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Bacillus strains as biocontrol agents against fungi associated with defoliation in *Pinus patula*, *Pinus montezumae*, and *Pinus pseudostrobus*

Abstract

Worldwide, forest loss is attributed primarily to fires, pests, and diseases. Among them, fungal pathogens are frequently associated with important diseases in pine forests, with dreadful consequences. In the mountain range of northern Puebla, México, phytopathogenic fungi cause defoliation in *Pinus patula* Schl. et Cham (Pinales: Pinaceae), *Pinus montezumae* Lamb. (Pinales: Pinaceae), and *Pinus pseudostrobus* Lindl. (Pinales: Pinaceae). The standard treatment for controlling fungal diseases is the application of highly toxic fungicides. Given that these products are inefficient and pose risks to biodiversity and human health, it is necessary to develop effective and environmentally friendly alternatives. In this context, this investigation proposed the use of rhizospheric forest bacteria as antagonists against phytopathogenic fungi under *in vitro*, greenhouse, and field conditions. Fungi associated with pine defoliation were isolated and identified to evaluate their responses against *Bacillus* and *Peribacillus* strains. Among the most frequently occurring fungal genera associated with the three pine species, three were shared: *Clonostachys*, *Curvularia*, and *Myrmaecium*. In addition, foliar application of *Bacillus thuringiensis* (Bacillales: Bacillaceae), and *Peribacillus simplex* (Bacillales: Bacillaceae) reduced lesion incidence and the severity index of symptoms associated with fungi in a forest system up to 50%. These results are promising for research on biological alternatives for the control of fungi in forest systems.

Keywords: Antagonism, Growth control, Lytic enzymes, Pinaceae, Rhizobacteria.

1. Introduction

The Mexican forestry sector provides resources for approximately 11 million people through forest harvesting (Zamora, 2016). However, pests and diseases have been documented across different forest species in Mexico, mainly in areas with substantial anthropogenic influence (SEMARNAT, 2016). Among the most prevalent diseases in conifers, fungal infections are the most common and manifest symptoms through different parts of the plant, compromising its health (Raitelaitytė *et al*., 2017). Pine pathogenic fungi can be transmitted between several species during their life cycles by natural or anthropogenic vectors (Gutiérrez-Flores *et al*., 2022). Moreover, fungi are mostly treated with synthetic fungicides, which can lead to resistance, low efficacy, and negative environmental consequences (Crissman, 2002). Considering these premises, alternative methods are being sought to control the presence of fungal pathogens in forest systems and contribute to their conservation.

Biological control has been demonstrated to be effective for managing fungal diseases in *Pinus* spp. under greenhouse and field conditions (Iturritxa *et al*., 2017). For instance, bacterial strains from the genera *Serratia* (Gutiérrez-Flores *et al*., 2023) and *Bacillus* have been shown to control fungal diseases caused by *Fusarium ciricinatum* in *Pinus taeda* (Soria *et al*., 2012). Additionally, *Phytophthora* infection in nursery plants can be achieved by the inoculation of *Bacillus*, *Pseudomonas*, and *Trichoderma* (Reglinski & Dick, 2005).

In this research, we investigated the antagonistic effects of *Bacillus simplex* (Bacillales: Bacillaceae), *Bacillus thuringiensis* (Bacillales: Bacillaceae), and *Peribacillus simplex* (Bacillales: Bacillaceae) on fungal pathogens associated with the defoliation of pine species found in northern Puebla, México as alternatives to chemical fungicide use in forests. First, sampling was performed at the study site to isolate and identify fungi from the pine plants

and bacteria through the use of biocontrol mechanisms, including chitinase activity and siderophore production. The effectiveness of these bacterial strains in controlling fungi was tested directly on isolated fungi, pine needles, seedlings, and mature pine trees. The results showed that the bacterial strains were effective at controlling fungal defoliation diseases in pine species.

2. Materials and methods

2.1 Study site

Sampling of the pine species *Pinus patula* Schl. *et* Cham (Pinales: Pinaceae), *Pinus montezumae* Lamb. (Pinales: Pinaceae), and *Pinus pseudostrobus* Lindl. (Pinales: Pinaceae) with defoliation symptoms was performed inside a forest polygon comprising six municipalities in northern Puebla, Mexico: Aquixtla, Chignahuapan, Ocotepec, Tetela de Ocampo, Zacatlán, and Zautla (Figure 1). In addition, a field study was conducted on a forest management property in Chignahuapan, located at the coordinates 19°45'15" N 98°06'21" W.

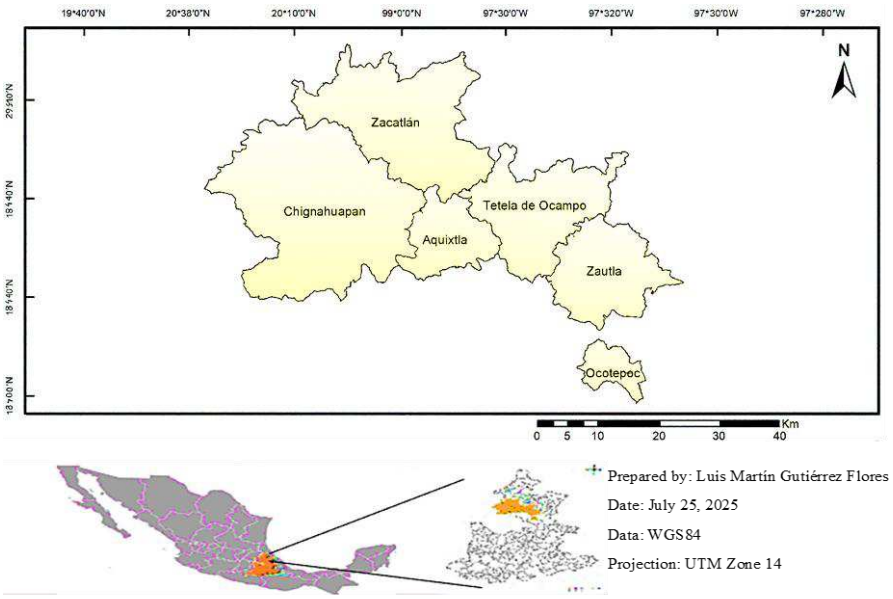


Fig. 1 Forest polygon under study in northern Puebla, Mexico

2.2 Isolation, characterization, and identification of *Bacillus* with antagonistic capacities obtained from forest soil

Bacterial strains were isolated from the rhizosphere of pine trees affected by defoliation in northern Puebla, Mexico. A soil suspension was mixed at a 1:10 ratio and heated to 80 °C for 30 min; the resulting suspension was subsequently plated onto nutrient agar and incubated at 37 °C for 24 h. Several colonies were isolated by the streak plate method (Oberá *et al*., 2005).

