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REVISÃO SISTEMÁTICA E ANÁLISE GENÔMICA DO POTENCIAL DE BIOCONTROLE DE BACTÉRIAS ENDOFÍTICAS

Belém – PA

Dezembro 2025

CLEYSON PANTOJA SERRÃO

REVISÃO SISTEMÁTICA E ANÁLISE GENÔMICA DO POTENCIAL DE BIOCONTROLE DE BACTÉRIAS ENDOFÍTICAS

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Pará, como requisito final para obtenção do título de Doutor em Genética e Biologia Molecular.
Área de concentração: Genética vegetal
Linha de Pesquisa: Prospecção e caracterização de organismos endofíticos

Orientadora: Prof. Dra Cláudia Regina Batista de Souza

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FOLHA DE APROVAÇÃO

CLEYSON PANTOJA SERRÃO

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Pará, como requisito final para obtenção do título de Doutor em Genética e Biologia Molecular. A comissão julgadora é composta pelos seguintes membros:

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SUMÁRIO

ESTRUTURA DA TESE

A tese apresentada está centrada na temática geral da aplicação de microrganismos endofíticos na agricultura. Este documento está dividido em uma introdução geral, dois capítulos e uma conclusão geral. A introdução geral visa interligar os dois capítulos que tem formato de artigo científico. O primeiro capítulo constitui uma revisão sistemática com meta-análise apresentada em formato de artigo já publicada em periódico científico. O segundo capítulo envolve a caracterização genômica de bactérias endofíticas isoladas da raiz de *Piper tuberculatum*, um capítulo finalizado também submetido em periódico científico. Este capítulo também é apresentado em formato de artigo. Após os dois capítulos há uma breve conclusão que visa unir os resultados de ambos os capítulos e dar fechamento ao documento de tese. Por fim temos o cronograma da tese e os anexos com a produção científica resultante dos trabalhos de doutorado.

Em resumo as 5 partes da tese são:

Introdução geral – Breve introdução ligando os dois artigos do trabalho.

Capítulo I: Bacillus as tools for biocontrol of plant diseases: A meta-analysis of two decades of research, 2000-2021.

Autores: Cleyson P. Serrão, Jean C. G. Ortega, Paulo C. Rodrigues, Cláudia R. B. de Souza.

Capítulo II: Caracterização genômica das endofíticas Pt12 e Pt13 isoladas da raiz de *Piper tuberculatum*.

Autores: Cleyson P. Serrão, Alex R. J. Lima, Cláudia R. B. de Souza.

Fechamento – Breve discussão dos resultados apresentados na tese e fechamento do documento.

Anexos

INTRODUÇÃO GERAL

As doenças de plantas constituem um dos principais desafios para a agricultura moderna, causando expressivas perdas econômicas e comprometendo a segurança alimentar mundial. Historicamente, o controle desses patógenos tem se baseado no uso intensivo de produtos químicos, os quais, embora eficientes em curto prazo, geram impactos negativos sobre o meio ambiente, a saúde humana e a biodiversidade. Diante das crescentes demandas por sistemas agrícolas mais sustentáveis, o biocontrole microbiano surge como uma alternativa ecologicamente viável e promissora, fundamentada no uso de microrganismos benéficos capazes de suprimir fitopatógenos e promover o crescimento vegetal.

Entre os microrganismos mais estudados nesse contexto, espécies dos gêneros *Bacillus* e *Pseudomonas* têm se destacado por sua ampla distribuição, adaptabilidade e variedade de mecanismos de ação, incluindo a produção de compostos antimicrobianos, sideróforos, fitormônios e enzimas hidrolíticas. Entretanto, apesar do grande número de estudos publicados, ainda existem lacunas quanto à síntese de informações sobre a eficácia de *Bacillus* em diferentes condições experimentais, bem como à base genética que sustenta o potencial de biocontrole em espécies de *Pseudomonas* endofíticas associadas a plantas tropicais.

Nesse contexto, esta tese foi estruturada em dois eixos complementares. O primeiro apresenta uma revisão sistemática e meta-análise sobre o potencial de biocontrole de *Bacillus* e espécies correlatas, compilando e analisando dados de mais de duas décadas de pesquisas. O estudo sintetiza evidências quantitativas sobre a eficiência desses agentes, identifica os fatores que influenciam seu desempenho e propõe diretrizes otimizadas para o uso de bioinoculantes na agricultura sustentável. Essa abordagem de caráter global fornece um panorama robusto sobre o estado atual do conhecimento e o potencial prático das bactérias do gênero *Bacillus* no manejo de doenças vegetais.

O segundo eixo da tese aprofunda a compreensão dos mecanismos genéticos de biocontrole e promoção de crescimento em uma bactéria endofítica isolada de pimenteira nativa da Amazônia, *Piper tuberculatum*, resistente à fusariose. Por meio do sequenciamento completo do genoma, análises filogenômicas e mineração de genes, foi possível identificar determinantes genéticos associados à Promoção de crescimento em plantas. Assim, esta tese integra uma abordagem abrangente — da síntese global de evidências empíricas à exploração genômica de isolados amazônicos — com o objetivo de ampliar a compreensão sobre o papel de bactérias endofíticas dos gêneros *Bacillus* e *Pseudomonas* no biocontrole e sua aplicação em sistemas agrícolas sustentáveis. Ao considerar as limitações destacadas por Kaminsky et al. (2019), especialmente os conflitos inerentes entre a seleção de microrganismos em condições laboratoriais e seu desempenho inconsistente em campo, este trabalho busca considerar tais barreiras na elaboração e eficiência em de bioinoculantes por meio de análises que contemplam diferentes escalas, condições experimentais. A tese combina a síntese da literatura sobre o gênero *Bacillus* considerando experimentos em campo, em ambiente controlado, diferentes métodos de inoculação, doses e espécies de plantas, isolados e patógenos, com à mineração de genes associados a funções de biocontrole, colonização, persistência e ocupação de nicho, de modo a avaliar os endófitos de forma mais realista e integrada. Dessa forma, as abordagens empregadas visam responder aos desafios ecológicos e funcionais apontados na literatura, oferecendo uma visão mais completa do potencial desses microrganismos como bioinoculantes eficientes.

CAPÍTULO I

BACILLUS AS TOOLS FOR BIOCONTROL OF PLANT DISEASES: A META-ANALYSIS OF TWO DECADES OF RESEARCH, 2000-2021

Autores: Cleyson P. Serrão, Jean C. G. Ortega, Paulo C. Rodrigues, Cláudia R. B. de Souza.

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REVIEW

Bacillus species as tools for biocontrol of plant diseases: A meta-analysis of twenty-two years of research, 2000–2021

Cleyson Pantoja Serrão[1,2] · Jean Carlo Gonçalves Ortega[1] · Paulo Canas Rodrigues[3] ·
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Abstract

The traditional way of dealing with plant diseases has been the use of chemical products, but these harm the environment and are incompatible with the global effort for sustainable development. The use of *Bacillus* and related species in the biological control of plant diseases is a trend in green agriculture. Many studies report the positive effect of these bacteria, but a synthesis is still necessary. So, the objective of this work is to perform a meta-analysis of *Bacillus* biocontrol potential and identify factors that drive its efficacy. Data were compiled from articles published in journals listed in two of the main scientific databases between 2000 and 2021. Among 6159 articles retrieved, 399 research papers met the inclusion criteria for a systematic review. Overall, *Bacilli* biocontrol agents reduced disease by 60% compared to control

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groups. Furthermore, experimental tests with higher concentrations show a strong protective effect, unlike low and single concentration essays. Biocontrol efficacy also increased when used as a protective inoculation rather than therapeutic inoculation. Inoculation directly in the fruit has a greater effect than soil drenching. The size of the effect of *Bacillus*-based commercial products is lower than the newly tested strains. The findings presented in this study confirm the power of *Bacillus*-based bioinoculants and provide valuable guidance for practitioners, researchers, and policymakers seeking effective and sustainable solutions in plant disease management.

Highlights

- *Bacillus* biological control agents significantly reduced disease by 60% when compared to negative control groups;
- The concentration of the inoculum drives the protection effect of Bacillus inoculation;
- Protective inoculation has a greater effect than therapeutic inoculation;
- Inoculation directly in the fruit has a greater effect than soil drenching;
- In comparison with test strains, commercial bioproducts have a lower protective effect.

Keywords Bacillales · Biotic stress · Systematic review · Endophytic bacteria · Size effect

Introduction

Plant diseases are a major threat to world food security, with the annual losses due to plant pathogens and pests representing 17–23% of the total yield (Savary et al. 2019). In addition, global warming and climate change are likely to aggravate the problem (He et al. 2021; Richard et al. 2022; Bebber et al. 2019; Delgado-Baquerizo et al. 2020; Ibáñez et al. 2023). The traditional way of managing plant diseases using synthetic pesticides has been questioned due to its impact on the environment (Pathak et al. 2022; Tudi et al. 2021). The indiscriminate use of pesticides also leads to the evolution of more resistant and aggressive pathogens

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Introduction

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In bacteria, the most representative phyla associated with plant roots are Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes (Liu et al., 2017; Rana et al., 2020). In Firmicutes, Gram-positive Bacilli bacteria (*Peanibacillus*, *Bacillus*, *Lactobacillus* and other genera) are very widespread because they colonize many habitats, including plant tissues (Radhakrishnan et al., 2017). The commercial value of Bacilli species resides in their diverse secondary metabolism (Fira et al., 2018). Some *Bacillus* species have approximately 4 to 5% of their whole genome dedicated to clusters of secondary metabolites such as bacteriocins, antimicrobial peptides and lipopeptides and polyketides (Cai et al., 2017; Deng et al., 2011; Chen et al., 2007).

Besides the vast machinery of antibiosis, Bacilli species are excellent competitors for space or nutrients, as they produce siderophores for iron acquisition (Dimkić et al., 2022; Yue et al., 2023) and organic acids for phosphate solubilization (Lin et al., 2023; Saeid et al., 2018). They also have genes for the biological fixation of nitrogen (Singh et al., 2020) and for motility via flagella and root attachment via teichoic acid production (Olanrewaju et al., 2021). Bacilli species also exert their biocontrol abilities by inducing systemic resistance (ISR) in the host using a set of pathogen-associated molecular patterns (PAMPs). The early recognition receptor leads to a downstream signaling cascade and the induction of basal defense systems and genes of proteins related to pathogenesis (Xie et al., 2018; Zhou et al., 2021a; Portieles et al., 2021). Finally, bacteria can promote plant growth by producing and modulating plant hormones such as auxins, gibberellins, jasmonic acid and ethylene (Shahzad et al., 2016; Khan et al., 2020, Misra and Chauhan, 2020).

Considering the strong biocontrol properties of Bacilli species, some commercial bioproducts available on the market are Serenade® based on *Bacillus velezensis* QST713, Rizhovital® based on *B. velezensis* FZB42 and Companion® based on *B. velezensis* D747 (Ngalimat et al., 2021). These and other commercial agents are recommended to control various types of diseases such as foliar, soilborne and post-harvest fruit diseases.

Despite the commercial alternatives, it is challenging to develop biocontrol products based on microorganisms. In general, biocontrol agents are less effective in the field environment compared to controlled conditions, such as *in vitro* or greenhouse essays (Kaminsky et al., 2019). These inconsistencies between laboratory and field studies occur due to poor quality of soil, the nature of interactions with other microorganisms in the environment, abiotic stress and contamination (Massa et al., 2022; Orozco-

Mozqueda et al., 2021; Kumar et al., 2022). Furthermore, studies have already shown that the same biocontrol agent has different efficiency towards different types of diseases, also depending on the inoculation method (Minchev et al., 2021; Dawar et al., 2010; Zhang et al., 2009). Additionally, *Bacillus* induces a distinctive physiological state called “priming”; in the primed state the preventive inoculation of the strains leads to a better or more rapid mounting of plant defense responses (Conrath et al., 2006; Madriz-Ordeñana et al., 2022). Consequently, the nature of the treatment, whether preventive or therapeutic, also influences the plant's response to inoculation and biocontrol efficacy of an isolate or bioproduct (Morales et al., 2022). Other studies show that the variation in the efficiency of biocontrol exerted by Bacilli species is also controlled by the concentration of the inoculum (He et al., 2022; Zhou et al., 2021b; Másmela-Mendoza and Moreno-Velandia, 2022). Finally, since extracellular metabolites have strong biocontrol properties directly or indirectly (Zhang et al., 2023), many studies comparatively evaluate these metabolites in the biocontrol of diseases. Lipopeptides, proteins, fermentation broth and supernatant may exhibit different effects on disease suppression compared to the bacterial suspension (Xu et al., 2019; Liu et al., 2020; Wang et al., 2023). The differences in experimental designs come from the different properties and biocontrol mechanisms of the strain in question, and from the interaction with hosts and the pathogens. Those different experimental designs add heterogeneity to the field of study and raise questions about the effect of the same isolate on other crops, pathogens and environments, in addition to which methodological factor can reduce or increase the effectiveness of a specific biocontrol agent (Leblanc, 2021). Asking those questions is the biggest challenge, and the answers may help in the development of future studies and commercial products.

A meta-analysis of the contribution of Bacilli biocontrol agents may suggest answers to these questions. In the last few years, meta-analysis has become a recurrent approach in the field of agronomy, with more than 1000 meta-analyses conducted, more than half of them after 2012 (Krupnik et al., 2019). Indeed, meta-analysis is one of the most objective and robust tools used in agricultural research to combine the results of many experiments. Combined with a systematic review, this approach provides a broad overview of the science field under study, based on a synthesis of multiple scientific reports with transparent search protocols, reproducible exclusion criteria and statistical tests to weigh the quality of studies (Borenstein et al., 2009, Gurevitch et al., 2018).

Endophytic bacteria have become an interesting object of study for systematic reviews. The power of the endophytic effect in inducing tolerance to abiotic stresses, such as salinity, drought and heavy-metal contamination, has been synthetized and analyzed in elegant meta-analyses in the last few years (Tufail et al., 2021; Tufail et al., 2022; Franco-Franklin et al., 2021; Rho et al., 2018). For example, Leblanc (2022) made a meta-analysis of biocontrol agents in the genus *Streptomyces* and the main experimental procedures that drive the size of the effect on pathogen antagonism. Similar analyses are missing on biocontrol agents of the Bacilli group, as it is necessary to understand the methodological guides to optimize the effect of inoculation of Bacilli to prevent plant diseases. In this context, the main goal of this work is to perform a meta-analysis of studies employing Bacilli species as biological control agents to identify methodological and experimental factors that influence their efficacy in plant disease management. Studies and reports like these are essential in understanding the state of the art in the field of biological control and thus to provide researchers with methodological guidelines to optimize the procedures/methods of their experiments.

Materials and Methods

Data mining

The data set was established based on the related scientific literature on biocontrol of plant diseases, following two guidelines for systematic review: the PICO framework (Miller and Forrest, 2001) and the PRISMA flower diagram (Page et al., 2021). Two databases were used to provide the set of studies, SCOPUS and Web of Science (WOS). Studies were searched in these two databases with the following keywords and Boolean arguments: Bacillus AND Endophyt* AND "Plant disease*" AND biocontrol OR "Biologic* Control". Data mining was conducted on August 18th, 2021, and filters were applied to language, period and type of report, selecting publications in English, date range from 2000 to 2021 and only research articles, thus excluding review and other types of reports.

The search resulted in a dataset of 6159 articles, 4383 provided by SCOPUS and 1776 provided by WOS. The selection workflow demanded two screening steps: (i) an initial abstract screening to discard the out-of-scope studies; and (ii) a full article screening to select the final set of studies that were considered in the meta-analysis. The selection criteria that combine the two screening steps included the following restrictions (Figure 1):

1. The study must report at least one bacterium of the Bacilli group, including *Bacillus*, *Peanibacillus*, *Lactobacillus* among others, properly identified.
2. The study must report biocontrol trials with information on global indicators of disease severity or incidence. Severity was used primarily.
3. Any study that combines the tested strains with compost, fertilizer, or adjuvant must report one treatment with the combined agent alone.
4. The study can include greenhouse, field, growth chamber and room conditions in pre- or post-harvest diseases. This includes fruit protection studies and excludes dual antagonism essays *in vitro*.
5. The study must clearly report details such as sample size, mean and a metric of variance (standard deviation, standard error, or confidence interval) in experimental treatments. When the type of variance is not specified, it was assumed that SD was reported.

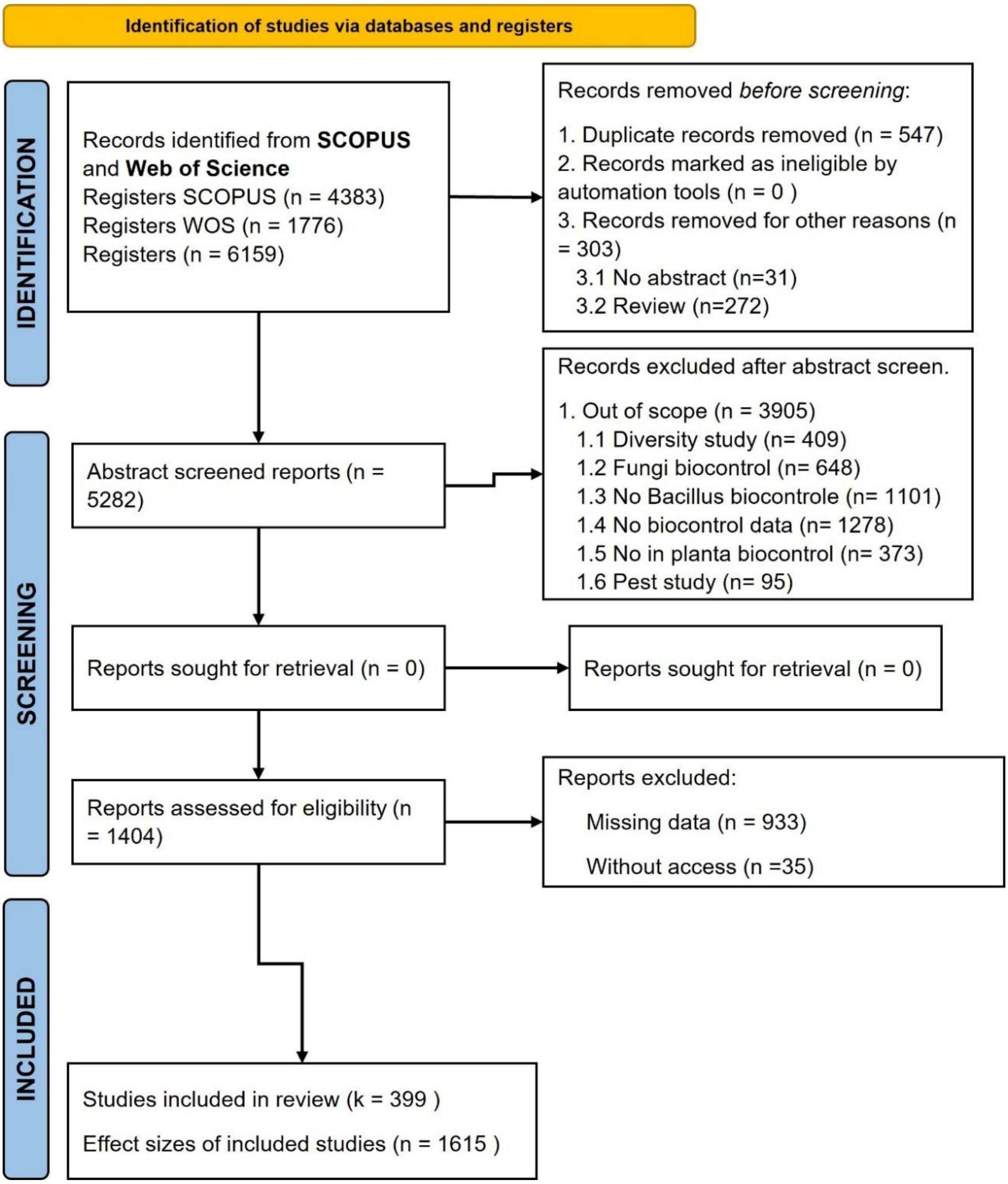

Figure 1 PRISMA 2020 flow diagram for new systematic reviews and meta-analysis including searches of databases and registers

Data on species of strain, plant species, pathogen species, type of pathogen and disease, environmental conditions, year and inoculation method were collected from the studies. Values from the response variables were extracted from graphical representations using the metaDigitise package in R software v4.0.2 (R Development Core Team. 2015) when not available in the main text. When the strain was inoculated before or at the same time as the pathogen, the effect was considered protective. Inversely, when the inoculation of the strain occurred after the pathogen, it was considered a therapeutic effect. In studies that tested different concentrations of the inoculum, the lowest and the highest concentrations were collected. For time-series experiments, data from the last timepoint were used. Experiments with commercial products, lipopeptides, proteins and supernatant of *Bacillus* species were identified to compare the size of the effect with tested strains.

