https://doi.org/10.17221/41/2025-PPS

Characterisation of *Colletotrichum* species associated with anthracnose disease in red chilli pepper (*Capsicum annuum* L.) in the South of Vietnam and the effectiveness of the consortium *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens*

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Citation: Ha V.T.N., Vuong H.T., Thang T.B., Hung H.T. (2026): Characterisation of *Colletotrichum* species associated with anthracnose disease in red chilli pepper (*Capsicum annuum* L.) in the South of Vietnam and the effectiveness of the consortium *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens*. Plant Protect. Sci.: 239–251.

Abstract: Anthracnose is one of the most destructive diseases that limits pepper production and quality worldwide. In this study, the causal agent of anthracnose in red chilli pepper in Southern Vietnam was collected and identified based on morphological characteristics and multilocus sequence regions (*ITS, β-tubulin, GPDH, ACT*). The antifungal activity of *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens* was evaluated *in vitro* and *in vivo* under greenhouse conditions. The results revealed that the morphological analysis categorised the *Colletotrichum* isolates into three species: *C. acutatum, C. gloeosporioides,* and *C. scovillei.* Sequence analysis of the four genes confirmed that *C. scovillei* was the causal agent of anthracnose in chilli pepper in Southern Vietnam. *B. amyloliquefaciens* and *P. fluorescens* bacteria demonstrated antifungal activity against *C. scovillei* in *vitro*, with mycelial growth inhibition rates ranging from 20.79% to 78.69%. The consortium of *B. amyloliquefaciens CC-LD2.4*, *P. fluorescens CC-FN1.1*, and *P. fluorescens O-BT1.2* achieved 84.4% control efficacy at 7 days after inoculation (DAI), which declined to 68.5% at 14 DAI and 41.7% at 21 DAI, at the flowering stage, and achieved 100% control efficacy at the fruiting stage. *B. amyloliquefaciens CC-LD2.4* showed very high chitinase, protease, and cellulase activities (halo diameter of 26.7 mm, 22.7 mm, 21.5 mm), whereas *P. fluorescens CC-FN1.1* was very high in protease and cellulase (14.3 mm, 12.4 mm) but weak in chitinase (5.1 mm), and *P. fluorescens O-BT1.2* exhibited overall lower activities (3.4–9.9 mm). There is still considerable room to optimise bacterial consortia to develop bio-fungicides that meet the requirements for an alternative or advanced solution for controlling anthracnose in red chilli peppers in sustainable agriculture.

Keywords: anthracnose; biological control; bacterial consortium; sustainable agriculture

Chilli peppers are an important vegetable in Vietnam, grown across the North and South. This crop is susceptible to the anthracnose disease caused by *Colletotrichum* spp., which can cause yield losses of up to 84% during cultivation and postharvest (Diao et al. 2017). These fungi are character-

Supported by the Minister of Science and Technology of Vietnam, under Grant No. NĐT/HU/22/20.

https://doi.org/10.17221/41/2025-PPS

ised by their ability to infect various stages of chilli growth, with the most severe symptoms occurring on ripening fruits.

In the North-Central of Vietnam, six species, including *C. scovillei, C. siamense, C. fructicola, C. brevisporum, Fusarium sulawesiense,* and *Epicoccum latusicollum*, were identified as the causative agents of chilli disease (Nguyen et al. 2023). In contrast, the causal agents in the South remain unknown. The lack of information about causal agents results in significant difficulties in disease management, as the species of this fungus is complicated (Than et al. 2008; Diao et al. 2017; Silva et al. 2017; Mongkolporn & Taylor 2018; Noor & Zakaria 2018; Khalimi et al. 2019; De Silva et al. 2019). That is because different species of *Colletotrichum* may exhibit varying responses to control measures; for example, *C. gloeosporioides* is highly susceptible to benzimidazole, while *C. acutatum* possessed moderate susceptibility against this fungicide (Peres et al. 2004; Ishii et al. 2022).

With an increasing preference for organic agricultural products worldwide, organic farming systems are gaining popularity, driving a high demand for biopesticides (Benbrook et al. 2021). Suppressing pathogens and inducing systemic resistance are effective biocontrol strategies for integrated plant disease management and offer a sustainable alternative to agrochemicals (Dikbas et al. 2023). Among beneficial microorganisms, *Bacillus* and *Pseudomonas* have been widely recognised as biocontrol agents in practical, sustainable agriculture (Suprapta et al. 2020; de Andrade et al. 2023; Sarfraz et al. 2023). The effects of using *Bacillus* and *Pseudomonas* individually depend on mechanisms such as the ability to secrete antibiotics, compete for nutrients and living space, or engage in parasitism; mechanisms of assimilation; and stimulation of plant disease resistance and iron competition (Shi et al. 2021). To increase the effectiveness of plant disease control of *Bacillus* and *Pseudomonas*, mixing these strains is necessary (Dimkić et al. 2022; Moreno-Velandia et al. 2024; Wang et al. 2025). However, little investigation has been conducted on using *Bacillus* and *Pseudomonas* to control anthracnose of chilli caused by *Colletotrichum* spp.

Based on the above-mentioned considerations, this study was done with the aim: *(i)* to identify the causal agents of anthracnose in the South of Vietnam; *(ii)* to evaluate the mycelium inhibition rate of the *Bacillus amyloliquefaciens*, and *Pseudomonas fluorescens* against *C. scovillei in vitro*; *(iii)* to investigate the bio efficacy of the bacterial combination in reducing the disease incidence and intensity of anthracnose in chili peppers under greenhouse condition.

MATERIAL AND METHODS

Isolation and characterisation of pathogens. Anthracnose-infected red chilli pepper (*Capsicum annuum* L.) fruits of cultivar F1-TN242 were collected from different provinces in the South of Vietnam from 2022 to 2023. Isolation was carried out using a tissue transplanting technique according to Nguyen et al. (2023) on potato dextrose agar [PDA; consisting of 200 g of potato, 20 g of agar (100%, Long Hai LTd., Vietnam), 20 g of D-glucose (99%, Bio Basic, USA), and 1 000 mL of distilled water]. The isolate was initially identified as a species by observation under an Olympus CX43 microscope (Olympus, Japan) morphological features, including colony color, sectoring pattern of surface mycelium, and nature of sporulation, the size and shape of 30 conidia harvested from each isolate were measured, the appressorium, and the presence of a seta or perithecium were examined (Sutton 1980; Cai et al. 2009). After that, the molecular identification of all the isolates was investigated.

