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Pseudomonas and Burkholderia inhibit growth and asexual development of Phytophthora capsici

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Pseudomonas and *Burkholderia* inhibit growth and asexual development of *Phytophthora capsici*

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Abstract: The objective of this study was to isolate and characterize antagonistic rhizobacteria from chili against a notorious phytopathogen *Phytophthora capsici*. Among the 48 bacteria isolated, BTLbbc-02, BTLbbc-03, and BTLbbc-05 were selected based on their inhibitory activity against *P. capsici*. They were tentatively identified as *Burkholderia metallica* BTLbbc-02, *Burkholderia cepacia* BTLbbc-03, and *Pseudomonas aeruginosa* BTLbbc-05, respectively, based on their 16S rRNA gene sequencing. All inhibited the growth of *P. capsici* at varying levels by inducing characteristic morphological alterations of *P. capsici* hyphae. The cell-free culture supernatant of all three isolates impaired motility (up to 100%) and caused lysis (up to 50%) of the halted zoospores. Bioassays revealed that *Pseudomonas* sp. had higher antagonism and zoospore motility-inhibitory effects against *P. capsici* compared with two other isolates, *Burkholderia* spp. and *B. metallica*, which caused vacuolation in mycelium. All three bacteria suppressed sporangium formation and zoosporogenesis of *P. capsici*, and improved the seed germination and growth of cucumber. Our findings suggest that epiphytic bacteria, *B. metallica*, *B. cepacia*, and *P. aeruginosa*, could be used as potential biocontrol agents against *P. capsici*. A further study is required to ensure conformity with the existing regulations for soil, plant, and human health.

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1 Introduction

Phytophthora blight is a deadly disease worldwide caused by *Phytophthora capsici*, affecting a wide range of vegetables such as chili, cucumber, and tomato. This devastating zoosporic pathogen can damage up to 30% of total vegetable production worldwide [1, 2]. It attacks mainly vegetables and weed species belonging to Cucurbitaceae, Fabaceae, and Solanaceae, and causes blight, damping-off, fruit rot, vine blight, and leaf lesion symptoms. Due to the unavailability of biorational and biocontrol agents for managing this pathogen, farmers use a wide range of fungicides that are hazardous to the environment and human health. Currently, metalaxyl, mefenoxam, fluopicolide, propamocarb, phosphonates, and the like, have shown efficacy to control *P. capsici*; however, long-term use poses the threat of control failure due to resistance development in the population [3]. Therefore, biocontrol is considered as an essential component of sustainable and integrated *P. capsici* management strategy. *P. capsici* infects host plants mostly through asexually generated characteristic biflagellate motile zoospores released from sporangia in a favorable (moist and high temperature) environment. The zoospores have powerful sensory transduction systems to locate potential infection sites on the host and then rapidly undergo the necessary morphological changes to invade the host tissues [4]. Moreover, this pathogen can survive in soil as oospores from months to several years until a favorable environment becomes available for sporulation [5]. The infection cycles of *P. capsici* are extremely rapid, resulting in an epidemic in a large area within a few days. The disruption of asexual development and/or motility of zoospores are considered effective strategies to control the disease caused by this peronosporomycete phytopathogen [6, 7]. Therefore, a crop rotation system should be effective in disrupting the disease cycle. However, the scarcity of suitable land for vegetable production in Bangladesh compels farmers to grow the same crop in the same piece of land for multiple years [8].

Several lines of evidence suggest that some plant root–associated microbes are capable of protecting the host plant from infection by pathogenic microorganisms through direct and/or indirect antagonism [7]. In addition, rhizoplane bacteria are known to suppress *P. capsici* by inhibiting the production of zoospores from sporangia and/or impair the motility of zoospores [9]. Because rhizoplane bacteria exist in the rhizosphere of their host plants, they can be easily isolated, mass produced on artificial substrates, and applied as inoculative release for protecting plants from disease [10–15]. Interestingly, rhizosphere bacteria were shown to suppress *P. capsici*, and some of them were also identified as plant growth–promoting bacteria [10–14]. However, insufficient data are available specifically on the biocontrol of *P. capsici* by native bacterial antagonists in Bangladesh. One recent study reported screening for antagonistic bacteria, i.e. *Pseudomonas* spp. against *P. capsici* from the rhizoplane of host plants [16] in Bangladesh, with positive results that encouraged us to isolate more antagonistic rhizobacteria from host plants and investigate the mode of action of these beneficial microbes in controlling *P. capsici*. For example, *Burkholderia* spp. and *Pseudomonas* spp. provided beneficial effects to plants through plant growth promotion and antagonistic activities against several plant pathogens [17, 18]. Although these species are key bacterial pathogens for causing cystic fibrosis in humans, the extraction of cell wall components or bioactive components and its application to plants make these safer alternatives to live organisms. In addition, the implementation of molecular approaches, e.g. DNA-based identification for accurate evaluation of pathogenic and nonpathogenic forms, helped determine safe strains for the biocontrol of plant pathogens [19]. The specific objectives of this study were to (i) isolate naturally occurring root-associated rhizobacteria from the native host plant of *P. capsici*; (ii) screen purified isolates based on their inhibitory effects on mycelial growth, formation of sporangia, release of zoospores, and motility of the released *P. capsici* zoospores and also test their ability for PGP activity; and (iii) identify potential bacterial isolates for biocontrol of *P. capsici* pathogen through 16S rRNA gene sequencing.

2 Materials and methods

2.1 Isolation of rhizoplane bacteria from host plants of *P. capsici*

Due to the prevalence of chili plants as one of the common hosts of *P. capsici*, the roots of different wild and cultivated species of chili plants grown in Gazipur and Barishal districts of Bangladesh were collected. To isolate rhizoplane bacteria from the collected roots, samples were washed under running tap water for 15 min followed by rinsing five times with sterilized distilled water (SDW). Root samples were then vortexed for 1 min in 20 mL sterile test tubes containing SDW to dislodge bacteria from the root surface. One milliliter of the bacterial suspension was then diluted with SDW up to 1×10^{-9} from which 100 µL aliquots of each sample (1×10^{-9} dilution series) were spread separately on nutrient agar plates and incubated at 25 °C for 48 h [20]. Morphologically distinct colonies were purified by repeated streak cultures on new plates containing the same medium [21].

2.2 Production of zoospores

Seven-day-old cultures of *P. capsici* grown in V8 Juice agar medium (SDW 800 mL, V8 Juice 200 mL, calcium chloride 2 g, and agar 15 g) were used for zoospore production. Five disks (each 7 mm diameter) of V8 Juice agar medium containing actively growing *P. capsici* were cut with a sterilized cork borer followed by placing them in sterilized Petri dishes, and 10 mL SDW was added to each Petri dish. The dishes were kept in the dark for 72 h at ambient room temperature (25 ± 2 °C) and then placed in a refrigerator (4 °C) for 60-min (cold treatment). After cold treatment, the Petri dishes were again placed in the dark at room temperature for 1 h to release zoospores from sporangia. The concentration of zoospores in the water suspension was observed and quantified under a light microscope [22].

