

UNIVERSIDADE FEDERAL DE VIÇOSA

**Potential for direct and indirect plant growth promotion and heterologous
expression of biocontrol enzymes from slow-growing bacteria**

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BLEND A DE FREITAS RODRIGUES JESUINO

Potential for direct and indirect plant growth promotion and heterologous expression of biocontrol enzymes from slow-growing bacteria

Dissertation submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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I dedicate this work, first and foremost, to little Blenda, who was captivated by National Geographic documentaries and became the biologist she once watched.

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ABSTRACT

JESUINO, Blenda de Freitas Rodrigues, M.Sc., Universidade Federal de Viçosa, August, 2025. **Potential for direct and indirect plant growth promotion and heterologous expression of biocontrol enzymes from slow-growing bacteria.** Adviser: Mateus Ferreira Santana. Co-advisers: Patricia Pereira Fontes and Caio Roberto Soares Bragança.

Economic factors, physical space limitations, and agricultural production losses due to phytopathogens pose significant challenges for sustainable agriculture. The main current responses to these challenges are the use of chemical fertilizers and pesticides. In addition to or instead of these challenges, more sustainable solutions have been developed, above all through the use of biofertilizers and biopesticides. With the growing demand for plant growth-promoting bacteria (PGPBs) and biocontrol bacteria, new microbial groups have been prospected. The promising indications of slow-growing bacteria for these purposes led to their further exploration in this work. However, given that their long life cycle can make scaling up difficult, the approach of heterologous expression of biocontrol enzymes encoded by slow-growing bacteria was also used. The diversity of the slow-growing isolates was evidenced through phylogenetic analysis, which revealed that the 11 sequenced isolates are distributed in seven distinct genera. A wide variety of genes related to plant growth-promoting traits (PGPT) were detected in all the isolates, with the most abundant genetic traits being related to heavy metal detoxification, osmotic stress neutralization, vitamin production, chemotactic motility, secretion systems, and siderophore production. The in vitro detection of PGPT, carried out to validate the genes predicted in silico, confirmed the ability of slow-growing bacteria to promote plant growth. The genes encoding biocontrol enzymes were also prospected in silico and validated in vitro. Biosynthetic gene clusters (BGC) prospecting detected 13 distinct BGC classes and a frequency of 57 clusters, as well as seven classified as "other". In the paired culture trials between the slow-growing bacterial isolates and phytopathogenic fungi, many bacterial isolates showed antagonism to *Fusarium equiseti* CCF422, *Fusarium oxysporum* CCF184, *Rhizoctonia solani* 2930, and *Sclerotinia sclerotiorum* 15B, with average inhibition above 20%. A total of 168 defense genes related to 30 defense systems were detected throughout the genomes of the 11 slow-growing isolates. In addition, a total of 39 resistance genes were also detected, distributed across the genomes of seven isolates and three isolates with virulence genes. In addition to the prolonged growth time, the presence of virulence genes also means that the biocontrol potential of the isolates has to be assessed indirectly, which can be done through the heterologous

expression of proteins. Heterologous expression of the enzymes chitinase, exo-beta-1,3-glucanase, and N-acetylmuramyl-L-alanine amidase was carried out. The recombinant enzymes proved to be active in the transformed *Escherichia coli* BL21, both in the degradation of the substrate and in the culture paired with phytopathogenic fungi. However, protein extraction revealed that the recombinant enzymes remained in the insoluble fraction, from which they were purified and then refolded. However, only glucanase showed enzymatic activity, making it necessary to modify the refolding buffer to recover the three-dimensional structure of chitinase and amidase. These preliminary results provide good prospects for the future application of a biopesticide composed of recombinant biocontrol enzymes.

Keywords: plant growth-promoting bacterias (PGPBs); enzyme prospecting; phytopathogenic fungus; dual culture; Recombinant protein

RESUMO

JESUINO, Blenda de Freitas Rodrigues, M.Sc., Universidade Federal de Viçosa, agosto de 2025. **Potencial de promoção do crescimento vegetal direto e indireto e expressão heteróloga de enzimas de biocontrole de bactérias de crescimento lento.** Orientador: Mateus Ferreira Santana. Coorientadores: Patricia Pereira Fontes e Caio Roberto Soares Bragança.

Os fatores econômicos, limitações de espaço físico e perdas da produção agrícola causadas por fitopatógenos representam grandes desafios para uma agricultura sustentável. As principais respostas atuais para esses desafios são o uso de fertilizantes e pesticidas químicos. Em adição ou substituição, esses desafios têm sido enfrentados com soluções mais sustentáveis, sobretudo com o uso de biofertilizantes e biopesticidas. Com a crescente demanda por bactérias promotoras do crescimento vegetal (PGPBs) e de biocontrole, novos grupos microbianos têm sido prospectados. Os indicativos promissores das bactérias de crescimento lento para esses fins basearam sua maior exploração no presente trabalho. Porém, pensando que o ciclo de vida prolongado pode dificultar o escalonamento, também foi utilizada a abordagem da expressão heteróloga de enzimas de biocontrole codificadas por bactérias de crescimento lento. A diversidade dos isolados de crescimento lento foi evidenciada através das análises filogenéticas, que revelaram que os 11 isolados estão distribuídos em sete gêneros distintos. Foi detectada uma grande variedade de genes relacionados aos traços de promoção do crescimento vegetal (PGPT) em todos os isolados, sendo os traços genéticos mais abundantes relacionados à detoxificação de metais pesados, neutralização de estresse osmótico, produção de vitaminas, motilidade quimiostática, sistemas de secreção e produção de sideróforos. A detecção *in vitro* dos PGPTs realizada para validação dos genes previstos *in silico* confirmou a capacidade das bactérias de crescimento lento em promover o crescimento vegetal. Os genes codificantes de enzimas de biocontrole também foram prospectados *in silico* e validados *in vitro*. A prospecção por clusters de genes biossintéticos (BGC) detectou 13 classes de BGCs distintas e frequência de 57 clusters, além de sete classificados como “outros”. Nos ensaios de cultura pareada entre as bactérias de crescimento lento e os fungos fitopatogênicos, muitos isolados bacterianos se mostraram antagonismo ao *Fusarium equiseti* CCF422, *Fusarium oxysporum* CCF184, *Rhizoctonia solani* 2930, and *Sclerotinia sclerotiorum* 15B, com inibição média acima de 20%. Foram detectados um total de 168 genes de defesa relacionados a 30 sistemas de defesa em todo o genoma dos 11 isolados de crescimento lento.

Além

disso, também foram detectados um total de 39 genes de resistência distribuídos no genoma de sete isolados e três isolados com genes de virulência. Além do tempo de crescimento prolongado, a presença de genes de virulência também faz com que o potencial de biocontrole dos isolados tenha que ser acessado de forma indireta, o que pode ser feito através da expressão heteróloga de proteínas. Foi realizada a expressão heteróloga das enzimas quitinase, exo-beta-1,3-glucanase e N-acetylmuramyl-L-alanine amidase. As enzimas recombinantes se mostraram ativas na *Escherichia coli* BL21 transformada tanto na degradação do substrato quanto na cultura pareada com fungos fitopatogênicos. Porém, a extração proteica revelou que as enzimas recombinantes ficaram na fração insolúvel, de onde foram purificadas e em seguida redobradas. Entretanto, apenas a glucanase apresentou atividade enzimática, sendo necessário modificar o tampão de redobrimento para recuperar a estrutura tridimensional da quitinase e amidase. Estes resultados preliminares dão boas perspectivas para a futura aplicação de um biopesticida composto por enzimas recombinantes de biocontrole.

Palavras-chave: bactérias promotoras do crescimento vegetal (PGPBs); prospecção enzimática; fungo fitopatogênico; Cultura dupla; proteína recombinante

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1. GENERAL INTRODUCTION

Agriculture is an essential practice for human survival and has been gaining increasing attention on a planet that reached eight billion people in 2022 (ONU News, 2022). In addition to its importance for food, agriculture is also responsible for generating jobs, especially in rural areas. However, economic factors, physical space limitations, and crop production losses caused by phytopathogens represent major challenges for sustainable agriculture (FAO, 2019; FAO, 2025). The primary responses to these challenges are the use of chemical fertilizers and pesticides, practices that are detrimental to the ecosystem and human health and which are being banned in many places (Drechsel et al., 2025). In addition or instead these challenges have been met with more sustainable solutions, especially with the use of biofertilizers and biopesticides. estimated that there was a 13% increase in the use of bioinputs in the 2024/2025 harvest. This growth is four times greater than the global average, positioning Brazil as a major player in sustainable agriculture (CropLife, 2025).

The growing demand for agricultural inputs, together with the loss of productivity and the growing desire and need for more sustainable planting and management practices, has led to new biological approaches being adopted in the era of Agriculture 2.0. Among these practices is the replacement of chemical fertilizers with microorganisms capable of promoting plant growth (Chandrasekaran & Paramasivan, 2024). Plant growth-promoting bacteria (PGPBs) are capable of causing this effect through various mechanisms, such as the production of plant growth regulators, the fixation of essential nutrients for crops, and the association with the plant, which improves its ability to absorb nutrients and minerals (Andrade et al., 2023).

Every year, between 20% and 40% of food production is lost due to a wide range of agricultural pests and diseases. Among the phytopathogenic organisms, fungi cause a loss in food production around 220 billion dollars every year (FAO, 2019; FAO, 2025), with phytopathogenic fungi being responsible for the loss of 20% to 40% of agricultural production. Of these losses, 10% to 20% occur after harvest (Agricultural Research Service, 2022; Phys, 2025; MPG, 2025).

Several strategies are implemented to control agricultural pests and diseases, including physical, chemical, and biological practices. Physical practices are not very

effective, chemical practices are the most common but costly and harmful to the environment, and biological practices, such as the use of bacteria to control plant diseases, are seen as a more environmentally friendly measure. The main biological mechanisms for controlling phytopathogens combine indirect forms, such as competition for an ecological niche or substrate, and direct forms, such as the production of inhibitory compounds and the induction of systemic resistance. Some bacterial genera are widely used as biocontrol agents (Compant et al., 2005; Soni, Rawal & Keharia, 2021).

Bacteria represent the most abundant and diverse living organisms in all domains of life. Yet it is believed that less than 1% of microorganisms are cultured under laboratory conditions, an estimate that is referred to as the great plate count anomaly (Staley & Konopka, 1985; Epstein, 2013). Currently, although the majority of information on bacteria comes from cultivated strains, more than half of the bacterial phyla do not have cultivated representatives, which may have a different biology from that observed so far (Lewis et al., 2020). The lack of knowledge about their growth conditions, nutritional requirements, dormancy, ecological interactions, and other factors makes the cultivation of the vast majority of bacteria in laboratory conditions challenging and often impossible. In addition to the lack of knowledge of their biology and all the evolutionary insights it could provide, this means that 99% of their biotechnological potential remains unexplored, a phenomenon known as microbial "dark matter" (Kapinusova, Marin & Uhlik, 2023).

One of the most challenging bacterial groups is the slow-growing bacteria, which, despite being very diverse phylogenetically, have some general characteristics and are often described as fastidious, living in oligotrophic environments, whether terrestrial or marine, and are usually classified as K-type strategists, i.e., they have slow growth, greater tolerance to stresses and adaptation to competition for resources. Norris et al. (2021) believe that slow-growing bacteria remain K-strategists even when provided with high levels of nutrients due to the intrinsic characteristics of their biology, especially due to the trade-offs between nutrient transport systems.

Because of this, their ecological role has raised doubts and hypotheses over time, although they remain a little-explored group. The genomic analyses carried out by the research group in the work by Gonçalves & Santana (2023), with five slow-growing isolates, revealed the presence of genes involved in various ecological

functions, including participation in biogeochemical cycles, the metabolization of various carbohydrates, the biosynthetic potential for secondary metabolites, and the presence of plant growth-promoting (PGP) related genes. In a subsequent study, Gonçalves et al. (2024) further explored slow-growing bacteria, more specifically *Acidobacterium* spp., and their possible role as PGPB. To this end, a genomic analysis was carried out looking for genes related to nitrogen fixation, exopolysaccharide production, root colonization by nodulation, tolerance to oxidative stress, iron acquisition, salt-potassium transport stress, plant embryogenesis, spermidine production, indole-3-acetic acid (IAA)-related tryptophan metabolism, and phosphorus solubilization. The authors' *in silico* analyses, in addition to detecting several genes related to the promotion of plant growth, also detected indications of potential for biocontrol related to induced systemic resistance of plants, secretion of antimicrobial compounds, co-colonization with phytopathogens, and lytic enzyme activities (Gonçalves et al., 2024). Other research groups also work with some slow-growing isolates. Dubey et al. (2021), for example, conducted a series of screenings for plant growth promotion and biocontrol of the isolate *Micrococcus luteus* AKAD 3-5. This species has previously been reported to be slow-growing (Environment and Climate Change Canada, 2018), although the authors who carried out the tests did not characterize the isolate as slow-growing. No direct reports of plant growth promotion and biocontrol trials with slow-growing bacteria were found. Therefore, the only direct reports found are the aforementioned *in silico* analyses conducted by our research group.

Given the above, considering the need for agricultural solutions to promote plant growth and biocontrol, as well as the indicated potential of slow-growing bacteria for these purposes, their use in sustainable agricultural practices is promising. However, even with these promising indications and the increased attractiveness of biofertilizers and biopesticides, there is still no diversification in the bacterial isolates manipulated. This obstacle is related both to the longer growth time, which means that this bacterial group continues to be neglected, and to the difficulties in mimicking its natural environment, meeting its nutritional requirements, which are often unknown, and difficulties in terms of incubation time, especially in microbial consortia (Epstein, 2013). One way of overcoming these obstacles is by

using the heterologous expression of biocontrol enzymes encoded by slow-growing bacteria.

The heterologous expression of proteins has several benefits, such as the production of the compound of interest on a large scale and in a short period of time. In addition, this approach makes it possible to overcome the difficulties related to the use of wild bacteria by dispensing with their cultivation in laboratory conditions; only the genetic information is needed to carry out heterologous expression (Kaur et al., 2018). Bioproducts based on microbial enzymes have become more attractive due to their high catalytic activity, large-scale production, and stability, as well as not having the adverse effects on the environment and human and animal health that synthetic products have (Arora et al., 2020). Biocontrol enzymes promote reactions that compromise the viability of phytopathogens and can inhibit or modify cell wall synthesis, perforate the cell membrane, or degrade the cell wall of phytopathogens (Mota et al., 2017).

Despite the importance of isolating these bacteria in order to study different aspects of their biology and expand the range of microorganisms used for PGP directly and indirectly, using an approach that does not require cultivation has its benefits. In order to contribute to sustainable efforts to promote plant growth and combat phytopathogens, this work aims to explore the potential of slow-growing bacteria in these approaches and use the heterologous expression of proteins from these bacteria for the biocontrol of phytopathogens.

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CHAPTER 1

INVESTIGATING THE PLANT GROWTH-PROMOTING POTENTIAL OF SLOW-GROWING BACTERIAL ISOLATES

Blenda de Freitas Rodrigues Jesuino¹; Luciano Nascimento de Almeida¹; Sumaya Martins Tupy¹; Maria Paula Soares de Figueiredo¹; Suelen Chaves Fernandes¹; Rodrigo da Silva Duarte¹; Caio Roberto Soares Bragança^{2*}; Mateus Ferreira Santana^{1*}. **Investigating the plant growth-promoting potential of slow-growing bacterial isolates**. In preparation.

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ABSTRACT

The growing demand for plant growth-promoting bacterias (PGPBs) due to incentives for more sustainable agriculture, in turn, encourages greater bacterial diversity to be explored. Despite the notorious bacterial diversity, the microorganisms that are cultivated in laboratory conditions represent only 1% of the diversity found in the environment. Many factors are involved in this cultivation limitation. Slow-growing bacteria are a group with high phylogenetic diversity and are only grouped together by their longer life cycle. This study aimed to investigate the possible role of 14 isolates of slow-growing bacteria with regard to their potential for application in plant growth promotion. The diversity of the slow-growing isolates was evidenced through phylogenetic analysis, which revealed that the 11 sequenced isolates are distributed in seven distinct genera; three isolates have not yet been sequenced. A wide variety of genes related to plant growth-promoting traits (PGPT) were detected in all the isolates, but some stood out. The most abundant genetic traits are related to heavy metal detoxification, osmotic stress neutralization, vitamin production, chemotactic motility, secretion systems, and siderophore production. The *in vitro* detection of PGPT, biological nitrogen fixation, solubilization of phosphate, solubilization of potassium, and production of siderophores, carried out to validate the genes predicted *in silico*, confirmed the potential of slow-growing bacteria to promote plant growth. Given the above, the bacterial group analyzed shows potential to meet the growing demand for sustainable agricultural solutions.

Keywords: Sustainable agriculture; Biotechnological potential of microorganisms; Biofertilization; Biological nitrogen fixation; Genomic analysis.

1. INTRODUCTION

In 2022, the Organization of United Nations (ONU) announced that the planet had reached eight billion people, which raised several discussions about the natural resources needed to meet this contingent, with emphasis on the topics of food and space optimization. Another point that has come into question is the need to adopt more sustainable practices, including in agriculture (ONU News, 2022; FAO, 2025). Currently, the most common way of trying to increase crop productivity is by applying chemical fertilizers. However, this practice is costly and inefficient over time, as well as leading to environmental pollution, especially of surrounding water bodies, which in turn negatively affects the vitality of crops due to its use in irrigation (Chandrasekaran & Paramasivan, 2024). Due to the harmful effects of chemical fertilizers, many of them are banned in various places, and, in Sri Lanka, their use has been banned throughout the country, which reveals the new direction of agricultural practices around the world (Drechsel et al., 2025).

A more ecological and sustainable way of dealing with the need to increase agricultural productivity is through the use of biofertilizers or bacterial inoculants. Many bacteria commonly found in the soil or in association with plants have plant growth-promoting traits (PGPT) (Glick, 2020). The main direct PGPTs are biological nitrogen fixation, phosphate, potassium, and zinc solubilization, production of siderophores, and phytohormones. There are also indirect plant growth promotion (PGP) mechanisms, such as inducing systemic resistance, increasing tolerance to abiotic stresses, modulating ethylene metabolism, and synthesizing antimicrobial compounds, which inhibit or prevent colonization by phytopathogens (Glick, 2020). Another indirect form of PGP is the degradation of pollutants in the soil by plant growth-promoting bacterias (PGPBs) (Kaldalu & Tenson, 2019). The growing demand for PGPBs due to incentives for more sustainable agriculture in turn, encourages greater bacterial diversity to be explored (Naamala & Smith, 2020).

Despite the notorious bacterial diversity, the microorganisms that are cultivated in laboratory conditions represent only 1% of the diversity found in the environment. This observed difference is called the "great plate count anomaly" (Staley & Konopka, 1985; Epstein, 2013). There are many factors involved in this limitation of cultivation, such as not knowing the nutritional requirements of the isolates and the physicochemical conditions needed. However, the demand is often

simply for more incubation time. Slow-growing bacteria are a group with high phylogenetic diversity and are only grouped together by their longer life cycle. Despite this, this bacterial group has some general characteristics; they tend to be fastidious, live in oligotrophic environments, whether terrestrial or aquatic, and are usually classified as K-strategists in ecological theories because they grow slowly and have a high biomass conversion rate (Norris et al., 2021).

There is little information on the cultivation of slow-growing bacteria and even less on the genomic information that can make this bacterial group a potential plant growth promoter. Despite the scarcity of information on slow-growing isolates, whether *in silico*, *in vitro*, or *in vivo*, it is plausible to assume that they have untapped biotechnological potential and could be highly promising for PGP. In addition, it is also possible to deduce that in certain situations, their prolonged growth time is a benefit. One example is about the degradation of pollutants in the soil and/or protection of the plants with which they are associated in these environments, because, as demonstrated by Kaldalu & Tenson (2019), the slow growth of bacteria is a decisive factor in having persistent ones growing in a medium with inhibitors and antimicrobials. Using genomic analysis to search for PGPTs can reveal many promising slow-growing isolates (Gonçalves & Santana, 2023).

In view of the above, slow-growing bacteria may have potential applications in plant growth promotion. Therefore, this study aimed to investigate the possible role of fourteen isolates of slow-growing bacteria in terms of their potential application in plant growth promotion.

2. MATERIAL AND METHODS

2.1 Isolates and cultivation conditions

The bacteria studied in this work are all those that were characterized as having slow growth by our research group in the work of Fernandes et al. (unpublished data). The isolates were given the codes E6/3, E1/2, E1/4, PARNA 23/36, PARNA 23/39, PARNA 1/3, PARNA 12/25, PARNA 23/25, PARNA 1/14, PARNA 11/4 and PARNA 19/9. In addition to these isolates that were sequenced, three others, PARNA 11/18, PARNA 19/11, and E2/6, that we have not yet been able to extract the DNA also belong to the bacterial library of the Laboratório de Genética

Molecular e de Microrganismos (LGMM) (BIOAGRO, UFV), and were characterized as slow-growing by Fernandes et al. (unpublished data).

The slow-growing bacteria were cultivated at $28 \pm 1^\circ\text{C}$ for seven days in Reasoner's 2A medium (R2A) at pH = 7, liquid or solid, and with or without agitation, depending on the objective (liquid medium and agitation to obtain greater cell mass and solid and without agitation when the objective is only to obtain a few bacterial colonies). R2A is made up of: 0.5 g/L peptone, 0.5 g/L yeast extract, 0.5 g/L acid casein digestion, 0.5 g/L glucose, 0.5 g/L soluble starch, 0.3 g/L dipotassium phosphate, 0.3 g/L magnesium sulphate, and 0.3 g/L sodium pyruvate. Each isolate was stocked in 50% glycerol and stored in freezers at -20 and -80°C (Fadanka et al., 2022).

2.2 DNA extraction and genomic sequencing

The genomic DNA of the isolates was extracted from pure cultures in the exponential growth phase using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. DNA quality and concentration were assessed by spectrophotometry using the NanoDrop™ 2000/2000c (Thermo Fisher Scientific Inc.), while integrity was checked by 0.8% agarose gel electrophoresis.

Sequencing was carried out on the MiSeq™ platform, generating short paired reads with a maximum read length of 2×300 bp (Illumina Inc.). The raw reads were assessed for quality using the FastQC software (Andrews, 2010), and the filtering and adapter removal steps were carried out using Trimmomatic (Bolger, Lohse & Usadel, 2014). The genomes were assembled using SPAdes (Bankevich et al., 2012), and the quality of the assemblies was checked using QUAST (Mikheenko et al., 2023) and CheckM v.1.2.3 (Parks et al., 2015). Functional annotation was carried out using Prokka (Seemann, 2014), and the assessment of genome quality, including estimates of completeness and contamination, was carried out using CheckM v. 1.2.3 (Parks et al., 2015). Finally, taxonomic classification was carried out using GTDB-Tk (Chaumeil et al., 2020).