Molecular characterisation of pathogens was done based on sequencing of the encoding region of internal transcribed spacer (*ITS*), with primers ITS4 (5'TCCTCCGCTTATTGATATGC3') and ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') (White et al. 2012); *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) GDF1 (5'GCCGTCAACGACCCCTTCATTGA3' and GDR1 (5'GGGTGGAGTCGTACTTGAGCATGT3') (Guerber et al. 2003); *actin* (*ACT*) ACT–512F (5'ATGTGCAAGGCCGGTTTCGC3') and ACT–783R (5'-TACGAGTCCTTCTGGCCCAT3') (Carbone & Kohn 1999), *beta-tubulin* (β-*tub2*) with primers T1 (5'AACATGCGTGAGATTGTAAGT3' and Bt2b (5'ACCCTCAGTGTAGTGACCCTTGGC3') (Glass & Donaldson 1995; O'Donnell & Cigelnik 1997).

Fungal isolates were grown on PDA medium for 7 days at 28 °C, and DNA was extracted using the commercial genomic DNA isolation kit TopPURE® Genomic DNA Extraction Kit (ABT Biotech Ltd., Vietnam). The polymerase chain reaction (PCR) was performed using the method described by Than et al. (2008) in a 25 µL reaction mixture,

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containing 2 µL DNA (1ng/µL) of extracted total genomic DNA template, 12.5 µL of 2x DreamTaq Red PCR MasterMix (Thermo Scientific Inc., USA), 6.5 µL of DNase-free water, and 2 µL (20 pM) of each primer (Apical Scientific, Malaysia). PCR amplification was performed in iCycler Thermal Cycler (Bio-Rad Laboratories Inc., USA) by following protocols: Denaturation was done at 95 °C for 5 min, 35 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s in the case of the ITS4/ ITS5 primer, at 57 °C for 30 s ATC-512F/ATC-783R primers, at 59 °C for 30 s GDF1/GDR1 primers and at 61.5 °C for 30 s Bt2b/T1 primers; and extension at 72 °C for 45 s) and final extension at 72 °C for 7 min. The PCR product was separated on a 1.5% agarose gel (40 min at 1.5 V/cm), stained with 6X GelRed (ABT Ltd., Vietnam), and photographed under UV light (E-BOX CX5TS Edge, Vilber Lourmat, France). After that, PCR products were sequenced using Sanger technology by ABT Biotech Ltd., Vietnam. All sequences were analysed using BioEdit software (version 7.2), and the similarity of these strains was checked against strains in the National Centre for Biotechnology Information (NCBI) database using a BLAST search. The sequence alignments were analysed using ClustalW methods, and multiple gene sequences were combined to construct a phylogenetic tree with a bootstrap coefficient of 1 000 using the distance method and neighbour-joining (NJ) in MEGA software (version 11) (Tamura et al. 2021).

Screening of Antagonistic Bacteria against *Colletotrichum sp. LD06* (later identified as *C. scovillei*). The antagonistic activity of *Pseudomonas fluorescens* and *Bacillus amyloliquefaciens* strains (Table 1) against *Colletotrichum* sp. *LD06* was evaluated using dual-plate assays on PDA medium (Raymaekers et al. 2020). The experiment followed a completely randomised design, with each bacterial strain as a treatment and PDA medium without bacteria as the control. Each treatment was replicated three times, and each replicate consisted of three Petri dishes. The diameter of the fungal growth inhibition zone around bacterial colonies

Table 1. Diameter of mycelial growth of *Colletotrichum scovillei LD06*, and antagonistic efficiency (AE)

Bacterial isolates		Diameter of mycelial growth (mm)				
		4 DAI	5 DAI	6 DAI	7 DAI	AE at 7 DAI (%)
Bacillus amyloliquefaciens	CC-LD1.1	$30.65^{c\text{-}f}$	42.22^{bcd}	44.65^{bc}	48.08^{bc}	39.90^{ef}
	CC-LD2.4	21.08^{gh}	24.28^{fg}	27.52^{efg}	29.10^{efg}	63.62^{abc}
	O-BT1.1	39.47^{b}	47.88^{b}	53.90^{b}	56.53^{b}	29.33^{fg}
	O-BT3.1	$34.77^{b\text{-}e}$	42.08^{bcd}	47.08^{bc}	51.33^{bc}	35.83^{efg}
	O-BT4.2	28.10^{efg}	33.42^{def}	36.98^{cdef}	44.92^{bcd}	43.86^{def}
	CC-LD1.3	39.22^{bc}	44.12^{bcd}	47.65^{bc}	51.10^{bc}	36.13^{efg}
	O-BT2.2	22.98^{fg}	25.23^{f}	26.97^{fg}	28.78^{efg}	64.02^{abc}
	CC-LD2.2	$35.18^{b\text{-}e}$	40.18^{bcd}	46.07^{bc}	49.18^{bc}	38.52^{efg}
Pseudomonas fluorescens	CC-LD1.2	21.48^{gh}	33.30^{def}	$40.32^{b\text{-}e}$	45.32^{bcd}	43.36^{def}
	CC-LD2.1	38.75^{bcd}	46.60^{bc}	53.55^{b}	58.50^{b}	26.88^{fg}
	CC-LD2.3	30.45^{def}	36.38^{cde}	42.68^{bcd}	47.33^{bcd}	40.83^{def}
	O-BT1.2	13.71^{hi}	14.65^{gh}	15.87^{gh}	20.52^{fg}	74.35^{ab}
	O-BT2.1	$35.58^{b\text{-}e}$	46.03^{bc}	51.03^{bc}	58.65^{b}	26.69^{fg}
	O-BT3.2	22.82^{fg}	26.97^{ef}	30.88^{def}	32.85^{def}	58.94^{bcd}
	O-BT4.1	20.33^{gh}	25.70^{ef}	29.57^{def}	36.33^{cde}	54.59^{cde}
	CC-FN1.1	10.27^{i}	11.60^{h}	14.18^{h}	17.05^{g}	78.69^{a}
	CC-FN1.2	$38.98^{b\text{-}e}$	47.46^{bcd}	57.52^{bcd}	63.36^{bcd}	20.79^{g}
Control		53.33^{a}	61.85^{a}	71.33^{a}	80.00^{a}	–
Critical value (%)		11.66	12.66	13.45	13.97	15.97
Levels of significance		**	**	**	**	**

$^{a-h}$ Similar letters above the means indicate a significant difference at $P < 0.01$ by ANOVA and Duncan's test; "–" did not evaluate; AE – antagonistic efficiency; DAI – day after inoculation

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was measured between 4 and 7 days after inoculation. Antagonistic efficiency (AE) was calculated using the formula: AE (%) = [(*D* – *d*) / *D*] × 100, where *D* is the diameter of fungal growth in the control (mm), and *d* is the diameter of fungal growth in the treatment (mm). Based on AE values, antagonistic activity was classified as follows: very high (≥ 76%), high (61–75%), moderate (46–60%), and low (< 45%).

