PLANT SCIENCE TODAY
Vol 12(2): 1-10
https://doi.org/10.14719/pst.6576

eISSN 2348-1900

RESEARCH ARTICLE

Metabolic compounds of biocontrol agents from organic inputs for managing chilli anthracnose caused by *Colletotrichum acutatum*

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Received: 06 December 2024; Accepted: 10 January 2025; Available online: Version 1.0: 28 April 2025; Version 2.0 : 19 June 2025

Cite this article: Manoranjitham SK, Naveen KK, Darshan K, Sendilvel V, Rajendran L, Shanmugapriya D, Arulsia A. Metabolic compounds of biocontrol agents from organic inputs for managing chilli anthracnose caused by *Colletotrichum acutatum*. Plant Science Today. 2025; 12(2): 1-10. https://doi.org/10.14719/pst.6576

Abstract

Chilli anthracnose caused by *Colletotrichum acutatum* L. is a major constraint to crop yield and quality. This study, 35 bacterial isolates were obtained from four organic bio-inputs, Panchagavya, Jeevamruth, fish amino acid and Arappu buttermilk extract, to develop a biocontrol agent against this disease. These isolates were biochemically and molecularly confirmed to be *Bacillus* spp. The isolates were evaluated for antagonistic potential and plant growth-promoting activity through *in vitro* assays. Most effective *Bacillus* isolates (PG12 and PG2) were analyzed using thin-layer chromatography, revealing antibiotics such as iturin (Rf = 0.3) and surfactin (Rf = 0.7). These compounds were further confirmed by PCR amplification of specific genes, producing amplicons of 647 bp and ~620 bp, respectively. Gas chromatography-mass spectrometry (GC-MS) analysis identified antimicrobial volatile compounds such as fatty acids, lipopeptides, peptides and aldehydes in these isolates. The effectiveness of these potent isolates against C. *acutatum* was compared with the existing bioagent (*Pseudomonas fluorescens*) and botanicals (e.g., Neem) under greenhouse and field conditions using various combinations. The potent isolates of *B. subtillis* proved effective in minimizing damage caused by chilli anthracnose in pot culture and natural conditions. The existing study focuses on adopting these isolates in commercial formulation with increased efficacy and shelf life to increase farmers' income.

Keywords: 16S rRNA; anthracnose; *Bacillus subtillis*; bioagent; chilli; GC-MS; TLC

Introduction

Chilli (*Capsicum* spp.), a member of the Solanaceae family, is an essential spice crop with significant market demand. It is mainly used for culinary purposes, adding flavour, colour and pungency. Nutrition-wise, it is also a great source of Vitamin C, Vitamin A, Vitamin B6, Vitamin K1, Vitamin E, Potassium, Manganese, Magnesium, Iron and Copper. Anthracnose (dieback) caused by *Colletotrichum* spp. is a significant threat to chilli production, leading to yield losses of up to 50 % (1). Prominent *Colletotrichum* species responsible for anthracnose include *C. gloeosporioides, C. capsici, C. acutatum, C. siamense, C. coccodes, C. karstii, C. dematium* and *C. scovillei* (2). For years, chilli anthracnose has been managed using chemical fungicides. However, their overuse has led to fungicide resistance, environmental pollution and adverse effects on farmers' health and finances. Thus, alternative management methods are required to suppress this existing problem. Organic approaches are eco-friendly cost-effective and ensure soil health and high-quality chilli production. This method is risky in application as many reports in the literature support the control of this devastating pathogen. For example, *Saccharomyces cerevisiae* and Bacillus subtilis can be used against the chilli pathogen (3). Similarly, research indicates THE antagonistic activity of *P. fluorescens* isolate Pf1 against *C. capsici in-vitro* (4). Recent studies have shown that plant growth-promoting rhizobacteria (PGPRs) can promote plant growth by fixing and solubilizing mineral phosphates and other nutrients (5).

Notably, PGPRs produce volatile compounds with proven antimicrobial properties and can induce systemic resistance in plants. Furthermore, they exhibit antagonistic activity against phytopathogens by producing a variety of antimicrobial compounds such as chitinases, β-1,3-glucanase, antibiotics, siderophores and cyanide. *Bacillus* species are considered the most efficient PGPRs due to their ability to produce spores that survive in adverse climatic conditions (6). In addition, an increase in photosynthesis and chlorophyll content has also been reported, leading to good growth in *Arabidopsis* seedlings exposed to VOCs from *B. subtilis* GBO3 (7). In organic agriculture, farmers use several Indigenous Technical

Knowledge (ITK) based strategies, including spraying organic bio-inputs such as Panchagavya (3 %), Jeevamruth drenching (3 %), fish amino acid extract (1 %) and Arappu buttermilk extract (10 %). The reason lies in the presence of PGPRs in these inputs. Therefore, the hour needs to isolate, enumerate and validate the promising bioagents in these organic inputs. Building on this background, the study aimed to isolate distinct bacterial strains, evaluate their antagonistic and plant growth-promoting activities and assess their efficacy in managing chilli anthracnose under greenhouse and field conditions.

Material and Methods

Experimental pathogen

The pathogen *Colletotrichum acutatum,* previously isolated and characterized in our laboratory (Plant Pathology, TNAU), was selected for this study. The isolate was identified by ITS amplification and its sequence was submitted to the NCBI database under accession number MF063316. The pure culture was aseptically grown on Potato dextrose agar slants for additional use.

Organic bio-inputs preparation method

Panchagavya preparation

Panchagavya is an organic product made from nine ingredients: milk, cow urine, cow dung, ghee, curd, tender coconut, banana, jaggery and water. It promotes plant growth and boosts immunity against biotic stress. Seven kilograms of cow dung and one kilogram of cow ghee were mixed thoroughly and kept for three days. Then, an equal amount of cow urine and water (10 L) was mixed continuously. After that, 3 L of cow milk, 2 L of cow curd, 3 L of tender coconut water, 3 kg of jaggery and 12 bananas were mixed thoroughly. The container was stored in the shade condition for 15 days, stirring regularly twice daily (8).