2.3 Phylogenetic and Average Nucleotide Identity (ANI) analysis

To build the phylogenetic tree of the isolates, a preliminary taxonomic classification was carried out using GTDB-Tk (Chaumeil et al., 2020) to guide the selection of reference genomes. Based on this classification, a reference genome and five genomes of phylogenetically close species, with more than 95% completeness and less than 5% contamination, were retrieved from the National Center for Biotechnology Information (NCBI), when available. CheckM v1.2.3 (Parks et al., 2015) was then used using the *tree_qa* command to extract 43 marker genes encoding single-copy proteins. The concatenated alignment of the protein sequences of these universal genes was used to reconstruct the phylogenetic tree by maximum likelihood, using IQ-TREE v1.6.11 (Minh et al., 2020), with the best-fit model LG+F+I+G4. The analysis was conducted with the parameters -m TEST -bb 1000, ensuring statistical robustness and high accuracy of the topology generated. The final tree was visualized on the iTOL platform (Letunic & Bork, 2019), where visual annotations such as heatmap and correlations with additional genomic characteristics were incorporated.

In order to complement the taxonomic delimitation of the isolates, an analysis of Average Nucleotide Identity (ANI) between genomes was carried out using the FastANI v1.34 software (Jain et al., 2018). The analysis was conducted in "many-to-many" mode, with the *-matrix* option, allowing the generation of a complete similarity matrix between all pairs of genomes. The results were visualized using a heatmap built with the *pheatmap* package in the R environment.

2.4 Identification of PGPT

2.4.1 *In silico* identification

To detect the presence of PGPTs in the genomes of slow-growing isolates, the comprehensive PGPg_finder pipeline (Pellegrinetti et al., 2024) was used, which combines direct sequence annotation with *de novo* assembly methods to accurately detect the presence of PGPTs in genomes. The tool cross-references the analyzed sequences with the PlaBAs database (Patz et al., 2021), allowing the identification and quantification of the PGPTs present in the genomic sets evaluated. The PGPTs were divided into the categories of bioremediation, phytohormones, biofertilization, and competitive exclusion.

The prediction of biosynthetic gene clusters (BGC) of secondary metabolites was carried out using antiSMASH version 7.0 online (Blin et al., 2023). The genomes of the slow-growing isolates were submitted to this software to detect the BGC, and the script used filtered them in Python version 3.11. After this, the BGCs were represented using a heatmap incorporated into the phylogenetic tree.

2.4.2 *In vitro* identification

To validate the presence of the predicted PGPTs in the genome of the slow-growing isolates *in silico*, the following *in vitro* tests were carried out.

2.4.2.1 Biological nitrogen fixation

The detection of the ability to fix nitrogen was carried out according to the protocol established by Baldani et al. (2014), in which 100 μ L of the bacterial suspension was inoculated in a test tube containing 5 mL of semi-solid Nitrogen-free Bromothymol Blue (NFb) medium. The test tubes were incubated at $28 \pm 1^\circ\text{C}$ for 14 days. The NFb medium is composed of 5 g/L malic acid, 0.5 g/L potassium phosphate dibasic, 0.2 g/L magnesium sulfate heptahydrate, 0.1 g/L sodium chloride, 0.02 g/L calcium chloride dihydrate, 2 mL/L micronutrient solution, 2 mL/L of bromothymol blue solution, 4 mL/L iron ethylenediaminetetraacetic acid (FeEDTA) solution, 1 mL/L vitamin solution, 4.5 g/L potassium hydroxide, and 1,7 g/L bacteriological agar. The positive result for nitrogen fixation could be observed by the formation of a whitish film just below the surface of the culture medium and a change in the color of the medium from green to blue.

2.4.2.2 Ammonia production

Detection of the ability to produce ammonia was carried out according to an adaptation of the protocol established by Cappuccino e Sherman (1992), in which 100 μ L of the bacterial suspension was inoculated into a centrifuge tube containing 10 mL of peptone water. The tubes were incubated at $28 \pm 1^\circ\text{C}$ for seven days, and after this period, the bacterial suspension was centrifuged for 3 minutes at 10,000 rpm. Then, 5 mL of the supernatant was transferred to test tubes and 2.5 mL of Nessler's reagent was used to reveal ammonia production. The peptone water consists of 10 g/L of bacteriological peptone and 5 g/L of sodium chloride. To prepare

150 mL of Nessler reagent, 15 g of Mercury Iodide II and 10.5 g of potassium iodide were diluted in 75 mL of distilled water and 75 mL of 32% sodium hydroxide. The positive result for ammonia production was determined by the change in color to a gradient from light yellow to orange.

2.4.2.3 Phosphate solubilization

Detection of the ability to solubilize phosphate was carried out according to the protocol established by Nautiyal (1999), in which 10 μ L of the bacterial suspension was inoculated into the center of a Petri dish containing 20 mL of National Botanical Research Institute's phosphate growth medium (NBRIP). The plates were incubated at $28 \pm 1^\circ\text{C}$ for 14 days. The NBRIP medium consists of 10 g/L glucose, 5 g/L calcium phosphate, 5 g/L magnesium chloride hexahydrate, 0,25 g/L magnesium sulfate heptahydrate, 0.2 g/L potassium chloride, 0.1 g/L ammonium sulfate, and 15 g/L bacteriological agar. The positive result for phosphate solubilization was determined by the development of a translucent halo around the bacterial colonies.

2.4.2.4 Potassium solubilization

Detection of the ability to solubilize potassium was carried out according to the protocol established by Hu, Chen & Guo (2006), in which 10 μ L of the bacterial suspension was inoculated into the center of a Petri dish containing 20 mL of modified Aleksandrov medium (MAM). The plates were incubated at $28 \pm 1^\circ\text{C}$ for 14 days. The MAM medium consists of 5 g/L glucose, 0.5 g/L magnesium sulphate heptahydrate, 0.005 g/L iron III chloride, 0,1 g/L calcium carbonate, 2 g/L calcium phosphate, 2 g/L of natural potassium-containing mineral, 0.1 g/L bromothymol blue, and 15 g/L bacteriological agar. The positive result for potassium solubilization was determined by the development of a yellow halo around the bacterial colonies.

2.4.2.5 Siderophore production

Detection of the ability to produce siderophores was carried out according to the protocol established by Pérez-Miranda et al. (2017), in which 10 μ L of the bacterial suspension was inoculated into the center of a Petri dish containing 20 mL of nutrient agar medium (NA). The plates were incubated at $28 \pm 1^\circ\text{C}$ for seven days,

and after this period, 10 mL of cromazurol S (CAS) medium was poured over the Petri dishes containing the bacterial cultures grown on NA medium, and then the plates were incubated again at 28°C overnight. The NA medium consists of 5 g/L peptone, 1.5 g/L yeast extract, 1.5 g/L meat extract, 5 g/L sodium chloride, and 15 g/L bacteriological agar. The CAS medium consists of solution A, containing 90 mL of distilled water, 60.5 mg of CAS, 70.9 mg of hexadecyltrimethylammonium bromide (HDTMA), 10 mL iron III chloride at 1 mM and hydrochloric acid at 10 mM, and in solution B, containing 900 mL distilled water, 30.24 g piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) and 9 g/L bacteriological agar. The positive result for siderophore production was determined by the development of a color change around the bacterial colonies.

3. RESULTS

3.1 Phylogenetic analysis of the slow-growing isolates

The genomic analysis of the sequencing of the isolates revealed that all of them showed completeness above 97.53%, reaching 100% in the isolates PARNA 1/3 and PARNA 12/25. The highest level of contamination was only 2.78% in isolates PARNA 23/36 and PARNA 23/39, while in PARNA E6/3 it was 0.00%. The genome size varied from 3.9 Mb in isolate PARNA 1/14 to 8.07 Mb in PARNA 19/9. The number of scaffolds varied from 56 in PARNA E6/3 to 317 in PARNA 23/36. The number of contigs varied from 58 in PARNA E6/3 to 348 in PARNA 23/36. GC content ranged from 35.13% in PARNA 23/36 to 74.04% in PARNA 1/3 (Tab. 1 and Fig. 1A).

Table 1 - Genomic characteristics of the slow-growing isolates

Isolate	Completeness	Contamination	Genome size	Scaffolds	Contigs	GC%	Predicted genes
E6/3	99.81	0.00	4.019819	56	58	46.42	3902
PARNA 23/36	97.53	2.78	5.958984	317	348	35.13	6108
PARNA 23/39	97.53	2.78	5.961133	273	304	35.14	6077
E1/2	99.73	1.40	7.003171	259	263	47.18	6903
PARNA 1/3	100.00	1.25	4.641309	95	104	74.04	4047
PARNA 12/25	100.00	1.62	4.775111	65	66	62.05	4361
PARNA 23/25	99.76	0.27	5.382108	144	147	67.93	4887
PARNA 1/14	98.76	0.25	3.924081	128	191	69.94	3824
E1/4	99.85	0.37	7.056943	173	176	46.36	6296
PARNA 11/4	99.68	0.84	8.065725	255	259	53.11	7303
PARNA 19/9	99.68	0.84	8.071847	268	281	53.12	7309

PARNA 12/25, PARNA 1/14 also obtained a low ANI value of 91.67%, which suggests that these two species are probably new. Only one other isolate, PARNA 1/3, had an ANI slightly below 95%, but all the others were above the reliability range (Fig. 1B).

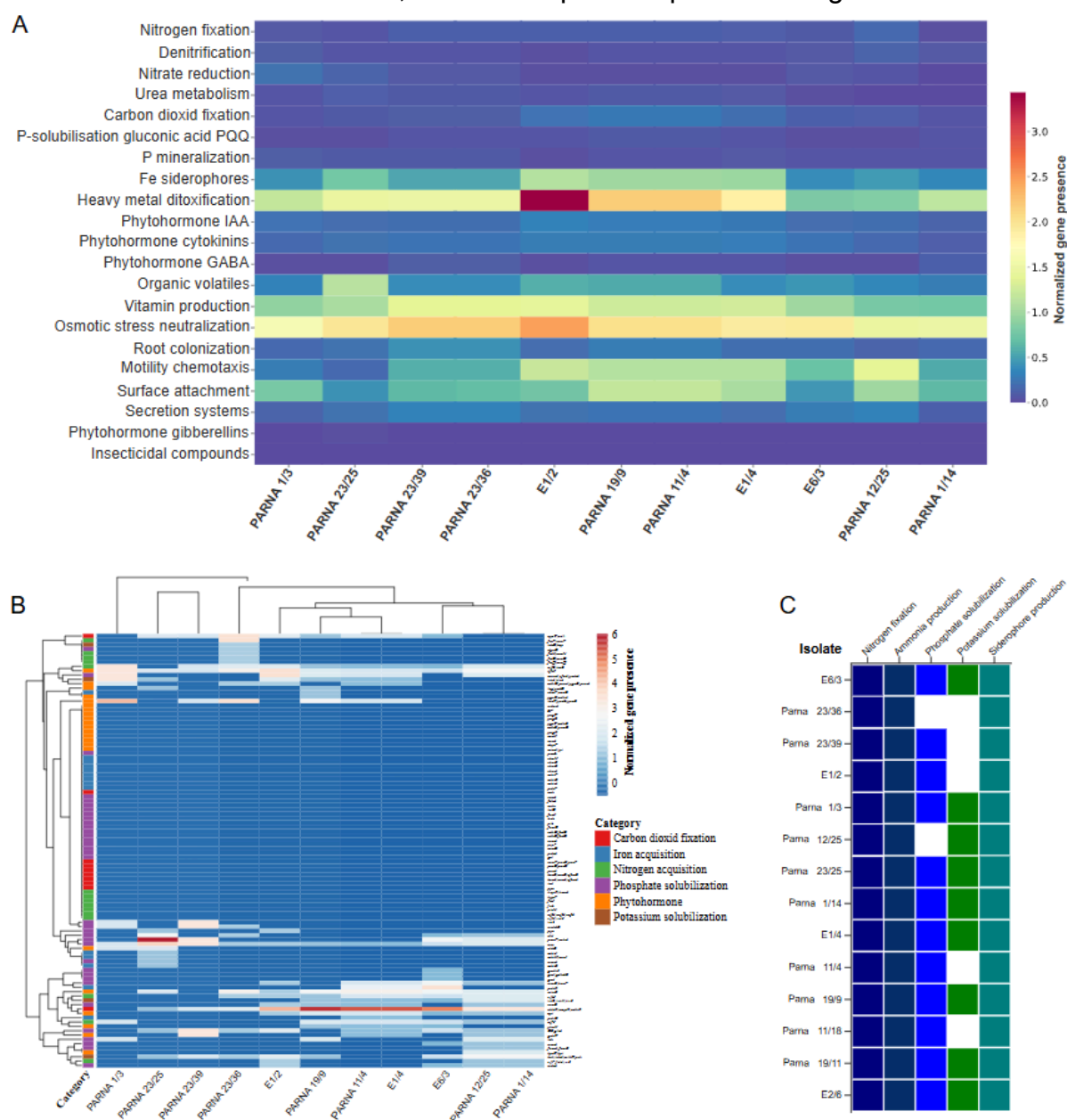
The diversity of the slow-growing isolates was evidenced through phylogenetic analysis, which revealed that the 11 sequenced isolates are distributed in seven distinct genera, namely *Bacillus* spp., *Brevibacillus* spp., *Cellulosimicrobium* spp., *Dyella* spp., *Gordonia* spp., *Leifsonia* spp., and *Paenibacillus* spp. There are three isolates from the *Bacillus* spp. genus, of which one is probably *B. velezensis* and two are *B. paramycoides*. *Paenibacillus* spp. has three isolates, of which one is *P. pabuli* E1/4 and two are *P. elgii*. The 11 isolates correspond to *Bacillus velezensis* E6/3, *Bacillus paramycoides* PARNA 23/36, *Bacillus paramycoides* PARNA 23/39, *Brevibacillus reuszeri* E1/2, *Cellulosimicrobium cellulans* PARNA 1/3, *Dyella* spp. PARNA 12/25, *Gordonia hongkongensis* PARNA 23/25, *Leifsonia* spp. PARNA 1/14, *Paenibacillus pabuli* E1/4, *Paenibacillus elgii* PARNA 11/4 and *Paenibacillus elgii* PARNA 19/9. The phylogenetic analysis had high support, with bootstrap values close to 100 in most branches (Fig. 1).

3.2 Detection of PGPTs

The content of PGPTs-related genes was divided into four categories: bioremediation, phytohormones, biofertilization, and competitive exclusion (Fig. 1A). The slow-growing bacteria with the most genes related to bioremediation were *G. hongkongensis* PARNA 23/25 and *B. reuszeri* E1/2, with 149 and 137 genes, respectively. The isolate *B. reuszeri* E1/2 also stood out in the phytohormones category, as did *P. pabuli* E1/4, with 219 and 211 genes, respectively. The isolates with the greatest potential for biofertilization were *P. elgii* PARNA 11/4 and *P. elgii* PARNA 19/9, both with 296; *B. reuszeri* E1/2 also had relevant results in this category, with 293 genes. In competitive exclusion, the highest number of genes, 384, was detected in the genomes of *C. cellulans* PARNA 1/3 and *P. elgii* PARNA 19/9. In general, *B. reuszeri* E1/2 had the highest abundance of PGPTs genes in 3 categories, *P. elgii* PARNA 11/4 and *P. elgii* PARNA 19/9 in 2, and *G. hongkongensis* PARNA 23/25 had the highest potential for bioremediation (Fig. 1A). In addition, the

analysis of BGCs in the genomes revealed that the highest abundance, 27, was found in the isolates *P. elgii* PARNA 11/4 and *P. elgii* PARNA 19/9 (Fig. 1A).

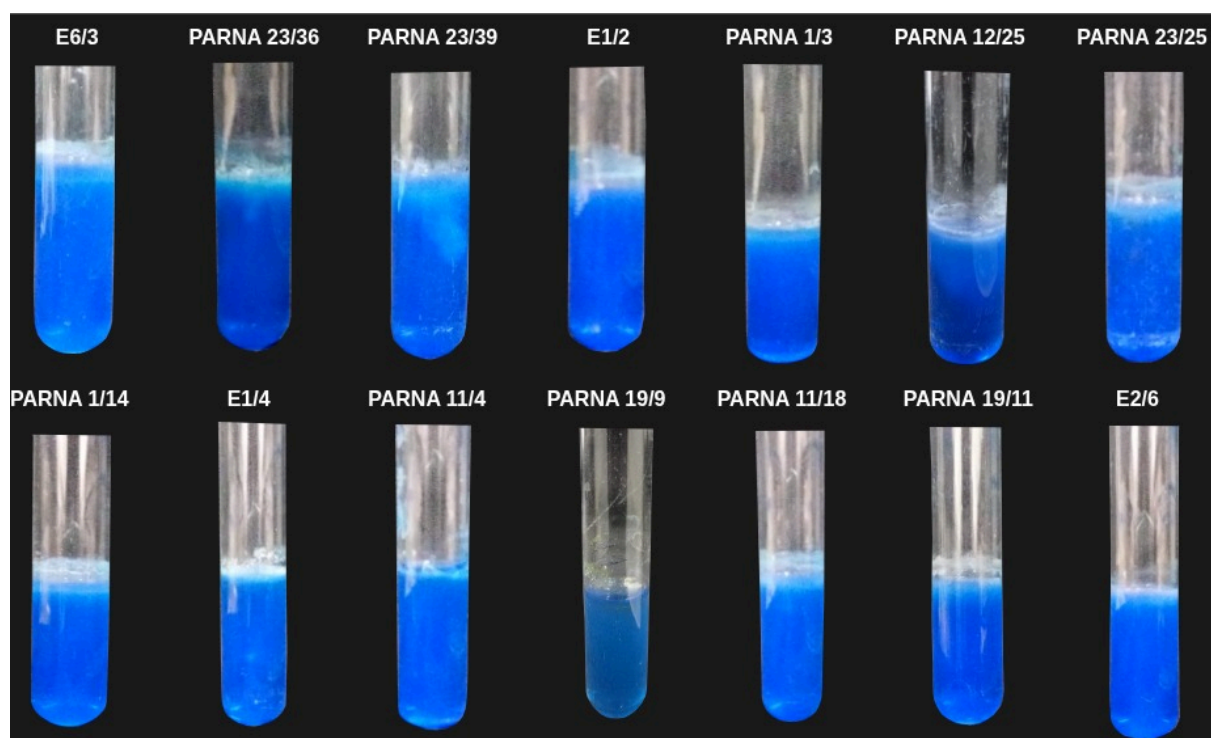
Fig. 2. The abundances of plant growth-promoting traits (PGPTs) classes (A) and genes (B) across the strain's genome. The heatmap represents the distribution of genes directly associated with plant growth-promoting. Gene presence was normalized, and rows and columns were grouped using correlation distance and average linkage methods. Only the genes most directly involved in PGPTs are represented. **C)** *In vitro* PGPTs assays (nitrogen fixation, ammonia production, phosphate solubilization, potassium solubilization, and siderophores production) in which each test received a color, and blank spaces represent a negative result.



The most abundant PGPTs detected *in silico* are related to heavy metal detoxification, osmotic stress neutralization, vitamin production, chemotactic motility, secretion systems, and siderophore production. The isolate with the highest abundance of PGPTs was *B. reuszeri* E1/2, followed by *P. elgii* PARNA 11/4 and *P. elgii* PARNA 19/9 (Fig. 2A). In agreement with plant growth-promoting traits, many genes detected in abundance were related to them (Fig. 2B).

The *in vitro* detection of PGPT, carried out to validate the genes predicted *in silico*, confirmed the ability of slow-growing bacteria to promote plant growth. These assays were carried out both with the 11 sequenced isolates and with the other three isolates, which were classified as slow-growing but whose genomes have not yet been sequenced. All PGPTs assays are summarized for each isolate in Fig. 2C. Of the 14 isolates, all proved to be effective at fixing nitrogen (Fig. 3) and were able to produce ammonia (Fig. 4), 12 solubilized phosphate (Fig. 5), nine solubilized potassium (Fig. 6), and all produced siderophores (Fig. 7). The isolates *Bacillus velezensis* E6/3, *P. elgii* PARNA 19/9, and the non-sequenced isolates 19/11 and E2/6 had positive results in all the PGPTs assays carried out *in vitro* (Fig. 2C, 3, 4, 5,6 and 7).

Fig. 3. Biological capacity of slow-growing isolates to fix atmospheric nitrogen. The test was carried out using NFb medium.



4. DISCUSSION

The isolation carried out on soil samples by our research group in the work by Fernandes et al. (unpublished data) classified the 14 bacterial isolates discussed in this work as slow-growing. Of the 14 isolates, 11 are sequenced and have been phylogenetically analyzed, while the other three, despite being of unknown phylogeny, showed potential for PGP in the tests carried out *in vitro*. The phylogenetic analysis carried out revealed great diversity among the slow-growing bacteria, with 7 different genera detected (Fig. 1).

Of the isolates discussed, only *B. velezensis* has a considerable number of representatives deposited in the NCBI (1,408). Some have just over a hundred deposits, such as *Dyella* spp. and *Leifsonia* spp. (106 and 125, respectively). However, most have few deposits, such as *B. paramycoides*, *B. reuszeri*, *C. cellulans*, *G. hongkongensis*, *P. pabuli*, and *P. elgii* (33, 8, 36, 8, 9, and 11, respectively). Despite the number of deposits for *Dyella* spp. and *Leifsonia* spp., a flexible phylogenetic analysis had to be carried out as they did not align with the genomes first selected, so it was necessary to download all the *Dyella* spp. and *Leifsonia* spp. deposits to carry out a phylogeny within the genus and identify the closest isolates. However, only one isolate aligned with *Dyella* spp. PARNA 12/25 and two isolates aligned with *Leifsonia* spp. PARNA 1/14, which were selected for the analysis carried out in this study (Fig. 1).

This isolation possibly resulted in two new species of slow-growing bacteria, *Dyella* spp. PARNA 12/25, with an ANI of 85.60%, and *Leifsonia* spp. PARNA 1/14, with an ANI of 91.67% (Fig. 1B). In addition to the low ANI value, in the phylogeny carried out with all the representatives of the genera deposited to identify the closest ones, both *Dyella* spp. PARNA 12/25 and *Leifsonia* spp. PARNA 1/14 appeared on segregated branches. *Dyella* spp. PARNA 12/25 aligned only with *Dyella silvae* RLT163 and *Leifsonia* spp. PARNA 1/14 aligned only with *Leifsonia* sp. Leaf336 and *Leifsonia* sp. McL0607 (data not shown).