Statistical methodology

The synthesis of primary studies' results is made in meta-analysis by approximating the results of different studies to a common scale. The quantity that makes such a transformation of studies' results is known as effect size, which quantifies the magnitude of a treatment or intervention across multiple studies (Borenstein et al., 2009; Gurevitch et al., 2018). It provides a standardized metric to express the strength and direction of the relationship between variables under investigation. The effect sizes summarizing studies' outcomes on a common scale were calculated as the log response ratio (LRR) using the equation $LRR = ln\frac{Vb}{Vc}$, where Vc is the mean of the control trials and Vb is the mean of the inoculated trials, also called biocontrol trials (Hedges et al., 1999). A positive effect size corresponds to an increase in the disease indicator following biological control application, and a negative effect size corresponds to a decrease in disease measures. Using LRR as a measure of effect size is highly recommended in this case, because severity and incidence can be measured on different scales, so a standardized index such as LRR is adequate, and the log transformation maintains the symmetry of the ratio measuring the study outcomes across different types of disease reports (Borenstein et al., 2009; Rosenberg et al., 2013). The variance of LRR (V_{LRR}) was calculated using: $V_{LRR} = \frac{SD_b{}^2}{N_b \times \overline{x_b}^2} + \frac{SD_c{}^2}{N_c \times \overline{x_c}^2}$, where SD is the standard deviation, N the sample size and $\overline{x}$ the mean for the inoculation (b) and control treatments (c) (Lajeunesse, 2011). The precision of the observed effects was accessed by interpretation of confidence intervals (Borenstein et al., 2009; Gurevitch et al., 2018; Cooper et al., 2009). For this, the 95% confidence intervals ($CI_{95\%}$) from each study were calculated with the usual equations: $CI_{95\%}$ = effect size ± 1.96 × SE, where SE was the square-root of V_{LRR}. For each study, an effect size and associated variance were calculated by the "escalc" function from the "metafor" package in the R environment (Viechtbauer, 2010).

The weighted average of all studies' effect sizes is called accumulated effect size. We weighted each effect size by the inverse of its variance. This procedure of weighting is done in meta-analyses to give a higher weight when estimating an average or another parameter for more precise studies (Borenstein et al., 2009). The accumulated effect size (weighted average of all studies' outcomes; *wES*) was computed by fitting a random-effects multilevel model (Nakagawa and Santos, 2012), where the parameters were estimated by restricted maximum likelihood (REML). The $CI_{95\%}$ for the wES was estimated as: $CI_{95\%}$ = wES ± 1.96 × SE. Cochran's Q test was used to quantify the heterogeneity between the studies in the random-effects model; the Q test assesses whether the observed variability in effect sizes is consistent with what would be expected by chance alone. Multi-level meta-analysis was applied to deal with multiple effect size estimates from the same study (Nakagawa and Santos, 2012). The multi-level meta-analysis was estimated using the "rma.mv" function in the "metafor" package (Viechtbauer, 2010). To facilitate the interpretation of results, back transformation was applied using the formula $\Delta\% = 100 \times (e^{LRR} - 1)$. This back transformation was made to return the effect size measured in log-scale of a given study to the relative scale representing the percentual reduction of disease.

The moderators were chosen based on the actors involved in experimental procedures in biocontrol trials: the bacterial inoculum, the plant host, the target pathogen and the type of disease measure, and are defined as follows:

1. Experimental moderators relative to bacterial inoculation: *Bacillus* species, concentration (High, single, or low), type of inoculation (protective or therapeutic), commercial bioproducts as comparative controls and type of biocontrol agents (cell suspension, fermentation broth, supernatant or lipopeptides).
2. Experimental moderators relative to the plants' host: plant species, growth environment, inoculation method;
3. Experimental moderators relative to the pathogens: pathogen species, pathogen type and type of disease;
4. Experimental moderators relative to measure of disease: disease severity or disease incidence.

The random-effects multi-level models described above were also used to test if the moderators can explain the variations in the effect size. In the case of the species of bacteria, host and pathogens, the most frequent agents in each class were used as moderators because of the low number of studies using the remaining agents. In order to explore the effects of moderator variables, a full model with all moderators available was fitted, and then the effect of each moderator was explored separately to detail the results.

The potential presence of publication bias in meta-analysis results was assessed using a funnel plot built in the ggplot2 (Wickham, 2016) package relating the effect size and its respective $\sqrt{Vi}$ for each study, followed by trim fill analysis using the “trimfill” function of the “metafor” package (Viechtbauer, 2010). Rosenthal fail-safe numbers were calculated by the “fsn” function of the “metafor” package (Viechtbauer, 2010) to estimate the number of studies that should be included to change the significance of the meta-analysis. If the fail-safe number exceeds 5k+10 (where k is the number of studies in the meta-analysis; Rosenthal, 1979), the evidence-of-bias publication was rejected (Rosenthal, 1991). Also, a cumulative meta-analysis was performed to evaluate the evolution of the evidence over time, assessing the impact of each new publication on the estimate of the biocontrol effect (Lau et al., 1995). The cumulative meta-analysis was executed by the “comu” function of the “metafor” package (Viechtbauer, 2010).

The meta-analysis and data representation were done in R software v4.0.2 (R Development Core Team, 2015), with “base” and “metafor” v4.2.0 (Viechtbauer, 2010), “ggplot2” v3.4.2 (Wickham, 2016) and “networkd3” (Allaire et al., 2017) packages.

Results

General summary and effect size

After the two screening steps, the initial dataset of 6159 articles was reduced to 399 peer-reviewed experimental articles. The results of the two screening steps are described in Supplementary Tables S1 and S2. The systematic review was carried out on 1615 experimental trials contained in the 399 articles of the final dataset. Almost 60 identified species of the Bacilli group are used as biocontrol agents; *Bacillus* is the most studied genus, followed by *Peanibacillus*. *Bacillus subtilis* and the operational group *Bacillus amyloliquefaciens* are the most studied species, representing 42% of the studies. A diversity of 107 species of host plants were collected in the dataset, of which *Solanum lycopersicum* was the most inoculated species, representing 15% of all studies. Rice and wheat have a high incidence as well. *Fusarium oxysporum*, *Botrytis cinerea* and *Magnaporthe oryzae* are the most common pathogens, representing 13.2%, 7.7% and 5.5% of all experimental trials, respectively. A total of 143 species of different pathogens were the focus of the experiments.

There is great diversity in the experimental design, with 599 different combinations of *Bacillus* inoculant, pathogen and host. Most combinations between pathogens, isolates and plants appear only once. The combination of *Bacillus subtilis*, *Solanum lycopersicum* and *Fusarium oxysporum* was the most frequent, present in 20 experimental designs (number of studies n= 20 (Figure 2). Some pathogens only affect specific crops, *Magnaporthe oryzae* is only studied in rice and *Puccinia striiformis* is only studied in wheat.

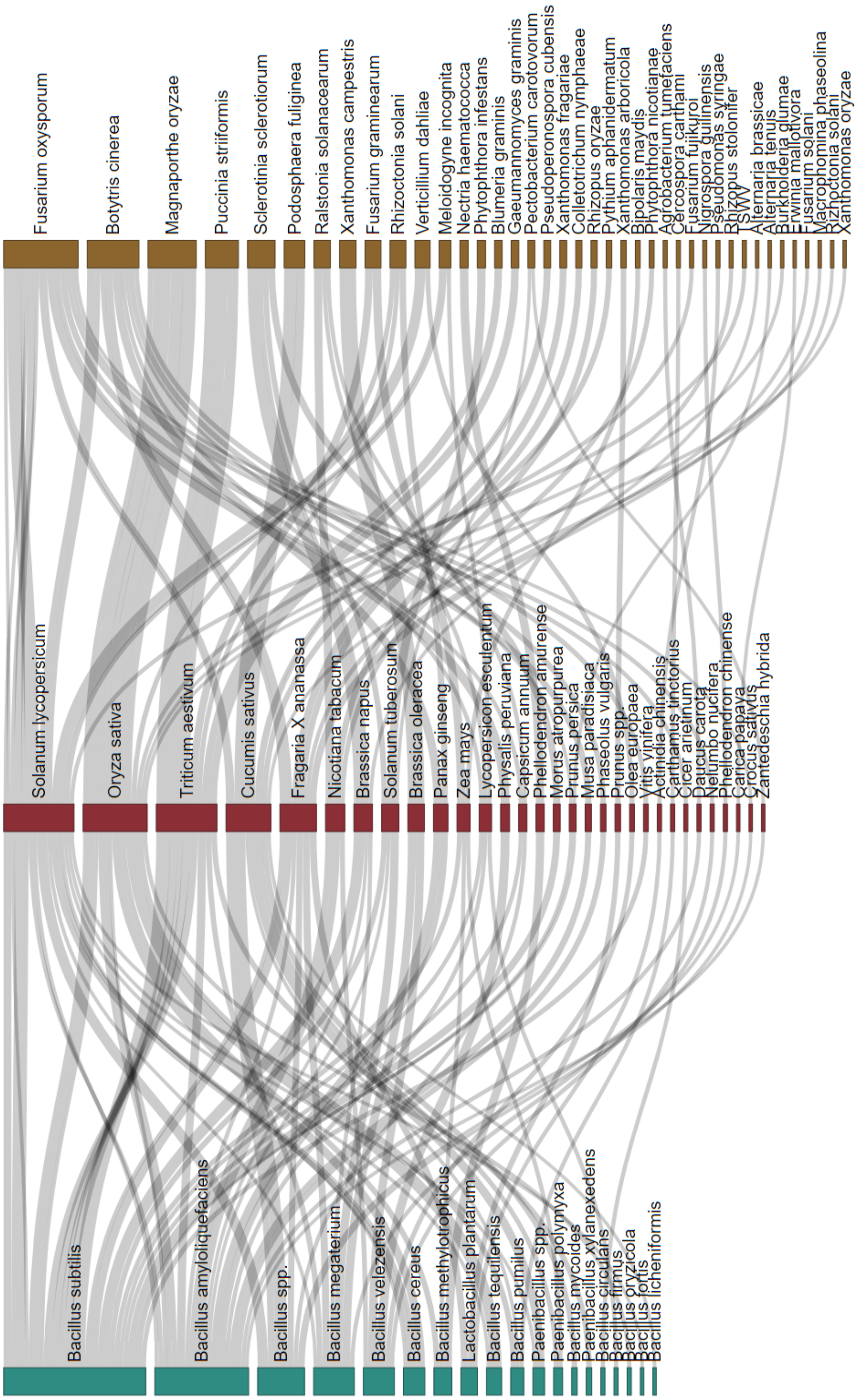
Fusarium oxysporum
Botytris cinerea
Magnaporthe oryzae
Puccinia striiformis
Sclerotinia sclerotiorum
Podosphaera fuliginea
Ralstonia solanacearum
Xanthomonas campestris
Fusarium graminearum
Rhizoctonia solani
Verticillium dahliae
Meloidogyne incognita
Nectria haematococca
Phytophthora infestans
Blumeria graminis
Gaeumannomyces graminis
Pectobacterium carotovorum
Pseudoperonospora cubensis
Xanthomonas fragariae
Colletotrichum nymphaeae
Rhizopus oryzae
Pythium aphanidermatum
Xanthomonas arboricola
Bipolaris maydis
Phytophthora nicotianae
Agrobacterium tumefaciens
Cercospora carthami
Fusarium fujikuroi
Nigrospora guilinensis
Pseudomonas syringae
Rhizopus stolonifer
SWV
Alternaria brassicae
Alternaria tenuis
Burkholderia glumae
Erwinia mallotivora
Fusarium solani
Macrophomina phaseolina
Rizhoctonia solani
Xanthomonas oryzae
Solanum lycopersicum
Oryza sativa
Triticum aestivum
Cucumis sativus
Fragaria X ananassa
Nicotiana tabacum
Brassica napus
Solanum tuberosum
Brassica oleracea
Panax ginseng
Zea mays
Lycopersicon esculentum
Physalis peruviana
Capsicum annuum
Phellodendron amurense
Morus atropurpurea
Prunus persica
Musa paradisiaca
Phaseolus vulgaris
Prunus spp.
Olea europaea
Vitis vinifera
Actinidia chinensis
Carthamus tinctorius
Cicer arietinum
Daucus carota
Nelumbo nucifera
Phellodendron chinense
Carica papaya
Crocus sativus
Zantedeschia hybrida
Bacillus subtilis
Bacillus amyloliquefaciens
Bacillus spp.
Bacillus megaterium
Bacillus velezensis
Bacillus cereus
Bacillus methylotrophicus
Lactobacillus plantarum
Bacillus tequilensis
Bacillus pumilus
Paenibacillus spp.
Paenibacillus polymyxa
Bacillus mycoides
Paenibacillus xylanexedens
Bacillus circulans
Bacillus firmus
Bacillus oryzicola
Bacillus fortis
Bacillus licheniformis

Figure 2 Sankey plot for the experimental setups included in the meta-analysis. The green group indicates the Bacilli species. The red group indicates the species of plants present in the dataset. The brown group shows the species of pathogens. The combination of *Bacillus subtilis, Solanum lycopersicum* and *Fusarium oxysporum* was the most frequent, with a total of number of experiments (n) =20, and the combinations with n < 5 were omitted for simplicity

It was possible to estimate 1615 effect sizes from the 399 studies. The efficacy of disease indicator outcome when using biocontrol compared to reference varied from – 6.19 to 1.61 in Log response ratio (Figure 3). In general, Bacilli biocontrol agents reduced plant diseases significantly (*wES* ± $CI_{95\%}$= -0.937 ± 0.061, p<0.0001, k = 399, number of effect sizes (n) = 1615). The percent change of the accumulated effect size indicated that inoculation of *Bacillus* and related species reduced disease by an average of 60.80%. Cochran's Q test was used to determine the residual heterogeneity in the random effects model, with the residual heterogeneity of the complete dataset using the meta-analysis multi-level being Q (df = 1614) = 112.9700, p < 0.0001.

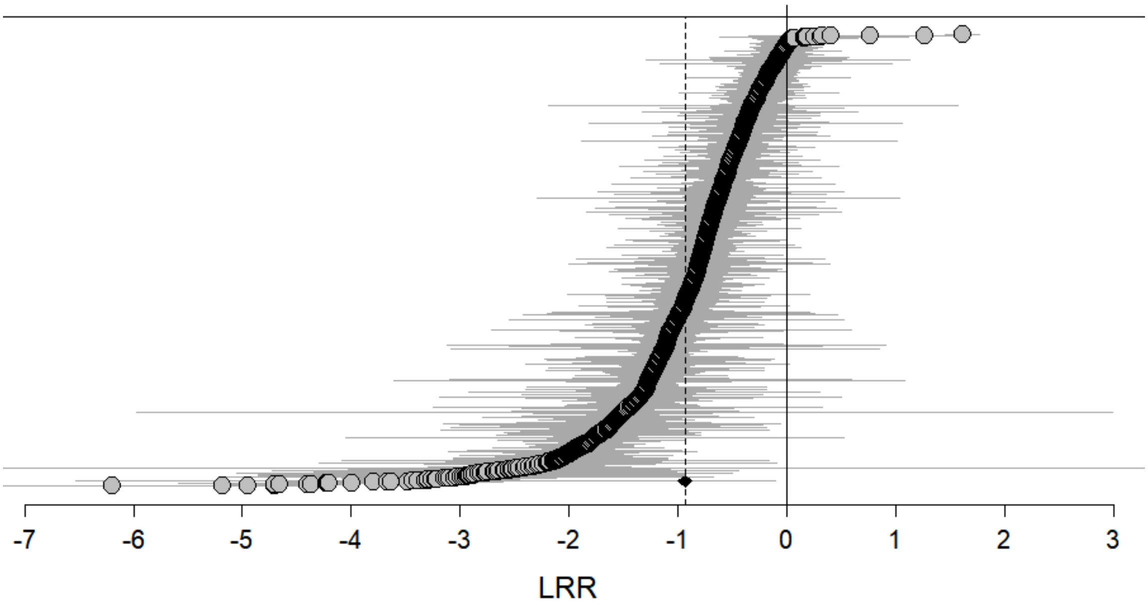

Figure 3 Disease reduction using *Bacillus* inoculation quantified by log Response Ratio (LRR). Negative values indicate a greater reduction of a disease indicator in the *Bacillus* inoculation than in the reference treatment. Circles and gray horizontal bars indicate LRR estimates for each experiment and their respective 95% confidence interval (CI_{95}). The diamond indicates the accumulated effect size (*wES*) estimate and its CI_{95}. The dashed and continuous vertical lines show the *wES* and a zero-effect size, respectively. Experiment outcomes (n=1615) are ordered by increasing LRR estimate

Experimental moderators relative to pathogens

Pathogens causing soilborne diseases constitute about half of the observations (n = 850), foliar disease accounted for 36% of all the observations (n = 588) and experimental data about fruit decay 11% of the observations (n = 177). The inoculation of Bacilli agents has a strong effect on all types of diseases, with the estimate for soilborne disease and foliar disease reduction 59.4% and 60.8%, respectively (Figure 4A). Although a high effect of Bacilli on the prevention of fruit decay (68.11%) was observed, there was no significant difference between the three types of disease in the meta-analysis, which means that the type of disease cannot explain the variation in effect sizes in the model (Q_M = 5.7422, p = 0.057; Figure 4A).