All bacterial strains were tested for chitinase activity and siderophore production in chitin-enriched minimal medium (Okumoto, 2001) and Chrome Azurol S (CAS) medium (Schwyn & Neilands, 1987), respectively. The productive efficiency (PE) of chitinase and siderophores was assessed based on the degradation halo diameter (DH) and bacterial colony diameter (DB) according to the formula $PE = (DH - DB)/DB \times 100$ (Campuzano *et al*., 2017; Parra-Cota *et al*., 2017).

Afterward, 16 bacteria with the highest siderophore and chitinase production were tested against the fungi isolated from the three pine species (*P. patula, P. montezumae,* and *P. pseudostrobus*). For the *in vitro* antagonism assay (treatments performed in triplicate), the bacterial strains were propagated in nutrient broth at 30 °C and 120 rpm for 24 h. The bacteria were adjusted to 10^{-5} colony-forming units (CFU) ml^{-1}, six radial streaks were subsequently made on antibiosis agar, and the plates were incubated for 24 h. Next, the fungal strains were inoculated onto plates containing the bacterial strains; a fungus-only control was plated, and all the plates were incubated and evaluated after 6 days.

The six bacteria with the greatest inhibition of fungal growth were identified by 16S rRNA gene sequencing. First, DNA was extracted from the strains according to the Genomic DNA Extraction Kit (Wizard, Promega) protocol. The 16S rRNA gene was amplified with the primers fD1 (5'-AGAGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGAGGTGATCCAGCC-3'). PCR was performed in a TC-3000 G system (Techne®), with a 50 µL reaction consisting of 12.5 µL of GoTaq Colorless Master Mix (Promega®), 1 µM of each primer, 4 µL of genomic DNA (20 ng mL^{-1}), and 23.5 µL of nuclease-free water. The amplification consisted of initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. The products were subjected to electrophoresis on agarose (1.5%), dyed with GelRed® Nucleic Acid Gel Stain, and visualized using a Wisd WUV-L50 transilluminator (WiseUV®). The PCR products were purified with a Wizard® Genomic DNA Purification Kit and sequenced by the LANGEBIO Genomic Services Unit. The sequences obtained were analyzed with Chromas Lite® and compared against the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool (BLAST). In addition, BioEdit and MEGA-X software programs were used to perform multiple sequence alignments and construct a phylogenetic tree using the neighbor–joining (NJ) method and the Jukes–Cantor evolutionary model, with bootstrap support from 1000 replicates.

2.3 Fungi associated with defoliation in *P. patula, P. montezumae,* and *P. pseudostrobus*

The fungi associated with defoliation in the three pine species were isolated from needles with evidence of fungal damage. The needles were disinfected with 3% NaOCl and incubated on potato dextrose agar (PDA) to stimulate fungal propagation. The fungal colonies that

developed were isolated and identified by ITS region sequencing with the primers ITS1 (5'-GAGGGAAGGGG-3') and ITS4 (5'-GAGAGAAG-3') following the methodology of Álvarez *et al*. (2013).

Fungal DNA was extracted with a Wizard Genomic DNA Purification Kit (Promega). The PCR was performed in a final volume of 50 µL: 25 µL of GoTaq Colorless Master Mix (Promega®), 0.4 µM of each primer, 4 µL of genomic DNA (20 ng µL), and 17 µL of nuclease-free water. The amplification consisted of initial denaturation at 110 °C for 10 min, followed by 33 cycles of denaturation at 94 °C for 1 min, annealing at 49 °C for 1 min, and extension at 72 °C for 3 min, with a final extension at 72 °C for 7 min. The amplified product (~600 bp) was verified by electrophoresis on a 3% agarose gel; the bands were stained with GelRed® Nucleic Acid Gel Stain and visualized using a Wisd WUV-L50 transilluminator (WiseUV®). The PCR products were subsequently purified, sequenced, and analyzed following previously described specifications.

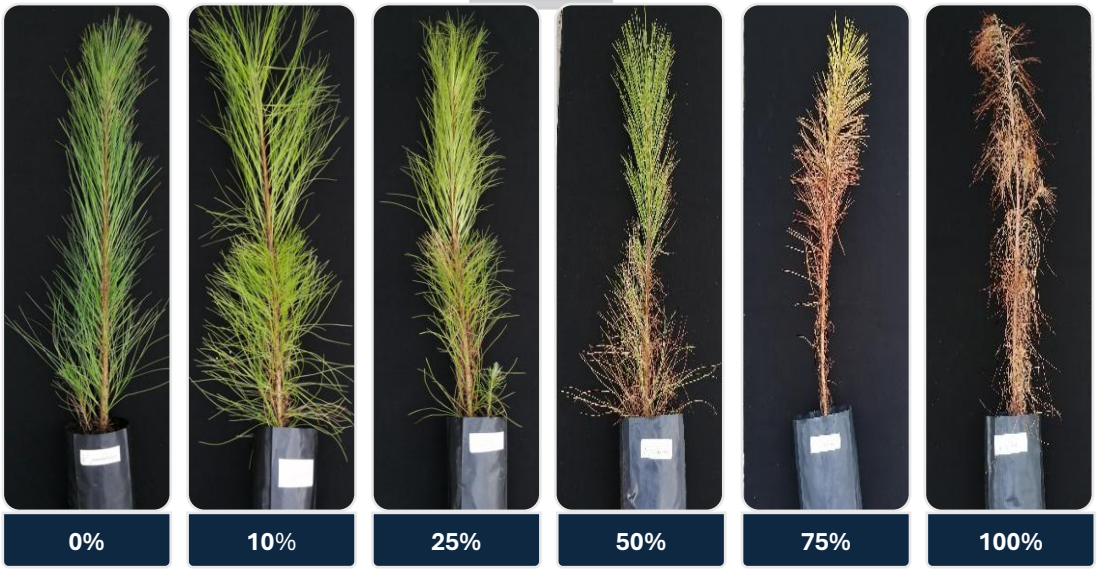
2.4 Effects of *Bacillus* strains on the control of pathogenic fungi of *Pinus* under greenhouse conditions