The detection of PGPTs in the slow-growing isolates explored in this work was carried out both through genomic analysis, which sought to identify the genes involved in plant growth promotion, and through qualitative *in vitro* tests to validate the genes prospected. The PGPTs were divided into different categories to analyze the abundance of genes present in each. The isolates with the most genes related to

bioremediation were *G. hongkongensis* PARNA 23/25 and *B. reuszeri* E1/2 (Fig. 2). Despite having few deposits of *G. hongkongensis*, this species has never been related to bioremediation before, nor to PGP more generally. The species *B. reuszeri* is better known and has been related to PGPTs before. Kamli et al. (2022) carried out the first exhaustive genomic analysis of *Brevibacillus* sp. and identified *B. reuszeri* J31TS6 as promising for polyurethane degradation. The slow-growing isolate *B. reuszeri* E1/2 also stood out in the phytohormone category, as did *P. pabuli* E1/4 (Fig. 2). Yildirim et al. (2011) inoculated an isolate of *Brevibacillus reuszeri* with manure in broccoli crops and detected a 24.3% increase in yield and a 13.7% increase in chlorophyll content, as well as an increase in the absorption of macro- and micronutrients. The species *P. pabuli* has already been associated with the production of phytohormones, but the authors concluded that the production was too low or a false positive occurred due to the sensitivity of the instrument, ruling out the species as promising in this category (Handy et al., 2021).

The isolates with the greatest potential for biofertilization were *P. elgii* PARNA 11/4 and *P. elgii* PARNA 19/9 (Fig. 2). No direct associations were found between this species and the classic biofertilization mechanisms, such as nitrogen fixation and phosphate solubilization. There have been reports of this in the genus, but for the *P. elgii* species, this work is probably the first direct correlation.

Interestingly, the genomic analyses detected a greater abundance of PGPT-related genes in the isolates *B. reuszeri* E1/2, *P. elgii* PARNA 11/4, and *P. elgii* PARNA 19/9 (Fig. 2). However, in the nitrogen fixation, ammonia production, phosphate solubilization, potassium solubilization, and siderophore production tests carried out *in vitro*, the isolates that stood out were, in addition to isolated *P. elgii* PARNA 19/9, *B. velezensis* E6/3, *C. cellulans* PARNA 1/3, *Dyella* spp. PARNA 12/25, *Leifsonia* spp. PARNA 1/14, *P. pabuli* E1/4, and the non-sequenced isolates 19/11 and E2/6 (Fig. 2C). However, the tests carried out were not directly targeted by the database used. Even so, noting that two of the three slow-growing isolates that are not sequenced stood out in the *in vitro* tests raises curiosity about what their genomes might reveal through *in silico* analysis.

In addition to the aforementioned forms of PGP, which use more direct mechanisms, there are other strategies adopted by bacteria to promote plant growth indirectly, such as biocontrol. There are many ways in which bacteria are able to

carry out biological control, and, although not the focus of this work, exclusive competition and BGCs whose secondary metabolites can have major inhibitory effects against phytopathogens have been identified. Siedler et al. (2020) demonstrated that competitive exclusion between cellulose-degrading bacteria shapes the composition of the microbial community in agricultural environments, preventing other microorganisms from establishing themselves. Almeida et al. (unpublished data), through an in-depth genomic analysis, detected the main mechanisms involved in the biological control capacity in the *Paenibacillus* spp. genus, among which secondary metabolites stood out; therefore, it was no surprise to find that the isolates *P. elgii* PARNA 11/4 and *P. elgii* PARNA 19/9 have a higher abundance of BGCs and genes related to exclusive competition mechanisms (Fig. 1A). In addition to the benefits of their own BGCs for plants, some isolates can stimulate the BGCs of plants with which they are associated. Trinh et al. (2018) found that *Arabidopsis thaliana* seedlings treated with *P. elgii* PP7S showed increased expression of key genes regulating the anthocyanin and flavonoid biosynthesis pathways, as well as having activated induction of systemic resistance in response to the presence of pathogens.

Fig. 4. Ability of slow-growing isolates to produce ammonia. The test was revealed through Nessler's reagent.



Fig. 5. Ability of slow-growing isolates to solubilize phosphate. The test was performed using NBRIP medium.

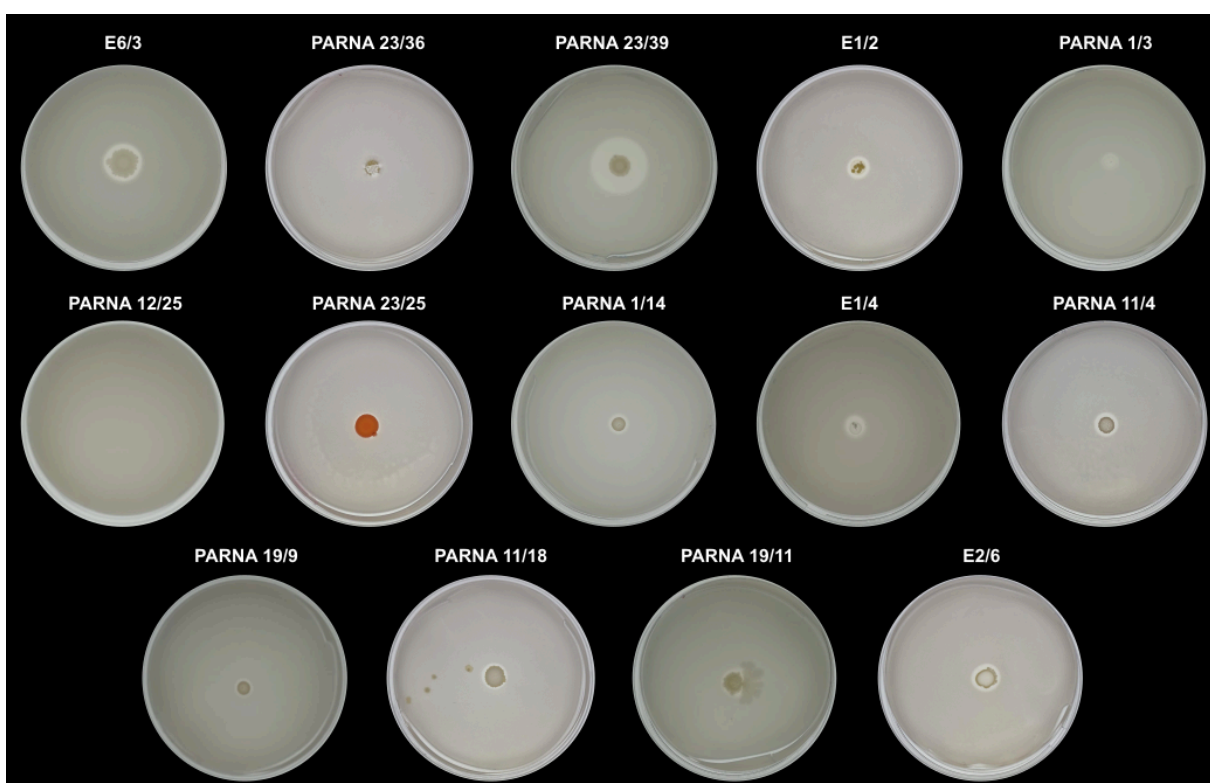


Fig. 6. Ability of slow-growing isolates to solubilize potassium. The test was performed using MAM medium.

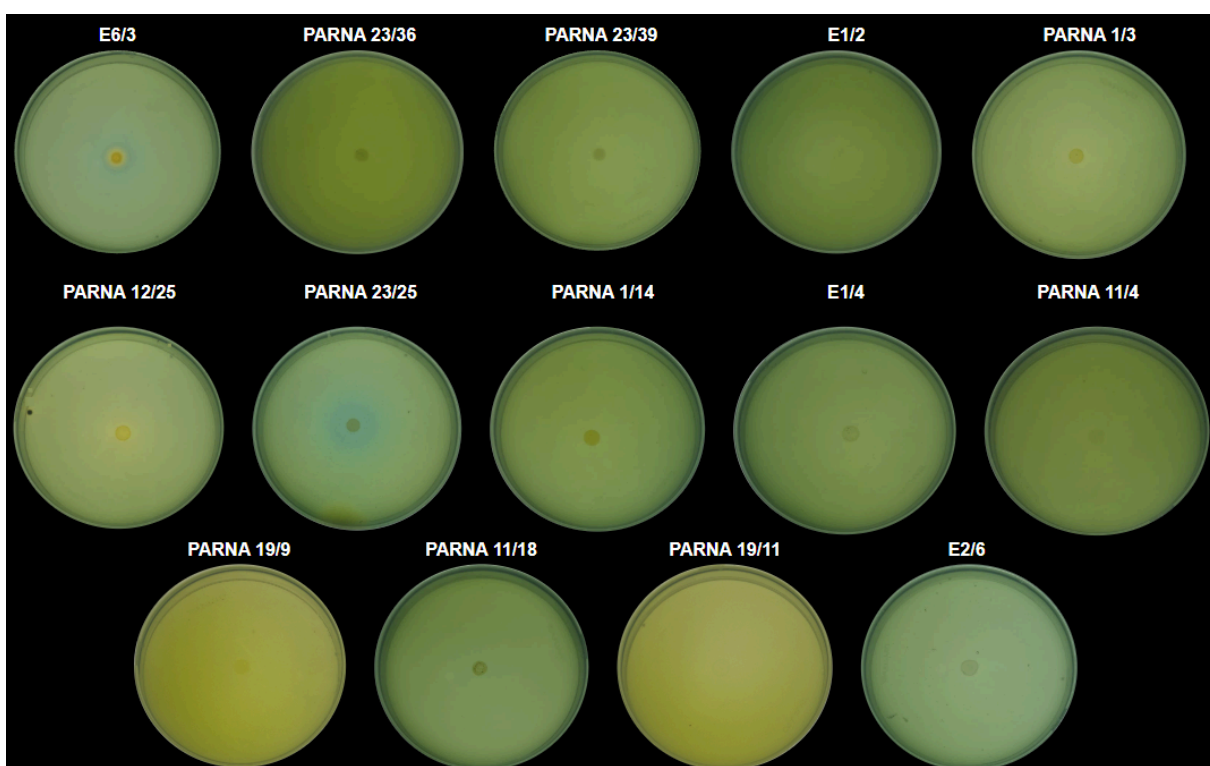
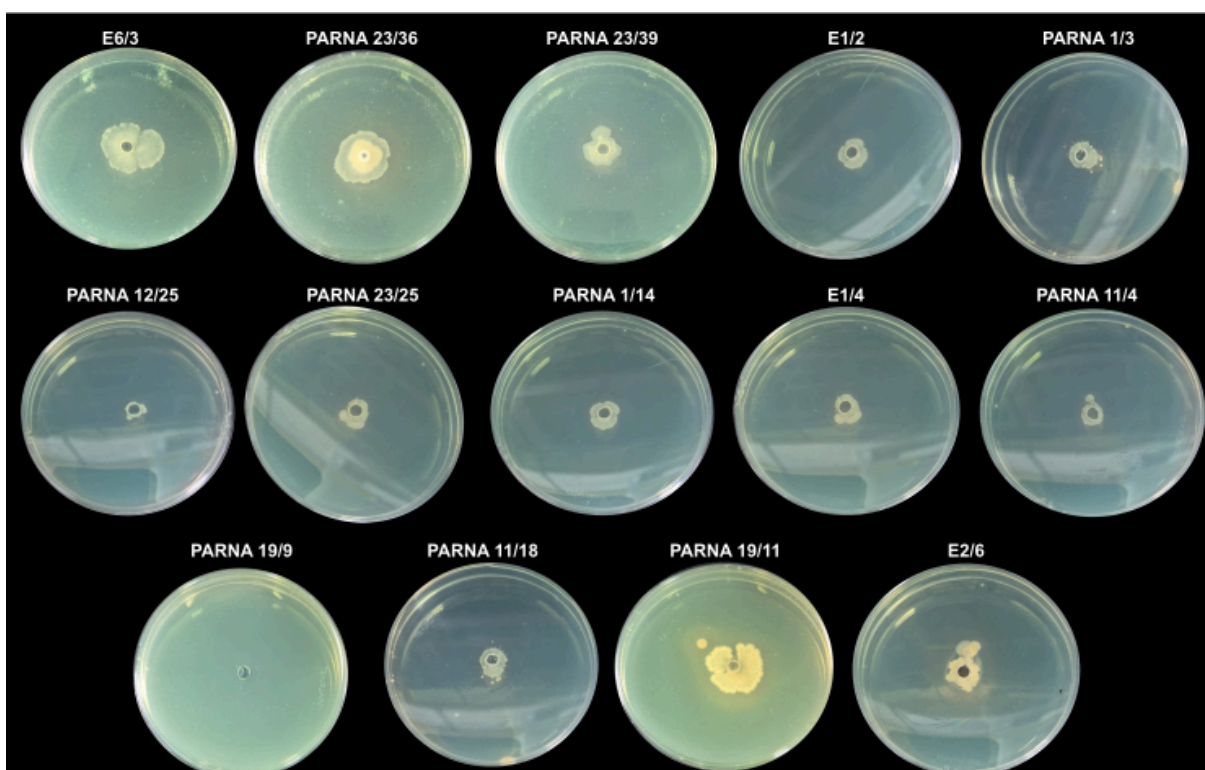


Fig. 7. Ability of slow-growing isolates to produce siderophores. The test was revealed through CAS medium.



5. CONCLUSION

The analyses carried out, both *in silico* and *in vitro*, revealed that slow-growing bacteria have a high capacity to promote plant growth, with satisfactory results in bioremediation, phytohormone production, biofertilization, mineral solubilization, and the production of compounds beneficial to plants. Given the above, the bacterial group analyzed shows potential to meet the growing demand for sustainable agricultural solutions.

Compliance with ethical standards

This study does not contain any experiment with human participants or animals performed by any of the authors.

Conflict of Interest

The authors have no conflicts of interest to disclose.

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CHAPTER 2

INVESTIGATING THE BIOCONTROL POTENTIAL OF SLOW-GROWING BACTERIAL ISOLATES

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ABSTRACT

Every year, between 20 and 40% of food production is lost due to a wide range of agricultural pests and diseases. This loss in food production represents around 220 billion dollars annually. Although synthetic pesticides are the most widely used method, there is a growing demand for phytopathogen control strategies that are more sustainable. Despite the good results in the use of microorganisms for biocontrol, few bacteria are known and used in this approach. Among the broad range of bacterial groups analyzed, slow-growing bacteria have attracted attention due to their potential to contribute to the biological control of crops. This study aimed to investigate the possible role of fourteen isolates of slow-growing bacteria in their potential application as biocontrol. Several genes encoding biocontrol enzymes were detected in the annotated genome of the slow-growing isolates. *In vitro* substrate degradation tests confirmed their enzymatic activity. Biosynthetic gene clusters (BGC) prospecting detected 13 distinct BGC classes and a frequency of 57 clusters, as well as seven classified as "other". In the paired culture tests between the slow-growing isolates and phytopathogenic fungi, many bacterial isolates showed antagonism to *Fusarium equiseti* CCF422, *Fusarium oxysporum* CCF184, *Rhizoctonia solani* 2930, and *Sclerotinia sclerotiorum* 15B, reaching 86,70% inhibition. In addition, a total of 168 defense genes related to 30 defense systems, 12 types of resistance genes, and 3 isolates with virulence genes were detected. The species *Paenibacillus elgii* was identified as having great potential for biocontrol for the first time. Given the above, it is concluded that slow-growing bacteria can contribute to meeting the growing demand for biopesticides that minimize the use of synthetic pesticides.

Keywords: Phytopathogens; Biopesticides; Biocontrol enzymes; Biosynthetic gene clusters (BGC); Microbial antagonism.

1. INTRODUCTION

Every year, between 20% and 40% of food production is lost due to a wide range of agricultural pests and diseases. This loss in food production represents around 220 billion dollars annually (FAO, 2019; Agricultural Research Service, 2022; FAO, 2025). Of these annual losses, 70 to 80% are due to the action of phytopathogenic fungi (Moore, Robson & Trinci, 2020; APBB, 2023) and 10% to 20% occur after harvest (Agricultural Research Service, 2022; Phys, 2025; MPG, 2025). Diseases caused by *Fusarium* spp. affect several cultivars and can compromise a significant portion of production. Diseases caused by fungi of this genus can compromise up to 80% of pepper production globally, for example (Ham et al., 2021). *Rhizoctonia solani* is another fungus with a wide host range and causes average losses of 30% to 60% of sugar beet production, for example, although there are also records of entire crop losses (Misra, Mall & Singh, 2023). *Sclerotinia sclerotiorum* is a highly relevant plant pathogen worldwide, particularly in legumes. In China, for example, it causes losses of up to 80% of cultivars (Duo, Yin & Wang, 2025).

Although synthetic pesticides are the most widely used method for controlling phytopathogens due to their rapid action and broad spectrum, their use involves high costs and can pose risks to the environment, human health, and other non-target organisms (Tudi et al., 2021; Drechsel et al., 2025). In this sense, there is a growing demand for more sustainable phytopathogen control strategies. One promising alternative is the use of microorganisms, which can inhibit phytopathogens and agricultural pests without causing environmental pollution or damage to human health (Soni, Rawal & Keharia, 2021).

Despite the good results in the use of microorganisms for biocontrol, few bacteria are known and used in this approach, which is due to the difficulties of cultivation, interactions established with other organisms, and growth time (Kato et al., 2018). On the other hand, some phylogenetically diverse and little-studied bacterial groups, called slow-growing bacteria, have been attracting attention due to their great potential to contribute to the biological control of crops (Gonçalves & Santana, 2023). Many slow-growing bacteria encode various lytic enzymes involved in biocontrol by degrading the cell wall of phytopathogens, as well as secondary metabolites, volatile compounds, and inhibitory non-volatile compounds, thus possessing great potential for biological control. Despite the scant literature on the

genomic information that could make this bacterial group a potential biocontrol agent, there are some indications of this capacity. This is the case with the presence of biosynthetic gene clusters (BGC) in the genomes of these bacteria, many of which are essential for their antagonistic capacity against phytopathogens (Nifakos et al., 2021).

Given the above, slow-growing bacteria have untapped potential for biological control of phytopathogens. This study aimed to investigate the possible role of fourteen isolates of slow-growing bacteria in their potential application as biocontrol.

2. MATERIALS AND METHODS

2.1 Slow-growing bacterial isolates and cultivation conditions

The bacteria studied in this work are all those that were characterized as having slow growth by our research group in the work of Fernandes et al. (unpublished data). The phylogenetic description of these bacteria was previously carried out in the work by Jesuino et al. (unpublished data). The isolates were *Bacillus velezensis* E6/3, *Bacillus paramycoides* PARNA 23/36, *Bacillus paramycoides* PARNA 23/39, *Brevibacillus reuszeri* E1/2, *Cellulosimicrobium cellulans* PARNA 1/3, *Dyella* spp. PARNA 12/25, *Gordonia hongkongensis* PARNA 23/25, *Leifsonia* spp. PARNA 1/14, *Paenibacillus pabuli* E1/4, *Paenibacillus elgii* PARNA 11/4 and *Paenibacillus elgii* PARNA 19/9. In addition to these isolates that were sequenced, three others, PARNA 11/18, PARNA 19/11, and E2/6, that we have not yet been able to extract the DNA from, were also characterized as slow-growing by Fernandes et al. (unpublished data) and used in the *in vitro* assays.

The slow-growing bacteria were grown at $28 \pm 1^\circ\text{C}$ for seven days in Reasoner's 2A medium (R2A) at pH = 7, liquid or solid, and with or without agitation, depending on the objective (liquid medium and agitation to obtain greater cell mass and solid and without agitation when the objective is only to obtain a few bacterial colonies). R2A is made up of: 0.5 g/L peptone, 0.5 g/L yeast extract, 0.5 g/L acid casein digestion, 0.5 g/L glucose, 0.5 g/L soluble starch, 0.3 g/L dipotassium phosphate, 0.3 g/L magnesium sulphate, and 0.3 g/L sodium pyruvate. The stock of each isolate was made in 50% glycerol and stored in freezers at -20°C and -80°C (Fadanka et al., 2022).

2.2 Phytopathogenic fungi and cultivation conditions

Of the fungi tested in this work, *Fusarium equiseti* CCF422 and *Fusarium oxysporum* CCF184 were kindly provided by the Laboratório de Micologia e Etiologia de Doenças Fúngicas de Plantas (Departamento de Fitopatologia, UFV), while *Rhizoctonia solani* 2930 is part of the collection of the Laboratório de Patologia Florestal (BIOAGRO, UFV), and *Sclerotinia sclerotiorum* 15B belongs to the collection of the Laboratório de Genética Molecular e de Microrganismos (LGMM) (BIOAGRO, UFV). The fungi were stocked according to Castellani's method (1963), while they were cultivated in Potato Dextrose Agar (PDA, Kasvi®), and the incubation temperature was $25 \pm 1^\circ\text{C}$.

2.3 Prospecting for biocontrol enzymes and BGC

2.3.1 *In silico* detection

In silico detection of the enzymes involved in biocontrol in the annotated genome of the 11 sequenced isolates was carried out manually by searching for keywords in the genomic annotation file. The enzyme names and variations of the enzyme group names were: chitinase, glucanase, N-acetylmuramyl-L-alanine amidase, protease, lipase, gelatinase, collagenase, lysozyme, glycosidase, galactosidase, and mannanase. In addition, the identification of biosynthetic gene clusters (BGC) was carried out using the antiSMASH software version 7.0 (Blin et al., 2023). The BGCs were represented by a heatmap incorporated into the phylogenetic tree. In addition, additional information provided by the software was collected.

2.3.2 *In vitro* detection

2.3.2.1 Chitinase

Detection of the ability to degrade chitin was carried out according to the protocol established by Skujins et al. (1965), in which 10 μL of the bacterial suspension was inoculated into the center of a Petri dish containing 20 mL of colloidal chitin medium. The plates were incubated at $28 \pm 1^\circ\text{C}$ for seven days. The colloidal chitin medium is composed of 1.0 g/L of ammonium sulphate, 0.2 g/L of monobasic potassium phosphate, 1.6 g/L of dibasic potassium phosphate, 0.2 g/L magnesium sulphate, 0.1 g/L sodium chloride, 0.01 g/L iron II sulphate heptahydrate, 0.02 g/L calcium chloride dihydrate, 1% (w/v) colloidal chitin, and 15 g/L

bacteriological agar. The positive result for the enzymatic activity of chitinase was established by the degradation halo in the culture medium, whose only carbon source was chitin.