Jeevamruth preparation

Jeevamruth facilitates the proliferation of helpful soil organisms and enhances crop yield. It consists of five ingredients, *viz.,* 200 L water, 10 kg of cow dung, 5 to 10 L cow urine, 1 to 2 kg of jaggery, 1 kg flour of the pulses and a handful of soil (undisturbed). All the ingredients were thoroughly mixed and regularly mixed in the morning and evening for 3-4 days (9).

Arappu buttermilk extract preparation

Arappu buttermilk extract helps plant growth, repels insects and prevents fungal diseases. The extract consists of four ingredients, *viz.,* 5 L buttermilk, one-litre tender coconut, 1 to 2 kg of arappu leaf powder and one-litre juice from waste fruit. Firstly, the fruit juice and arappu leaf powder were mixed thoroughly, then placed in a nylon mesh and tied. Later, the mixture in nylon mesh was immersed in a buttermilk-tender coconut solution and mixed regularly in the morning and evening for seven days.

Fish amino acid preparation.

Fish amino acids stimulate plant growth and microorganism activity as they are a great source of nutrients and various amino acids. Fish amino acid contains two ingredients: fish waste and 1 kg of solid jaggery 1 kg. This mixture was mixed thoroughly and kept for 30 days in shade condition. After 30 days, the liquid portion was filtered and used for assay (10).

Isolation of bacterial strains from organic bio-inputs

The bacterial isolates were isolated using the serial dilution technique from the four organic bio-inputs prepared earlier. To prepare the sample, 1.0 mL of four different organic bio inputs were added to 10 mL of distilled water, thoroughly mixed and vortexed for 15 min. Individual suspensions were serially diluted from 10^{-1} to 10^{-6} on plates containing PDA (Hi -Media) and nutrient agar media (NAM) (Hi-Media). To establish a pure culture, a part of the growing culture was aseptically transferred to fresh nutrient medium plates and incubated at 25 ± 2 °C.

Study of growth promotion activity by bacterial isolates

Preparation of inoculum

The bacteria isolates were cultured in nutrient agar broth (NABat room temperature with constant shaking for two days. Bacterial cells were extracted through centrifugation at 10000 rpm for 15 min and further re-immersed in phosphate buffer (0.01 M, pH 7.0). The cell concentration was altered to 10^8 CFU/mL using a spectrophotometer.

Seed bacterization

Chilli seeds (K1) were disinfected for 30 sec in 1 % sodium hypochlorite, washed in sterile distilled water and allowed to air dry instantly with a sterile atmosphere flow. Seeds were submerged for 2 hr in a suspension of bacteria at optimum concentration (10^8) and shade-dried.

Plant growth-promotion

The plant development increased the activities of the numerous strains of bacteria, which were evaluated using a standard roll towel method on chilli seedlings based on the seed vigour index (11). Twenty chilli seeds were put on the soaked sprouting material beforehand (Saran Hand-Made Paper Industry, India). Another pre-soaked germination paper strip was placed to hold the seeds in position and pressed gently. The polythene sheet was then rolled in and incubated for 14 days in the growth chamber. For each treatment, three replications were maintained. The germination percentage of seeds was determined and individual seedlings' shoot and root lengths were measured. The seedling vigour index was documented using an Equation 1 formula (12).

Vigor index = Germination % × (Mean shoot length + Mean root length) (Eqn. 1)

Dual culture technique

Isolated bacteria were screened for antagonistic effects using the dual culture method. The freshly grown bacterial isolate was streaked at one side of the petri plate and on the opposite side, a seven-day-old fungal mycelial disc of *Colletotrichum acutatum*. The control plate was maintained without an antagonist. Mycelial reduction by bacterial isolates was assessed by applying an Equation 2 formula.

$$PI = \frac{C-T}{C} \times 100 \qquad \text{(Eqn. 2)}$$

Where, PI: Percent growth inhibition; C: Control (Pathogen alone); T: Treatment (Pathogen and Antagonist)

Biochemical characterization of potential biocontrol-bacterial isolates

Different basic biochemical tests were conducted to identify the bacterial isolates isolated from Panchagavya, Jeevamruth, Arappu buttermilk extract and fish amino acids. The promising bioagents were inoculated into nutrient broth and kept at room temperature until inoculum turbidity exceeded 1.0 OD at 620 nm. The tests included Gram staining, KOH test, Catalase test, Growth in NaCl, HCN production and Siderophore production (13). Promising bioagents showing maximum pathogen growth inhibition and plant growth promotion were further biochemically characterized using an identification kit (HIMEDIA-Hi *Bacillus* Identification Kit-KB013).

Molecular characterization of potential biocontrol-bacterial isolates

Total genomic DNA of bacterial isolates PG12 and PG2 was extracted using the cetyl trimethyl ammonium bromide technique with minor changes and further treated with DNAse-free RNAse (10mg/mL) (14). The 16S rRNA intervening sequence of isolates was amplified using specific 27F (5 'AGAGTTTGATCCTGGCTCAG3') and 1392R (5' GGTTACCTTGTTACGACTT 3') to confirm the identity of isolates. The PCR reaction was performed by a cycle gradient thermal cycler with the condition set at a hold of initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 60 sec, annealing at 55 °C for 45 sec, primer extension of 72 °C for 60 sec, final extension time 72 °C of 7 min followed by cooling at 4 °C. The resulting fragments were displayed on 1.2 % agarose at 80V stained with ethidium bromide (0.5 µL mL) and unpurified PCR product (30 µL) was sent to the sequencing facility, S. Agrigenome Labs Pvt. Ltd, Cochin, Kerala. The resulting sequences were then subjected to BLAST analysis and deposited in the GenBank.

