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RESEARCH ARTICLE

Co-cultivation of *Bacillus amyloliquefaciens* and *Trichoderma harzianum*: Synergistic effects on plant growth and biocontrol of jasmine collar rot

Arshath Khan A[1], Yesuraja I[1*], Revathy N[1], Chandramani P[2]& Merina Prem Kumari S[3]

[1]Department of Plant Pathology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai 625 104, Tamil Nadu, India

[2]Department of Agricultural Entomology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai 625 104, Tamil Nadu, India

[3]Department of Biotechnology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai 625 104, Tamil Nadu, India

*Email: yesupatho@gmail.com

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Abstract

Jasmine (*Jasminum officinale*) is a valuable and culturally significant flowering crop in India. Still, its cultivation is greatly impacted by collar rot disease, caused by *Sclerotium rolfsii* Sacc., resulting in yield losses of up to 50%. This study explores co-culture technology, utilizing *Trichoderma harzianum* TR(D)-4 and *Bacillus amyloliquefaciens* BC(M)-2, as an integrated single-product solution for the biological control of this pathogen. Field surveys conducted across major jasmine-growing districts in Tamil Nadu revealed a range of disease severity (20% - 52%) and incidence (29% - 64%). The collar rot pathogen, and biocontrol agents were isolated from surveyed locations and identified through morphological characteristics and molecular phylogenetic analysis based on ITS and 16S rDNA sequences. *In vitro* assays using dual-plate and paired-plate methods demonstrated that *Bacillus amyloliquefaciens* BC(M)-2 achieved inhibition rates of 71.34% and 78.27%, respectively, while *Trichoderma harzianum* TR(D)-4 exhibited inhibition rates of 86.27% and 75.43% against *Sclerotium rolfsii* SR(D)-5. Co-culturing these antagonistic strains synergistically improved antifungal effectiveness, achieving an 87% inhibition rate against *S. rolfsii* compared to their separate cultures. The synergistic interaction in co-culture promoted the production of novel compounds, including alpha-bisabolol (AB), bis(2-ethylhexyl) phthalate, and harziandione, which enhanced plant growth and inhibited *S. rolfsii*. Further planta studies confirmed that the co-culture significantly reduced disease incidence and enhanced plant growth in both pre-and post-inoculation strategies. This research highlights the potential of co-culturing *T. harzianum* TR(D)-4 and *B. amyloliquefaciens* BC(M)-2 as an effective and sustainable approach for managing collar rot disease in jasmine.

Keywords

alpha-bisabolol; biocontrol; bis(2-ethylhexyl) phthalate; co-culture; metabolic pathways; synergistic effect

Introduction

India's favorable agro-climatic conditions support cultivating a wide variety of flower crops, including jasmine (*Jasminum officinale*), often referred to as the "queen of flowers" and belonging to the Oleaceae family. Jasmine is prized for its ornamental appeal and use in perfumery and medicine, with nearly 200 species found worldwide. Among these, only four species hold commercial

value: *Jasminum auriculatum*, *Jasminum sambac*, *Jasminum pubescens* and *Jasminum grandiflorum*. Jasmine cultivation in India produces approximately 2.41 million tonnes annually, with an average yield of 3.5 tonnes per hectare, significantly contributing to the economy with annual sales exceeding ₹20 crore. Despite its economic significance, jasmine is highly vulnerable to various diseases, especially those caused by fungi, bacteria, viruses and phytoplasmas, which pose substantial biotic challenges to its cultivation.

One of the most destructive diseases affecting jasmine is collar rot, caused by the soil-borne fungus *Sclerotium rolfsii* Sacc. (Telomorph: *Athelia rolfsii*). This necrotrophic pathogen causes significant yield losses, potentially reducing production by up to 52%. The disease is characterized by wilting, with whitish mycelial growth at the plant's collar region, often accompanied by sclerotia formation. This fungal infection hampers nutrient absorption and ultimately leads to plant death.

