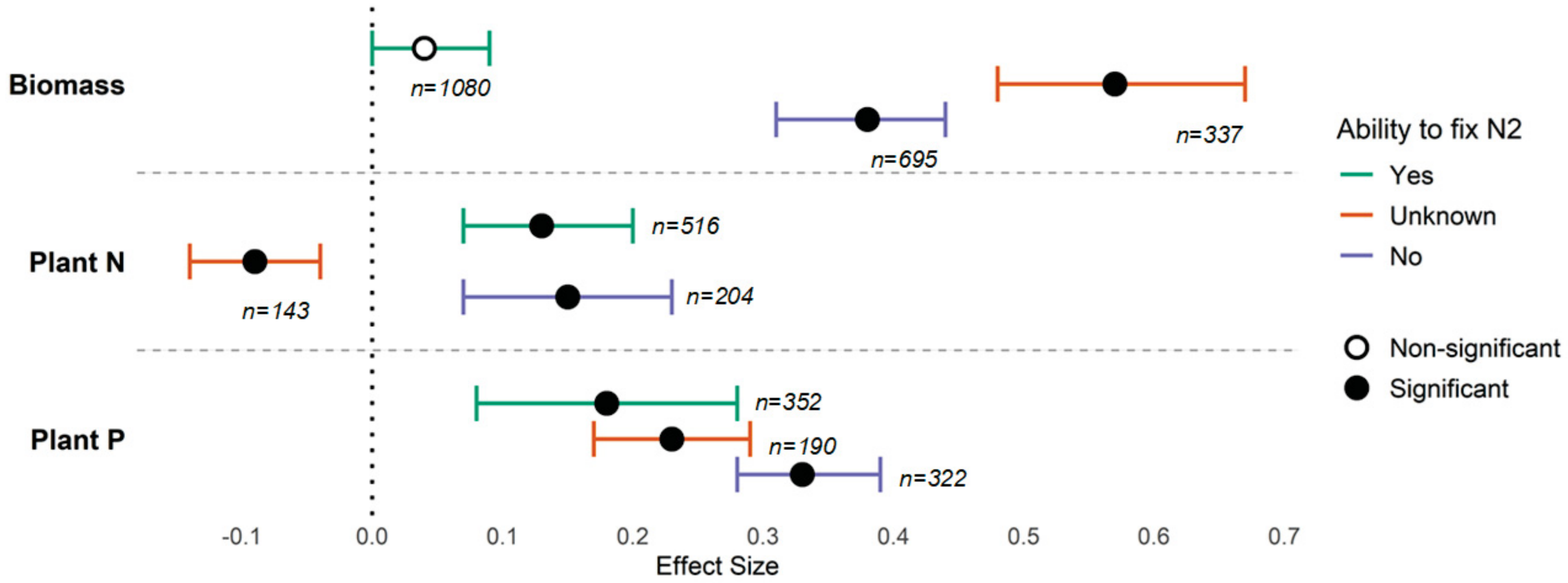
2.5.2 Responses to functional traits, inoculant complexity, substrate conditions, and fertilization

PGPM inoculation significantly increased N and P contents in N_2-fixing leguminous species, but had no significant effect on biomass. In legume species of unknown N_2-fixing capacity, inoculation increased biomass and P content but reduced N content. Non-leguminous species showed the most consistently positive responses to PGPM inoculation across all measured variables (Figure 1).

Inoculation with a single PGPM strain significantly increased all three variables: biomass, N and P contents. Multiple PGPM inoculum increased biomass and P content but did not significantly affect N content (Figure 2).

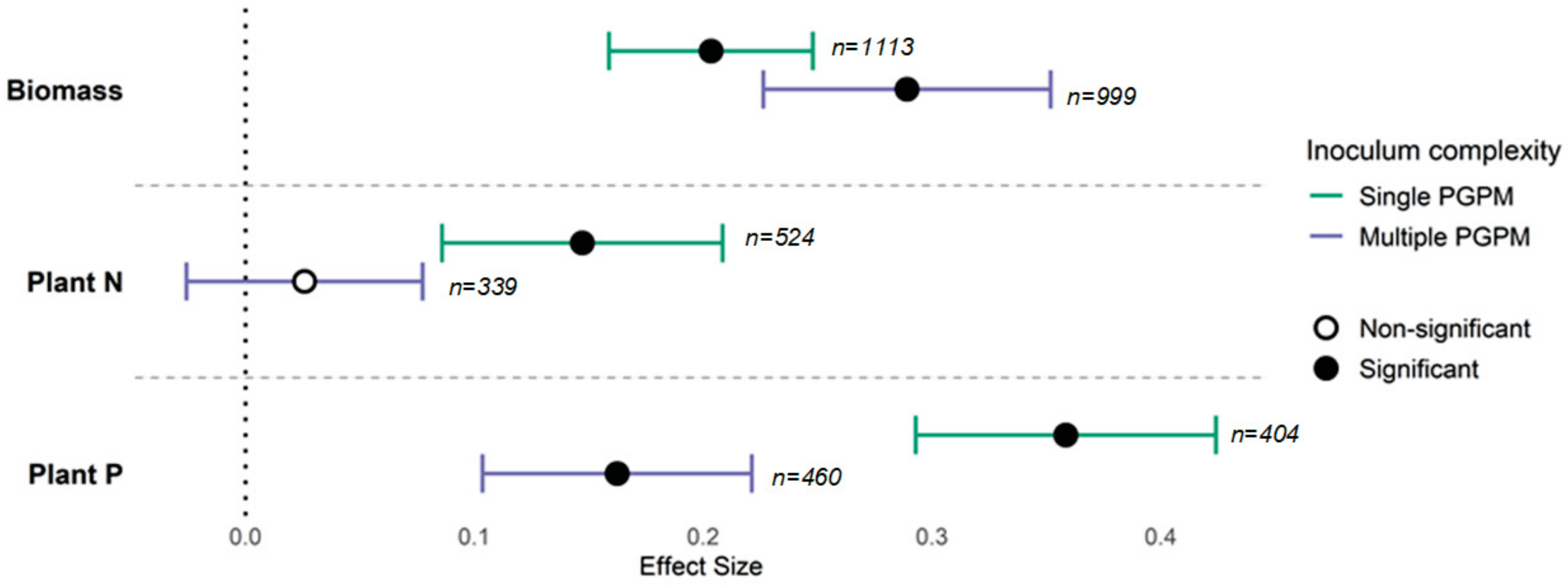
Substrate sterilization strongly influenced plant responses (Figure 3). In sterilized substrates, PGPM inoculation increased all measured variables, with biomass showing nearly a 50% increase (lnRR = 0.496). In non-sterile substrates, inoculation increased N and P contents but had no significant effect on biomass.

FIGURE 1 – EFFECT SIZE (*lnrr)* OF PGPM INOCULATION ON BIOMASS, N, AND P IN ATLANTIC FOREST TREE SEEDLINGS BY N_2-FIXATION ABILITY. BARS INDICATE 95% CONFIDENCE INTERVALS ($p<0.05$); N DENOTES THE NUMBER OF OBSERVATIONS.



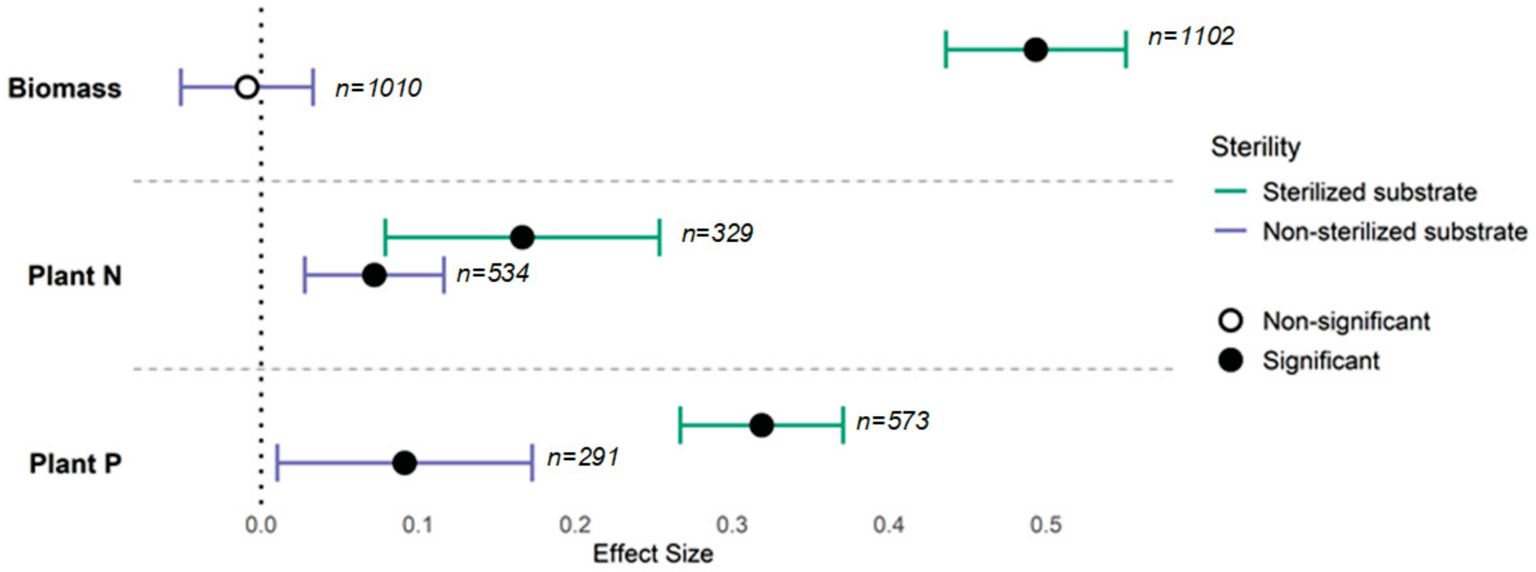
SOURCE: The author (2025).

FIGURE 2 – EFFECT SIZE (*lnrr)* OF PGPM INOCULATION ON BIOMASS, N, AND P IN ATLANTIC FOREST TREE SEEDLINGS BY INOCULUM COMPLEXITY. BARS INDICATE 95% CONFIDENCE INTERVALS (p<0.05); N DENOTES THE NUMBER OF OBSERVATIONS.



SOURCE: The author (2025).

FIGURE 3 – EFFECT SIZE (*lnrr)* OF PGPM INOCULATION ON BIOMASS, N, AND P IN ATLANTIC FOREST TREE SEEDLINGS BY SUBSTRATE CONDITION. BARS INDICATE 95% CONFIDENCE INTERVALS (p<0.05); N DENOTES THE NUMBER OF OBSERVATIONS.



SOURCE: The author (2025).

Nutrient fertilization also modulated PGPM effects (Table 2). Negative responses in biomass and nutrient content occurred when inoculation (without fertilization) was used instead of N or P fertilization. However, when both inoculated and uninoculated seedlings received similar fertilizer additions, PGPM inoculation consistently increased biomass, N, and P contents compared with controls.

Table 3 shows the most significant outcomes that can guide strategies to enhance seedlings vigor in BAF restoration.

TABLE 2 – LOG RESPONSE RATIOS OF PGPM INOCULATION ON BIOMASS, N, AND P IN ATLANTIC FOREST TREE SEEDLINGS UNDER FERTILIZATION COMBINATIONS.

Fertilizer Combination	**Plant Biomass**				**Plant N**				**Plant P**			
	n	***lnRR***	***95% C.I.***	***p-value***	***n***	***lnRR***	***95% C.I.***	***p-value***	***n***	***lnRR***	***95% C.I.***	***p-value***
N only												
I+N / C+N	711	0.238	0.175; 0.301	<0.0001	244	0.031	-0.014; 0.076	0.1738	327	0.286	0.211; 0.361	<0.0001
I+N / C-N	106	0.154	-0.015; 0.323	0.0746	51	0.607	0.477; 0.737	<0.0001	21	-1.118	-1.508; -0.709	<0.0001
I-N / C+N	304	-0.234	-0.370; -0.103	0.0005	219	-0.125	-0.212; -0.038	0.0050	57	0.223	0.032; 0.413	0.0224
I-N / C-N	991	0.355	0.304; 0.407	<0.0001	349	0.212	0.130; 0.294	<0.0001	459	0.273	0.219; 0.327	<0.0001
P only												
I+P / C+P	1088	0.276	0.230; 0.322	<0.0001	371	0.155	0.082; 0.229	<0.0001	482	0.281	0.231; 0.330	<0.0001
I+P / C-P	123	1.114	0.871; 1.357	<0.0001	51	-0.079	-0.281; 0.123	0.4437	70	0.337	0.172; 0.501	<0.0001
I-P / C+P	129	-0.631	-0.805; -0.457	<0.0001	53	0.122	0.064; 0.180	<0.0001	72	-0.414	-0.611; -0.218	<0.0001
I-P / C-P	772	0.197	0.140; 0.254	<0.0001	388	0.074	0.016; 0.131	0.0125	240	0.320	0.234; 0.406	<0.0001
N + P												
I+N+P / C+N+P	550	0.295	0.228; 0.363	<0.0001	159	-0.039	-0.087; 0.008	0.1065	251	0.322	0.251; 0.393	<0.0001
I+N-P / C+N-P	127	0.399	0.286; 0.512	<0.0001	69	0.154	0.055; 0.253	0.0023	60	0.380	0.175; 0.586	0.0003
I-N+P / C-N+P	371	0.402	0.334; 0.471	<0.0001	131	0.467	0.277; 0.656	<0.0001	190	0.282	0.207; 0.357	<0.0001
I-N-P / C-N-P	474	0.213	0.145; 0.281	<0.0001	164	0.153	0.073; 0.233	0.0002	177	0.315	0.217; 0.412	<0.0001
I+N+P / C-N-P	8	2.782	2.157; 3.407	<0.0001	4	0.188	0.023; 0.353	0.0255	4	-0.151	-0.213; -0.089	<0.0001
I+N-P / C-N+P	30	-0.924	-1.223; -0.619	<0.0001	14	0.262	0.166; 0.357	<0.0001	14	-1.628	-1.860; -1.396	<0.0001
I-N+P / C+N-P	30	0.441	-0.008; 0.891	0.0542	14	-0.056	-0.218; 0.106	0.4959	14	1.018	0.394; 1.644	0.0014

Abbreviations: *n* = number of observations; *lr* = log response ratio, calculated as the value of the inoculated treatment divided by the non-inoculated control; 95% *C.I.* = confidence interval at $p < 0.05$; I = inoculated; C = control (not inoculated); +N = fertilized with N; -N = not fertilized with N; +P = fertilized with P; -P = not fertilized with P.

Interpretation: Negative *lr* values indicate reductions due to inoculation, positive values indicate increases, and effects are significant when *lr* and both *C.I.* limits share the same sign.

SOURCE: The author (2025).

TABLE 3 – BEST OUTCOMES OF PGPM INOCULATION IN TREE SEEDLINGS FOR BRAZILIAN ATLANTIC FOREST RESTORATION.

PGPM	AMF	NFB	PGPB	All PGPM combined
Increase	biomass, P content	biomass, N content	N and P contents	
Ecological group	pioneer; early secondary	early secondary	pioneer	
Functional group	-	-	-	Species lacking N_2-fixation ability
Inoculum complexity	-	-	-	Single PGPM inoculum
Substrate conditions	-	-	-	Sterilized substrate
Fertilization	-	-	-	P only

Abbreviations: PGPM = plant growth-promoting microorganisms; AMF = arbuscular mycorrhizal fungi; PGPB = plant growth-promoting bacteria; NFB = nitrogen-fixing bacteria.
Predictors: Ecological group: pioneer, early secondary, late secondary, and climax; Functional group: N_2-fixing leguminous trees, leguminous trees with unknown N_2-fixing ability, non-leguminous/non–N_2-fixing trees; *Inoculum complexity*: single PGPM inoculum, multiple PGPM inoculum; Substrate condition: sterilized, non-sterilized; Fertilization: fertilization with N and P (two levels: fertilized or non-fertilized) and general fertilization (four levels: N only, P only, N + P, or non-fertilized).
SOURCE: The author (2025).

2.6 DISCUSSION

2.6.1 Effects of PGPM inoculation on BAF tree seedlings across ecological successional groups

Among the three PGPM types, AMF performed best. Therefore, based on the strong positive responses of BAF tree seedlings to AMF in our study, we recommended producing AMF-inoculated seedlings for restoration. However, the availability of commercial AMF inoculants in the Brazilian market remains very limited – for example, only one inoculant, based on *Rhizophagus intraradices*, is currently officially available (Stoffel et al. 2020). Production of AMF inoculants is constrained by the fact that AMF are obligate biotrophic symbionts, implying that they can only grow when colonizing living host plants. Globally, commercial AMF products often fail to enhance crop yields due to low propagule viability or pathogen contamination (Salomon et al. 2022; Koziol et al. 2024a; Koziol et al. 2024b). Thus, the greatest challenge in harnessing the benefits of AMF for BAF restoration lies in the large-scale production of viable inoculants. At the same time, an important concern is the invasive potential of exotic AMF strains produced by the global inoculant industry (Hart et al. 2018), whereas the use of indigenous inoculants can support local biodiversity (Koziol et al. 2018). Notably, most studies included in our meta-analysis evaluated indigenous strains, underscoring the strong potential of native AMF to promote the growth of BAF tree seedlings. We therefore recommend prioritizing indigenous AMF species when producing inoculated seedlings for restoration.

We found that the most light-dependent ecological groups (i.e., pioneer and early secondary species) were also the most responsive to PGPM inoculation. The AMF inoculation resulted in significantly greater responses in pioneer and early secondary species than in late secondary and climax species. Among leguminous trees, NFB and AMF + NFB inoculation increased biomass only in pioneer species. These findings align with previous reports of positive effects of AMF inoculation on species from the early phases of tropical succession, both in BAF species (Zangaro et al. 2012a; Zangaro et al. 2012b; Zangaro et al. 2013) and in other ecosystems worldwide (Wang et al. 2024; Wang et al. 2025).

The “successional stage hypothesis” provides insight into this preferential association between ecological groups and specific PGPM (Liu & Zhao, 2023). The hypothesis establishes that net plant–soil microbial feedback varies in direction and intensity depending on successional stage, thereby explaining why shrub and tree species emerge in sequence following deforestation. Pioneer and early secondary seedlings readily form associations with AMF because their rapid growth requires large nutrient inputs, increasing their dependence on AMF. In addition, their sun-loving, fast-growing habitat sustains high photosynthetic rates, enhancing carbon availability to support symbiosis (Zangaro et al. 2012b; Zangaro et al. 2013). Consequently, AMF colonization is generally greater under high light conditions, whereas low light increases the carbon cost and may reduce the symbiotic benefit – supporting the idea that heliophytic species are better able to maintain mycorrhizal symbioses (Wood et al. 2023).

