ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/351428053

Evaluation of Bacillus spp. isolates as potential biocontrol agents against charcoal rot caused by Macrophomina phaseolina on common bean

Article *in* Journal of General Plant Pathology · May 2021

DOI: 10.1007/s10327-021-01019-4

CITATIONS
13

READS
271

6 authors, including:

Juan Carlos Martínez-Álvarez
Instituto Politécnico Nacional
20 PUBLICATIONS **267** CITATIONS
SEE PROFILE

Castro Martínez Claudia
Instituto Politécnico Nacional
27 PUBLICATIONS **404** CITATIONS
SEE PROFILE

Yolani De Jesús Bojórquez-Armenta
Instituto Politécnico Nacional
3 PUBLICATIONS **14** CITATIONS
SEE PROFILE

Ignacio Eduardo Maldonado-Mendoza
Instituto Politécnico Nacional
116 PUBLICATIONS **3,726** CITATIONS
SEE PROFILE

All content following this page was uploaded by Juan Carlos Martínez-Álvarez on 29 November 2021.

The user has requested enhancement of the downloaded file.

Journal of General Plant Pathology
https://doi.org/10.1007/s10327-021-01019-4

DISEASE CONTROL

Evaluation of *Bacillus* spp. isolates as potential biocontrol agents against charcoal rot caused by *Macrophomina phaseolina* on common bean

Yolani de J. Bojórquez-Armenta[1] · Guadalupe A. Mora-Romero[2] · Melina López-Meyer[1] · Ignacio E. Maldonado-Mendoza[1] · Claudia Castro-Martínez[1] · Cecilia de los A. Romero-Urías[2] · Jesús D. Cordero-Ramírez[3] · Juan C. Martínez-Álvarez[1]

Received: 19 December 2020 / Accepted: 27 April 2021
© The Phytopathological Society of Japan and Springer Japan KK, part of Springer Nature 2021

Abstract
The fungus *Macrophomina phaseolina*, the causal agent of charcoal rot of common beans, damages the roots, stems, and leaves of seedlings and plants and forms resistant structures, so that chemicals are not sufficient for disease control. Integrated management systems associated with the use of biological control techniques are a sustainable alternative. Here we collected 37 native bacterial isolates from the common bean rhizosphere and screened them for antagonistic activity against *M. phaseolina*. Four isolates (BA97, BN17, BN20, and BR20) identified as *Bacillus* spp. showed antagonism in vitro against *M. phaseolina*, inhibiting its growth by 62.5–85%. In an *in planta* antagonistic assay, isolate BN20 reduced disease severity the most. Isolates BA97, BN17, BN20, and BR20 produced volatile compounds as a mechanism of antagonism. They also produced indole acetic acid in vitro (1.98–3.87 µg/ml). These results suggest that seed bacterization with the rhizobacterial isolates for field planting may be an effective means to reduce crop damage by *M. phaseolina*.

Keywords Biological control · Charcoal rot · Common bean · *Macrophomina phaseolina*

Introduction

Common bean (*Phaseolus vulgaris* L.) can be affected by a variety of pathogenic organisms (Singh and Schwartz 2010), including the fungus *Macrophomina phaseolina* (Tassi) Goid. Worldwide, it parasitizes more than 500 plant species (Kunwar et al. 1986) including several important crops such as common bean, soybean, sorghum, maize, alfalfa, and cotton (Islam et al. 2012; Shrivastava et al. 2017). This fungus is the causal agent of charcoal rot, a disease that can produce significant yield losses in infested soils (Sabaté et al. 2017).

In common bean, charcoal rot can affect the root at any growth stage of the plant (Kaur et al. 2012). Fungal diseases in crop fields are controlled through integrated management that includes monitoring and preventive control and chemical products when symptoms of infections occur (Lindsey et al. 2020). However, the incorrect use of such products can result in a minimal effect on the control of the pathogen, a negative impact on the environment, a potential risk for human health and an unnecessary cost to the grower (Bolzonella et al. 2019; Mendoza Cantú and Ize Lema 2017).

Other options for prevention are thus needed, such as resistant common bean varieties, seed treatments, crop rotation, and water management (Radwan et al. 2014). Sustainable strategies are needed for more eco-friendly alternatives to reduce the use of fertilizers and pesticides (Ab Rahman et al. 2018; Sabaté et al. 2017; Shrivastava et al. 2017). Biological control by beneficial microorganisms is considered a promising method for sustainable integrated management

Yolani de J. Bojórquez-Armenta and Guadalupe A. Mora-Romero contributed equally to this work.

✉ Juan C. Martínez-Álvarez
jcmartinezal@ipn.mx

[1] Departamento de Biotecnología Agrícola, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, Unidad Sinaloa, Instituto Politécnico Nacional, Guasave, Sinaloa, Mexico

[2] Unidad de Investigación en Ambiente y Salud, Universidad Autónoma de Occidente, Los Mochis, Sinaloa, Mexico

[3] Departamento Académico de Ciencias Naturales y Exactas, Universidad Autónoma de Occidente, Guasave, Sinaloa, Mexico

Published online: 21 July 2021

of diseases (Ab Rahman et al. 2018). For example, root-associated and endophytic bacteria, known as plant growth-promoting rhizobacteria (PGPR), can positively influence plant growth (Ali et al. 2020; Etesami and Maheshwari 2018). These bacteria develop in the rhizosphere or on the rhizoplane and on the surface and interior of root nodules (Bhattacharyya and Jha 2012; Kloepper 1978). PGPRs can also act as biocontrol agents against pathogens (Alijani et al. 2019).

In the present study, we isolated bacteria from the rhizosphere and nodules of common bean plants and analyzed their antagonistic potential against *M. phaseolina* in vitro and *in planta*.