2.3 In vitro interaction by dual culture assay

All bacterial isolates were tested for antagonistic activity against mycelial growth of *P. capsici* on potato dextrose agar (PDA) (potato extract 200 g, dextrose 20 g, agar 15 g, and SDW up to 1 L) using dual culture assay. One mycelial disk of 6 mm diameter from the periphery of a 5-day-old *P. capsici* colony was placed on a PDA plate 3 cm apart from the bacterium inoculation on the same plate. After inoculation, the Petri dish was kept at 25 °C in the dark for 5 days. For each bacterium, three Petri dishes containing PDA were inoculated following the same methods. Inhibition zone was determined after 7 days from each plate. For light microscopic observation, dual culture plates were placed under the lens to focus the colony edge growing toward the bacterial colonies after 7 days. The growth inhibition percentage was calculated by taking the average of three replicates following the formula of Chilpa et al. [23]:

$$\% \text{ Inhibition} = (A - B) / A \times 100$$

where, A = mycelia growth (diameter in cm) in control, and B = mycelia growth (diameter in cm) in PDA containing bacterium. Based on dual culture assay activity, three of the isolated bacteria were chosen for further investigation.

2.4 Biochemical characterization of the isolates

A series of biochemical tests (KOH test, Gram staining test, catalase test, oxidase test) were conducted using previously characterized methods [24].

2.5 Molecular and phylogenetic identification of bacteria

For the identification of selected bacterial isolates, chromosomal DNA was extracted from pure cultures of actively growing isolates according to Park et al. [25]. The 16S rRNA fragments were amplified by PCR using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [26]. Amplification was performed in a thermocycler programmed (Mastercycler® Gradient, Eppendorf, Hamburg, Germany) at 94 °C for 5 min, followed by 30 cycles of 40 s at 94 °C, 40 s at 55 °C, and 1 min at 72 °C with a final extension at 72 °C for 10 min. A 5 µL aliquot of each PCR amplicon was electrophoresed on a 1.5% agarose gel in 0.5′ Tris-Borate-EDTA buffer at 100 V for 40 min, stained with ethidium bromide solution for 20 min, and the PCR products were visualized with a UV transilluminator (BioDoc-IT System, Japan). Amplified products were purified using Quick PCR purification column (Promega, Madison, WI, USA) and directly sequenced using the Big Dye terminator cycle sequencing Ready Reaction kit (Applied Biosystems, Forster City, CA, USA) in an ABI Prism® 310 Genetic Analyzer (Applied Biosystems). The sequences were compared with the GenBank database of the NCBI at http://www.ncbi.nlm.nih.gov using the BLASTN [27] and reference sequences were retrieved to perform phylogenetic analysis. Molecular analysis by the 16S rRNA gene sequencing technique was followed in this study to identify three potential antagonistic bacteria (BTLbbc-02, 03, and 05). Ribosomal RNA possesses useful markers for the identification of bacterial phylogeny. Identification of the phylogenetic neighbors and calculation of pairwise 16S rRNA gene sequence similarity were obtained using the EzTaxon server (EzTaxon, http://www.eztaxon.org/) [28]. The neighbor-joining tree was constructed by bootstrapping (Figure 3).

2.6 Effect of bacterial cells on sporangium formation in vitro

To study the inhibitory effect of the isolated bacteria on sporangia formation by *P. capsici*, an agar block containing *P. capsici* was removed with a 7-mm-cork borer from the periphery of 7-day-old colonies, and the blocks were distributed in a multidish containing 1 mL of clarified agar (15 g agar/liter SDW). Each bacterium was grown in nutrient broth for 3 days at room temperature on a benchtop shaker set at 120 rpm. One milliliter of SDW was added to freshly harvested and washed (2 times) bacterial cells from 1.5 mL of bacterial culture broth. One milliliter of the bacterial suspension (10^9–10^7 CFU/mL) was added to each cell of the multidish containing *P. capsici* disk on agar medium. In a control plate, only SDW was added to the cells of the multidish. The dish was incubated in the dark for 72 h at 25 °C to allow sporangia to develop. The number of sporangia was counted in each treatment and control under a light microscope. To evaluate sporangium formation, four mycelial plugs (0.95 cm^2 each) were taken from each well and each mycelial plug was transferred into a sterile 2-mL Eppendorf tube containing 1 mL of SDW. The 2 mL Eppendorf tube containing the mycelial plug was vortexed vigorously for 2 min to release the sporangia. The numbers of sporangia were counted microscopically in each 3-µL droplet at ×100 magnification 3 times. Each treatment was replicated 3 times and the whole experiment was repeated 3 times.

2.7 Effect of bacteria on zoosporogenesis of *P. capsici* in vitro

Phytophthora capsici was grown on V8 Juice agar medium at 25 °C for 7 days. Agar blocks containing *P. capsici* were removed with a sterilized 7-mm-cork borer from the periphery of 7-day-old colonies, and the blocks were distributed in a multidish containing 1 mL of clarified agar (15 g agar/1 L SDW). Then, washed bacterial suspension was added to the multidish as described previously. The dishes were kept in the dark for 72 h at room temperature (approximately 25 °C) and then placed in a refrigerator (4 °C) for 60 min (cold treatment). After that, the Petri dishes were placed in the dark at room temperature for 1 h. The zoospores were released nearly after 1 h and broken sporangium were observed and counted under a

light microscope [22]. After 60 min, three random microscopic fields from each cell of the multidish were observed under a microscope and the broken sporangium, which released zoospores, were also counted from three different fields randomly selected under a light microscope.

2.8 Motility inhibition and lysis of zoospore in vitro

The motility-inhibitory activity of epiphytic bacterial isolates against *P. capsici* zoospores was tested according to Islam et al. [6]. Briefly, selected bacterial isolates were cultured in nutrient broth for 72 h at room temperature in a shaking incubator at 120 rpm. Then, broth cultures were centrifuged at 15,000 rpm for 1 min from which 360 µL of zoospore suspensions were taken into a multidish plate. Then, 40 µL cell-free culture filtrates of the bacterial isolate were added with a micropipette. Motility halting and lysis of zoospore by bacterial isolates were investigated under a compound light microscope at different time intervals (15, 30, 45 and 60 min).

2.9 Determination of indole 3 acetic acid production

The production of indole 3 acetic acid (IAA) was determined according to Bric et al. [29] with minor modifications. For the determination of IAA production, epiphytic bacterial isolates were inoculated in Jensen's broth (sucrose 20 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, NaCl 0.5 g, $FeSO_4 \cdot 7H_2O$ 1 g, $Na_2MoO_4 \cdot 2H_2O$ 0.005 g, $CaCO_3$ 2 g), and incubated at 29 ± 2 °C for 48 h. After incubation, 1 mL of inoculated broth culture was transferred into new 50 mL fresh Jensen's broth culture containing 2 mg mL^{-1} of L-tryptophan and incubated at 29 ± 1 °C for 72 h. Approximately 2 mL of the culture solution was centrifuged at 15,000 rpm for 1 min and the supernatant was used to detect the IAA concentration of bacteria. One milliliter of the supernatant was mixed with 2 mL of Salkowsky's reagent (2 mL 0.5 M $FeCl_3$, 49 mL SDW, and 49 mL 70% perchloric acid) according to Gordon and Weber [30]. After 20–25 min, a pink color that developed in the solution indicated IAA production. The absorbance of each solution was measured using a spectrophotometer at 530 nm. An IAA standard curve was prepared by making a series of pure IAA solutions (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, and 65 µg mL^{-1} of pure IAA per solution) and the absorbances of these solutions were taken in a spectrophotometer at 530 nm. In the controls, the supernatants of noninoculated test tubes were used, and no color was observed.