The successional stage hypothesis likewise explains the benefits of NFB and AMF + NFB inoculation for pioneer legumes, since active biological N_2 fixation is an energetically costly and light-limited process (Taylor & Menge, 2018). If light is available, legumes may increase their photosynthetic rate to sustain NFB in their nodules (Kaschuk et al. 2010). However, although some studies have shown that co-inoculation with AMF and NFB can be especially beneficial for legumes (Andreata et al. 2024; Kalamulla & Yapa, 2024), co-inoculation AMF + NFB decreased shoot biomass in early secondary species and did not improve it in late secondary species. Thus, a major challenge is disentangling how the taxonomic diversity of AMF interacts with that of NFB, and how these interactions ultimately influence plant performance (Kavadia et al. 2021).

2.6.2 Biomass and nutritional responses by functional traits, inoculant complexity, substrate condition, and fertilization

With respect to N_2-fixation, PGPM inoculation increased N and P contents in both non-leguminous species and nodulated N_2-fixing legumes. However, while inoculation enhanced biomass in non-leguminous species and in legumes with unknown N_2-fixation ability, it had no significant effect on the biomass of nodulated N_2-fixing legumes, indicating differences in resource-use strategies among seedling species. Although increased nutrient status generally enhances photosynthetic capacity and growth (Kaschuk et al. 2010), the biomass of nodulated N_2-fixing legumes may be constrained by P-dependent energy process (Míguez-Montero et al. 2020) and by the diversion of photosynthates required to sustain nodulation and biological N_2 fixation (Taylor & Menge, 2018). Differences in N_2-fixation ability likely reflect distinct ecological strategies: N_2-fixing leguminous tree species contribute to system-level nutrient enrichment and are valuable in restoring plantations (Chaer et al. 2011; Jhariya et al. 2018), whereas non-leguminous species primarily contribute to the rapid establishment of vegetation cover.

Considerable effort has been directed toward assembling complex inoculants, including synthetic or simplified microbial communities (SynComs) that represent key ecological functions in soil and plant microbiomes (Souza et al. 2019). Mixing several taxa aims to maximize plant growth under controlled conditions (Shayanthan et al. 2022). Using multiple PGPM inoculum may be advantageous because it mimics the redundancy and resilience of native soil microbial communities. However, our meta-analysis shows that inoculation of both single and multiple PGPM strains increased seedlings biomass, while only single-strain inoculation improved the N and P status of BAF seedlings. These results suggest that using inoculum containing single instead of multiple PGPM resulted in greater responses than multiple PGPM inoculum. Further studies are needed to optimize the selection of compatible microbial groups and determine appropriate inoculum concentrations for BAF tree seedlings.

Our findings that inoculated sterilized soil enhanced seedling biomass and nutrient status is consistent with previous work showing that, when inoculated beneficial microbes (e.g., AMF) can freely colonize roots without competition, their performance is maximized compared to inoculations of non-sterile soil (Aggangan & Moon, 2013). However, sterilization often alters soil abiotic properties (e.g., N, P, pH), which may also contribute to growth differences (Hu et al. 2020). Nonetheless, the results are unlikely to be explained by soil sterilization alone, as this process also removes native microbial communities that may interact with the inoculum (Li et al. 2023). Including treatments with microbial filtrates or leachates would help disentangle these effects, but such experimental designs were not used in studies included in this meta-analysis. In natural (non-sterile) soils, PGPM must compete with indigenous microorganisms, and their efficacy depends strongly on abiotic conditions and inoculation strategies (Lopes et al. 2021; Malgioglio et al. 2022). Furthermore, the efficiency of PGPM inoculants depends not only on their ability to colonize, survive, and persist in the environment but also on the complex network of interactions within the rhizosphere (Verbruggen et al. 2013; Kong et al. 2025). In this context, indigenous

PGPM are often considered more adaptable because they benefit from a “home-field advantage” (Kong et al. 2025). Therefore, further research on inoculating BAF seedlings with PGPM is needed to identify strains that can effectively compete with native soil microorganisms and thereby enhance both biomass production and nutrient status in seedlings.

Given the environmental impacts and high production costs of N and P fertilizers (Aziz et al. 2015; Tyagi et al. 2022), there is an urgent need to minimize their use by integrating more sustainable practices (Guignard et al. 2017; Brownlie et al. 2022; Tyagi et al. 2022). In our analysis, PGPM inoculation alone resulted in lower responses compared to N and P fertilization, indicating that PGPM cannot fully replace fertilizers. However, in all other cases, where both inoculated and control treatments share similar fertilizers conditions, PGPM inoculation increased seedling biomass and improved nutrient status, showing that PGPM are also beneficial under fertilizer conditions.

Although our study provides valuable insight into PGPM use for BAF tree seedlings in greenhouse settings, field validation is still needed. Overall, our findings represent a first step toward evidence-based decision-making in PGPM-assisted restoration of BAF ecosystems, serving as a guide for selecting the most promising PGPM under greenhouse conditions. This selection is essential given the long developmental cycle of trees in the field and the need for long-term experiments to assess restoration outcomes. Moreover, even when greenhouse inoculation does not result in significant plant growth promotion, valuable insights can still be gained regarding inoculum performance (Gu et al. 2020).

We proposed a set of best practices for inoculating BAF tree seedlings. Our findings indicate that AMF are the most responsive PGPMs for these species. Accordingly, we recommend the use of suitable indigenous AMF inoculants in nurseries producing seedlings for restoration campaigns. For improved nursery performance, the use of single PGPM inocula and P fertilization is also advised. Nevertheless, future studies should assess how associations between native BAF seedlings and AMF develop under field conditions, particularly in relation to plant–microbe and microbe–microbe interactions.

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3 CHAPTER 2: COMPETITIVENESS OF PLANT-GROWTH PROMOTING BACTERIA INOCULATED INTO *Araucaria angustifolia* ASSESSED BY qPCR AMPLIFICATION

3.1 RESUMO

O pinheiro-brasileiro ou araucária (*Araucaria angustifolia* (Bertol.) Kuntze) é uma espécie arbórea sul-americana criticamente ameaçada de extinção, cujas mudas podem se beneficiar da inoculação com bactérias promotoras de crescimento vegetal (BPCV). No entanto, a viabilidade da aplicação de BPCV em mudas de árvores ainda é pouco compreendida. Este estudo avaliou o crescimento e as respostas nutricionais de mudas de araucária à inoculação com BPCV em três substratos diferentes e avaliou a persistência das cepas inoculadas nas raízes das mudas cultivadas em areia esterilizada ou solo natural. Duas formulações de BPCV foram testadas: (i) *Azospirillum brasilense* Ab-v5 e Ab-v6, e (ii) *Bacillus subtilis* CCTB04, *B. pumilus* CCTB05 e *B. amyloliquefaciens* CCTB09. A colonização radicular foi avaliada por diluição seriada com plaqueamento em superfície e por qPCR direcionado às cepas Ab-v6 e CCTB04. Utilizando qPCR, detectamos Ab-v6 e CCTB04 em areia esterilizada, mas apenas CCTB04 em solo natural. Esses resultados confirmam que a qPCR é uma ferramenta confiável para monitorar a colonização por BPCV em raízes de araucária e sugerem que CCTB04 é mais competitiva que Ab-v6 na interação com a comunidade microbiana nativa do solo. Além disso, ambos os inoculantes melhoraram o estado nutricional e o crescimento radicular, embora os efeitos tenham variado dependendo do substrato de crescimento. De modo geral, o estudo destaca que a persistência de BPCV na rizosfera depende da competitividade da cepa introduzida. Portanto, estratégias eficazes de seleção e inoculação de BPCV devem levar em consideração as interações com as comunidades microbianas da rizosfera.

Palavras-chave: Microbiologia florestal. Persistência de inoculantes. Sementes de pinheiro. Monitoramento molecular microbiano. Relação diversidade–invasibilidade.

3.2 ABSTRACT

Brazilian pine or araucaria (*Araucaria angustifolia* (Bertol.) Kuntze) is a critically endangered South American tree species whose seedlings may benefit from inoculation with plant growth-promoting bacteria (PGPB). However, the feasibility of PGPB application in tree seedlings remains poorly understood. This study evaluated the growth and nutritional responses of araucaria seedlings to PGPB inoculation in three different substrates and assessed the persistence of inoculated strains on seedling roots grown in sterilized sand or natural soil. Two PGPB formulations were tested: (i) *Azospirillum brasilense* Ab-v5 and Ab-v6, and (ii) *Bacillus subtilis* CCTB04, *B. pumilus* CCTB05, and *B. amyloliquefaciens* CCTB09. Root colonization was evaluated by serial dilution with surface plating and by qPCR targeting strains Ab-v6 and CCTB04. Using qPCR, we detected Ab-v6 and CCTB04 in sterilized sand, but only CCTB04 in natural soil. These findings confirm that qPCR is a reliable tool for monitoring PGPB colonization in araucaria roots and suggest that CCTB04 is more competitive than Ab-v6 when interacting with the native soil community. Moreover, both inoculants enhanced nutrient status and root growth, although effects varied depending on the growth substrate. Overall, the study highlights that PGPB persistence in the rhizosphere depends on the competitiveness of the introduced strain. Therefore, effective PGPB selection and inoculation strategies must account for interactions with rhizosphere microbial communities.

Keywords: Forestry microbiology. Inoculant persistence. Pine seeds. Microbial molecular monitoring. Diversity–invasibility relationship.

3.3 INTRODUCTION

The conservation of endangered tree species remains one of the greatest challenges in conservation biology forestry (Szaro, 2008; Potter et al. 2017). *Araucaria angustifolia* (Bertol.) Kuntze, a conifer endemic to the Atlantic Forest in South America, commonly known as Brazilian pine or araucaria, holds fundamental ecological and socioeconomic importance (Bogoni et al. 2020). However, it is critically endangered due to deforestation and overexploitation (Marchioro et al. 2020). Innovative strategies that integrate conservation, high-quality seedling production, and ecosystem restoration are urgently required to ensure the survival of this species (Tagliari et al. 2021).

Plant growth-promoting bacteria (PGPB), particularly species of *Azospirillum* and *Bacillus*, have attracted increasing interest for their ability to enhance the growth of forest seedlings (Kondo et al. 2020; 2024; Calzavara et al. 2021; Lopez et al. 2021). These microorganisms act through direct mechanisms, such as nitrogen fixation and phosphorus solubilization, and indirect mechanisms, including competition with pathogens and induction of systemic resistance (Olanrewaju et al. 2017). While their benefits are well established in agricultural crops (Reed & Glick, 2023), knowledge about their effectiveness in slow-growing and threatened tree species remains limited. For example, the effective root colonization by inoculated bacteria or their persistence in the rhizosphere are rarely assessed, hindering causal links between microbial presence and plant growth promotion. Successful associations require that inoculants survive, colonize roots, and compete effectively with native microbiota (Benizri et al. 2001; Rilling et al. 2019; Santoyo et al. 2021).

Quantifying bacterial colonization is thus essential to validate inoculation outcomes. Quantitative PCR (qPCR) offers a sensitive and specific method for detecting inoculated strains and correlating their abundance with plant growth responses (Soares et al. 2021). Another key factor is the growth substrate: sterile media facilitate establishment by reducing microbial competition (Li et al. 2019), whereas non-sterile soil better reflects field conditions and challenges microbial persistence (Li et al. 2023).

In this study, we evaluated the viability of PGPB inoculation in araucaria seedlings by monitoring root colonization in sterile and non-sterile substrates. We hypothesized that qPCR is a feasible tool for tracking the persistence of introduced strains in the araucaria rhizosphere. Such an approach can contribute to advancing the understanding of the mechanisms underlying positive plant responses to PGPB. Our ultimate goal was to support the development of more efficient seedling production strategies and to promote the application of PGPB in conservation and forest restoration programs.

3.4 MATERIALS AND METHODS

3.4.1 Araucaria seeds

Seeds were collected from the ground after natural cone abscission of araucaria trees for the first experiment (2020-2021) in Campo Magro, PR, Brazil (25º22’30’’ S, 49º22’46’’ W), and for the second experiment, from Quitandinha, PR, Brazil (25º54’58.1’’ S, 49º35’00.2’’ W). Seeds were classified by viability, without size sorting, by water immersion, discarding floating seeds (Wendling & Zanette, 2017).

3.4.2 Inoculation

Two commercial inoculants were used in the experiments. The first contained *Azospirillum brasilense* Ab-v5 and Ab-v6 (Biotrop – Soluções Biológicas, Curitiba, PR, Brazil) at 2 x 10^8 CFU mL^{-1}, and the second contained *Bacillus subtilis* CCTB04, *B. pumilus* CCTB05, and *B. amyloliquefaciens* CCTB09 (Biotrop – Soluções Biológicas, Curitiba, PR, Brazil) at 1 x 10^8 CFU mL^{-1}. Inoculation was carried out by applying 50 mL of inoculant solution, diluted in filtered water, to the substrate around each seedling. Control seedlings received 50 mL of filtered water only. In 2020-2021, inoculants were applied at a concentration of 0.8% (Kondo, 2022). In 2023, the concentrations were 0.9% for *A. brasilense* and 1.0% for *Bacillus* spp., following Kondo et al. (2024).

3.4.3 Greenhouse experiments

The experiments were conducted in a greenhouse in Curitiba, Paraná, Brazil (25º24’45.7’’ S, 49º14’57.0’’ W) during 2020/2021 and 2023. Except for precipitation and wind, greenhouse conditions closely reflected the external climate. Climatic data for the experimental periods (June 2020 to January 2021, and June to December 2023) were obtained from the Meteorological Database for Teaching and Research (BDMEP) of the National Institute of Meteorology (INMET), using the Curitiba automatic station A807. Climatic data and substrate conditions are reported in Table 4.

TABLE 4 – EXPERIMENTAL CONDITIONS FOR THE GREENHOUSE EXPERIMENTS.

Year	**2020-2021**	**2023**	**2023**
Substrate	**Carolina Substrate®**	**Sterilized Sand**	**Natural Soil**
pH in $CaCl_2$	6.3	5.1	4.2
pH in SMP	-	7.2	5.0
Al (cmol$_c$ dm^{-3})	-	0	0.7
H+Al (cmol$_c$ dm^{-3})	-	2	12.1
Total organic C (g dm^{-3})	10.14	13.2	56.1
K_2O (%, m/m)	<0.25	-	-
K (cmol$_c$ dm^{-3})	-	0.06	0.2
Ca (cmol$_c$ dm^{-3})	80	1.8	2.2
Mg (cmol$_c$ dm^{-3})	35	0.4	0.9
P (mg dm^{-3})	-	23.3	9.4
P_2O_5 (%, m/m)	0.35	-	-
CEC (cmol$_c$ dm^{-3})	3	4.3	15.4
Clay (%, m/m)	-	-	42
Silt (%, m/m)	-	-	19
Sand (%, m/m)	-	-	39
Greenhouse			
Mean maximum greenhouse temperature (°C)	30.6	31	31
Mean minimum greenhouse temperature (°C)	10.9	8.4	8.4
Sowing date	Jun 30, 2020	Jun 19, 2023	Jun 21, 2023
Inoculation date	Sep 23, 2020	Nov 20, 2023	Nov 23, 2023
Seedling age at inoculation	3 months	5 months	5 months
Plant analyses	7 months	6 months	6 months
Pot size	0.5 L bags	0.8 L pots	0.8 L pots

SOURCE: The author (2025).

The experiments followed a completely randomized design with three seedling treatments: (1) control (not inoculated), (2) inoculation with *A. brasilense*, and (3) inoculation with *Bacillus* spp. In the 2020-2021 pilot experiment, treatments comprised six replicates with one harvest. In 2023, experiments with sterilized sand comprised 32 replicates per treatment, and those with non-sterile soil comprised 36 replicates per treatment. For each substrate, 12 seedlings per treatment were harvested at 7 and 21 days after inoculation for root colonization analyses: six seedlings for the surface plating method (CFU) and six for DNA amplification by qPCR. Remaining seedlings were harvested 28 days after inoculation. Each experimental unit consisted of a single araucaria seedling grown in one bag/pot.

In 2020, seeds were sown in 0.5 L polystyrene bags filled with Carolina Soil Substrate® (hereafter referred to as Carolina substrate), purchased from a commercial supplier. Seeds were not disinfected prior to sowing. Inoculation was performed after seedling emergence, when seedlings had reached approximately 5 cm in height. Harvesting was carried out 215 days of age.

In 2023, seeds were sown in 0.8 L pots containing either sterilized sand or non-sterile soil, with characteristics shown in Table 4. Inoculation was performed when seedlings reached a shoot height 1.5 times greater than the container height. Seedlings were harvested at 7, 21, and 28 days after inoculation.

For sterilized sand treatments, medium-sized sand was purchased from a builder's merchant and sieved through a 2 mm mesh. The sand was washed by submerging in tap water (10 cm column), stirring for three minutes, and draining, repeated five times. It was then rinsed four times with deionized water under the same procedure. Next, the sand was submerged in a 3% HCl solution (5 cm column), stirred, and left to stand for 24 h to desorb nutrients. The following day, the acid was drained, and the sand was washed four additional times with deionized water to remove residual HCl. The washed sand was spread in polyethylene trays, air-dried on greenhouse benches, packed in cotton cloth bag, and sterilized by autoclaving at 120º for 30 minutes. The sterilized sand was dried again on greenhouse benches and transferred to pots on the day of sowing. A subsample was dried at 105 ºC to constant mass and analyzed chemically (Table 4). Seeds used in the sterilized sand treatments were surface-sterilized by immersion in 70% ethanol for 30 seconds, followed by 2% sodium hypochlorite for 15 seconds, and rinsed five times with sterile deionized water before sowing.

For the natural soil treatment, soil was collected from the 0-20 cm layer of a Haplic Cambisol, according to SiBCS (Santos et al. 2025), corresponding to an

Inceptisol in the USDA Soil Taxonomy (Soil Survey Staff, 2022), at the Canguiri Experimental Farm in Pinhais, Paraná, Brazil (25º23'24.5'' S, 49º08'36.5'' W). Ten individual samples were collected at different points within the area and combined to form a composite sample. The soil was sieved, transferred to 0.8 L pots, and used without seed surface disinfection.