2.3.2.2 Glucanase

The ability to degrade glucan was detected by inoculating 10 μ L of the bacterial suspension in the center of a Petri dish containing 20 mL of minimal medium developed in this work, which is composed of 10 g/L of oat flour, 1.0 g/L monobasic potassium phosphate, 0.5 g/L magnesium sulfate heptahydrate, 5.0 g/L sodium chloride, and 15 g/L bacteriological agar. The plates were incubated at $28 \pm 1^\circ\text{C}$ for seven days. The positive result for the glucanase enzyme activity was established by the degradation halo in the culture medium, whose carbon source is the beta-glucan in the oat cell wall.

2.3.2.3 N-acetylmuramyl-L-alanine amidase

The ability to degrade N-Acetyl-D-Glucosamine was detected by inoculating 10 μ L of the bacterial suspension in the center of a Petri dish containing 20 mL of minimal medium developed in this work, which is composed of 10 g/L of N-Acetyl-D-Glucosamine, 1.0 g/L potassium phosphate monobasic, 0.5 g/L magnesium sulfate heptahydrate, 5.0 g/L sodium chloride, and 15 g/L bacteriological agar. The plates were incubated at $28 \pm 1^\circ\text{C}$ for seven days. The positive result for enzymatic activity was established by the degradation halo in the culture medium, whose only carbon source was N-Acetyl-D-Glucosamine.

2.3.2.4 Protease

The ability to degrade the protein of skim milk was detected by inoculating 10 μ L of the bacterial suspension in the center of a Petri dish containing 20 mL of minimal medium developed in this work, which is composed of 10 g/L of skim milk, 1.0 g/L potassium phosphate monobasic, 0.5 g/L magnesium sulfate heptahydrate, 5.0 g/L sodium chloride, and 15 g/L bacteriological agar. The plates were incubated at $28 \pm 1^\circ\text{C}$ for seven days. The positive result for enzymatic activity was established by the degradation halo in the culture medium, whose only carbon source was skim milk.

2.3.2.5 Lipase and/or esterases

The ability to degrade lipids was detected by inoculating 10 µL of the bacterial suspension in the center of a Petri dish containing 20 mL of minimal medium developed in this work, which is composed of 10 mL/L of olive oil, 1.0 g/L potassium phosphate monobasic, 0.5 g/L magnesium sulfate heptahydrate, 5.0 g/L sodium chloride, and 15 g/L bacteriological agar. The plates were incubated at $28 \pm 1^\circ\text{C}$ for seven days. The positive result for enzymatic activity was established by the degradation halo in the culture medium, whose only carbon source was olive oil.

2.3.2.6 Gelatinase

The ability to degrade gelatin was detected by inoculating 10 µL of the bacterial suspension in the center of a Petri dish containing 20 mL of minimal medium developed in this work, which is composed of 10 g/L gelatin, 1.0 g/L potassium phosphate monobasic, 0.5 g/L magnesium sulfate heptahydrate, 5.0 g/L sodium chloride, and 15 g/L bacteriological agar. The plates were incubated at $28 \pm 1^\circ\text{C}$ for seven days. The positive result for enzymatic activity was established by the degradation halo in the culture medium, whose only carbon source was gelatin.

2.5 Biocontrol of phytopathogenic fungi

The detection of the ability of slow-growing bacterial isolates to inhibit or prevent the development of phytopathogenic fungi through paired culture was carried out according to an adaptation of the protocol established by Slama (2019), in which a pure bacterial suspension was streaked 1 cm away from the left side of a 9 cm diameter Petri dish containing PDA and then a 6 mm disk of fungal mycelium was placed on the right side of the Petri dish. The plates were incubated at $25 \pm 1^\circ\text{C}$ until the mycelium of the fungal control, inoculated without the streak of the bacterial suspension, reached the point at which the streak was made. Only the isolate *C. cellulans* PARNA 1/3 required adaptation, as it was unable to grow on PDA medium. Therefore, the test was conducted on R2A, and another fungal control was carried out on this medium. Inhibition of mycelial growth was calculated using the formula:

$$PI = \frac{C-T}{C} \times 100$$

where C and T represent the diameters of the fungal colonies on the control and paired culture plates, respectively.

2.8 Defense systems, resistance genes, and virulence factors

The presence of defense systems in the genome of the slow-growing isolates was detected using DefenseFinder (Tesson et al., 2024). Antibiotic resistance genes were searched for by combining the gene sequences with the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2023), ResFinder (Zankari et al., 2012), MEGARes (Lakin et al., 2017), and ARG-ANNOT (Gupta et al., 2014) databases. Virulence factors were detected using the Virulence Factor Database (VFDB) (Chen et al., 2016), which, like the databases used to detect the presence of antibiotic resistance genes, is integrated with Anti-Biotic Resistance (ABRicate) (Seemann, 2018).

3. RESULTS

3.1 Prospecting for biocontrol enzymes

In silico enzyme prospecting was carried out with the 11 isolates that were phylogenetically analyzed in a previous work, which investigated their associations with plant growth-promoting traits (PGPT) (Jesuino et al.) (unpublished data). Of the 11 enzymes prospected, variations of chitinase and protease were detected in all genomes. Lipase, galactosidase, and amidase were also detected in an almost homogeneous way. *B. velezensis* E6/3, *B. paramycoides* PARNA 23/36, *B. paramycoides* PARNA 23/39, *B. reuszeri* E1/2, *G. hongkongensis* PARNA 23/25, and *Leifsonia* spp. PARNA 1/14 presented at least six of the 11 biocontrol enzymes prospected. The other enzymes were not as abundant in the isolates' genomes. However, some enzymes appeared in great diversity, as was the case with the eight chitinase variations in the genome of *C. cellulans* PARNA 1/3 (Fig. 1A).

In addition to these 11 isolates, *in vitro* prospecting included three others that have not yet been sequenced (PARNA 11/18, PARNA 19/11, and E2/6). The semi-quantitative assay carried out to detect substrate degradation by chitinase, glucanase, amidase, lipase, and/or esterase and gelatinase had a high correlation with what was expected from the enzymatic prospecting carried out *in silico* (Fig. 1B). Most of the isolates showed enzymatic activity. More specifically, of the 14 isolates,

only three did not degrade chitin (Fig. 2), in four isolates, no glucanase and amidase activity was detected (Fig. 3 and 4, respectively), all isolates demonstrated activity of protease (Fig. 5), only one did not form a halo with lipase and/or esterases (Fig. 6), and two did not degrade gelatin (Fig. 7). All the other slow-growing bacteria showed enzymatic activity on these substrates (Fig. 1B).

Fig. 1. Enzyme prospecting *in silico* (A) and *in vitro* (B). The colors represent the enzymes in the genome of the isolates, and the size of the square represents the abundance of the same enzyme. Semi-qualitative *in silico* prospecting was performed with the 11 sequenced isolates, while *in vitro* prospecting included, in addition to them, the three isolates not yet sequenced. Assays were performed with culture media in which the only carbon source is the enzyme substrate.

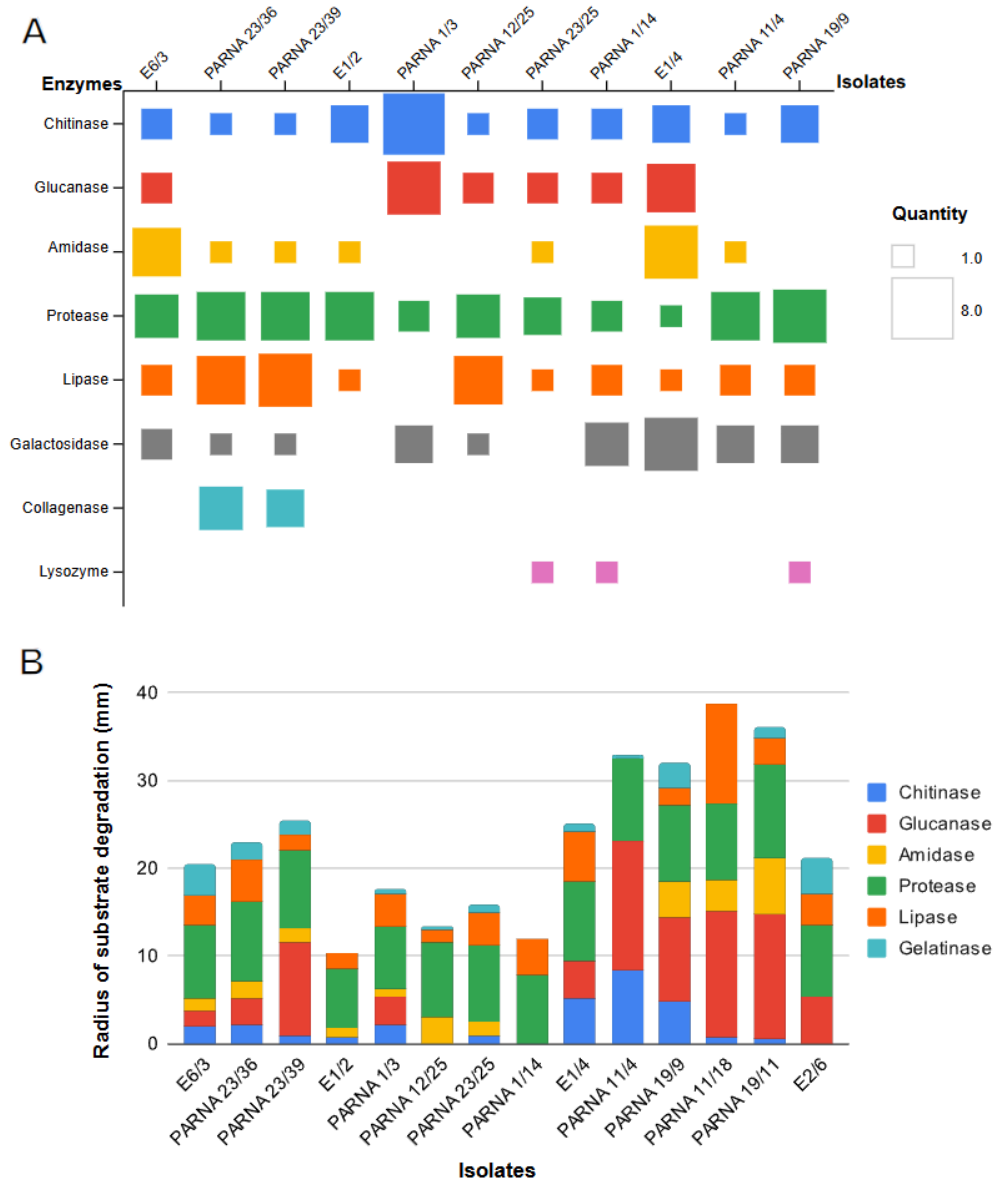


Fig. 2 - Ability of slow-growing isolates to degrade chitin. The test was performed using a culture medium in which chitin was the only carbon source.

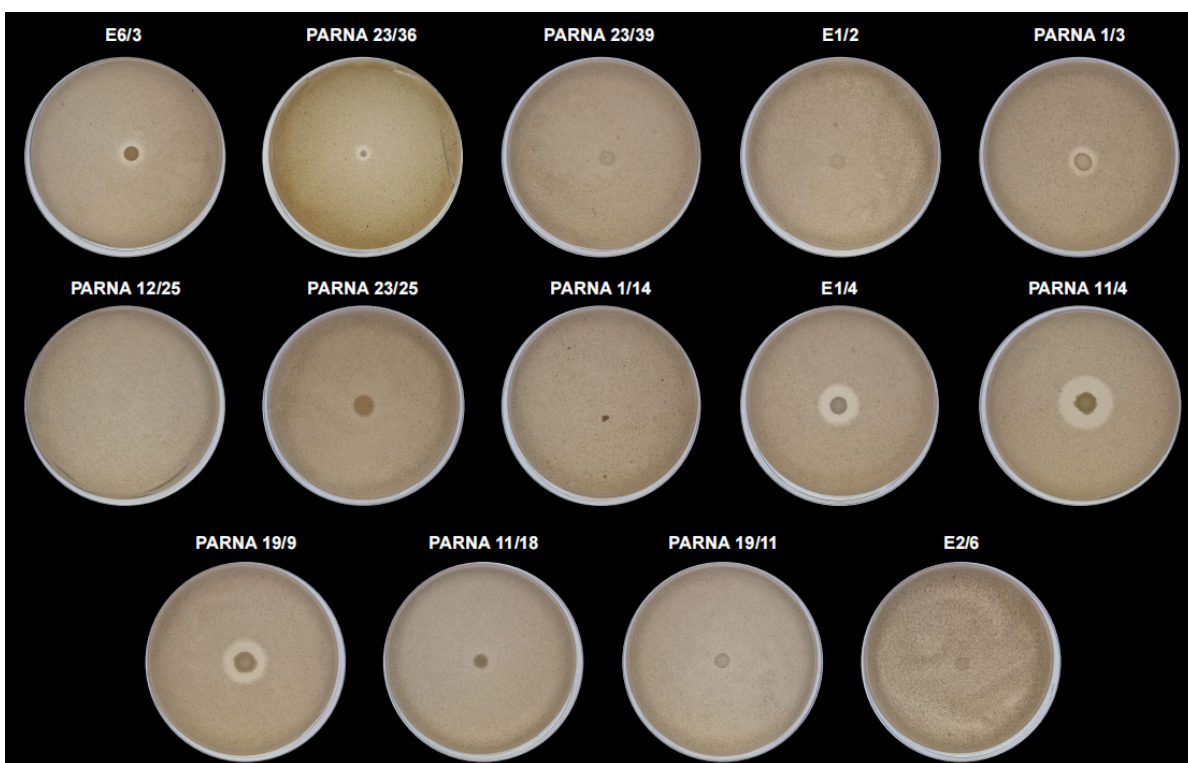


Fig. 3 - Ability of slow-growing isolates to degrade glucan. The test was performed using a culture medium in which glucan was the only carbon source.

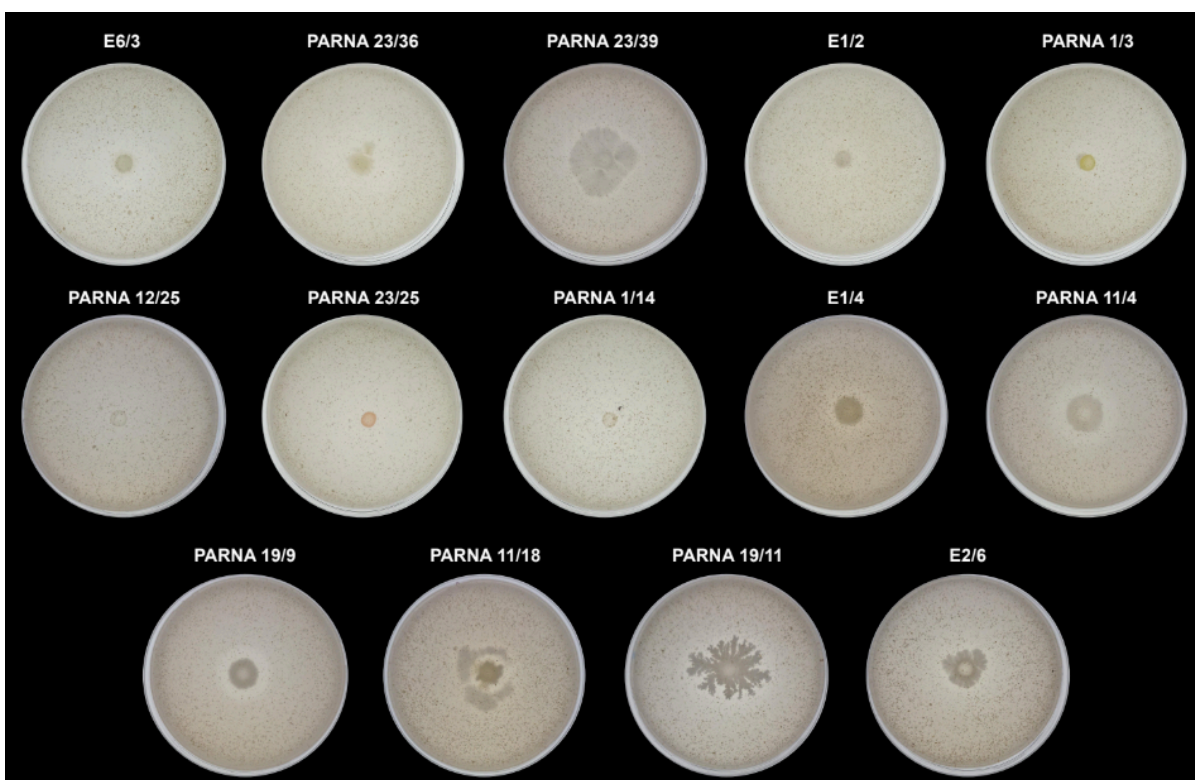


Fig. 4 - Ability of slow-growing isolates to degrade N-Acetyl-D-Glucosamina. The test was performed using a culture medium in which N-Acetyl-D-Glucosamina was the only carbon source.

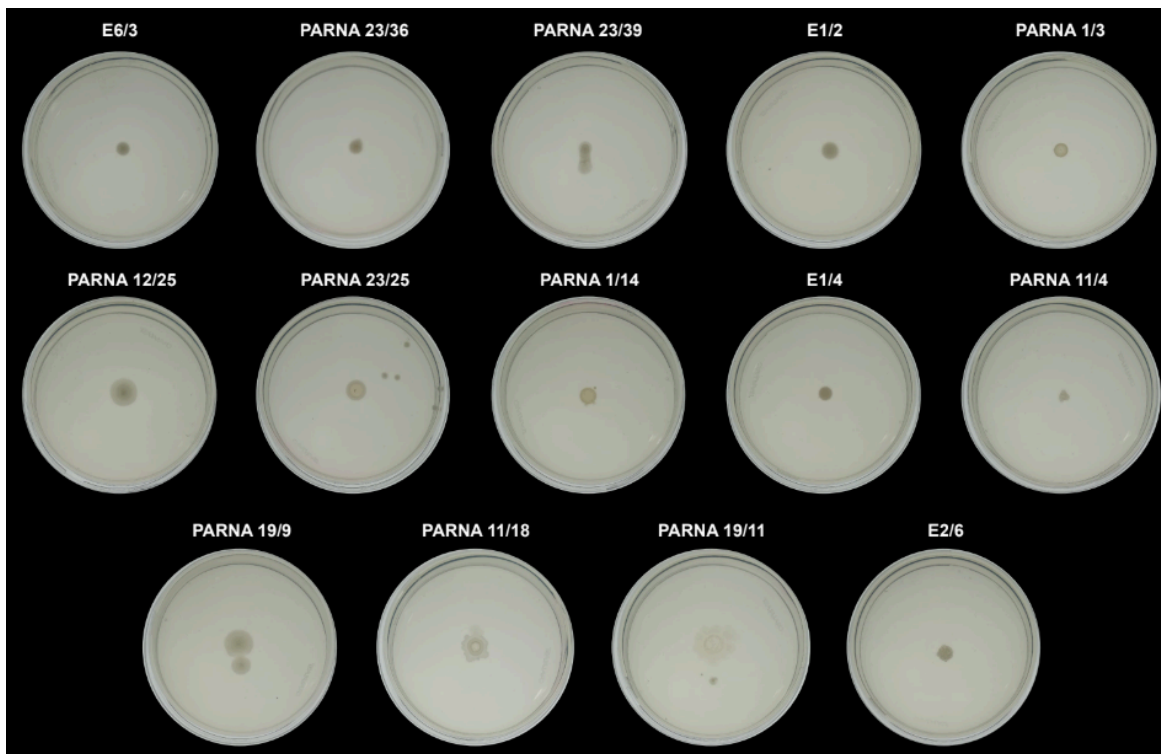


Fig. 5 - Ability of slow-growing isolates to degrade skim milk. The test was performed using a culture medium in which skim milk was the only carbon source.

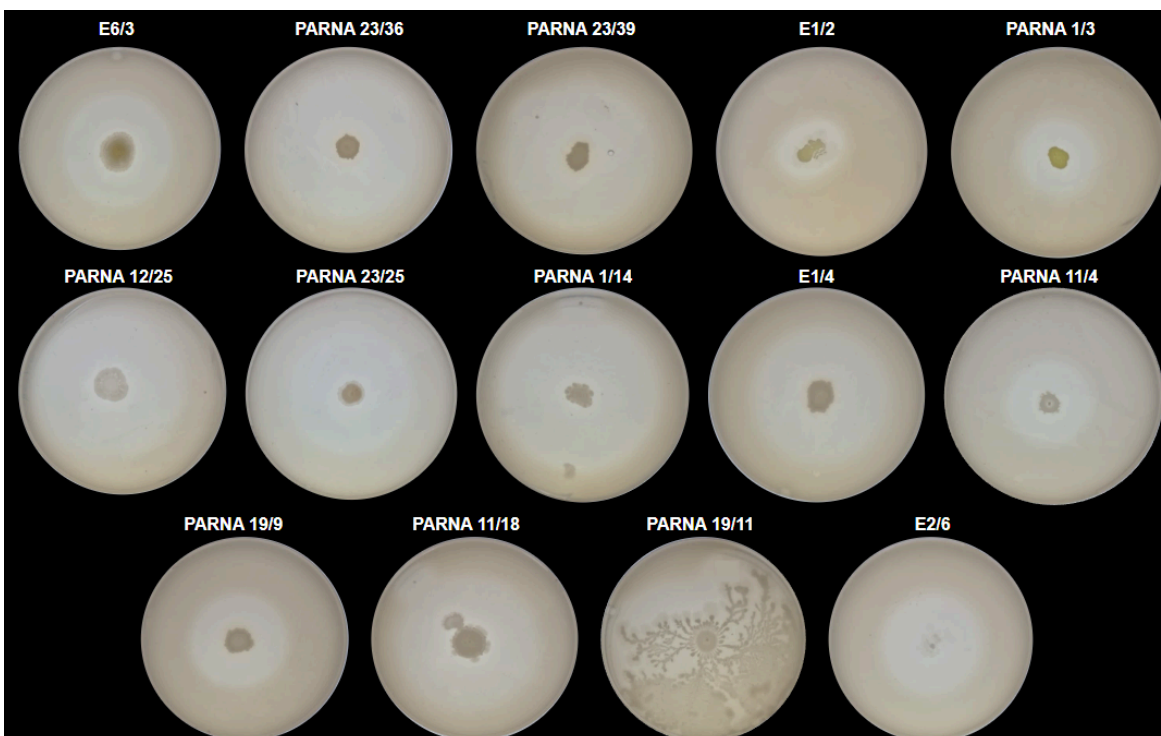


Fig. 6 - Ability of slow-growing isolates to degrade lipids. The test was performed using a culture medium in which lipids were the only carbon source.