Experiments with fungi include most of the data, namely 72.1% (n = 1165), followed distantly by bacteria with 14.9% (n = 240), oomycetes with 6.75% (n = 109), nematodes with 3.9% (n = 63) and, lastly, viruses with only 2.23% (n = 36) (Figure 4B). The size of the accumulated effect of pathogen type varies between -0.96 in fungal pathogens and -0.81 in nematodes, which is equivalent to a reduction between 61.7% and 55.6% in disease parameters. However, there is no significance in the difference between the mean effect sizes of subgroups of type of pathogens, meaning that Bacilli inoculation is equally effective on the different types of target phytopathogens ($Q_M = 2.0197$, $P = 0.732$) (Figure 4B).

As mentioned above, a total of 143 species of different pathogens appear in the dataset, so a test was done to explore whether the species of pathogen explained the variation in the effect size data. The ten most common pathogens of all types were used as moderators; they represent more than 50% of the observations. The Bacilli biocontrol agents reduced the disease in all ten species of pathogens, but none differed significantly from another ($Q_M = 8.8693$, $p = 0.44$). The two most studied species of fungi and bacteria are *Fusarium oxysporum* (n = 214) and *Ralstonia solanacearum* (n = 75), respectively. *Bacillus* and related species effectively reduce the impact of *Fusarium oxysporum* by 58.04%, while diseases caused by *Ralstonia solanacearum* were reduced by 58.41% (Figure 4C).

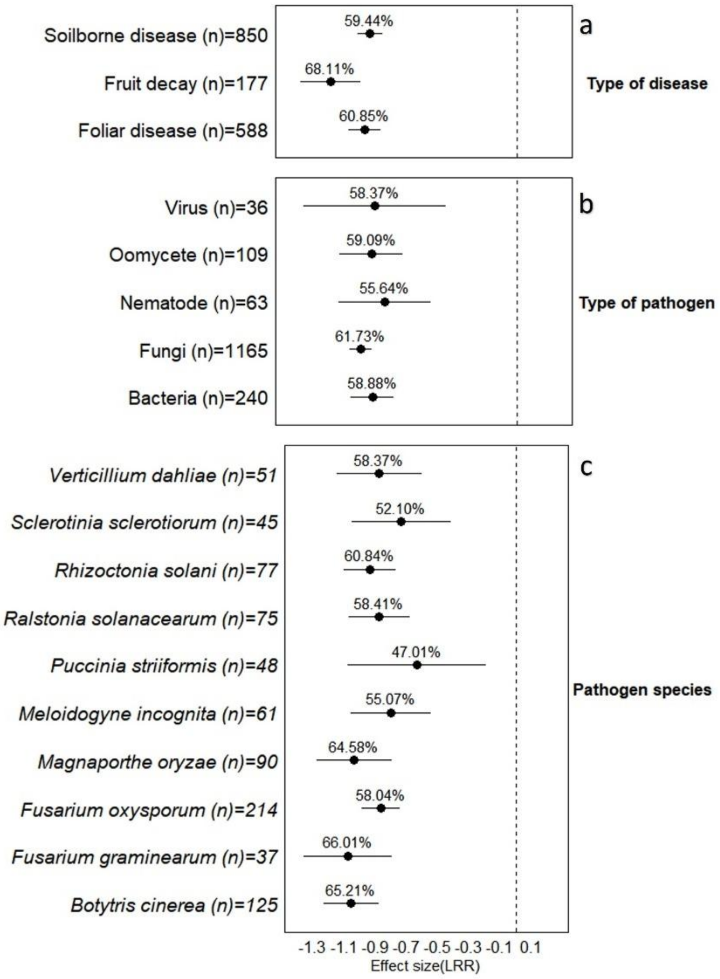

Figure 4 Average weighted effect size by subgroups of Type of disease (A), Type of pathogen (B) and Pathogen species (C). X-axis shows the effect size estimate as the log Response Ratio (LRR). Negative values indicate a greater reduction of a disease indicator in the *Bacillus* inoculation than in the reference treatment. The circles and horizontal bars indicate average weighted effect sizes and their respective 95% confidence intervals (CI_{95}). The dashed vertical line indicates a zero effect. The percentage above the bars shows the reduction of disease due to the Bacilli biocontrol agents, which means a positive effect on the biocontrol of plant diseases. The number of observations is indicated by n

Experimental moderators relative to the plant host

The experiments with Bacilli biocontrol agents were conducted in four types of environments: greenhouse, field, growth chambers and laboratory conditions, also referred to as room conditions. About half of the experiments were conducted in a greenhouse (n = 811) and around 16% in the field (n = 413) (Figure 5A). The environmental setup of the trials can also explain the variation in the data (Q_M= 9.3366, p = 0.025). Biocontrol trials under greenhouse conditions have 60.5% biocontrol efficacy against 57.1% in field conditions, but there is no statistical significance between field and greenhouse environments. However, there is a slight difference between the experiments' efficacy in room conditions, with 65.7% (n = 258), and in field conditions (Figure 5A).

The plant inoculation method is the methodological moderator that varies the most among those used. The different plant inoculation methods used in the dataset were classified into six categories. Seed soaking (n = 371) and foliar application (n = 549) are the two most common methods, comprising more than half of the studies (Figure 5B).

Bacillus and related species exhibit a significant variation in biological control efficacy toward different methods of inoculation (Q_M= 10.7764, p = 0.029). Biocontrol using postharvest fruit inoculation (n = 123) displays a high level of efficacy, at 72.05%, in contrast with the biocontrol efficacy of soil drenching (n = 357) which shows the lowest effectiveness of all methods, 57.5%. All the other methods presented an average of 60% effectiveness (Figure 5B).

To evaluate the competence of the interaction of *Bacillus* agents with a specific host, we used the most frequent plant species as moderators of biocontrol efficacy. From the 107 plant species, we considered the 10 most frequent to explain the variation in the dataset, and it was concluded that there is no evidence that plant species can drive the biocontrol properties of Bacilli species (Q_M= 6.2580, p = 0.713). Disease was reduced by 64.25% in *Solanum lycopersicum*, based on 248 observations (n = 248). The highest disease suppression occurred in *Fragaria x Ananassa* with 68.65% and the lowest occurred in *Solanum tuberosum* with 53.6% (Figure 5C).

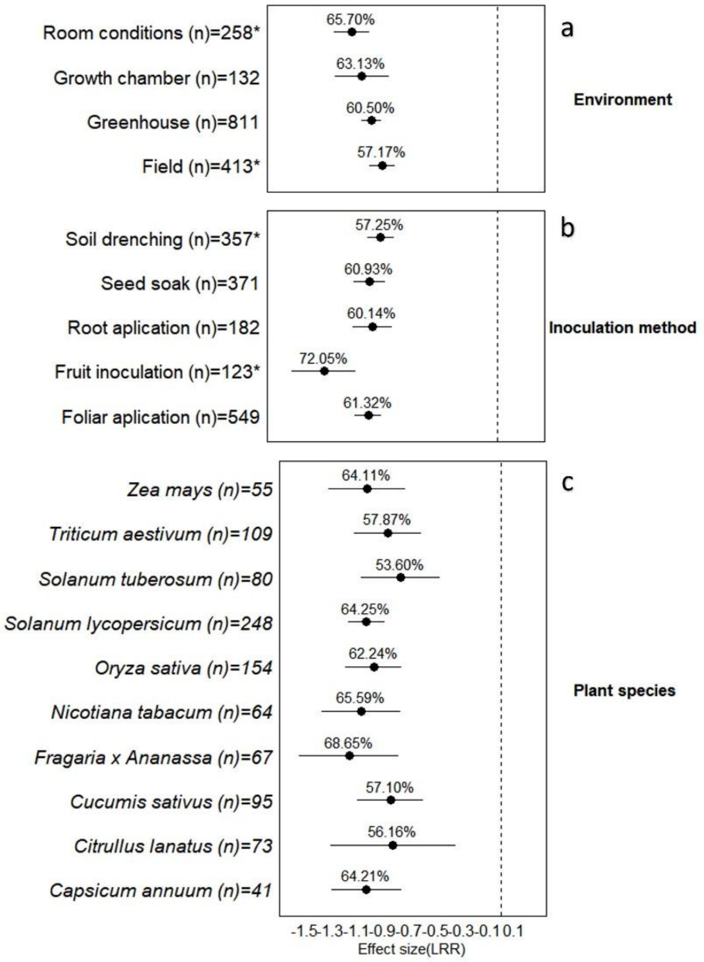

Figure 5 Average weighted effect size by subgroups of Environment (A), Inoculation method (B) and Plant species (C). X-axis shows the effect size estimate as the log Response Ratio (LRR). Negative values indicate a greater reduction of a disease indicator in the *Bacillus* inoculation than in the reference treatment. The circles and horizontal bars indicate average weighted effect sizes and their respective 95% confidence intervals (CI95). The dashed vertical line indicates a zero effect. The percentage above the bars shows the reduction of disease due to the Bacilli biocontrol agents, which means a positive effect on the biocontrol of plant diseases. The number of observations is indicated by n

Experimental moderators relative to Bacilli inoculum

A total of 85.5% of the observations are derived from studies testing single concentrations, with about 14.5% including dilutions in the volume of inoculum. The inoculum concentration exerts an important influence on the effectiveness of the biocontrol of *Bacillus* agents (Q_M= 65.1510, $p < 0.001$). In the experiments with serial dilution of bacterial suspension, the highest concentrations (n = 109) reduced plant disease by 69.6%, whereas the lowest concentrations reduced it by about 48.4% (n = 109), with an efficiency decrease of 21%. Studies with a single concentration in the experiments had a median effect of about 60% (Figure 6A).

Only 16.5% of the observations are therapeutic approaches, with the most popular being protective applications, 84.5%. The application type also explains the variation in the biocontrol efficiency (Q_M = 17.0959, $p < 0.001$). Protective treatments (n = 1348) reduced diseases by 62.3% compared to an average reduction of diseases by 53.5% by therapeutic management (n = 267, Figure 6B).

Only 17% of the experimental designs applied isolated extracellular metabolites as biocontrol agents. Free cell metabolic extracts (n = 95), fermentation products (n = 94) and lipopeptides (n = 37) are the most studied types of inoculums. Most studies used bacterial suspension in biocontrol trials (n = 1346). The nature of the inoculum, whether a fermentation broth, supernatant, lipopeptide or bacterial suspension, does not drive the variation in the data (Q_M= 4.5088, p = 0.211), meaning that inoculation of bacterial suspension, or not, has similar effects on *Bacillus* biocontrol efficacy (Figure 6C).

Studies assessing biocontrol efficiency using new strains present a higher average effect compared with commercial products based on *Bacillus* (Q_M = 8.1851, p = 0.042). Diseases were reduced by 43% in trials using commercial bioproducts (n = 126) and 61% when newly isolated strains were applied (n = 1489). However, it is noteworthy that *Bacillus* biocontrol agents still differ from the negative controls used in those experiments, whether they are newly isolated strains or commercial products (Figure 6D).

The seven most used species of Bacilli biocontrol agents, representing 67% of the observations, were analyzed to identify differential effects on disease management. *Peanibacillus polymixa* has the highest levels of disease reduction with 65.7% (n = 31) and *Bacillus pumilus* has the lowest biocontrol potential with 56.5% (n = 96). However, none of the species has a differential effect on disease biocontrol, as there is no statistical significance in the variations of disease reduction estimates toward any Bacilli species assessed (Q_M=5.0725, p = 0.534; Figure 6E).

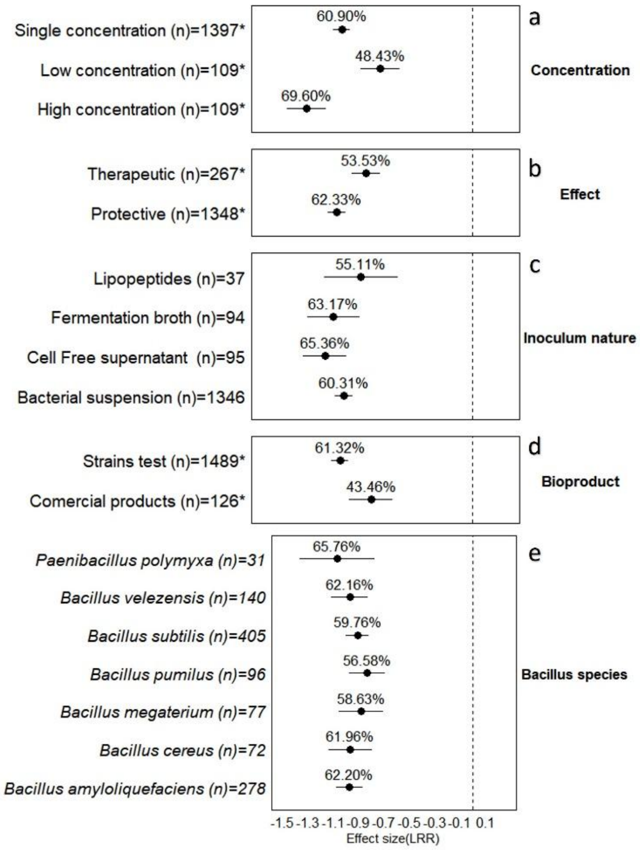

Figure 6 Average weighted effect size by subgroups of Concentration (A), Effect type (B), Inoculum nature (C), (D) Bioproduct and (E) *Bacillus* species. X-axis shows the effect size estimate as the log Response Ratio (LRR). Negative values indicate a greater reduction of a disease indicator in the *Bacillus* inoculation than in the reference treatment. The circles and horizontal bars indicate average weighted effect sizes and their respective 95% confidence intervals (CI95). The dashed vertical line indicates a zero effect. The percentage above the bars shows the reduction of disease due the Bacilli biocontrol agents, which means a positive effect on the biocontrol of plant diseases. The number of observations is indicated by n

Experimental moderators relative to disease measure

The type of disease measure assessed in the experiments does not influence the variation in study outcomes (Q_M= 1.0405, p = 0.307). The effect of bacilli inoculation on reduction of disease severity was 60.11% and disease incidence reduction was 62.7% on average.

Publication bias

The funnel plot shows a symmetrical distribution between the variation in effect size and the square root of the variance, and no study was included to balance the model by the trim fill method (Figure 7). The number of studies with non-significant results required to change the results of the meta-analysis was 24,353,833, greater than the cut-off of 2000. Taken together, the findings permit the result of the meta-analysis to be validated and conclude that publication bias has a marginal influence on variation of data.

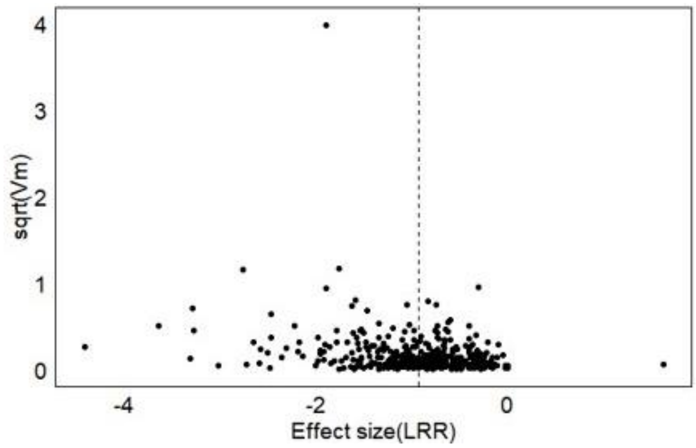

Figure 7 Funnel plot to evaluate the bias in the dataset of meta-analysis, showing the symmetrical distribution of the effect size around the dashed line, which represents the accumulated effect of -0.93. The x-axis shows the mean of LnRR per publication and the y-axis shows the root square of the variance sqrt (Vm)

The cumulative meta-analysis shows a great variation in the accuracy of the studies in the early years. In the initial years, the effect size of *Bacillus* biocontrol agents was higher, but the accuracy of the study was weak. Over the years, the evidence of biocontrol traits of *Bacillus* became stronger. The tendency stabilized after 2010, so the data range used in the search is appropriate to represent the literature in the Bacilli biocontrol area (Figure 8).

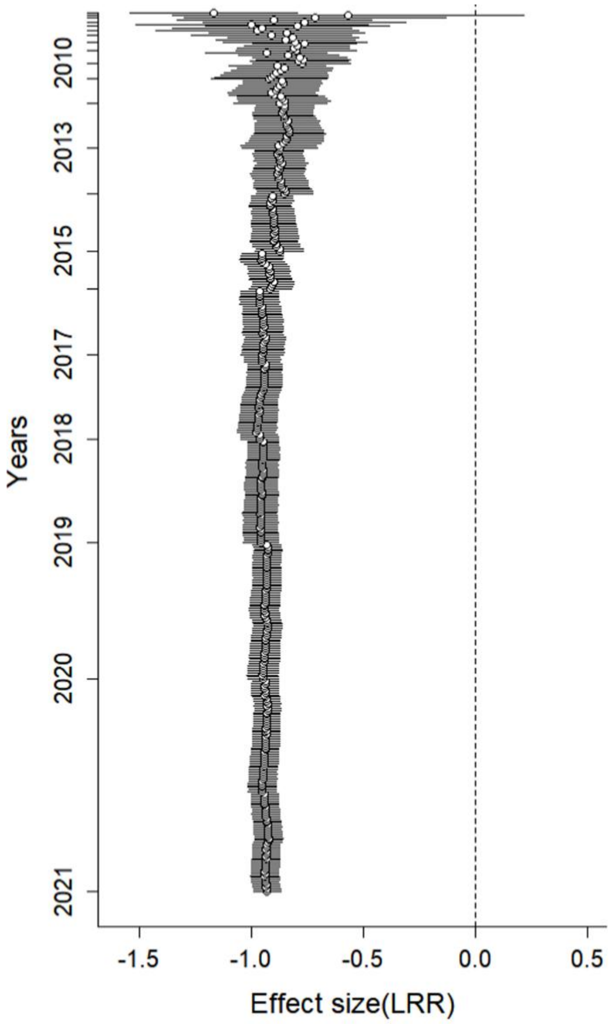

Figure 8 Cumulative Forest plot for size effect in disease reduction of Bacilli biocontrol agents. The dots and bars indicate log-scale effect sizes and their respective 95% confidence intervals. The vertical dashed line represents an effect size of 0; if the confidence interval of the effect sizes touches the dashed line, it means no difference between the Bacilli-inoculated treatments and the control treatments of the study. The x-axis presents the Log response ratio and the y-axis presents the years of the publications included in the study

Discussion

The main goal of this work is to use a meta-analysis to evaluate the biocontrol power of Bacilli and identify experimental factors that influence their efficacy in plant disease management. Although extensive literature already consolidates the role of *Bacillus* species in biocontrol, an updated overview of the area of knowledge was necessary. Overall, this meta-analysis confirms the strong potential of *Bacillus* and some related species for the biocontrol of plant diseases via systematic synthesis and statistical methods, using a dataset of more than 1600 trials from 399 publications. Our meta-analysis not only confirms the potential of Bacilli agents but also provides analysis of a broader panel of host species, pathogens and experimental setups already extensively used in the field.