3.4.4 Substrate analyses

Substrate analyses were performed in accordance with the official rules of Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) for substrate testing, as established by SDA/MAPA Normative Instruction No. 37/2017 (Brasil, 2017) and Normative Instruction No. 24/2007 (Brasil, 2007). The pH in $CaCl_2$ was determined using a 0.01 mol L^{-1} $CaCl_2$ solution in a 1:2.5 substrate-solution ratio. Exchangeable Ca, Mg, and Al were extracted with 1 mol L^{-1} KCl, while K and P were extracted with Mehlich-1 solution. Potential acidity (H+Al) was determined using the SMP buffer method. Organic carbon content was measured by the Walkley-Black method (Walkley & Black, 1934). For soil samples, physical analyses included determination of particle-size distribution using the Bouyoucos hydrometer method (Bouyoucos, 1962).

3.4.5 Microbiological analyses

3.4.5.1 Serial dilution and plate counting of colony forming unities (CFU)

At each harvest, the seedlings were taken to the laboratory and removed from the pots for analyses. Bacterial counting by surface plating method were performed using a sample composed of rhizosphere sand or soil. To obtain CFU values, approximately 10 g of rhizosphere were weighed in duplicate (one portion for bacterial counting and the other for determining dry mass). The sample was transferred to a 250 mL Erlenmeyer flask containing five glass beads and 90 mL of sterile saline solution (0.85% NaCl), yielding a 1:10 solution. The suspension was shaken on a rotatory shaker (Tecnal Equipamentos Científicos, Brazil) for 15 minutes to disperse the CFU and then allowed to settle. From this suspension, a 1:100 dilution was prepared and used for rhizosphere sand, and a 1:1000 dilution was prepared and used for rhizosphere soil. From each dilution, 0.1 mL was plated in triplicate on Petri dishes containing Tryptic Soy Agar (TSA) and Rojo Congo (RC) culture medium. The inoculum was spread using a disposable sterile Drigalsky loop (Synth, Brazil). Plates were sealed with parafilm, inverted, and placed in a BOD incubator (Solab Científica, Brazil) at 28 ºC for three days for rhizosphere sand, and five days for rhizosphere soil. After that, the colonies were counted with a digital colony counter (Tecnal Equipamentos Científicos, Brazil). The Petri dishes was performed in triplicate and in all treatments both culture media were used.

The culture media used were TSA and RC, commonly used by the Brazilian inoculant industry for quality control of *Bacillus* spp. and *Azospirillum* spp. inoculants, respectively. The TSA medium was obtained as a commercially available, ready-to-use formulation (pre-weighted powder), according to the manufacturer's instructions (Kasvi, Brazil). The RC medium was prepared following the protocol described in Brazilian Ministry of Agriculture Normative Instruction No. 14 (Brasil, 2018) and contained 0.5 g K_2HPO_4 (Synth, Brazil), 0.2 g $MgSO_4.7H_2O$ (Synth, Brazil), 0.1 g NaCl (Synth, Brazil), 0.5 g yeast extract (Kasvi, Brazil), 0.015 g $FeCl_3.6H_2O$ (Dinâmica, Brazil), 5 g DL-malic acid (Êxodo Científica, Brazil), 4.8 g KOH (Synth, Brazil), 15 mL Congo Red Solution (Êxodo Científica, Brazil) and 20 g Agar (Kasvi, Brazil). The components were diluted in 1 L of deionized water in the mentioned order and the pH was adjusted to 7.0 using KOH.

3.4.5.2 qPCR

For qPCR analyses, 250 mg samples of rhizosphere sand or soil were insert into 15 mL Falcon tubes (Synth, Brazil) and frozen at -20 ºC. DNA extraction was performed using the DNeasy® PowerSoil® Pro Kit (Qiagen, Germany), following the manufacturer's instructions. The steps included chemical and mechanical cell lysis by shaking in a bead beating tube, followed by removal of inhibitors and DNA purification with IRT (Inhibitor Removal Technology) and by binding to silica membrane in centrifuge columns. DNA was eluted in 50 µL of elution solution provided by the kit and stored at -20 ºC until use. The integrity and presence of the extracted DNA were verified by electrophoresis in a 1% agarose gel prepared with TAE 1X and stained with SYBR Safe® (Thermo Fischer Scientific, USA) (Supplementary Figure S1). For loading onto the gel, 5 µL mixtures were prepared, containing 2 µL of extracted DNA (50 ng/ µL), 2 µL of ultrapure water and 1 µL of sample distribution, according to the dye used

(Blue Juice 10X or original 2X). Thus, approximately 100 ng of DNA were applied per well. The run was performed at 150 V with 400mA amperage for 30 minutes in TAE 1X. The molecular weight marker DNA ladder 1 Kb (Thermo Fischer Scientific, USA) was used as a reference (positive control), and the fragments were visualized under a UV transilluminator (Loccus, Brazil). qPCR quantifications were performed in a QuantStudio 3^{TM} Real-Time PCR System thermocycler (Applied Biosystems, USA). Species-specific primers were used to detect *A. brasilense* strain Ab-v6 or *B. subtilis* strain CCTB04. Primers for detecting *A. brasilense* Ab-v6 by qPCR were used as described by Stets et al. (2015). The *B. subtilis* CCTB04 primers were designed by the BIOTROP research team and kindly provided for this study. Reactions were conducted in a final volume of 12.5 µL, containing 6.25 µL of SYBR Green GoTaq qPCR Master Mix (Promega, Ref A6001, USA), 0.2 µM of each primer, 2 µL of template DNA and ultrapure water to complete the volume. Thermal cycling conditions included initial denaturation at 95 ºC for 10 minutes, followed by 40 cycles of 95 ºC for 15 seconds and 60 ºC for 1 minute. Amplification specificity was verified by analyzing the dissociation curve (melting curve) at the end of the run. The relative quantification was based on the threshold cycle (Ct) values and the samples were analyzed in triplicate.

3.4.6 Plant morphological and nutritional analyses

Seedlings were separated from the shoot and roots parts, and the roots were washed in tap water to remove excess sand or soil. The shoot part was placed to dry at 60 ºC until constant mass was obtained, and the fresh roots were scanned in WinRHIZO (Regent Instruments, Canada). After that, the roots were placed to dry (Tecnal Equipamentos Científicos, Brazil) at 60 ºC until constant mass was reached, and the dry mass of shoot and roots was then determined.

The dried shoot samples were ground and subjected to analyses of the nutrients N, P, K, Ca, Mg, S, Cu, Fe, Mn, Zn and B. The N concentration was measured after digestion of 0.20 g of plant tissue sample with 2 mL of H_2SO_4 (Êxodo Científica, Brazil) and 1 mL of deionized water, which was progressively heated in a digestion block (Tecnal Equipamentos Científicos, Brazil) at 350 ºC for 1 h (Tedesco et al. 1995). Its determination was carried out in a Kjeldahl steam distillation still (Tecnal Equipamentos Científicos, Brazil) (Bremner, 1965). The concentrations of P, K, Ca, Mg, S, Cu, Fe, Mn, and Zn were measured after digestion of 0.25 g of tissue sample with 3 mL of HNO_3 (Neon, Brazil) and 1 mL of $HClO_4$ (Neon, Brazil) and progressively heated in a digestion block (Tecnal Equipamentos Científicos, Brazil) at 180 ºC for 2 h. In the extract, the concentrations of Ca, Mg, S, Cu, Zn, Fe, and Mn were determined by atomic absorption spectrophotometer (Perkin Elmer, USA). The P concentration was determined by colorimetry, based on the methodology described by Murphy and Riley (1962), in a spectrophotometer (Tecnal Equipamentos Científicos, Brazil). For K, the concentration in the digested extract was determined in a flame photometer (Tecnal Equipamentos Científicos, Brazil). Finally, the B concentration was determined by burning 0.50 g of plant tissue in a muffle furnace (Eletrolab, Brazil) at 600 ºC for 1 h. After cooling, 10 mL of 0.18 M H_2SO_4 (Êxodo Científica, Brazil) was added, stirred, and allowed to settle for 3 h. Subsequently, 4 mL of the supernatant was removed and 4 mL of azomethine-H buffer (Neon, Brazil) was added, and the determination was performed in a spectrophotometer (Tecnal Equipamentos Científicos, Brazil) (Gaines & Mitchell, 1979; Krug et al. 1981).

3.4.7 Statistical analyses

The data were analyzed using R software, version 4.5.1 (R Core Team, 2020). First, the ANOVA assumptions were tested. The verification of normality, homoscedasticity and independence of the residuals was performed using the Shapiro-Wilk, Bartlett and Durbin-Watson tests, respectively. Specifically, the CFU data were transformed (log+1) due to the presence of zero and/or very discrepant values and, when the assumptions were not met, the Box-Cox transformation was applied from the original data. For all data, when there was a significant difference between the treatments ($p<0.05$), the Tukey test was applied to compare the means.

3.5 RESULTS

3.5.1 Microbial quantification (CFU and qPCR)

There was abundant bacterial growth in both TSA and RC culture media across all greenhouse treatments (Table 5). Colony counts ranged from 2.85×10^3 to 7.70×10^4 CFU g^{-1} of rhizosphere mass, with few significant differences among treatments. In sterilized sand, at seven days after inoculation, seedlings inoculated with

A. brasilense exhibited higher CFU g^{-1} in TSA medium compared with those inoculated with *Bacillus* spp. Conversely, in natural soil at twenty-one days after inoculation, CFU counts in RC medium were similar between *A. brasilense* and *Bacillus* spp. inoculated seedlings. The *A. brasilense* CFU did not differ from the non-inoculated treatments.

TABLE 5 – COLONY-FORMING UNITS (CFU) OBTAINED FROM 1:100 AND 1:1000 SERIAL DILUTIONS OF *Araucaria angustifolia* RHIZOSPHERE SAMPLES COLLECTED AT SEVEN AND TWENTY-ONE DAYS AFTER INOCULATION (DAI), FROM SEEDLINGS INOCULATED WITH *A. brasilense* Ab-v5 AND Ab-v6 OR *B. subtilis* CCTB04, *B. pumilus* CCTB05, AND *B. amyloliquefaciens* CCTB09, GROWN IN STERILIZED SAND AND NATURAL SOIL, UNDER GREENHOUSE CONDITIONS IN CURITIBA, BRAZIL, IN 2023.

	Sterile sand			
Serial dilution: 1:100	**7 DAI**		**21 DAI**	
Treatment	**Culture medium**			
	TSA	**RC**	**TSA**	**RC**
	CFU g^{-1*}	**CFU g^{-1}**	**CFU g^{-1*}**	**CFU g^{-1}**
A. brasilense	4.26 x 10^{4} a	6.60 x 10^{4} ^{ns}	1.47 x 10^{4} ^{ns}	1.77 x 10^{4} ^{ns}
***Bacillus* spp.**	1.53 x 10^{4} b	5.16 x 10^{4} ^{ns}	8.83 x 10^{3} ^{ns}	1.60 x 10^{4} ^{ns}
Control	7.70 x 10^{4} a	6.80 x 10^{4} ^{ns}	1.40 x 10^{4} ^{ns}	2.07 x 10^{4} ^{ns}
CV (%)	35.3	3.7	22.7	7.7
	Natural soil			
Serial dilution 1:1000	**7 DAI**		**21 DAI**	
Treatment	**Culture medium**			
	TSA	**RC**	**TSA**	**RC**
	CFU g^{-1*}	**CFU g^{-1}**	**CFU g^{-1*}**	**CFU g^{-1*}**
A. brasilense	7.84 x 10^{3} ^{ns}	1.48 x 10^{4} ^{ns}	5.29 x 10^{3} ^{ns}	1.20 x 10^{4} ab
***Bacillus* spp.**	1.46 x 10^{4} ^{ns}	1.70 x 10^{4} ^{ns}	1.34 x 10^{4} ^{ns}	3.27 x 10^{4} a
Control	1.23 x 10^{4} ^{ns}	1.23 x 10^{4} ^{ns}	2.85 x 10^{3} ^{ns}	1.04 x 10^{4} b
CV (%)	48.7	8.7	43.8	0.1

Notes: Means followed by different letters within a column differ significantly according to Tukey's test at 5% probability, based on log-transformed data or *Box-Cox transformed data. ns indicates non-significant differences at 5% probability in ANOVA. Reported means are presented in the original (non-transformed) scale, whereas the coefficient of variation (CV) refers to the transformed data (log or Box-Cox). Each treatment was tested with 8 replicates for sterilized sand and 12 replicates for natural soil.
SOURCE: The author (2025).

Based on the Ct and Tm values, it was possible to identify *A. brasilense* Ab-v6 and *B. subtilis* CCTB04 by qPCR (Table 6). In sterilized sand, DNA amplification of both strains occurred at seven and twenty-one days after inoculation. In natural soil, only the DNA of *B. subtilis* CCTB04 was amplified at seven and twenty-one days after inoculation. In both experiments, no DNA from any of the PGPB of interest was amplified in the control treatments samples.

TABLE 6 – QUANTIFICATION CYCLE (Ct) VALUES AND MELTING TEMPERATURES (°C) OBTAINED BY qPCR FOR THE IDENTIFICATION OF *A. brasilense* STRAIN Ab-v6 AND *B. subtilis* STRAIN CCTB04 OF *Araucaria angustifolia* RHIZOSPHERE SAMPLES COLLECTED AT SEVEN AND TWENTY-ONE DAYS AFTER INOCULATION (DAI), FROM SEEDLINGS INOCULATED WITH *A. brasilense* Ab-v5 AND Ab-v6 OR *B. subtilis* CCTB04, *B. pumilus* CCTB05, AND *B. amyloliquefaciens* CCTB09, GROWN IN STERILIZED SAND AND NATURAL SOIL, UNDER GREENHOUSE CONDITIONS IN CURITIBA, BRAZIL, IN 2023, AND COLLECTED AT SEVEN AND TWENTY-ONE DAYS AFTER INOCULATION.

Sterilized Sand					
Treatment	**Target strain**	**7 DAI**		**21 DAI**	
		Ct value	**Tm (ºC)**	**Ct value**	**Tm (ºC)**
A. brasilense	*A. brasilense* Ab-v6	37.03	83.60	36.56	83.37
***Bacillus* spp.**	*B. subtilis* CCTB04	33.11	75.10	34.85	75.50
Control	*A. brasilense* Ab-v6 and *B. subtilis* CCTB04	ND	ND	ND	ND
Natural Soil					
Treatment	**Target strain**	**7 DAI**		**21 DAI**	
		Ct value	**Tm (ºC)**	**Ct value**	**Tm (ºC)**
A. brasilense	*A. brasilense* Ab-v6	ND	ND	ND	ND
***Bacillus* spp.**	*B. subtilis* CCTB04	34.03	75.15	33.11	75.20
Control	*A. brasilense* Ab-v6 and *B. subtilis* CCTB04	ND	ND	ND	ND

Notes: ND = DNA of the target strain not detected despite amplification. The qPCR standard curve for *A. brasilense* Ab-v6 had $R^2 = 0.996$, slope = -3.40, amplification efficiency = 96.58%, and melting temperature = 83.6 °C. The qPCR standard curve for *B. subtilis* CCTB04 had $R^2 = 0.997$, slope = -3.32, amplification efficiency = 100.03%, and melting temperature = 75.3 °C.
SOURCE: The author (2025).

3.5.2 Plant growth and nutritional analyses

The inoculation of PGPB did not affect shoot biomass in either the 2020-2021 or 2023 experiments (data not shown), but it significantly influenced root development (Table 7) and shoot nutrients status (Table 8).

In 2020-2021, inoculation of araucaria seedlings with *A. brasilense* and *Bacillus* spp. increased total root length compared with non-inoculated seedlings (Table 7). In 2023, inoculation with *A. brasilense* also affected root development, but the response depended on the growth substrate. In sterilized sand, inoculation with *A. brasilense* reduced total root length, root surface area, and the abundance of fine roots

(0-0.5 cm, 0.5-1.0 cm, and 2.0-2.5 cm). In contrast, in natural soil, inoculation with *A. brasilense* enhanced the development of fine roots (0-0.5 cm and 0.5-1.0 cm).

Nutrient status was also influenced by inoculation. In sterilized sand, *A. brasilense* increased P and K concentrations, while *Bacillus* spp. increased Mn. In natural soil, *A. brasilense* increased Zn levels in seedlings shoots (Table 8).

TABLE 7 – ROOT DRY MATTER (RDM), TOTAL ROOT LENGTH (TRL), TOTAL LENGTH OF THE TAPROOT (TLT), ROOT SURFACE AREA (RSA), ROOT VOLUME (RV), AND THE LENGTH (L) OF FINE ROOTS WITHIN THE DIAMETER CLASSES OF 0–0.5 cm, 0.5–1.0 cm, 1.0–1.5 cm, 1.5–2.0 cm, AND 2.0–2.5 cm EVALUATED IN *Araucaria angustifolia* SEEDLINGS INOCULATED WITH *A. brasilense* Ab-v5 AND Ab-v6 OR *B. subtilis* CCTB04, *B. pumilus* CCTB05, AND *B. amyloliquefaciens* CCTB09, GROWN UNDER GREENHOUSE CONDITIONS IN CURITIBA, BRAZIL, DURING THE YEARS 2020-2021 AND 2023.

Year		**2020-2021**				**2023**				**2023**			
Substrate		**Carolina Substrate**				**Sterilized Sand**				**Natural Soil**			
PGPB		***Azospirillum***	***Bacillus***	**Control**	**CV (%)**	***Azospirillum***	***Bacillus***	**Control**	**CV (%)**	***Azospirillum***	***Bacillus***	**Control**	**CV (%)**
Root Atributes													
RDM	g	1.13 ^{ns}	1.06 ^{ns}	1.16 ^{ns}	4.6	0.98 ^{ns}	1.03 ^{ns}	1.02 ^{ns}	19.6	1.23 ^{ns}	1.18 ^{ns}	1.07 ^{ns}	23.8
TRL	cm	24.33 a	27.80 a	17.50 b	22.6	-	-	-	-	-	-	-	-
TLT	cm	-	-	-	-	510.00 b	747.21 a	828.81 a	22.8	762.32 a	585.01 b	603.70 ab	26.1
RSA	cm^2	-	-	-	-	127.20 b	175.24 ab	195.21 a	24.5	191.23 ^{ns}	189.03 ^{ns}	187.11 ^{ns}	24.7
RV	cm^3	-	-	-	-	2.86 ^{ns}	3.48 ^{ns}	3.65 ^{ns}	28.1	3.84 b	4.92 a	4.39 ab	21.4
Fine root classes													
Ø L (0 – 0.5)	cm	-	-	-	-	242.17 b	352.09 ab	434.95 a	39.0	397.91 a	251.41 b	295.73 b	32.0
Ø L (0.5 – 1.0)	cm	-	-	-	-	157.34 b	262.85 a	300.34 a	27.2	250.76 a	191.58 b	179.46 b	24.1
Ø L (1.0 – 1.5)	cm	-	-	-	-	28.12 ^{ns}	43.17 ^{ns}	50.00 ^{ns}	45.0	50.23 ^{ns}	54.16 ^{ns}	46.30 ^{ns}	33.6
Ø L (1.5 – 2.0)	cm	-	-	-	-	10.25 ^{ns}	17.04 ^{ns}	16.42 ^{ns}	42.9	20.51 b	28.31 a	24.27 ab	23.8
Ø L (2.0 – 2.5)	cm	-	-	-	-	5.34 b	9.90 a	9.47 a	35.1	10.60 b	15.64 a	13.88 ab	36.3

Notes: Means followed by different letters within a row differ significantly according to Tukey's test at 5% probability. ns = non-significant differences within a row at 5% probability in ANOVA. Legend: CV = coefficient of variation. Experiments were conducted with 6 replicates in Carolina substrate, 8 replicates in sterilized sand, and 12 replicates in natural soil.
SOURCE: The author (2025).