Extraction and thin layer chromatography analysis of antifungal compounds and PCR amplification to trace antibiotic gene

The crude antibiotics from *Bacillus* spp. (as detected) were obtained through a methodology (15) with some modifications. The concentrated crude extract of the extracellular antifungal compounds was then mixed in 1 mL methanol for further antifungal activity assay *in vitro*. Biologically active compounds extracted from *Bacillus* isolates were spotted on silica gel TLC gel plate at a rate of 10 µl /spot. Separation was achieved using chloroform: methanol: ethanol: water in the ratio of 70:30:35:5 on TLC plates after drying (16). The spots were visualized under ultraviolet rays (Millipore Sigma™) by using an Equation 3 formula.

$$\text{Rf value} = \frac{\text{Distance moved by the solute from origin}}{\text{Distance moved by the solvent from the origin}} \quad \text{(Eqn. 3)}$$

To confirm the presence of antibiotic genes, such as Iturin C and Surfactin were amplified using specific primers (17). PCR amplification was set at the following conditions: Initial denaturation at 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min followed by primer extension at 72 °C for 1 min and a final extension of 72 °C for 10 min.

Extraction of effective Bacillus spp crude metabolites and GC-MS profiling

Crude metabolites of the two highly effective bacterial strains, PG 2 and PG 12, were extracted using ethyl acetate. The resultant product (100 mg) was dissolved in 1 mL of HPLC-grade methanol. The extract's efficacy was assessed with two replications by the paper disc diffusion technique at 50, 75 and 100 MI concentrations of crude extracts (18, 19). Paper discs were treated with sterile water and were maintained as the control. The plates were incubated at 20 ± 2°C for 9 days and an area of inhibition was recorded.

The presence of volatile compounds in crude extracts was identified by GC/MS QP-2010 plus system (Agilent 7890B) in the Department of Nano Science and Technology, TNAU, Coimbatore, India. RTx-5 Sil MS column (30 m × 0.25 mm id × 0.25 film thickness) fitted with MS (Mass Selector Detector).

Extraction of plant extracts from botanicals

Antagonistic activity of twelve botanicals such as *Lantana camera, Vitex negundo*, *Tubernaemontana divaricate*, *Melia azedarach*, *Euphorbia antiquorum* L, *Pongamia pinnata, Cassia alata*, *Abrus precatorium*, *Azadirachta indica*, *Aegel marmelos*, *Prosopis juliflora* and *Rauwolfia serpentine* was analyzed *in-vitro* against *C. acutatum*. Fresh plant material was thoroughly rinsed with sterilized water to remove dirt particles and debris. Tissue was ground with an equal volume of autoclaved distilled water @ 1 g/mL and the extract was filtered through a muslin cloth to remove bacterial contaminants. The concentrated plant extract solution (100 %) was diluted with sterilized distilled water to the appropriate concentrations.

Testing the *in-vitro* efficacy of plant extracts against C. acutatum

In-vitro mycotoxic activity of plant extracts against the pathogens was determined by the method (20) at 10 % concentration. The concentrated plant extract (100 %) was diluted by mixing about 10 mL of plant extract to sterilized warm 90 mL of PDA medium and plated on a sterilized petri dish and allowed to solidify. Three replications of each treatment and control without plant extract were maintained. A culture disc (9 mm size) of *Colletotrichum* spp. from pure culture was placed onto the centre of the medium. The colonies' radial growth was measured regularly and percent growth inhibition was calculated.

Talc-based formulation

The NAB containing inoculum concentration of 9×10^8 CFU/ mL is considered ideal. The method was used to prepare talc-based bio-formulation: one kg of the purified talc powder (sterilized at 105 °C for 12 hr), 15 g of calcium carbonate (pH adjustment to neutral) and 10 g of adhesive

carboxymethyl cellulose (CMC) were mixed with 400 mL of bacterial suspension under sterile conditions, dried overnight and packed in a polypropylene bag and sealed (21). During application, the bacterial population was estimated at 2.5 to 3 × 10^8 CFU/g in the talc formulation.

Evaluation of promising bioagent and botanical against chilli anthracnose under glasshouse conditions

A completely randomized design was followed to test the efficacy of promising bioagents and botanicals for managing chilli anthracnose pathogen. Three replications for each treatment were maintained. Eight seeds of chilli cultivar K1 were sown in each pot and arranged on a slab in a row and pots were watered periodically up to saturation level. A sterile needle scrapped the virulent isolate (C1) conidial mass from the Petri plate with sterile water. The conidial concentration was adjusted with sterile distilled water using a haemocytometer (Thermo Fisher Scientific). The conidial suspension with 10^6 conidia/mL was challenge inoculated after one day of application of different treatments (Table 1) by foliar spray. The seedlings were maintained at 85 percent relative humidity and the disease incidence was noted after the development of the symptoms at seven-day intervals and expressed as percent disease incidence.

Evaluation of promising bioagent and botanical against chilli anthracnose pathogen under field conditions

Field experiments were conducted from October to April 2017 at the Eastern Block Farm, TNAU, Coimbatore, to evaluate the effectiveness of bioagents and botanicals against the chilli anthracnose pathogen. Chilli K1 variety was sowed and the trail was laid out in a randomized block design (RBD) with three replications maintaining a plot size of 2.5 × 2.5 m. Nine treatments previously used in greenhouse trials and standard treatments, such as the application of enriched farmyard manure @ 12.5 t/ha, humic acid drenching @ 2 mL/L and biofertilizers, were used. Three sprays were given according to respective treatments after 35 days of sowing at 15-day intervals. A standard score chart recorded Disease severity after the last sprays. The weight of fruits was recorded and yield per hectare was calculated. The percent increase in yield (PIY) was calculated as below Equation 4.

$$PIY = \frac{T\text{-}C}{C} X\ 100 \qquad \text{(Eqn. 4)}$$

Results and Discussion

Isolation and morphology characters of bacterial isolates

Thirty-five bacterial strains were isolated from organic bio inputs such as Panchagavya, Jeevamruth, fish amino acid and arappu buttermilk extract. The isolated bacterial strains were phenotypically different, ranging from creamy white to light brown colour and serrated and wavy margins. The colony characters of most of the isolates were rough with a serrated margin. Few isolates produced creamy, translucent, slimy and smooth-surfaced colonies. The colony colour ranged from creamy white to light brown (Table 2).