In planta trials against *Colletotrichum* sp. *LD06* under greenhouse conditions. Red chilli pepper (*Capsicum annuum* L.) cultivar TN242 (Trang Nong Ltd., Vietnam), which is susceptible to anthracnose, was used in this study. Three bacteria, including *B. amyloliquefaciens* CC-LD2.4, *P. fluorescens* CC-FN1.1, and *P. fluorescens* O-BT1.2, were used. The efficacy of bacterial strains against *Colletotrichum* sp. *LD06* was evaluated under greenhouse conditions at both the flowering and fruiting stages, with separate experiments conducted for each stage following the method described by Yanti et al. (2020). Each experiment was conducted in a completely randomised design with a single factor "treatment". In total, 14 treatments were evaluated, each with 3 independent replicates. The experimental unit was a single pot containing a single chilli plant; each replicate comprised 10 pots (10 plants), for a total of 30 plants per treatment. The potting substrate was sterilised twice at 121 °C for 20 min.

The treatments involved a negative control (no pathogen was applied), a positive control (pathogen only inoculated with *Colletotrichum* sp. *LD06*), the chemical fungicide Ridomil Gold 68WP (Syngenta, Vietnam), and the biological pesticide Biobac (UPL Ltd., Vietnam) applied at the manufacturer's label rate, and bacterial treatments (e.g., *B. amyloliquefaciens CC-LD2.4*; *P. fluorescens CC-FN1.1*; full list in Table 2). The pathogen *Colletotrichum* sp. *LD06* was cultured on PDA medium for 7 days, after which spores were harvested and suspended in sterile water to a final concentration of 10^6 spores/mL plus 0.05% Tween 80. Spore viability was assessed by plating a 100 µL aliquot on PDA and calculating germination rates after 24 h (> 90% germination required for use). The standardised conidial suspension was applied with a hand-held atomiser, spraying until run-off onto both sides of leaves, stems, and peduncles. The bacterial suspensions, cultivated for 48 h in liquid LB medium and suspended with sterile water to a concentration of 10^7 CFU/mL plus 0.05% Tween 80, were applied similarly after 24 h of pathogen in-

Table 2. Disease incidence, AUDPC value, disease severity index (DSI), and the control effect at the flowering stage

Variants	Disease incidence (%)			AUDPC	DSI (%)			Control effect (%)		
	7 DAI	14 DAI	21 DAI		7 DAI	14 DAI	21 DAI	7 DAI	14 DAI	21 DAI
Pseudomonas fluorescens O-BT1.2	13.7^{a}	18.7^{b}	41.4^{ab}	162.6^{b}	3.3^{b}	7.2^{b}	16.7^{b}	26.7^{f}	21.0^{f}	20.6^{c}
Bacillus amyloliquefaciens CC-LD2.4	12.3^{a}	18.4^{bc}	38.1^{bc}	152.5^{b}	2.8^{bc}	6.5^{bc}	12.8^{cde}	36.2^{f}	28.1^{ef}	27.5^{bc}
P. fluorescens CC-FN1.1	12.0^{a}	18.9^{b}	37.9^{bcd}	153.4^{b}	2.6^{c}	6.1^{bc}	13.9^{cd}	42.3^{ef}	33.5^{e}	33.6^{ab}
P. fluorescens O-BT1.2 + B. amyloliquefaciens CC-LD2.4	8.6^{b}	16.2^{bc}	29.3^{bcd}	122.9^{c}	2.0^{d}	5.2^{cd}	14.4^{bc}	54.4^{de}	40.5^{de}	33.0^{ab}
P. fluorescens O-BT1.2 + *P. fluorescens* CC-FN1.1	9.0^{b}	15.4^{c}	27.4^{cd}	116.9^{cd}	1.6^{de}	4.5^{de}	13.2^{cd}	63.5^{cd}	51.4^{cd}	37.7^{ab}
B. amyloliquefaciens CC-LD2.4 + *P. fluorescens* CC-FN 1.1	8.3^{b}	16.0^{bc}	30.3^{bcd}	123.4^{c}	1.3^{e}	3.8^{e}	12.7^{de}	70.3^{bc}	59.5^{bc}	39.9^{a}
P. fluorescens O-BT1.2 + *B. amyloliquefaciens* CC-LD2.4 + *P. fluorescens* CC-FN1.1	5.3^{c}	9.9^{d}	27.2^{cd}	91.3^{de}	0.7^{f}	2.7^{f}	12.3^{cde}	84.4^{ab}	68.5^{ab}	41.7^{a}
Ridomil Gold 68WP	3.3^{d}	10.5^{d}	23.5^{d}	83.7^{e}	0.5^{g}	2.5^{f}	11.1^{e}	90.0^{a}	75.7^{a}	44.5^{a}
Biobac 50WP	4.4^{cd}	10.9^{d}	25.6^{cd}	90.7^{de}	0.6^{fg}	2.6^{f}	11.8^{de}	85.1^{ab}	71.3^{ab}	43.6^{a}
Positive control	13.6^{a}	22.7^{a}	77.6^{a}	198.7^{a}	4.5^{a}	9.3^{a}	33.0^{a}	–	–	–
Critical value (%)	21.2	16.5	37.1	32.2	24.6	20.3	12.9	23.5	22.8	21.5
Levels of significance	**	**	**	**	**	**	**	**	**	**

$^{a–g}$ Similar letters above the means indicate a significant difference at $P < 0.01$ by ANOVA and Duncan's test; "–" did not evaluate; AUDPC – area under the disease progress curve; DSI – disease severity index; DAI – day after innoculation

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oculum, either singly or as mixtures (consortium). Chilli pepper (*Capsicum annuum* cv. TN242) seedlings were grown under identical conditions until reaching the target stage. For the flowering stage, plants were inoculated and treated at the early flowering stage (75% of plants in the experiment opened their first flowers). For the fruiting stage, a separate cohort was inoculated and treated at the early fruiting stage (75% of plants in the experiment reached fruit set at 0.5–1.0 cm). Monitoring and recording the disease rate, disease index, and control efficacy at 7, 14, and 21 days after treatment as described by (Ro et al. 2021). During the experiment, the temperature in the greenhouse was 28 ± 2 °C, the humidity was 75 ± 5%, and there were 12 h of light daily.