TABLE 8 – NUTRIENTS EVALUATED IN *Araucaria angustifolia* SEEDLINGS INOCULATED WITH *A. brasilense* Ab-v5 AND Ab-v6 OR *B. subtilis* CCTB04, *B. pumilus* CCTB05, AND *B. amyloliquefaciens* CCTB09, GROWN UNDER GREENHOUSE CONDITIONS IN CURITIBA, BRAZIL, DURING THE YEARS 2020-2021 AND 2023.

Year		**2020-2021**				**2023**				**2023**			
Substrate		**Carolina Substrate**				**Sterilized Sand**				**Natural Soil**			
PGPB		***Azospirillum***	***Bacillus***	**Control**	**CV (%)**	***Azospirillum***	***Bacillus***	**Control**	**CV (%)**	***Azospirillum***	***Bacillus***	**Control**	**CV (%)**
Nutrients	**Unities**												
N	g kg^{-1}	10.50 a	13.20 a	5.60 b	39.5	7.85 a	4.93 b	6.89 ab	22.1	7.62 ^{ns}	8.52 ^{ns}	8.78 ^{ns}	29.0
P	g kg^{-1}	2.26 a	2.30 a	1.21 b	32.2	2.44 a	2.02 ab	1.76 b	16.1	1.50 ^{ns}	1.19 ^{ns}	1.20 ^{ns}	20.5
K	g kg^{-1}	-	-	-	-	12.20 a	9.70 ab	9.42 b	14.7	5.60 ^{ns}	16.20 ^{ns}	15.40 ^{ns}	11.5
Ca	g kg^{-1}	-	-	-	-	6.09 ^{ns}	5.16 ^{ns}	5.79 ^{ns}	12.5	6.22 ^{ns}	6.41 ^{ns}	6.32 ^{ns}	12.7
Mg	g kg^{-1}	-	-	-	-	2.04 ^{ns}	1.76 ^{ns}	1.84 ^{ns}	12.6	2.56 ^{ns}	2.34 ^{ns}	2.42 ^{ns}	13.7
S	g kg^{-1}	-	-	-	-	0.77 ^{ns}	0.46 ^{ns}	0.46 ^{ns}	35.0	0.76 ^{ns}	0.92 ^{ns}	0.85 ^{ns}	16.6
B	mg kg^{-1}	-	-	-	-	9.34 ^{ns}	9.76 ^{ns}	4.54 ^{ns}	75.2	10.97 ^{ns}	8.65 ^{ns}	10.56 ^{ns}	36.9
Cu	mg kg^{-1}	-	-	-	-	3.56 ^{ns}	4.60 ^{ns}	6.80 ^{ns}	40.4	4.50 ^{ns}	3.62 ^{ns}	2.98 ^{ns}	24.9
Fe	mg kg^{-1}	-	-	-	-	43.04 ^{ns}	40.96 ^{ns}	58.88 ^{ns}	30.0	50.56 ^{ns}	46.08 ^{ns}	58.72 ^{ns}	37.2
Mn	mg kg^{-1}	-	-	-	-	109.44 ab	137.92 a	91.84 b	19.4	149.44 ^{ns}	168.32 ^{ns}	153.92 ^{ns}	22.6
Zn	mg kg^{-1}	-	-	-	-	17.92 ^{ns}	17.92 ^{ns}	18.12 ^{ns}	12.5	14.58 a	12.42 ab	10.98 b	15.7

Notes: Means followed by different letters within a row differ significantly according to Tukey's test at 5% probability. ns = non-significant differences within a row at 5% probability in ANOVA. Legend: CV = coefficient of variation. Experiments were conducted with 6 replicates in Carolina substrate, 8 replicates in sterilized sand, and 12 replicates in natural soil.
SOURCE: The author (2025).

3.6 DISCUSSION

Our results confirm the importance of adopting a multi-method approach for tracking and monitoring inoculated strains, as proposed by Manfredini et al. (2021). In this study, it became clear that counting CFU on plates is not a reliable method for rhizosphere bacterial quantification, since both inoculated and non-inoculated seedlings exhibited high bacterial growth (Table 5), despite our efforts to maintain sterile conditions during the analyses. In contrast, the qPCR technique did not detect the strains of interest (*A. brasilense* Ab-v6 or *B. subtilis* CCTB04) in the rhizosphere of non-inoculated seedlings, but did detect in inoculated seedlings at both seven and twenty-one days after inoculation (Table 6). Similarly, Cortese et al. (2023) employed qPCR to monitor *Bacillus altitudinis* T5S-T4 and *B. altitudinis* 19RS3 inoculated into *Ilex paraguariensis* seedlings under nursery conditions. These results corroborate the notion that qPCR with strain-specific primers is a robust approach for detecting the presence and persistence of inoculated strains in native tree seedlings.

The qPCR technique was also effective in assessing the competitiveness of the PGPB under different substrates (Table 6). To date, *B. subtilis* CCTB04 was consistently detected in both sterilized sand and natural soil substrates, at seven and twenty-one days after inoculation, whereas *A. brasilense* Ab-v6 was only detected in sterilized sand. Indeed, natural soils represent a highly inhospitable environment for introduced strains. According to the diversity–invasibility relationship (DIR) hypothesis, increased species richness and community evenness enhance the ability of the soil microbiome to exploit and compete for resources, leaving fewer niches and resources available to the “invader” (Mawarda et al. 2022). Consequently, a strain must effectively compete for natural resources in order to survive and persist in soil. Recent evidence further supports this interpretation, showing that the high complexity of native soil microbiomes can reduce the efficiency of *A. brasilense* strain Ab-v5 inoculation, whereas controlled simplification of microbial communities (through dilution or sterilization) favors inoculant persistence and enhances plant growth promotion (Ferrarezi et al. 2023). Our findings are consistent with this framework, suggesting that *B. subtilis* CCTB04 was more competitive in the rhizosphere under natural soil conditions than *A. brasilense* Ab-v6. This advantage may be attributed to the ability of *B. subtilis* to form endospores, which likely improved its survival in natural soil. As a matter of fact, among *Bacillus* species, *B. subtilis* is a well-established model for elucidating the molecular mechanisms underlying spore longevity and resistance to environmental stressors (Nicholson et al. 2000; Mahapatra et al. 2022).

In the experiment during 2020-2021, inoculation with either *A. brasilense* or *Bacillus* spp. increased total root length of araucaria seedlings cultivate in Carolina Substrate (Table 7). Conversely, in 2023, inoculation with *Bacillus* spp. did not significantly affect the growth of any root class, whereas inoculation with *A. brasilense* promoted either positive or negative growth responses depending on the substrate (Table 7). This indicates that the growth substrate plays a decisive role in shaping plant responses to inoculation. Interestingly, inoculation with *A. brasilense* in seedlings grown in natural soil increased the development of finer roots (Table 7), even though the DNA of strain Ab-v6 was not amplified by qPCR in this treatment (Table 6). This apparent contradiction is likely explained by a short-lived “primer effect” of *A. brasilense* (Takahashi et al. 2024), which is rapidly suppressed by the competitive indigenous microbial community. This case illustrates the need to account for complex interactions between PGPB genotype, host plant genotype, and the environment (Duflos et al. 2024). Therefore, our results indicate that the efficiency of inoculation depends not only on bacterial application, but also on the ecological context of plant-microbe interactions. This underscores the importance of evaluating PGPB-plant associations under multiple growth conditions when selecting efficient strains.

Consistent with our previous study (Kondo et al. 2024), inoculation with PGPB enhanced the nutritional status of araucaria seedlings shoots (Table 8). However, in this study, the nutritional benefits of PGPB inoculation were more evident in seedlings cultivated in Carolina substrate and sterilized sand than in natural soil (Table 8). This highlights the critical role of strain competitiveness against indigenous microbial communities in determining the efficacy of PGPB inoculation. Following the DIR hypothesis, and given the resource limitation imposed by high microbial diversity (Mahapatra et al. 2022), indigenous plant growth-promoting microorganisms possess a “home-field advantage” compared to introduced strains (Kong et al. 2025). However, indigenous strains lack the efficient ability of introduced strains to grow and modulate plant physiology.

To our knowledge, this is the first study to apply qPCR to monitor the persistence of PGPB in *A. angustifolia* seedlings under greenhouse conditions, enabling us to directly link bacterial presence to improvements in plant nutritional status. This molecular approach, combined with morphological evaluation, revealed clear contrasts between growth substrates: while PGPB were able to proliferate and promote nutritional gains in commercial substrate and sterilized sand, they faced strong competition and limited survival in natural soil. These findings reinforce that the success of inoculation depends on the ability of PGPB to withstand complex microbial interactions under realistic growth conditions. For this reason, protection strategies such as encapsulation (Rojas-Sánchez et al. 2022) are essential to extend the viability and effectiveness of inoculants in the field.

In addition to qPCR, genome-based diagnostic tools such as multiplex PCR, targeting strain-specific genes, have recently been proposed as a cost-effective alternative for large-scale screening and confirmation of inoculant identity (Rolla-Santos et al. 2024). Although not quantitative, this approach may represent a valuable complementary tool for future studies aiming to monitor the association for *A. brasilense* Ab-v6 and *B. subtilis* CCTB04 in seedlings of different tree species, particularly under nursery conditions.

Furthermore, our results emphasize the need for advanced molecular tools to fully capture rhizosphere dynamics after inoculation. Approaches such as shotgun metagenomics could provide comprehensive understanding of the impacts of inoculated strains on the native microbiome. At the same time, elucidating the composition and function of root exudates across different substrates will be critical to clarifying how these compounds facilitate or restrict bacterial establishment. Together, these lines of research will support the optimization of PGPB inoculation in araucaria seedlings, improving the efficiency and reliability of these associations and, ultimately, contributing to the successful cultivation and conservation of this species.

3.7 CONCLUSION

This study demonstrates that qPCR is an effective tool for monitoring PGPB persistence in the rhizosphere and for assessing their competitiveness against indigenous soil communities. Our results suggest that inoculation success depends not only on bacterial application but also on interactions with the environment and the host. These findings underscore the importance of integrating microbial ecology into PGPB selection to improve seedling production strategies and support the conservation and restoration of forest species.

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4 CHAPTER 3: PLANT GROWTH-PROMOTING BACTERIA INOCULATION ENHANCES SHOOT NUTRIENT CONTENTS OF *Araucaria angustifolia* SEEDLINGS UNDER DROUGHT STRESS[2]

4.1 RESUMO

A *Araucaria angustifolia* é uma espécie de conífera criticamente ameaçada de extinção, e o déficit hídrico pode se tornar uma grande limitação para seu crescimento e sobrevivência. Estratégias silviculturais que aumentam a resiliência das mudas, como a inoculação com bactérias promotoras de crescimento vegetal (BPCV), podem ser essenciais para garantir a persistência da espécie sob estresse hídrico. Este estudo avaliou os efeitos de regimes hídricos no solo e da inoculação com BPCV sobre o crescimento e características fisiológicas e bioquímicas de mudas de *A. angustifolia*. As plantas foram submetidas a três níveis de umidade do solo (100%, 70% e 30% da capacidade de retenção de água, CRA) e inoculadas com BPCV 1 (*Azospirillum brasilense* Ab-v5 e Ab-v6) ou BPCV 2 (*Priestia aryabhattai* CBMAI1120, *Bacillus haynesii* CCT7926 e *Niallia circulans* CCT0026). As mudas foram avaliadas aos 7 e 21 dias após a inoculação (DAI). Aos 7 DAI, observou-se interação significativa entre umidade do solo e inoculante para o teor de Zn na parte aérea, com a maior concentração nas plantas inoculadas com BPCV 1 sob 30% da CRA. Aos 21 DAI, os teores de açúcares solúveis e Cu também apresentaram interações, com valores mais altos com BPCV 2 sob 30% da CRA e BPCV 1 sob 100% da CRA, respectivamente. Aos 7 e 21 DAI, a inoculação isolada aumentou os teores de Mg, S, Cu e N, enquanto 100% da CRA aumentou os teores de K, Fe e Zn, e o déficit hídrico promoveu um maior diâmetro de raízes finas apenas aos 21 DAI. De modo geral, a inoculação com BPCV melhorou a absorção de nutrientes pela parte aérea e contribuiu para uma maior tolerância à seca em mudas de *A. angustifolia*, destacando seu potencial como uma ferramenta biotecnológica promissora para a conservação da espécie e o manejo silvicultural.

Palavras-chave: Rizobactérias. Inoculantes bacterianos. Mudas de árvores. Estresse abiótico. Absorção de nutrientes.

4.2 ABSTRACT

Araucaria angustifolia is a critically endangered conifer species, and water deficit may become a major constraint for its growth and survival. Silvicultural strategies that enhance seedlings resilience, such as inoculation with plant growth-promoting bacteria (PGPB), may be key to ensuring the species' persistence under drought stress. This study evaluated the effects of soil water regimes and PGPB inoculation on growth, physiological, and biochemical traits of *A. angustifolia* seedlings. Plants were subjected to three soil water levels (100%, 70%, and 30% of water container capacity, WCC) and inoculated with PGPB 1 (*Azospirillum brasilense* Ab-v5 and Ab-v6 or PGPB 2 (*Priestia aryabhattai* CBMAI1120, *Bacillus haynesii* CCT7926, and *Niallia circulans* CCT0026).

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[2] Under revision in New Forests

Seedlings were evaluated at 7 and 21 days after inoculation (DAI). At 7 DAI, a significant interaction between soil water and inoculant was observed for shoot Zn content, with the highest concentration in plants inoculated with PGPB 1 under 30% WCC. At 21 DAI, soluble sugars and Cu content also showed interactions, with higher values in PGPB 2 under 30% WCC and PGPB 1 under 100% WCC, respectively. At 7 and 21 DAI, inoculation alone increased Mg, S, Cu, and N contents, while full soil water enhanced K, Fe, and Zn, and water deficit promoted a greater fine-root diameter only at 21 DAI. Overall, PGPB inoculation improved shoot nutrient uptake and contributed to greater drought tolerance in *A. angustifolia* seedlings, highlighting its potential as a promising biotechnological tool for species conservation and silvicultural management.

Keywords: Rhizobacteria. Bacterial inoculants. Tree seedlings. Abiotic stress. Nutritional uptake.

4.3 INTRODUCTION

Agroecological systems – including forestry, agriculture, and livestock farming – are increasingly affected by extreme climatic events such as droughts, floods, heat waves, and unseasonal frosts (Altieri et al. 2015; Brunner, 2023). These phenomena compromise plant productivity, natural regeneration, and ecosystem stability, representing one of the major global challenges for sustainable land management (Lobell & Gourdi, 2012; Liu, 2025a). Developing strategies that enhanced plant tolerance to abiotic stress is therefore a crucial step toward climate-resilient production systems (Rivero et al. 2022). Among the most promising approaches are the use of beneficial microorganisms, the selection of tolerant genotypes, and the optimization of genetic engineering strategies (Marco et al. 2015; Guzmán et al. 2022; Rivero et al. 2022).

Araucaria angustifolia, commonly known as Brazilian pine, Paraná pine, or simply araucaria, is an emblematic conifer native to southern Brazil and classified as a critically endangered species due to deforestation, habitat fragmentation, and overexploitation (Scipioni et al. 2019). In addition to anthropogenic pressures, this species may face additional risks arising from climate change, particularly the lack of connectivity between adult individuals, hindering the species' reproduction and natural regeneration (Tagliari et al. 2021). Therefore, experiments assessing drought effects on araucaria seedlings are crucial for elucidating the physiological limits of this species and supporting evidence-based conservation and reforestation programs under future climate scenarios.

Although symbiotic associations such as mycorrhizae can improve drought tolerance in seedlings of forest species, such as *Araucaria Araucana* (Chávez et al. 2023), commercial mycorrhizal inoculants are not readily available, and studies have shown that many commercial inoculants are of low quality (Salomon et al. 2022; Koziol et al. 2024). In this context, the use of plant growth-promoting bacteria (PGPB) represents a feasible and accessible alternative for enhancing the resilience of tree seedlings in reforestation programs (Kondo et al. 2024; Kondo et al. 2026). However, experimental data for araucaria remains scarce.

We hypothesize that inoculation with selected PGPB strains could enhance the drought tolerance of araucaria seedlings by improving their physiological and biochemical responses. The aim of this study was to verify the effects of soil water regimes and PGPB inoculation on growth, physiological, and biochemical traits of araucaria seedlings, in order to improve the establishment of this species in restoration programs.

4.4 MATERIALS AND METHODS

4.4.1 Soil characterization

Soil was collected from a depth of 0-20 cm at the Canguiri Experimental Farm, Pinhais, Paraná, Brazil (25º23'24.5'' S; 49º08'35.5'' W). On field, the soil was classified as a Haplic Cambisol (SiBCS; Santos et al. 2025), corresponding to an Inceptisol (Soil Survey Staff, 2022). Soil samples were air-dried, sieved, and subjected to the following analyses: soil pH($CaCl_2$) was measured in 0.01 mol L^{-1} $CaCl_2$ (soil:solution ratio 1:2.5); exchangeable Ca, Mg, and Al, extracted with 1 mol L^{-1} KCl; K and P with Mehlich-1; potential acidity (H+Al), extracted with the SMP buffer; soil organic carbon (SOC), determined by the Walkley-Black method (Walkley & Black, 1934); particle-size distribution, determined using the Bouyoucos hydrometer method (Bouyoucos, 1962), and bulk density by the volumetric ring method (Gee & Bauder, 1986). The soil was used without liming or mineral fertilization in the two experimental phases. Soil chemical characteristics were as follows: pH (in $CaCl_2$): 4.2; pH (in SMP): 5.0; Al ($cmol_c$ dm^{-3}): 0.7; K ($cmol_c$ dm^{-3}): 0.2; Ca ($cmol_c$ dm^{-3}): 2.2; Mg ($cmol_c$ dm^{-3}): 0.9; Al+H ($cmol_c$ dm^{-3}): 12.1; Organic C (g dm^{-3}): 56.1; and P (mg dm^{-3}): 9.4. Soil physical characteristics were as follows: clay (%): 42.5; silt (%): 18.8; sand (%): 38.8; and, density (g cm^{-3}): 1.0.