2.10 Preparation of bacterial inocula

Bacterial strains were cultured in 250 mL conical flasks containing 150 mL of nutrient broth medium on an orbital shaker at 120 rpm for 48 h at 27 °C. The bacterial cells from the broth were collected by centrifugation at 10,000 rpm for 10 min and washed twice with SDW. The bacterial pellets were suspended in SDW and the inoculum of isolated bacteria was adjusted to a cell number of 10^8 mL^{-1} using a spectrophotometer. The suspension was vortexed for 45 s prior to use for seed treatment.

2.11 Biological treatments

Thirty *Cucumis sativus* seeds (Baromashi variety from Lal Teer Seed Company, Dhaka, Bangladesh) were taken and surface sterilized with 1% (v/v) sodium hypochlorite solution for 10 min. The bacterial inoculum was then poured onto surface-sterilized seeds and mixed for 30 min by agitation. Seeds were then placed in 9-cm Petri dishes and dried overnight at room temperature to ensure better coating of the seeds with bacteria.

2.12 Seed germination in Petri dish

Seeds treated with three bacterial isolates in three replicates for each isolate were placed in Petri dishes with two layers of moistened filter paper and kept for 5 days at 25 °C. Water was added to the filter papers on alternate days as needed to keep them moist.

2.13 Assessment of seed germination

After growing for 2 weeks inside the Petri dish, cucumber seedlings were harvested. Fresh and dry biomass of shoots and roots (g) as well as length of shoots and roots (mm) were recorded. Germination percentage was calculated by using the formula [31]:

$$\text{Germination (\%)} = \frac{\text{No. of seeds germinated}}{\text{Total no. of seeds sown}} \times 100\%$$

2.14 Vigor index determination

Vigor index is a clear indication by which the plant growth–promoting activity of rhizospheric bacterial isolates could be assessed [32]. Vigor index was calculated by using the following formula [33]:

Vigor index
= {mean shoot length (cm) + mean root length (cm)}
× germination %

Each experiment was conducted 3 times for assessment of seed germination and determination of vigor index.

2.15 Statistical analysis

The statistical analysis of data was performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test in SPSS 15 software for mean separation. All experiments were repeated 3 times with three replications of each treatment. Results are presented as mean ± standard error.

3 Results

3.1 Isolation of epiphytic rhizobacteria

A total of 48 epiphytic bacteria were isolated from the roots of field-grown chili plants, from which three isolates displayed potent antagonistic activities against *P. capsici* in vitro in dual culture assays. Based on the initial antagonistic capability of isolates, three bacteria (BTLbbc-02, 03, and 05) were selected for this experiment.

3.2 In vitro investigation by dual culture bioassay

In dual culture assay on PDA medium, the isolates BTLbbc-02, 03, and 05 inhibited 76%, 83%, and 89% of mycelial growth of *P. capsici*, respectively (Figure 1). The isolate BTLbbc-05 caused significantly ($p < 0.001$) higher levels of antiperonosporomycetal activity with 89% inhibition of *P. capsici* hyphae. The other two isolates, BTLbbc-02 and BTLbbc-03, also significantly ($p < 0.001$) inhibited the growth of *P. capsici* hyphae compared with untreated controls (Figure 2A). Interestingly, all three antagonistic bacteria caused hyphal growth inhibition by inducing characteristic morphological alterations in the hyphae of *P. capsici* approaching toward the bacterial colony (Figure 1E–G). These remarkable variations in morphology of the tip of approaching hyphae toward the bacterial colonies were observed with the aid of light microscopy. Various kinds of morphological alterations observed in hyphae approaching toward the bacterial colonies were the swelling of tips, excessive branching, curling, necrosis, and irregular branching. Interestingly, BTLbbc-03 caused high levels of vacuolation in *P. capsici*

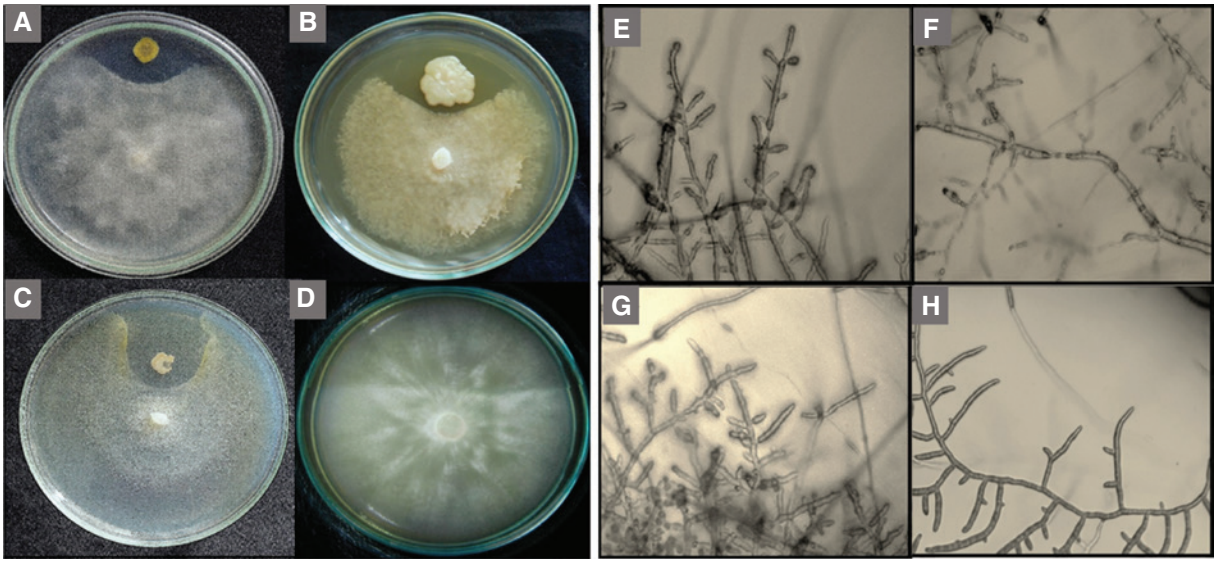

Figure 1: In vitro interaction between isolated bacteria and *P. capsici* in dual culture plate on PDA, all isolates were inoculated on one site of the PDA plate with equal distance apart from the agar plug of *P. capsici* (A) BTLbbc-02, (B) BTLbbc-03, (C) BTLbbc-05, and (D) control with plug of *P. capsici* at the center of the PDA plate. Microphotographs showing hyphal morphological alterations in *P. capsici* hyphae were caused by rhizoplane bacteria/bacterial metabolites (E–H). Interactions were studied on PDA – (E) BTLbbc-02: irregular branching and curling; (F) BTLbbc-03: swelling and vacuolation; (G) BTLbbc-05: inhibition zone with overbranching and necrosis; (H) *P. capsici* normal mycelium growth without any alteration in control. Scale bar = 50 μm.