Evaluation of antifungal activity

Thirty-six bacterial isolates were evaluated against *C. acutatum* under laboratory conditions (Table S1). The isolates PG 12 isolate showed a maximum inhibition of 77.33 %, followed by PG 2 at 65.00 %. JM 1 and PG 7 showed an inhibition rate of 64.00 % and 62.00 %, respectively (Fig. 1 and 2). Research indicates comparable findings: *Bacillus subtilis* isolates EPCO 16 were efficient towards *C. gloeosporioides* (22).

Plant growth promotion activity of bacterial isolates

The effect of 35 bacterial strains on chilli seed growth promotion was studied using the roll towel method under *in -vitro* conditions (Table S2). The bacterial isolates, namely PG 2, PG 12, JM7, AB 5, JM 1 and AB 4, significantly increased the vigour of the chilli seedling. An optimum vigour index of 2493.92 was observed in the PG 2 isolate. This was followed by the isolates PG 12, JM 7, JM 1 and AB 4, which accounted for vigour index of 2112.00, 2018.75, 1883.76, 1594.32 and 1872 respectively. Seed treatment with *P. fluorescens* (Pf1) resulted in a vigour index of 1594.32, compared to the untreated control, which had the lowest vigour index of 1003.08. Similarly, research indicates endophytic *Bacillus* (EPC 5) inhibited the growth of *Ganoderma lucidum* in coconut (23, 24).

Biochemical characterization of bacterial isolates

The isolated bacterial strains were identified by various biochemical tests: Gram staining, KOH test, 7 % NaCl, malonate utilization test, Voges Proskauer's acetonin production and citrate utilization test. Biochemical and morphological characterization revealed that 28 strains were Gram-positive and 8 were Gram-negative, all of which tested positive in the above tests. Based on tests, these isolates were tentatively identified as *Bacillus* spp. and *Pseudomonas* spp. (Table 3). Two isolates, PG2 and PG12,

Table 1. Different combinations of treatments using promising biocontrol (PG2, PG12 and Pf1) and botanical (*Azadirachta indica*)

S.No	Treatments
T_1	Seed Treatment (ST) @ 10 g/kg of *Bacillus subtilis* (PG 12) @10g/kg and Seedling dip (SD) @ 0.5 % for 30 min
T_2	ST @ 10 g/kg *Bacillus subtilis* (PG 2) @10g/kg and SD @ 0.5 % for 30 min
T_3	ST @ 10 g/kg *Bacillus subtilis* (PG 12) + SD @0.5 % +Foliar spray (FS) @ 0.2 % of *Bacillus* (PG 12) at bimonthly interval
T_4	ST @ 10/kg Bacillus *subtilis* (PG 2) + SD @0.5 % +Foliar spray (FS) @ 0.2 % of *Bacillus* (PG 2)
T_5	ST @ 10 g/kg *Bacillus subtilis* (PG2) + SD @0.5 % + Soil Application (SA) @ 2.5 kg/ha + Foliar spray (FS) @ 0.2 % of *Bacillus* (PG 2) at bimonthly interval
T_6	ST @ 10 g/kg of *Bacillus subtilis* (PG 12) +SA @ 2.5 kg/ha + SD @0.5 % +FS @ 0.2 % of *Bacillus* (PG 12) at bimonthly interval
T_7	Foliar spray @ 10 % botanical (*Azadirachta indica*)
T_8	Standard check as ST@ 10 g/kg of *Pseudomonas fluorescens* (Pf 1) + SD @0.5 % +FS @ 0.2 % +SA @ 2.5 kg/ha of (Pf 1)
T_9	Inoculated control (pathogen)

Table 2. Phenotypic colony characters of bacterial isolates

S. No	Isolate	Phenotypic colony characters	S. No	Isolate	Phenotypic colony characters
1	PG 1	Creamy white with a serrated margin	19	JM 6	Pinkish white, dry, with a serrated margin
2	PG 2	Creamy light white with a serrated margin	20	JM 7	Creamy white, dry with a serrated margin
3	PG 3	Light brown with a serrated margin	21	JM8	White, dry, with a wavy margin
4	PG 4	Light brown with a serrated margin	22	AB 1	Creamy white with a serrated margin
5	PG 5	Pure white, slimy with a wavy margin	23	AB 2	Creamy light white with a serrated margin
6	PG 6	Light brown with a serrated margin	24	AB 3	Light brown with a serrated margin
7	PG 7	Pure white, slimy with a wavy margin	25	AB 4	Light brown with a serrated margin
8	PG 8	Pure white, slimy with a wavy margin	26	AB 5	Pure white, slimy with a wavy margin
9	PG 9	Pure white, slimy with a wavy margin	27	AB 6	Light brown with a serrated margin
10	PG 10	Light brown with a serrated margin	28	FS 1	Pure white, slimy with a wavy margin
11	PG 11	Slimy, light yellow with a serrated margin	29	FS 2	Pure white, slimy with a wavy margin
12	PG 12	Light brown with a serrated margin	30	FS 3	Pure white, slimy with a wavy margin
13	PG 13	Pure white, slimy with a wavy margin	31	FS 4	Light brown with a serrated margin
14	JM 1	Pure white, creamy with a wavy margin	32	FS 5	Slimy, light yellow with a serrated margin
15	JM 2	Pure white, creamy with a wavy margin	33	FS 6	Pure white, creamy with a wavy margin
16	JM 3	Pure white, slimy with a wavy margin	34	FS 7	Pure white, creamy with a wavy margin
17	JM 4	Dull white, dry with a serrated margin	35	FS 8	Pure white, slimy with a wavy margin
18	JM 5	Light brown with a serrated margin	36	PF 1	Dull white, dry with a serrated margin