4.4.2 PGPB inoculants

Liquid inoculants containing plant growth-promoting bacteria (PGPB) were obtained from the manufacturer (Biotrop – Soluções Biológicas, Curitiba, PR, Brazil). Consortium 1 (PGPB 1) contained two strains of *Azospirillum brasilense* (Ab-v5 and Ab-v6) at 2×10^{8} CFU mL^{-1}. Consortium 2 (PGPB 2) contained three species: *Priestia aryabhattai* CBMAI1120 (formerly *Bacillus aryabhattai*) at 2.1×10^{12} viable endospores L^{-1}, *Bacillus haynesii* CCT7926 at 8.8×10^{11} viable endospores L^{-1}, and *Niallia circulans* CCT0026 (formerly *Bacillus circulans*) at 3.0×10^{11} viable endospores L^{-1}.

4.4.3 Soil water regimes

The maximum soil water availability for plants was estimated by soil water container capacity (WCC) following this procedure: five 7 L pots were filled with 6 L of air dry soil (measured with a 1 L graduated cylinder), corresponding to 6 kg (confirmed by weighing). The initial volumetric water content (θv) of the dry soil mean value was 0.040 m^{3} m^{-3}, measured by a TEROS 11 soil moisture sensor (METER Group, USA). Then, it was added 3 L of water to achieve saturation in 24 hours, in which the mean θv was 0.440 m^{3} m^{-3}. After free drainage of the pots per 24, and 48 hours, the mean θv was 0.307 and 0.257 m^{3} m^{-3}, respectively, which the later was defined as the soil WCC.

The soil water regimes were established considering θv = 0.257 m^{3} m^{-3} as 100% WCC, θv = 0.18 m^{3} m^{-3} as 70% WCC, and θv = 0.08 m^{3} m^{-3} as 30% WCC. Maintenance of the WCC regimes was performed daily using the TEROS 11 sensor and the following calculation:

Amount of water (mL) = *Sv* x (Dθv – Mθv),

where: *Sv* is the volume of soil in the pot (mL), Dθv is the desired volumetric moisture content (m^{3} m^{-3}), and Mθv is the measured volumetric moisture content (m^{3} m^{-3}).

4.4.4 Experimental design

The experiment was conducted under a completely randomized design with a 3 x 3 factorial (six replicates per treatment; each replicate was one pot with a single araucaria seedling). Factor 1 was defined by inoculation of plant growth-promoting bacteria (PGPB) consortia (PGPB 1: *A. brasilense* Ab-v5 + Ab-v6; PGPB 2: *P. aryabhattai* CBMAI1120, *B. haynesii* CCT7926, *N. circulans* CCT0026; and control). Factor 2 was defined by soil water regime (100% water container capacity: WCC – no water stress; 70% WCC – moderate stress; 30% WCC – severe stress).

4.4.5 Plant growth conditions

The experiment was conducted in a greenhouse at the Federal University of Paraná (UFPR), Department of Soils and Agricultural Engineering (Curitiba, PR, Brazil; 25º24’45.7’’ S; 49º14’57.0’’ W). Soil and air moisture were monitored; indoor temperatures varied according to outdoor conditions. During the implementation period of the WCC regimes, the average maximum and minimum temperatures were 34.0 ºC and 14.6 ºC, respectively. Detailed weather conditions for the experimental period are presented in Supplementary Figure S2.

Araucaria seeds were collected from the ground after cone fall near a mother tree, known for its excellent pine nut quality, in Quitandinha, PR, Brazil (25º54’58.1” S, 49º35’00.2’’ W). The seeds were immersed in water to test their viability, and those that floated were discarded. Seeds were not size-selected or surface-disinfected. Sowing was done on 26 June 2023 (one seed per pot).

Seedlings were grown in 0.8 L pots filled with soil described 4.4.1 section, under no water stress, until shoot height reached ~1.5 x root length (pot length used as root proxy), considered suitable for field planting (Wendling et al. 2017). Seedlings were transplanted to pots on 15 January 2024. Immediately after transplant, each pot was submitted to soil water regimes; then, the soil moisture was measured daily with the TEROS 11 and adjusted according to treatments no water stress, moderate stress, severe stress, always in the evenings.

After 15 days under the different soil water regimes, on 31 January 2024, inoculation of PGPBs was done by applying 50 mL of diluted inoculants in filtered water on the soil next to each seedling. Inoculant of PGPB 1 was diluted to 0.9% (v/v) and PGPB 2 to 1.0% (v/v) (Kondo, 2022; Kondo et al. 2024). Control seedlings received 50 mL filtered water.

4.4.6 Plant analyses

Three seedlings per treatment were sampled at 7 and 21 days after inoculation (DAI), on 7 and 21 February 2024, respectively. At the days of harvest, plants were removed from soil. Shoots were weighed immediately to obtain shoot fresh mass (*SFM*). Tips were excised, wrapped in aluminum foil, snap-frozen in liquid N_2, and stored at -20 ºC for biochemical analyses. Remaining shoots were immersed in deionized water for 16 h to obtain shoot turgid mass (*STM*), then dried at 60 ºC to constant mass for shoot dry mass (*SDM*). Relative water content (*RWC*) was calculated as:

$$\text{RWC (\%)} = (SFM - SDM)/(STM - SDM) \times 100.$$

Fresh roots were washed and preserved in 70% tap water, then they were scanned and analyzed with WinRHIZO (Regent Instruments, Canada), dried, and weighed.

Frozen shoot tips were used to determine soluble sugars using the extraction method described by López-Hidalgo et al. (2021), and quantification with the anthrone method (Yemm & Willis, 1954). Proline was determined as in Bates et al. (1973).

Dried shoots were milled and analyzed for macronutrients (N, P, K, Ca, Mg, S) and micronutrients (Cu, Fe, Mn, Zn, B). Total N was determined after digestion of 0.20 g tissue with 2 mL H_2SO_4 and 1 mL deionized water, heated at 350 ºC for 1 h (Tedesco et al. 1995), followed by Kjeldahl steam distillation (Bremner, 1965). For P, K, Ca, Mg, S, Cu, Fe, Mn and Zn, 0.25 g was digested with 3 mL HNO_3 and 1 mL $HClO_4$ at 180 ºC for 2 h; Ca, Mg, S, Cu, Zn, Fe and Mn were quantified by atomic absorption spectrophotometry (PerkinElmer, USA); P was determined colorimetrically (Murphy & Riley, 1962), using a spectrophotometer (Tecnal, Brazil); K was measured by flame photometry (Tecnal, Brazil). Boron was determined separately: 0.50 g was ashed at 600 ºC for 1 h; ash was extracted with 10 mL 0.18 M H_2SO_4. After 3 h settling, 4 mL supernatant was mixed with 4 mL azomethine-H buffer (Neon, Brazil), and absorbance was read spectrophotometrically (Tecnal, Brazil) (Gaines & Mitchell, 1979; Krug et al. 1981).

4.4.7 Statistical analyses

Analyses were performed in R (v4.5.1; R Core Team, 2020). ANOVA assumptions were checked by tests of residuals: Shapiro-Wilk (normality), Bartlett (homoscedasticity), and Durbin-Watson (independence). When treatment effects were significant ($p<0.05$), means were compared by Tukey's test.

4.5 RESULTS

Seedlings developed normally during the experiment. The ANOVA showed that soil water regimes and PGPB inoculation had no significant effect on overall plant growth, expected for fine roots (0-0.5 cm diameter), which were influenced by soil water (Supplementary Table S2). The soil water regime influenced the contents of soluble sugars, K, Cu, Fe, and Zn at 21 days of inoculation (DAI). PGPB inoculation significantly affected shoot nutrient contents (Mg, S, Cu, and Zn) at 7 DAI, and soluble sugars and N at 21 DAI. The interaction between soil water regimes and PGPB inoculation affected shoot Zn content at 7 DAI, and soluble sugars Cu contents (Supplementary Table S2). Only the results being affected by soil water regimes, PGPB inoculants, or the interaction of both are shown.

At 21 DAI, the soil regimes affected the contents of K, Fe and Zn contents, regardless of PGPB inoculants. Plants grown under 100% WCC accumulated more K, Fe, and Zn contents than plants undergoing moderate and severe drought (Table 9). Severe water stress (30% WCC) induced the growth of fine roots in relation to no stress (100% WCC), while moderate stress (70% WCC) did not affect growth of them (Table 9).

TABLE 9 – FINE ROOTS LENGTH AND SHOOT NUTRIENT CONTENTS OF *Araucaria angustifolia* SEEDLINGS, INOCULATED WITH OR WITHOUT PLANT GROWTH-PROMOTING BACTERIA (PGPB) INOCULANTS, AND SUBJECTED TO THREE SOIL WATER REGIMES (100%, 70%, AND 30% OF CONTAINER WATER CAPACITY-WCC) IN POTS UNDER GREENHOUSE CONDITIONS, 21 DAYS AFTER INOCULATION.

Soil water regimes	**Fine roots**	**Shoot Nutrient Contents**		
	Ø 0 to 0.5 (cm)	**K (g kg^{-1})**	**Fe (mg kg^{-1})**	**Zn (mg kg^{-1})**
100% WCC	338.0 b	16.4 a	140.1 a	14.2 a
70% WCC	421.2 ab	15.2 b	99.5 b	11.7 b
30% WCC	454.2 a	14.2 b	103.9 ab	12.7 ab

Notes: Inoculation was performed by applying 50 mL of diluted PGPB inoculants [1] or [2] into the furrow at seedling transplantation. PGPB [1] contained *Azospirillum brasilense* Ab-v5 and Ab-v6, whereas PGPB [2] contained *Priestia aryabhattai* CBMAI1120, *Bacillus haynesii* CCT7926, and *Niallia circulans*

CCT0026. Means followed by different lowercase letters within columns differ significantly according to Tukey’s test ($p<0.05$). Only affected nutrients are presented.
SOURCE: The author (2025).

The results of Zn (at 7 DAI), soluble sugars, and Cu contents (at 21 DAI) were explained by the interaction of soil water regimes and PGPB inoculation (Table 10). The inoculation of PGPB 1 increased shoot Zn content in plants grown under severe stress, with severe drought. At 21 DAI, soluble sugar content was enhanced under by inoculation with PGPB 2, while it was not affected by PGPB 1. However, inoculation of PGPB 1 favored the accumulation of Cu contents in plants grown under no stress in relation to PGPB 2 (Table 10).

TABLE 10 – INTERACTION RESPONSES OF *Araucaria angustifolia* SEEDLINGS TO INOCULATION OF PLANT GROWTH PROMOTING BACTERIA (PGPB) AND SOIL WATER REGIMES (100%, 70%, AND 30% WATER CONTAINER CAPACITY).

Harvest	**7 DAI**		
Plant variable	**Zn (mg kg^{-1})**		
Soil water regimes	**100% WCC**	**70% WCC**	**30% WCC**
Inoculants			
PGPB [1]	14.0 AB a	11.6 B a	16.0 A a
PGPB [2]	12.9 A ab	12.6 A a	11.7 A b
Control	10.5 A b	12.6 A a	11.4 A b
Harvest	**21 DAI**		
Plant variable	**Soluble sugars (mg g^{-1})**		
Soil water regimes	**100% WCC**	**70% WCC**	**30% WCC**
Inoculants			
PGPB [1]	3.1 A a	3.3 A a	3.6 A b
PGPB [2]	3.0 B a	3.2 B a	4.7 A a
Control	3.0 A a	2.6 A a	2.9 A b
Harvest	**21 DAI**		
Plant variable	**Cu (mg kg^{-1})**		
Soil water regimes	**100% WCC**	**70% WCC**	**30% WCC**
Inoculants			
PGPB [1]	5.0 A a	2.9 B a	2.7 B a
PGPB [2]	3.2 A b	3.1 A a	4.1 A a
Control	3.8 A ab	3.2 A a	3.3 A a

Notes: Means followed by different lowercase letters within columns and different uppercase letters within rows differ significantly according to Tukey’s test ($p<0.05$). Only affected nutrients are presented. Sugar content is based on fresh weight, while Cu and Zn content are based on dry weight.
SOURCE: The author (2025).

There were nutrients that were not affected by soil water regimes, but they were affected by PGPB inoculation. At 7 DAI, inoculation of either PGPB 1 or PGPB 2 increased the shoot contents of Mg, S, and Cu in relation to the control (Table 11). At 21 DAI, the N contents for plants inoculated with PGPB 2 (*P. aryabhattai*, *B. haynesii*, and *N. circulans*) were higher than those of plants inoculated with PGPB 1 (*A. brasilense*) and non-inoculated plants (Table 11).

TABLE 11 – SHOOT NUTRIENT CONTENTS OF *Araucaria angustifolia* SEEDLINGS, INOCULATED WITH OR WITHOUT PLANT GROWTH-PROMOTING BACTERIA (PGPB) INOCULANTS, AND SUBJECTED TO THREE SOIL WATER REGIMES IN 7-L SOIL POTS UNDER GREENHOUSE CONDITIONS, AT 7 AND 21 DAYS AFTER INOCULATION.

Harvest	**7 DAI**			**21 DAI**
Nutrient	**Mg (g kg^{-1})**	**S (g kg^{-1})**	**Cu (mg kg^{-1})**	**N (g kg^{-1})**
Inoculant				
PGPB [1]	2.4 ab	1.2 a	3.6 a	13.5 b
PGPB [2]	2.7 a	1.0 ab	3.4 a	18.9 a
Control	2.3 b	0.9 b	2.4 b	14.3 b

Notes: Inoculation was performed by applying 50 mL of diluted PGPB inoculants [1] or [2] into the furrow at seedling transplantation. PGPB [1] contained *Azospirillum brasilense* Ab-v5 and Ab-v6, whereas PGPB [2] contained *Priestia aryabhattai* CBMAI1120, *Bacillus haynesii* CCT7926, and *Niallia circulans* CCT0026. Means followed by different lowercase letters within columns and different uppercase letters within rows differ significantly according to Tukey's test ($p<0.05$). Only affected nutrients are presented.
SOURCE: The author (2025).

4.6 DISCUSSION

Araucaria seedlings showed symptoms of drought stress. To date, severe drought stress stimulated the growth of fine roots (Table 9) at second harvest, corresponding to the soil water treatments at 21 days after inoculation (DAI). Reallocating carbon belowground and investing in new fine roots is a common strategy in mature trees subjected to drought stress, which shed older roots and produce new ones to enhance water uptake (Brunner et al. 2015). According to Mehra et al. (2025) when soil dries out, roots often respond by growing in search of water. This adaptative response depends on the cost-benefit balance between water acquisition and carbons expenditure, as well as the plant's capacity to afford this investment (Eissenstat et al. 2000). However, young fine roots are generally more efficient at absorbing water than older roots, thereby improving the plant's ability to acclimate to water stress (Brunner et al. 2015).

Another piece of evidence that araucaria seedlings were suffering from drought stress was the decrease in shoot K, Fe, and Zn contents (Table 9). Higher K contents in plants maintained at no stress are consistent with the dependence of this nutrient on diffusion, which is directly affected by water availability (Xu et al. 2021). Lower K contents may compromise cellular osmotic balance and other essential functions (e.g., stomatal opening and closing), reducing the plant's ability to grow under water stress conditions (Wang et al. 2013). Likewise, the deprivation of other nutrients may impair plant metabolism and growth. Thus, maintaining shoot nutrient levels may represent a strategy to mitigate drought stress promoted by PGPB inoculation.

In this study, seedlings subjected to severe stress and inoculated with PGPB 2 accumulated more soluble sugars in shoots (Table 10). The higher accumulation of soluble sugars suggests an active role of these bacteria in modulating seedling metabolism. This effect is likely associated with the induction of stress tolerance mechanisms, as soluble sugars function as compatible osmolytes that help maintain cellular osmotic potential, stabilize membranes and proteins, and protect against oxidative damage (Singh et al. 2022). Moreover, the accumulation of sugars indicates that seedlings maintained their photosynthetic activity under water deficit, thereby avoiding metabolic collapse (Jogawat, 2019). The positive interaction with PGPB 2 consortium – compromising *P. aryabhattai*, *B. haynesii*, and *N. circulans* – whose members have previously been classified as *Bacillus* spp. (Gupta et al. 2020), may be explained by their ability to produce phytohormones, exopolysaccharides, and antioxidant enzymes. These compounds play crucial roles in signaling and activating metabolic pathways associated with soluble carbohydrate accumulation (Lee et al. 2024). In this context, the increase in soluble sugars can be interpreted as physiological marker of the contribution of these PGPB to osmotic adjustments and enhanced resilience to water stress in araucaria seedlings.

Moreover, seedlings exposed to severe water stress and inoculated with the PGPB 1 consortium showed increased Zn content as early as 7 DAI (Table 10). This result may be related to the ability to PGPB to increase Zn availability, either by exudation of organic acids and siderophores or by stimulating root growth and consequent exploration of still-wet micropores in the soil (Singh et al. 2024; Liu et al. 2025b). Thus, inoculation appears to have compensated for the limitation imposed by the lower mobility of Zn under water deficit.

Our previous study showed that inoculation with PGPB increases the nutrient content of seedlings, regardless of the inoculation method (Kondo et al. 2024). Similarly, in this study, PGPB inoculation increased Mg, S, and Cu at 7 DAI and N at 21 DAI, regardless of soil moisture conditions (Table 11). In the case of Cu, these effects can be attributed to the fact that Cu uptake, unlike Zn, relies heavily on active transport and the physiological integrity of the plant for absorption and translocation (Marschner, 2011). Inoculation with either PGPB may have stimulated phytohormone production that enhances root activity, along with the exudation of organic ligands (e.g., amino acids and organic acids) that mobilize Cu to the soil solution, thereby promoting its uptake (Gureeva & Gureev, 2023; Giri et al. 2025).

Furthermore, it is important to mention that Mg, S, Cu, and N are part of metalloproteins, which play a role in photosynthesis, respiration, and the biosynthesis of compounds important for plant growth (Lilay et al. 2024). This study showed that PGPB may help maintain or even enhance nutrient acquisition. Elsewhere, PGPB may increase nutrients through mechanisms such as improved root growth, nutrient solubilization, and the production of plant growth-promoting regulators (Abdelaal et al. 2021). Supposing a plant is subject to a transient drought condition, seedlings will be likely benefited from PGPB inoculation, as their metabolism and root systems remained relatively intact until the water soil conditions are reestablished.