The most efficient bacterial strains (*P. simplex* (M2CC2), *B. thuringiensis* (M4PN3), and *P. simplex* (M5EL3)) that inhibited fungal growth in the *in vitro* test were selected to evaluate their effects as a mixture on pine plants infected with fungi associated with defoliation (treatments performed in triplicate). To achieve this, seedlings of *P. montezumae, P. patula,* and *P. pseudostrobus* (70 cm tall) were separately infected with the fungi *C. coatesiae, M. rubricosum,* and *C. rosea*. The fungi were propagated in potato dextrose broth for 5 days and then the seedlings were then foliar-sprayed, and the plants were kept at 30 °C and 60% humidity (Kim *et al*., 2024).

158 The bacteria were propagated in nutritive broth at 30 °C and 120 rpm for 24 h, and the
159 population was adjusted to 108 CFU mL−1. The bacterial treatments (15 mL) were foliar
160 sprayed twice at 15 and 30 days after the seedling infection. The incidence (I) and severity
161 index (SI) attributable to the presence of fungi were evaluated at 3 and 9 months post-
162 infection. Disease incidence was assessed by the number of lesions present on each pine
163 needle (1). To evaluate the disease severity index (SI), the severity scale developed in this
164 study (Figure 2) was used, and the formula proposed by Bock *et al*. (2022) (2) was applied.

165 I = Incidence of lesions = Σ lesions in each needle (1)

166 $$IS = Severity\ index = \frac{\Sigma(class\ frequency \times rating\ class\ score)}{(total\ number\ of\ observations \times \max imum\ disease\ index)} \times 100 \quad (2)$$



167

168 **Fig. 2** Disease severity scale in *Pinus* sp. plants. The percentage on the scale indicates the
169 extent of damage to the plant caused by fungi associated with defoliation

170 2.5 Effects of *Bacillus* as a biocontrol agent of pathogenic fungi on *Pinus* under field
171 conditions.

The pine trees selected for the test were in a forest management system with a predominant presence of *Pinus patula*. The selected trees were 10 m tall and showed evidence of fungal lesions on the needles and leaves and defoliation.

Three fungal control treatments were applied: biological, synthetic fungicide, and control (ten repetitions per treatment). The biological treatment consisted of a mixture of *P. simplex* (M2CC2), *B. thuringiensis* (M4PN3), and *P. simplex* (M5EL3) adjusted to 10^8 CFU mL^{-1}, the three most efficient bacteria for fungal biocontrol in previous tests; the synthetic fungicide was chlorothalonil (Tetrachloroisophthalonitrile), and a control group with no treatment was added. After seven months, the disease incidence (I) and severity index (SI) parameters were evaluated. Disease incidence was assessed by the number of lesions present on each pine needle, and to evaluate the disease severity index (SI), the formula proposed by Bock *et al*. (2022) was applied.

3. Results

3.1 *Pinus*-associated rhizobacteria

The bacterial isolation method targeted the genus *Bacillus* and yielded 27 gram-positive bacillus-shaped strains. All the strains produced siderophores, and 74% of the strains metabolized chitin. The 27 strains were identified first by the BioMérieux API system, and the most recurrent species were *Bacillus cereus* and *Bacillus mycoides* (Table 1).

Table 1 Chitinase and siderophore production of bacteria isolated from forest soil

Biochemical identification	Molecular identification (access number)	Strain	Productive efficiency of chitinases (%)	Productive efficiency of siderophores (%)
Bacillus cereus		BUAP M1CG1	11.90±1.71c	10.90±1.17d
Bacillus cereus		BUAP M2CC1	16.16±2.50c	14.16±5.20d
Bacillus cereus	*Bacillus mycoides* (OR452329.1)	BUAP M3H1	64.44±9.18a	37.18±3.47c
Bacillus cereus	*Bacillus mycoides* (OR452330.1)	BUAP M3H3	28.18±1.57b	83.33±5.89a

Bacillus cereus		BUAP M4PN2	17.26±06.76	31.75±2.24c
Bacillus cereus		BUAP M5EL1	14.81±06.41c	11.61±4.64d
Bacillus cereus	*Peribacillus simplex* (OR452332.1)	BUAP M5EL3	00.00±0.00d	57.41±6.93b
Bacillus cereus		BUAP M6MZ1	✓	✓
Bacillus cereus		BUAP M6MZ3	26.19±2.06b	33.33±11.78c
Bacillus cereus	*Bacillus cereus* (OR452333.1)	BUAP M6MZ4	33.33±0.00b	20.45±03.21c
Bacillus cereus		BUAP M8T1	✓	✓
Bacillus cereus		BUAP M8T2	00.00±0.00d	07.27±1.95d
Bacillus cereus		BUAP M8T4	06.14±0.45c	16.98±2.34d
Bacillus cereus		BUAP M8T5	11.57±0.80c	13.88±1.96d
Bacillus firmus		BUAP M4PN1	00.00±0.00d	41.66±6.80b
Bacillus mycoides		BUAP M3H2	21.21±5.25c	20.37±6.54c
Bacillus mycoides	*Bacillus thuringiensis* (OR452331.1)	BUAP M4PN3	✓	✓
Bacillus mycoides		BUAP M5EL2	14.02±3.54c	12.04±0.65d
Bacillus mycoides		BUAP M6MZ2	20.50±5.56b	25.00±0.00c
Bacillus mycoides		BUAP M8T3	13.03±6.05c	31.75±2.44c
Bacillus mycoides		BUAP M8T6	00.00±0.00d	15.08±1.12d
Bacillus mycoides		BUAP M8T7	✓	✓
Bacillus mycoides		BUAP M8T8	✓	✓
Bacillus mycoides		BUAP M8T9	26.06±6.82b	20.37±6.54c
Bacillus no reactive		BUAP M2CC3	00.00±0.00d	14.29±2.21d
Brevibacillus	*Peribacillus simplex* (OR452328.1)	BUAP M2CC2	32.19±2.40b	24.07±4.28c
Geobacillus		BUAP M1CG2	00.00±0.00d	37.30±4.05c