Bacillus agents have had their biocontrol potential extensively verified and revised (Luo et al., 2022; Rabbee et al., 2023). The hi-seq genomic approach confirms the vast secondary metabolism of the group (Dunlap et al., 2019; Kiesewalter et al., 2021). Moreover, RNA sequencing data confirm that *Bacillus* strains also drive gene expression in host plant defense to respond to pathogen inoculation (Cheng et al., 2022; Portieles et al., 2021). Overall, these biocontrol traits lead to a reduction of 60.8% in disease indicators based on the effect sizes of all studies included in the meta-analysis. Similar results were found in the biocontrol of fungal diseases by Actinomycetes in the genus *Streptomyces,* with 56% disease reduction (Leblanc et al., 2022). In another report, a meta-analysis of *Aphanomyces* root rot biocontrol shows higher disease reduction by bacteria of the *Bacillus* genus as well (Godebo et al., 2022). In addition, the effect of a varied group of biocontrol agents was evaluated in reducing bacterial wilt, with a reduction in the global effect of more than 49%. However, species of the genus *Bacillus* had a lower effect compared to *Pseudomonas* (Chandrasekaran et al., 2016).

In the context of Bacillus bioinoculants, the present meta-analysis reveals a notable overall increase in effectiveness. However, it is imperative to approach these findings with a critical perspective. While the aggregated data may suggest higher efficacy, several studies show great variation in the effectiveness of biocontrol exerted by *Bacilli* species. Considering a specific study involving 20 strains of *B. cinerea*, which varied in geographic origin, host of isolation, and aggressiveness levels, the protective effectiveness of *B. subtilis* QST713 was observed to span from 40% to 86% on tomato leaves and from 0% to 80% on lettuce leaves (Bardin et al., 2013). In addition, *B. amyloquofaciens* and *B. subtilis* were effective in controlling clubroot caused by *Plasmodiophora brassica* in cabbage, but not in canola under field conditions (Peng et al., 2011). In a different investigation, Serenade demonstrated significant effectiveness in controlled conditions, leading to a nearly 90% reduction in early blight caused by *Alternaria solani* in potatoes. However, the impact diminished considerably in hand-sprayed field trials, resulting in a disease reduction of 20–25%. Notably, the effects nearly disappeared in full-scale field trials conducted with tractor sprayers (Stridh et al., 2022). Those findings exemplify the variability in outcomes that contributes to the overall critical assessment of *Bacillus* bioinoculants, emphasizing the need for a nuanced interpretation of their efficacy in diverse settings.

The results show that none of the moderators related to pathogens explain the variation in results of studies on the efficacy of biocontrol of *Bacillus* and related species against diseases. Based on the high number of studies, *Bacillus* species have a broad range of action and mitigate the deleterious effects of a diverse group of phytopathogens equally.

There is no statistical difference between the uses of *Bacillus* in different disease types. Fruit decay undergoes the highest reduction among disease types, even though most studies were aimed at soilborne diseases. Previous reports show that *Bacillus* biocontrol agents have a lower effect on soilborne diseases. Ojiambo and Scherm (2006) conducted a meta-analysis of different plant biocontrol agents, and no differences in biocontrol effectiveness were found between soil-borne and aerial diseases in the total meta-analysis dataset, but a low effectiveness was found when the analysis was carried out using only *Bacillus* agents against soilborne diseases. This discrepancy between our results and the literature can perhaps be explained by the difficulty in obtaining a reliable classification of disease typing from different publications. The study also corroborates the finding that there is no significance in the effect of biocontrol agents on the different types of pathogens, like fungi, bacteria and others (Ojiambo and Scherm, 2006).

Fungi were the most studied pathogen group, while viruses had only few studies. Although there are studies that prove the antiviral properties of *Bacillus* strains, there are still no commercial bioproducts to control plant viral diseases based on *Bacillus* on the market (Manjunatha et al., 2022), which illustrates the requirement for further studies and optimization of application methodologies. In plants, one of the main responses to viral infections of biocontrol agents is the production of extracellular ribonucleases (Mandadi and Scholthof, 2013). The production of extracellular ribonucleases, together with induction of systemic resistance, are the main mechanisms of control of viral diseases by biocontrol agents like *Bacillus* (Veselova et al., 2022; Sorokan et al., 2020). More studies of Bacilli biocontrol agents on the management of plant viral diseases can overcome this gap in the field of research.

In the subgroup relative to the plant's role in biocontrol trials, the testing environment explained the variation in the dataset. Most experimental settings involve greenhouse and field studies and there is no statistically significant difference between these two categories, although field studies have a lesser effect on disease reduction. There is a significant reduction in disease biocontrol efficacy in the experiments conducted in laboratory conditions and field conditions. The results confirm that *Bacillus* biocontrol agents are effective both in the greenhouse and in the field. The trials involving laboratory conditions include detached leaf assays, fruit protection and *in vitro* plant growth. This type of setup is very similar to *in vitro* conditions of antagonism, and it is a poor predictor of biocontrol effects in the field (Kaminsky et al., 2019), which explains the slight difference between the two environments. Numerous studies report a reduced efficacy in field applications against controlled greenhouse and/or *in vitro*-like conditions (Bardin et al., 2015; Leblanc, 2022). The factors that explain the variation in effectiveness are generally climate conditions in field experiments, a lack of ecological competence of the biocontrol agent, intrinsic traits of the antagonistic microbe and the poor quality of bioproducts (Kaminsky et al., 2019; Abd-Elgawad and Askary, 2020; Collinge et al., 2022; Bardin et al., 2015).

Additionally, the soil-drenching inoculation method was least efficient in biocontrol trials, differing significantly from fruit inoculation. The complex network of interactions in the soil community may explain this. Experiments using high-throughput metagenome sequencing show that inoculation of *Bacillus* positively or negatively affects the diversity of the microbiome, which may affect the biocontrol efficacy in the trial (Gu et al., 2022; Kröber et al., 2014; Fan et al., 2023; Mahapatra et al., 2022). Fruit inoculation had the highest level of disease suppression. The ability of biocontrol agents to control post-harvest diseases of fruits and vegetables is well documented and it is carried out in two main mechanisms: (i) competition for nutrients and space, (ii) via production of metabolites with antibiotic properties (Irtwange 2005; Sharma et al., 2009). *Bacillus* species have a diverse secondary metabolism and are excellent competitors, which explains their wide application in the prevention of post-harvest diseases (Lastochkina et al., 2019).

There is no statistical difference between the size effect in the different species of plants. Some studies show the variation in the biocontrol effect across different plant groups. The efficacy of fungal biocontrol agents in controlling root-knot nematodes varies significantly across different crops (Peiris et al., 2020). Ojiambo and Scherm (2006) and Chandrasekaran et al. (2016) also suggest that microbial biocontrol agents are more effective in perennial crops than in annual crops. These studies group different plant species by the characteristics of their cultures; perhaps grouping the species of the *Bacillus* dataset can help explain the variation in the data. These analyses show that moderators relative to plant hosts have a small influence on the variation of data. The difference between the experimental categories is minimal, and the *Bacillus* species can similarly protect all the plant species.

Three of the five moderators related to bacterial inoculum explained the variations in the data. These findings illustrate how to optimize the effects of *Bacillus* inoculation by means of the experimental design. The different types of inoculum concentration or dosage explained the variation in the size effect. In studies with serial dilutions, the higher concentration achieved better results in biocontrol data than the low concentrations. Wang et al. (2022) suggest that concentrations of 10^6 and 10^7 CFU/mL did not achieve suitable inhibitory activities and recommend higher concentrations for appropriate effect. The interpretation of these outcomes requires caution, and consideration must be given to the concentration of *Bacillus* when utilizing it as a biofertilizer, as higher concentrations can influence biogeochemical cycles, the carbon sequestration process, and humus production (Mahapatra et al., 2022). Másmela-Mendoza and Moreno-Velandia (2022) provided evidence of such detrimental effects attributed to the concentration of the *B. velezensis* Bs006 inoculum. The authors demonstrated that elevated concentrations of the bacterial suspension and supernatant led to alterations in the tomato rhizosphere community by depleting carbon sources, resulting in a reduction of the strain's biocontrol efficacy against Fusarium wilt. Additionally, while *Bacillus* species are commonly known for their beneficial roles in promoting plant health and biocontrol, there is emerging evidence suggesting that under certain conditions, some strains within the *Bacillus* genus may cause plant diseases (Aly et al., 2022; Jin et al., 2022; Wang et al., 2017).

Therapeutic inoculation has an 8% reduction in biocontrol efficacy compared to protective inoculation. The dynamics of the occupation of plant space and nutrients is an important factor in biocontrol. It has been suggested that *Bacillus* bacteria can colonize tissues, occupy space and deplete nutrients before pathogen infection, thus gaining competitive advantages (Perea-Molina et al., 2022; Legein et al., 2020; Wang et al., 2022). Studies demonstrated that the modulation of the secondary metabolism is induced upon interaction with pathogens, which reveals competitive efficiency regarding antibiosis traits (Andrić et al., 2020). Additionally, *Bacillus* strains produce siderophores, form biofilms and show significant motility (Blake et al., 2021; Bais et al., 2004), which are attributes directly involved in niche occupancy in plants. The efficiency in modulating their metabolism for antibiosis, the ability to induce host defenses and competence in occupation/persistence in the environment are characteristics that highlight the greatest potential of protective inoculations in the biocontrol of plant diseases.

There is no difference in biocontrol efficacy between the different types of biocontrol agents, with the most efficient inoculum being the Cell free supernatant of *Bacillus*. Most of the studies used bacterial suspension in biocontrol trials, besides lipopeptides or supernatant. Wang et al., (2020) found higher biocontrol efficacy in isolated metabolites of *Bacillus* species but also noted that most studies utilize all-bacterial suspensions. *Bacillus* species are endospore-forming bacteria, so they persist in latent form in the soil or inside the host for a long time (Yánez-Mendizabal et al., 2012; Mckenney et al., 2013). This feature is important to add value and develop bioproducts for application in the field (Martínez-Álvarez et al., 2016; Sepúlveda et al., 2020). That explains the use of bacterial suspension in biocontrol trials, besides other types of inoculums. It is also important to find alternative strategies to increase the persistence and production of biocontrol agents without bacterial suspension, such as improving culture medium, method isolation and quality of preparations.

The different species of biocontrol agents have a marginal effect on the variation of data. *B. subtilis*, *B. amyloliquefaciens* and *B. velezensis* are the three most used species of bacteria. These three bacteria are very closely related, and their taxonomic classification is undergoing updates (Soni and Keharia, 2021). Some strains of *B. subtilis* and *B. amyloliquefaciens* were reclassified into *B. velezensis* (Dunlap et al., 2016; Fan et al., 2018). Recently, Fan et al., (2017) proposed an updated classification for *B. amyloliquefaciens* as a taxonomic group above species level, assigned as "operational group *B. amyloliquefaciens*", consisting of three species of endospore-forming bacteria, the soil-borne *B. amyloliquefaciens*, plant-associated *B. siamensis* and *B. velezensis.* So, the taxonomic differences between species can be artificial artifacts, and their similarity is reflected in the effectiveness of biocontrol agents in the trials. In contrast, a comparison at the level of a genus of Bacilli bacteria may explain the variation in the dataset.

As a positive control, *Bacillus* commercial products were compared with newly tested strains. There is a significant reduction of 17% in the biocontrol efficacy of the bioproducts compared to tested strains. Cook (1993) suggests that arbitrary use of biocontrol agents like Serenade may result in poor efficacy in different crops. In addition, root colonization is more efficient in native strains of *Bacillus* compared to strains cultivated in a laboratory or those used commercially (Miljaković et al., 2020), which highlights the importance of endophytic or rhizospheric lifestyle traits of the strains. Furthermore, the resistance of the plant pathogens to plant symbiont bioinoculants is an underexplored field (De Souza et al., 2016). Theoretical evolution studies indicate that interactions with protective symbionts lead to raised virulence traits in pathogens, reducing host populations (Smith and Ashby, 2023), which means a reduction in biocontrol agent efficacy. General resistance mechanisms involve active efflux, metabolization of antimicrobial compounds, and interference with the regulation and biosynthesis of antimicrobial metabolites produced by biocontrol agents (Raaijmakers et al., 2009; Duffy et al., 2003). Studies conducted with *B. subtilis* CL27 shows that after eight successive treatments on plants of *Astilbe hybrida*, the protective effect against *B. cinerea* strongly decreased and, after ten treatments, the control became completely ineffective (Li and Leifert, 1994). Furthermore, fungal pathogens can produce mycotoxins capable of reducing the colonization efficiency of biocontrol agents. *Fusarium* spp. secondary metabolites are involved in disrupting quorum signaling in bacteria (Venkatesh and Keller, 2019), which may lead to a reduction in the presence of biocontrol agents in the plant microbiome, including Bacilli species (Zhang et al., 2021).

The results show that studies on Bacilli as biocontrol agents present outcomes that are more variable than expected by chance (Cochran's Q test). However, subgroup analysis showed that two moderators related to the host plant explained the variation in the data: the environment and the inoculation method. In addition, subgroups related to the inoculum are also a source of variation in the data, such as concentration, type of inoculation or commercial strains. Furthermore, none of the moderators relative to bacteria, host, or pathogens species explained the variation in the data, which means a general effect and wide range of interactions of Bacilli bacteria. The outcomes of this meta-analysis should be interpreted in the context of several limitations. A significant constraint is the exclusion of a substantial number of initially screened studies. Among the studies meeting the biological criteria, many lacked adequate statistical reporting, a common issue in meta-analysis data compilation (Ngugi et al., 2011). Also, another key challenge lies in the differences in study design, participant characteristics, and measurement methods among the included studies.

Additionally, publication bias is another limitation of meta-analysis, where studies with positive findings are more likely to be published, which can influence the overall effect size and lead to an overestimation of treatment effects. Publication biases were tested and rejected using multiple methods, and the cumulative meta-analysis showed that evidence of the effect size of *Bacillus* on disease biocontrol was established after 2015, when the scientific community was effectively studying the subject. Zhao et al., (2024) delineated three distinct stages in bioinoculant research using a bibliometric analysis: (I) an early phase characterized by q-exponential growth spanning from 1980 to 2005; (II) an intermediate period marked by linear growth from 2006 to 2015; and (III) a subsequent phase characterized by p-exponential growth from 2016 to 2022. Over the last six years alone, the count of annual publications about bioinoculants has surged significantly, rising from 668 in 2016 to 1,775 in 2022 (Zhao et al., 2024). Notably, the publications in 2022 represent a twentyfold increase compared to the output in 2000. This substantial growth emphasizes the increased interest and attention dedicated to research on biofertilizers in recent years and reflects the evolution of scientific evidence on the size of the effect of Bacilli species on biocontrol.

Concluding remarks

The present meta-analysis confirms the considerable efficacy of *Bacillus* and certain associated species in reducing plant diseases. This conclusion is portrayed from an extensive dataset comprising over 1600 trials sourced from 399 publications, revealing a noteworthy reduction of 60% in disease values.

Additionally, we examined the variability in biocontrol data by considering the primary agents utilized in biocontrol trials. This analysis involved moderators associated with pathogens, host plants, and variations in inoculation methodologies as subgroups. Our analysis reveals that the effect size of *Bacillus* biocontrol agents in plant disease management remains stable across pathogen-related subgroups, suggesting a consistent and generalizable pattern in different types of pathogens and diseases.

Moreover, the subgroup analyses, focusing on Plant Host-Related factors, reveal variations in the effectiveness of biocontrol agents based on the environmental context in which the studies were conducted. Our analysis reveals that under field experimental conditions, biocontrol efficacy is significant lower compared to controlled settings such as laboratories, which highlights the importance of considering the competence of the isolates in colonizing the host and the resilience of the strains in adverse conditions. Additionally, differences in inoculation methods within the Plant Host-Related subgroups demonstrate significant implications for the efficacy of *Bacillus* biocontrol.

In addition, subgroups associated with inoculum characteristics play a central role in the variation of data. Fluctuations in inoculum concentration and the nature of inoculation (therapeutic or protective) have been especially identified as noteworthy factors, influencing the effectiveness of *Bacillus* biocontrol agents in plant disease management. This implies that inoculations with elevated concentrations and a protective approach tend to enhance the biocontrol potential of Bacillus genus bacteria. Finally, our meta-analysis concludes that commercial Bacillus-based products exhibit a minor effect when compared to the impact observed with newly strains of *Bacillus*. Together, these results indicate that it is important to understand the biocontrol characteristics of the strains and to focus on the ecological capabilities of the agents to guarantee the effectiveness of the products in the field. Our findings contribute to a deeper understanding of the dynamics surrounding *Bacillus* biocontrol, providing valuable guidance for practitioners, researchers, and policymakers seeking effective and sustainable solutions in plant disease management.

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Declarations

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Competing interests

The authors declare no competing interests.

Data Availability

The data that support the findings of this study is provided within the manuscript or supplementary information files.

Author Contributions

Conceptualization: C.P. Serrão, J.C.G Ortega, P.C. Rodrigues and C.R.B. de Souza. Material preparation, data collection and analysis: C.P. Serrão and J.C.G. Ortega. Writing—original draft preparation: C.P. Serrão. Writing—review and editing: J.C.G Ortega, P.C. Rodrigues and C.R.B. de Souza. Reading and approval of the final version of the manuscript: C.P. Serrão, J.C.G Ortega, P.C. Rodrigues and C.R.B. de Souza.