Biocontrol-related enzymes. The ability of bacterial strains to produce chitinase, cellulase, and protease was assessed using universal salts medium (Masi et al. 2023) containing $MgSO_4 \cdot 7H_2O$ (0.3 g), $(NH_4)_2SO_4$ (3 g), KH_2PO_4 (2 g), and agar (15 g). The medium was supplemented with 1% (*w/v*) colloidal chitin (≥ 75% deacetylated, USA), 1% (*w/v*) carboxymethyl cellulose (CMC, viscosity 300–800 mPa·s, Xilong Scientific Co., Ltd., China), or 1% (*w/v*) casein (Sigma-Aldrich, USA) to detect chitinase, cellulase, and protease activity, respectively. All assays were conducted in triplicate, with three Petri dishes per replicate. The enzyme activity was qualitatively evaluated by measuring the clear zone (halo) surrounding the colony, and classified as follows: low (halo diameter < 6 mm), moderate (6–< 10 mm), high (10–< 12 mm), and very high (≥ 12 mm) (Ray et al. 2010).

RESULTS

Isolation and characterisation of *Colletotrichum* causing anthracnose on chilli pepper. A total of 12 *Colletotrichum* isolates were collected and then divided into 3 groups. Group I, identified as *Colletotrichum acutatum* species complex, involving 4 isolates: *LD01*, *LD02*, *LD03*, and *LD06*. Colonies irregular in morphology, with abundant white-greyish aerial mycelium, reverse dark brown, conidial masses salmon pink. Sclerotia are abundant, scattered, and separate. Setae present. Conidia are straight and transparent, cylindrical, obtuse at the apices, 9.13–14.33 µm × 2.17–5.00 µm. Appressoria are sparse, initially colourless, gradually

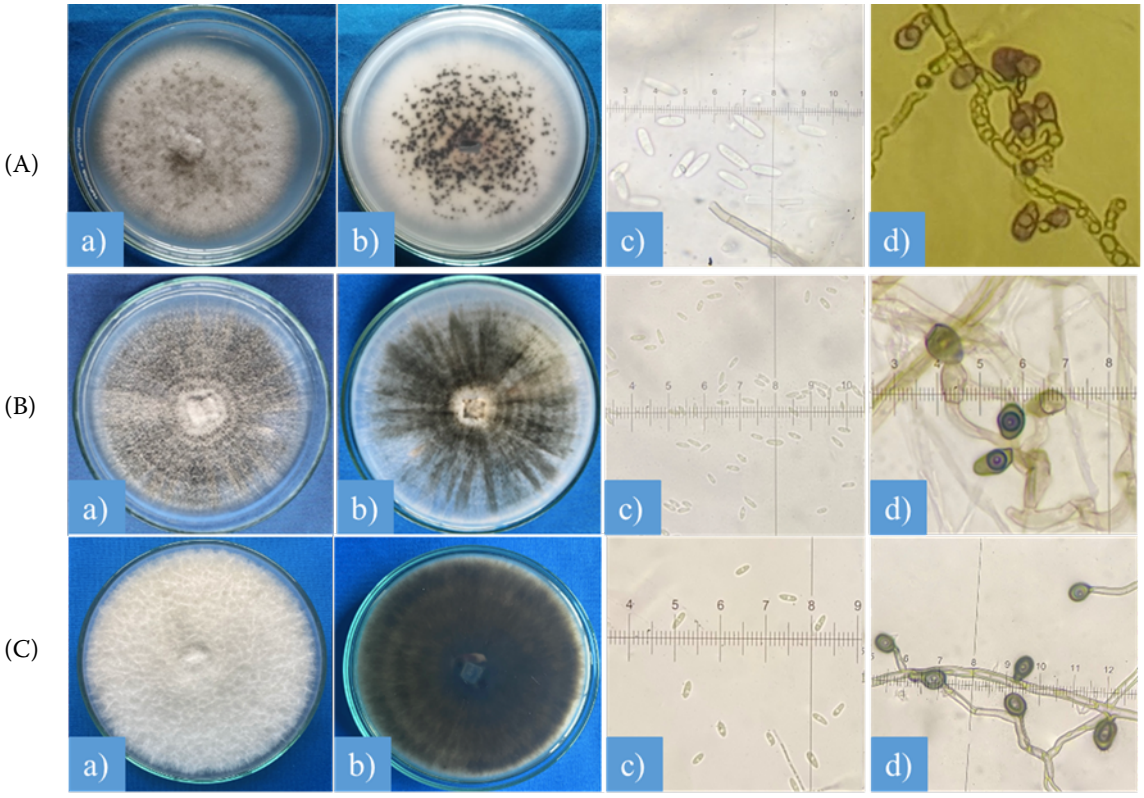

Figure 1. Morphological characterisations of *Colletotrichum* sp.
(A) *C. acutatum*; (B) *C. scovillei*; (C) *C. gloeosporioides*; (a–b) colony (c) conidia; (d) appressoria

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turning brown to black, clavate, or slightly irregular, with an average size of 7.33–13.33 μm × 2.77–5.33 μm, and often becoming complex (Figure 1A).

Group II, identified as *C. scovillei,* involving *BR13*, *DN14*, *DN16* and *LD12* isolates. The colony of black sclerotia, only rarely with diffuse white aerial mycelium; mycelium is thin, light greyish white, and conidial masses are honey. Sclerotia abundant, black, setose, globose, separate, or confluent. Setae present. Conidia are fusiform, straight, consistently constricted, 7.75–14.08 μm × 3.42–6.75 μm. Appressoria common, medium brown, long clavate, occasionally irregular, and edge almost crenate, 7.00–12.67 μm × 4.08–7.75 μm (Figure 1B), rarely becoming complex.

Group III, identified as *C. gloeosporioides,* involving 4 isolates: *TN04*, *TN09*, *TN10*, and *TN11*. The colony is greyish-white to greyish-black, and the mycelium is thin, greyish-white to black, growing high and evenly. Conidia are straight, unicellular, short, cylindrical, and obtuse at the apex; the average size is 5.50–14.38 μm × 3.63–6.63 μm. Appressoria are inverted ovoid, club-shaped or of indeterminate shape, light brown to dark brown, thin-walled, with a lot of yellow nuclei distributed irregularly inside, with an average size of 6.25–12.38 μm × 6.00–7.88 μm (Figure 1C), clavate or irregular, sometimes becoming complex.

The classification of *Colletotrichum* fungi based solely on morphological characteristics usually causes much confusion, and the role of molecular analysis has become increasingly important. The DNA of *Colletotrichum* spp. isolates were successfully amplified, with PCR products of about 600–700 bp for the ITS region, 700–800 bp for β-*tubulin*, 200–300 bp for GPDH, and 200–300 bp for ATC. The results of searching for high-similarity available sequences in GenBank showed that the studied fungal isolates all belonged to the species Colletotrichum scovillei, with similarity levels ranging 98.02–100.00% and coverage ranging 98–100%.