Although the experimental design initially included determining the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX), these analyses could not be performed. During preliminary tests, no color change was observed in the SOD reaction, as the protocol of Giannopolitis & Ries (1977), and absorbance values of the samples were lower than those of the blank samples, indicating strong interference from leaf compounds. Since the assays for CAT (Aebi, 1984), POD (Chance & Maehly, 1955), and APX (Nakano & Asada, 1981) also rely on sensitive optical reactions, such interference likely compromised their accuracy as well. This limitation may be related to the high levels of phenolic, biflavonoid, and resinous (terpenoid) compounds reported in *A. angustifolia* (Perotti et al. 2015; Frezza et al. 2020), which can react with colorimetric reagents, chelate metal ions, or inhibit enzymes (Christodoulou et al. 2022), leading to inconsistent spectrophotometric readings (Weydert & Cullen, 2010). Therefore, enzymatic activity data were not included, as the chemical composition of

A. angustifolia poses specific challenges for the standardization of colorimetric biochemical assays under water stress conditions.

Overall, this study provides positive evidence for the potential of PGPB to mitigate water stress in araucaria seedlings. However, further studies are needed, particularly those exploring different durations and intensities of water stress in combination with PGPB inoculation. As a slow-growing tree species, araucaria presents a significant challenge, since experimental designs must balance the need to impose stress intense enough to elicit physiological responses without compromising seedling survival. Future research should also assess the intrinsic capacity of araucaria seedlings to tolerate and adapt to drought stress, in order to achieve a better understanding of the species' physiological responses to climate change and the role of biotic interactions in this process (Castro et al. 2020; Marchioro et al. 2023).

4.7 CONCLUSION

Our hypothesis could be confirmed, highlighting the potential of PGPB inoculation to mitigate drought stress in araucaria seedlings by sustaining nutrient uptake and activating key metabolic pathways linked to drought resilience, offering a promising biotechnological approach for the conservation and restoration of this vulnerable species.

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5 CHAPTER 4: EDAPHOCLIMATIC CONDITIONS AFFECT THE FIELD GROWTH OF *Araucaria angustifolia* SEEDLINGS INOCULATED WITH PLANT GROWTH-PROMOTING MICROORGANISMS

5.1 RESUMO

Araucaria angustifolia (pinheiro-brasileiro; araucária) é uma conífera nativa da América do Sul de grande importância ecológica e socioeconômica, contribuindo para a conservação da biodiversidade, o suporte à fauna silvestre, a alimentação humana por meio da produção de sementes e a renda dos agricultores. Como essa espécie apresenta crescimento lento e altas taxas de mortalidade no campo, estratégias para aprimorar o desenvolvimento inicial são de particular interesse. Neste estudo, sementes e mudas de *A. angustifolia* foram inoculadas com microrganismos promotores de crescimento vegetal (MPCV) –*Azospirillum brasilense*, *Bacillus* spp. e *Rhizophagus* spp.– em dois momentos distintos: em casa de vegetação e no campo. Os inoculantes foram aplicados uma vez (inoculação apenas em casa de vegetação), duas vezes (inoculação em casa de vegetação e no campo) ou nenhuma vez (sem inoculação). No campo, antes do transplante, metade das mudas foi reinoculada na cova de plantio. Após o estabelecimento completo das mudas no campo (90 dias após o transplante), os parâmetros de crescimento foram medidos mensalmente até a colheita (599 dias após o início das mensurações). A inoculação com MPCV não afetou a altura ou a biomassa das mudas; no entanto, a taxa de aplicação ao longo do tempo apresentou efeitos significativos no diâmetro do caule das mudas. De modo geral, o crescimento da araucária pareceu ser fortemente influenciado pelas condições edáficas e climáticas. A inoculação com MPCV pode ter contribuído para aspectos específicos do desenvolvimento inicial das árvores, mas experimentos adicionais em campo, sob diversas condições ambientais, são necessários para esclarecer os potenciais benefícios dos MPCV para o estabelecimento de mudas de *A. angustifolia*.

Palavras-chave: Fungos micorrízicos arbusculares. Bactérias promotoras de crescimento vegetal. Experimento de campo com mudas. Disponibilidade de P. Pinheiro-brasileiro.

5.2 ABSTRACT

Araucaria angustifolia (Brazilian pine; araucaria) is a native South American conifer of high ecological and socioeconomic importance, contributing to biodiversity conservation, wildlife support, human nutrition through seed production, and farmers' income. Because this species exhibits slow growth and high rates of mortality in the field, strategies to enhance early development are of particular interest. In this study, *A. angustifolia* seeds and seedlings were inoculated with plant growth-promoting microorganisms (PGPM) –*Azospirillum brasilense*, *Bacillus* spp., and *Rhizophagus* spp.– in two different moments: at the greenhouse and in the field. The inoculants were applied once (inoculation occurred only in greenhouse), twice (inoculation occurred both in greenhouse and in the field) or none (without inoculation). In the field, before the transplanted, half of the seedlings were reinoculated in the hole. After the seedlings were fully established in the field (90 days after transplanting), growth parameters were measured monthly until harvest (599 days after the start of measurements). Inoculation with PGPM did not affect plant height or biomass; however, application rate over time showed significant effects on stem diameter of the seedlings. Overall, araucaria growth appeared to be strongly influenced by soil and climatic conditions. PGPM inoculation could have contributed to specific aspects of early tree development, but additional field experiments under diverse environmental conditions are necessary to clarify the potential benefits of PGPM for *A. angustifolia* seedling establishment and growth.

Keywords: Arbuscular mycorrhizal fungi. Plant growth-promoting bacteria. Seedling experimental field. P availability. Brazilian pine.

5.3 INTRODUCTION

Araucaria angustifolia (Brazilian pine; araucaria) is a native South American conifer of high ecological and socioeconomic importance. Because of intensive lodging and exploitation since the nineteen centuries, the species has been threatened with extinction (Scipioni et al. 2019). The species plays a key role in biodiversity conservation and wildlife support and provides nutritional and economic benefits through seed production, which contributes to farmers' income (Kondo et al. 2024). Despite its importance, *A. angustifolia* exhibits slow early growth and high rates of mortality in the field (Wendling & Zanette, 2017), posing challenges for seedling establishment and restoration of natural habitats.

Recent evidence indicates that the inoculation of tree seedlings with plant growth-promoting microorganisms (PGPM) represents a promising strategy to enhance seedling nutrition and vigor, thereby supporting reforestation and restoration efforts (Kondo et al. 2026). PGPM comprise a diverse group of bacteria and fungi that colonize the rhizosphere or internal plant tissues and promote plant growth through multiple mechanisms, including phytohormone production, modification of the rhizosphere environment, improved nutrient availability, and increased resilience to edaphoclimatic stresses (Teiba et al. 2023; Laishram et al. 2025; Kondo et al. 2026).

Although the beneficial effects of PGPM on plant growth have been widely documented (Berruti et al. 2016; Olanrewaju et al. 2017; Ansabayeva et al. 2025), most studies involving forest species have been conducted under controlled nursery or greenhouse conditions (Faria et al. 1995; Zandavalli et al. 2004; Bergottini et al. 2015; Barcellos et al. 2021; Kondo et al. 2024). Evidence regarding the effectiveness of PGPM inoculation under field conditions remains limited, particularly for native tree species from biodiversity-rich and threatened biomes such as the Atlantic Forest. Moreover, information on the persistence of PGPM effects after seedling transplantation and the potential benefits of reinoculation strategies is still scarce. Addressing these gaps is essential to support the application of PGPM-based approaches in large-scale restoration and conservation programs.

In this study, we hypothesized that araucaria seedlings inoculated and/or reinoculated with PGPM would exhibit improved survival and growth following transplantation to field conditions. Therefore, the objective of this work was to evaluate the potential of PGPM inoculation as a biological strategy to enhance the establishment and early growth of *A. angustifolia* seedlings under field conditions.

5.4 MATERIALS AND METHODS

5.4.1 Experimental conditions

The first phase of the study was conducted in a greenhouse from 2022 to 2023 at the Agricultural Sciences Sector of the Federal University of Paraná (UFPR), Curitiba, Paraná State, Brazil (25°24′45.7″ S; 49°14′57.0″ W). The second phase was carried out from 2023 to 2025 in an experimental area near the Department of Soil Science and Agricultural Engineering (DSEA) within the same sector (25°24’46.8” S; 49º14’58.3” W), under field-like conditions.

5.4.2 Seeds acquisition

In 2022, seeds of *A. angustifolia* were collected from the ground after natural cone fall in a rural area of Cerro Verde, municipality of Quitandinha, Paraná (25º54’58.1’’ S; 49º35’00.2’’ W). The mother tree was previously known for producing high-quality seeds.

Seed viability was assessed by the flotation method, discarding seeds that floated (Wendling et al. 2017). No size-based selection was performed; only viable seeds were retained. Seeds were not disinfected before sowing.

5.4.3 Soil characterization

The soil used in the greenhouse phase was collected from the 0-20 cm layer at the Canguiri Experimental Farm, municipality of Pinhais, Paraná (25°23′24.5″ S; 49°08′36.5″ W). According to the Brazilian Soil Classification System (SiBCS), this material corresponds to a Cambisol, equivalent to an Inceptisol in the Soil Survey Staff system (Soil Survey Staff, 2022; Santos et al. 2025). A composite sample was formed for standard physical and chemical characterization.

In the second phase, prior to establishing the field experiment, three subsamples were randomly taken from the same soil depth (0-20 cm) at the planting site and combined into a composite sample for routine analyses. This soil was identified as a Haplic Cambisol (SiBCS), also classified as an Inceptisol according to Soil Survey Staff (Soil Survey Staff, 2022; Santos et al. 2025).

Soil samples were air-dried, passed through a sieve, and then subjected to several routine assessments. Soil pH ($CaCl_2$) was determined in a 0.01 L^{-1} $CaCl_2$ solution using a 1:2.5 soil-to-solution ratio. Exchangeable Ca, Mg, and Al were obtained using 1 mol L^{-1} KCl, whereas K and P were extracted via the Mehlich-1 solution. Potential acidity (H+Al) was estimated using the SMP buffer method. Soil organic carbon was quantified by the Walkley-Black procedure (Walkley & Black, 1934). Particle-size fractions were measured using the Bouyoucos hydrometer technique (Bouyoucos, 1962). The soil was employed without liming or addition of mineral fertilizers during both experimental stages (greenhouse and field). The chemical and physical properties were described in Table 12.

TABLE 12 – SOIL ATTRIBUTES USED FOR THE INITIAL GROWTH OF *Araucaria angustifolia* SEEDLINGS IN THE GREENHOUSE (2022-2023) AND FOR GROWTH AND DEVELOPMENT IN THE FIELD EXPERIMENT (2023-2025).

Chemical properties of soil	**Greenhouse**	**Field**
pH in $CaCl_2$	4.3	4.9
pH in SMP	4.9	5.3
Al ($cmol_c$ dm^{-3})	2.5	0.2
K ($cmol_c$ dm^{-3})	0.1	0.1
Ca ($cmol_c$ dm^{-3})	3.7	8.1
Mg ($cmol_c$ dm^{-3})	1.5	4.5
H+Al ($cmol_c$ dm^{-3})	13.2	8.5
Total organic C (g dm^{-3})	38.4	58.8
Available P (mg dm^{-3})	6.5	35.5
Physical properties of soil	**Greenhouse**	**Field**
Clay (g kg^{-1})	638	642
Silt (g kg^{-1})	150	225

Sand (g kg^{-1})	213	134

SOURCE: The author (2025).

5.4.4 PGPB and AM inoculants

The liquid PGPB inoculants were provided by the manufacturers (commercial products). The bacteria used were *Azospirillum brasilense* Ab-v5 and Ab-v6, and *Bacillus pumilus* CCTB05, *B. subtilis* CCTB04, and *B. amyloliquefaciens* CCTB09. The AM inoculant used in the greenhouse was *Rhizophagus clarus* RJN 102 A (no commercial product), and the AM used in the field was *Rhizophagus intraradices* (commercial powdered product).

5.4.5 Inoculation in the greenhouse

Seedlings were grown in 0.8 L pots. A small planting hole (“mini pit”, ~5 cm deep) was made in each pot to apply the inoculants. Each seedling received 50 mL of inoculant solution prepared with filtered water at the following concentrations: 0.9% for the *A. brasilense* inoculant, and 1.0% for the *Bacillus* spp. inoculant (Kondo, 2022; Kondo et al. 2024). For the AM treatment, *R. clarus* inoculant was sprinkled into the pit before sowing. The inoculum provided an average concentration of 1.4 spores g^{-1} of substrate, obtained from 7 g of weighed spores. Control pots received 50 mL of filtered water only.

Sowing and inoculation were performed on 27 July 2022, using one seed per pot. After planting, pots were randomly in the greenhouse.

5.4.6 Seedling growth and acclimatization

Seeds remained in the greenhouse until they reached a shoot height approximately 1.5 times the estimated root length (root length based on pot depth), considered an ideal growth stage for field transplantation (Wendling et al. 2017).

On 6 February 2023, seedlings were moved to full-sun conditions for a 90-day acclimatization period, during which their appearance and vigor were monitored. Height and stem diameter of pre-inoculated and non-inoculated seedlings were measured just before transplantation, when they were still in the pots. There were no significant differences between inoculated and non-inoculated seedlings that were transplanted to the field (Supplementary Table S3).

5.4.7 Field reinoculation and planting conditions

Half of the previously inoculated seedlings received a second inoculation in the planting pits, following the same concentrations and application methods used in the greenhouse. Seedlings inoculated with *R. clarus* in the greenhouse were inoculated in the field using *R. intraradices*. A total of 100 mg of commercial inoculant containing 166667 AM propagules per g were weighed and diluted in 450 mL of filtered water, providing 255 propagules per seedling. Control seedlings received 50 mL of filtered water.

Field planting took place on 6 May 2023. Seedlings were spaced 85 x 85 cm apart.

5.4.8 Experimental design

The field experiment followed a randomized block design in a 3 x 3 factorial arrangement, totaling nine treatments and nine replicates, totaling 81 experimental units. The factor 1 was the inoculant type that contained three levels: *A. brasilense*, *Bacillus* spp., and *Rhizophagus* spp. The factor 2 was the number of applications of the inoculant, and contained three levels: one, two and none (control).

5.4.9 Growth monitoring in the field

Height and stem diameter measurements began three months after planting, and were taken monthly using a centimeter-graded ruler and a digital caliper, respectively. Seedlings remained in the field for 22 months. No pesticide or disease control practices were performed, except for periodic cutting of spontaneous vegetation. Figure 4 represents the experiment from the selection of viable *A. angustifolia* seeds to the final measurement before harvest.

FIGURE 4 – OVERVIEW OF THE EXPERIMENTAL SETUP USING *Araucaria angustifolia* SEEDLINGS INOCULATED IN THE GREENHOUSE AND IN THE FIELD WITH *Azospirillum brasilense* Ab-v5 AND Ab-v6 (INOCULANT 1), *Bacillus pumilus* CCTB05, *B. subtilis* CCTB04, AND *B. amyloliquefaciens* CCTB09 (INOCULANT 2), AND *Rhizophagus clarus* RJN 102 A AND *R.*

intraradices (INOCULANT 3). **(A)** *A. angustifolia* SEEDS USED FOR POT PLANTING (AFTER VIABILITY TEST); **(B)** AM FUNGAL POWDER INOCULANT (*R. clarus*) APPLIED PRIOR TO SOIL ADDITION AND *A. angustifolia* SEED PLANTING; **(C)** EARLY SEEDLING GROWTH STAGE; **(D)** SEEDLINGS READY FOR ACCLIMATIZATION UNDER FULL SUNLIGHT; **(E)** SEEDLINGS APPEARANCE AFTER 90 DAYS OF ACCLIMATIZATION; **(F)** INOCULATION OF THE PLANTING HOLES; **(G)** SEEDLING PLANTED AFTER HOLE INOCULATION; **(H)** SEEDLINGS GROWING UNDER FIELD CONDITIONS; **(I)** HEIGHT OF THE TALLEST TREE IN THE EXPERIMENT (INOCULATED ONCE WITH *Bacillus* spp.) AT THE FINAL MEASUREMENT.

SOURCE: The author (2026).

5.4.10 Weather conditions

The weather conditions recorded during the experimental period (599 days), including the measurement dates, the intervals between consecutive assessments, and the average, minimum, and maximum monthly temperatures, as well as precipitation (Table 13), were obtained from the Meteorological Database for Teaching and Research (BDMEP) of the National Institute of Meteorology (INMET) (INMET, 2023, 2024, 2025), using the A807 automatic station in Curitiba.

TABLE 13 – WEATHER CONDITIONS DURING THE INTERVALS OF MEASUREMENTS DURING THE DURATION OF THE FIELD EXPERIMENT OF *Araucaria angustifolia* BEING INOCULATED (ONCE), REINOCULATED (TWICE) OR NON-INOCULATED WITH PLANT GROWTH PROMOTING MICROORGANISMS *Azospirillum brasilense* Ab-v5 AND Ab-v6 (INOCULANT 1), *Bacillus pumilus* CCTB05, *B. subtilis* CCTB04 AND *B. amyloliquefaciens* CCTB09 (INOCULANT 2) AND *Rhizophagus clarus* RJN 102 A AND *R. intraradices* (INOCULANT 3) IN CURITIBA, PARANÁ, BRAZIL.

Date	**Interval (days)**	**Average Temperature (°C)**	**Maximum Temperature (°C)**	**Minimum Temperature (°C)**	**Precipitation accumulated (mm)**
06/05/2023	Transplantation	-	-	-	-
04/08/2023	90	15.4	27.1	3.5	106.4
04/09/2023	31	16.2	29.6	6.2	142.8
10/10/2023	36	19.0	33.0	11.0	251.0
06/11/2023	27	17.4	31.3	8.7	308.2
10/12/2023	34	21.4	34.4	8.7	232.4
08/01/2024	29	22.0	35.3	13.7	46.2
08/02/2024	31	22.0	35.3	13.3	212.0
01/03/2024	22	22.2	31.7	16.5	133.8
09/04/2024	39	21.6	32.6	13.8	68.4
17/05/2024	38	20.7	29.5	9.3	123.6
04/06/2024	18	15.5	26.4	5.3	128.2
27/07/2024	53	16.4	26.8	2.6	112.4
27/08/2024	31	15.8	31.0	1.3	53.2
26/09/2024	30	19.3	33.1	2.5	93.6
29/10/2024	33	18.7	33.1	10.8	98.4
27/11/2024	29	19.6	31.8	12.1	48.4
18/12/2024	21	20.5	31.8	13.1	187.8
31/01/2025	44	21.2	29.8	13.1	290.4
25/03/2025	53	22.2	31.4	14.1	357.8
Amount (days, mm)	689	-	-	-	2995
Average (ºC)	-	19.6*	31.5	9.5	-

*Final average temperature (19.6 ºC) was based on the average of all measurements throughout the experiment (20,446 measurements, since there are several measurements throughout a single day).
SOURCE: The author (2026).