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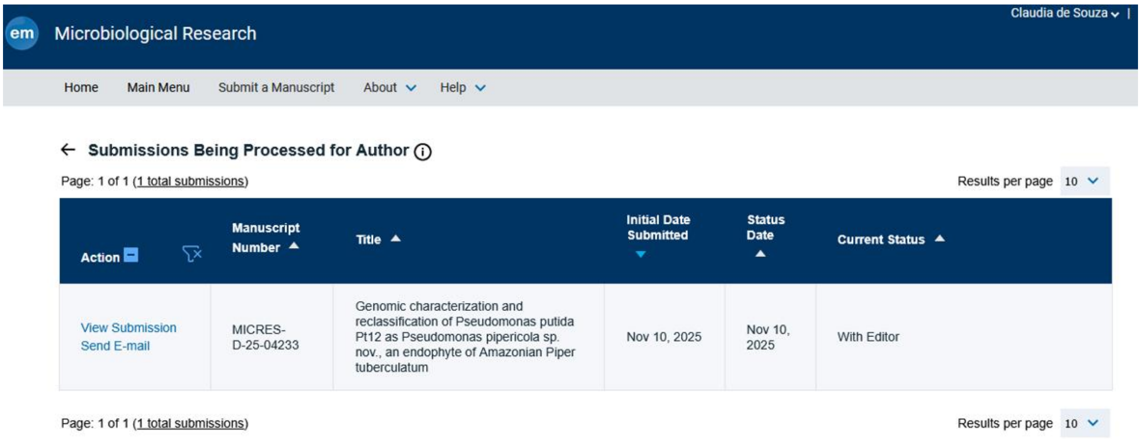
CAPÍTULO II

CARACTERIZAÇÃO GENÔMICA DAS ENDOFÍTICAS Pt12 ISOLADA DA RAIZ DE *Piper tuberculatum*

O capítulo II desta tese segue formatação da revista na qual o artigo foi submetido:



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Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status
View Submission Send E-mail	MICRES-D-25-04233	Genomic characterization and reclassification of Pseudomonas putida Pt12 as Pseudomonas pipericola sp. nov., an endophyte of Amazonian Piper tuberculatum	Nov 10, 2025	Nov 10, 2025	With Editor

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Genomic characterization and reclassification of *Pseudomonas putida* Pt12 as *Pseudomonas pipericola* sp. nov., an endophyte of Amazonian *Piper tuberculatum*

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Abstract

Black pepper (*Piper nigrum* L.) is a major tropical crop severely affected by fusariosis caused by *Fusarium solani* f. sp. *piperis*. In the absence of effective control methods, beneficial endophytic bacteria represent a sustainable alternative. In previous studies, the *Pseudomonas putida* Pt12 was isolated from roots of *Piper tuberculatum*, a Piperaceae occurring in the Amazon region with resistance to the pathogen infection. Moreover, Pt12 showed *in vitro* antifungal activity and plant growth-promoting traits; however, the genetic basis of these traits remains unclear. Thus, this study explores the genome of Pt12 through whole-genome sequencing and pan-genome analysis to uncover the genetic determinants of its biocontrol potential. Genomic DNA from the endophytic strain *Pseudomonas* sp. Pt12 was sequenced using the Illumina platform. The genome was assembled with Unicycler and annotated using the RASTtk and Prokka pipelines. Comparative and phylogenomic analyses were performed against related *Pseudomonas* species using ANI, MLSA, and pan-genome approaches. The Pt12 genome presents 6.14 Mb with GC 62.8% content and encodes 5,460 genes. Functional annotation revealed traits associated with root colonization, biocontrol, and plant growth promotion, including siderophore and indole-3-acetic acid biosynthesis, phosphate solubilization, and acetoin/2,3-butanediol metabolism. Genome mining identified 11 biosynthetic gene clusters and 17 genomic islands. Comparative analyses showed that Pt12 does not group withing *Pseudomonas* groups, suggesting a distinct lineage, supporting the reclassification of *P. putida* Pt12 as a new species, *Pseudomonas pipericola* sp. nov. Pan-genome analysis indicated a highly open genome with 433 strain-specific genes, including those related to motility, chemotaxis, and naringenin degradation. In conclusion, this endophyte harbors genes linked to root colonization, hormone synthesis, and antifungal activity. Its unique gene content and specialized metabolism suggest ecological adaptation as a mutualistic endophyte.

Keywords: *Pseudomonas*, Biocontrol, Endophyte, Genome, Plant niche

Introduction

Black pepper (*Piper nigrum* L.), widely known as the "King of Spices," is a tropical climbing plant from the Piperaceae family, native to India and cultivated worldwide for culinary, medicinal, and cosmetic purposes (Prabhakaran, 2001). In Brazil, its cultivation began over 200 years ago in backyard systems, expanding commercially in the 1950s with Japanese immigration to Tomé-Açu (Pará) and rising global market demand (Duarte et al., 2004). A major limitation in black pepper production is fusariosis, caused by *Fusarium solani* f. sp. *piperis*, which damages the plant's root system and reduces its lifespan (Tremacoldi et al., 2010). There are no chemical control or resistant *P. nigrum* cultivars available, and management in the Amazon relies solely on preventive practices. Thus, alternative and sustainable strategies are urgently needed (Tremacoldi et al., 2010). In this context, Nascimento et al. (2015) reported the identification of *Pseudomonas* strains Pt12 and Pt13, isolated from the roots of *Piper tuberculatum*, a Piperaceae occurring in the Amazon region with resistance to the pathogen infection (Albuquerque et al., 2001). *Pseudomonas* Pt12 and Pt13 have shown promising antifungal activity, inhibiting *F. solani* f. sp. *piperis in vitro* growth by up to 55% (Nascimento et al., 2015). Moreover, Pt12 also exhibited plant growth-promoting traits, such as indole-3-acetic acid (IAA) production, phosphate solubilization, and siderophore synthesis (Oliveira et al., 2021). *P. nigrum* plants inoculated with Pt13 have shown increased chlorophyll content, CO_2 assimilation, leaf area, and biomass (Pereira et al., 2019).

Pseudomonas is a diverse genus of Gram-negative, rod-shaped bacteria within the γ-proteobacteria. These organisms are highly adaptable, inhabiting a wide range of environments including soil, water, and plant tissues (Hesse et al., 2018). While some species, such as *P. aeruginosa* and *P. syringae*, are pathogenic to humans and plants respectively (Grace et al., 2022; O'Brien et al., 2011), most *Pseudomonas* spp. are beneficial, functioning as mutualists or commensals with potential for biocontrol, biostimulation, and bioremediation (Sah et al., 2021). Endophytic *Pseudomonas* strains are among the most commonly used bacteria in the biological control of plant diseases. Several species have been commercialized as bioproducts, such as Bio-Save©, BioJect Spot-Less©, AtEze© (Mehmood et al., 2023; Stockwell et al., 2007). Their effectiveness against *Fusarium*-induced root diseases in crops like wheat and tomato has been demonstrated under greenhouse and field conditions (Hu et al., 2014; Ibrahim et al., 2023; Miftakhov et al., 2023). In addition to *Fusarium*, *Pseudomonas* species have shown efficacy against other phytopathogens such as *Phytophthora*, *Botrytis cinerea*, and *Ralstonia solanacearum* (Yang and Hong, 2020; Wang et al., 2021; Clough et al., 2022). This broad-spectrum potential justifies deeper investigation of Pt12 and Pt13, particularly regarding their genetic mechanisms of plant growth promotion and biocontrol.

The *Pseudomonas* genus exhibits remarkable genomic plasticity, reflecting its ability to thrive in diverse habitats. A study analyzing 166 *Pseudomonas* genomes from various environmental sources reported genome sizes ranging from 3.03 Mbp (*P. caeni*) to 7.38 Mbp (*P. saponiphila*), with an average of 5.63 Mbp (Hesse et al., 2018). More recently, Girard *et al.* (2021) proposed 43 new species and highlighted the extensive genomic diversity within the genus based on 270 *Pseudomonas* genomes, ranging from 3.03 Mbp to 10.4 Mbp. These findings underscore the vast genomic variability within *Pseudomonas*, especially among endophytic strains. Comparative genomic studies have revealed extensive pan-genomes across different clades, with core genes complemented by large accessory gene pools linked to host colonization, antimicrobial metabolites production, and environmental adaptation (Hesse et al., 2018; Girard et al., 2021). For example, only ~1% of genes in the pan-genome of 1,311 *P. aeruginosa* strains were conserved across all isolates (Freschi et al., 2023), while over 93% of genes in *P. syringae* were part of the accessory genome (Ranković et al., 2023). In *Pseudomonas fluorescens*, a core genome of 1,334 genes was identified across 93 strains, with many plant growth-promoting and biocontrol traits located in the accessory genome (Garrido-Sanz et al., 2016). For *P. putida*, the core genome comprises 3,386 genes, largely associated with nutrient and amino acid transport (Udaondo et al., 2016). These findings indicate that the combined core and accessory genomes reflect the ecological and evolutionary pressures experienced by each species and strain, including interactions with both prokaryotic and eukaryotic organisms (Loper et al., 2012). Thus, constructing the pan-genome of endophytic bacteria like Pt12 can provide key insights into niche adaptation, host colonization, and co-evolution—factors essential for developing effective bioinoculants. The pan-genome framework is essential for understanding the ecological and evolutionary dynamics of endophytes. Accessory genes often encode traits involved in host interaction, such as motility, niche adaptation, and symbiosis (Silby et al., 2011; Gong et al., 2023). Whole genome sequencing also enables the identification of genes responsible for biocontrol and plant growth promotions such as those involved in antibiotic production, hormone biosynthesis, and stress resistance. For instance, genomes of beneficial strains like *P. aeruginosa* DJ06 and *P. putida* LWPZF revealed genes for siderophores, IAA production, phosphate solubilization, and nitrogen fixation (Guo et al., 2023; Jin et al., 2022). Conversely, the absence of biocontrol genes in *P. pergaminensis* emphasizes the diversity of functional roles even among growth-promoting strains (Díaz et al., 2022).

Despite the confirmed biotechnological potential of *P. putida* Pt12, the molecular mechanisms underlying their antifungal activity remain poorly understood. This study aims to explore the genomic basis of Pt12's biocontrol and plant growth-promoting capabilities through whole-genome sequencing and pan-genome analysis. By identifying key genes and metabolic pathways involved in niche colonization, antifungal defense, and host interaction, we seek to uncover the potential of Pt12 as a sustainable bioinoculant for black pepper cultivation in the Amazon region.

Material and methods

Bacterial culture

The *P. putida* Pt12 isolated from *P. tuberculatum* roots by Nascimento et al. (2015) was stored at the Molecular Biology Laboratory of the Federal University of Pará (UFPA, Belém, Brazil). Pt12 was grown on tryptone soy agar (Kasvi, Brazil) plates at 28°C for 12 h. Isolated bacterial colonies were inoculated into tryptone soybean broth (Kasvi, Brazil), growth under shaking (180–200 rpm) at 28°C for 12 h and then used in DNA extraction procedure.

Genomic DNA extraction and whole genome sequencing

Bacterial genomic DNA was extracted using the PureLink Microbiome DNA Purification Kit (ThermoFisher, USA). DNA samples were evaluated via agarose gel electrophoresis and quantified using Qubit fluorometer (Invitrogen, USA).

Sequencing was executed using paired-end Illumina technology (150 bp reads) performed by GenOne Biotechnologies (Rio de Janeiro-RJ, Brazil). Quality control was done with FastQC. Genome assembly was performed using SPAdes via Unycle v0.5.1 pipeline with multiple k-mers (Wick et al., 2017). Genome assembly quality was evaluated using QUAST v5.2.0, based on a total of 80 contigs. Scaffolding was performed using MeDuSa v1.5, with *Pseudomonas* sp. LRP2, *P. putida* AA7, and *P. putida* W5 as reference genomes, selected due to their high phylogenetic similarity (Bosi et al. 2015).

Genome annotation and secondary metabolite prediction

Gene initial annotation was performed using the RASTtk pipeline (Brettin et al., 2015). The genome annotation was refined using Prokka v.1.14.15 (Seemann 2014), and the predicted protein sequences were subsequently classified using COGclassifier V2.0 and BlastKOALA to assign functional categories and metabolic pathways (Kanehisa et al., 2016. Additionally, biosynthetic gene clusters (BGCs) involved in the production of secondary metabolites were identified using antiSMASH v8.0 (Blin et al., 2025). Genomic islands (GIs) were predicted using GIPSy to identify symbiotic islands (Soares et al., 2016). Plant growth promotion traits were predicted using PlaBAse database (Patz et al., 2021). The circular genomic representation was generated using CIRCOS integrating multiple genomic features, including gene annotation, predicted GIs, sequencing depth, GC content, GC skew, and genome alignments with *Pseudomonas sp.* LRP2, *Pseudomonas putida* AA7, *P. putida* W5, and *Pseudomonas florecens* ZL22 which were used during the genome scaffolding and circularization process.

Comparative genomic

Following the taxonomic framework of Udaondo et al. (2024), which reclassified 413 genomes of the *Pseudomonas putida* group into nine cliques, five representative species from each clique were selected for comparative analyses, including type strains when available. To specifically address the Pt12 isolate, a set of closely related species was identified based on pairwise alignment scores of the housekeeping gene *rpoD*, hereafter referred to as the “Pt12 group”, comprising four previously described species together with *Pseudomonas promysaligenes*. The *rpoD* gene was used due to its established phylogenetic resolution within *Pseudomonas* (Girard et al., 2021). Additional *Pseudomonas* lineages and the genus *Cellvibrio* were included as outgroups (Girard et al., 2021). A total dataset of 63 genomes was included in the phylogenomic analysis.

For phylogenomic comparisons, the dataset of 63 genomes was analyzed using two complementary approaches. A multilocus sequence analysis (MLSA) was performed with the VBCG pipeline, which uses a set of conserved 20 core genes to infer phylogenetic relationships (Tian and Imanian, 2023). In parallel, pairwise genomic relatedness among isolates was assessed using Average Nucleotide Identity (ANI), providing quantitative measures of genome-wide similarity (Jain et al., 2018).

The Pangenome was constructed analyzing a dataset of 53 *Pseudomonas putida*–related genomes using Roary, to characterize shared and variable gene content across our selected strains v.3.11.2 (Page et al. 2015). The pangenome statistical analyses were performed in R (R Core Team, 2024) using the micropan package (Snipen & Liland, 2015) to estimate gene diversity and presence–absence relationships among genomes. Similarity between strains was calculated with the Jaccard index, and multivariate analyses were conducted with the vegan package (Oksanen et al., 2022).

Results

Pseudomonas Pt12 genome consists of 5 contigs with a total length of 6,147,762 base pairs and an N50 of 6,142,191 bp. The GC content is 62.79%, and sequencing coverage reached an average depth of 372×, with 99.94% of the genome covered at ≥10×. Genome completeness was estimated at 100%, with no detectable contamination. A total of 5,460 coding sequences (CDS) were predicted, of which 3,482 (63.8%) correspond to protein-coding genes with functional assignments, while 1,959 (35.9%) were annotated as hypothetical proteins. Additionally, 14 mobile or transposable elements and 5 phage-related genes were identified. The genome also contains 78 tRNA and 3 rRNA genes, indicating a complete and well-annotated bacterial genome assembly. Table 1 resume Pt12 genome features.

The functional annotation based on the COG database revealed a broad distribution of genes across various metabolic and cellular categories, the most represented functions included transcription (421 genes), amino acid transport and metabolism (487 genes), general function prediction only (315 genes), and signal transduction mechanisms (314 genes). Additional abundant categories comprised cell wall/membrane/envelope biogenesis (311 genes), energy production and conversion (283 genes), and translation, ribosomal structure and biogenesis (262 genes). A circular representation of the *Pseudomonas* Pt12 genome structure and its genomic features is shown in Fig. 1.

Functional annotation using the PLaBAse database revealed that a substantial portion of the genome is associated with traits involved in plant–microbe interactions. Approximately 25% of the identified genes were related to plant colonization, indicating a strong potential for root surface attachment and endophytic behavior. Genes associated with competitive exclusion represented 21%, suggesting mechanisms for niche establishment and suppression of rival microorganisms. Additionally, 20% of the genes were linked to biocontrol activities and genes involved in bioremediation accounted for 10%, implying a role in the degradation or transformation of environmental contaminants, while 8% were associated with the production of plant hormones, which may contribute to plant growth promotion. The predicted plant growth–promoting traits (PGPTs) of strain Pt12, separated into direct and indirect effects, are presented in Fig. 2.

The genomic analysis revealed the presence of key gene clusters associated with plant growth promotion. The *PvdAELHMVPY genes*, responsible for pyoverdine biosynthesis, was identified, indicating the bacterium's potential to enhance iron acquisition in the rhizosphere. Additionally, the *PqqBCDEF* and *Gdh* genes, involved in phosphate solubilization, were detected, suggesting an efficient mechanism for improving plant phosphorus availability. The complete set of genes related to IAA biosynthesis via indole-3-acetamide (IAM) pathway and tryptophan biosynthesis were also found, supporting the bacterium's ability to modulate plant hormonal balance and stimulate root development. Also, genes are involved in chemotaxis and quorum sense, like, *Che*ABZY and methyl-accepting chemotaxis proteins, also genes *LapA*BCDEP involved Type I secretion system for biofilm formation. '

Genes associated with heavy metal resistance and bioremediation were also identified in the genome. The presence of the *copABZ* operon and *mco* genes suggests a system for copper homeostasis and detoxification through efflux and oxidation mechanisms. Moreover, the detection of the *czcABC* gene cluster, which encodes an RND-type efflux transporter for cobalt, zinc, and cadmium, indicates a capacity for heavy metal tolerance.

Furthermore, *Pseudomonas* Pt12 genome harbors a complete set of genes for both the synthesis and degradation of acetoin and 2,3-butanediol, including *als*S (1–3), *als*D, *bdh*A, *aco*ABC, *Aco*R, and *Acu*C and the *acd*S gene for 1-aminocyclopropane-1-carboxylate deaminase.

Genome mining using antiSMASH identified a diverse set of secondary metabolite BGCs in strain Pt12. A total of 11 clusters were detected, including six RiPP-type clusters, two NRPS clusters and one cluster each of T1PKS, NAGGN, and terpene-precursor types. The predominance of RiPP and NRPS clusters suggests strong potential for the biosynthesis of bioactive compounds, including antimicrobial peptides and nonribosomal siderophores such as pyoverdine. A total of 16 genomic islands were predicted in the *Pseudomonas* Pt12 genome, encompassing regions associated with diverse ecological and adaptive functions. These include seven putative genomic islands, four pathogenicity islands, two resistance islands and two symbiotic islands. The genome mining features are represented in table 2 and genomic island are also represented in circus plot on Fig. 1.