C. scovillei isolate CAUA13, *C. scovillei* isolate PHL2, *C. scovillei* isolate PHPG7, *C. scovillei* strain LC8, *C. scovillei* strain XFH1Y1, *C. acutatum* isolate Col-9-ALB, *C. acutatum* strain B13-16, *C. acutatum* strain P77, *C. acutatum* strain P185, *C. truncatum* strain DLFC2303, *C. truncatum* isolate AVCT02, *C. truncatum* strain GQMYTJ24, *C. gloeosporioides* isolate qzpgz161, *C. gloeosporioides* isolate zgmly1, *C. gloeosporioides* isolate SXC11, *C. gloeosporioides* isolate qzpgz2, *C. musae* isolate LPPC389, *C. musae* isolate PKV C01, *C. musae* strain C26, *C. fructicola* strain CLMYTJ59, *C. fructicola* strain LXMYTJ29, *C. fructicola* strain AHMYTJ2, *C. siamense* isolate CT5-1, *C. siamense* isolate SD-3, *C. siamense* isolate SD-2, *C. siamense* isolate Cs35 were used to construct a phylogenetic tree, *Corynespora cassiicola* was considered as an outgroup (Figure 2). This phylogenetic tree had the highest bootstrap rate of 99% and the lowest of 68%. The sequences of 12 isolates in this study were grouped into *Colletotrichum scovillei*, including isolates CAUA13, PHL2, PHPG7, LC8, and XFH1Y1, which caused anthracnose on chilli pepper. In addition, this *C. scovillei* group is in a clade with *C. accutatum*, suggesting that *C. scovillei* may belong to a *C. accutatum* species complex. This result confirms that the 12 isolates investigated in this study were *C. scovillei.* For the next experiments, the *C. scovillei LD06* was used.

Antifungal activity of bacteria against *C. scoville LD06* under laboratory conditions. In co-culture treatments with bacteria, colony diameters at all observed time points were significantly lower than in the control, varying depending on the bacteria's ability to inhibit hyphal growth of *C. scoville LD06*. The smallest colony diameter was observed when co-cultured with *P. fluorescens CC-FN1.1,* as 10.27 mm at 4 DAI and slowly increased to 17.05 mm at 7 DAI, followed by co-cultured with bacteria *P. fluorescens O-BT1.2*, and *B. amyloliquefaciens O-BT2.2* and *B. amyloliquefaciens CC-LD2.4* with colony diameters of 20.52 mm, 28.72 mm, and 29.10 mm, respectively, at 7 DAI (Table 1).

At 7 DAI, all bacterial isolates demonstrated a significant capacity to suppress the mycelial growth of *C. scovillei LD06*, with AE ranging from 20.79% to 78.69% compared to the control. The bacterium P. fluorescens CC-FN1.1 exhibited the most pronounced inhibitory effect on fungal mycelia, with an antagonistic efficiency (AE) of 78.69%, significantly higher than other treatments and the control ($P \leq 0.01$). Followed by *P. fluorescens O-BT1.2*, *B. amyloliquefaciens O-BT2.2*, and *B. amyloliquefaciens CC-LD2.4* with high antagonistic activity, the AE of 74.35%, 63.62%, and 64.02%, respectively. *B. amyloliquefaciens O-BT4.2*, *P. fluorescens CC-LD1.2, P. fluorescens CC-LD2.3, P. fluorescens O-BT4.1,* and *P. fluorescens O-BT3.2* showed moderate inhibitory effects (AE of 43.8%6, 43.36%, 40.83%, 54.59% and 58.94%), while the remaining bacteria exhibited low antagonistic activity against *C. scovillei LD06* (Table 1, Figure 3).

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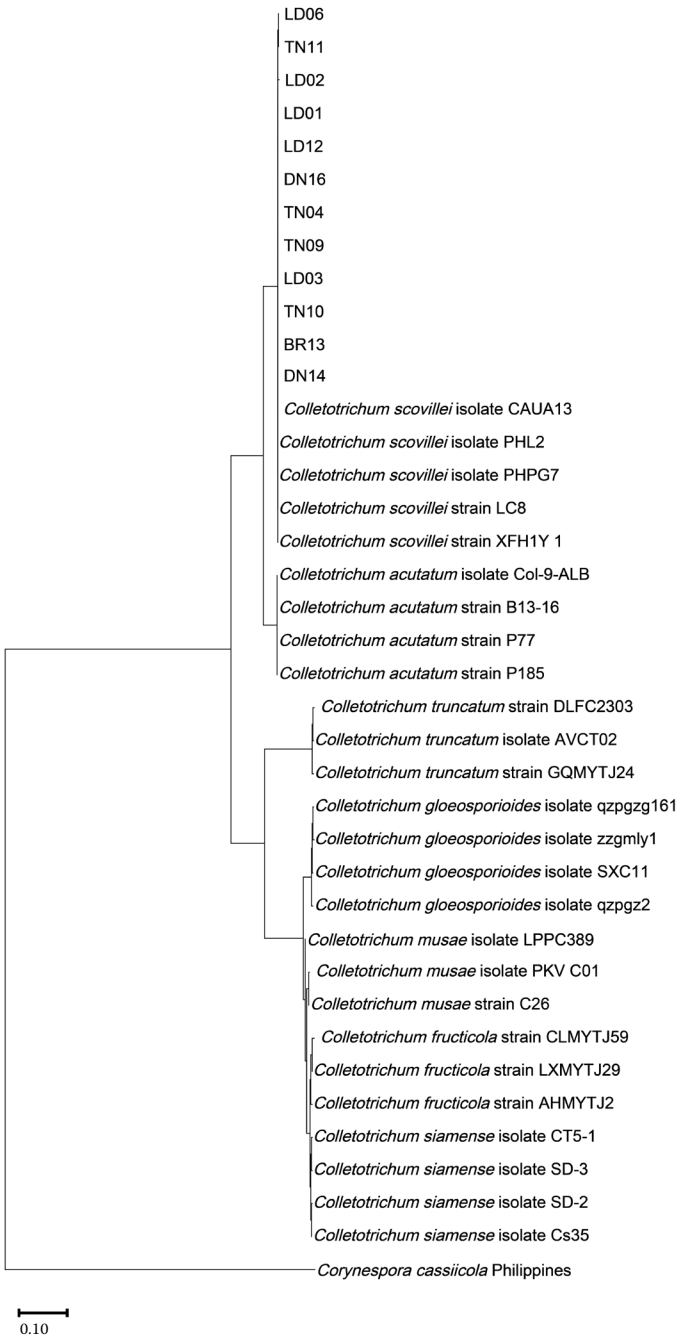