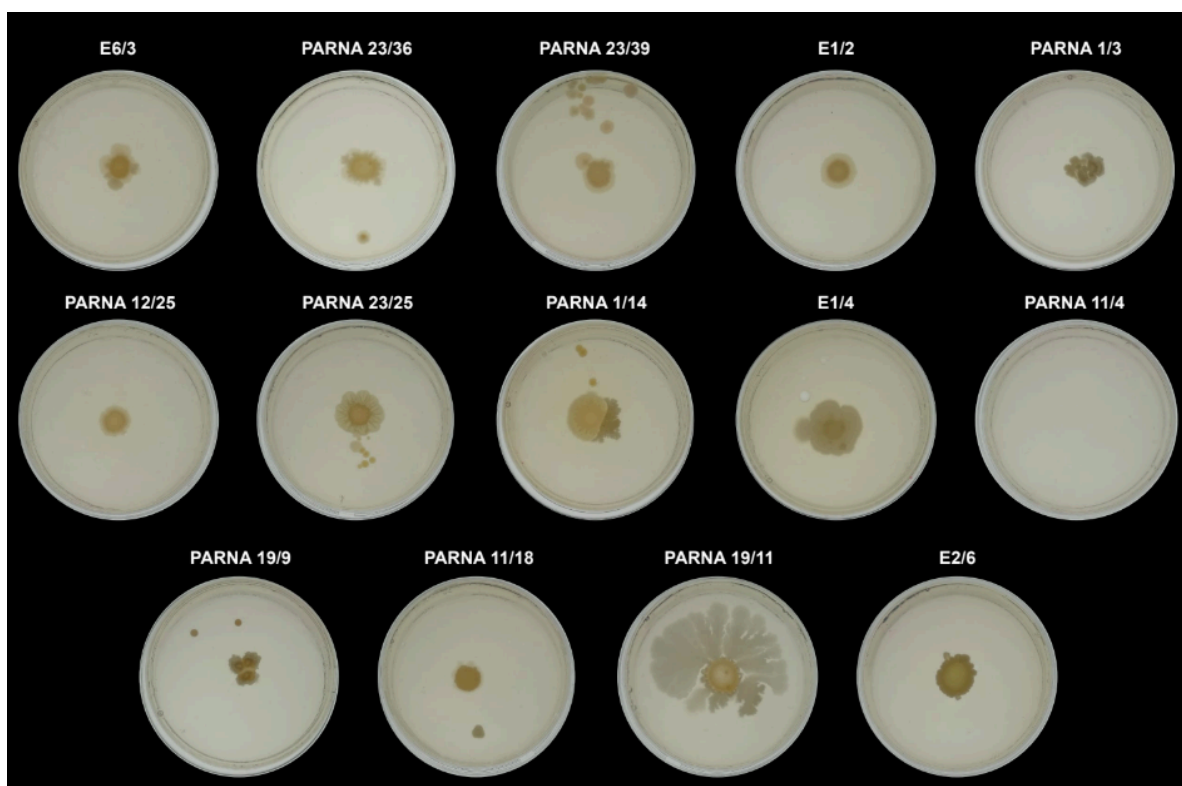
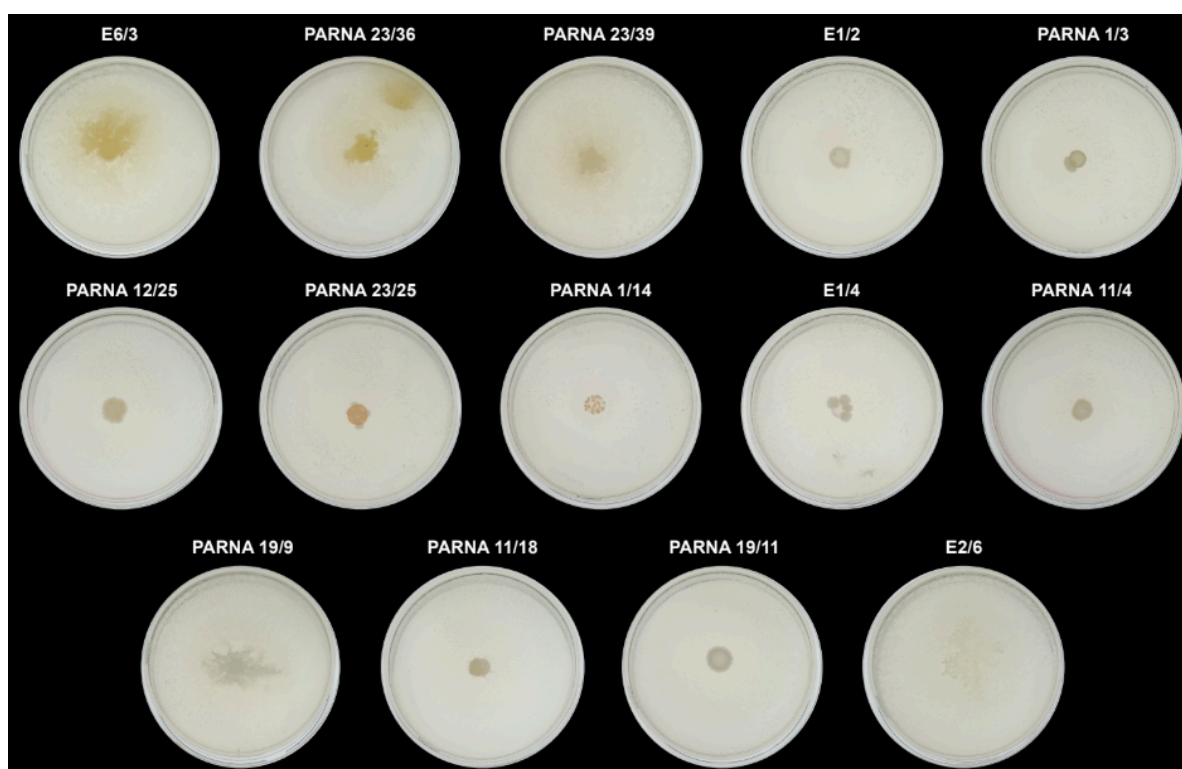


Fig. 7 - Ability of slow-growing isolates to degrade gelatin. The test was performed using a culture medium in which gelatin was the only carbon source.



3.2 Prediction of BGCs in the genomes

The slow-growing isolates with the highest number of BGCs were *P. elgii* PARNA 11/4 and *P. elgii* PARNA 19/9, with 27 annotations in their genomes. There were 13 distinct BGC classes and a frequency of 57 clusters, as well as seven classified as other. The most frequent clusters were non-ribosomal peptide (NRP), Polyketide, NRP+Polyketide, Ribosomally synthesized and Post-translationally modified Peptides (RiPP), and Terpene, with 20, 7, 6, 5, and 5 occurrences, respectively. 135 cluster types were detected, among which the most frequent were Nonribosomal Peptide Synthetase (NRPS), RiPP-like, cyclic-lactone-autoinducer, and terpene, with 33, 16, 10, and 9 occurrences, respectively. In addition to the 7 classes of BGCs that were not identified, 16 types of clusters showed similarity with other known clusters below 15%, while 10 are in the range of 15 to 49%, 6 between 50 and 74% and only 25 show similarity above 75% (Fig. 8).

3.3 Biocontrol of phytopathogenic fungi

The paired culture test between the slow-growing bacterial isolates and the phytopathogenic fungi revealed that the phytopathogens were inhibited by many of the bacteria compared to the control, which consisted of inoculating the fungus without the bacterial streak. Of the 14 isolates, only two showed no inhibitory power against any of the four plant pathogens tested: *F. equiseti* CCF422 (Fig. 9), *F. oxysporum* CCF184 (Fig. 10), *R. solani* 2930 (Fig. 11), and *S. sclerotiorum* 15B (Fig. 12). All the others were able to inhibit all four fungi to a greater or lesser extent. The isolate PARNA 19/11 was the one with the highest average percentage inhibition values; it inhibited *S. sclerotiorum* 15B by 86.7%, *R. solani* 2930 by 74.49%, *F. oxysporum* CCF184 by 37.87% and *F. equiseti* CCF422 by 29%. *C. cellulans* PARNA 1/3 also stood out, having been able to inhibit all phytopathogenic fungi by more than 25% (Fig. 13).

3.4 Defense systems, resistance genes and virulence factors

The analysis carried out using DefenseFinder detected a total of 168 defense genes related to 30 types of defense systems throughout the genome of the 11 slow-growing sequenced isolates. The isolates with the most defense genes are *G. hongkongensis* PARNA 23/25, *P. elgii* PARNA 11/4, *P. elgii* PARNA 19/9, and *B.*

reuszeri E1/2, with 37, 27, 27 and 18 genes, respectively. The isolates with the greatest diversity of defense systems were *G. hongkongensis* PARNA 23/25 and *B. reuszeri* E1/2, with 10 and 8 systems, respectively. The most abundant defense system was Restriction-Modification (RM), Wadjet, and Cas, whose frequency was 12, 7 and 4, respectively (Fig. 14).

A total of 12 types of resistance genes were detected, the most frequent being *FosB*, *clbB*, *Bla1*, and *RPH*, with frequencies of eight, six, six and four, respectively (Fig. 15A). The products of these genes are thiol transferase for resistance of phosphomycin, 23S rRNA methyltransferase, beta-lactamase and rifampin phosphotransferase, respectively (Fig. 15B). The isolates with the most resistance genes are *B. paramycoides* PARNA 23/36, *B. paramycoides* PARNA 23/39, *P. elgii* PARNA 11/4 and *P. elgii* PARNA 19/9, with eight, eight, six and six genes, respectively (Fig. 15C).

The detection of virulence gene products was identical in the *B. paramycoides* PARNA 23/36 and *B. paramycoides* PARNA 23/39 isolates, with exotoxin, nutritional/metabolic factor, effector release system, and exoenzyme being encoded by seven, five, four, and two genes, respectively. In *G. hongkongensis* PARNA 23/25, only one gene for isocitrate lyase and one gene associated with stress survival were detected (Fig. 1 to 6).

4. DISCUSSION

Enzyme prospecting revealed that all the isolates sequenced contain variations of chitinase and protease, two enzymes highly associated with biocontrol. The vast majority also have lipase, galactosidase, and amidase. In addition, some enzymes appeared in great abundance, as was the case with the eight chitinase variations in the genome of *C. cellulans* PARNA 1/3 (Fig. 1A). The semi-quantitative assay carried out to detect substrate degradation by chitinase, glucanase, amidase, lipase, and/or esterase and gelatinase had a high correlation with what was expected from the enzyme prospecting carried out *in silico* (Fig. 1B). Therefore, the genes predicted *in silico* were validated *in vitro*.

The presence of these lytic enzymes in the genome of the isolates and the confirmation of their enzymatic activity support the theory that these bacteria have potential for biocontrol. Riseh et al. (2024) highlight the importance of biological

control of phytopathogens through bacteria with lytic enzymes that destabilize the cell wall of phytopathogens, as well as degrade their proteins and/or DNA. The authors highlight chitinases, cellulases, proteases, lipases, glucanases, and amylases as the most important. In addition, these enzymes can also interfere with the *quorum-sensing* system of phytopathogens or destabilize the signaling molecules used in this system, which inhibits the potential for pathogenicity on the plant.

In the substrate degradation test, which was initially only qualitative, the degradation halo measurements were noted because it was observed that some isolates that grow more slowly than others were forming larger degradation halos; this was the case with *P. elgii* PARNA 11/4, *P. elgii* PARNA 19/9, and PARNA 19/11 (Fig. 1B). This observation may be related to greater enzymatic efficiency, greater catalytic activity, and/or greater catalytic specificity. As there are only 11 deposits in the NCBI of the *P. elgii* species, it is not very well known, and the phylogeny of PARNA 19/11 is not yet known. Their enzymes may be more effective, even if they are synthesized more slowly, following their life cycle.

The literature review by Riseh et al. (2024), which associates bacteria that produce lytic enzymes with biocontrol, highlights the genera *Bacillus*, *Halomonas*, *Marinobacter*, and *Microbulbifer* as having the greatest enzymatic activity against phytopathogenic fungi. The chitinase from *P. elgii* HOA73 is mentioned as being active against *Botrytis cinerea*, but the *P. elgii* species was not highlighted in the review beyond this citation. Chitinase prospecting in the genome of the isolate *P. elgii* PARNA 11/4 detected only three putative chitinases, which is five variations less than *C. cellulans* PARNA 1/3 (Fig. 1A). Even so, even though *P. elgii* PARNA 11/4 had a longer generation time than *C. cellulans* PARNA 1/3, it showed a chitin degradation halo 3.99 times larger with the same incubation time (Fig. 1B and Fig. 2). This isolate is therefore promising for biotechnological applications involving chitinase.

Along with more efficient enzymes, an enzyme consortium is a desired characteristic, often resulting in synergy. Ajuna et al. (2023) discussed the advantages of species of the genus *Bacillus* spp. in having multiple lytic enzymes, so that they are effective against various phytopathogens and capable of degrading multiple polymers that make up their cell wall. The authors highlighted chitinases, proteases, glucanases, lipases, amylases, and cellulases as the best for an enzymatic consortium. Thus, it is interesting that isolates have multiple lytic enzymes,

as is the case with *B. velezensis* E6/3, PARNA 23/36, *B. paramycoides* PARNA 23/39, *B. reuszeri* E1/2, *G. hongkongensis* PARNA 23/25, and *Leifsonia* spp. PARNA 1/14, all of which have at least six of the 11 biocontrol enzymes prospected. In addition to enzyme diversity, these isolates also had a high abundance within each enzyme category (Fig. 1A).

Fig. 8. Biosynthetic gene clusters (BGC) identified in the genomes. A) Distribution and frequency of BGCs Types in the genome of slow-growing isolates indicated by the heatmap. **B)** The heatmap indicates similarity with other BGCs in the antiSMASH database. Only similarities above 75% are truly reliable.

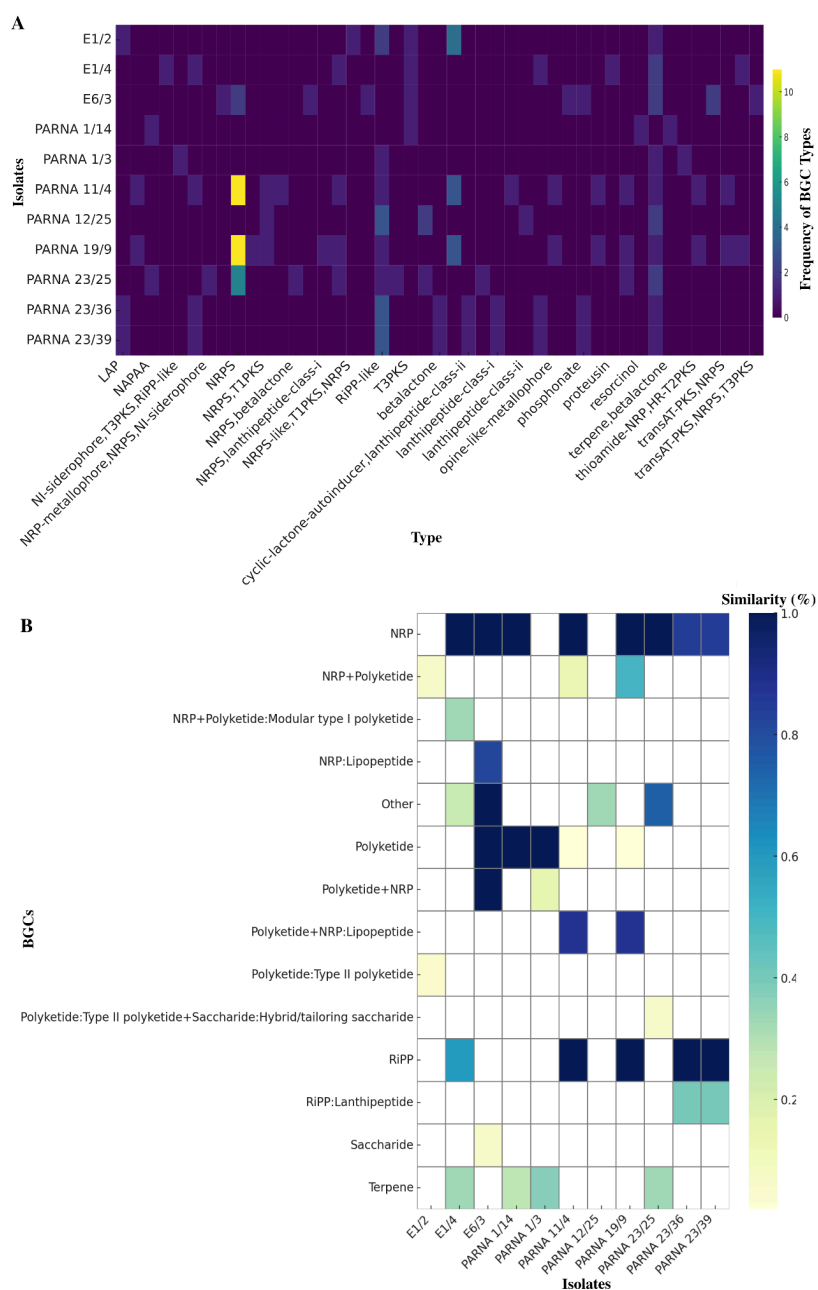


Fig. 9. Antagonism of slow-growing isolates against *Fusarium equiseti* CCF422.
The biocontrol test was performed using the paired culture technique.

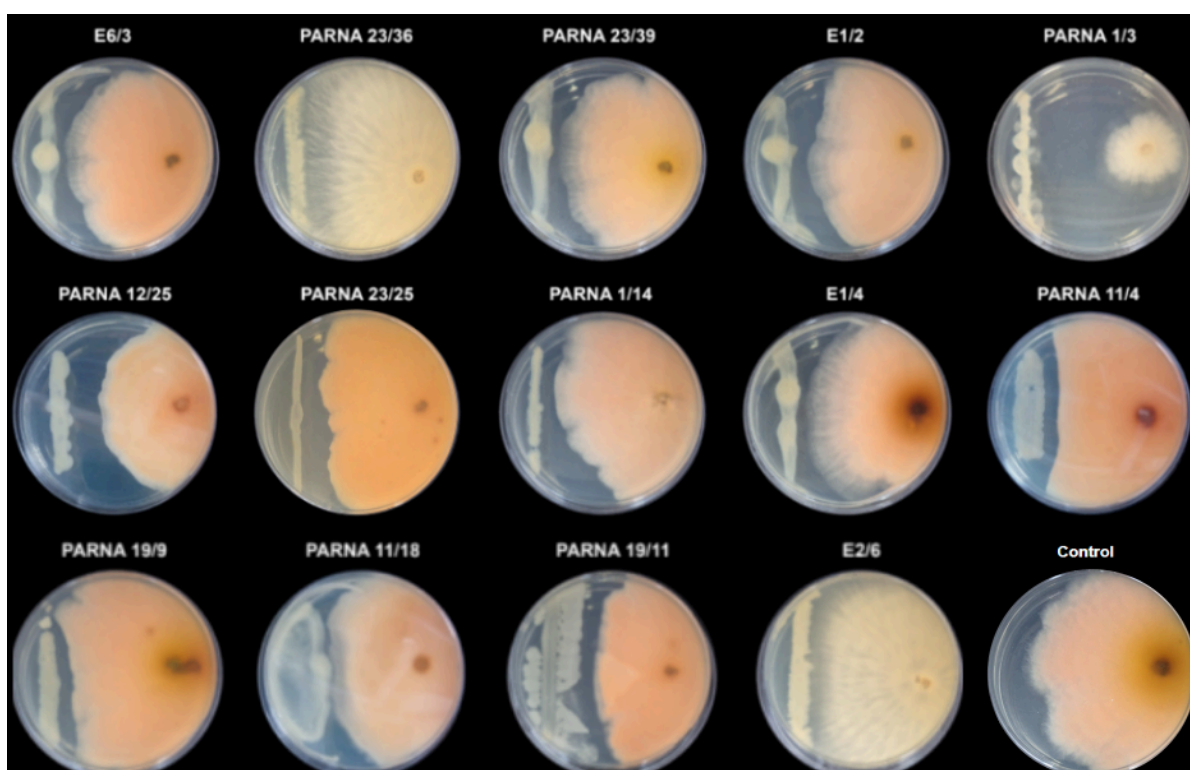


Fig. 10. Antagonism of slow-growing isolates against *Fusarium oxysporum* CCF184.
The biocontrol test was performed using the paired culture technique.

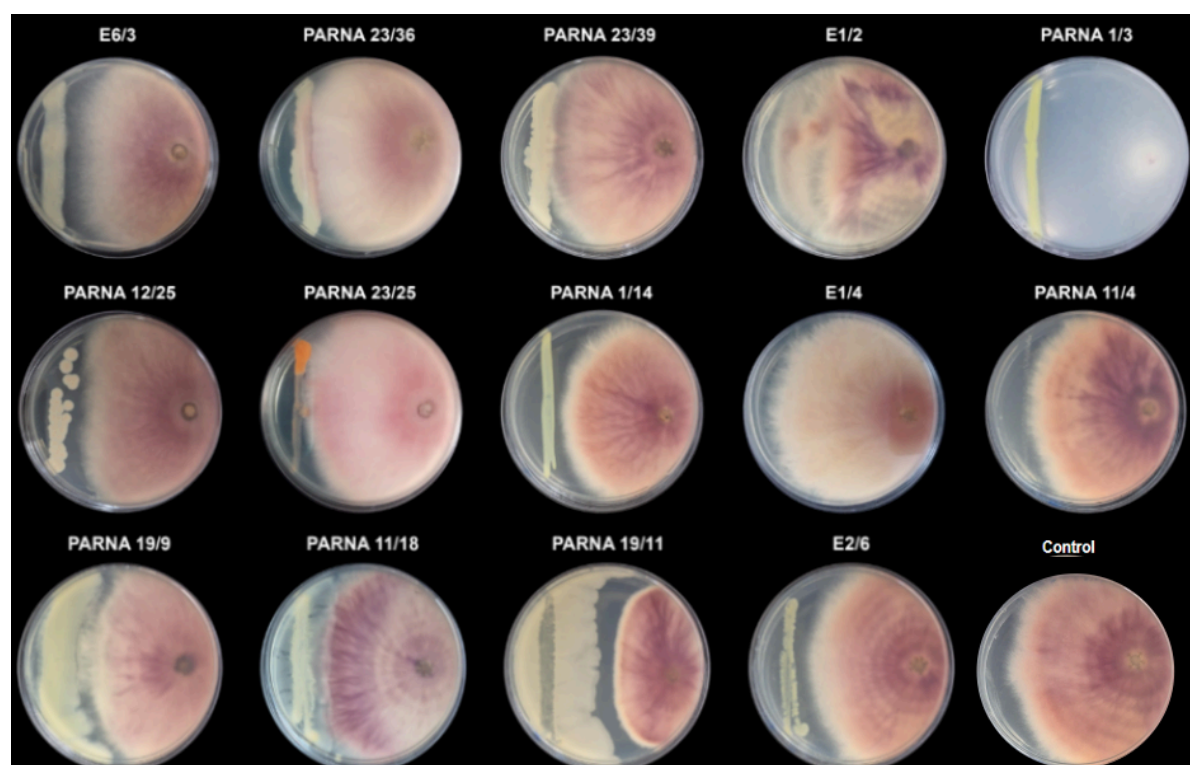


Fig. 11. Antagonism of slow-growing isolates against *Rhizoctonia solani* 2930.
The biocontrol test was performed using the paired culture technique.