The ANI analysis indicates that none of the species within the Pt12 group exhibited ANI values above the 95% threshold, supporting that they do not belong to the same species. The strains in Pt12 group exhibited ANI values ranging from 91% to 93%. In addition, these groups showed low ANI values when compared with the outgroup strains, such as *Cellvibrio* sp. and *Pseudomonas fluorescens*. Similarly, ANI values below 95% were also observed between Pt12 and strains from cliques 1 and 2, suggesting that Pt12 is distinct from both *Pseudomonas putida* and *Pseudomonas alloputida* groups. In contrast, clades 1, 2, 5, and 9 displayed higher ANI similarities between themselves. The genomic similarity among *Pseudomonas* strains, based on ANI, is depicted in Fig. 3.

The phylogenomic tree constructed using VBCG data using MSLA approach revealed the clustering of 53 *Pseudomonas* strains along with two outgroup strains belonging to the genus *Cellvibrio* and other *Pseudomonas* species known for plant-associated interactions. The phylogenetic relationships among *Pseudomonas* strains inferred from conserved genes are illustrated in Fig. 4. Strain Pt12 is grouped with four other *Pseudomonas* isolates and three *Pseudomonas promysaligenes* strains, forming a distinct clade highlighted in red, supported by a high bootstrap value. In contrast, cliques 1, 2, 5, and 9 are clustered together, with clique 1 corresponding to *Pseudomonas alloputida* and clique 2 to *Pseudomonas putida*, consistent with the clustering observed on the heapmap using ANI approach, further supporting their close phylogenetic. Both the ANI heatmap and the phylogenetic tree based on the MLSA approach indicate that *Pseudomonas sp.* Pt12 does not cluster with any of the known *P. putida* species groups or closely related taxa.

The pangenome analysis revealed a total of 58,832 genes, of which 706 genes (1.2%) were classified as core genes (present in 99–100% of the strains), representing the conserved and essential genetic repertoire of the group. Additionally, 122 genes (0.2%) were identified as soft-core genes (present in 95–99% of the strains), indicating a small subset of nearly ubiquitous genes. The shell genome comprised 4,776 genes (8.1%), corresponding to genes of intermediate frequency among strains and likely associated with adaptive functions. Finally, the cloud genome accounted for most of the dataset, with 53,228 genes (90.5%), representing strain-specific or rare genes that contribute to genetic variability and ecological adaptation. Altogether, these results indicate that the analyzed pangenome is highly open, reflecting substantial genomic diversity among the strains. Pt12.

Based on the gene rarefaction analysis, the Heap's Law model was fitted to describe the dynamics of the pangenome. The estimated Heap's α value was 0.5 and indicates that the pangenome remains open, suggesting that the inclusion of additional genomes continues to significantly increase the total gene repertoire. The intercept ($k \approx 5000$) represents the estimated core genome size, reflecting the average baseline gene content among the analyzed strains, Fig. 5A represent de rarefaction curve of pangenome genes. Moreover, the Pseudomonas putida Pt12 strain was found to possess 433 unique genes, some of them coding proteins related to flagella biogenesis, motility and naringenin degradation. To gain a better understanding of the genomic relationships among the analyzed *Pseudomonas* strains, a Principal Coordinates Analysis (PCoA) based on Jaccard distances of the pangenome presence/absence matrix was performed. The resulting plot revealed that *Pseudomonas* sp. Pt12 is positioned apart from *P. putida* (clique 1) and *P. alloputida* (clique 2), reflecting its distinct gene content. The separation observed in the PCoA space indicates a low level of shared gene families with these cliques, Fig. 5B shows PCoA distribution.

Discussion

The genus *Pseudomonas* comprises highly versatile bacteria that inhabit diverse ecological niches, from soil and water to plant and animal hosts due to its genome plasticity (Zaki et al., 2022). This remarkable diversity of ecological niches is reflected in the genomic plasticity of *Pseudomonas* species, as well as in their capacity for horizontal gene transfer. *Pseudomonas* genome sizes range from 3.19 to 7.76 Mb with GC content varying between 38% to 68% (Yi and Dalpke, 2022). The number of predicted genes varies from 2,831 to 7,360 highlighting the extensive genetic variability that likely underpins the adaptability of these bacteria to diverse environments. Pt12 genome, with 6.18 Mb and a GC content of 62.77%, aligns with values typical for *Pseudomonas putida* species (Udaondo et al., 2016). Strains of this group are widely recognized for their beneficial roles in plant growth promotion and potential use in biofertilizer development.

These rhizospheric Pseudomonads possess specific traits like motility, chemotaxis, specialized pili for attachment, LPS biosynthesis to be able to colonize plant environment. Genes for those traits were found in Pt12 genome. The identification of *cheA*, *cheB*, *cheZ*, *cheY, CheR, CheV and CheW* genes in the genome of *Pseudomonas* Pt12 suggests that this strain possesses the core components of a chemotaxis signaling system. This system includes ligand-binding sensors known as methyl-accepting chemotaxis proteins (MCPs), a methylation-based sensor memory module (CheR and CheB), a kinase-based signal transduction module (*Che*A, *Che*Y, and *Che*Z) and structural connection proteins scaffolding the cell pole sensory array (CheV and CheW) (Yang et al., 2025). In *P. aeruginosa*, chemotaxis-related genes are distributed across multiple clusters, with at least three containing complete sets of *che* genes that are functionally relevant to motility and environmental sensing. Previous studies have shown that cluster II genes, including *cheB2* and *cheA2*, play key roles in modulating chemotactic responses toward organic compounds and inorganic nutrients such as phosphate and magnesium (Abel et al., 2022). Notably, *mcpA* and *mcpB*, which encode MCPs responsible for detecting specific chemical gradients, were also identified in the Pt12 genome. These MCPs are essential in *P. aeruginosa* for sensing environmental signals, including magnesium and diverse chemoattractants (Abel et al., 2022).

Also, genes related to the Type I secretion system (T1SS), responsible for exporting large adhesion proteins (LAPs; *Lap*A, *Lap*B, *Lap*C, *Lap*D, *Lap*E, and *Lap*P), and the Type IV secretion system (T4P/TIVSS), involved in pili biogenesis (*Pil*C, *Pil*E, and *Pil*M), were identified in the *Pseudomonas putida* Pt12 genome. These secretion systems are responsible for exporting proteins that mediate surface attachment and promote biofilm formation. In *Pseudomonas* species, both T1SS and T4P systems are essential for stable surface colonization and effective interaction with plant roots (Zboralski and Filion, 2020, Krzyżanowska et al., 2024, Sampedro *et al.*, 2015). Mutations in genes encoding MCPs or components of secretion systems significantly reduce rhizosphere colonization and biocontrol effectiveness. For instance, in *Pseudomonas chlororaphis* O6, mutants with disrupted MCP genes showed up to a 100-fold decrease in root colonization efficiency on tobacco plants, demonstrating the pivotal role of chemotaxis in early plant–microbe interactions (Han *et al.*, 2010). Similarly, in *Pseudomonas putida* KT2440, deletion of type VI secretion system (T6SS) genes led to impaired root colonization and decreased competitiveness in the tomato rhizosphere, confirming that these systems contribute to stable niche establishment and efficient plant protection (Vázquez-Arias *et al.*, 2025). Their presence in Pt12 genome suggests an adaptive advantage for establishing persistent associations within the rhizosphere and enhancing environmental resilience. Similar gene clusters have also been identified in the genomes of other rhizospheric *Pseudomonas* strains (Berendsen *et al*., 2015).

Genes associated with nutrient acquisition and metabolic versatility are key determinants of the biofertilization potential of *Pseudomonas* species. Udaondo *et al.* (2024) suggested that another set of genes relevant for root colonization in *Pseudomonas alloputida* KT2440 includes several high-affinity transport systems for iron and phosphate uptake, indicating an adaptation to nutrient-limited environments commonly found in the rhizosphere. Similarly, the genome of *Pseudomonas* Pt12 harbors numerous genes involved in siderophore biosynthesis and phosphate solubilization. These results are consistent with Oliveira et al. (2021), who reported the general growth promotion traits in *Pseudomonas* Pt12 isolate using colorimetric methods, highlighting their potential to enhance plant nutrient availability and promote growth under limiting soil conditions.

The *Pseudomonas* Pt12 genome harbors a complete *pqq* operon responsible for the biosynthesis of pyrroloquinoline quinone (PQQ), together with the *gdh* gene encoding the PQQ-dependent glucose dehydrogenase (GDH) (Bargaz et al., 2021). This enzymatic system catalyzes the oxidation of glucose to gluconic acid, which solubilizes inorganic phosphate by acidification and chelation of metal cations, enhancing phosphate availability for plant uptake (Bargaz et al., 2021). In addition, Pt12 possesses a complete *PhoU–PstABC* cluster, comprising the phosphate transport system regulatory protein (*PhoU*), the ATP-binding protein (*PstB*), and the permease proteins (*PstA* and *PstC*), which form a high-affinity ABC transporter for phosphate uptake under limiting conditions (Bargaz et al., 2021). The PQQ–GDH system identified in Pt12 genome may contribute not only to phosphate solubilization but also to potassium release through soil acidification mechanisms. Additionally, the PLaBase annotation indicates that Pt12 harbors genes involved in the production of various organic acids, further supporting its potential to mobilize mineral-bound potassium. The genome also encodes potassium transport-related genes, including Potassium-transporting ATPase A, B, and C chains and TrkA and TrkH potassium uptake system proteins, suggesting a coordinated role in potassium uptake and solubilization that enhances nutrient availability in the rhizosphere (Guo et al., 2009, Zhao et al., 2024).

The *Pseudomonas* Pt12 genome harbors substantial gene clusters associated with pyoverdine siderophore biosynthesis. Pyoverdine (*Pvd* genes) biosynthesis is orchestrated by a large biosynthetic pathway, the peptide precursor, ferribactin, is synthesized by non-ribosomal peptide synthetases (NRPSs) and subsequently modified by tailoring enzymes (Schalk et al., 2020). While *PvdL*, the NRPS responsible for synthesizing the chromophore-derived peptide moiety, is conserved, the NRPSs forming the peptide backbone vary greatly across fluorescent *Pseudomonas (*Granã-miraglia *et al.,* 2023*)*. In *Pseudomonas putida* Pt12, the genes responsible for pyoverdine biosynthesis are distributed across several genomic regions, reflecting the complex modular architecture typical of siderophore pathways in *Pseudomonas*. The main biosynthetic cluster identified by antiSMASH includes *pvdE, pvdO, pvdN, pvdM,* and *pvdP*, with genes involved in the maturation of pyoverdine in the periplasm. Additional genes such as *pvdY, pvdS, pvdL, pvdH, pvdQ,* and *pvdA* are in distinct loci, together forming an extensive repertoire for pyoverdine production, modification, and regulation. This distributed organization is consistent with reports for other *Pseudomonas fluorescens* complex members, where pyoverdine biosynthetic genes often appear in multiple clusters due to evolutionary rearrangements and horizontal gene transfer events. Such genomic plasticity may contribute to the structural diversity of pyoverdines and the ecological versatility of *Pseudomonas* spp. (Loper et al., 2015, Granã-miraglia et al*.,* 2023).

In plant-associated bacteria, IAA biosynthesis can proceed through several distinct pathways that mainly differ in their intermediates and key enzymes. The tryptophan-dependent routes are the most widely characterized and include the indole-3-pyruvate (IPA), indole-3-acetamide (IAM), tryptamine (TAM), indole-3-acetonitrile (IAN), and tryptophan side-chain oxidase (TSO) pathways (Tang et al., 2023). Pt12 genome contains all genes required for tryptophan biosynthesis (*Trp*AB CDEFG). Pt12 also harbors *iaaM* and *ramA_3* in tandem, encoding tryptophan 2-monooxygenase and stereoselective amidase, respectively, which catalyze the two steps of IAA production via the IAM pathway, those data are consistent with Oliveira et al. (2021), who demonstrated that Pt12 produce IAA mainly under high L-tryptophan concentrations, suggesting a tryptophan-dependent mechanism, such the IAM pathway. Mutants of *Bacillus thuringiensis* RZ2MS9 deficient in IAA production exhibit a markedly reduced capacity to promote maize growth compared to the wild-type strain (Figueredo et al., 2023). Also, in *Pseudomonas putida* GR12-2, plays a key role in promoting host plant root development, as evidenced by experiments in which an *ipdC* insertional mutant—deficient in indolepyruvate decarboxylase activity—failed to promote root elongation and adventitious root formation in canola and mung bean compared to the wild-type strain (Patten and Glick, 2002).

The genomic annotation of *Pseudomonas* Pt12 through the PLaBAse platform revealed that approximately 8% of its genes are associated with bioremediation functions. Genes conferring resistance to copper (*copAB* copper resistance proteins and *mco* multicopper oxidase) to arsenic (*arsH*, *arsB* and *arsR2*) resistance and transport systems for zinc (zinc ABC transporter components), *zur* (zinc uptake regulator) and the *czcCBA* efflux complex together with *czcD*, which mediate cadmium resistance. Furthermore, the cobalt-specific ABC transporter system composed of *cbtJ*, *cbtK*, and *cbtL*. Many of these proteins belong to the Resistance–Nodulation–Cell Division (RND) efflux pump family, which enables bacteria to tolerate toxic substances by actively exporting them out of the cell, effectively bypassing the periplasm and reducing intracellular toxicity (Udaondo et al., 2024). Also, Several *Pseudomonas* species, including Pt12, harbor the complete set of *cob* and *cbi* genes involved in the cobalamin biosynthetic pathway, highlighting their potential role in cobalt assimilation and vitamin B_{12} production (Li et al.,2008, Fang et al., 2017).

The *Pseudomonas* Pt12 genome harbors a complete set of genes for both the synthesis and degradation of acetoin and 2,3-butanediol, including *als*S, *als*D, *bdh*A, *aco*ABC, *Aco*R, and *Acu*C. Acetoin and 2,3-butanediol are volatile organic compounds produced during the stationary phase of bacterial growth, known to trigger induced systemic resistance (ISR) in plants and enhance defense responses against pathogens (Rudrappa et al., 2010). Genes involved in acetoin and 2,3-butanediol biosynthesis are commonly found in *Pseudomonas* genomes. For instance, *Pseudomonas protegens* ML15 utilize these compounds to suppress *Botrytis cinerea* infection in tomato plants (Ajijah et al., 2023), while *Pseudomonas aeruginosa* PGPR2 has demonstrated effective biocontrol of *Macrophomina phaseolina* in mung bean through similar mechanisms (Illakkiam et al., 2014).

The presence of an ACC deaminase–related gene in the genome of *Pseudomonas* Pt12 suggests a potential role in mitigating plant ethylene stress. Although the *acdS* gene typically ranges from 1,014 to 1,017 bp in *Pseudomonas* species (Glick & Nascimento, 2021), the Pt12 homolog is shorter (899 bp), indicating possible structural variation. Nonetheless, BLASTp revealed homologous sequences of similar length in plant-associated bacteria, such as *Pseudomonas* MNF1, isolated from coffee, showing 90.97% identity (de Sousa et al., 2022).

Four pathogenicity islands were identified in the *Pseudomonas putida* Pt12 genome, located approximately around 3 Mb and 6 Mb. These regions harbor genes related to the type VI secretion system (T6SS) and flagellar biosynthesis. The presence of T6SS-associated genes suggests a potential role in bacterial competition and interaction with other microorganisms or host cells, as this system is commonly involved in interbacterial antagonism and effector delivery. Likewise, the flagellar biosynthesis genes indicate motility-associated functions that may enhance environmental adaptation and host colonization. Together, these features highlight that the predicted pathogenicity islands are likely to contribute to ecological competitiveness rather than virulence, consistent with the lifestyle of Pseudomonads as a versatile rhizosphere-associated bacterium. In addition, a symbiotic island was detected containing mostly hypothetical genes, which may represent uncharacterized elements involved in plant–microbe interactions or niche adaptation.

Among the BGCs predicted by antiSMASH, most did not match any characterized secondary metabolite, indicating the presence of potentially novel biosynthetic pathways in *Pseudomonas sp.* Pt12. Only one cluster showed similarity to a known compound, corresponding to the pyoverdine biosynthetic pathway, a siderophore typically associated with iron acquisition and plant growth promotion. This finding suggests that, while Pt12 retains classical traits of the *Pseudomonas* genus related to iron metabolism, it also harbors unexplored biosynthetic potential that may contribute to its ecological fitness and biotechnological relevance.

The analysis based on the rpoD marker places *Pseudomonas* sp. Pt12 in close relation to *Pseudomonas* sp. LRP2-20, *P. putida* W5, *P. putida* AA7, *P. fluorescens* ZL22 and three *Pseudomonas promysalinigenes* strains. As demonstrated by Girard et al. (2020), sequencing the *rpoD* gene allowed accurate classification of 145 environmental isolates into 20 known *Pseudomonas* species, confirming its effectiveness as a diagnostic marker. In agreement with previous reports highlighting the frequent misclassification of genomes within the *Pseudomonas putida* group (Tran et al., 2017, Morimoto et al., 2020), our hypothesis proposes that those strains may be wrongly classified as *Pseudomonas putid*a and *fluorescens*. Such findings reinforce the ongoing need for taxonomic revision in the *P. putida* complex, as proposed for well-known strains like KT2440 and BIRD-1, which were reclassified as *Pseudomonas alloputida* due to their genetic divergence from the type of strain *P. putida* NBRC 14164^T (Keshavarz-Tohid et al., 2019). This underscores that Pt12, like many plant-associated isolates, may represent a distinct lineage within the *Pseudomonas* genus, whose classification requires careful genomic comparison and species-level validation.

To address this taxonomic uncertainty, we used the framework proposed by Udaondo et al. (2024), which delineates nine major clusters encompassing 413 *Pseudomonas* species. This classification, based on ANI and Multilocus Sequence Analysis (MLSA), provides a robust comparative basis to evaluate the phylogenetic position of *Pseudomonas* sp. Pt12 within the *P. putida* complex.

The ANI-based comparison revealed that *Pseudomonas* sp. LRP2-20 shared the highest genomic similarity with *Pseudomonas* sp. Pt12, with an ANI value of 98.9%, suggesting that both strains belong to the same species. Other closely related strains, including *Pseudomonas putida* W5, *P. putida* AA7, *P. fluorescens* ZL22, and three *Pseudomonas promysalinigenes* isolates, exhibited ANI values ranging from 90% to 95%, indicating a close phylogenetic relationship but distinct species-level divergence. These results indicate that the use of the *rpoD* gene was an appropriate approach to identify strains closely related to the *Pseudomonas* sp. Pt12 isolate, as it effectively reflected the phylogenetic proximity later confirmed by ANI analysis. Furthermore, the ANI values between *Pseudomonas* sp. Pt12 and strains from cliques 1 and 2, which comprise the *P. putida* and *P. alloputida* groups, ranged from 87% to 90%, indicating that Pt12 shows low similarity and does not belong to these cliques. These results are supported by the VBCG analysis, which shows that *Pseudomonas* sp. Pt12 does not gather with species from cliques 1 and 2, further confirming its genetic divergence from the *P. putida* and *P. alloputida* groups. Together, these findings suggest that the Pt12 isolate may represent a novel *Pseudomonas* species.