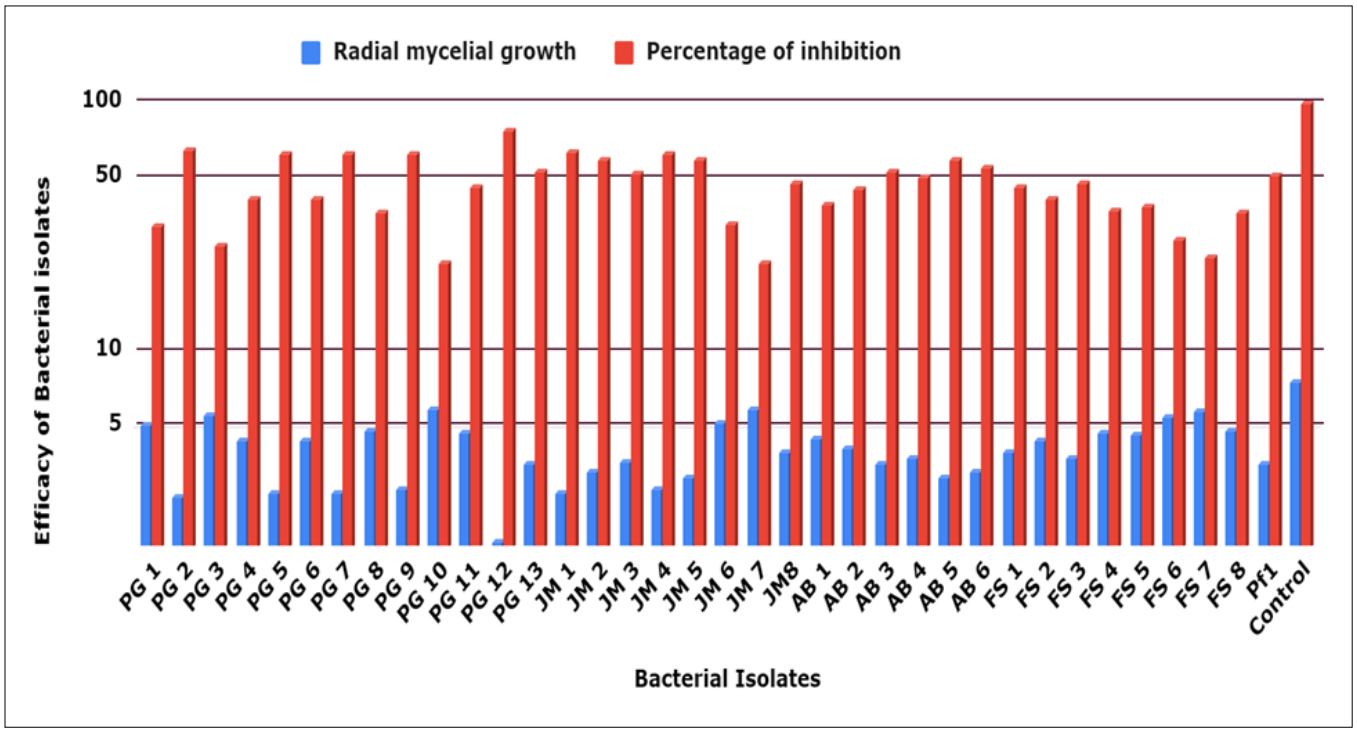

Fig. 1. *In vitro* efficacy of bacterial isolates against *Colletotrichum acutatum.*

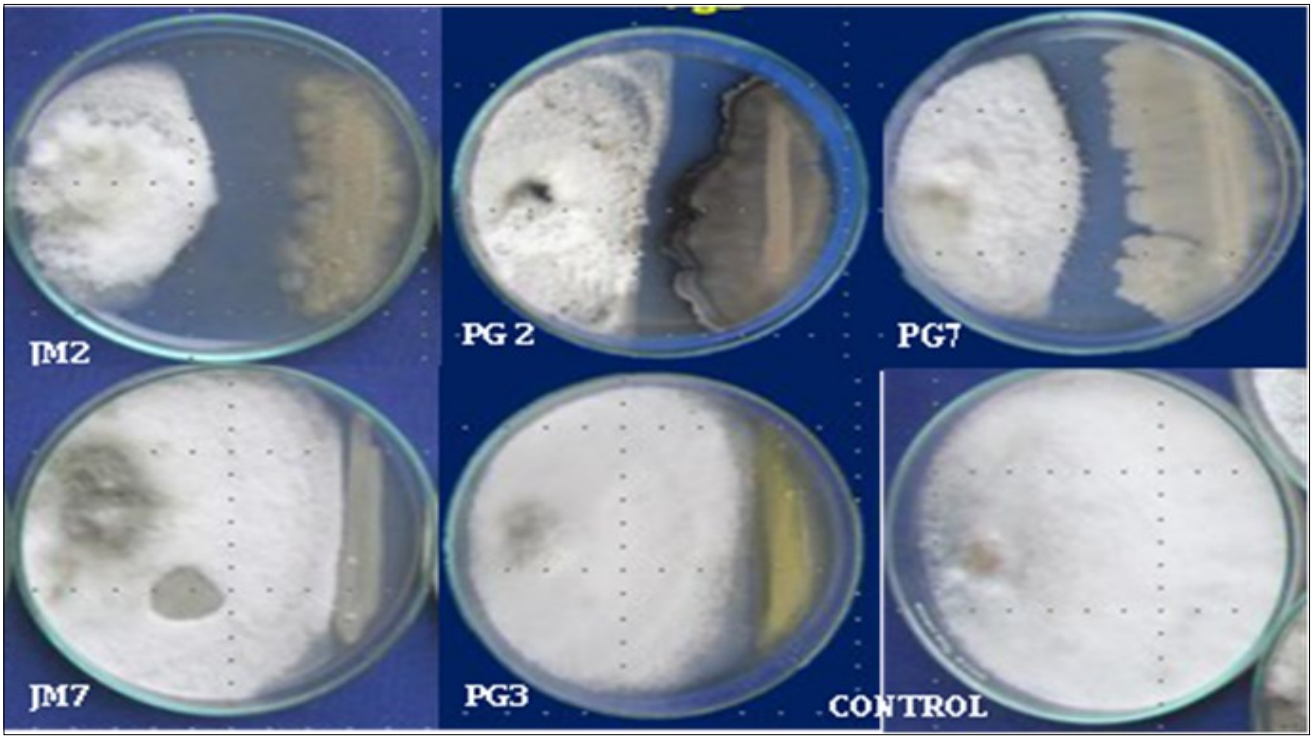

Fig. 2. *In vitro* testing of bacterial strains (JM2, PG2, PG7, JM7 and PG3) on inhibition of *Colletotrichum acutatum* mycelial growth.