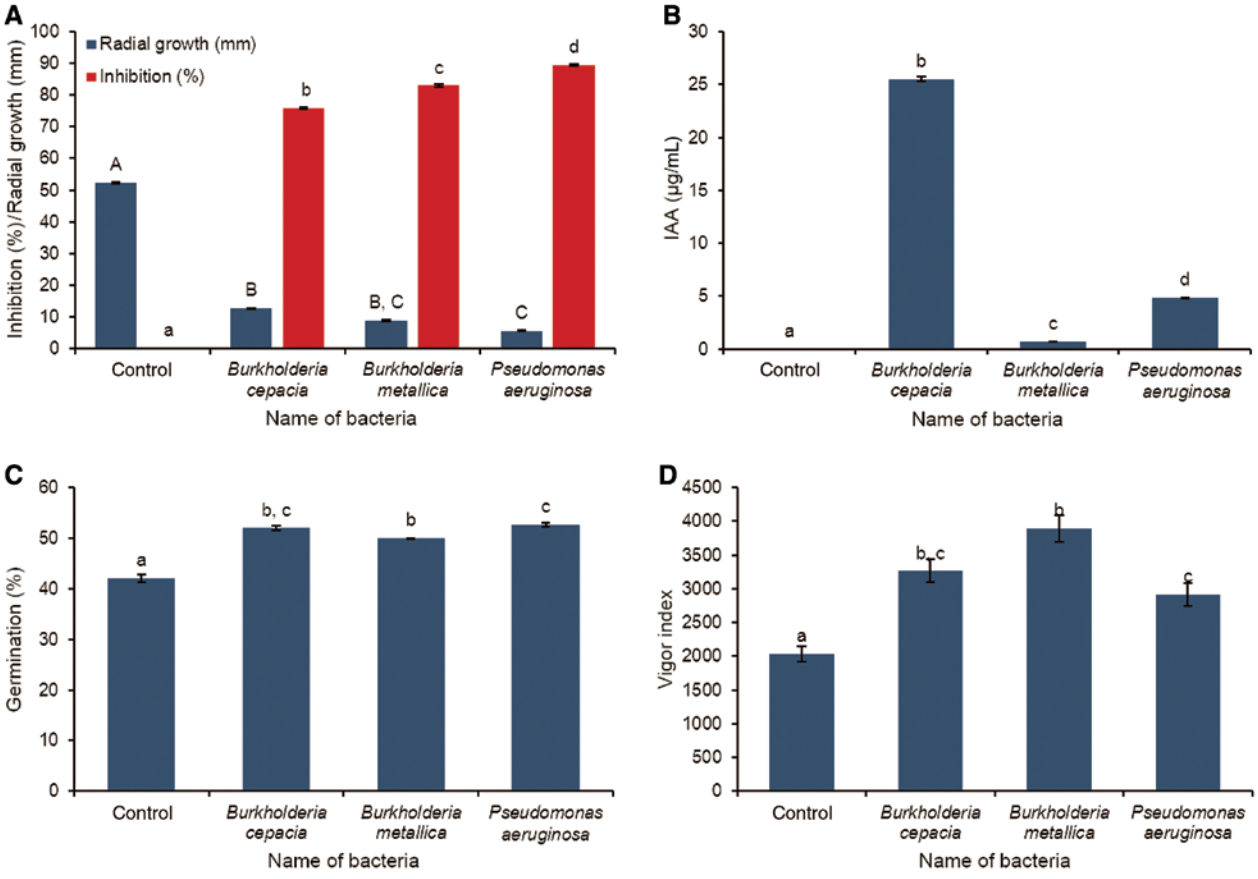

Figure 2: Effect of isolated bacteria on mycelium growth of *P. capsici*, germination percentage, and vigor index of *C. sativus* seeds in vitro. (A) Mycelium inhibition of *P. capsici*, (B) IAA production by three epiphytic rhizobacterial isolates, (C) germination percentage, and (D) vigor index at 12 days after treatment. Data are presented as the mean ± SEM (n = 3). Different superscripts ($^{a, b, c}$) within the same row indicate significant differences ($p < 0.05$) obtained by one-way ANOVA with multiple comparisons and Tukey's multiple comparison test.

hyphae with unusual branching and swelling of the tips (Figure 1F; Supplementary Figure S2).

3.3 Biochemical characterization of isolates

Morphological and biochemical characterization of the bacterial isolates were done using routine bacteriological tests such as colony morphology, Gram reaction, and oxidase and catalase tests. All these isolates showed a negative response to Gram staining test (Table 1). Colonies from all bacterial isolates were irregular and showed positive results in oxidase and catalase tests except for BTLbbc-05, which showed negative results in the catalase test (Table 1; Figure 1).

3.4 Molecular identification of the isolates

To identify the selected bacteria, we used 16S rRNA gene sequencing followed by phylogenetic analysis (Figure 3).

Table 1: Morphological and biochemical characters of rhizobacteria.

Epiphytic Rhizoplane bacterial isolates	Biochemical character			
	Colony margin	KOH test	Oxidase test	Catalase test
BTLbbc-02	Irregular	–	+	–
BTLbbc-03	Irregular	–	+	–
BTLbbc-05	Irregular	–	+	+

+, Positive; –, negative.

The phylogenetic tree showed 98–100% confidence levels among the three isolated antagonistic bacteria from the β-subclass proteobacteria BTLbbc-02 (*Burkholderia cepacia*) and BTLbbc-03 (*Burkholderia metallica*), and another one from the γ-subclass proteobacteria BTLbbc-05 (*Pseudomonas aeruginosa*) (Table 2; Figure 3). All the bacterial 16S rRNA gene sequences have higher than 97% similarity with the inquired sequences. BTLbbc-02 and BTLbbc-03 were tentatively identified as *Burkholderia* spp.