For the productive efficiency of chitinases and siderophores each value represents a mean ± standard error (n=5). ✓ = Strain producing chitinases and/or siderophores with non-quantifiable productive efficiency. Means with different letters within each column indicate significant differences according to Tukey's test ($p \leq 0.05$)

Sixteen bacterial strains with the highest siderophore and chitin production rates were selected for the Petri dish biocontrol assays against fungi (Table 1). All the strains inhibited the growth of *Myrmaecium rubricosum* (83.64% to 97.53%), *Curvularia coatesiae* (28% to 76%), and *Clonostachys rosea* (55%) (Figure 3).

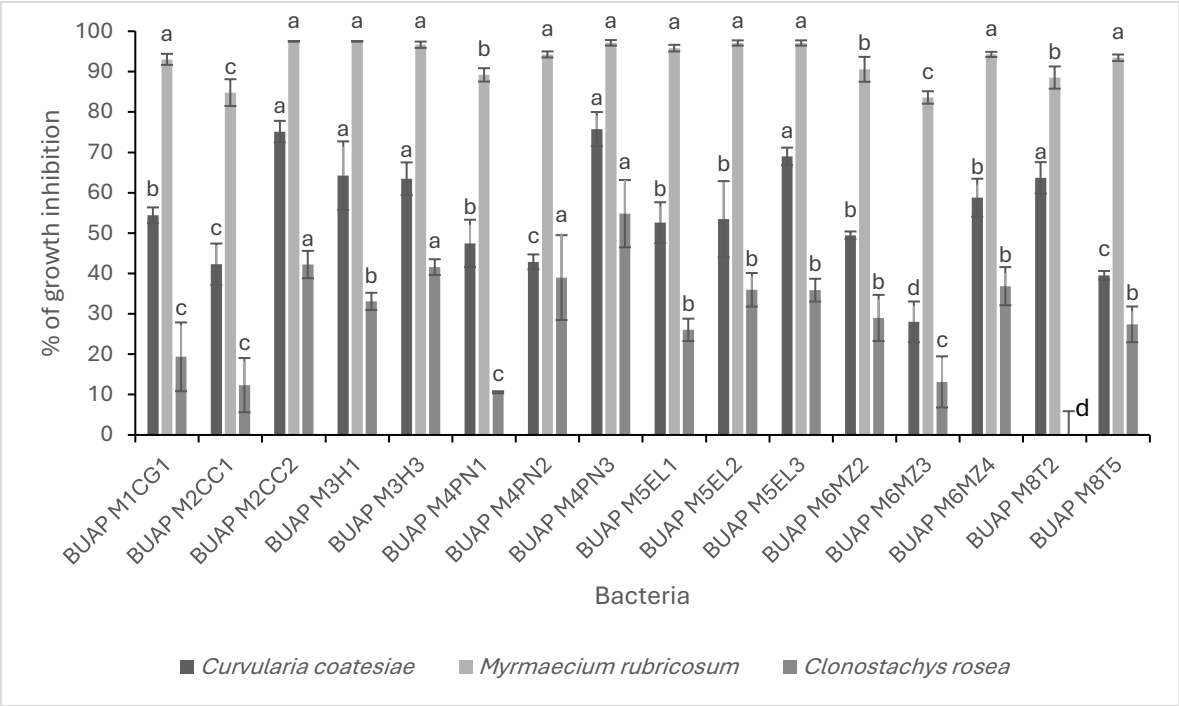
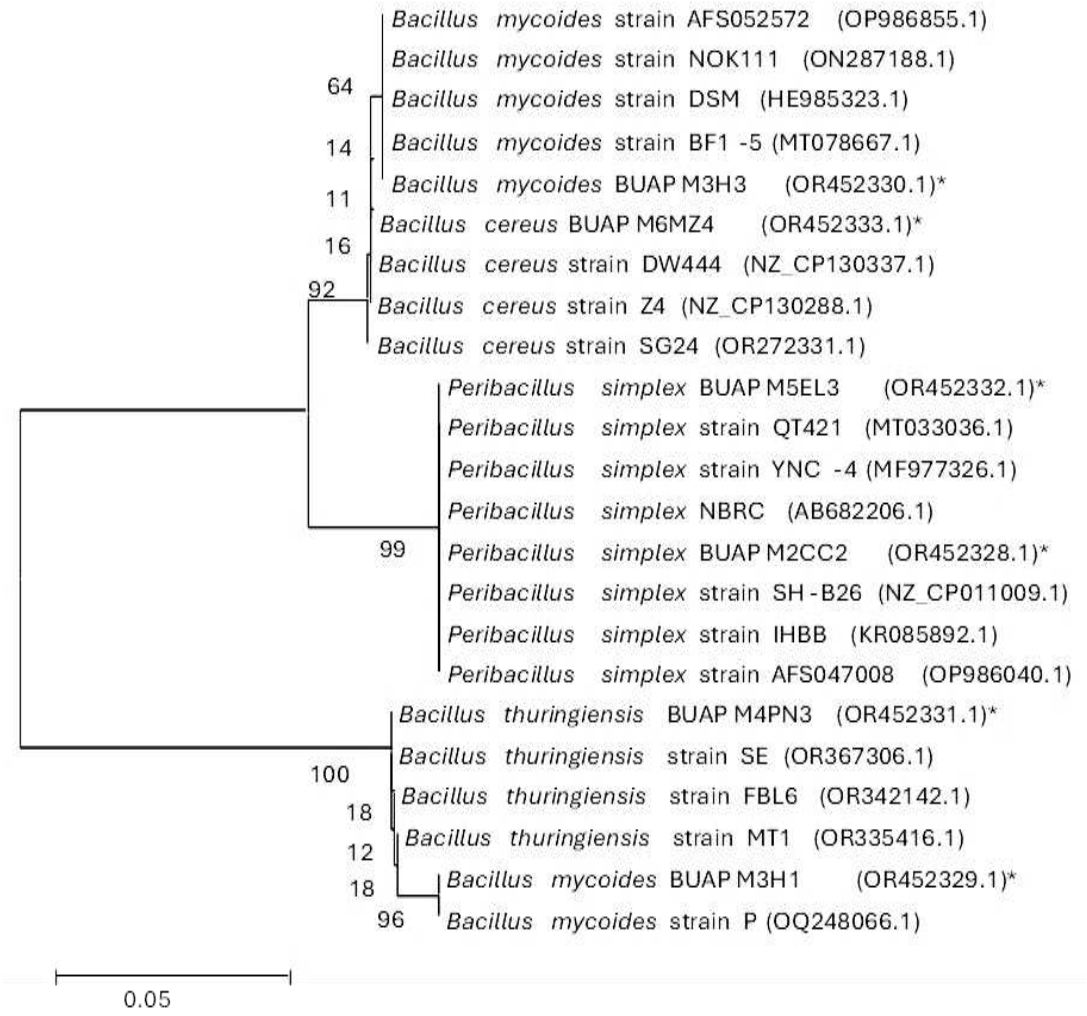