5.4.11 Harvest

At the end of the experiment, surviving seedlings were counted and harvested in their entirety on 25 March 2025. After harvest, the aerial parts of the trees were air-dried inside the greenhouse for 60 days until reaching a constant mass. Due to the substantial growth of the seedlings over the 22-month experimental period, individuals developed partially lignified tissues and attained sizes that prevented the complete aerial biomass from being dried in a temperature-controlled oven. Under these conditions, biomass determination was therefore based on air-dried shoot mass rather than oven-dried mass.

5.4.12 Growth rates

Growth rates were calculated by dividing the difference between successive measurements (cm for height and mm for diameter) by the corresponding time interval (in days).

5.4.13 Statistical analyses

Data were analyzed in R (R Core Team, 2020). For height and stem diameter, linear mixed-effect models were fitted using the lmerTest package. Model assumptions for normality and homoscedasticity were checked through residuals diagnostics. Time was treated as a repeated-measures factor, and plants were included as random intercepts to account for within-subject correlation. Fixed effects included inoculant, application rate, time, and their interactions. Type III ANOVA with Satterthwaite’s correction was used to assess significance. When the application rate x time interaction was significant, pairwise comparisons among application rates were performed within each month using Tukey-adjusted p-values. In addition, growth rates data were analyzed using linear models including inoculant and application rate as fixed effects since random effects associated with plant identity and block negligible variance and resulted in singular model fits.

5.5 RESULTS

Throughout the experimental field period (22 months), only four seedlings died. Three of these mortalities occurred in treatments involving *A. brasilense*: two in seedlings inoculated once and one in a non-inoculated seedling. In treatments involving *Rhizophagus*, one seedling died in the treatment inoculated once.

Time had a highly significant effect on both height and diameter (p<0.001) (Table 14). However, no significant effects of inoculant or its interaction with time were detected. Application rate showed a marginal effect on diameter (0.085) and a significant interaction with time (0.029), while the corresponding effects for height were not significant.

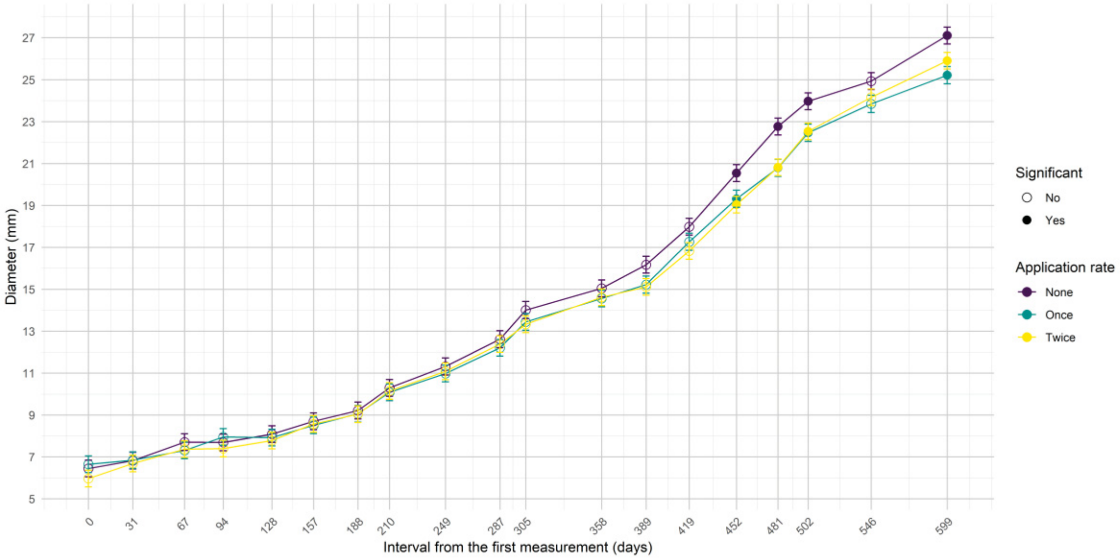
TABLE 14 – P-VALUES OF ANOVA FROM MIXED-EFFECTS MODELS FOR HEIGHT AND STEM DIAMETER OVER 19 MEASUREMENTS OF *Araucaria angustifolia* INOCULATED (ONCE), REINOCULATED (TWICE) OR NON-INOCULATED WITH PLANT GROWTH-PROMOTING MICROORGANISMS *Azospirillum brasilense* Ab-v5 AND Ab-v6 (INOCULANT 1), *Bacillus pumilus* CCTB05, *B. subtilis* CCTB04 AND *B. amyloliquefaciens* CCTB09 (INOCULANT 2) AND *Rhizophagus clarus* RJN 102 A AND *R. intraradices* (INOCULANT 3) AFTER 599 DAYS.

Variable	**Height**	**Stem diameter**
Source of variation	**p-value**	**p-value**
Inoculant	0.48	0.56
Application rate	0.18	0.08
Time	<0.001	<0.001
Inoculant x Application rate	0.82	0.76
Inoculant x Time	1.00	0.97
Application rate x Time	0.06	0.02
Inoculant x Application rate x Time	0.97	0.81

SOURCE: The author (2025).

The interaction between application rate and time significantly affected stem diameter (Table 14). This pattern is illustrated in Figure 5, where diameter increased steadily over 22-month period for all treatments. Although the trajectories were similar during the early months, differences among application rates became more evident as growth progressed. Seedlings receiving two applications showed slightly greater diameters in several intermediate and later months, whereas the single-application and non-inoculated treatments followed comparable but slightly lower trends (Figure 5).

FIGURE 5 – SIGNIFICANT TEMPORAL VARIATION IN STEM DIAMETER OF *Araucaria angustifolia* OVER 19 MEASUREMENTS WITHIN 599 DAYS IN THE FIELD FOR THREE APPLICATION RATES (NONE, ONCE, AND TWICE) OF PLANT GROWTH-PROMOTING MICROORGANISMS *Azospirillum brasilense* Ab-v5 AND Ab-v6 (INOCULANT 1), *Bacillus pumilus* CCTB05, *B. subtilis* CCTB04 AND *B. amyloliquefaciens* CCTB09 (INOCULANT 2) AND *Rhizophagus clarus* RJN 102 A AND *R. intraradices* (INOCULANT 3).



SOURCE: The author (2026).

Growth rates of both height and stem diameter were not significantly influenced by inoculant type, application rate, or their interaction (Table 15), indicating similar growth dynamics among treatments during the evaluation period (p>0.05).

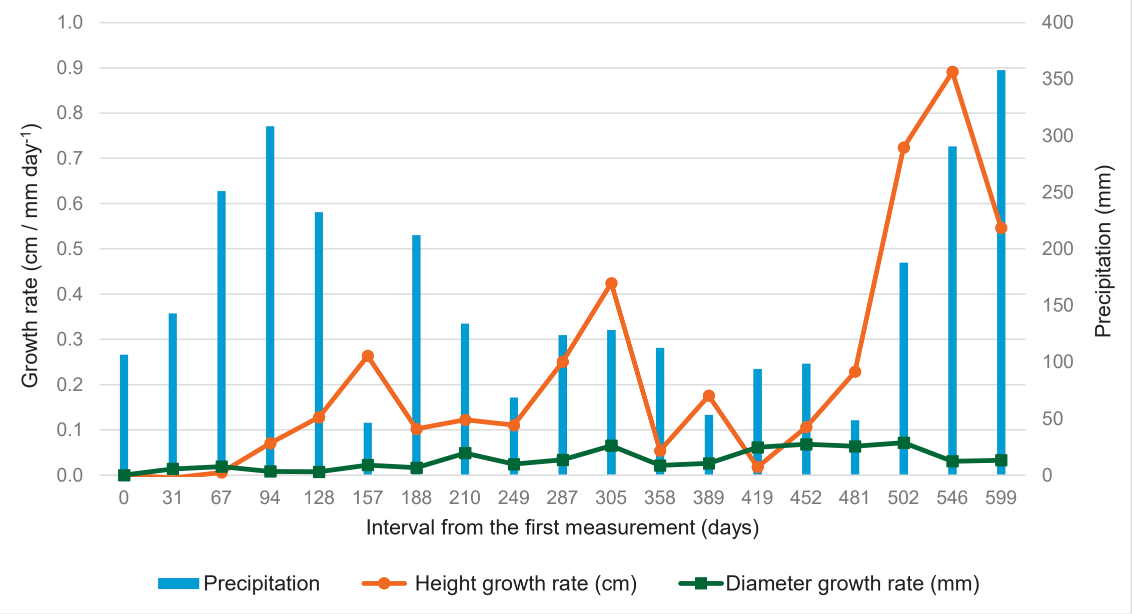
TABLE 15 – P-VALUES OF ANOVA FROM HEIGHT GROWTH RATE AND DIAMETER GROWTH RATE IN *Araucaria angustifolia* SEEDLINGS INOCULATED WITH *Azospirillum brasilense* Ab-v5 AND Ab-v6 (INOCULANT 1), *Bacillus pumilus* CCTB05, *B. subtilis* CCTB04 AND *B. amyloliquefaciens* CCTB09 (INOCULANT 2) AND *Rhizophagus clarus* RJN 102 A AND *R. intraradices* (INOCULANT 3) APPLIED ONCE, TWICE, AND NONE IN THE FIELD.

Variable	**Height**	**Diameter**
Source of variation	**p-value**	**p-value**
Inoculant	0.58	0.07
Application rate	1.40	0.36
Inoculant x Application rate	0.40	0.11

SOURCE: The author (2026).

Growth rates of height and stem diameter varied over time throughout 19 measurements in the field experiment. In addition, the precipitation accumulated between measurements also showed marked temporal variation during the experimental period (Figure 6).

FIGURE 6 – TEMPORAL VARIATION IN HEIGHT AND STEM DIAMETER GROWTH RATES OF *Araucaria angustifolia* SEEDLINGS AND PRECIPITATION ACCUMULATED BETWEEN 19 CONSECUTIVE MEASUREMENTS UNDER FIELD CONDITIONS. GROWTH RATES REPRESENT MEAN VALUES ACROSS TREATMENTS. PRECIPITATION IS SHOWN ON THE SECONDARY Y-AXIS DUE TO DIFFERENCES IN MAGNITUDE AMONG VARIABLES.



SOURCE: The author (2026).

Finally, the dry whole tree mass showed no differences between treatments (Table 16).

TABLE 16 – MASS OF WHOLE DRIED *Araucaria angustifolia* TREES PLANTED IN THE FIELD AND INOCULATED ONLY IN A GREENHOUSE (ONCE) OR REINOCULATED IN THE FIELD (TWICE) WITH *Azospirillum brasilense* Ab-v5 AND Ab-v6 (INOCULANT 1), *Bacillus pumilus* CCTB05, *B. subtilis* CCTB04 AND *B. amyloliquefaciens* CCTB09 (INOCULANT 2) AND *Rhizophagus clarus* RJN 102 A AND *R. intraradices* (INOCULANT 3) AFTER 599 DAYS.

Inoculant	**Application rate**	**Dry mass of the whole tree (kg plant^{-1})**
***A. brasilense* Ab-v5 and Ab-v6**	Once	0.57 ^{ns}
	Twice	0.71 ^{ns}
	None	0.74 ^{ns}
***B. pumilus* CCTB05, *B. subtilis* CCTB04 and *B. amyloliquefaciens* CCTB09**	Once	0.74 ^{ns}
	Twice	0.64 ^{ns}
	None	0.66 ^{ns}
R. clarus* RJN 102 A and *R. intraradices	Once	0.61 ^{ns}
	Twice	0.62 ^{ns}
	None	0.71 ^{ns}

ns: not significant in the ANOVA.
SOURCE: The author (2026).

5.6 DISCUSSION

Despite previous evidence indicating that PGPM enhance early tree seedling performance under controlled conditions (Kondo et al. 2020; 2021; 2024; 2026), the present long-term field experiment did not reveal consistent effects of inoculant type on the height and stem diameter (Table 14), growth rates (Table 15), or final biomass (Table 16) of *A. angustifolia*. This lack of response under field conditions reinforces the notion that positive effects observed in nursery or greenhouse conditions studies do not necessarily translate into measurable advantages after outplanting, especially for slow-growing forest species exposed to high environmental variability. Similar patterns have been reported in field studies with tree seedlings, in which environmental factors often override microbial inoculation effects once plants are established in the field (Chanway & Holl, 1994; Chanway et al. 2000).

Although inoculant type did not influence growth variables, the interaction between application rate and time significantly affected stem diameter, with seedling receiving two applications exhibiting slightly greater diameters in some intermediate and later periods (Table 14, Figure 5). In contrast, plant height was not affected by inoculation or application frequency throughout the experimental period (Table 14). This dissociation between height and diameter responses is not uncommon in tree species, as height-diameter allometry can vary with environmental and growth stage, and carbon allocation to axial versus radial growth is influenced by physiological and mechanisms constraints (e.g., tree height-diameter allometry varies across sites and species; height and diameter increments are often not proportional) (King, 2005; Zhang et al. 2020). Thus, small and transient differences in stem diameter may reflect short-term physiological adjustments rather than sustained improvements in overall plant performance. Minor and inconsistent fluctuations observed in stem diameter in specific treatments or periods were not further explored, as they were not sustained over time and did not translate into differences in growth rate (Table 15) or biomass (Table 16).

One plausible explanation for the absence of pronounced inoculation effects lies in the relatively favorable soil chemical conditions at the field site, particularly regarding P availability (Table 12). Previous studies have shown that *A. angustifolia* responds strongly to P supply, with marked improvements in growth and nutritional status under increased P availability (Moreira-Souza & Cardoso, 2002; Vilcatoma-Medina, 2021; 2024). In the present study, soil P levels were substantially higher in the field phase than in the greenhouse phase (Table 12), potentially reducing plant dependence on microbial-mediated nutrient acquisition. Under such conditions, the function contribution of PGPM may become less apparent, as nutrient limitation (a key driver of microbial benefits) is alleviated (Standish et al. 2021). This interpretation does not imply that *A. angustifolia* is unresponsive to microbial inoculation per se, but rather than positive responses may be context-dependent and less evident when soil fertility is not limiting.

Throughout the experimental period, *A. angustifolia* seedlings exhibited continuous growth, with periods of faster and slower increments in both height and diameter (Figure 6). In general, growth rates tended to be higher during intervals with lower precipitation, whereas prolonged periods of intense rainfall coincided with reduced growth increments (Figure 6). The experimental period was marked by unusually high cumulative precipitation (Table 13), likely associated with large-scale climatic anomalies such as El Niño – Southern Oscillation (ENSO) events (Cai et al. 2020). Consequently, excess soil moisture may impair root aeration, and overall plant performance (Zhang et al. 2025), potentially masking treatment effects and limiting growth even under otherwise favorable nutritional conditions.

Although inoculation treatments did not result in significant growth advantages, our study reinforces the need to evaluate the performance of inoculated seedlings in the field, since the benefits of inoculation observed under controlled conditions (nurseries or greenhouses) will not always be observed in the field. Take together, these results highlight the complexity of translating microbial inoculation benefits from controlled environments to long-term field conditions and underscore the need for integrated management strategies that account for soil fertility, and climatic variability in *A. angustifolia* conservation efforts.

5.7 CONCLUSION

The continuous growth and high survival of seedlings across all treatments indicate that *A. angustifolia* can establish successfully under field conditions when soil fertility is adequate, even during periods of adverse weather conditions. However, the absence of strong inoculation effects suggests that environmental factors, particularly soil nutrient availability and water dynamics, may play a dominant role in regulating seedling growth, potentially overriding the benefits of microbial inoculation observed under controlled conditions.

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6 GENERAL CONCLUSION

There is a positive interaction between PGPM and seedlings of native forest species from the BAF biome, with particular emphasis on *A. angustifolia*. This interaction contributes to the early development of seedlings and to the modulation of physiological responses associated with plant establishment. Thus, the results reinforce the potential application of PGPM as a promising strategy to produce native seedlings and for forest conservation and restoration initiatives.

7 FINAL CONSIDERATIONS

From the perspective of seedling production, this thesis has a simultaneously process-oriented and applied character. The meta-analysis showed that the greatest benefits of inoculation occur in pioneer and early secondary species, and P fertilizer is the best input to produce the seedlings, while experiments with *A. angustifolia* demonstrated variable responses depending on the substrate, water regime, and timing of inoculant application. These findings indicate that there are no universal solutions, and the adoption of PGPB in restoration programs should consider the compatibility between microorganism, plant species, and growing environment.

The results also encourage a broader reflection on the need to strengthen a soil-forest microbiology oriented toward ecological restoration. Most microbiological knowledge has been from agricultural systems, and its direct transfer to forest ecosystems is limited. Developing approaches specifically designed for forest soils (considering the complexity of plant-microorganism interactions, ecological succession, and environmental heterogeneity) is essential to transform PGPM into technologies effectively applicable to biodiversity conservation. In this sense, advancing from simplified inoculation models to context-dependent strategies represents a key challenge for the field.

Finally, the data generated represent an initial path, however, several approaches within forest microbiology remain to be investigated. Among these, the use of shotgun metagenomics stands out as promising strategy to achieve a deeper understanding of the interactions between PGPM and the rhizosphere of tree seedlings. In addition, metabolomic approaches may assist in the initial elucidation of the signaling processes involved in PGPM-seedling interactions, while transcriptomics may enable the identification of differentially expressed genes resulting from this association. In this context, omics-based tools represent promising strategies for a detailed understanding of these interactions and may contribute to the development of more effective approaches aimed at the conservation of forest species.

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APPENDIX 1 – ARTICLE 1

Table S1 – Summary of studies included in the meta-analysis, considering the following abbreviations: PGPM = plant growth-promoting microorganisms; AMF = arbuscular mycorrhizal fungi; PGPB = plant growth-promoting bacteria; NFB = nitrogen-fixing bacteria; PI = pioneer; ES = early secondary; LS = late secondary; CL = climax; BFN = biological N_2 fixation.