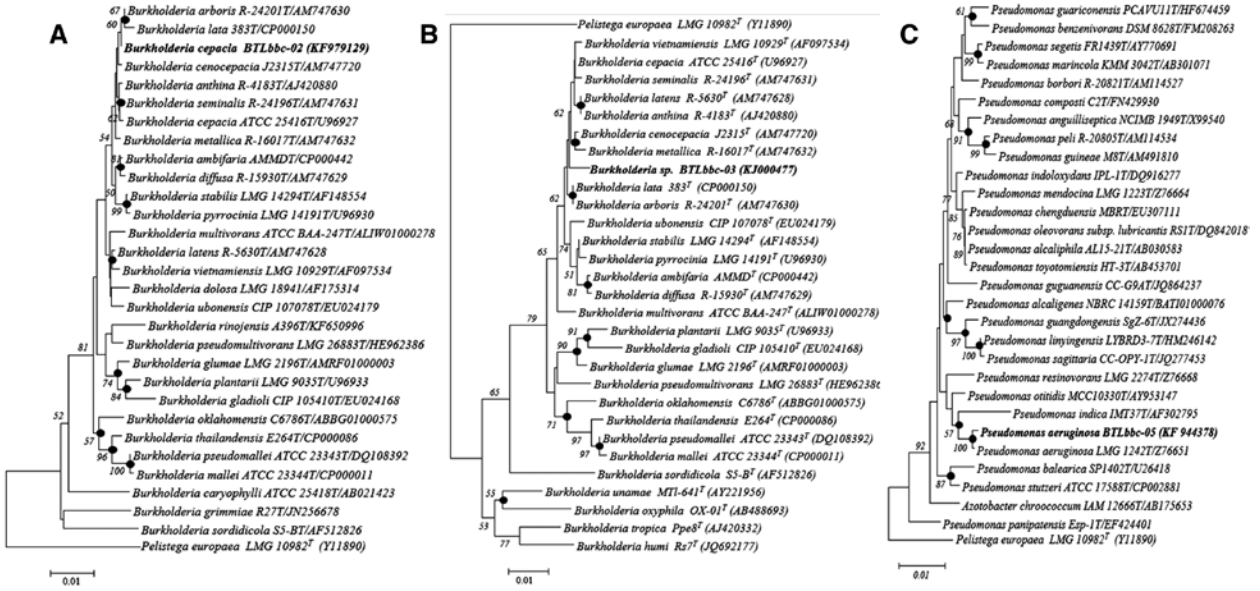

Figure 3: Phylogenetic tree of identified bacteria, *B. cepacia* BTLbbc-02, *B. metallica* BTLBBC-03, and *P. aeruginosa* BTLbbc-05. Genetic distances were computed by the Jukes-Cantor model. Percentages at nodes are levels of bootstrap support >50% based on neighbor-joining analyses of 1000 resampled data sets. Solid circles are the corresponding nodes (groupings) that were recovered in the maximum-likelihood tree. Scale bar 0.01 is nucleotide substitution per position. (A) Neighbor-joining phylogenetic tree representing relationships of *B. cepacia* BTLbbc-02 among the genus *Burkholderia* and related taxa based on nucleotide sequence of 16S rRNA gene. *Pelistega europaea* LMG 10982^{T} (Y11890) was used as an out group. (B) Neighbor-joining phylogenetic tree representing relationships of *B. metallica* BTLBBC-03 among the genus *Burkholderia* and related taxa based on nucleotide sequence of 16S rRNA gene. *P. europaea* LMG 10982T (Y11890) was used as an out group. (C) Neighbor-joining phylogenetic tree representing relationships of *P. aeruginosa* BTLbbc-05 among the genus *Pseudomonas* and related taxa based on nucleotide sequence of 16S rRNA gene. *P. europaea* LMG 10982^{T} (Y11890) was used as an out group.

Table 2: List of identified bacteria with isolate name, GenBank accession number, closest match according to 16S rRNA gene sequence (accession number), number of bases, maximum score, and % match with closest sequences in GenBank.

Isolate name	GenBank accession number	Closest match according to 16S rRNA gene sequence (accession number)	No. of bases	Maximum score	% Match
B. cepacia BTLbbc02	KF979129	*B. cepacia* strain 4APE (AB695353)	1246	2457	99.00
B. metallica BTLbbc03	KJ000477	*B. metallica* strain R-16017^{T} (AM747632)	1056	1162	99.03
P. aeruginosa BTLbbc05	KF944378	*P. aeruginosa* strain LMG 1242^{T} (Z76651)	1068	2340	97.02

with accession number KF979129 and KJ000477, respectively, and BTLbbc-05 was identified as *Pseudomonas* sp. with accession number KF944378.1 (Table 1). The gene sequence data of the isolates were submitted to the NCBI GenBank.

3.5 Suppression of *P. capsici* sporangia formation by antagonistic bacteria

The formation of abundant multinucleate sporangia under favorable conditions is critical for pathogenesis by *Phytophthora* pathogens. To see whether the bacterial antagonists have any inhibitory effects on the formation of sporangia in *P. capsici* hyphae, we conducted an in vitro assay for all isolates against *P. capsici*. Microscopic observations revealed that all bacterial isolates significantly decreased the number of sporangia formation in *P. capsici* hyphae compared with controls (Figure 4). All three identified antagonists, *B. cepacia* BTLbbc-02 ($p < 0.005$), *B. metallica* BTLbbc-03 ($p < 0.001$), and *P. aeruginosa* BTLbbc-05 ($p < 0.001$) inhibited sporangium formation significantly by 39%, 69% and 57%, respectively, compared with untreated controls (Figure 4C).

3.6 Antagonistic bacteria suppress zoosporogenesis of *P. capsici*

The release of biflagellate motile zoospores from sporangia is called zoosporogenesis, which is also a critical