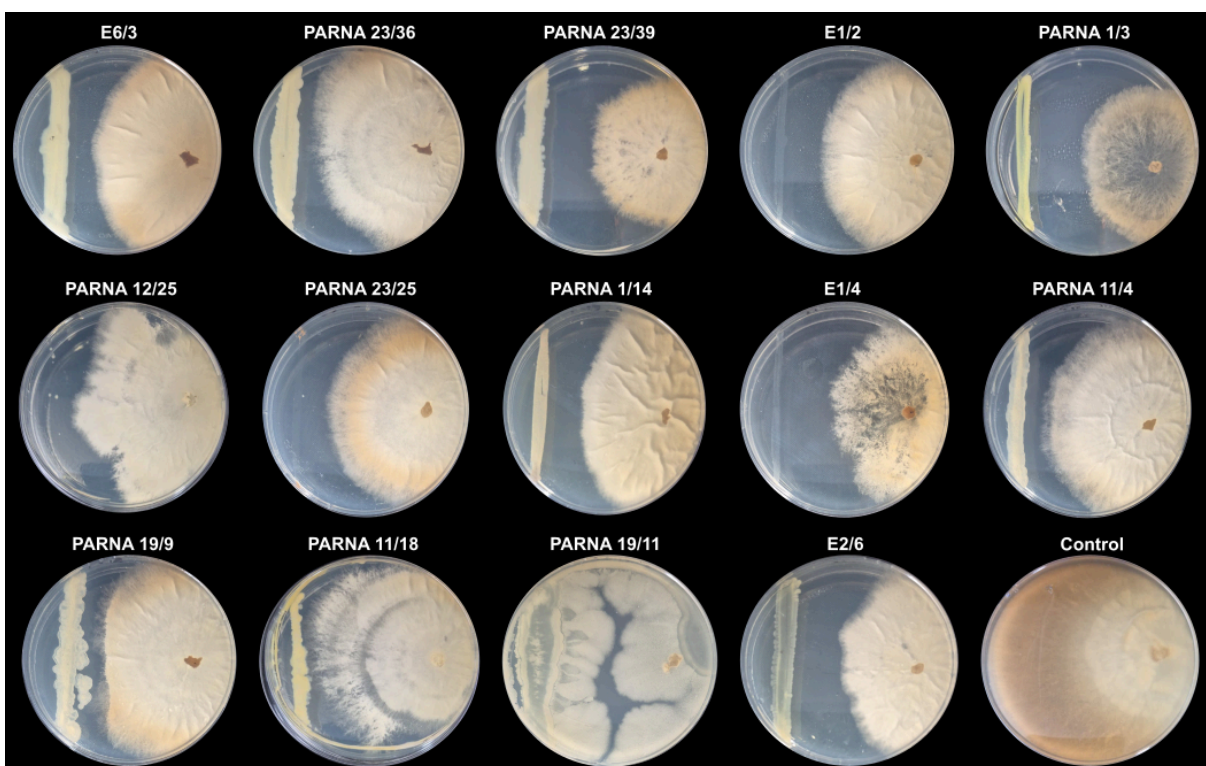


Fig. 12. Antagonism of slow-growing isolates against *Sclerotinia sclerotiorum* 15B. The biocontrol test was performed using the paired culture technique.

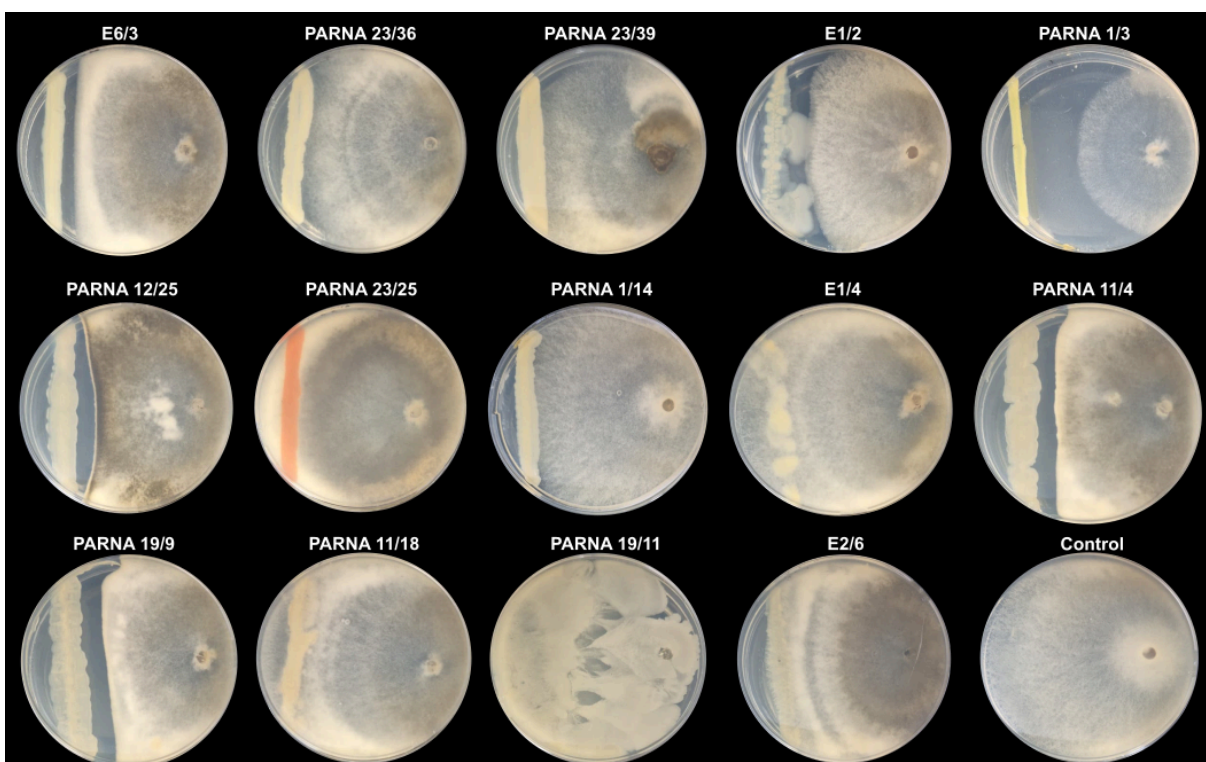


Fig. 13. Biocontrol of phytopathogenic fungi. Percentage of inhibition of phytopathogenic fungi by slow-growing bacterial isolates in paired culture.

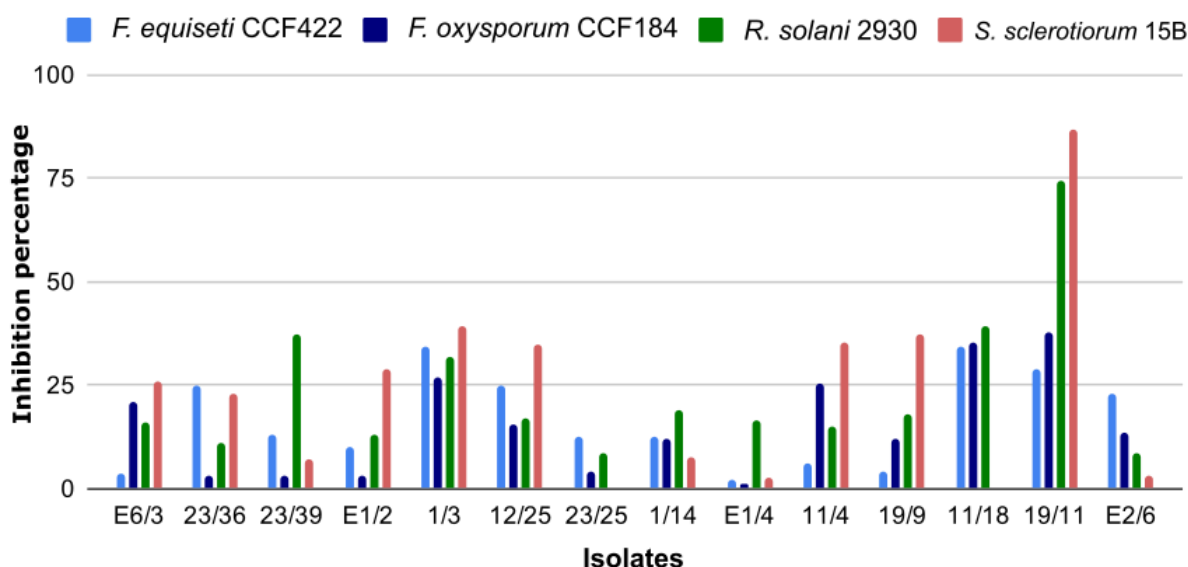
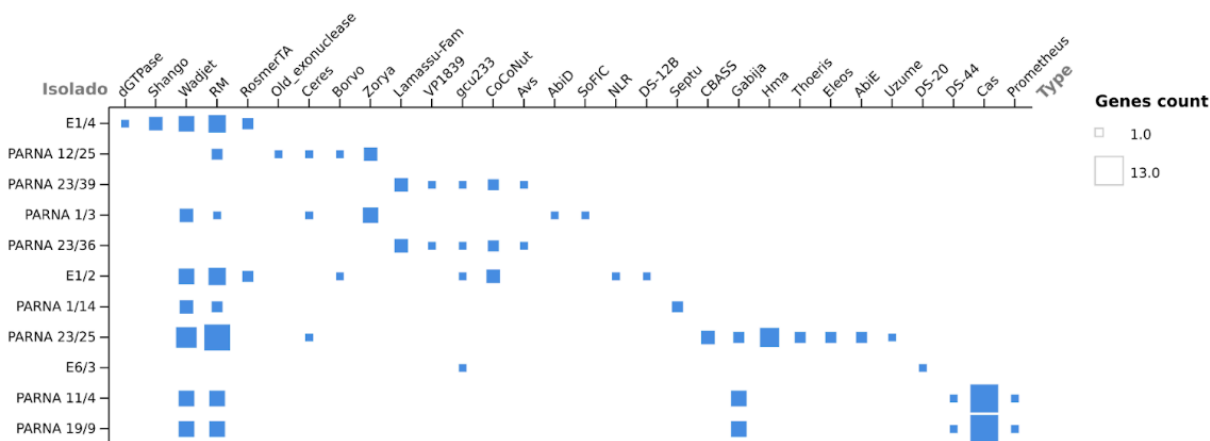


Fig. 14. Defense systems types. Distribution of defense system types in the genomes of slow-growing bacterial isolates. The size of the square is directly proportional to the number of genes associated with each type of defense system.



Another desired characteristic in antagonist isolates is the presence of BGC, because many types synthesize secondary metabolites with inhibitory action against phytopathogens. The isolates with the highest number of BGCs were *P. elgii* PARNA 11/4, *P. elgii* PARNA 19/9, and *G. hongkongensis* PARNA 23/25, with 27, 27, and 17 BGC, respectively (Fig. 8). The comparative genomic analysis within the *Paenibacillus* phylum carried out by Almeida et al. (unpublished data) had already highlighted the relevance of secondary metabolites in the biocontrol capacity of

members of this phylum, especially regarding the *P. larvae*, *P. polymyxa*, and *P. mucilaginosus* species. However, there were no mentions of the species *P. elgii*, probably because it only has 11 representatives deposited in the NCBI, and possibly some of them did not pass the filtering for completeness and quality of the genome. As the isolates in question, *P. elgii* PARNA 11/4 and *P. elgii* PARNA 19/9, had great phylogenetic support and showed excellent completeness and quality values (Jesuino et al.) (unpublished data), this work points to this species as promising for the synthesis of secondary metabolites with biocontrol potential. Another promising species is *G. hongkongensis*, whose isolate PARNA 23/25 also showed a high diversity of BGCs and has no history of relating secondary metabolites to biocontrol. For the *G. hongkongensis* species, there are only a few mentions of carotenoid production (Rhenals-Montoya et al., 2024).

The NRPS cluster was the most frequently detected BGC in the *P. elgii* PARNA 11/4, *P. elgii* PARNA 19/9, and *G. hongkongensis* PARNA 23/25 genomes, as well as in the other slow-growing isolates. NRPS represents 24.44% of the BGCs types found. There is a strong association between NRPS and biotechnological applications, including biocontrol. In their literature review, Iacovelli, Bovenberg & Driessen (2021) highlighted the antimicrobial activity of NRPS through the synthesis of penicillin, vancomycin, daptomycin, and echinocandin, mainly. Gaudelli, Long & Townsend (2015) also highlighted the involvement of NRPS in the synthesis of β -lactam antibiotics.

Seven cluster types were classified as other, and 16 cluster types showed less than 15% similarity to other known clusters. Blin et al. (2025), discussing the updates to the new version of antiSMASH, established 3 levels of confidence for the similarity between BGC, with the range of equal to or greater than 75% classified as reliable, below 75% to 50% as intermediate confidence, and below 50% to 15% with low similarity. They also clarified that cluster similarities of less than 15% are not considered reliable. Therefore, there are possibly 16 new types of BGCs among the slow-growing isolates (Fig. 8).

The detection of potentially new BGC, together with their validation, is of great relevance for finding new isolates that promote biological control. Fatema et al. (2018) observed that gene expression of the 31 putative genes encoding polyketide synthases (PKSs) was increased in the mycoparasitic fungus *Clonostachys rosea*, a

biocontrol agent that was tested against the phytopathogenic fungi *Botrytis cinerea* and *Fusarium graminearum*. To confirm the importance of PKS in antagonism, the authors constructed mutants with deletions in the genes that encode it and carried out the paired culture assay. It was observed that the inhibitory capacity of the deleterious mutant was reduced by 20% to 40% compared to the wild strain.

Fig. 15. Resistance genes. A) Frequency of resistance genes. B) Resistance gene products. C) Number of resistance genes in the genome of slow-growing isolates.

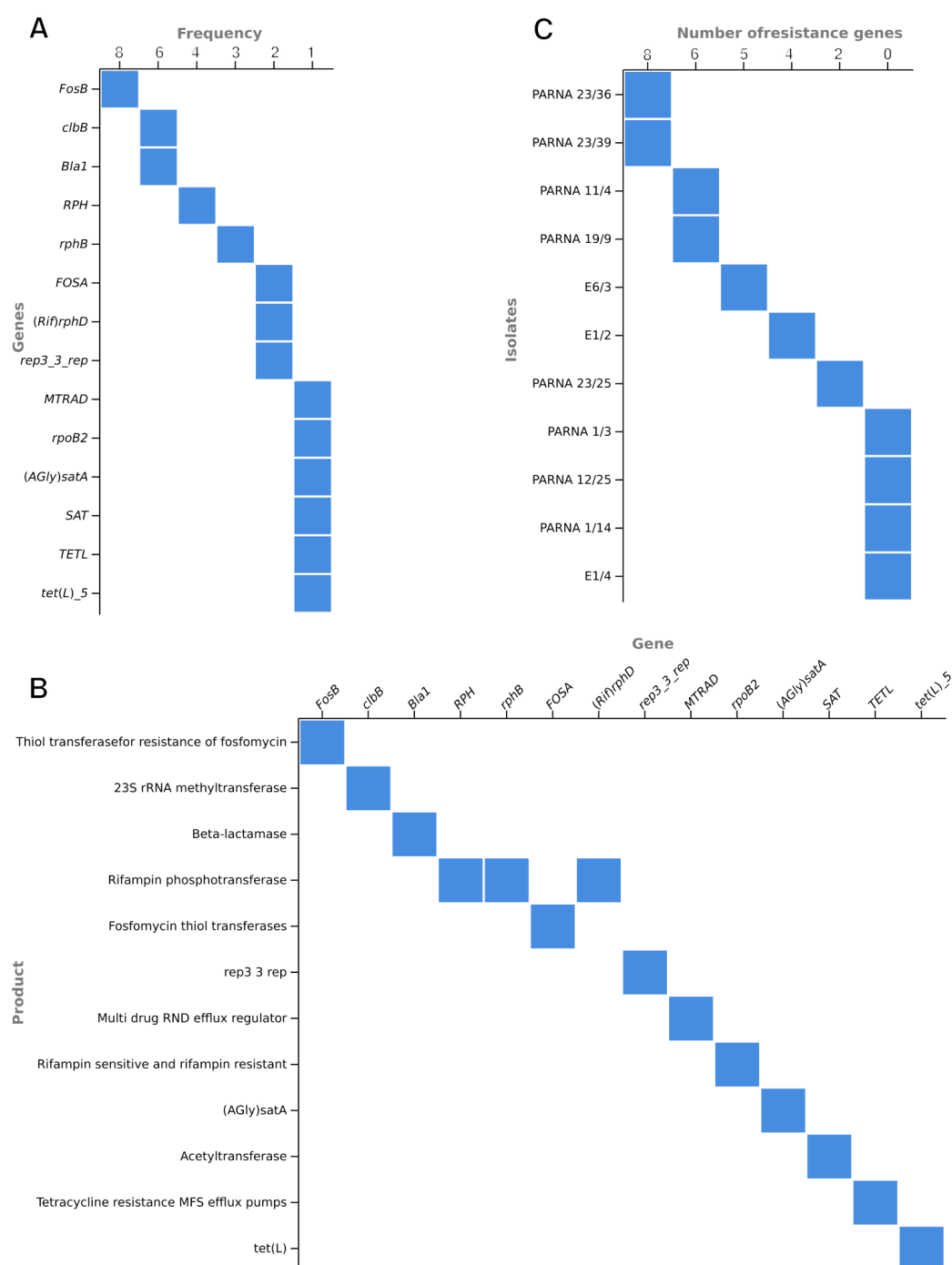
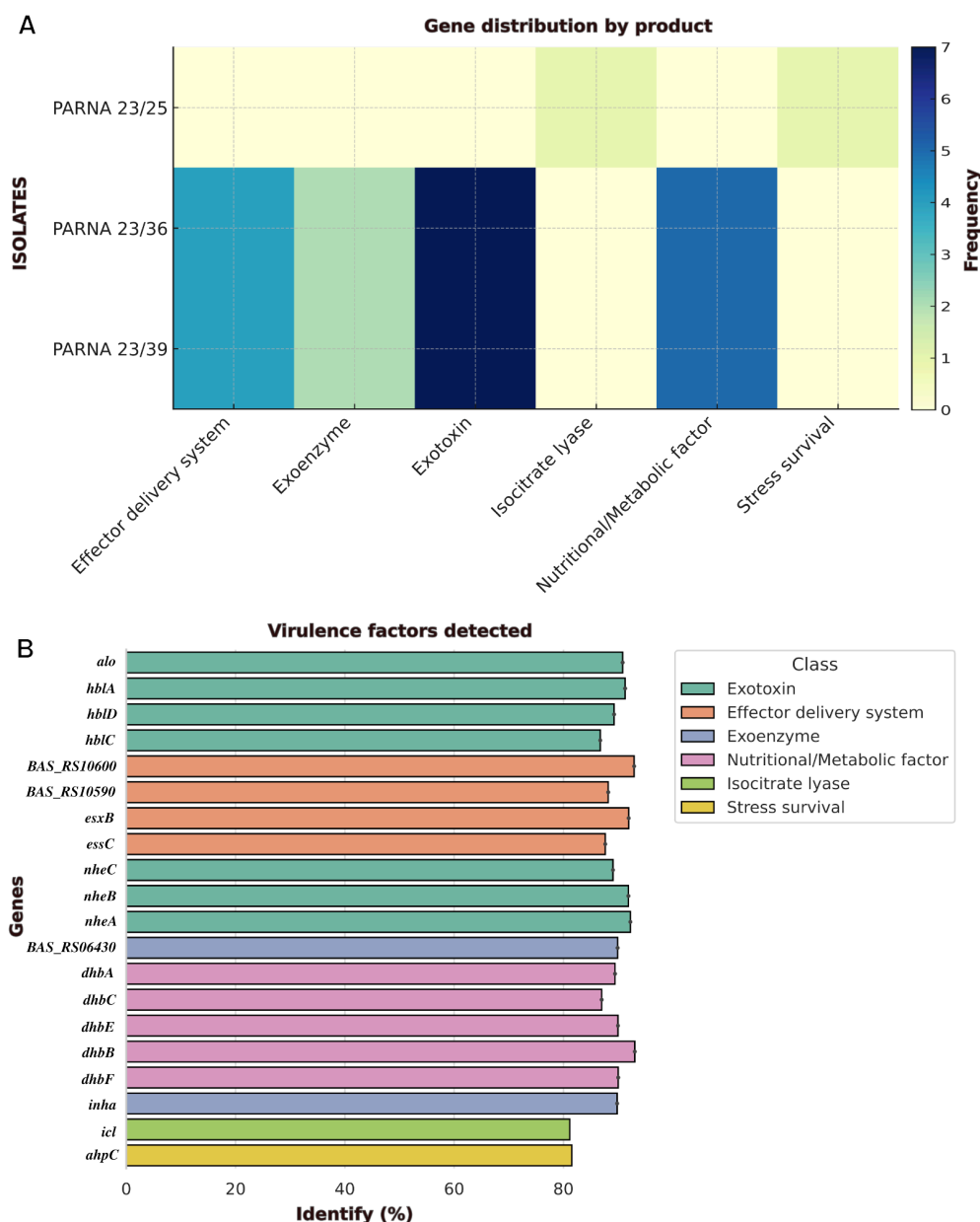


Fig. 16. Virulence genes products. A) Frequency of virulence genes associated with the products and their presence in the isolates. **B)** Frequency of virulence gene product classes, associated genes, and similarity with the database.



In the paired culture tests carried out in this study, many growth isolates proved to be antagonistic to the phytopathogens *F. equiseti* CCF422 (Fig. 9), *F. oxysporum* CCF184 (Fig. 10), *R. solani* 2930 (Fig. 11), and *Sclerotinia sclerotiorum* 15B (Fig. 11). However, we do not know whether the inhibition observed is due to secondary metabolites, hydrolytic enzymes, volatile compounds, or a mixture of factors. Isolate 19/11 was the only one that managed to inhibit the four fungi tested and obtained the highest levels of inhibition, and it has already been observed that its

enzymes have high catalytic power, which raises questions about its phylogeny and what its genome may reveal (Fig. 1B e Fig. 13). The bacterial isolate PARNA 19/11 demonstrated this great antagonistic potential against phytopathogenic fungi even though the paired culture assay was carried out under ideal conditions for the fungi (cultivation in PDA and incubation at $25 \pm 1^\circ\text{C}$) (Slama, 2019), to the detriment of the best cultivation conditions for the bacteria (cultivation in R2A and incubation at $28 \pm 1^\circ\text{C}$) (Fernandes et al.) (unpublished data).