Materials and methods

Sampling and bacteria isolation

Ten soil samples were collected from different agricultural crop fields in Guasave, Sinaloa, Mexico. The soil samples were mixed and placed in 20-cm-diameter pots. Common bean seeds (var. Azufrado Higuera) were sown in five pots (five seeds per pot) and used as a trap culture. Pots were then kept at 25 °C with 16 h light/8 h dark for 30 days. Plants were then uprooted, and the roots were placed in 50 ml polypropylene tubes containing 30 ml of 0.85% (w/v) sterile saline solution to make serial dilutions. Dilutions were spread onto Luria Bertani (LB) agar (Sigma-Aldrich, St. Louis, MO, USA and incubated at 30 °C for 24 h until bacterial colonies were observed. Concurrently, and before roots were placed in the 50 ml tubes, five nodules were removed from each plant and disinfected in 95% (v/v) ethanol for 1 min, 3% (v/v) sodium hypochlorite for 3 min, then rinsed three times with sterile distilled water. Each nodule was placed in 50 µl of sterile distilled water in a 1.5 ml microcentrifuge tube and crushed aseptically. Subsequently, 100 µl of nodule contents were streaked onto LB agar plates, and plates were incubated at 30 °C for 24–48 h until bacterial colonies were observed. Bacterial colonies were chosen according to differences in colony morphology, then purified by single-colony streaking on LB plates and incubated as described. They were maintained at − 70 °C in LB broth with 15% (v/v) glycerol (Daigle et al. 2002; Lakshmi et al. 2015; Patil 2014).

Phytopathogenic fungi

The *M. phaseolina* strain, kindly provided by the Unidad Tecnológica Fitosanitaria Integral (UTEFI-SAGARPA), was isolated from common bean plants with charcoal rot symptoms from a common bean field located in the El Fuerte Valley, Sinaloa, Mexico, and stored frozen (− 70 °C) as a stock in 15% (v/v) glycerol. The strain was previously characterized as a pathogen for common bean plants according to Koch's postulates.

Fusarium verticillioides, *F. oxysporum* f. sp. *radicis-lycopersici*, *Rhizoctonia solani*, and *Sclerotium rolfsii* were isolated from different plant species (maize, tomato, and common bean, respectively) in local agricultural fields by our work group, but are all considered potential common bean pathogens (Paparu et al. 2020; Sánchez-García et al. 2017). They were stored frozen (− 70 °C) as a stock in 15% (v/v) glycerol.

Hemolysis test

For this initial screen to eliminate any bacterial strains that can be potentially harmful to human health, an individual bacterial colony from each isolate was grown in LB broth at 30 °C and 250 rpm for 24 h. Cultures were centrifuged at 13,000 rpm for 5 min, then 100 µl of a bacterial culture was added to a 5-mm-diameter well in a blood agar plate and incubated at 37 °C for 24 h. Isolates that removed the color from the blood agar around the wells were designated as hemolytic bacteria; isolates that did not change the color of the blood agar around the wells were designated as non-hemolytic bacteria. The experiments were performed with six replicates per isolate (Forbes et al. 2002).

Antagonistic activity assay

The bacteria were tested for antagonism against *M. phaseolina* using a dual culture technique (Paneerselvam et al. 2012) with modifications. Briefly, a mycelial plug (5 mm in diameter) from the edge of a 7-day-old colony of *M. phaseolina* on potato dextrose agar (PDA) (Difco, Lawrence, KS, USA) was placed in the center of a 90-mm-diameter Petri dish containing. A bacterial isolate was spotted at four equidistant locations 2 cm from the mycelial plug, and plates were incubated at 28 °C for 3–5 days. When the mycelial diameter on the control plates (without bacteria) was 4 cm, the radius of the fungal colonies on the test plates was measured. Percentage of inhibition (PI%) was calculated as

$$\mathrm{PI\%} = [(C - T)/(C)] \times 100, \quad (1)$$

where C is the radius of the fungus in the control plate and T is the radius of the fungus in the presence of the bacterial isolates (Paneerselvam et al. 2012). Experiments were performed twice, with four replicates per bacterial isolate. Isolates that inhibited the pathogen ≥ 50% were selected for further testing. Four selected isolates were then evaluated in vitro using the same dual culture technique described in this section for antagonistic potential against four potential

common bean phytopathogenic fungi: *F. verticillioides*, *F. oxysporum* f. sp. *radicis-lycopersici*, *R. solani*, and *S. rolfsii*.

Volatile compounds assay

Two-compartment agar plates were set up with LB agar in one section and PDA in the other (Fernando et al. 2005). A bacterial isolate was streaked on the LB agar, then a mycelial plug (5 mm in diameter) of *M. phaseolina* was placed on the PDA. Plates were then sealed and incubated at 28 °C for 3–5 days. When the control mycelial growth was 4 cm in diameter, the radius of the mycelial colony was measured to calculate PI% (Eq. 1). The experiments were done twice, with three replicate plates per bacterial isolate.

Phosphate solubilization assay

The bacterial isolates were incubated in 5 ml of LB broth for 18 h at 30 °C and 200 rpm, then 1 ml of each culture was centrifuged three times at 16,000×*g* for 10 min to obtain a supernatant. Then 50 µl of the supernatant for an isolate was placed in each of three 5-mm-diameter wells in a Pikovskaya's agar plate [dextrose 10 g; $Ca_3(PO_4)_2$ 5 g; yeast extract 0.5 g; $(NH_4)_2SO_4$ 0.5 g; KCl 0.2 g; $MgSO_4$ $7H_2O$ 0.1 g; $FeSO_4$ $7H_2O$ 0.0001 g; $MnSO_4$ 0.0001 g; agar 15 g; distilled water 1 l]. The Petri dishes were incubated for 8 days at room temperature (25 ± 2 °C). Isolates with a clear halo in the medium around the well were selected as phosphate-solubilizers (Pikovskaya 1948). The experiments were performed with six replicates per isolate (2 plates/isolate, 3 wells/plate).

Siderophore plate assay

One hundred microliters of a bacterial culture was added to a 5-mm-diameter well in a chrome azurol S (CAS) agar plate (Schwyn and Neiland 1987) and incubated at room temperature (25 ± 2 °C) in the dark for 16 days. Isolates that turned the blue CAS agar around the wells to a transparent orange or yellow color were designated as siderophore producers. The experiments were performed with six replicates per isolate (2 plates/isolate, 3 wells/plate).

Indole-acetic acid (IAA) production

For an IAA colorimetric analysis, an individual bacterial colony from each isolate was grown in LB broth at 30 °C and 200 rpm for 24 h. Cultures were centrifuged at 16,000×*g* for 10 min, then 100 µl of the bacterial supernatant was removed, and 100 µl of Salkowski's reagent [($FeCl_3$ 0.5 M, $HClO_4$ 35% (v/v)] was added. The mixture was incubated for 30 min at room temperature (25 ± 2 °C) in the dark. IAA was quantified by spectrophotometry at 530 nm, and concentrations were calculated from an indole-3-acetic acid (Sigma-Aldrich, St. Louis, MO, USA) standard curve (Gordon and Weber 1951). The experiments were performed with three colony replicates per bacterial isolate.