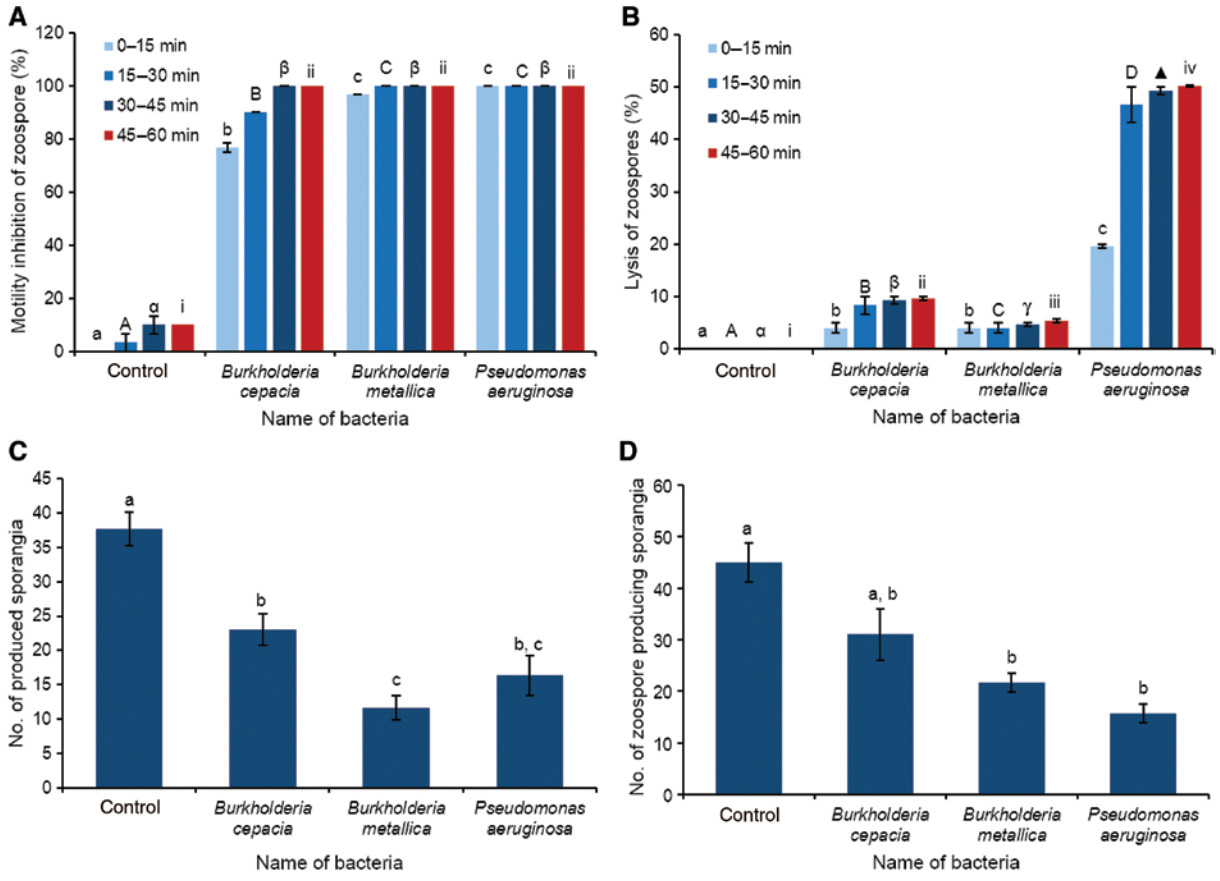

Figure 4: Effect of isolated bacteria on zoospore motility, zoospore lysis, sporangium formation, and zoosporogenesis of *P. capsici*. (A) Motility of *P. capsici* zoospore, (B) lysis of *P. capsici* zoospore, (C) sporangia formation by *P. capsici*, and (D) the ability of sporangium *P. capsici* to release zoospore. Data are presented as the mean ± SEM (n = 3). Different superscripts ($^{a, b, c; A, B, C; \alpha, \beta, \gamma, \blacktriangle; i, ii, iii, iv}$) within the same row indicate significant differences ($p < 0.05$) obtained by one-way ANOVA with multiple comparisons and Tukey's multiple comparison test.

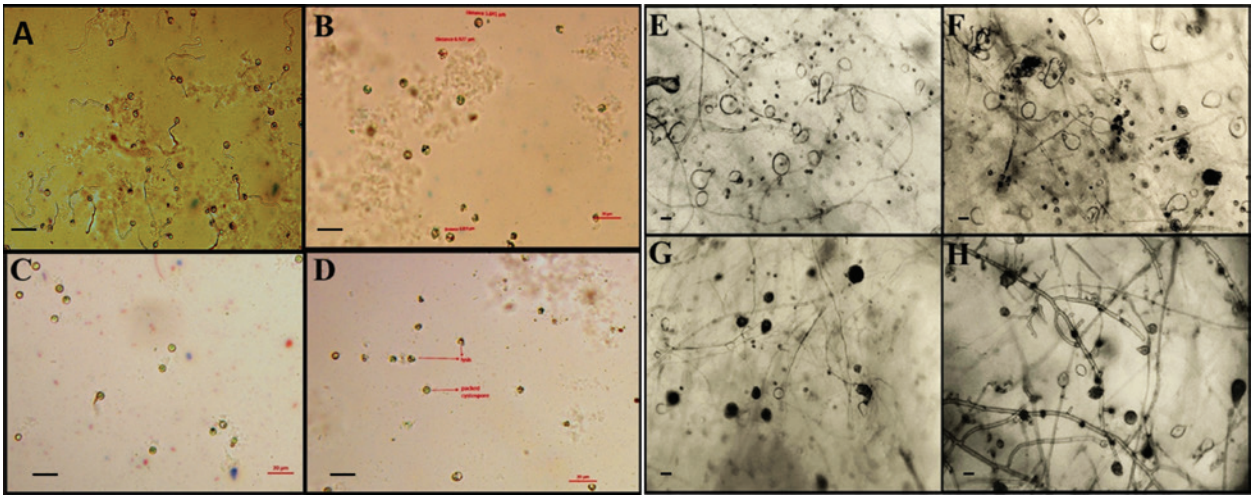

Figure 5: Microphotographs showing the effect of bacterial suspension on zoospore and sporangia of *P. capsici*. (A) Intact cystospore of control plate produced germ-tube; zoospore halting and lysis by (B) BTLbbc-02, (C) BTLbbc-03, and (D) BTLbbc-05; sporangium development and releasing of zoospore in (E) control plate without treatment, (F) BTLbbc-02 inoculated plate, (G) BTLbbc-03 inoculated plate, and (H) BTLbbc-05 inoculated plate (scale bar = 20 μm).

stage for the successful infection of host plants by *P. capsici*. We tested whether culture filtrates of antagonists have any effect on the release of zoospores from *P. capsici* sporangia (Figures 4 and 5). After adding culture filtrates of bacteria onto *P. capsici* mycelium taken from the multi-dishes, the dishes were incubated for zoospore release and then the effects of the antagonists were observed by using a light microscope (5E–H). The bioassay revealed that all antagonists inhibited the release of zoospores from the sporangia. The lowest number of sporangia that gave birth to zoospores was achieved by the treatment of *P. aeruginosa* BTLbbc-05, followed by *B. metallica* BTLbbc-03. Suppression of zoospore release by both isolates were significantly different from untreated controls ($p > 0.001$) (Figure 4D). As expected, control *P. capsici* mycelia (treated with only SDW) produced the highest number of zoospores.

3.7 Motility inhibition and lysis of zoospore

To test whether the bacterial isolates could inhibit the motility of zoospores, time-course changes in the motility of zoospores were observed after the addition of cell-free culture filtrates to the suspension of freshly released motile *P. capsici* zoospores. Interestingly, the zoospores of *P. capsici* became immotile (up to 100%) within 15 min of exposure to the cell-free culture supernatant of all three identified bacterial isolates (BTLbbc-02, 03, and 05) (Figure 5A–D). Moreover, subsequent lysis of some immotile zoospores was observed within 15 min in all three treated zoospore-containing plates. Moreover, within 60 min, zoospore lysis was caused by *B. cepacia* BTLbbc-02, *B. metallica* BTLbbc-03, and *P. aeruginosa* BTLbbc-05 at 10%, 5%, and 50%, respectively (Figure 4A and B). These results indicated that *P. aeruginosa* BTLbbc-05 caused significant cell lysis compared with controls ($p < 0.005$). In contrast, no lysis (Figure 5A) was observed in the control dishes; moreover, the zoospore became cystospores and produced germ-tubes after some time.

3.8 IAA by epiphytic rhizoplane bacteria

All three isolates produced IAA ranging from 0.7 to 26.0 µg/mL. High levels of IAA were produced by *B. cepacia* BTLbbc-02 (260 µg/mL), which was followed by *P. aeruginosa* BTLbbc-05 (4.8 µg/mL) and *B. metallica* strain BTLbbc-03 (0.7 µg/mL). *B. cepacia* BTLbbc-02 and *P. aeruginosa* BTLbbc-05 showed significantly higher levels ($p < 0.001$) of IAA production compared with controls (Figure 2B, Supplementary Figure S2E–H).