which showed maximum *in-vitro* mycelial growth inhibition activity and PGPR activity, were further biochemically characterized using KB013 HiBacillus identification kit and KB009 HiCarbohydrate™ Kit (Fig. 3). Previously used biochemical characteristics and fatty acids profile of *Bacillus* sp (24).

Molecular characterization of promising bioagents

Additional PCR analysis was carried out using 27 F (AGAGTTTGATCCTGGCTCAG) and 1392 R (GGTTACCTTGTTACGACTT). To find the bacterial antagonist *Bacillus* sp., the 16S rRNA region-specific primer amplified a ~1350 bp fragment of the 16S-23S rRNA intervening sequence for *Bacillus* sp., and sequences were determined for effective isolates PG 2 and PG 12 (Fig. 4). The sequence received was subjected to BLAST analysis and isolates revealed 80-95 % nucleotide sequence identity of with the *Bacillus* spp. and submitted in NCBI bearing the accession number *Bacillus subtilis* PG 12 (MF150000), *Bacillus* sp. PG 2 (KY929121).

In-vitro analysis of the crude antibiotic activity of promising isolates

The crude antibiotic extract from PG 2, PG 12, PF 1 and JM 1 isolates were screened against *C. acutatum to* test their antagonistic activity by paper disc method. Isolate PG 12 showed a maximum inhibition of 65.00 %, followed by PG 2 with 53.41 % and further analyzed using TLC and GC/MS.

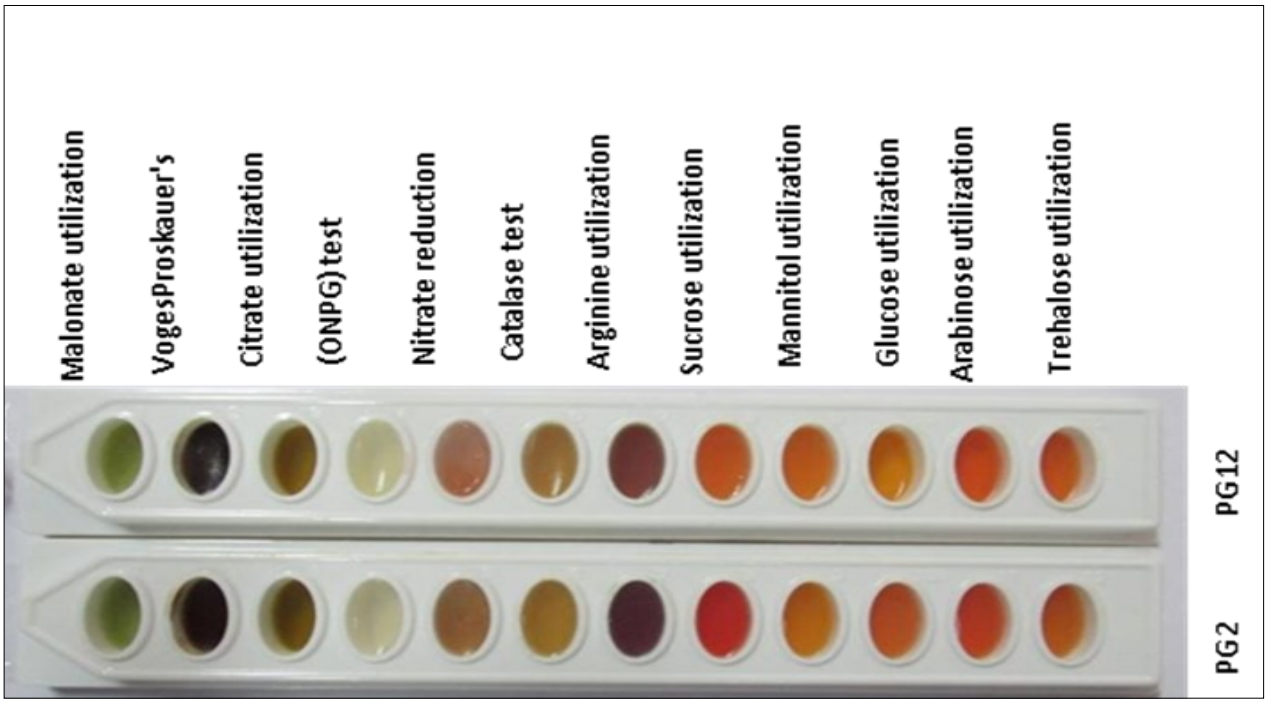

Fig. 3. Identification and characterization of promising bacterial strains using the biochemical kit (KB013).