Disease severity assessment

To evaluate disease severity caused by *M. phaseolina* on bean, we planted one common bean seed (var. Azufrado Higuera) in each of 70 polystyrene pots (355 cm^3, 20 cm diameter) containing a sterile vermiculite–sand mix (1:1). Ten pots of soil received 1 ml of sterile distilled water for the absolute controls, and 60 received 1 ml of a suspension of microsclerotia and hyphal fragments (3.4×10^3 CFU/ml). The pots were placed on steel shelves and kept in a growth room (12 m^2) at 25 °C under a 16 h light:8 h dark photoperiod for 8 days.

We rated the severity of charcoal rot in common bean plants 8 days after inoculation with *M. phaseolina* using a scale of 0 to 5: 0 = no damage; 1 = minor chlorosis and necrotic lesions on cotyledons; 2 = necrosis on cotyledons, stem base, and roots, and seedlings with two true leaves (in some cases deformed); 3 = cotyledons completely necrotic, with necrosis nearing the stem, small leaves and shorter plants compared to levels 1 and 2; 4 = seedlings without leaves, cotyledons and stems necrotic; 5 = seeds not germinated or stems short, roots completely necrotic, and in some cases, microsclerotia present (Fig. S1a–f).

In planta assays of bacterial antagonism of *M. phaseolina*

Common bean seeds were surface-disinfected by soaking in a 1% (w/v) NaOCl solution for 3–6 min, then rinsed six times in sterile distilled water and allowed to dry at room temperature before inoculation (Somasegaran and Hoben 1994). To produce the bacterial inoculum, we started a fresh culture of each strain using the frozen glycerol stock (− 70 °C): one colony was placed in an assay tube with 5 ml of LB broth and grown on an orbital shaker at 200 rpm and 30 °C for 18 h. A 500-ml Erlenmeyer flask containing 100 ml of LB medium was then inoculated with 1 ml of this culture (1% v/v) and incubated at 30 °C and 200 rpm for 14 h. To produce the fungal inoculum, we subcultured *M. phaseolina* in Petri dishes for 7–10 days, then flooded the dishes with sterile distilled water and agitated the surface with a sterile glass rod to obtain a suspension of microsclerotia and hyphal fragments, estimating the CFUs by dilution on PDA. Then 1 ml of the bacterial suspension (1×10^6 CFU/ml) and 1 ml of the fungal suspension (3.4×10^3 CFU/ml) were poured onto the seeds. The treated seeds were sown in pots containing sterile vermiculite/sand substrate (1:1, v/v). Two control treatments were included: an absolute control, in which

the seeds were treated in the same way with LB broth; and a pathogen control, in which seeds were treated only with *M. phaseolina*. The trial was done twice in a completely randomized design, with five replicates per treatment in a growth room at 25 °C with 16 h light/8 h dark for 8 days. Each pot received 15–20 ml of sterile distilled water every 48 h. Disease incidence (*I*) was calculated using Eq. 2 (Eke et al. 2016).

$$I(\%) = \frac{\text{No. infected plants}}{\text{Total no. plants}} \times 100. \quad (2)$$

The disease index (DI) was calculated based on the disease severity index (described above) using Eq. 3 (Eke et al. 2016).

$$\text{DI}(\%) = \frac{\sum (ab)}{AK} \times 100, \quad (3)$$

where *a* is the number of plants with the same disease severity scale, *b* is the disease severity scale, *A* is the total number of plants, and *K* is the highest disease severity scale.

The biocontrol effect (BE%) was calculated using Eq. 4 (Chen et al. 2010).

$$\text{BE}\ (\%) = \frac{\text{DIP} - \text{DIB}}{\text{DIP}} \times 100, \quad (4)$$

where DIP is the disease index for the pathogen control and DIB is the disease index for the bacterial treatment plus fungal inoculation. In addition, shoot and root length were measured, and plant fresh mass was recorded.

Molecular identification

The bacterial isolates were identified by sequencing the 16S rRNA gene. The isolates were grown in LB broth for 18 h at 200 rpm and 30 °C. Genomic DNA was extracted using the DNAzol kit (Thermo Fisher Scientific, Carlsbad, CA, USA), according to the manufacturer's recommendations. The 16S rDNA gene was amplified by polymerase chain reaction (PCR) using oligonucleotides F_2C (5′-AGAGTTTGATCA TGGCTC-3′) and C (5′- ACGGGCGGTGTGTAC-3′) (Shi et al. 1997), which amplify a region of ~ 1400 bp. The PCR mix contained 1 µl (50–100 ng) of DNA, 1 × of reaction buffer, 1 mM of $MgCl_2$, 0.5 mM of each oligonucleotide, 500 µM of deoxynucleotide triphosphate (dNTPs) and 0.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) in a total volume of 25 µl. The PCR was performed using a MyCycler thermal cycler (BioRad; Hercules, CA, USA) with one cycle of 95 °C for 4 min; 30 cycles at 95 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min; and a final extension at 72 °C for 5 min. The PCR products were electrophoretically separated in agarose (0.8%, w/v) in 0.5 × Tris–acetate-EDTA (TAE) buffer and visualized with UV light after ethidium bromide staining. The PCR products were sequenced in both directions using an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA, USA) at the National Genomic Laboratory (Langebio, CINVESTAV-Irapuato, Mexico). The sequences were edited using BioEdit, version 7.0.5.3 and compared with the NCBI database using the BLASTn platform and the megablast algorithm.

For identifying *M. phaseolina*, the ITS region was amplified with the oligonucleotides ITS-1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′), which amplify a region of ~ 600 bp (White et al. 1990). The thermocyling program consisted of one cycle of 4 min at 95 °C; 34 cycles of 1 min denaturation at 95 °C, 1 min at 55 °C, and 2 min at 72 °C; and a final step at 4 °C for 5 min.