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C. cellulans PARNA 1/3 also stood out in the paired culture assays, being able to inhibit *F. equiseti* CCF422, *F. oxysporum* CCF184, *R. solani* 2930, and *S. sclerotiorum* 15B by 34.60%, 27.04%, 31.86%, and 39.51% respectively.

These findings are in agreement with those of Yeon and Shim (2014), who carried out the same test with *C. cellulans* SG-1 and the phytopathogen *Pythium ultimum* KACC No. 40705 and detected inhibition capacity of mycelial growth. In addition, the authors associate this antagonism with the ability to produce and secrete secondary metabolites, after finding a series of BGCs in the isolate's genome, especially anthranilic acid, cyclo-(dehydroala-L-Leu), cyclo-(LPro-L-Tyr), L-phenylalanine, cyclo-(L-Pro-L-Leu), and cyclo-(L-Pro-L-Val).

In addition to enzymatic activity and secondary metabolites, the successful inhibition of phytopathogens by slow-growing bacterial isolates may be associated

with defense genes. Defense genes are related to the protection conferred against antagonistic organisms and/or factors. A total of 168 defense genes related to 30 types of defense systems throughout the genome of the 11 slow-growing isolates was detected. The isolate with the most defense genes and with the greatest diversity of defense systems was *G. hongkongensis* PARNA 23/25. The most frequent type of defense system was RM (Fig. 14). The RM defense system corresponds to an innate immune system that limits the acquisition of mobile genetic elements (MGE), which in turn impacts genomic evolution. This defense system is very common, including in the genome of the species *Paenibacillus polymyxa*, widely used as a biocontrol agent and to promote plant growth (Chen et al., 2021).

The antibiotic resistance genes, in turn, are related to the survival of the isolates in the presence of such compounds. Mahdi, Fahsi & Hijri (2022) carried out a literature review on antibiotic resistance in plant growth-promoting bacteria and found that there are advantages and disadvantages to this issue. While bacteria used as biocontrol agents have advantages in antibiotic resistance to withstand antagonism from phytopathogens, they can transmit resistance genes to other bacteria. This can be a problem if pathogenic bacteria receive the resistance genes by horizontal gene transfer, for example. In addition to the resistance genes found in abundance in the genomes of *B. paramycoides* PARNA 23/36 and *B. paramycoides* PARNA 23/39, 12 genes of resistance and six defense systems were also detected in each (Fig. 15). The isolates *B. reuszeri* E1/2 and *P. pabuli* E1/4 also stood out, with five and four defense systems, respectively (Fig. 15). Smith et al. (2023) discussed the relationship between defense systems and how they are favorable for antagonism by bacteria, including against phytopathogens. However, they highlighted how this can be a problem in the case of potential human pathogens.

Virulence factors are related to the microorganism's ability to cause infections or greater vulnerability to them. Prospecting for virulence genes detected their presence in the genome of the isolates *B. paramycoides* PARNA 23/36, *B. paramycoides* PARNA 23/39, and *G. hongkongensis* PARNA 23/25, with 18, 18, and three genes, respectively (Fig. 16). The detection of virulence gene products was identical in the *B. paramycoides* PARNA 23/36 and *B. paramycoides* PARNA 23/39 isolates, with exotoxin, nutritional/metabolic factor, effector release system, and exoenzyme being encoded by seven, five, four, and two genes, respectively. In *G.*

hongkongensis PARNA 23/25, only one gene for isocitrate lyase and one gene associated with stress survival were detected (Fig. 16). Isocitrate lyase is not directly involved in virulence, but may contribute to the persistence of virulent isolates by enabling them to utilize lipids as a carbon source during chronic infections when they are inside macrophages (Muñoz-Elías & McKinney, 2005). The second virulence factor identified in the *G. hongkongensis* PARNA 23/25 isolate is related to stress survival and consists of the product of the alkyl hydroperoxide reductase gene (*ahpC*). This gene enables pathogens, such as *Mycobacterium tuberculosis*, to survive antimicrobial products generated by the host cell in response to infection and neutralizes oxidative stress (Springer et al., 2021). Despite the possible association of isocitrate lyase and stress survival with virulence, these factors can only increase pathogenicity, not make an isolate virulent. Therefore, in the absence of other virulence factors, isolate PARNA 23/25 is not capable of causing virulence on its own.

The isolates *B. paramycoides* PARNA 23/36 and *B. paramycoides* PARNA 23/39 have a great abundance of virulence genes, so it is more interesting to assess their potential for biocontrol indirectly, for example, through heterologous expression. However, *G. hongkongensis* PARNA 23/25 only has 2 virulence-related genes, and their products are not related to the determining virulence factors (Chen et al., 2016). Thus, investigating further whether these genes are really harmful and whether they are active ensures greater safety in the application of the isolate and generating deleterious mutants, if this is the case, does not make its safe application as a biocontrol agent unfeasible. *G. hongkongensis* PARNA 23/25 is very promising as an antagonist, showing multiple lytic enzymes and BGC. No virulence genes were detected in the genome of the other isolates, which ensures their possible application as biological control agents.

5. CONCLUSION

The enzymatic analyses carried out in this study showed that the enzymes of the slow-growing isolates were more effective in degrading the substrate. In addition, *P. elgii* spp. were identified as promising biocontrol agents for the first time because they have a wide variety of hydrolytic enzymes and, above all, BGCs involved in the biocontrol of phytopathogens. Given the above, it is concluded that slow-growing

bacteria can contribute to meeting the growing demand for biopesticides that minimize the use of synthetic pesticides.

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Compliance with ethical standards

This study does not contain any experiment with human participants or animals performed by any of the authors.

Conflict of Interest

The authors have no conflicts of interest to disclose.

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CHAPTER 3

HETEROLOGOUS EXPRESSION OF ENZYMES FROM SLOW-GROWING BACTERIA FOR BIOLOGICAL CONTROL OF PHYTOPATHOGENS

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ABSTRACT

Heterologous protein expression is a promising biotechnological approach to exploit bacteria with potential in the biological control of phytopathogens, but which present difficulties in scaling up. This is the case with slow-growing bacteria, a group with high phylogenetic diversity that is relegated due to its prolonged life cycle. However, these bacteria can be exploited indirectly. This work set out to carry out the heterologous expression of chitinase from *Acidobacteria bacterium* AB60, exo-beta-1,3-glucanase from *Cellulosimicrobium cellulans* PARNA 1/3, and N-acetylmuramyl-L-alanine amidase from *Bacillus velezensis* E6/3. The genetic transformation of *Escherichia coli* BL21 and the induction of heterologous expression were successful for all the recombinant enzymes. Protein extraction revealed that the recombinant enzymes remained in the insoluble fraction, from which they were purified and then refolded. However, only glucanase showed enzymatic activity. The paired culture test between *E. coli* BL21 transformed with chitinase and glucanase and phytopathogenic fungi resulted in inhibition. Transformation with chitinase controlled *F. equiseti* CCF422 by 19.43% and *F. oxysporum* CCF184 by 15.49%. Transformation with glucanase controlled *F. equiseti* CCF422 by 25.52% and *F. oxysporum* CCF184 by 22.35%. Transformation with both enzymes did not inhibit *R. solani* 2930 and *S. sclerotiorum* 15B. These preliminary results provide good prospects for the future application of a biopesticide composed of recombinant biocontrol enzymes.

Keywords: Biotechnology; Chitinase; Glucanase; Amidase; Protein purification.

1. INTRODUCTION

Agricultural production will need to gradually increase by approximately 50% by 2050 to keep up with projected population growth. However, some factors pose obstacles to this, such as phytopathogens, one of the main threats to global food security. Approximately 40% of agricultural production is lost due to the direct action of phytopathogens (Wang et al., 2024). The proportion of crop loss varies depending on the cultivar and its associated phytopathogens. Phytopathogens, for example, impair 10.1% to 28.1% of wheat production, 24.6% to 40.9% of rice production, and 19.5% to 41.4% of corn production (Savary et al., 2019; Shukla et al., 2022). To respond to the threats to food security posed by phytopathogens, new biotechnological approaches are being explored.

A sustainable practice that is increasingly being used is biological control using plant growth-promoting bacteria (PGPB). However, there are major limitations to the bacterial isolates known and used as PGPB, such as their survival after being inoculated into the plant and their success in expressing the compounds of interest under the adverse conditions found in the field (Massa et al., 2022). One way to overcome this difficulty is to heterologously express biocontrol proteins. Heterologous protein expression consists of introducing the genetic information for protein synthesis of interest from a naturally coding organism via an expression vector into a heterologous host (Terpe, 2006).

As the heterologous expression of proteins does not depend on naturally coding organisms, it is possible to use this approach in various applications. This includes using the genetic information of slow-growing bacteria that are promising for biocontrol of phytopathogens, but which are difficult to scale up due to their long life cycle. Due to the ecological strategy K-type of slow-growing bacteria, their enzymes tend to be more stable and effective, even if synthesized in smaller proportions, than those of bacteria with a fast life cycle (Ramin & Allison, 2019; Norris et al., 2021). The recombinant biocontrol enzymes can be used for multiple phytopathogens, simply by changing the biocontrol enzyme or creating an enzyme cocktail. Bioproduct alternatives have been increasingly sought after due to the environmental and health problems caused by chemical pesticides (Fabian et al., 2020).

According to data from BCC Research (2021), the global market for enzymes for industrial applications will reach 8.7 billion dollars by 2026, with an annual growth

rate of 6.3%. Although there is no data on the proportion of how many of these enzymes are purified from naturally coding organisms and how many are expressed heterologously, most publications refer to the purification of enzymes that are synthesized naturally. However, many tests are needed to obtain the enzyme from the organism that codes for it naturally, with problems relating to low expression rates, high production costs, inadequate growth conditions, lack of knowledge of the factors that induce synthesis, changes to the culture medium depending on the organism you are working with at the time and a lack of standardization in incubation times and the proportion of inoculum. In contrast, the heterologous expression approach does not present these obstacles, since the heterologous host already has standardized cultivation conditions and expression can be induced in order to obtain a high rate of the protein in question (Arora et al., 2020).

To exploit the biocontrol potential of slow-growing bacterial isolates and overcome the challenges of their direct use, heterologous protein expression will be used in this work to enable slow-growing bacteria, laboriously isolated and sequenced by our research group, to have their biocontrol potential exploited through the heterologous expression of the enzymes chitinase, glucanase, and amidase.

2. MATERIALS AND METHODS

2.1 Isolates and cultivation conditions

The *E. coli* BL21 (DE3), used for heterologous protein expression, was grown at $37 \pm 1^\circ\text{C}$ *overnight* in Luria-Bertani (LB) medium, liquid or solid, and with or without agitation, depending on the objective. The LB is composed of 10 g/L bacterial tryptone, 5 g/L yeast extract, and 5 g/L sodium chloride. Chloramphenicol (25 mg/mL) was added to the LB as it is a resistance marker for *E. coli* BL21, and ampicillin (50 mg/mL) was sometimes added to select transformant strains. Each isolate was stocked in 50% glycerol and stored in freezers at -20 and -80°C (Fadanka et al., 2022).

Of the fungi tested in this work, *Fusarium equiseti* CCF422 and *Fusarium oxysporum* CCF184 were kindly provided by the Laboratório de Micologia e Etiologia de Doenças Fúngicas de Plantas (Departamento de Fitopatologia, UFV), while *Rhizoctonia solani* 2930 is part of the collection of the Laboratório de Patologia Florestal (BIOAGRO, UFV), and *Sclerotinia sclerotiorum* 15B belongs to the

collection of the Laboratório de Genética Molecular e de Microrganismos (LGMM) (BIOAGRO, UFV). The fungi were stocked according to Castellani's method (1963), while they were cultivated in Potato Dextrose Agar (PDA, Kasvi®), and the incubation temperature was $25 \pm 1^\circ\text{C}$.

2.2 Cloning and transformation

The isolates naturally encoding the genes used in the cloning for the heterologous expression of the proteins were previously characterized as slow-growing by Fernandes et al. (unpublished data). The phylogenetic description of these isolates and the genomic search for the genes encoding the enzymes chitinase, glucanase, and amidase, as well as other enzymes involved in biocontrol, was previously carried out in the work by Jesuino et al. (unpublished data), except *Acidobacteria bacterium* AB60, which was isolated and characterized in the study by Rodrigues et al. (2020). The selected coding genes were chitinase from *Acidobacteria bacterium* AB60, exo-beta-1,3-glucanase from *Cellulosimicrobium cellulans* PARNA 1/3, and N-acetylmuramyl-L-alanine amidase from *Bacillus velezensis* E6/3.

The cloning of each selected coding gene was preceded by codon optimization so that there would be no codon bias because the organisms were different. Once this was done, each gene was cloned individually into the pET-21a(+) vector, and artificial gene synthesis was carried out by FastBio (FastBio Ltda, Ribeirão Preto, SP, Br). The pET-21a(+) vector has 5,443 base pairs (bp), pBR322 as the replication origin, T7 as the promoter, *lacO* as the IPTG-controlled regulation system, several restriction sites, the selected ones being 5'-GCTAGC (*NheI*) and 3'-CTCGAG (*XhoI*), C-terminal His-tag as the purification tag, and *ampR* as the resistance gene (NOVAGEN, 2003).

The genetic transformation of *E. coli* BL21 (DE3) was carried out individually for each artificially synthesized vector, using an adaptation of the protocol by Sambrook & Russel (2000). In this adaptation, *E. coli* BL21 was activated and grown in an Erlenmeyer flask with LB medium plus chloramphenicol under shaking at 150 rpm in a shaker at $37 \pm 1^\circ\text{C}$ overnight. The cells were then transferred to Eppendorf and centrifuged at 3,500 g for two minutes at $4 \pm 1^\circ\text{C}$. The supernatant was discarded, and the pellet was resuspended in 1 mL of 0.1 M CaCl_2 . This suspension

was centrifuged under the same conditions, the supernatant was discarded, the pellet was resuspended in 150 μL of 0.1 M CaCl_2 , and then divided into two 75 μL aliquots. 1 μL of the vectors was added individually at a concentration of 10 ng, except for the control. Once this was done, the heat treatment was carried out, which consisted of leaving the cells on ice for 20 minutes, incubating for one minute at $42 \pm 1^\circ\text{C}$ in the water bath, and transferring to ice for 2 minutes. Once the heat treatment was complete, 1 mL of LB medium without antibiotics was added, and the cells were incubated at $37 \pm 1^\circ\text{C}$ under agitation at 150 rpm in a shaker for 2 to 4 hours to recover. After this period, the cells were centrifuged at 3,500 g for two minutes at $4 \pm 1^\circ\text{C}$ and concentrated to 50 μL . This aliquot was divided to make spread plates in solid LB with only the antibiotic of resistance of the bacteria (chloramphenicol) as a viability control and in solid LB with the antibiotic of resistance of the bacteria and the vector (chloramphenicol and ampicillin) for the selection of transformants. The plates were incubated in B.O.D. at $37 \pm 1^\circ\text{C}$ overnight. After this period, a colony of transformant bacteria was transferred to another solid LB plate with the two antibiotics as a safety measure so as not to select satellite cells.

2.3 Induction of heterologous protein expression

The bacteria transformed with the expression vector were induced to express the biocontrol enzyme by adding Isopropyl β -D-1-thiogalactopyranoside (IPTG). To do this, a transformant colony was transferred to an Erlenmeyer flask containing LB medium plus the antibiotics ampicillin and chloramphenicol. The Erlenmeyer flask was incubated in a shaker at $37 \pm 1^\circ\text{C}$ and 150 rpm until it reached an optical density (OD_{600}) of 0.5. After this, 5 mL of the bacterial suspension was transferred to 15 mL centrifuge tubes, and each received different concentrations of IPTG (0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, 0.80, 0.90, and 1.00 mM), with the exception of the control. The bacteria were induced *overnight*, and then a sample was taken and analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the *Nine Evenly Spaced Bands of 10-225 kDa for Use with SDS-PAGE* marker.

2.4 Total and protein fraction extraction and purification

In order to analyze whether the recombinant enzymes were present in the soluble or insoluble fraction, an extraction of protein fractions was carried out. To do this, 1 mL of the induced bacterial aliquot was centrifuged in an Eppendorf at 6,500 g and $4 \pm 1^\circ\text{C}$ for 15 minutes, and the supernatant was discarded. The cells were transferred to ice, resuspended in 500 μL of extraction buffer (50 mM Tris-HCl, pH = 8.2 mM EDTA), and treated with 10 mg/mL of lysozyme and 0.1% Triton 10%. After this, the cells were incubated at $30 \pm 1^\circ\text{C}$ for 15 minutes and then transferred to ice for a further 30 minutes before being ultrasonicated. Sonication was carried out in the Elmasonic Easy water bath ultrasonicator in 5 pulses of 2 minutes with equal intervals between each one. The cells were then centrifuged at 12,000 g and $4 \pm 1^\circ\text{C}$ for 15 minutes, the supernatant, which consists of the soluble fraction, was transferred to another eppendorf, and the pellet, which consists of the insoluble fraction, was resuspended in 100 μL of 1x SDS buffer (50 mM Tris-HCl, 100 mM DTT, 2% SDS, 0.1% coomassie blue and 10% glycerol). A sample of each protein fraction was collected and analyzed by SDS-PAGE.

Once the protein fraction in which each enzyme was found had been identified, they were purified. To do this, the IPTG-induced cells were centrifuged for 10 minutes at 10,000 rpm, the supernatant was discarded and the pellet was resuspended in 5 mL of denaturing lysis buffer (NaH_2PO_4 100 mM, Tris 10 mM, Urea 10 mM), plus lysozyme (1 mg/mL), 0.8 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% v/v Triton X-100; the mixture was kept on ice for 30 minutes. The cells were ultrasonicated under the same conditions and centrifuged at 14,000 g for 20 minutes at $4 \pm 1^\circ\text{C}$. The supernatant was collected in a new tube, and to it was added 500 μL of Ni Sepharose™ resin (Cytiva®) previously equilibrated with the denaturing lysis buffer. The sample was shaken overnight at $4 \pm 1^\circ\text{C}$ and then centrifuged at 1,600 g for 2 minutes. The resin was washed with 10 mL of denaturing wash buffer (NaH_2PO_4 100 mM, Tris 10 mM, Urea 10 mM, and Imidazole 20 mM) and centrifuged at 1600 g for 2 minutes; this step was carried out three times, and all washes were analyzed on SDS-PAGE. After the washes, 500 μL of denaturing elution buffer (NaH_2PO_4 100 mM, Tris 10 mM, Urea 10 mM, and Imidazole 500 mM) was added to the resin, and the mixture was incubated under agitation at $4 \pm 1^\circ\text{C}$ overnight. Subsequently, the sample was eluted three times, spaced one hour apart, and all the supernatants were

analyzed in SDS-PAGE. Elution consisted of centrifuging the sample at 1,600 g for two minutes.

2.5 Refolding of proteins from insoluble fractions

The refolding of the recombinant enzymes was carried out according to the protocol established by Vasconcellos et al. (2014), in which a tenfold (10x) dilution of the eluate was carried out in refolding buffer (100 mM Tris pH 8.0, 600 mM NaCl, 1 mM GSSG, 2 mM GSH, and 33% glycerol). The samples were stored at $4 \pm 1^\circ\text{C}$ for 24 hours, and after this period could be manipulated for enzymatic activity and SDS-PAGE.

2.6 Detection of enzyme activity

To confirm that the enzymes predicted *in silico* have *in vitro* activity, a substrate degradation test was first carried out with the naturally coding organisms, which were inoculated into a culture medium whose only carbon source was chitin for chitinase, glucan for glucanase, and N-Acetyl-D-Glucosamine for amidase (Jesuino et al.) (unpublished data). This test was also carried out heterologously with transformed *E. coli* BL21 and the purified enzyme. To do this, 10 μL of the bacterial suspension or the enzyme suspension was inoculated into the center of a Petri dish containing 20 mL of medium. The plates were incubated at $28 \pm 1^\circ\text{C}$ for seven days. The positive result for enzyme activity was established by the degradation halo in the culture medium.

The colloidal chitin medium is composed of 1.0 g/L of ammonium sulphate, 0.2 g/L of monobasic potassium phosphate, 1.6 dibasic potassium phosphate, 0.2 g/L magnesium sulphate, 0.1 g/L sodium chloride, 0.01 g/L iron II sulphate heptahydrate, 0.02g/L calcium chloride dihydrate, 1% (w/v) colloidal chitin, and 15 g/L bacteriological agar. The minimum medium for glucan consists of 10 g/L of oat flour, 1.0 g/L of monobasic potassium phosphate, 0.5 g/L of magnesium sulphate heptahydrate, 5.0 g/L of sodium chloride, and 15 g/L of bacteriological agar. The minimum medium for amidase is composed of 10 g/L N-Acetyl-D-Glucosamine, 1.0 g/L potassium phosphate monobasic, 0.5 g/L magnesium sulfate heptahydrate, 5.0 g/L sodium chloride, and 15 g/L bacteriological agar.

2.7 Biocontrol of phytopathogenic fungi

The detection of the ability of *E. coli* BL21 transformed with a vector for chitinase and the *E. coli* BL21 transformed with a vector for glucanase to inhibit or prevent the growth of the phytopathogenic fungi *Fusarium equiseti* CCF422, *F. oxysporum* CCF184, *Rhizoctonia solani* 2930, and *Sclerotinia sclerotiorum* 15B through paired culture was carried out separately according to an adaptation of the protocol established by Slama (2019). In this adaptation, a streak of bacterial suspension was made 1 cm away from the left side of a 9 cm diameter Petri dish containing PDA, and then a 6 mm disk of fungal mycelium was placed on the right side of the Petri dish. The streaked bacteria were induced to express the recombinant enzymes when they were at $OD_{600} = 0.5$ and were grown under shaker agitation in their standard conditions overnight. The test plates were incubated at $25 \pm 1^\circ\text{C}$ until the mycelium of the fungal control, inoculated without the streak of the bacterial suspension, reached the point at which the streak was made. Inhibition of mycelial growth was calculated using the formula:

$$PI = \frac{C - T}{C} \times 100\%$$

where *C* and *T* represent the diameters of the fungal colonies on the control and dual culture plates, respectively.