Table 3. Identification and characterization of bacterial strains by biochemical characteristics

S. No	Isolates	Biochemical tests						Tentatively identified as
		Gram staining	KOH	Catalase test	Growth in 7 % NaCl	Siderophore production	HCN production	
1	PG 1	+	-	+	+	-	+	*Bacillus* sp.
2	PG 2	+	-	+	+	++	-	*Bacillus* sp.
3	PG 3	-	+	+	+	-	-	*Pseudomonas* sp.
4	PG 4	+	-	+	+	-	-	*Bacillus* sp.
5	PG 5	+	-	+	+	+	++	*Bacillus* sp.
6	PG 6	+	-	+	+	-	++	*Bacillus* sp.
7	PG 7	+	-	+	+	+	-	*Bacillus* sp.
8	PG 9	+	-	+	+	-	++	*Bacillus* sp.
9	PG 10	+	-	+	+	+	+	*Bacillus* sp.
10	PG 11	+	-	+	+	-	++	*Bacillus* sp.
11	PG 12	+	-	+	+	-	-	*Bacillus* sp.
12	PG 13	-	+	+	+	+++	+	*Pseudomonas* sp.
13	JM 1	+	-	+	+	-	-	*Bacillus* sp.
14	JM 2	+	-	+	+	+++	+	*Bacillus* sp.
15	JM 3	+	-	+	+	-	++	*Bacillus* sp.
16	JM 4	-	+	+	+	-	-	*Pseudomonas* sp.
17	JM 5	+	-	+	+	+	-	*Bacillus* sp.
18	JM 6	+	-	+	+	-	-	*Bacillus* sp.
19	JM 6	+	-	+	+	-	-	*Bacillus* sp.
20	JM 7	+	-	+	+	-	++	*Bacillus* sp.
21	JM8	+	-	+	+	-	-	*Bacillus* sp.
22	AB 1	+	-	+	+	-	++	*Bacillus* sp.
23	AB 2	+	-	+	+	+	-	*Bacillus* sp.
24	AB 3	-	+	+	-	-	-	*Pseudomonas* sp.
25	AB 4	-	+	+	-	-	-	*Pseudomonas* sp.
26	AB 5	-	+	+	-	++	-	*Pseudomonas* sp.
27	AB 6	+	-	+	+	-	-	*Bacillus* sp.
28	FS 1	-	+	+	-	+	+	*Pseudomonas* sp.
29	FS 2	+	-	+	+	+	-	*Bacillus* sp.
30	FS 3	-	+	+	-	-	+	*Pseudomonas* sp.
31	FS 4	+	-	+	+	-	-	*Bacillus* sp.
32	FS 5	-	+	+	-	-	-	*Pseudomonas* sp.
33	FS 6	+	-	+	+	-	-	*Bacillus* sp.
34	FS 7	+	-	+	+	-	-	*Bacillus* sp.
35	FS 8	+	-	+	+	-	-	*Bacillus* sp.
36	PF1	-	+	+	-	+++	+++	*Pseudomonas* sp.

(+) - Positive (-) – Negative

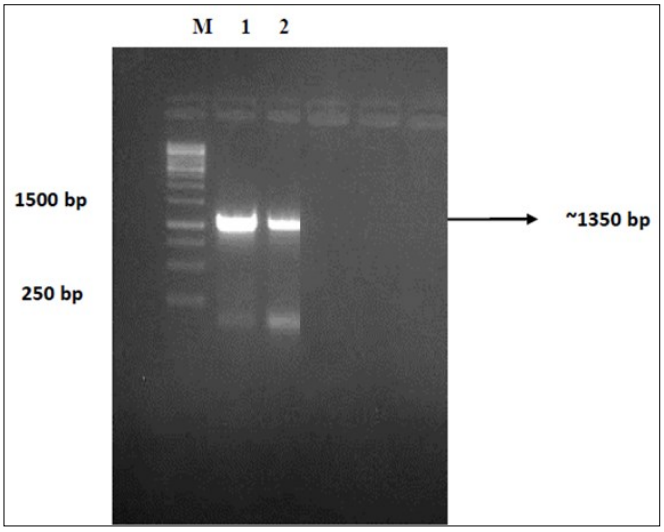

Fig. 4. PCR amplification of 16S-23S rRNA intervening sequence of *Bacillus* sp. isolates (Lane 1- PG2; Lane 2- PG12) showing amplicon size of 1350bp and lane M represents DNA ladder.

Detection of antifungal compounds through thin-layer chromatography (TLC) and PCR confirmation

Promising Bacillus spp., PG 12 and PG 2 isolates were analyzed through TLC for antibiotic production. The result revealed the production of surfactin and iturin with the *Rf* value of 0.3 and 0.7, respectively, in strains PG 12 and PG 2. Iturin, surfactin and fengysin played a significant role in the suppression of *C. acutatum* pathogen. Further, to confirm the existence of antibiotic biosynthetic genes in ineffective isolates of *Bacillus* sp., different antibiotic gene-specific primers corresponding to iturin and surfactin were used. The effective *Bacillus* isolate PG 2 and PG 12 showed the presence of iturin C and surfactin with an amplification size of ~647 bp and ~620 bp. Previously, many workers have reported the antibiotic activity of surfactin and fengysin C produced by *B. subtillis* against different pathogens effectively. Similarly, suppression of cucumber *Fusarium* wilt and *Phytophthora* pepper blight by *Bacillus subtilis* isolate ME488. The isolates were found to produce iturin, bacilysin and mersacidin antibiotics. The iturins antibiosis-based biocontrol activity by *Bacillus* strains against several diseases has been observed in diverse plant species. Potential production of antifungal lipopeptides such as iturin, fengycin, surfactin, bacillomycin and bacilysin in bacterial endophyte *Bacillus subtilis* EPCO16 isolated from the cotton plant when analyzed by thin layer chromatography (25).

Identification of volatile compounds from potential strain by GC-MS analysis

Based on the *in vitro* efficacy of bacterial antagonists against fungal pathogens and production of a higher number of crude antibiotics, *viz.,* PG 2 and PG 12 were selected for the GC/MS profiling to identify the volatile compounds. The GC-MS chromatogram detects the novel secondary metabolites, which may support the antifungal action against pathogens (supplementary information S3). Sixty-one chemical constituents in the isolate *B. subtilis* PG12 were detected at different retention times (RT). Among these, the major compounds were 1,2-Ethanediol, Hydroxylamine, Benzeneacetic acid, Propanedioic acid, Cyclooctane, Piperazine, Diisopropylpiperazine, 2-Propanone etc. Similarly, eighteen different chemical compounds could be differentiated in isolate *B. subtilis* PG2 GC/MS. Most were Hydroxylamine, Difluoromethane, Piperazine, Acetamide, Propanenitrile and Uramil-diacetic acid. A similar group of compounds was identified and plant growth promotion and antifungal activity were confirmed. The biological activity and chemical structure of phytocompounds were identified. Thus, the presence of lipopeptide and fatty acid-based substances in *B. subtilis* PG 12 and *Bacillus* sp. PG 2 suggests a possible biocontrol agent against the chilli anthracnose pathogen.