For phylogenetic analysis of the bacterial isolates, multiple sequence alignments were performed using the CLUSTAL W software (Larkin et al. 2007). A phylogenetic tree based on the 16S rRNA sequence was constructed in MEGA X (Kumar et al. 2018) using the maximum likelihood method and the Kimura two-parameter model (Kimura 1980), with starting values based on 1000 repetitions. The 16S rRNA sequences of the four bacterial isolates (BA97, BN17, BN20, and BR20) and reference strains of the closest genera were included, and *Streptomyces* sp., *Escherichia coli*, *Rhizobium leguminosarum* and *Pseudomonas* sp. were used as outgroups.

Statistical analyses

All data were analyzed using SPSS software (IBM SPSS Statistics for Windows, version 25.0. IBM, Armonk, NY, USA). The data obtained from in vitro antifungal assays, volatile compounds and biochemical assays were analyzed by one-way ANOVA and Tukey's test. The data from *in planta* antagonism tests were analyzed using the Kruskal–Wallis test and Dunn's procedure with Bonferroni's correction. Significance was evaluated using $\alpha < 0.05$ in all tests.

Results

Isolation and selection of antagonistic bacteria, in vitro assays of antagonism

Of the 243 isolates obtained from roots and nodules and screened in the hemolysis assay, 37 isolates were non-hemolytic and tested further for antagonism against *M. phaseolina*. Six of the 37 isolates inhibited the growth of *M. phaseolina* by 55–85% (Table 1); the four best antagonists comprised two rhizospheric isolates (BA97 and BR20) and two isolates from nodules (BN20 and BN17), which inhibited radial growth of the fungus by 85–62.5% compared to

Table 1 Effect of 37 bacterial isolates on mycelial growth of *Macrophomina phaseolina* in vitro

Isolate	Inhibition (%)[a]	Isolate	Inhibition (%)	Isolate	Inhibition (%)
BA14	0	BA103	0	BR15	0
BA35	0	BA104	0	BR20	62.5
BA48	0	BA105	0	BR41	0
BA89	0	BA106	0	BR52	35.0
BA90	0	BA108	0	BR53	23.5
BA91	0	BA109	0	BR56	45.0
BA92	0	BA110	0	BR95	0
BA93	55	BA111	0	BN2	0
BA94	0	BN13	35	BN3	35.0
BA95	0	BN14	0	BN5	0
BA97	85	BN16	0	BN8	30.0
BA100	60	BN17	85		
BA101	0	BN20	80.0		

[a]Inhibition $\% = [(C-T)/C] \times 100$, where C = radius of mycelium in the control, T = radius of mycelium in the presence of the bacterium

the fungus with no bacteria (Fig. 1a, b; Table 2). Additionally, the selected isolates did not inhibit mycelial growth of the four other fungi as effectively as they did *M. phaseolina*; inhibition percentages ranged from 0 to 52%, depending on the fungus (Table 2).

In planta assays

In the *in planta* assays using seeds inoculated with isolates BA97, BN17, BN20, and BR20 and the fungus or the fungus alone, BA97 and BN17 had positive effects on plant root length, whereas BN20 had a positive effect on root fresh mass and root and shoot length, as compared to the Mp treatment. Nevertheless, the increase in plant root and shoot length was similar to the control plants without treatment. Plants treated with BR20 did not differ significantly from the Mp control plants (Table 3); thus, BR20 did not provide any disease control. For plants inoculated with isolates BA97, BN17, and BR20, the disease index (DI) ≥ 50% (54.4–68.6%) and biocontrol effect (BE%) ≤ 50% (11.1–14.8%). Isolate BN20 gave the highest control over the pathogen; the DI% was 34.3% and BE was 55.5% (Table 3).

Assays for volatile compounds, phosphate solubilization, siderophores, and IAA

The four selected bacterial isolates produced inhibitory volatile compounds in the in vitro antagonistic assays; mycelial diameters were inhibited between 37.5 and 75% (Table 4). On the other hand, none of the four isolates solubilized phosphate or produced siderophores. The isolates produced between 1.98 and 3.87 µg/ml indole acetic acid (Table 4).

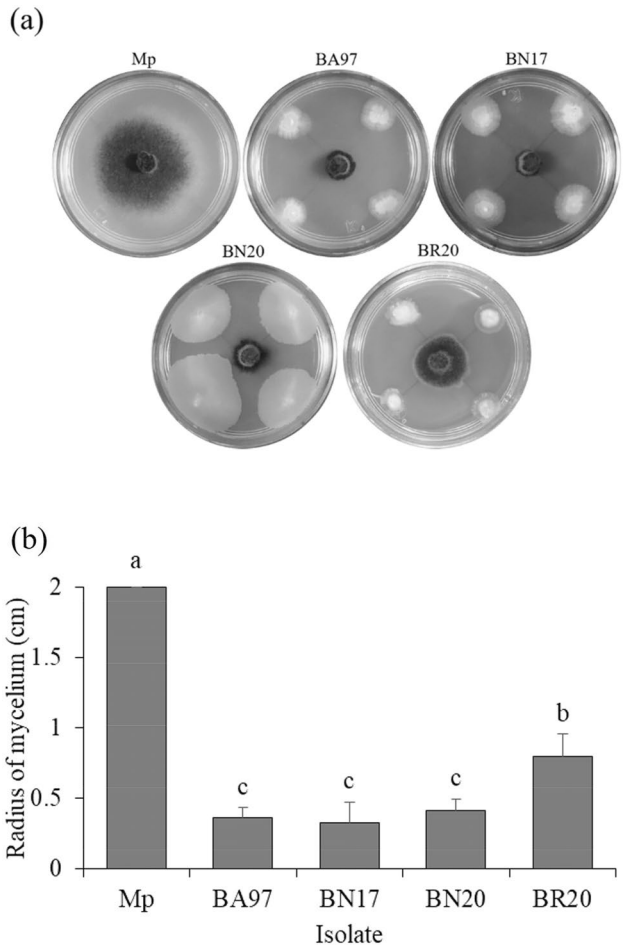

Fig. 1 Inhibitory effect of bacterial isolates BA97, BN17, BN20, and BR20 against *Macrophomina phaseolina* (Mp) in vitro. **a** Assay of selected isolates after 5 days. A mycelial plug is in the center of the PDA; four bacterial colonies are at the outer edge. **b** Radius of mycelium after 5 days in the assay shown in (**a**). Error bars at the top of histobars are standard deviation of the mean. Different letters indicate significant differences between isolates ($P \leq 0.05$)

Identification of the selected isolates

The PCR of the 16S rDNA regions from the BN20, BA97, BN17, and BR20 isolates yielded products of 1242–1297 bp. Based on comparison of the sequences with the NCBI GenBank database, all isolates are members of the genus *Bacillus*. Isolates BA97 and BR20 shared high identity (99.06–99.72%) with *Bacillus* sp. and *B. cereus* (100% coverage, E-value = 0.0). Isolate BN17 had the highest identity (96.83%) with *B. aryabhattai* and *B. megaterium* (100% coverage, E-value = 0.0), isolate BN20 had 95.94–96.00% identity with *B. aryabhattai* (99–100% coverage, E-value = 0.0). All sequences were deposited in the GenBank database (accessions MW269582–MW269585).