Presently, systemic fungicides like as propiconazole, hexaconazole and carbendazim are employed to effectively control jasmine collar rot disease. However, these chemicals pose significant environmental risks, including soil persistence, contamination and potential toxicity to aquatic organisms. Their application can disrupt soil health and biodiversity by negatively impacting non-target microorganisms and promoting the development of resistant fungal strains, highlighting the need for alternative, sustainable management strategies. Using antagonistic microbes, such as *Bacillus* spp. and *Trichoderma* spp., presents a promising alternative to chemical fungicides. Researchers have demonstrated that rhizosphere microorganisms possess antifungal properties that effectively inhibit soil-borne plant pathogens, including *Fusarium* spp. and *Sclerotium rolfsii* (1).

Interestingly, the genera *Trichoderma* and *Bacillus*, a filamentous fungus and bacterium, respectively, are known to colonize root surfaces or reside within root tissues as endophytes (2). Species such as *T. harzianum* and *B. amyloliquefaciens* are particularly effective in suppressing the growth of phytopathogenic fungi, including *Sclerotinia sclerotium*, *Macrophomina phaseolina*, *Fusarium* spp., and *S. rolfsii*. They achieve this through various antagonistic mechanisms, including the production of lytic enzymes that degrade pathogen cell walls, competition for nutrients and space and the enhancement of plant defense responses through induced systemic resistance. *Trichoderma* species operate via multiple pathways, including antibiosis, mycoparasitism and systemic resistance induction. At the same time, their rapid growth rate and tolerance to abiotic stresses enhance their effectiveness as biocontrol agents (3). Conversely, *Bacillus* species, particularly *B. amyloliquefaciens*, are renowned for their genetic ability to produce a range of antimicrobial compounds, especially cyclic lipopeptides. Among these, fengycin, iturin and surfactin are the most prominent, with specific isoforms in the iturin and fengycin families exhibiting potent antimicrobial properties, which contribute significantly to the biocontrol efficacy of *Bacillus* strains (4).

In recent years, volatile compounds produced by antagonistic microbes during metabolism-called microbial Volatile Organic Compounds (mVOCs)-have attracted considerable attention for their dual role in antimicrobial activity and plant growth stimulation. These small molecules play critical roles in plant-microbe interactions by inhibiting pathogens, promoting plant growth and modulating soil microbial communities. mVOCs suppress pathogen growth by disrupting cellular functions and activating plant defense responses. Additionally, some mVOCs facilitate nutrient mobilization, enhancing nutrient availability to plants, while others stimulate the production of plant growth hormones, encouraging robust root and shoot development. Identifying the specific mVOCs responsible for these antagonistic actions could lead to innovative and sustainable biocontrol solutions for organic farming.

Various volatile metabolites produced by *Trichoderma* spp.-including 2H-Pyran-2-one (6-pentyl-), eicosane, dodecanol, thymine and ribitol-as well as non-volatile secondary metabolites, such as atroviridin, konginginin, viridiol and trichodermin, are well-known for their antimicrobial properties against numerous plant pathogens (5). Similarly, certain *Bacillus* species produce a complex array of mVOCs, such as n-decanal, 2-undecanone, benzothiazole, dimethyl trisulfide and pyrazine, all of which have demonstrated biocontrol potential (6). Given the advantages of *T. harzianum* and *B. amyloliquefaciens* in plant stimulation and biocontrol, this study aimed to establish a consortium of these species using co-culture technology.

In recent years, only a few studies have explored the co-culturing of *Bacillus* and *Trichoderma* species for the biological control of phytopathogens (7, 8). Although the biocontrol and phytostimulating mechanisms of *T. harzianum and B. amyloliquefaciens* are distinct, both are promising candidates for co-culture technology. Various biosynthetic gene clusters responsible for synthesizing volatile and non-volatile metabolites often remain inactive under standard laboratory conditions. In natural environments, microbes interactions and competition for resources and space trigger the release of these metabolites. By replicating such interactions through co-culture, dormant gene clusters can be activated, potentially producing new antimicrobial compounds that are usually absent in monocultures due to specific microorganism interactions (9, 10).