Figure 2. The phylogenetic tree of *Colletotrichum scovillei* is based on sequences of 4 gene regions (*ITS*, *β-tubulin*, *GPDH*, *ATC*)

Bootstrap values of 1 000 were presented as percentages

In treatments with highly antagonistic bacteria, *C. scovillei LD06* hyphae exhibited a bushy, erect growth pattern and limited radial expansion on the agar surface, indicating potent inhibition by the bacteria. In contrast, hyphae in other treatments spread more horizontally, suggesting less inhibition (Figure 3). Due to high antifungal activity against *C. scovillei LD06*, four bacterial isolates involving *P. fluorescens CC-FN1.1*, *P. fluorescens O-BT1.2*, and *B. amyloliquefaciens CC-LD2.4*, *B. amyloliquefaciens O-BT2.2*, were selected for the greenhouse experiments.

***In planta* trials against *C. scovillei LD06* under greenhouse conditions.** *C. scoville LD06 showed a high virulence in* the red chilli pepper cultivar TN 242, causing anthracnose with the highest disease incidence and area under the disease progress

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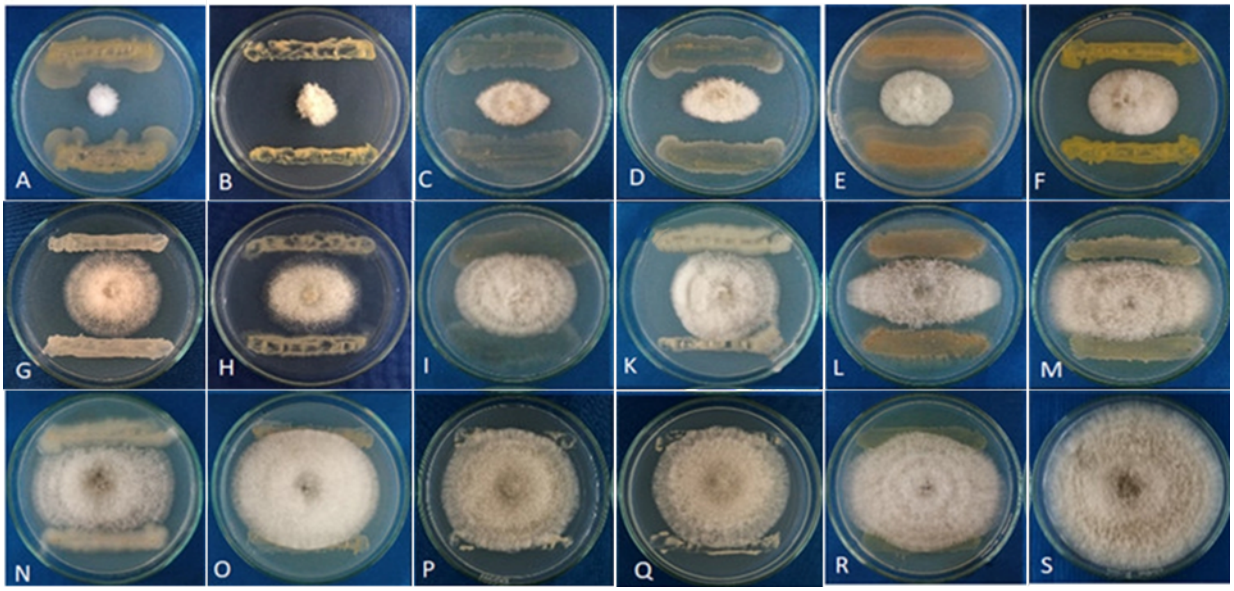

Figure 3. Mycelial growth of *Colletotrichum scovillei LD06* at 7 day after co-culture with bacteria
(A) – CC-FN1.1; (B) – O-BT1.2; (C) – O-BT2.2; (D) – CC-LD2.4; (E) – O-BT3.2; (F) – CC-LD1.2; (G) – O-BT4.1; (H) – O-BT4.2; (I) – O-BT3.1; (K) – CC-LD2.3; (L) – CC-LD1.3; (M) – O-BT1.1; (N) – CC-LD2.2; (O) – CC-LD1.1; (P) – CC-LD2.1; (Q) – O-BT2.1; (R) – CC-FN1.2; (S) – control

curve (AUDPC) value, indicating severe disease progression without treatment. *In vivo* pretest: *B. amyloliquefaciens* O-BT2.2 affected red chilli pepper plants; therefore, this bacterium was excluded from further evaluation. In the treatments with only the antagonistic bacteria *B. amyloliquefaciens CC-LD2.4*, *P. fluorescens CC-FN1.1*, *P. fluorescens O-BT1.2*, and the negative control (uninoculated plants), the disease incidence was 0% at all observation times, indicating that these bacteria were not phytotoxic and that there was no cross-contamination between treatments (Table 2).

Flowering stage. In all treatments, disease incidence increased over time (7–21 DAI), suggesting that repeated applications are needed to maintain control. The commercial pesticides Ridomil Gold 68WP and Biobac 50WP effectively controlled anthracnose, with the lowest disease incidence at all time points (7 DAI, 14 DAI, and 21 DAI) and AUDPC values, significantly lower than those of other treatments (Table 2).

When individual or combined bacteria *B. amyloliquefaciens CC-LD2.4*, *P. fluorescens CC-FN1.1*, and *P. fluorescens O-BT1.2* were applied to inoculated red chilli plants with *C. scovillei LD06*, disease incidence was reduced. A significant control effect was observed in the treatment with a combination of three bacteria and remained throughout the experiment. The disease incidence treated by the three bacteria at 21 DAI was 27.2%, which was not significantly different from Biobac 50WP (25.6%) (UPL Ltd., Vietnam) and Ridomil Gold 68WP (23.5%) (Syngenta, Vietnam) ($P \leq 0.01$)(Table 2).

At the flowering stage, the disease control efficacy at 7 DAI of the treatment with a consortium of bacteria *B. amyloliquefaciens CC-LD2.4*, *P. fluorescens CC-FN1.1*, and *P. fluorescens O-BT1.2* reached 84.4% and then gradually decreased to 68.5% at 14 DAI and to 41.7% at 21 DAI (Table 2), which was not significantly different from the chemical control Ridomil Gold 68WP (44.5%) and the biological control Biobac 50WP (43.6%) ($P \leq 0.01$).