In-vitro antagonistic activity of botanical against *C. acutatum*

In vitro screening of different botanical extracts against chilli anthracnose pathogen, *C. acutatum,* revealed that 10 % leaf extract of *A. indica* recorded the lowest mycelial growth of 33.66 mm followed by 10 % leaf extracts of *V. negundo* with mycelial growth of 26.33 mm as compared to 59.25 mm in the control. They accounted for 60.33 % and 55.55 % *vitro* reduction in pathogen mycelial growth, respectively. The remaining plant extracts recorded mycelial growth ranged between 52 to 36 mm and mycelial growth inhibition ranged between 12 to 38 percent over control. Before our experiment, many workers revealed similar results using different botanicals. Research indicates the mycostasis property of crude extracts extracted from palmarosa oil, sweet flag and neem oil against the growth of anthracnose fungus. The maximum reduction of spore formation, as well as the mycelial formation of *Colletotrichum capsici* and *Alternaria alternate* when leaf extracts (10 %) of *Abrus precatorius* (Gundumuthu) and *Aegle marmelos* (vilvum) were used (26).

Efficacy of bioagent and botanicals against chilli anthracnose pathogen under glasshouse and field conditions

Talc-based bioformulation of *Bacillus* strains, PG 12 and PG 2, *P. fluorescence* (Pf1) and plant extract of *A. indica* was applied to the plants with different methods of application as previously described to detect antagonism against anthracnose pathogen, *C. acutatum* under glasshouse condition. Among the different treatments, seed treatments of *B. subtilis* strain PG 12 + seedling dipping @ 0.5 % + soil application @ 2.5 kg/ha + foliar spray @ 0.2 % of *B. subtilis* strains (PG 12) at bimonthly intervals recorded a lesser disease incidence of 21.83 %, which was found to be 62.47 % reduction over control. This was followed by standard check as seed treatment of *P. fluorescens* (Pf1) + seedling dipping @ 0.5 % +foliar spray @ 0.2 % + soil application @ 2.5 kg/ha recorded the anthracnose disease incidence of 24.64 %, which was found to be 57.63 % reduction over control. Meanwhile, the inoculated control recorded 58.16 % disease incidence.

In field trials, in addition to the percent disease reduction yield due to treatment was also recorded. The results were similar to greenhouse trials, among the different treatments, application of seed treatment of *B. subtilis* strains PG 12 + seed dipping @ 0.5 % + soil application @ 2.5 kg/ha + foliar spray @ 0.2 % of *B. subtilis* PG 12 at bimonthly interval recorded the minimum disease incidence of 19.34 PDI accounting to 57.91 % reduction over control. Followed by standard check as seed treatment of *P. fluorescens* (Pf 1) + seed dipping @ 0.5 % + foliar spray @ 0.2 % + soil application @ 2.5 kg/ha of (Pf 1) treatment recorded the anthracnose disease incidence of 23.41 PDI accounting to 49.06 % reduction over control. The maximum disease incidence was observed in untreated control, which recorded an incidence of 45.96 PDI. The highest yield was also recorded in the treatment, seed treatment of *B. subtilis* strain (PG 12) + seed treatment @ 0.5 % + soil application @ 2.5 kg/ha + foliar spray @ 0.2 % of *Bacillus* (PG 12) at bimonthly interval recorded a higher fruit yield of 3673.58 kg/ha which was followed by the application of Pf1 with a fruit yield 3546.98 kg/ha. The control treatment recorded the minimum yield attributes of 2545.95 t/ha (Table S3 & S4).

Similarly, using different *Pseudomonas* strains for managing plant diseases has been reported directly as bacterial suspensions or in various formulations. Research indicates the activity of *P. fluorescens* Pf1 against chickpea wilt and pigeon pea wilt pathogens (27). Similarly, the fungitoxic activity of *Bacillus* sp. (BNA 57) against tomato wilt was also noticed (28). The above evidence clearly emphasises the antagonistic property of endophytic bacteria against major pathogens infecting significant crops. In addition to disease management, *B. subtilis* (PG 12) treated plots also registered a significantly higher fruit yield of nearly 30.84 % (mean of two trials) increase over untreated control plots. These findings revealed that endophytes also enhanced protection and productivity. The study demonstrates that applying *Bacillus subtilis* (PG 12) using various methods in chilli effectively protects against anthracnose, as these methods ensure an adequate load of antagonist bacteria throughout crop growth.

Conclusion

Indiscriminate usage of plant protection chemicals has increased environmental pollution and the risk of soil residue persistence, indirectly affecting the food chain through biomagnification. With the increasing concern for physical health and mental well-being, an organic approach to control the disease is the best option as it's environmentally friendly and economical. IKT has been used for ages by farmers, but its exact potential to suppress pathogens is unknown. The study presently has evidenced the presence of endophytes that act as a plant protection

agent. Biochemical and molecular characterization has shown the existence of *Bacillus subtilis* and *P. fluorescence* in organic bio-inputs. It reduces disease severity and plays a critical part in promoting plant growth and increasing yield, as it stimulates the synthesis of plant hormones and helps break down phosphorus and other nutrients. Scientific evidence of the presence of biocontrol in organic input encourages farmers to apply and allows the scientific community to develop new, more efficient formulations.

Acknowledgements

The authors acknowledge the Department of Plant Pathology and Agro Climate Research Centre, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India for providing the necessary facilities to carry out the above research program.

Authors' contributions

SKM and KNK conceived and planned the study design, carried out the experiment. VS reviewed the manuscript. LR and KD performed statistical analysis and prepared the manuscript. DS gave the inputs for the manuscript writing. AA helped in writing the manuscript. All the authors approved and read the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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