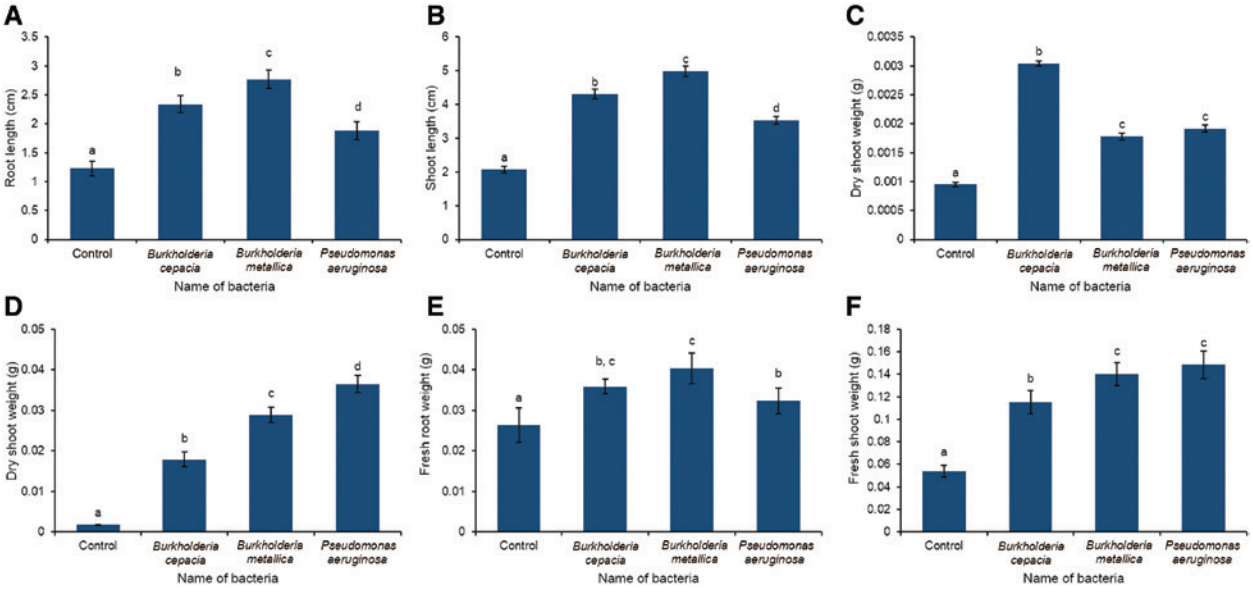

Figure 6: Effect of isolates on *C. sativus* growth by increasing shoot length, root length, fresh and dry weight of both root and shoot. (A) Root length, (B) shoot length, (C) dry root weight, (D) dry shoot weight, (E) fresh root weight, and (F) fresh shoot weight. Data are presented as the mean ± SEM ($n = 10$). Different superscripts ($^{a, b, c}$) within the same row indicate significant differences ($p < 0.05$) obtained by one-way ANOVA with multiple comparisons and Tukey's multiple comparison test.

3.9 Effect of epiphytic rhizoplane bacteria on germination and seedling vigor index of *C. sativus*

3.9.1 Effect on germination

Treatment of *C. sativus* seeds with rhizoplane bacteria significantly promoted the germination of seeds (Figure 2C; Supplementary Figure S1A–D). Untreated control seeds showed the lowest germination percentage (43%) under the conditions tested. However, *P. aeruginosa* BTLbbc-05–treated seeds gave 53% germination, which was followed by *B. cepacia* strain BTLbbc-02 (52%) (Figure 2C). There were highly significant differences ($p < 0.001$) among the bacterial treatments on *C. sativus* seed germination percentage.

3.9.2 Vigor index and growth promotion

Our data clearly show that isolated antagonists significantly promote root and shoot lengths compared with untreated controls (Figure 6). We also observed that the differences in the treatments for seedling vigor index, shoot growth, root growth, shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight were highly significant compared with untreated controls (Figure 6). Both *B. cepacia* BTLbbc-02–treated and *B. metallica* BTLbbc-03–treated seeds showed highly significant seedling vigor ($p < 0.001$) compared with untreated controls. *P. aeruginosa* BTLbbc-05–treated seeds also showed significantly ($p < 0.005$) higher vigor index (291). Moreover, all three identified isolates showed higher root length than untreated controls, where *B. metallica* BTLbbc-03 gave significantly ($p < 0.005$) higher root lengths compared with others (Figure 6A). All isolates promoted higher shoot length compared with controls. *Burkholderia metallica* BTLbbc-03 gave the highest shoot length of 4.995 cm compared with controls at 2.055 cm (Figure 6B). *B. cepacia* BTLbbc-02, *B. metallica* BTLbbc-03, and *P. aeruginosa* BTLbbc-05 produced higher root fresh weight (Figure 6C–F) than control seedlings ($p < 0.001$). In addition, *B. cepacia* BTLbbc-02, *B. metallica* BTLbbc-03, and *P. aeruginosa* BTLbbc-05 showed significant higher shoot fresh weight than untreated seedlings (Supplementary Figure 2).

4 Discussion

In recent years, epiphytic rhizobacteria from host plants have drawn the attention of researchers using them as biological control agents against *P. capsici* due to their ability to suppress pathogenic dispersing agents such as zoospores, cystospores, and hyphae. In the current study, three potential epiphytic rhizobacteria were isolated based on in vitro agar–based screening. We identified BTLbbc-02, BTLbbc-03, and BTLbbc-05 based on 16S rRNA gene sequencing, which showed 97–99% similarities with *Burkholderia* sp. (two) and *P. aeruginosa* (one). All these epiphytes displayed antagonistic effects on mycelial growth, zoospore motility and lysis, production of sporangia, zoospore release, and beneficial effects for the growth and development of cucumber seedlings along with IAA production. Because rhizobacteria can easily colonize plant roots and act as native biocontrol agents of the pathogen, our isolated bacterial strains could be used as potential biocontrol agents against *P. capsici*. Nevertheless, further research should be focused on in vivo experiments to confirm their ability as effective biopesticides for *P. capsici* in farmer's fields. It is important to note that isolates from the same species of these bacteria are recognized as human pathogens that cause cystic fibrosis [34]. Therefore, whether or not the plants identified in this study as *Burkholderia* spp. and *Pseudomonas* sp. are pathogenic to humans need to be confirmed through molecular studies at the genomovar level. In a recent study, the recA gene was investigated to identify the genomovar of *Burkholderia* spp. for safety evaluation. Hence, further studies are required to differentiate the pathogenic or nonpathogenic forms of these three isolates to confirm these as safe and potential biopesticides for controlling *P. capsici*. However, although several studies have reported *Burkholderia* spp. as biocontrol agents for plant disease [17, 35]; this study, for the first time, describes *Burkholderia* spp. from native chili roots of Bangladesh as biocontrol agents against *P. capsici*. Furthermore, *P. aeruginosa* as a biocontrol agent against *P. capsici* has also recently been described [16].