Although Nascimento et al. (2015) initially classified the Pt12 isolate as *Pseudomonas putida* based on 16S rRNA sequencing, several studies have demonstrated that this single gene has limited resolution for distinguishing closely related Pseudomonas species (Lalucat et al., 2022, Udaondo et al., 2024), supporting the need for genome-based approaches such as ANI and MLSA to refine taxonomic placement. The genus *Pseudomonas* is continuously expanding, largely driven by advances in genome sequencing technologies. Girard et al. (2021) described 43 new Pseudomonas strains with low similarity to the usual type strains, suggesting that type strains represent only a small fraction of the genus diversity and the rapid increase in available genome sequences has led to the frequent description of new *Pseudomonas* species, highlighting the dynamic and evolving nature of *Pseudomonas* taxonomy. Similarly, Udaondo et al. (2024) emphasized the taxonomic complexity of the genus, noting that although 336 *Pseudomonas* species are validly published more than 28,000 strains remain classified only as *Pseudomonas sp*. Supporting this, Udaondo et al. (2024) analyzed 26,363 genomes labeled as *Pseudomonas* in GenBank categorizing them into 435 distinct species-level clusters. Among these, 224 strains originally deposited as *Pseudomonas putida* were distributed across 31 different cliques, revealing extensive taxonomic inconsistencies. Those studies underscore the ongoing challenges in defining species boundaries within this highly diverse group. Taking that into consideration, we propose that *P. putida* Pt12 should be reclassified as a new species, *Pseudomonas pipericola* sp. nov. (Pi.pe.ri'co.la. L. masc. n. Piper, L. suff. -cola (from L. n. incola, which means resident).

The pangenome of the analyzed *Pseudomonas* group comprised 58,832 genes, including a small core genome (706 genes; 1.2%) and a large cloud component (53,228 genes; 90.5%), indicating high genomic plasticity and an open pangenome (Heap's α = 0.55). These findings are consistent with previous studies showing that *Pseudomonas* species possess highly dynamic genomes driven by horizontal gene transfer and niche specialization (Zaki et al., 2022). The PCoA based on Jaccard distances further supports the ecological selectivity of the *P. pipericola* sp. nov. genome, as this strain clusters far from other *Pseudomonas* species, reflecting the specialization of genes that may facilitate adaptation and interaction with Piperaceae plants in the Amazon region. Hypotheses regarding niche specialization of microbial accessory genomes have been confirmed in host–symbiont interactions, both in mutualistic and parasitic relationships, as demonstrated in plant-associated kosakonia (da Silva et al., 2024) and in the wheat pathogen *Zymoseptoria tritici* (Plissonneau et al., 2018), where accessory genome content is correlated to host adaptation. In line with these observations, *P. pipericola* sp. nov. displays genomic features consistent with niche adaptation, possessing 433 strain-specific genes, including a complete cluster for naringenin degradation. Naringenin is a plant-derived flavonoid that can act as a chemical signal in the rhizosphere, inducing chemotaxis and enhancing bacterial motility, adherence, and biofilm formation (Upadhyay et al., 2022). In addition, genes involved in flagellum biogenesis, motility toward chemotactic attractants (e.g., *Mcp*, *motA*, *motB*), and adhesion (*LapA*) are located within the *P. pipericola* sp. nov. strain-specific genome. These data suggest that the mechanisms underlying *P. tuberculatum* colonization are specific to the *P. pipericola* sp. nov. genome. Similar mechanisms have been observed in *Aeromonas* sp. H1, where naringenin triggers chemotaxis, enhances flagellar assembly, and induces fimbrial protein expression to facilitate host colonization (He et al., 2022). Together with other studies, these findings confirm that naringenin can induce changes in bacterial colonization patterns within the microbiome, acting like a "cry for help" mechanism (Uhlík et al., 2013, Shi et al., 2024), highlighting the ecological relevance of *P. pipericola* sp. nov. -specific genes for adaptation to Piperaceae plants in the Amazon region.

Concluding remarks

In conclusion, strain *P. pipericola* sp. nov. harbors a complete set of genes for chemotaxis, pili formation for adhesion, and biofilm production—traits that are crucial for root colonization of *P. nigrum*. Additionally, this endophyte exhibits plant growth-promoting potential through genes involved in siderophore production, phosphate solubilization, and IAA synthesis. The strain also shows genetic biocontrol potential, possessing multiple BGCs and the ability to induce plant immune responses via production of volatile organic compounds such as acetoin and 2,3-butanediol. Moreover, this endophyte likely represents a novel species, displaying niche specialization as evidenced by the presence of strain-specific genes associated with plant-derived chemical signals. Together, these genomic features highlight *P. pipericola* sp. nov. as a highly adapted endophytic bacterium with multiple mechanisms supporting colonization, growth promotion, and defense in pepper plants.

Conflict of interest

The authors declare no competing interests.

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Table 1 General genome assembly and annotation features of Pseudomonas sp. Pt12. The table summarizes key assembly metrics and functional annotation statistics of the draft genome

General Assembly Features		General anotations features	
Total sequence length (bp)	6147762	CDS	5460
Contigs N50 (bp)	6142191	Protein Functional Assignment	3482
Contig count	5	Hipotetic Protein	1959
Coverage >= 10x (%)	99.94	Mobile or transposition element	14
GC (%)	62.79	Phage	5
Avg. coverage depth	372	tRNA	78
Completeness (%)	100	rRNA	3
Contamination (%)	0	CRISPr	0

Table 2 Genomic islands and secondary metabolite biosynthetic gene clusters identified in the genome. The table lists the type of island and cluster predicted by GIPSy and antiSMASH, respectively, along with

their genomic positions (start and end coordinates) and classification according to functional or biosynthetic type.

GIPSy Genomic Island prediction				**Antismash prediction**		
Type of Island	Confidenc	Start	End	**Type**	Start	End
Genomic Island	NA	816695	835312	NRPS	1648293	1721377
Genomic Island	NA	1536378	1581870	NRPS	1878414	1931355
Genomic Island	NA	1857383	1869736	RiPP-like	2663802	2674647
Genomic Island	NA	2199189	2213488	RiPP-like	2689736	2700569
Genomic Island	NA	2221609	2230294	RiPP-like	2889492	2901678
Pathogenicity Island	Normal	2550815	2584129	ranthipeptide	3449412	3470842
Pathogenicity Island	Normal	2758831	2867809	RiPP-like	4003865	4014704
Pathogenicity Island	Strong	3033421	3048415	T1PKS	4728188	4775606
Genomic Island	NA	4118384	4146820	NAGGN	4808129	4823121
Resistence Island	Strong	4150577	4210549	terpene-precursor	5626443	5647330
Genomic Island	NA	4533157	4543541	redox-cofactor	5772734	5794881
Resistence Island	Strong	4676109	4685479			
Simbiotic Island	Normal	4717802	4760153			
Simbiotic Island	Strong	4764087	4775339			
Genomic Island	NA	5121436	5167289			
Pathogenicity Island	Weak	6041811	6082327			

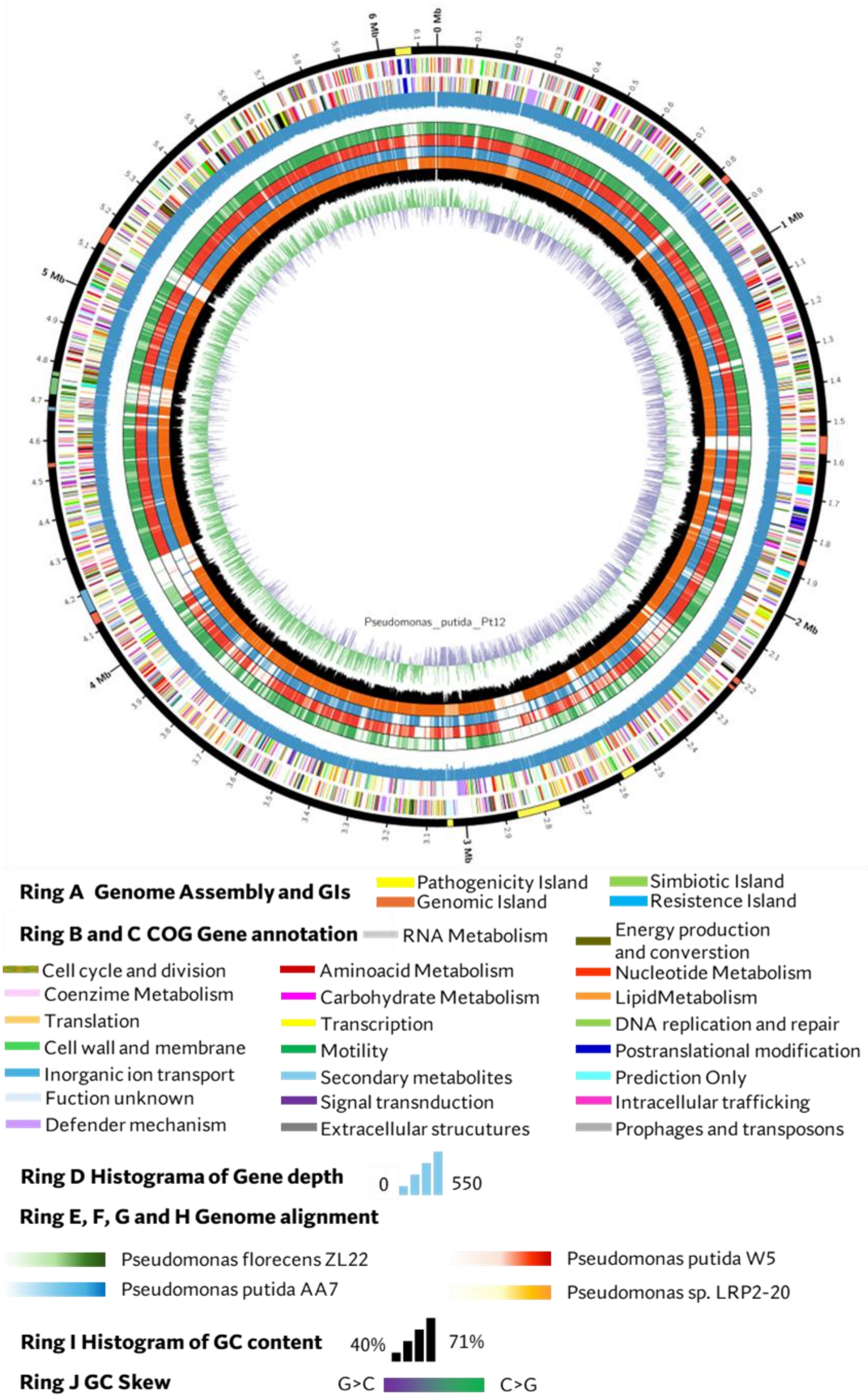


Fig. 1. Circular representation of the Pseudomonas sp. Pt12 genome generated with Circos. The outermost ring displays the genomic coordinates, followed by coding sequences (CDSs) on the forward and reverse strands. Inner tracks represent coverage depth across the genome, the alignment with reference strains, GC content, and GC skew.

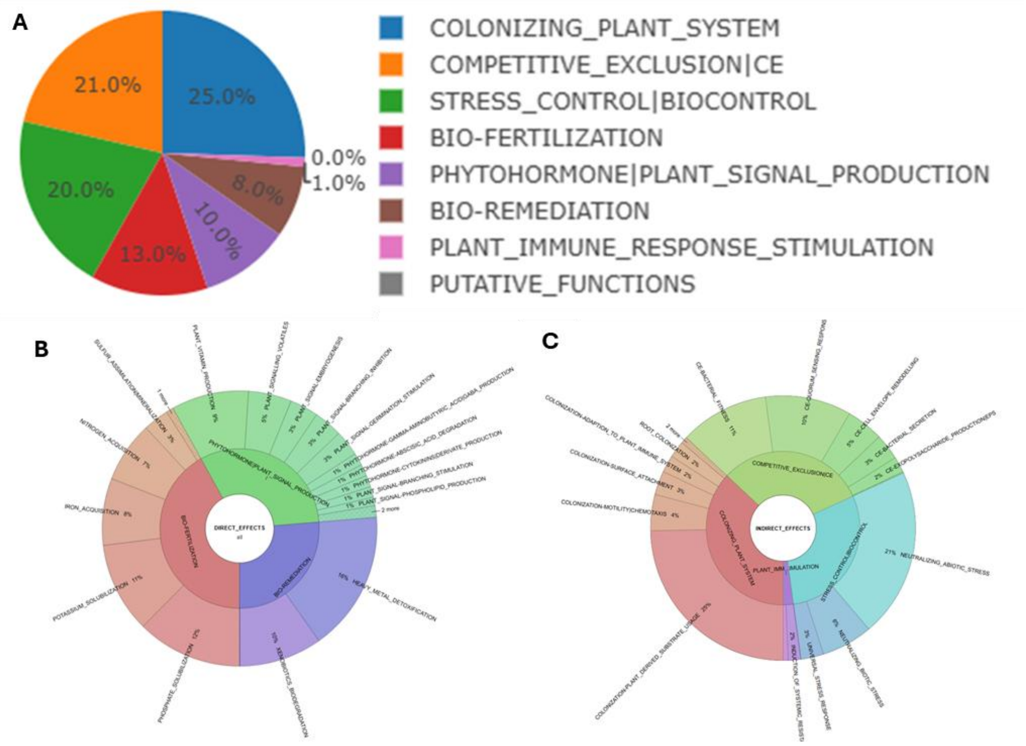


Fig. 2. PLaBAse-guided prediction of plant growth–promoting traits (PGPTs) in Pseudomonas putida Pt12. (A) Pie chart showing functional classification generated in strict mode (BLASTp + HMMER). (B) Krona plot illustrating direct effects (biofertilization, phytohormone and signal production), and (C) indirect effects (stress control, colonization, and competitive exclusion). Sector areas indicate relative abundance of each functional category.

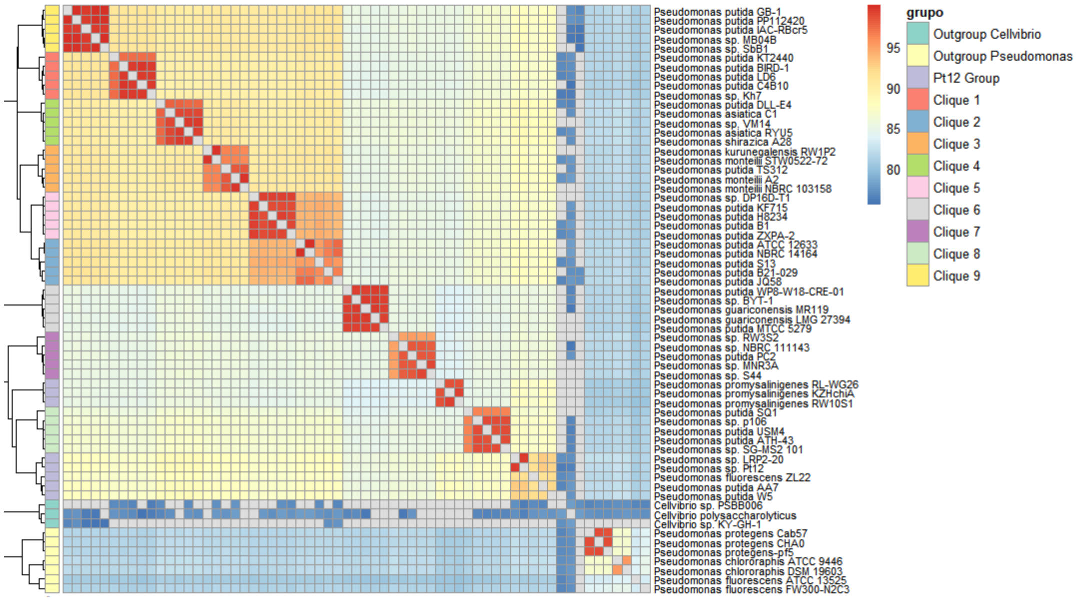


Fig. 3. Heatmap of Average Nucleotide Identity (ANI) values among Pseudomonas strains. The heatmap represents a pairwise comparison matrix, where each strain is compared to all others along both rows and columns. ANI values above 95% indicate strains belonging to the same species, whereas lower values correspond to distinct species. Increasing redness denotes higher genomic similarity (greater ANI), while increasing blueness indicates lower similarity and more distant relationships.

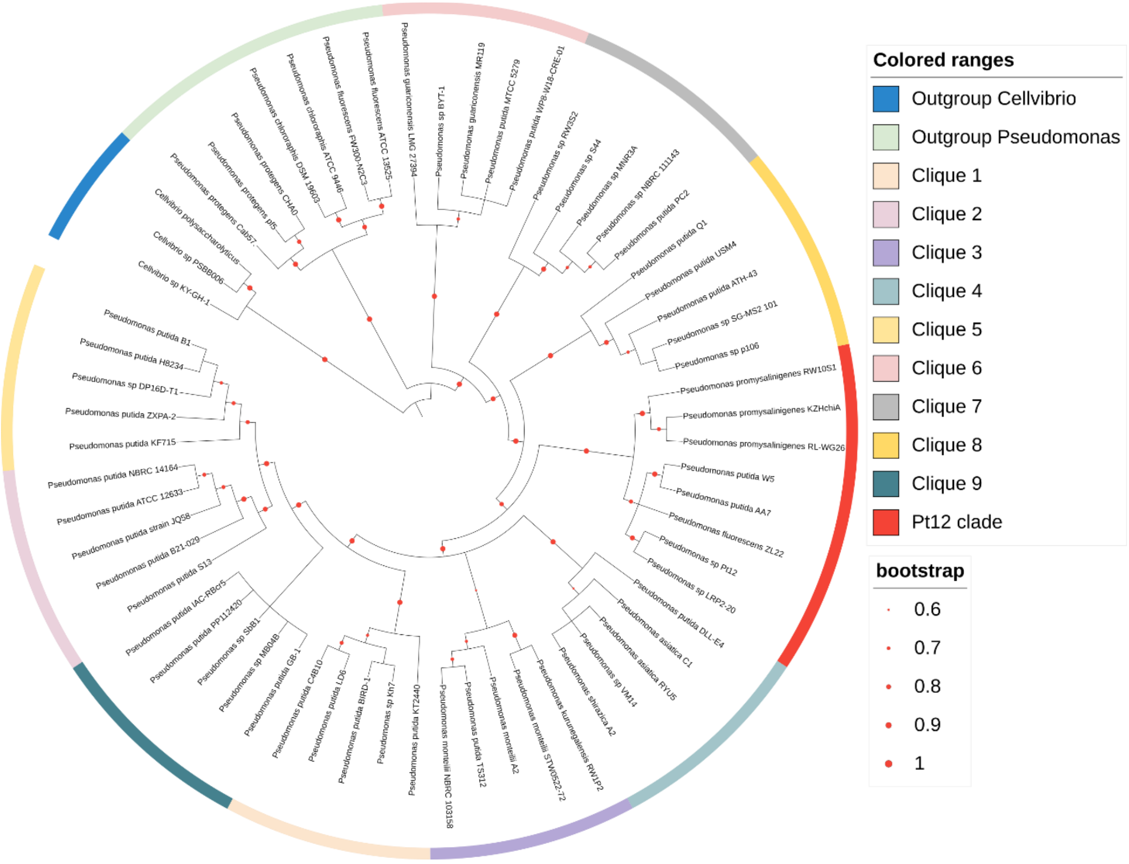


Fig. 4. Phylogenetic tree of *Pseudomonas* strains based on core gene sequences obtained using the Bacterial Core Genome tool (VBCG). The tree topology reflects evolutionary relationships inferred from conserved genomic regions. he tree was inferred with FastTree using the approximate maximum likelihood method and visualized with the Tree of Life app.