Fig. 3 *In vitro* inhibition of the mycelial growth of *C. coatesiae, M. rubricosum*, and *C. rosea* by *Bacillus* strains. Values represent the mean and standard deviation of the percentage of inhibition of mycelial growth of phytopathogenic fungi by *Bacillus* bacteria with respect to control treatment (n=3). Means with different letters between bacteria indicate significant differences according to Tukey's test ($p \leq 0.05$)

Based on these results, the six most efficient bacteria were identified by 16S rRNA sequencing (Table 1). The sequences of these strains were submitted to GenBank, and a phylogenetic tree was constructed to assess their relationships with other species reported in NCBI GenBank (Figure 4).



208

209 **Fig. 4** Phylogenetic tree of bacteria with enhanced fungal antagonism isolated from the
210 rhizosphere *of Pinus* spp. based on neighbor–joining (NJ) distance. The scale bar indicates
211 the number of base substitutions per site. Bootstrap node values as well as bacterial species
212 are shown, and numbers in parentheses indicate GenBank accession numbers of rDNA
213 sequences in GenBank. * Bacteria with greater fungal antagonistic capacity were isolated
214 from forest soil

215 3.2 Fungi associated with defoliation

216 With respect to phytopathogenic fungi, eight fungal strains were isolated from *P.*
217 *montezumae*, 25 from *P. patula,* and 10 from *P. pseudostrobus*. The nine fungi most
218 frequently isolated from needles were *Aspergillus flavus* (OQ975446), *Chaetomium*

globosum (OQ975448), *Clonostachys rosea* (OQ975449), *Coprinellus disseminates* (OQ975676), *Curvularia coatesiae* (OQ975457), *Hypoxylon texense* (OQ975509), *Myrmaecium rubricosum* (OQ975458), *Paecilomyces Formosus* (OQ975451), and *Sordaria fimicola* (OQ975675); three of them, *Curvularia coatesiae* (OQ975457), *Myrmaecium rubricosum* (OQ975458), and *Clonostachys rosea* (OQ975449), were found in common among the three pine species. A phylogenetic tree was also constructed from the sequences of these microorganisms to assess their relationships with species reported in NCBI GenBank (Figure 5).