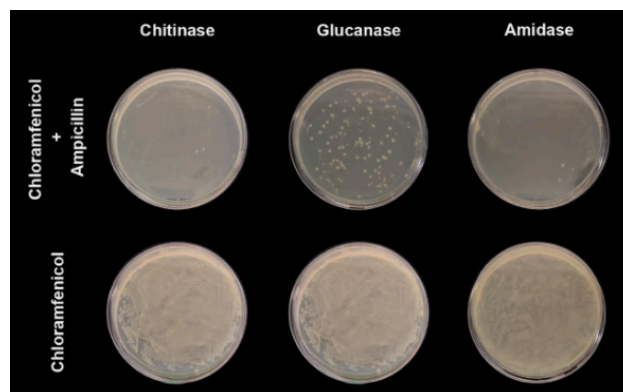
3. RESULTS

3.1 Genetic transformation, induction of heterologous expression, and protein extraction

The chitinase, glucanase, and amidase were individually cloned into the pET-21a(+) vector, which was artificially synthesized. These vectors were used for the genetic transformation and selection of *E. coli* BL21 transformants for the ampicillin resistance marker (Fig. 1).

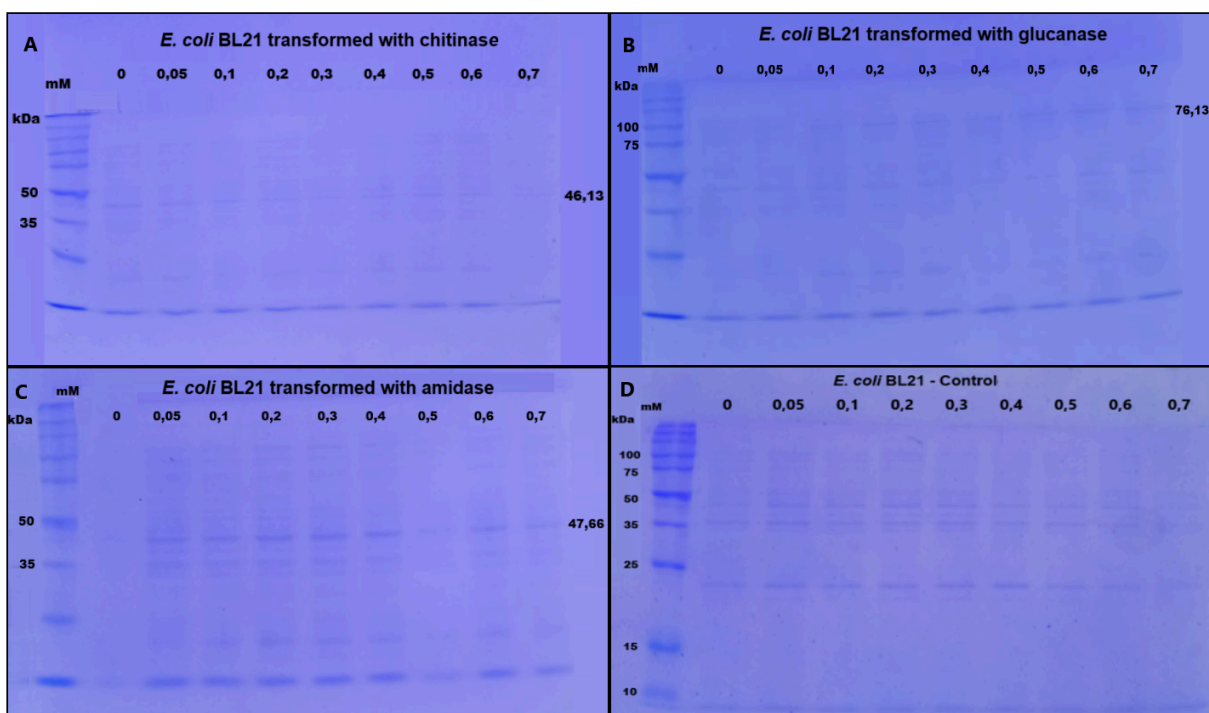
Fig. 1 - Transformation of *E. coli* BL21.

The row of plates above shows the colonies of transformant cells with chloramphenicol (resistance marker) and ampicillin (resistance conferred by the heterologous expression vector). The bottom row, whose plate did not contain ampicillin, served to verify cell viability.



E. coli BL21 was induced to express the recombinant enzymes by IPTG over a wide concentration range. In all the total protein extraction gels, with the exception of the control with untransformed *E. coli* BL21, the band corresponding to the size of the recombinant enzyme appeared. Chitinase has a molecular weight of 46,133.73 Da, exo-beta-1-3-glucanase 76,130.02 Da, and N-acetylmuramyl-L-alanine amidase 47,661.73 Da (Fig. 2).

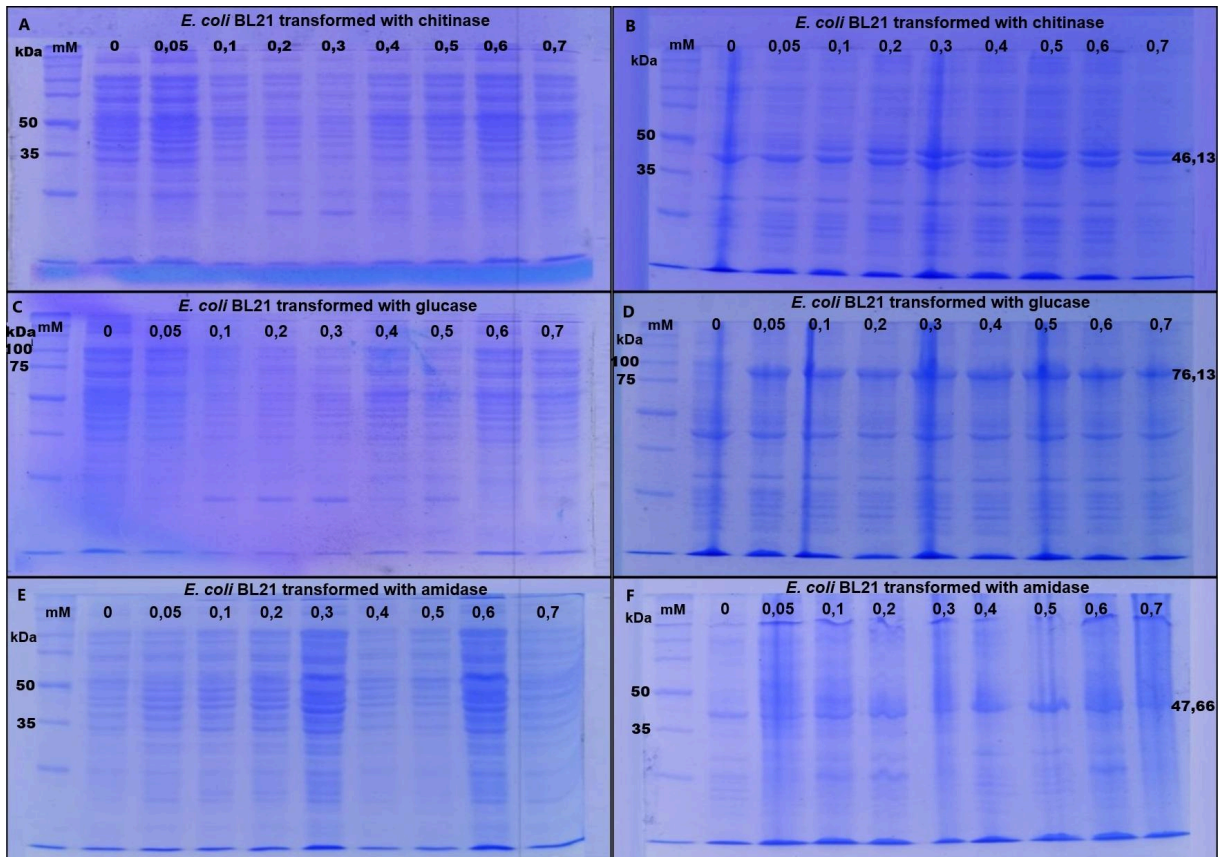
Fig. 2. SDS-PAGE with total protein extraction. The molecular mass of the protein is compared with the marker in the first well of each gel. The IPTG concentration used is indicated in molarity above each well, except for the first, which contains the marker. The run gels contain protein samples from bacteria transformed with the vector for chitinase (A), glucanase (B), and amidase (C). The control consisted of total protein extraction of untransformed bacteria (D).



The extraction of the soluble and insoluble protein fractions revealed that the three recombinant enzymes are present in the insoluble fraction, at least predominantly, as the gel analysis of the soluble fraction was inconclusive because it had too many bands (Fig. 3).

Fig. 3. SDS-PAGE with the extraction of soluble and insoluble fractions of protein. The molecular mass of the protein is compared with the marker in the first

well of each gel. The IPTG concentration used is indicated in molarity above each well, except the first, which contains the marker. The run gels contain samples of the soluble (left side) and insoluble (right side) fractions of the proteins from the vector-transformed bacteria for chitinase (**A** and **B**), glucanase (**C** and **D**), and amidase (**E** and **F**).



3.2 Purification, refolding, and detection of enzyme activity

The recombinant enzymes were purified using Ni Sepharose™ resin (Cytiva) and then washed and eluted. The substrate degradation test with the naturally coding organisms confirmed *in vitro* the presence and functionality of the biocontrol enzymes predicted *in silico*. Similarly, this test was conducted heterologously using *E. coli* BL21 transformed with the recombinant enzymes, which also proved capable of forming substrate degradation halos. Finally, this test was conducted with the purified enzymes under the same conditions. However, only glucanase recovered enzymatic activity. As the enzymes were active in both naturally coding organisms and transformed bacteria, this indicates that the refolding buffer used was unable to recover the three-dimensional structure of chitinase and amidase (Fig. 4).

3.3 Biocontrol of phytopathogenic fungi

The paired culture test carried out separately between *E. coli* BL21 transformed with chitinase and *E. coli* BL21 transformed with glucanase revealed that the phytopathogenic fungi were controlled compared to the control, which consisted of untransformed *E. coli* BL21. Transformation with chitinase controlled *F. equiseti* CCF422 by 19.43% and *F. oxysporum* CCF184 by 15.49%. Transformation with glucanase controlled *F. equiseti* CCF422 by 25.52% and *F. oxysporum* CCF184 by 22.35%. Transformation with both enzymes did not inhibit *R. solani* 2930 and *S. sclerotiorum* 15B, although it did prevent them from reaching the other end of the plate, as happened with the control (Figs. 5).

Fig. 4. Substrate degradation by naturally enzyme-encoding bacteria, transformed bacteria, and purified enzymes. Degradation of colloidal chitin by chitinase, glucan by glucanase, and N-Acetyl-D-Glucosamine by amidase.

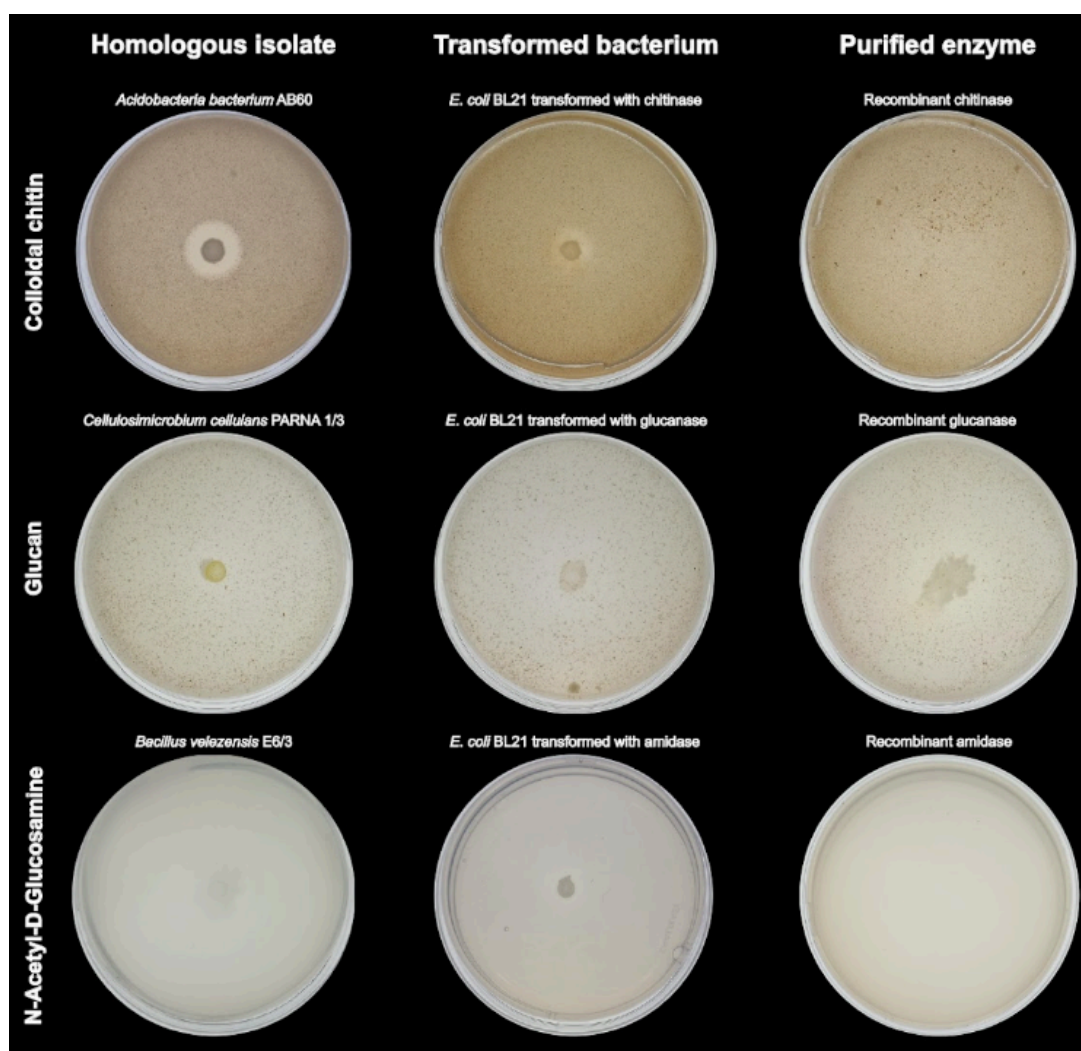
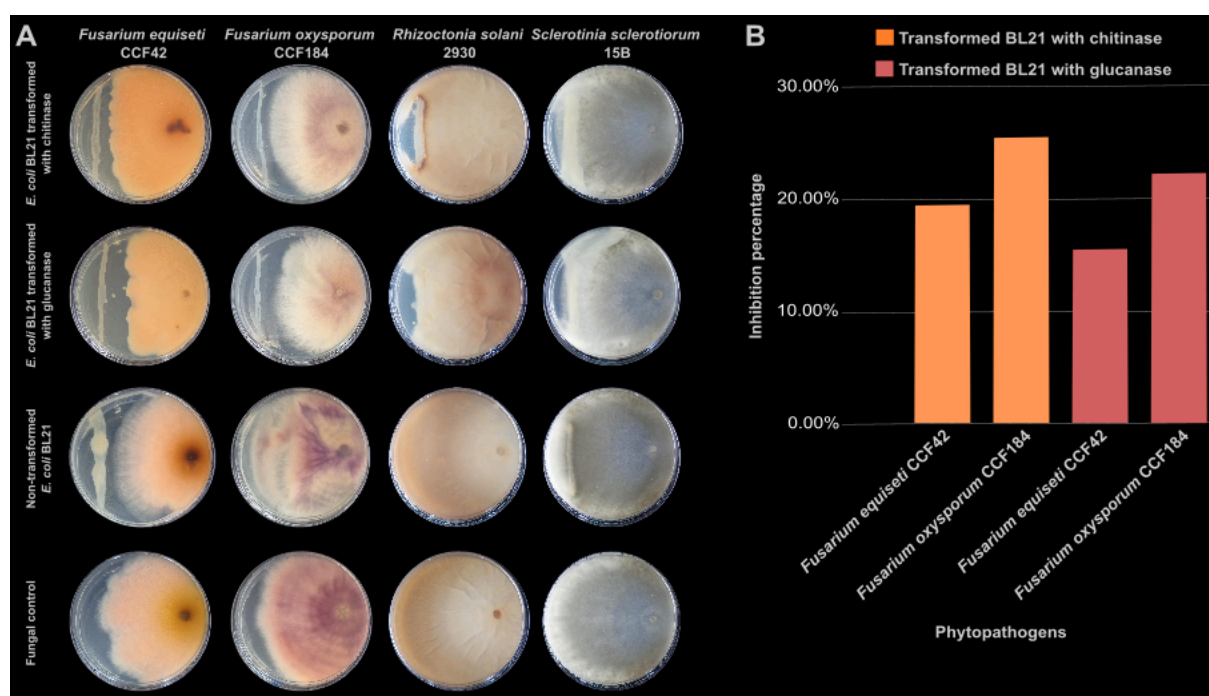


Fig. 5. Biocontrol of phytopathogenic fungi. Inhibition of phytopathogenic fungi by transformed *E. coli* BL21 with chitinase and by transformed *E. coli* BL21 with glucanase in paired culture. **A)** Petri dishes of the antagonism assay. **B)** Percentage inhibition of phytopathogenic fungi by bacteria transformed with vectors containing biocontrol enzymes.



4. DISCUSSION

With a view to greater production of enzymes of interest for biocontrol and being able to exploit the biotechnological control potential of slow-growing bacteria without having to deal with the obstacles related to their long life cycle, this work started with the approach of heterologous protein expression. The target phytopathogens for biocontrol are fungi. Therefore, in order to degrade the cell wall of these phytopathogens, the enzymes expressed heterologously were chitinase, glucanase, and amidase.

All the enzymes were induced to express (Fig. 2 and Fig.3). However, with the refolding protocol tested, only glucanase showed enzymatic activity, while the same folding buffer did not prove effective for chitinase and amidase (Fig. 4). This may be due to the intrinsic characteristics of the proteins themselves, such as the interference of the isoelectric point with the pH of the solution or even the half-life time. In the enzyme activity test by substrate degradation, the glucanase proved to

be active and, although the same test with the naturally coding organisms *C. cellulans* PARNA 1/3 was qualitative, it was incubated for a week to observe the degradation halo, while the same test carried out with both *E. coli* BL21 transformed with the exo-beta-1,3-glucanase and the purified recombinant enzyme was incubated only overnight (Fig. 4). A similar result was observed by Morales et al. (2020), who transformed *E. coli* BL21 with the vector containing the gene encoding laccase. They observed that the transformed bacterium degraded lignin faster than the naturally coding organisms, *Bacillus ligniniphilus* L1.

In addition to *E. coli* BL21 transformed with the exo-beta-1,3-glucanase being able to degrade the glucan source, it was also able to inhibit *F. equiseti* CCF422 by 25.52% and *F. oxysporum* CCF184 by 22.35%. This inhibition was greater than that observed by *E. coli* BL21 transformed with chitinase, which inhibited *F. equiseti* CCF422 by 19.43% and *F. oxysporum* CCF184 by 15.49% (Fig. 5). This is probably due to the very constitution of the fungal cell wall, as glucan is a thicker outer layer than chitin (Kang et al., 2018). These results demonstrate enzymatic activity. Zhang et al. (2011) did something similar by transforming the endophytic bacterium *Enterobacterium cloacae* SJ-10 with a vector containing the gene encoding the plant lectin. After transformation, the authors inoculated the transformed bacteria into rice seedlings and verified the activity of the recombinant product. The rice seedlings acquired insecticidal capacity and were able to biologically control leafhoppers, which cause the disease known as "suction burn". The tests were carried out with *Sogatella furcifera* WBPH.

Although optimizations still need to be made to increase heterologous expression, the gels (Fig. 2 and Fig. 3) showed that none of the enzymes required more than 0.5 mM IPTG to synthesize the recombinant enzymes. An additional step in the process of obtaining the purified enzyme with catalytic activity is the refolding, since both recombinant enzymes remained in the insoluble fraction. This makes it necessary to recover them from the inclusion bodies and find a refolding buffer and/or conditions that return the proteins to their three-dimensional structure (Bastos et al., 2017). Therefore, modifications that make the recombinant enzymes stay in the soluble fraction are more advantageous biotechnologically. Paraskevopoulou & Falcone (2018), thinking about the biotechnological advantages of directing the expression of recombinant proteins to the soluble fraction, tested a series of peptide

markers for this purpose. In addition, they observed that polyionic markers increased the solubility of recombinant enzymes.

5. CONCLUSION

The search for genes encoding biocontrol enzymes revealed their presence in slow-growing bacterial isolates. The prospected genes were validated *in vitro* by substrate degradation. The *E. coli* BL21 transformed with the vectors containing the recombinant enzymes was able to degrade the substrate differently from the non-transformed *E. coli* BL21, which shows the enzymatic activity. In addition, the bacteria transformed with chitinase and glucanase were able to control the phytopathogenic fungi *F. equiseti* CCF422 and *F. oxysporum* CCF184. The enzymes chitinase, glucanase, and amidase were induced and purified. However, in the protocol used, only the glucanase recovered its enzymatic activity with the refolding buffer. Although it is still necessary to find the refolding conditions for chitinase and amidase, these preliminary results provide good evidence for the use of heterologous protein expression in the biocontrol of phytopathogens.

The biotechnological approach explored in this work demonstrated that the biocontrol enzymes were active and had good prospects for use in the biological control of phytopathogens. The heterologous expression systems can serve as efficient platforms for producing and testing recombinant enzymes with potential in biocontrol applications. By validating the antifungal activity of recombinant chitinase and glucanase against important phytopathogens, this study highlights a promising alternative to chemical fungicides, contributing to more sustainable agriculture biocontrol strategies.

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Compliance with ethical standards

This study does not contain any experiment with human participants or animals performed by any of the authors.

Conflict of Interest

The authors have no conflicts of interest to disclose.

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GENERAL CONCLUSIONS

The analyses carried out, both *in silico* and *in vitro*, revealed that slow-growing bacteria have a high capacity for promoting plant growth and biocontrol. In terms of promoting plant growth, satisfactory results were found in bioremediation, production of phytohormones, biofertilization, solubilization of minerals, and production of compounds beneficial to plants. In terms of biocontrol capacity, it was observed that some of the enzymes from the slow-growing isolates were more effective at degrading the substrate. In addition, the species *P. elgii* spp. was identified as promising biocontrol agents for the first time because they present a wide variety of hydrolytic enzymes and, above all, BGCs involved in biocontrol. To access the biocontrol potential of these isolates indirectly, heterologous expression was used. *E. coli* BL21 was separately transformed with genes for chitinase, glucanase, and amidase, the products of which were able to degrade the substrate and control the phytopathogenic fungi *Fusarium equiseti* CCF422 and *Fusarium oxysporum* CCF184. The recombinant enzymes were induced and purified. However, in the protocol used, only the glucanase recovered its enzymatic activity with the refolding buffer. These preliminary results provide good indications of the use of slow-growing bacteria for plant growth promotion and the biological control of phytopathogens, both directly and through the heterologous expression of proteins.