In the phylogenetic analysis of several *Bacillus* species, isolates BA97 and BR20 clustered with *B. cereus*, and

Table 2 In vitro antifungal activity of bacterial isolates against *Macrophomina phaseolina* and other common bean fungal pathogens

Isolate	*M. phaseolina*		*Fusarium verticillioides*		*F. oxysporum* f. sp. *lycopersici*		*Rhizoctonia* sp.		*Sclerotium rolfsii*	
	Colony diameter (cm)	Inhibition (%)	Radial growth (cm)	Inhibition (%)	Colony diameter (cm)	Inhibition (%)	Colony diameter (cm)	Inhibition (%)	Colony diameter (cm)	Inhibition (%)
Control	2.00 ± 0.00^{a}	0	2.00 ± 0.00^{a}	0	2.00 ± 0.00^{a}	0	2.00 ± 0.00^{a}	0	2.00 ± 0.00^{a}	0
BA97	0.36 ± 0.07^{c}	85.00	0.96 ± 0.09^{c}	52.0	1.20 ± 0.14^{b}	40.0	1.41 ± 0.08^{b}	30.0	2.00 ± 0.00^{a}	0
BN17	0.32 ± 0.15^{c}	85.00	1.08 ± 0.07^{b}	46.5	1.18 ± 0.08^{b}	41.5	1.18 ± 0.01^{b}	26.5	2.00 ± 0.00^{a}	0
BN20	0.41 ± 0.08^{c}	80.00	1.04 ± 0.05^{bc}	48.5	1.20 ± 0.07^{b}	40.0	1.34 ± 0.17^{b}	35.0	$1.11 \pm 0.08^{**}$	44.5
BR20	0.80 ± 0.16^{b}	62.50	1.06 ± 0.07^{b}	47.0	1.23 ± 0.04^{b}	39.0	1.40 ± 0.10^{b}	30.5	2.00 ± 0.00^{a}	0

Control plate was inoculated only with the fungus. Different letters after means within a column indicate a significant difference between bacterial isolates ($P \leq 0.05$)

isolates BN17 and BN20 clustered with *B. megaterium* and *B. aryabhattai* (Fig. 2).

The PCR of the ITS rDNA region of the phytopathogenic fungus yielded a 505-bp product. Comparison of the sequence with the GenBank database, confrmed that the fungal isolate is *M. phaseolina* (99.8% identity, 100% coverage, E-value = 0.00). The sequence was deposited in GenBank as accession MW559069.

Discussion

In our study, four of 37 non-hemolytic bacterial isolates inhibited mycelial growth of *M. phaseolina* in vitro and enabled infected plants to grow as well as the control plants without the fungus. These four *Bacillus* sp. isolated showed potential effectiveness in controlling four other phytopathogenic fungi in vitro. Species of *Bacillus* are among the most frequently used biological agents to control phytopathogens (Fira et al. 2018), and they are widely distributed and well adapted to soil environments (Liu et al. 2019). They can also be found in root nodules as reported by Ferreira et al. (2020), who identified a broad diversity of bacterial genera such as *Bacillus*, *Paenibacillus*, *Burkholderia*, and *Pseudomonas* that cohabit nodules of common bean, cowpea bean (*Vigna unguiculata*), and siratro (*Macroptilium atropurpureum*). Even though we isolated BN17 and BN20 from nodules in this study, we still need to demonstrate the endophytic origin of these bacteria. *Bacillus* species are. In particular, they have diverse strategies to control plant fungal pathogens, such as the production of extracellular antibiotics and lytic enzymes and release of volatile organic compounds (Abdelmoteleb et al. 2017; Khan et al. 2018). Abdelmoteleb et al. (2017) reported that *Bacillus subtilis* produced antibiotics and hydrolytic enzymes that provided a broad spectrum of antifungal activities against the phytopathogenic fungi *Alternaria alternata*, *Macrophomina* sp., and *Colletotrichum gloeosporioides*. In our two-compartment assays to test for the production of volatile compounds, exposure to volatiles produced by the four best antagonistic isolates significantly inhibited (37.5–75%) the development of the phytopathogenic fungus. This inhibition is greater than that obtained by Gacitúa et al. (2009), who reported a growth reduction of *M. phaseolina* of 4–44% by volatile compounds produced by *B. stearothermophilus* strain TM 008 and *B. amyloliquefaciens* strain VIII 016. Volatile compounds are secondary metabolites that can alter the structure of hyphae and reduce their growth and eliminate the production of sclerotia (Ab Rahman et al. 2018; Sridharan et al. 2020). More studies are needed to identify the type of volatile and diffusible metabolites produced by the bacterial strains that we isolated.