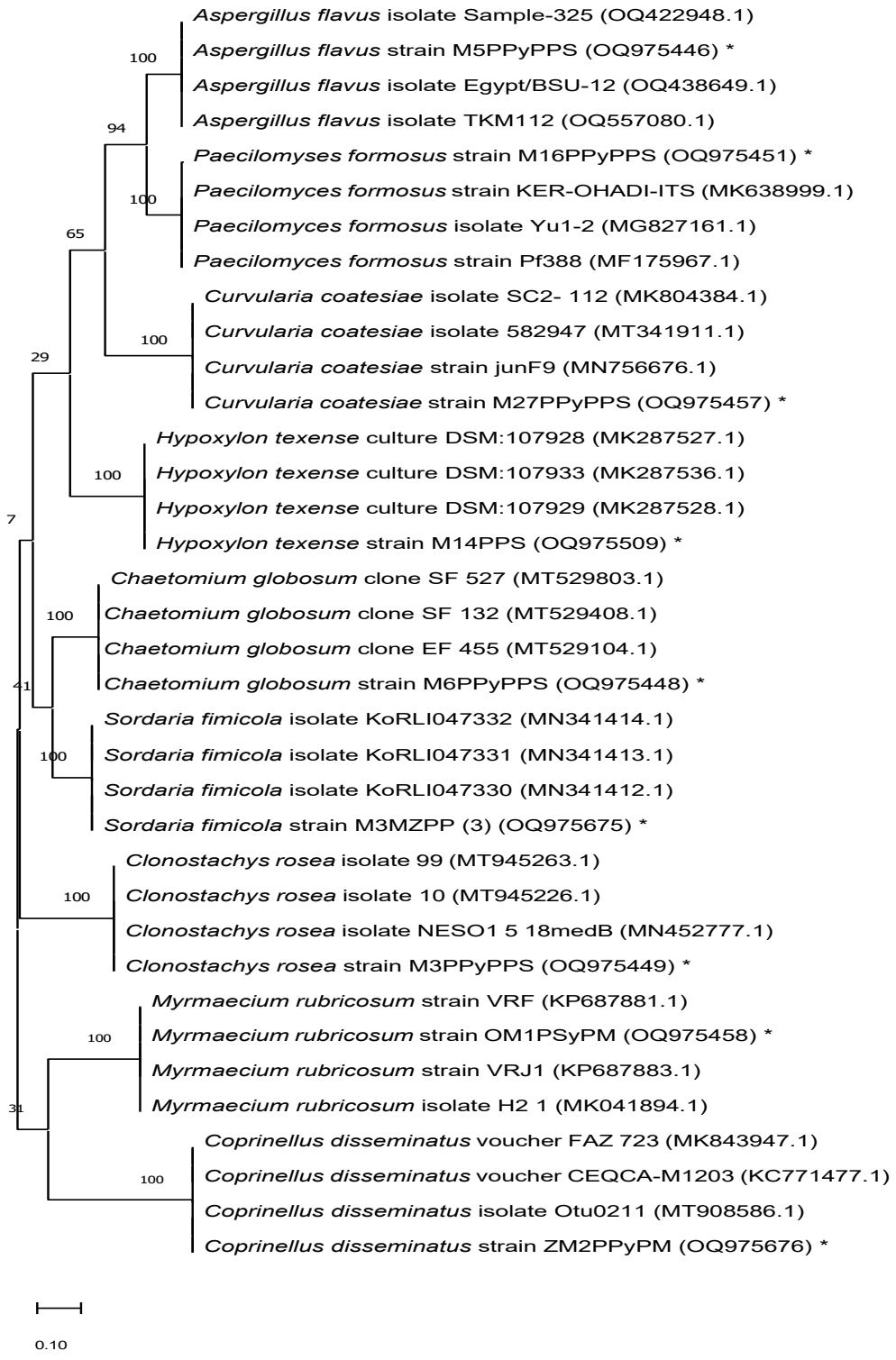


Fig. 5 Phylogenetic tree of fungi associated primarily with defoliation in *Pinus* spp. of mountain range of northern Puebla, México, based on neighbor–joining (NJ) distance. The scale bar indicates the number of base substitutions per site. Bootstrap values are shown next

231 to nodes, and numbers in parentheses indicate accession numbers for rDNA sequences in
232 GenBank. * Fungi most frequently isolated from the Puebla

233 3.3 Biocontrol of fungi associated with pine defoliation with antagonistic rhizobacteria

234 under greenhouse conditions

235 Fungal infection of pine seedlings manifested as damage to the needles, defoliation, and, in
236 the most severe cases, plant death. After three months of infection, *P. montezumae* had the
237 lowest disease severity index following inoculation by *C. coatesiae* (28±0.45%) and *M.*
238 *rubricosum* (24±0.55%), with lesion incidence reaching 2.66±0.58 and 2.5±0.59,
239 respectively. Moreover, *P. pseudostrobus* had the lowest severity index (28±0.55%) with *C.*
240 *rosea* fungus.

241 After nine months of treatment, similar behaviors were observed: *P. montezumae* presented
242 the lowest number of lesions, with 10.8±0.83 lesions, which were due to the presence of *C.*
243 *coatesiae*; 9.2±0.83 lesions, which were due to *M. rubricosum*; and 8.6±0.89 lesions, which
244 were due to *C. rosea*; and 44±11.18% disease severity, which was against *C. coatesiae*;
245 44±11.18% against *M. rubricosum*; and 56±20.91% against *C. rosea*. Conversely, *P. patula*
246 was the most susceptible pine species to pathogenic damage, as it presented the greatest
247 number of lesions per needle and the highest severity index. Moreover, bacterial treatments
248 decreased fungal damage in the three pine species under greenhouse conditions (Table 2);
249 for instance, *P. pseudostrobus* reported a reduction in disease severity of up to 30%.

250 **Table 2** Bacterial biocontrol of fungi under greenhouse conditions

Pine species	Treatment	Incidence (lesions)		Severity (%)	
		3 months	9 months	3 months	9 months
Patula	Control	02.20 ± 0.45d	08.33 ± 0.57d	48 ± 13.69bc	80 ± 17.68bc

Pine species	Treatment	Incidence (lesions)		Severity (%)	
		3 months	9 months	3 months	9 months
	C. coatesiae	16.33 ± 2.52c	24.00 ± 0.71a	56 ± 11.18b	92 ± 12.36a
	Bacillus + *C. coatesiae*	15.00 ± 1.41bc	21.00 ± 1.41ab	68 ± 13.69a	82 ± 12.36b
	M. rubricosum	06.66 ± 0.58b	17.66 ± 1.53bc	48 ± 13.69bc	72 ± 11.70cd
	Bacillus + *M. rubricosum*	05.66 ± 1.53ab	11.80 ± 0.83cd	40 ± 14.40c	60 ± 00.00d
	Clonostachys sp.	12.00 ± 2.00a	18.00 ± 1.73bc	64 ± 11.18ab	88 ± 12.36a
	Bacillus + *Clonostachys* sp.	09.33 ± 2.51a	13.33 ± 1.53cd	52 ± 13.69bc	80 ± 15.36bc
	Control	01.20 ± 0.84d	06.20 ± 0.83d	24 ± 6.71c	48 ± 13.69bc
	C. coatesiae	12.33 ± 1.53a	17.20 ± 2.59a	32 ± 8.22bc	52 ± 13.69b
	Bacillus + *C. coatesiae*	02.66 ± 0.58cd	10.80 ± 0.83c	28 ± 8.22c	44 ± 11.18c
Montezumae	*M. rubricosum*	08.66 ± 0.58b	14.80 ± 1.30ab	32 ± 8.22bc	56 ± 11.18ab
	Bacillus + *M. rubricosum*	02.50 ± 0.58cd	09.20 ± 0.83cd	24 ± 6.71c	44 ± 11.18c
	Clonostachys sp.	05.50 ± 0.58c	12.50 ± 1.73bc	44 ± 17.54a	64 ± 17.38a
	Bacillus + *Clonostachys* sp.	02.75 ± 0.96cd	08.60 ± 0.89cd	36 ± 6.71ab	56 ± 20.92ab
	Control	02.00 ± 0.71e	07.25 ± 0.95d	40 ± 14.40bc	60 ± 20.62c
	C. coatesiae	11.00 ± 1.00b	16.33 ± 1.53a	44 ± 11.18bc	80 ± 13.69ab
	Bacillus + *C. coatesiae*	08.33 ± 0.58c	13.40 ± 1.14b	36 ± 06.71c	52 ± 13.69cd
Pseudostrobus	*M. rubricosum*	09.00 ± 1.00c	14.60 ± 1.67ab	48 ± 13.69b	64 ± 11.18bc
	Bacillus + *M. rubricosum*	03.66 ± 0.58d	10.20 ± 2.16c	40 ± 20.12bc	48 ± 13.69d
	Clonostachys sp.	13.50 ± 1.29a	15.33 ± 1.53a	64 ± 20.92a	88 ± 13.69a
	Bacillus + *Clonostachys* sp.	04.33 ± 0.58d	09.33 ± 0.58cd	28 ± 08.22d	56 ± 11.18cd