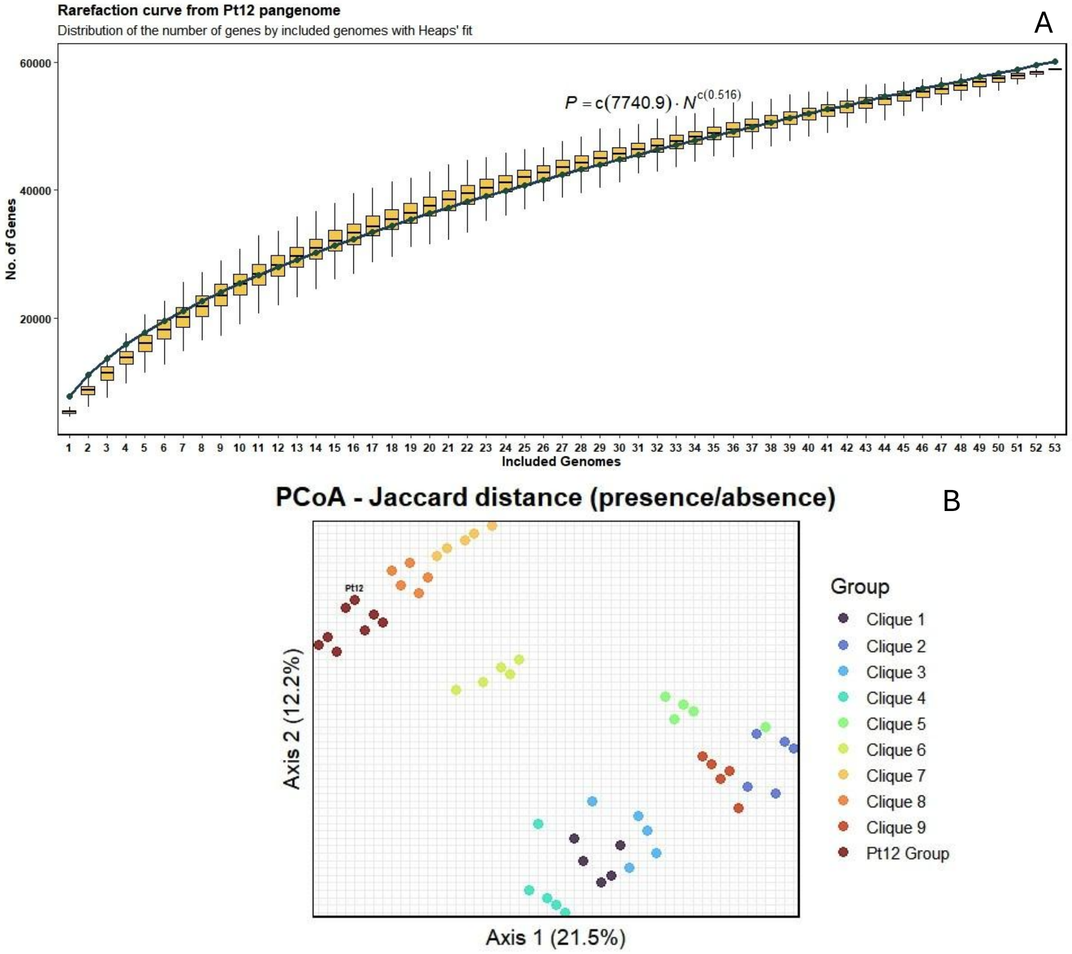


Fig. 5. Pangenome rarefaction curve (A) generated using the Roary pipeline v3.11.2, showing a total of 58,832 genes, including 706 (1.2%) core, 122 (0.2%) soft-core, 4,776 (8.1%) shell, and 53,228 (90.5%) cloud genes, indicating an open and highly diverse pangenome (Heap's α = 0.5). *Pseudomonas sp.* Pt12 harbored 433 unique genes, mostly encoding hypothetical proteins. (B) Principal Coordinates Analysis (PCoA) based on gene presence/absence matrix revealed clear clustering among Pseudomonas groups, with Pt12 separated from *P. putida* (clique 1) and *P. alloputida* (clique 2), reflecting its distinct genomic composition.

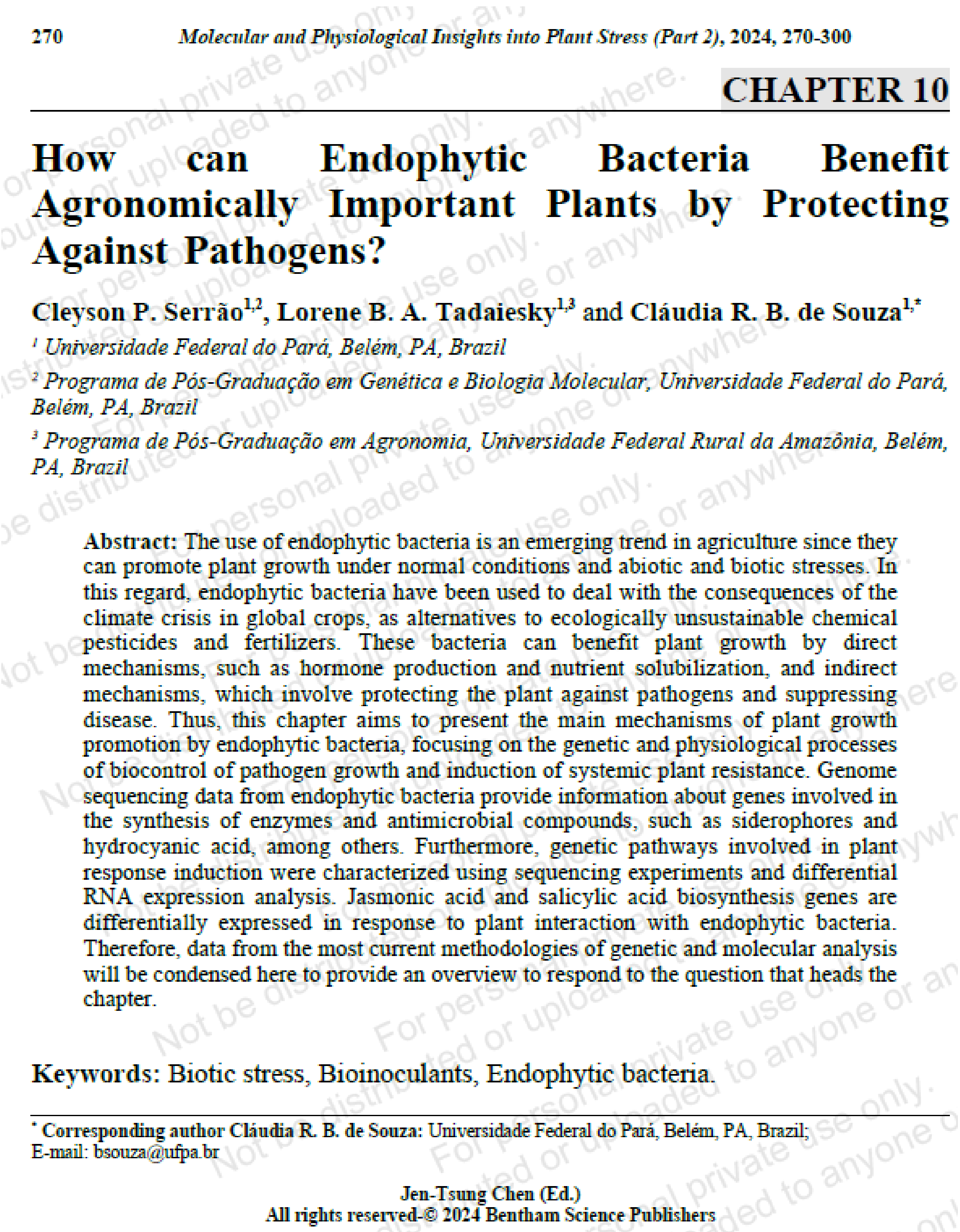
CONSIDERAÇÕES FINAIS

Os resultados reunidos nesta tese revelam de forma clara que a integração entre sínteses globais de evidências e abordagens genômicas aprofundadas é essencial para compreender e aprimorar o uso de microrganismos benéficos no manejo sustentável de doenças de plantas. A meta-análise conduzida sobre o potencial de biocontrole de espécies de *Bacillus* demonstra, com elevado grau de consistência (344 artigos), que esses microrganismos representam uma das abordagens mais robustas e eficazes já avaliadas para a supressão de fitopatógenos. A redução média de 60% na severidade das doenças, observada em um conjunto amplo e diversificado de estudos, evidencia não apenas a força desse grupo bacteriano, mas também a importância de variáveis metodológicas — como concentração do inóculo, tipo de aplicação e estágio fenológico — que modulam diretamente seu desempenho. A constatação de que produtos comerciais apresentam efeitos mais modestos que novas linhagens em teste indica que ainda existe um amplo espaço para inovação, seleção e desenvolvimento de bioinoculantes mais eficientes, especialmente quando se considera a crescente demanda mundial por práticas agrícolas de baixo impacto ambiental.

Esse cenário de alta eficácia associada a *Bacillus* encontra um paralelo complementar no estudo genômico de *Pseudomonas putida* Pt12, isolada de *Piper tuberculatum*, uma espécie amazônica naturalmente resistente à fusariose. A caracterização genômica de Pt12 amplia a compreensão sobre os mecanismos moleculares que sustentam o biocontrole, revelando um repertório gênico complexo, marcado por diversos clusters biossintéticos, múltiplas ilhas genômicas e um conjunto expressivo de genes exclusivos. Esses elementos sustentam sua capacidade de produzir sideróforos, hormônios vegetais, voláteis bioativos e metabólitos antifúngicos, além de reforçar sua competência em processos fundamentais como quimiotaxia, motilidade e colonização radicular. A posição filogenômica singular da Pt12, situada fora dos principais grupos de *Pseudomonas*, aliada à abertura do seu pangenoma e à presença de 433 genes específicos, reforça a hipótese de que se trata de uma linhagem adaptada a interações mutualísticas altamente especializadas, justificando sua proposta de reclassificação como *Pseudomonas pipericola sp. nov.*

Em conjunto, os dois eixos de investigação apresentados — a síntese global sobre *Bacillus* e a mineração genômica detalhada do isolado Pt12 — demonstram que o avanço no desenvolvimento de inoculantes microbianos exige tanto a consolidação do conhecimento disponível quanto a identificação de novas linhagens dotadas de propriedades funcionalmente relevantes. A convergência dos achados sugere que a eficácia no biocontrole não é fruto de um único mecanismo, mas da combinação de atributos ecológicos, metabólicos e evolutivos que emergem em microrganismos adaptados a interações íntimas com plantas hospedeiras, propriedades importantes tanto em bactérias do gênero *Bacillus* quanto em *Pseudomonas*. Portanto, esta tese reforça que o futuro do manejo biológico de doenças reside na união entre evidências integradas em escala global e descobertas genômicas de alta resolução, permitindo a seleção racional, o aprimoramento biotecnológico e a aplicação estratégica de bactérias benéficas capazes de sustentar sistemas agrícolas mais resilientes, produtivos e ambientalmente responsáveis.

ANEXOS



270 *Molecular and Physiological Insights into Plant Stress (Part 2)*, 2024, 270-300

CHAPTER 10

How can Endophytic Bacteria Benefit Agronomically Important Plants by Protecting Against Pathogens?

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Abstract: The use of endophytic bacteria is an emerging trend in agriculture since they can promote plant growth under normal conditions and abiotic and biotic stresses. In this regard, endophytic bacteria have been used to deal with the consequences of the climate crisis in global crops, as alternatives to ecologically unsustainable chemical pesticides and fertilizers. These bacteria can benefit plant growth by direct mechanisms, such as hormone production and nutrient solubilization, and indirect mechanisms, which involve protecting the plant against pathogens and suppressing disease. Thus, this chapter aims to present the main mechanisms of plant growth promotion by endophytic bacteria, focusing on the genetic and physiological processes of biocontrol of pathogen growth and induction of systemic plant resistance. Genome sequencing data from endophytic bacteria provide information about genes involved in the synthesis of enzymes and antimicrobial compounds, such as siderophores and hydrocyanic acid, among others. Furthermore, genetic pathways involved in plant response induction were characterized using sequencing experiments and differential RNA expression analysis. Jasmonic acid and salicylic acid biosynthesis genes are differentially expressed in response to plant interaction with endophytic bacteria. Therefore, data from the most current methodologies of genetic and molecular analysis will be condensed here to provide an overview to respond to the question that heads the chapter.

Keywords: Biotic stress, Bioinoculants, Endophytic bacteria.

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Jen-Tsung Chen (Ed.)

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Plant bZIP Proteins: Potential use in Agriculture - A Review

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Affiliations + expand

PMID: 37815184 DOI: 10.2174/0113892037261763230925034348

Abstract

With global climate changes and the increased demand for food due to expected world population growth, genetic improvement programs have aimed at producing crops with increased yield and tolerance to environmental stresses, such as drought, salinity, and pathogens. On the other hand, genetic improvement programs via biotechnology require candidate genes that confer traits of interest to be incorporated into improved crops. In this regard, genes encoding transcription factors (TFs) can be promising since they are proteins that transcriptionally regulate the expression of target genes related to the most diverse roles in the plant, including defense against stresses. Among TFs, bZIP (basic leucine zipper) proteins regulate many developmental and physiological processes in the plant, such as seed formation, fruit ripening, nutrient assimilation, and defense response to abiotic and biotic stresses. In this review, we aim to highlight the main advances in the potential use of bZIP TFs in the genetic improvement of crops. We address this potential mainly regarding crop tolerance to stresses and other agricultural traits, such as increased yield and fruit features.

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AGRONOMY AND FORESTRY | SHORT COMMUNICATION

Klebsiella endophytic bacteria control cassava bacterial blight in the eastern Amazon

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ABSTRACT

Cassava bacterial blight (CBB), caused by *Xanthomonas phaseoli* pv. *manihotis*, is one of the most important diseases affecting cassava production worldwide, including regions of Brazil in the eastern Amazon. The use of beneficial microorganisms, such as endophytic plant growth-promoting bacteria, has emerged as an effective tool for controlling diseases in many crops. Here, two *Klebsiella* endophytic isolates (26Y and 29Y) isolated from cassava were evaluated for the control of CBB through antagonistic assays and biological control of the disease in plants inoculated by irrigating the substrate and by foliar spray under greenhouse conditions. The two isolates were able to inhibit the *in vitro* growth of the pathogen, as well as to control the disease severity by at least 90% in plants inoculated by both inoculation methods. We report the first *Klebsiella* strains to control CBB in the eastern Amazon, though their risk assessment for drug-resistance in humans is still pending.

KEYWORDS: antagonistic activity, biological control, *Manihot esculenta*, plant growth-promoting bacteria, *Xanthomonas phaseoli*

Chapter 3

Beneficial *Pseudomonas* bacteria: Genomics-related mechanisms of plant growth promotion and biocontrol

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Chapter Outline

1. Introduction

In their habitat, plants are subjected to adverse environmental conditions, which interfere with their growth and development, including biotic stresses caused by different pathogenic microorganisms, such as bacteria, viruses, fungi, and oomycetes. In this regard, pathogens can disrupt the physiological and metabolic functioning of plants, resulting in losses in their yield and quality (Kumar and Verma, 2018). Losses of up to 40% due to attack by pathogens and pests have been estimated in major food crops, such as rice and maize (Savary et al., 2019). Furthermore, stressful conditions faced by plants can be significantly worsened by increasing global climate change (Delgado-Baquerizo et al., 2020; Zandalinas et al., 2021). To cope with stresses caused by pathogens, plants evolved by developing defense systems through physiological, cellular, and molecular mechanisms. In general, when in contact with pathogens, the plant responds through signaling pathways, containing several messenger molecules, such as reactive oxygen species (ROS), calcium (Ca^{2+}), and

oomycetes. In this regard, pathogens can disrupt the physiological and metabolic functioning of plants, resulting in losses in their yield and quality (Kumar and Verma, 2018). Losses of up to 40% due to attack by pathogens and pests have been estimated in major food crops, such as rice and maize (Savary et al., 2019). Furthermore, stressful conditions faced by plants can be significantly worsened by increasing global climate change (Delgado-Baquerizo et al., 2020; Zandalinas et al., 2021). To cope with stresses caused by pathogens, plants evolved by developing defense systems through physiological, cellular, and molecular mechanisms. In general, when in contact with pathogens, the plant responds through signaling pathways, containing several messenger molecules, such as reactive oxygen species (ROS), calcium (Ca^{2+}), and

nitric oxide (NO), leading to a defense response. The main phytohormones that play essential roles in the modulation of plant immunity include salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Pieterse et al., 2012).

In defense against pathogens, the plant uses its innate immunity system in defense against pathogens, classified into two types: pathogen-associated pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006). PTI comprises the first level of plant immunity, where the pathogen-associated molecular patterns (PAMPs), such as flagellins and lipopolysaccharides, are recognized by the pattern recognition receptors (PRRs) of the plant. To get past PTI defense, pathogens have developed effector proteins that can help in their penetration and survival within the host plant. In

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