Tree species	Ecological Group	Ability to fix N_2	References			
			AMF	NFB	PGPB	AMF+NFB
Alchornea glandulosa Poepp. & Endl.	PI	no	1			
Anadenanthera colubrina (Vell.) Brenan	PI	yes	2	2		2
Anadenanthera colubrina var. cebil (Griseb.) Altschul	ES	yes	3	3		3
Anadenanthera peregrina (L.) Speg.	ES	yes	4, 5, 6, 7, 8	4, 5, 45, 46		4, 5
Anadenanthera peregrina var. falcata (Benth.) Altschul	ES	yes	9, 10	47		47
Annona cacans Warm.	ES	no	1			
Apuleia leiocarpa (Vogel) J.F.Macbr.	LS	unknown	11, 12			
Araucaria angustifolia (Bertol.) Kuntze	ES	no	13		57	
Aspidosperma parvifolium A.DC.	CL	no	9, 10			
Aspidosperma polyneuron Müll.Arg.	LS	no	14			
Bauhinia spp	PI	unknown	9, 10			
Cabralea canjerana (Vell.) Mart.	LS	no	1		58	
Cassia grandis L.f.	ES	unknown	9, 10			
Cecropia pachystachya Trécul	PI	no	15, 16		58	
Cedrela fissilis Vell.	ES	no	1, 8, 9, 10, 17			
Ceiba speciosa (A.St.-Hil.) Ravenna	LS	no	9, 10, 18			
Cenostigma pluviosum var. *peltophoroides* (Benth.) Gagnon & G.P.Lewis	LS	unknown	9, 10			
Centrolobium robustum (Vell.) Mart. ex Benth.	PI	yes	19			
Citharexylum myrianthum Cham.	PI	no	1			
Colubrina glandulosa Perkins	ES	no	20, 21, 22			
Copaifera langsdorffii Desf.	CL	unknown	9, 10, 18			

Continue...

Table S1 (Continued)

Tree species	**Ecological Group**	**Ability to fix N_2**	**References**			
			AMF	**NFB**	**PGPB**	**AMF+NFB**
Croton floribundus Spreng.	PI	no	18			
Dahlstedtia muehlbergiana (Hassl.) M.J.Silva & A.M.G.Azevedo	ES	yes	23	23		23
Dalbergia nigra (Vell.) Allemão ex Benth.	ES	yes	24	48		48
Enterolobium contortisiliquum (Vell.) Morong	LS	yes	6, 16, 23, 25	23, 49, 50		23
Garcinia gardneriana (Planch. & Triana) Zappi	LS	no	19			
Genipa americana L.	ES	no	26			
Handroanthus impetiginosus (Mart. ex DC.) Mattos	LS	no	18			
Handroanthus serratifolius (Vahl) S.Grose	ES	no	18			
Heliocarpus popayanensis Kunth	ES	no			58	
Hymenaea courbaril L.	CL	unknown	9, 10, 18			
Ilex paraguariensis A.St.-Hil.	LS	no	27		59	
Inga edulis Mart.	ES	yes		51, 52		
Inga laurina (Sw.) Willd.	ES	yes	23	23		23
Inga vera Willd.	ES	yes		53		
Luehea divaricata Mart.	LS	no	19			
Luehea grandiflora Mart.	ES	no	9, 10, 16			
Machaerium nyctitans (Vell.) Benth.	ES	yes		54		
Magnolia ovata (A.St.-Hil.) Spreng.	LS	no	1, 9, 10			
Maytenus boaria Molina	LS	no	28			
Mimosa bimucronata (DC.) Kuntze	PI	yes	2	2, 55		2
Mimosa scabrella Benth.	PI	yes		55, 56		
Mimosa schomburgkii Benth.	PI	yes	29, 30			
Myrcia strigipes Mart.	LS	no	1			
Myroxylon peruiferum L.f.	CL	unknown	18			

Continue...

Table S1 (Continued)

Tree species	**Ecological Group**	**Ability to fix N_2**	**References**			
			AMF	**NFB**	**PGPB**	**AMF+NFB**
Ormosia arborea (Vell.) Harms	LS	yes	10	54		
Parapiptadenia rigida (Benth.) Brenan	ES	yes	2	2, 55		2
Peltophorum dubium (Spreng.) Taub.	PI	unknown	9, 10, 31	31		31
Piptadenia gonoacantha (Mart.) J.F.Macbr.	ES	yes	32	32		32
Plathymenia reticulata Benth.	ES	yes	6, 33			
Platycyamus regnellii Benth.	CL	yes	9, 10			
Platypodium elegans Vogel	ES	yes	18, 23	23, 54		23
Psidium cattleyanum Sabine	PI	no	34			
Pterogyne nitens Tul.	PI	unknown		45		
Sapindus saponaria L.	PI	no	9, 10, 18			
Sarcomphalus joazeiro (Mart.) Hauenschild	ES	no	35			
Schinus terebinthifolia Raddi	PI	no	1, 9, 10, 19, 36, 37, 38,			
Schizolobium parahyba (Vell.) Blake	PI	unknown	9, 10			
Senegalia polyphylla (DC.) Britton & Rose	ES	yes	39			
Senna macranthera (DC. ex Collad.) H.S.Irwin & Barneby	PI	unknown	7, 9, 10, 16, 40			
Senna multijuga (Rich.) H.S.Irwin & Barneby	PI	unknown	7, 9, 10, 16, 18, 41			
Senna spectabilis (DC.) H.S.Irwin & Barneby	PI	unknown	9, 10			
Sesbania virgata (Cav.) Poir.	PI	yes	3, 16, 42, 43, 60	42		42
Tocoyena sellowiana (Cham. & Schltdl.) K.Schum.	PI	no	44			
Trema micranthum (L.) Blume	ES	no	3, 10, 40, 60			

SOURCE: The author (2025).

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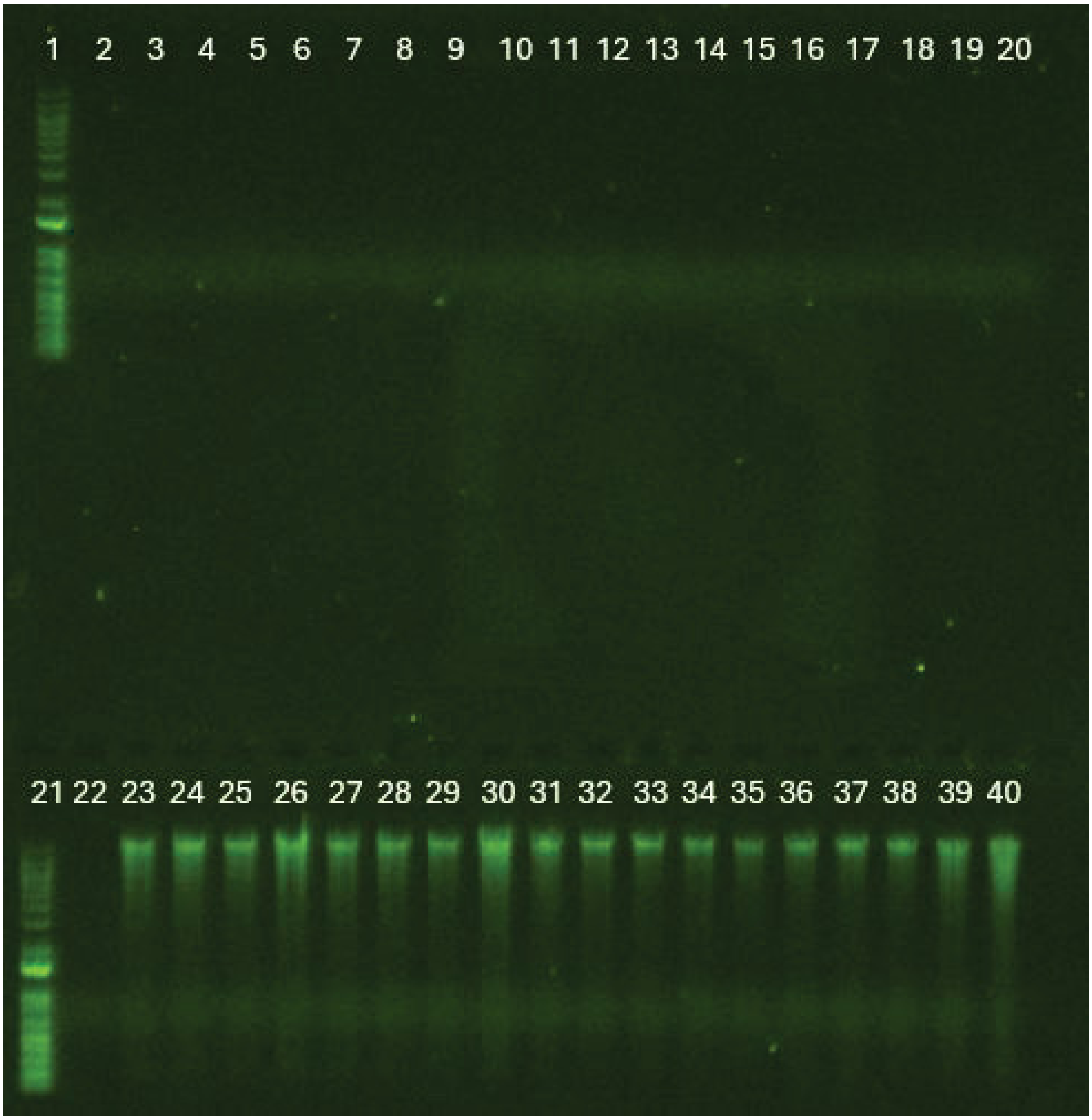
APPENDIX 2 – ARTICLE 2

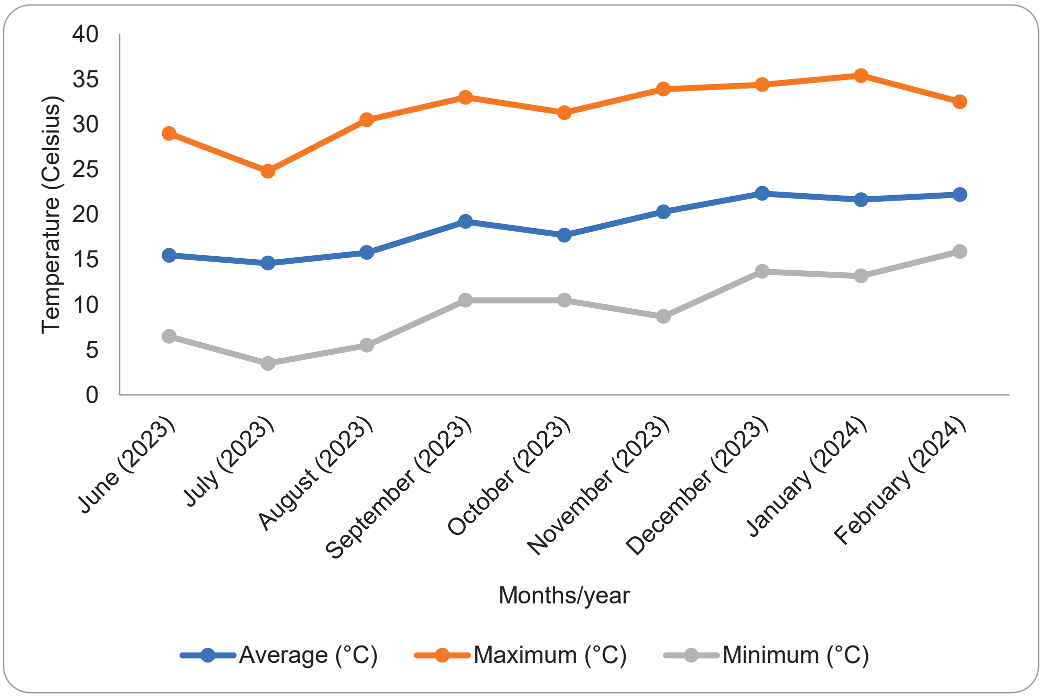
Figure S1 – Agarose gel electrophoresis (1%) showing the integrity and presence of DNA extracted from rhizospheric samples of sterile sand (lanes 2-20) and natural soil (lanes 22-40), at 7 and 21 days after inoculation (DAI) with *Azospirillum brasilense* Ab-v5 and Ab-v6 (inoculant 1) and *Bacillus pumilus* CCTB05, *B. subtilis* CCTB04 and *B. amyloliquefaciens* CCTB09 (inoculant 2) in *Araucaria angustifolia* seedlings. The DNA samples (approximately 100 ng per well) were separated in TAE 1X buffer and stained with SYBR Safe®.



Notes: **Identification of lanes:** lane 1 and 21: 1 kb DNA ladder. **Sterile sand rhizosphere:** lane 2, blank; lanes 3-5, *A. brasilense* at 7 days after inoculation (DAI); lanes 6-8, *Bacillus* spp. at 7 DAI; lanes 9-11, control (non-inoculated) at 7 DAI; lanes 12-14, *A. brasilense* at 21 DAI; lanes 15-17, *Bacillus* spp. at 21 DAI; lanes 18-20, control at 21 DAI. **Natural soil rhizosphere:** lane 22, blank; lanes 23-25, *A. brasilense* at 7 DAI; lanes 26-28, *Bacillus* spp. at 7 DAI; lanes 29-31, control at 7 DAI; lanes 32-34, *A. brasilense* at 21 DAI; lanes 35-37, *Bacillus* spp. at 21 DAI; lanes 38-40, control at 21 DAI.
SOURCE: The author (2024).

APPENDIX 3 – ARTICLE 3

Figure S2 – Air temperatures in the campus of Federal University of Paraná during the experiment. Temperature data were considered for two periods: (1) seedling formation (June-December 2023; winter-summer) and (2) experimental period (January-February 2024; summer). In period 1, mean daily maximum/minimum temperatures were 31.0/8.4 °C, respectively; in period 2, 34.0/14.6 °C, respectively. Data were obtained from the National Institute of Meteorology (INMET) BDMEP database, automatic station Curitiba, PR (code A807) (INMET, 2025). The regional climate is CFB (Köppen-Geiger; Peel et al. 2007).



SOURCE: The author (2025).

Table S2 – Significance of ANOVA and coefficients of variation (CV%) for plant growth of *Araucaria angustifolia* seedlings inoculated with two plant growth-promoting bacteria (PGPB) inoculants and subjected to three irrigation regimes in a greenhouse, after 7 and 21 days of inoculation.

Harvest	**7 DAI**				**21 DAI**			
Factor	**[1]**	**[2]**	**[1]×[2]**	**CV%**	**[1]**	**[2]**	**[1]×[2]**	**CV%**
Variable								
Shoot fresh mass (g)	ns	ns	ns	15	ns	ns	ns	18
Dry shoot mass (g)	ns	ns	ns	20	ns	ns	ns	19
Root fresh mass (g)	ns	ns	ns	18	ns	ns	ns	11
Root dry mass (g)	ns	ns	ns	16	ns	ns	ns	15
Relative Water Content (%)	ns	ns	ns	3	ns	ns	ns	5
Total root length (cm)	ns	ns	ns	23	ns	ns	ns	13
Root surface area (cm^2)	ns	ns	ns	21	ns	ns	ns	13
Root volume (cm^3)	ns	ns	ns	27	ns	ns	ns	18

Fine roots classes								
Ø 0 to 0.5 mm (cm)	ns	ns	ns	32	ns	*	ns	21
Ø 0.5 to 1.0 mm (cm)	ns	ns	ns	22	ns	ns	ns	20
Ø 1.0 to 1.5 mm (cm)	ns	ns	ns	31	ns	ns	ns	16
Ø 1.5 to 2.0 mm (cm)	ns	ns	ns	25	ns	ns	ns	15
Ø 2.0 to 2.5 mm (cm)	ns	ns	ns	31	ns	ns	ns	22
Soluble sugars (mg g^{-1})	ns	ns	ns	24	*	*	*	13
Proline (µmol g^{-1})	ns	ns	ns	44	ns	ns	ns	42
N (g kg^{-1})	ns	ns	ns	27	*	ns	ns	28
P (g kg^{-1})	ns	ns	ns	15	ns	ns	ns	15
K (g kg^{-1})	ns	ns	ns	9	ns	*	ns	6
Ca (g kg^{-1})	ns	ns	ns	12	ns	ns	ns	8
Mg (g kg^{-1})	*	ns	ns	12	ns	ns	ns	10
S (g kg^{-1})	*	ns	ns	22	ns	ns	ns	13
B (mg kg^{-1})	ns	ns	ns	17	ns	ns	ns	28
Cu (mg kg^{-1})	*	ns	ns	16	ns	*	*	20
Fe (mg kg^{-1})	ns	ns	ns	29	ns	*	ns	29
Mn (mg kg^{-1})	ns	ns	ns	24	ns	ns	ns	15
Zn (mg kg^{-1})	*	ns	*	12	ns	*	ns	12

Notes: **(PGPB) inoculants:** [1] *Azospirillum brasilense* Ab-v5 and Ab-v6; [2] *Bacillus haynesii* CCT7926, *Priestia aryabhatta*i CBMAI1120, and *Niallia circulans* CCT0026. **Soil water regimes:** [100%] full container capacity, [2] 70% container capacity, and [3] 30% container capacity. ns: not significant; *: $p<0.05$; Six-months old seedlings were transferred into 7 L soil pots, in greenhouse, inoculated with PGPB, submitted to water regimes and harvested after 7 and 21 days of inoculation. Soluble sugars and proline are based on fresh weight, while nutrients are based on dry weight.
SOURCE: The author (2025).

APPENDIX 4 – ARTICLE 4

Table S3 – Heights and diameters of *Araucaria angustifolia* seedlings grown in a greenhouse and were inoculated at sowing with *Azospirillum brasilense* Ab-v5 and Ab-v6 (inoculant 1), *Bacillus pumilus* CCTB05, *B. subtilis* CCTB04 and *B. amyloliquefaciens* CCTB09 (inoculant 2) and *Rhizophagus clarus* RJN 102 A (inoculant 3), after 90 days of acclimatization and one day before planted in the field in Curitiba, Paraná, Brazil.

Variable			
Height (cm)			
Inoculant		Application rate	
A. brasilense	25.2 ^{ns}	None	26.2 ^{ns}
Bacillus spp.	26.2 ^{ns}	Once	26.3 ^{ns}
Rhizophagus spp.	26.4 ^{ns}	Twice	25.1 ^{ns}
CV (%)		11.9	
Diameter (mm)			
Inoculant		Application rate	
A. brasilense	6.3 ^{ns}	None	6.2 ^{ns}
Bacillus spp.	6.2 ^{ns}	Once	6.5 ^{ns}
Rhizophagus spp.	6.4 ^{ns}	Twice	6.3 ^{ns}
CV (%)		10.1	

Notes: ns: not significant in the ANOVA.
SOURCE: The author (2025).