Values expressed as mean ± standard deviation (n = 5). Means with different letters within each column indicate significant differences according to Tukey's test ($p \leq 0.05$). Comparisons were performed independently for each pine species

3.4 Biocontrol of defoliation-associated fungi under field conditions

Under field conditions, the needles of the pine plants in the control and chemical treatments (chlorothalonil) developed fungal infection into chlorosis, wilting, and leaf fall (Figure 6).



Fig. 6 *Pinus patula* needles with different treatments. Treatment with *Bacillus* promoted the health of pine trees (right), reducing damage and necrosis in the needles shown in the control (left)

In contrast, compared with the control treatment (51.11±6.86), biological treatment with *P. simplex* (M2CC2), *B. thuringiensis* (M4PN3), and *P. simplex* (M5EL3) reduced the severity index to 25.12±1.17. Additionally, the number of lesions per needle in the biological treatment group (1.17±0.73) was lower than those in the chemical fungicide (7.17±2.85) and control treatment (7.33±2.36) groups (Table 3).

Table 3 Health parameters of *Pinus patula* plants under field conditions

Treatment	Severity index	Incidence of lesions
Control	51.11±6.86 a	7.33±2.36 a
Chemical (2,4,5,6-tetrachloroisophthalonitrile)	43.89±7.12 a	7.17±2.85 a
Biological (*P. simplex* (2)*, B. thuringiensis*)	25.12±1.17 b	1.17±0.73 b

For the Severity index and Incidence of lesions each value represents a mean ± standard error (n=10). Equal letters indicate that there is no significant difference between treatments according to ANOVA and Tukey test ($p \leq 0.05$)

4. Discussion

Molecular identification of fungi revealed that fungal species affecting *P. patula, P. montezumae*, and *P. pseudostrobus* in northern Puebla belong to different genera. One of

the fungi identified was *Sordaria fimicola*, which has been reported as a biocontrol agent of phytopathogenic fungi *in vitro*, as it inhibited the mycelial growth of *Pestalotiopsis guepinii*, *Colletotrichum capsici, Curvularia lunata*, *Alternaria alternata*, and *Fusarium oxysporum* (Jeamjitt *et al*., 2007). *Aspergillus flavus*, another fungus isolated from the pine, is recognized as a soil saprophyte and opportunistic pathogen of humans and plants. This fungus causes food poisoning and produces carcinogenic metabolites known as aflatoxins (Majumdar *et al*., 2018). Additionally, *Clonostachys rosea* is associated with foliar loss; however, it has been described as an essential biocontrol agent of plant fungi through the production of chitinase, glucanases, and secondary metabolites that induce systemic responses in plants (Roberti *et al*., 2008; Rodriguez *et al*., 2011). *Hypoxylon* is a fungal genus comprising more than 200 species, some of which are associated mainly with angiosperm trees such as saprophytes and endophytes present in forest ecosystems worldwide (Pourmoghaddam *et al*., 2020). Similarly, the fungus *Paecilomyces formosus*, a known plant pathogen identified in the forestry sector, is the causal agent of oak dieback, causing chlorosis and death of branches and stems (Sabernasab *et al*., 2019). This species was found to be associated with needle loss in *Pinus* sp. from the site study. Moreover, a fungal strain of the genus *Curvularia* was identified; this genus comprises cosmopolitan saprophytes, including pathogenic species of plants, animals, and humans (Marin-Felix *et al*., 2020). In particular, *C. coatesiae* has been described as the causal agent of rice plant blight (Liu *et al*., 2020). Similarly, *M. rubricosum* was also found to be associated with defoliation in pine species. Members of the genus *Myrmaecium* are distributed worldwide and are saprophytes that affect immunocompromised conifers and broadleaves (Jaklitsch *et al*., 2015). In addition, *M. rubricosum* has been reported to be a pathogen of agave plants that causes wilting, spotting, and chlorosis (Campos-Rivero *et al*., 2019). Similarly, the

295 fungus *Coprinellus disseminatus* was identified and has been shown to provide nutrients
296 that promote seed germination and seedling development in *C. appendiculata* (Gao *et al*.,
297 2022). *Chaetomium globosum* is a fungus reported as an endophytic fungus of medicinal
298 plants that can produce bioactive compounds and has potential industrial applications. It
299 represents an essential resource for biotechnological applications in agriculture, industry,
300 and medicine (Lata *et al*., 2020).