Bacteria can promote plant growth by enhancing the absorption of nutrients such as P and producing IAA and

Table 3 Effect of bacterial isolates BA97, BN17, BN20, and BR20 on charcoal rot disease and seedling development in common bean

Treatment	Shoot length (cm)	Root length (cm)	Seedling fresh mass (g)	Root fresh mass (g)	DI (%)	BE (%)
Control	9.74 ± 1.26^{a}	20.80 ± 1.94^{a}	2.587 ± 0.220^{a}	1.334 ± 0.260^{a}	–	–
Mp + BA97	7.02 ± 0.27^{ab}	23.30 ± 4.29^{a}	2.093 ± 0.267^{ab}	0.888 ± 0.386^{ab}	54.42	33.30
Mp + BN17	7.40 ± 1.77^{ab}	19.12 ± 5.53^{a}	1.402 ± 0.525^{b}	0.533 ± 0.217^{ab}	68.57	11.11
Mp + BN20	9.64 ± 2.04^{a}	24.14 ± 2.36^{a}	2.037 ± 0.520^{ab}	1.077 ± 0.412^{a}	34.28	55.50
Mp + BR20	7.18 ± 1.95^{ab}	14.30 ± 6.37^{ab}	1.513 ± 0.789^{b}	0.720 ± 0.599^{ab}	65.71	14.81
Mp	4.92 ± 2.47^{b}	5.50 ± 5.81^{b}	1.381 ± 0.295^{b}	0.209 ± 0.213^{b}	77.14	–

Control = seedlings without *Macrophomina phaseolina* or bacteria; Mp = *M. phaseolina*. Disease severity index, DI(%) = $\frac{\sum(ab)}{AK} \times 100$ where a = number of plants with the same severity rating, b = severity rating, A = total number of analyzed plants, K = highest severity rating. Biocontrol effect, BE (%) = $\frac{\text{DIP}-\text{DIB}}{\text{DIP}} \times 100$, where DIP = disease severity index in the control with pathogen only, DIB = disease severity index with bacterial treatment. Different letters after means within a column indicate a significant difference between isolates ($P \leq 0.05$)

Table 4 Inhibitory effect of volatile compounds (VOCs) and amount of indolacetic acid (IAA) produced by bacterial isolates BA97, BN17, BN20, and BR20

Isolate	Inhibition (%)	IAA (µg/ml)
Control	0^{e}	0^{e}
BA97	75.0 ± 0.0^{a}	2.16^{c}
BN17	55.0 ± 0.0^{c}	3.87^{a}
BN20	62.5 ± 0.5^{b}	2.67^{b}
BR20	37.5 ± 0.5^{d}	1.98^{d}

Controls: Plates for VOC tests contained only *Macrophomina phaseolina*; tubes for IAA production contained only culture medium without bacteria. Different letters after values within a column indicate significant differences between isolates ($P \leq 0.05$)

siderophores and indirectly help control fungal diseases (Alijani et al. 2019; Rodríguez et al. 2019). IAA, the most commonly produced auxin by several endophytic and rhizospheric bacteria, stimulates cell division, cell and tissue differentiation, cell elongation, and lateral root formation (Bhutani et al. 2018). In our study, we demonstrated that the four selected isolates produced IAA (1.98–3.87 µg/ml), similar to seven *Bacillus* isolates from maize that Zahid et al. (2015) found produced IAA (0.9–5.39 µg/ml) and promoted plant growth. These findings suggest that the bacterial isolates might protect the plant against the pathogen indirectly, through the promotion of root development. However, further study is required to confirm this hypothesis.

Optimum VOCs and IAA concentrations are required for these compounds to be effective in promoting growth and inhibiting pathogens (Campos et al. 2010; Ogwu 2018), but the biosynthesis of VOCs and IAA by bacteria varies depending on the environmental conditions (Nieto-Jacobo et al. 2017). In the present study, four *Bacillus* strains inhibited mycelial growth of *M. phaseolina* by VOCs and produced different quantities of IAA in vitro. However, the relationship between the production of these compounds and the biocontrol effect on charcoal rot of common bean in growth room experiments was unclear. Although BA97 reduced mycelial growth of *M. phaseolina* the most in the VOC assay and BN17 produced the most IAA, BN20 was the most effective at controlling charcoal rot *in planta*. This observation suggests that either the type of VOCs produced in vitro and *in planta* are not the same or that additional mechanisms not evaluated in this study are present in the interaction of the bacteria with the fungus and the plant. In fact, bacteria that are highly antagonistic in vitro do not necessarily have the same effectiveness when tested in the plant host or field (Egamberdiyeva 2007; Fan et al. 2012). Additional research is needed to identify and quantify the VOCs and IAA synthesized by *Bacillus* isolates BA97, BR20, BN17, and BN20 in growth room assays and determine the bacterial mechanisms that affect pathogen growth and establishment during its interaction with common bean plants.

In summary, our study of the in vitro and *in planta* control of *M. phaseolina* by *Bacillus* sp. BN20 isolate provides evidence that this control might be due to the production of diffusible and volatile compounds. To our knowledge, no disease index had yet been developed to assess the severity of charcoal rot disease for the conditions of our tests; therefore, we developed a scale of disease severity and used it to show that seed bacterization with isolate BN20 reduces charcoal severity and enhances the biocontrol efficacy in growth room tests of plants grown from the treated seeds. Our results indicate that seed bacterization with these rhizobacterial isolates may be an effective way to reduce crop damage by *M. phaseolina* in common bean fields. Studies are now needed to determine the efficacy of the selected isolates as seed treatments in the field.