Fruiting stage. The chilli pepper plant is most vulnerable to anthracnose during the fruiting period, especially when the fruit ripens and changes colour. In this experiment, anthracnose symptoms appeared at 6 DAI with *C. scovillei* LD06, with a disease incidence of 73.3%, and persisted until the end of the experiment. The fungicides Ridomil Gold 68WP and Biobac 50WP still demonstrated the ability to prevent infection and damage caused by *C. scovillei* LD06 in chilli. No disease symptoms were observed on the fruits in these treatments.

In treatments with single bacteria, disease incidence was relatively high: 46.7% for *P. fluorescens* O-BT1.2, 53.3% for *B. amyloliquefaciens* CC-LD2.4, and 53.3% for *P. fluorescens* CC-FN1.1, with no significant difference compared to the control. The dis-

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Table 3. Anthracnose disease incidence, Disease severity index, and control effect at the fruiting stage

Variants	Disease incidence (%)	Disease severity index (%)				Control effect (%)			
		6 DAI	9 DAI	12 DAI	15 DAI	6 DAI	9 DAI	12 DAI	15 DAI
Pseudomonas fluorescens O-BT1.2	46.7^{ab}	8.6^{ab}	8.6^{b}	8.6^{b}	8.6^{b}	62.3^{cd}	59.0^{cd}	57.4^{cd}	56.1^{cd}
Bacillus amyloliquefaciens CC-LD2.4	53.3^{ab}	9.9^{ab}	9.9^{b}	9.9^{b}	9.9^{b}	46.9^{d}	44.4^{d}	42.3^{d}	40.4^{d}
P. fluorescens CC-FN1.1	53.3^{ab}	9.9^{ab}	9.9^{b}	9.9^{b}	9.9^{b}	60.8^{cd}	57.6^{cd}	56.0^{cd}	53.7^{cd}
P. fluorescens O-BT1.2 + *B. amyloliquefaciens CC-LD2.4*	26.7^{b}	5.0^{b}	5.0^{b}	5.0^{bc}	5.0^{bc}	76.7^{bc}	76.1^{bc}	75.3^{bc}	74.6^{bc}
P. fluorescens O-BT1.2 + *P. fluorescens* CC-FN1.1	6.7^{c}	1.2^{c}	1.2^{c}	1.2^{cd}	1.2^{cd}	92.8^{ab}	92.3^{ab}	92.1^{ab}	92.1^{ab}
B. amyloliquefaciens CC-LD2.4 + *P. fluorescens* CC-FN 1.1	6.7^{c}	1.2^{c}	1.2^{c}	1.2^{cd}	1.2^{cd}	94.7^{ab}	94.8^{ab}	94.7^{ab}	94.4^{ab}
P. fluorescens O-BT1.2 + *B. amyloliquefaciens* CC-LD2.4 + *P. fluorescens* CC-FN1.1	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{d}	0.0^{d}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}
Ridomil Gold 68WP	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{d}	0.0^{d}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}
Biobac 50WP	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{d}	0.0^{d}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}
Positive control	73.3^{a}	16.1^{a}	23.5^{a}	30.9^{a}	30.9^{a}	–	–	–	–
Critical value (%)	34.4	21.1	21.7	24.3	24.3	10.3	11.2	11.8	12.2
Levels of significance	**	**	**	**	**	**	**	**	**

$^{a–d}$ Similar letters above the means indicate a significant difference at $P < 0.01$ by ANOVA and Duncan's test; "–" did not evaluate; DAI – day after inoculation

ease incidence decreased to 26.7% when treated with a combination of two bacteria, *P. fluorescens O-BT1.2* and *B. amyloliquefaciens CC-LD2.4*, and was only 6.7% when combining the other pairs of bacteria (*P. fluorescens* O-BT1.2 + *P. fluorescens* CC-FN1.1 or C CC-LD2.4 + *P. fluorescens* CC-FN1.1). Furthermore, combining all three bacteria completely inhibited the infection and damage caused by *C. scovillei LD06* (Table 3).

In treatments with individual bacteria or combinations of bacteria, the disease index remained unchanged over the observation period, while in the control, the disease index increased from 16.1% at 6 DAI to 30.9% at 15 DAI (Table 3). The highest efficacy in reducing fruit anthracnose was observed in treatments with the Ridomil Gold 68WP, Biobac 50WP, and the consortium of three bacteria. Efficacy reached 100% and then gradually decreased in treatments with combinations of two bacteria, with the lowest efficacy observed in treatments with a single bacterium (Table 3).

Ability to synthesise enzymes capable of controlling plant pathogenic fungi. Three bacteria, V O-BT1.2, V CC-FN1.1, and *B. amyloliquefaciens* CC-LD2.4, showed the ability to synthesise extracellular enzymes with varying levels of activity (Figure 4).

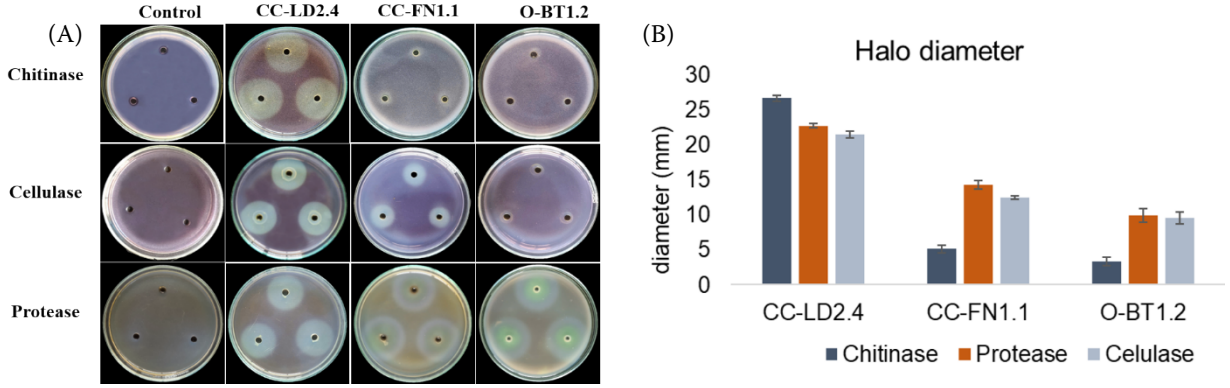

Figure 4. The result of the enzymatic assay
(A) on agar plates; (B) the halo diameter

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B. amyloliquefaciens CC-LD2.4 exhibited very high chitinase, protease, and cellulase activities, with halo zone diameters of 26.7 mm, 22.7 mm, and 21.5 mm, respectively. In contrast, *P. fluorescens CC-FN1.1* showed low chitinolytic activity (halo diameter of 5.1 mm) but very high proteolytic and cellulolytic activities (halo diameters of 14.3 mm and 12.4 mm, respectively). Similarly, *P. fluorescens O-BT1.2* displayed low chitinolytic activity and moderate proteolytic and cellulolytic activities, with halo diameters of 3.4 mm, 9.9 mm, and 9.6 mm, respectively (Figure 4B).