301 Under *in vitro* and *in situ* conditions, the efficacy of a consortium of *Bacillus* isolated from
302 forest soil was demonstrated for the control of fungi associated with defoliation in *Pinus* sp.
303 Similar results have been reported previously for *Bacillus* strains against *Fusarium*
304 *sambucinum* infection in the seeds and seedlings of *Pinus elliottii* (Maciel *et al*., 2014). The
305 biocontrol capacity of *Bacillus* species is partially attributable to their ability to produce
306 lytic enzymes, as this behavior has been observed in *B. subtilis* and *B. thuringiensis*, which
307 produce chitinases that hinder the development of *Rhizoctonia solani*, *Penicillium glaucum*,
308 and *Sclerotinia fuckeliana* by inhibiting spore germination (Liu *et al*., 2010; Yan *et al*.,
309 2011). Similarly, siderophore-producing bacteria isolated from the rhizosphere of pine
310 plants in Sierra Norte de Puebla are of great interest for their potential in biological control,
311 as these compounds sequester iron and reduce its availability to phytopathogens; moreover,
312 these bacteria can increase plant systemic resistance and promote growth (Aguado-
313 Santacruz *et al*., 2012). In addition, *Bacillus* strains, whose mechanisms involve both
314 siderophore and lytic enzyme production, inhibited the mycelial growth of *Fusarium solani,*
315 *Fusarium oxysporum, Fusarium moniliforme*, and *Alternaria alternata* by more than 50%
316 (Govin *et al*., 2019).

317 Among the strains identified and tested for their ability to control defoliating fungi, *B. cereus*
318 is described as an opportunistic pathogen, and it is often studied for its potential in the
319 agricultural sector as a chitinolytic bacterium, which has been demonstrated over the
320 phytopathogenic fungi *Botrytis* and *Cercospora arachidicola* (Huang *et al*, 2005; Kokalis-
321 Burelle *et al*., 1992; Kotiranta *et al*., 2000). *B. thuringiensis* produces Cry and Cyt proteins
322 during its sporulation phase, with biological activity used for pest control in agriculture; in
323 addition, the bacteria also produce chitinases (Adang *et al*., 2014; Bravo *et al*., 2011;
324 Sánchez & Barboza, 2017). *B. thuringensis* is the primary microorganism used worldwide
325 for the biological control of insect pests, driven by the growing demand for pesticide and
326 chemical-free food and the use of environmentally friendly control agents (Almeida *et al*.,
327 2016). *B. mycoides* has been described as a nonpathogenic agent that colonizes the
328 phyllosphere and can induce systemic resistance in plants, as it promotes the activity of
329 enzymes related to pathogenesis, such as chitinase, β-1,3-glucanase, and peroxidase
330 (Bargabus *et al*., 2002). This bacterium can control *Botrytis cinerea* in crops by secreting
331 volatile and nonvolatile inhibitory compounds and by collaborating in the activation of host
332 defense systems (Guetsky *et al*., 2007). In addition, it is an effective biocontrol agent against
333 fungal pathogens that affect plant roots (Ghazal *et al*., 2013). The genus *Peribacillus*
334 comprises 17 species isolated from diverse ecological niches, and some species, such as
335 *Peribacillus castrilensis*, are associated with plant growth promotion and biological control
336 due to their ability to produce inhibitory compounds, including antifungal lipopeptides,
337 thereby limiting the development of pathogens such as *D. solani* and *E. amylovora*
338 (Rodríguez *et al*., 2022). *Bacillus simplex* reduced the symptoms of black cumin root rot
339 caused by *Fusarium camptoceras* (PHYF1) in *Nigella sativa* up to 60% under greenhouse
340 and field conditions (Al-Sman *et al*., 2019).

)

341 Biocontrol treatments under greenhouse conditions have demonstrated that the use of
342 bacteria isolated from the rhizosphere of plants of the genus *Bacillus*, such as *B. subtilis, B.*
343 *megaterium*, and *B. brevis*, is efficient for the biocontrol of the development of fungi of the
344 genus *Fusarium* in rosemary plants, as they reduce the severity of infection by 20–38%
345 (Haggag, 2008; Saleh *et al*., 2021). *B. simplex* has also been reported to minimize the
346 incidence of infection by the fungi *Heterobasidion annosum* and *Armillaria mellea* by 55%
347 and 46.9%, respectively, in *Pinus radiata* seedlings (Mesanza *et al*., 2016). Similarly, in
348 natural environments, the use of bacteria for biological control is effective in combating
349 diseases. For example, under *in vitro* conditions, isolates of *P. fluorescens, E. billingiae*,
350 and *B. simplex* have antagonistic effects on *F. circinatum* in young *P. radiata* trees and can
351 reduce the growth of the fungus up to 29%; in addition, under natural conditions, the
352 incidence of lesions decreases up to 25% (Iturritxa *et al*., 2017). Furthermore, the strain *B.*
353 *simplex* S11R41 inhibited the pine root rot fungi *Heterobasidion annosum* and *Armillaria*
354 *mellea* in young *P. radiata* trees (Mesanza *et al*., 2019).

355 **5. Conclusion**

356 Defoliation in *Pinus* sp. from the Sierra Norte of the state of Puebla is mainly related to the
357 presence of nine fungi: *A. flavus, C. globosum, C. rosea*, *C. disseminatus, C. coatesiae, H.*
358 *texense, M. rubricosum, P. formosus,* and *S. fimicola.* In addition, the fungi *C. coatesiae, M.*
359 *rubricosum,* and *C. rosea* are common pathogens that damage *P. patula, P. montezumae,*
360 and *P. pseudostrobus*. The selection of six strains with biocontrol characteristics of *B.*
361 *mycoides* (2), *B. cereus, B. thuringiensis,* and *P. simplex* (2) species and their application in
362 *in vitro* and *in vivo* biological control assays demonstrates that bacteria isolated from forest
363 soil can limit fungal growth under controlled conditions and contribute to the disease in

greenhouse and field pines under forest management. The results demonstrate that biological control of fungal pathogens by antagonistic bacteria promotes pine health and represents an alternative to fungicide treatment in natural systems.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Luis Martín Gutiérrez-Flores, Lucía López Reyes, Amparo Mauricio-Gutiérrez, and Moisés Graciano Carcaño-Montiel performed preparation of materials, data collection, and analysis. The first draft of the manuscript was written by Luis Martín Gutiérrez-Flores, and all authors provided feedback on earlier versions. The statistical analysis was performed by Guadalupe Medina-de la Rosa. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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