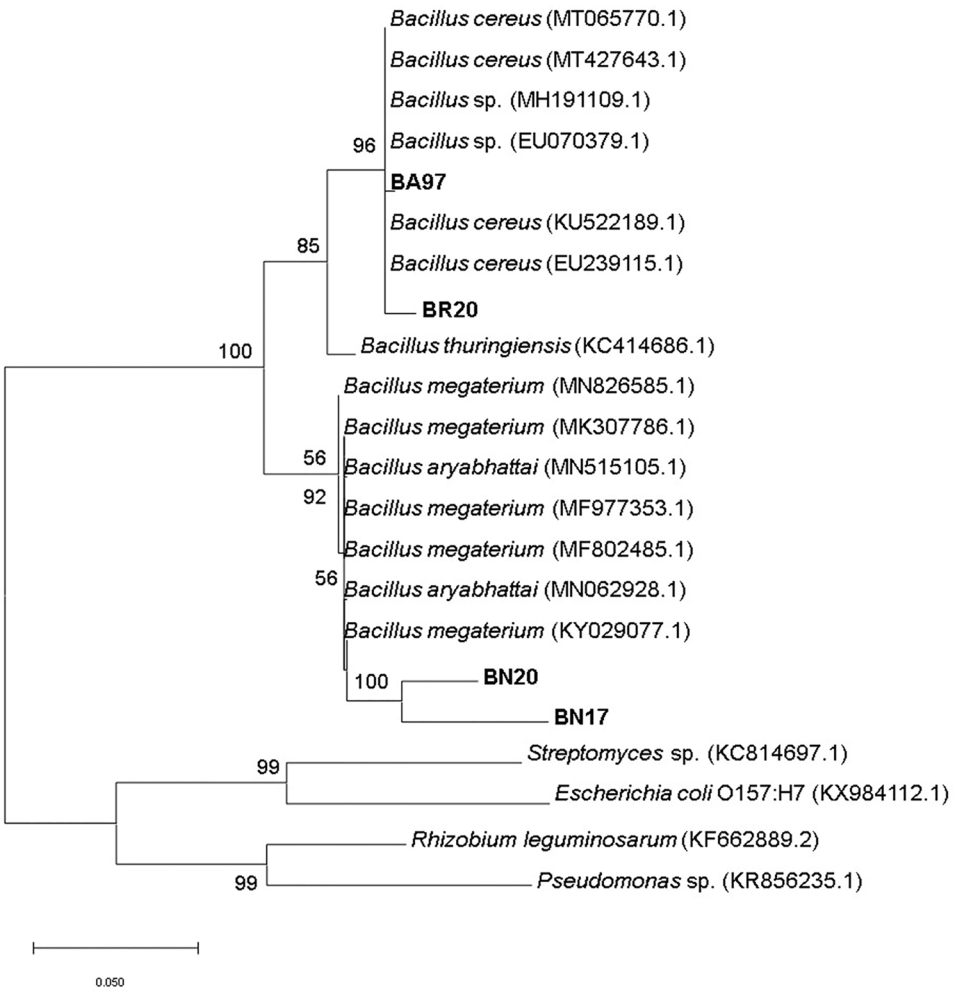

Fig. 2 Maximum likelihood tree based on 16S rDNA sequences of *Bacillus* sp. and isolates BA97, BR20, BN20, and BN17. The tree was constructed with MEGA X. Bootstrap percentages were calculated using 1000 replicates and the Kimura 2-parameter model and six gamma categories to model the among-site rate variation. *Streptomyces* sp., *Escherichia coli*, *Rhizobium leguminosarum*, and *Pseudomonas* sp. were used as outgroups. Bootstrap percentages are shown at the branches. The scale bar (0.050) indicates the number of nucleotide substitutions per site

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10327-021-01019-4.

Acknowledgements The authors are grateful to Quintin Armando Ayala for providing the *R. solani* strain used in the in vitro antagonistic assays. They thank Dr. Brandon Loveall of Improvence for English proofreading of the manuscript. The authors are also grateful to the Instituto Politécnico Nacional (SIP 20180963, SIP 20196294) and the Universidad Autónoma de Occidente (PIFIP-2019-16) for supporting this research. YJBA received support from COFAA (IPN) and a master's fellowship from CONACyT (706421).

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Ab Rahman SFS, Singh E, Pieterse CM, Schenk PM (2018) Emerging microbial biocontrol strategies for plant pathogens. Plant Sci 267:102–111

Abdelmoteleb A, Troncoso-Rojas R, Gonzalez-Soto T, González-Mendoza D (2017) Antifungical activity of autochthonous *Bacillus subtilis* isolated from *Prosopis juliflora* against phytopathogenic fungi. Mycobiology 45:385–391

Ali S, Hameed S, Shahid M, Iqbal M, Lazarovits G, Imran A (2020) Functional characterization of potential PGPR exhibiting broad-spectrum antifungal activity. Microbiol Res 232:385–391

Alijani Z, Amini J, Ashengroph M, Bahramnejad B (2019) Antifungal activity of volatile compounds produced by *Staphylococcus sciuri* strain MarR44 and its potential for the biocontrol of *Colletotrichum nymphaeae*, causal agent strawberry anthracnose. Int J Food Microbiol 307:108276

Bhattacharyya PN, Jha DK (2012) Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J Microbiol Biotechnol 28:1327–1350

Bhutani N, Maheshwari R, Negi M, Suneja P (2018) Optimization of IAA production by endophytic *Bacillus* spp. from *Vigna radiata* for their potential use as plant growth promoters. Isr J Plant Sci 65:83–96

Bolzonella C, Lucchetta M, Teo G, Boatto V, Zanella A (2019) Is there a way to rate insecticides that is less detrimental to human and environmental health? Glob Ecol Conserv 20:1–9

Campos VP, Pinho RSCD, Freire ES (2010) Volatiles produced by interacting microorganisms potentially useful for the control of plant pathogens. Cienc Agrotec 34:525–535

Chen F, Wang M, Zheng Y, Luo J, Yang X, Wang X (2010) Quantitative changes of plant defense enzymes and phytohormone in biocontrol of cucumber Fusarium wilt by *Bacillus subtilis* B579. World J Microbiol 26:675–684

Daigle DJ, Connick WJ, Boyetchko SM (2002) Formulating a weed-suppressive bacterium in "Pesta." Weed Technol 16:407–413

Egamberdiyeva D (2007) The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. Appl Soil Ecol 36:184–189

Eke P, Chatue GC, Wakam LN, Kouipou RMT, Fokou PVT, Boyom FF (2016) Mycorrhiza consortia suppress the fusarium root rot (*Fusarium solan*i f. sp. *phaseoli*) in common bean (*Phaseolus vulgaris* L.). Biol Control 103:240–250

Etesami H, Maheshwari DK (2018) Use of plant growth promoting rhizobacteria (PGPRs) with multiple plant growth promoting traits in stress agriculture: action mechanisms and future prospects. Ecotoxicol Environ Saf 156:225–246

Fan B, Carvalhais LC, Becker A, Fedoseyenko D, von Wiren N, Borriss R (2012) Transcriptomic profiling of *Bacillus amyloliquefaciens* FZB42 in response to maize root exudates. BMC Microbiol 12:1471–2180

Fernando WD, Ramarathnam R, Krishnamoorthy AS, Savchuk SC (2005) Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil Biol Biochem 37:955–964

Ferreira LDVM, Carvalho F, Andrade JFC, Oliveira DP, Medeiros FHV, Moreira FMDS (2020) Co-inoculation of selected nodule endophytic rhizobacterial strains with *Rhizobium tropici* promotes plant growth and controls damping off in common bean. Pedosphere 30:98–108

Fira D, Dimkić I, Berić T, Lozo J, Stanković S (2018) Biological control of plant pathogens by *Bacillus* species. J Biotechnol 285:44–55

Forbes BA, Sahm DF, Weissfeld AS (2002) Bailey and Scott's diagnostic microbiology, 11th edn. Mosby, St Louis