DISCUSSION

Chilli is one of the most valuable vegetables grown across Vietnam, with major production areas concentrated in the southern and central regions. The total area harvested in 2023 was 69 372 ha, and the average yield was 1 446.7 kg/ha (FAOSTAT 2024). However, its production and quality have been mainly limited by anthracnose, caused by fungi of the genus Colletotrichum. Identifying *Colletotrichum* spp. is crucial for effective disease management and involves a combination of morphological, cultural, and molecular approaches. However, species identification based on morphological criteria is inadequate due to overlap in morphological characters, especially conidial shape and colony characteristics (Anderson et al. 2024). In addition, morphological features can vary under different environmental conditions, such as changes in growth medium and temperature, leading to inconsistencies in identification (Khodadadi et al. 2020).

Based on complex gene regions (*ITS, β-tubulin, GPDH, ACT*), this study confirmed *Colletotrichum scovillei* as the causal agent of red chilli anthracnose in Southern Vietnam. These results are consistent with regional reports from Japan, Thailand, China, the Philippines, and the North of Vietnam, where *C. scovillei* has displaced *C. gloeosporioides* and *C. acutatum* in epidemiological dominance over the past decade (Than et al. 2008; Kanto et al. 2013; Liu et al. 2016; De Silva et al. 2019; Nguyen et al. 2023). Although, the number of strains included in this study was relatively limited, the *ITS, β-tubulin, GPDH,* and *ACT* gene regions have provided valuable insights into the taxonomic placement and functional potential of the isolates, underscoring the importance of accurate identification in biocontrol research, thank to variable regions that can differentiate species with overlapping morphological traits and provide high-resolution data for distinguishing closely related or cryptic species of *Colletotrichum* (Diao et al. 2017; Anderson et al. 2024).

In terms of biocontrol, previous chilli anthracnose studies using single *Bacillus* or *Pseudomonas* strains achieved 40–75% reduction in disease severity under greenhouse conditions (Suprapta et al. 2020; Yanti et al. 2020), but the consortium of three strains, *B. amyloliquefaciens* CC-LD2.4, *P. fluorescens* O-BT1.2, and CC-FN1.1, achieved an effect of 84.4% at 7 DAI at the flowering stage, comparable to commercial chemical and biological pesticides, and completely suppressed fruit infection. This consortium's efficacy can be supported by meta-analyses in biocontrol research by Raymaekers et al. (2020) and Sarfraz et al. (2023), which show that cross-genus microbial consortia outperform single strains. According to Minchev et al. (2021), bacterial consortia not only exhibited stronger antagonistic effects against pathogens but also demonstrated greater persistence, maintaining higher colony-forming unit (CFU) counts than single strains. *B. amyloliquefaciens*, with its ability to form endospores, is highly resilient to adverse environmental conditions, including UV radiation, desiccation, and pH fluctuations. This resilience likely enhanced the survival and persistence of *P. fluorescens* in both the phyllosphere and rhizosphere under greenhouse conditions. Robust sporulation and efficient colonisation may have enhanced the consortium‘s ability to compete with pathogens for nutrients and space (Niu et al. 2020; Dimkić et al. 2022; Moreno-Velandia et al. 2024).

In addition, *B. amyloliquefaciens* CC-LD2.4 exhibited strong extracellular enzyme activities. This is consistent with previous studies showing that *B. subtilis TD11* produced high levels of chitinase and cellulase, which are directly linked to biocontrol efficacy against fungal pathogens such as *Fusarium* and *Rhizoctonia* (Malik et al. 2023). Whereas *P. fluorescens* CC-FN1.1 and O-BT1.2 varied in enzyme expression (Kumar et al. 2022), and *P. fluorescens* CC-FN1.1 and O-BT1.2 via inducing systemic resistance, producing siderophores, and exhibiting plant growth-promoting traits Niu et al. 2020; Dimkić et al. 2022; Moreno-Velandia et al. 2024). According to Hegde and Monisha (2024), these complementary effects make the con-

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sortium more consistent and resilient in controlling disease than single-strain applications, resulting in a more reliable and effective biocontrol strategy.

Despite the promising results, the collection of pathogens in this study was small, and the antibacterial activity of bacteria was tested with only one isolate, *C. scovillei* LD06. Given the substantial genetic diversity within the *Colletotrichum* genus (e.g., *C. siamense*, *C. fructicola*), pathogen aggressiveness and sensitivity to biocontrol agents may vary considerably. Future work should therefore expand both the sampling scale and the number of strains examined to provide a more comprehensive understanding of microbial diversity and biocontrol potential under different agroecosystems. Such expanded investigations will enable more robust conclusions on the efficacy and generalizability of beneficial microbial consortia for controlling anthracnose in red chilli. Addressing these gaps will provide a more comprehensive understanding of the robustness and applicability of microbial consortia in anthracnose management. Nevertheless, the findings of this study highlight a promising strategy for robust biocontrol of *C. scovillei*, enhancing pathogen suppression, promoting plant growth and resilience, and offering an effective, sustainable alternative to chemical fungicides with potential for long-term application.

CONCLUSION

This study confirmed that *Colletotrichum* scovillei is the causal agent of chilli pepper anthracnose in the southern regions of Vietnam. All tested *B. amyloliquefaciens* and *P. fluorescens* strains demonstrated antifungal activity against *C. scovillei LD06* in vitro, with inhibition rates ranging 20.79–78.69%. Among them, *B. amyloliquefaciens* CC-LD2.4 demonstrated very high chitinase, protease, and cellulase activities, whereas *P. fluorescens* CC-FN1.1 was very high in protease and cellulase but weak in chitinase, and *P. fluorescens* O-BT1.2 exhibited overall lower activities. Notably, the consortium of these three strains achieved significantly higher control efficacy than the single strains, reducing disease severity and incidence to levels comparable to those of commercial chemical and biological products. Future research should expand pathogen collections, conduct cross-testing with additional *Colletotrichum* species, develop stable product formulations, and evaluate efficacy in multi-location field trials under natural disease pressure. Addressing these aspects will be crucial to advancing the consortium toward practical application and ensuring it meets agronomic, ecological, and commercial requirements as an effective solution for controlling anthracnose in chilli pepper production.

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Received: March 14, 2025
Accepted: December 10, 2025
Published online: May 6, 2026