A previous study reported that in vitro dual-culture bioassay is a justified method for comprehensive screening of biocontrol bacteria against plant pathogens [36]. In our study, *P. aeruginosa* BTLbbc-05 showed the highest inhibition of mycelial growth by 89% in dual-culture bioassay. Furthermore, because the isolates were collected from native chili plants, these isolates have the potential to be used effectively for controlling *Phytophthora* blight of chili and other vulnerable vegetables due to acquainted host-microbe interaction. Inhibitory activities of *Burkholderia* spp. [35] and *P. aeruginosa* against mycelial growth of *P. capsici* [37–40] have been reported. Kim et al. [41] reported that *P. aeruginosa* B5 isolated from pepper-growing soils biocontrol *P. capsici*. *Pseudomonas*

spp. are known to produce various antimicrobial secondary metabolites such as pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin, and phenazine to suppress phytopathogens [42, 43]. The production of antibiotics might be involved in the suppression of *P. capsici* mycelia and inhibition of asexual spores by the bacterial antagonists shown in our study. Both strains of *Burkholderia* spp. showed significant mycelial growth inhibition by inducing excessive branching and swelling of the tips of approaching hyphae of *P. capsici*. Moreover, *B. metallica* BTLbbc-03 caused apical branching and extensive vacuolation of *P. capsici* mycelium. Mao et al. [44] showed that *Burkholderia* sp. strain MP-1 has the ability to suppress *P. capsici* by producing antiperonosporomycetal compounds. Interestingly, *B. metallica* BTLbbc-03 caused vacuolation in *P. capsici* hyphae, which was supported by the preceding research (Supplementary Figure S1). Previous studies reported that vacuole biogenesis is a common feature of fungi, which is an energetically less costly mechanism during nutrient scarcity and other stresses. Nonetheless, cells try to make vacuoles to detoxify toxic components [45]. Extensive vacuole formation in mycelium induces defect virulence in fungi. *Trichoderma harzianum* was tested to control *P. capsici*; it caused vacuolation in *P. capsici* mycelium and acted as a strong biocontrol agent for this notorious phytopathogen [46]. Although there are differences between fungi and peronosporomycetes, both of them showed high vacuole biogenesis under adverse conditions. Therefore, *B. metallica* BTLbbc-03 altered and inhibited *P. capsici* mycelial growth, and development seemed to be associated with highly vacuolated hyphae. A further bioassay-guided chemical fractionation of filtrated cultures is needed to isolate the active principal(s) involved in the inhibitory activities of our antagonists.

Inhibition of mycelial growth, inhibition of motility, and lysis of zoospore are considered critical traits of bacterial antagonists for controlling *P. capsici*. Interestingly, *P. aeruginosa* BTLbbc-05 caused up to 50% zoospore lysis and also impaired the motility of zoospores by 100%. The stopped zoospores and cystospores exposed to BTLbbc-05 did not germinate after an hour of treatment. *B. cepacia* BTLbbc-02 and *B. metallica* BTLbbc-03 also impaired zoospore motility and promoted subsequent lysis of zoospores in vitro. It has been reported that *Burkholderia* sp. and *Pseudomonas* sp. produce rhamnolipids, which is a class of effective zoospore-lytic biosurfactants and could be highly effective against *Phytophthora* sp. [47].

In an earlier study, the inhibitory effect of *Paenibacillus polymyxa* GBR-462 was demonstrated on *P. capsici*, in which culture supernatant was used to study the effect of antagonistic bacteria on sporangium development. Inhibition of nuclear separation in the sporangia was proposed to inhibit zoospore formation within sporangium by the GBR-462 [48]. To confirm as effective biocontrol agent against *P. capsici*, we also investigated the ability of our antagonists to suppress sporangium formation by this pathogen. In our study, culture supernatants of antagonists were tested on sporangia formation by *P. capsici* mycelium. Our results indicate that the isolates might produce some antiperonosporomycetal compounds that remarkably inhibit the formation of *P. capsici* sporangia. Further studies showed that the bacterial culture filtrate significantly interrupted the process of zoospore release from the *P. capsici* sporangia. This investigation inspired further studies to understand the underlying mechanism of biocontrol of *P. capsici* by our isolates.

Many rhizobacteria were reported to produce IAA or auxin and promote plant growth. Interestingly, our all bacterial isolates produced IAA and enhanced seed germination and growth of cucumber seedlings. Bioassay revealed that all three isolates of rhizobacteria identified in this study significantly increased seed germination percentage, vigor index, shoot and root length, as well as fresh and dry weights of cucumber seedlings compared with untreated controls. Enhanced root and shoot growth of plants through producing IAA and improving water and nutrient uptake by *P. aeruginosa* and *Burkholderia* sp. have been reported [17, 18, 49, 50]. It is important to note here that *B. metallica* BTLbbc-03 seemed to be the best growth promoter for *C. sativus*, whereas it produced the least amount of IAA compared with the other two isolates. As known, plant growth–promoting bacteria could be effective through production or regulation of different hormones, i.e. auxin, cytokinin, or ethylene, and even regulating the expression of growth-related genes of the host plants. Our results suggest that *B. metallica* BTLbbc-03 not only promotes IAA production but also applies some additional strategies to promote the germination and growth of cucumber seedlings. Further investigations are required to elucidate the precise mechanism of action of this isolate.

It has been established that bacteria are an important member of the organisms on earth and play a crucial role to keep balance within nature. Accordingly, plant-microbe interactions are also crucial for plants in the production of protective defenses as well as growth stimulators. However, as biocontrol is a developing issue in the research area to decrease hazardous pesticide usage, our study focused on multilevel in vitro studies to discover some novel efficient antagonists to control *P. capsici*. To our knowledge, this is the first report of the isolation and

molecular identification of *Pseudomonas* and *Burkholderia* spp. from the native environment of Bangladesh in which both vegetative and reproductive stages of *P. capsici* were inhibited. Nonetheless, our study demonstrated that *B. cepacia* BTLbbc-02, *B. metallica* BTLbbc-03, and *P. aeruginosa* BTLbbc-05 have high potential to control *P. capsici*.

5 Conclusions

In summary, this study identified three epiphytic bacteria, that is, *B. cepacia* strain BTLbbc-02, *B. metallica* strain BTLbbc-03, and *P. aeruginosa* strain BTLbbc-05, from the roots of chili. These epiphytes exhibited potent biocontrol activities against a notorious peronosporomycete phytopathogen *P. capsici* by suppressing mycelial growth and inhibiting asexual development of the pathogen. Furthermore, they significantly promoted seed germination, increased seedling vigor, and growth of cucumber seedlings compared with the untreated controls. Therefore, our isolates of *Burkholderia* and *Pseudomonas* spp. could be used as biopesticides to control *P. capsici* in various vegetable crops as an alternative to hazardous synthetic pesticides. The study clearly opens an opportunity for utilizing native rhizobacteria as a biocontrol agent to promote ecofriendly sustainable agriculture. Further studies are needed to elucidate the underlying molecular mechanism of their exhibited activities and also to check whether the application of these plant-associated bacteria to the practical field is safe for humans and other organisms.

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Author contributions: A.K. performed experiments, analyzed data, and drafted the manuscript. T.F. and A.A.S performed data collection. T.I., S.M.N.I., M.R., and H.M.W. supervised the design of the study and data analysis, and revised the manuscript. All authors critically reviewed the manuscript for intellectual content and gave final approval for the version to be published.

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