Gacitúa S, Valiente CF, Díaz KP, Hernández JC, Uribe MM, Sanfuentes EV (2009) Identification and biological characterization of isolates with activity inhibitive against *Macrophomina phaseolina* (Tassi) Goid. Chil J Agric Res 69:526–533

Gordon SA, Weber RP (1951) Colorimetric estimation of indoleacetic acid. Plant Physiol 26:192–195

Islam MS, Haque MS, Islam MM, Emdad EM, Halim A, Hossen QMM (2012) Tools to kill: genome of one of the most destructive plant pathogenic fungi *Macrophomina phaseolina*. BMC Genom 13:493

Kaur S, Dhillon GS, Brar SK, Vallad GE, Chand R, Chauhan VB (2012) Emerging phytopathogen *Macrophomina phaseolina*: biology, economic importance and current diagnostic trends. Crit Rev Microbiol 38:136–151

Khan N, Martínez-Hidalgo P, Ice TA, Maymon M, Humm EA, Nejat N (2018) Antifungal activity of *Bacillus* species against *Fusarium* and analysis of the potential mechanisms used in biocontrol. Front Microbiol 9:2363

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kloepper JW (1978) Plant growth-promoting rhizobacteria on radishes. In: Proceedings of the 4th international conference on plant pathogenic bacteria, vol 2. Angers, France, pp 879–882

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA, X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549

Kunwar IK, Singh T, Machado C, Sinclair J (1986) Histopathology of soybean seed and seedling infection by *Macrophomina phaseolina*. Phytopathology 76:532–535

Lakshmi V, Kumari S, Singh A, Prabha C (2015) Isolation and characterization of deleterious *Pseudomonas aeruginosa* KC1 from rhizospheric soils and its interaction with weed seedlings. J King Saud Univ Sci 27:113–119

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948

Lindsey APJ, Murugan S, Renitta RE (2020) Microbial disease management in agriculture: current status and future prospects. Biocatal Agric Biotechnol 23:101468

Liu J, Cui X, Liu Z, Guo Z, Yu Z, Yao Q (2019) The diversity and geographic distribution of cultivable *Bacillus*-like bacteria across black soils of northeast China. Front Microbiol 10:1424

Mendoza-Cantú A, Ize-Lema IAR (2017) Las sustancias químicas en México. Perspectivas para un manejo adecuado. Rev Int Contam Ambie 33:719–745

Nieto-Jacobo MF, Steyaert JM, Salazar-Badillo FB, Nguyen DV, Rostás M, Braithwaite M (2017) Environmental growth conditions of *Trichoderma* spp. affects indole acetic acid derivatives, volatile organic compounds, and plant growth promotion. Front Plant Sci 8:102

Ogwu MC (2018) Effects of indole-3-acetic acid on the growth parameters of *Citrullus lanatus* (Thunberg) Matsum and Nakai. Momona Ethiop J Sci 10:109–125

Paneerselvam A, Kumar D, Thenmozhi R, Anupama P, Nagasathya A, Thajuddin N (2012) Selection of potential antagonistic *Bacillus* and *Trichoderma* isolates from tomato rhizospheric soil against *Fusarium oxysporum* f. sp. *lycoperscisi*. J Microbiol Biotechnol Res 2:78–89

Paparu P, Acur A, Kato F, Acam C, Nakibuul J, Nkuboye A (2020) Morphological and pathogenic characterization of *Sclerotium rolfsii*, the causal agent of southern blight disease on common bean in Uganda. Plant Dis 104:2130–2137

Patil VS (2014) Isolation, characterization and identification of rhizospheric bacteria with the potential for biological control of *Sida acuta*. J Environ Res Develop 8:411

Pikovskaya R (1948) Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Microbiology 17:362–370

Radwan O, Rouhana LV, Hartman GL, Korban SS (2014) Genetic mechanisms of host–pathogen interactions for charcoal rot in soybean. Plant Mol Biol Rep 32:617–629

Rodríguez AMD, Cota FIP, Santoyo G, de Los Santos Villalobos S, (2019) Chlorothalonil tolerance of indole producing bacteria associated to wheat (*Triticum turgidum* L.) rhizosphere in the Yaqui Valley. Mexico Ecotoxicol 28:569–577

Sabaté DC, Brandan CP, Petroselli G, Erra-Balsells R, Audisio MC (2017) Decrease in the incidence of charcoal root rot in common bean (*Phaseolus vulgaris* L.) by *Bacillus amyloliquefaciens* B14, a strain with PGPR properties. Biol Control 113:1–8

Sánchez-García BM, Espinosa-Huerta E, Villordo-Pineda E, Rodríguez-Guerra R, Mora-Avilés MA (2017) *Trichoderma* spp. native strains molecular identification and in vitro antagonistic evaluation of root phytopathogenic fungus of the common bean (*Phaseolus vulgaris* L.) cv. Montcalm Agrociencia 51:63–79

Schwyn B, Neilands J (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

Shi T, Reeves R, Gilichinsky D, Friedmann E (1997) Characterization of viable bacteria from Siberian permafrost by 16S rDNA sequencing. Microb Ecol 33:169–179

Shrivastava P, Kumar R, Yandigeri MJS (2017) In vitro biocontrol activity of halotolerant *Streptomyces aureofaciens* K20: a potent antagonist against *Macrophomina phaseolina* (Tassi) Goid. Saudi J Biol Sci 24:192–199

Singh SP, Schwartz HF (2010) Breeding common bean for resistance to diseases: a review. Crop Sci 50:2199–2223

Somasegaran P, Hoben HJ (eds) (1994) Handbook for rhizobia. Methods in legume–Rhizobium technology. Springer-Verlag, New York

Sridharan A, Thankappan S, Karthikeyan G, Uthandi S (2020) Comprehensive profiling of the VOCs of *Trichoderma longibrachiatum* EF5 while interacting with *Sclerotium rolfsii* and *Macrophomina phaseolina*. Microbiol Res 236:1–13

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, Orlando, pp 315–322

Zahid M, Abbasi MK, Hameed S, Rahim N (2015) Isolation and identification of indigenous plant growth promoting rhizobacteria from Himalayan region of Kashmir and their effect on improving growth and nutrient contents of maize (*Zea mays* L.). Front Microbiol 6:207

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

View publication stats