Thus, co-culture techniques offer a valuable experimental approach for increasing the production and diversity of secondary metabolites. Previous studies have shown that the co-culture fermentation filtrate of *B. subtilis* and *T. atroviride* can reduce the growth of the head blight fungus by 54% *in vitro*, a result attributed to the synergistic production of antifungal compounds like mevastatin and koningin A (11).

Many *in vitro* and *in vivo* studies have explored microbial interactions with plants. However, numerous studies have focused on the synergistic effects without thoroughly examining the underlying mechanisms. For instance, co-culturing *T. asperellum* with *B. amyloliquefaciens* in BP broth (containing 0.3% beef extract and 0.5% peptone) exhibited a more substantial inhibitory effect on *Botrytis cinerea* than when each was applied individually (8).

Similarly, pot experiments showed that a spore suspension of *Trichoderma* spp. combined with *Bacillus* spp. enhanced nutrient uptake and plant growth in beans by promoting root nodule formation (12). This suggests that microbial co-cultures can offer benefits beyond pathogen control, significantly contributing to plant health and productivity.

In wheat, an *in vitro* co-culture of *B. amyloliquefaciens* and *T. asperellum* led to the production of unique compounds such as α-Cuprenene, 1,2-Benzisothiazol-3(2H)-one and L-Proline N-pivaloyl-ethyl ester, which were not present in pure cultures. The application of this co-culture filtrate significantly stimulated plant growth and improved resistance to multiple *Fusarium* strains and *Botrytis cinerea* (7). In a nutshell, these results highlight that co-culture approaches can serve as effective instruments for enhancing both biocontrol and phytostimulation against various phytopathogens .

In this study, *Bacillus* and *Trichoderma* isolates from the rhizosphere were co-cultivated to evaluate their antagonistic effects against the jasmine collar rot pathogen. Additionally, monocultures and co-cultures of these potential biocontrol strains were analyzed to investigate their synergistic interactions in producing novel secondary metabolites. The impact of these interactions was then assessed for their effects on phytostimulation and biocontrol activity in *in planta* conditions.

Materials and Methods

Survey and purification of Pathogen

In 2022 and 2023, a survey was conducted to collect samples from collar rot-infected jasmine plants in major jasmine-growing districts of Tamil Nadu, including Madurai, Theni, Dindugal, Ramanathapuram and Virudhunagar. The collar rot pathogen was isolated from infected plants displaying whitish mycelial growth and multiple sclerotia on the collar region, using the tissue segmentation method (13). The samples were cultured on Petri plates and incubated at 28±2°C for 5 to 10 days. Following isolation, the fungal culture was purified and stored at 4°C for future studies. To assess morphological variability, a 9 mm fungal disc from a 7-day-old culture was inoculated onto PDA plates and incubated for 15 days at 28± 2°C. A pathogenicity test was conducted to evaluate the virulence of all *S. rolfsii* isolates, with the SR(D)-5 isolate showing the highest virulence and an incidence rate of 83% (Accession number: PQ145541)

Isolation and purification of the native bioagents

During the survey, soil samples were collected from the rhizosphere of healthy jasmine plants to isolate *Trichoderma* spp. and *Bacillus* spp. In particular, a *Trichoderma* selective medium (TSM) was used to isolate various *Trichoderma* isolates using the soil dilution technique (14). Following this, general biochemical tests and morphological analysis of conidiophores and the arrangement of phialides were performed to confirm the identification of the *Trichoderma* biocontrol isolates. Bacterial isolates from the rhizospheric soil were originally identified through their culture morphology and subsequently characterized biochemically according to Bergey's Manual of Systematic Bacteriology, which provides comprehensive descriptions of *Bacillus* species, including biochemical tests such as the catalase test, oxidase test and fermentation tests (15).

Molecular Confirmation of Pathogen and Antagonists

For genomic DNA extraction, both the pathogen and antagonist cultures were grown separately in Potato Dextrose Broth (PDB) and Nutrient Broth (NB) at 27±1°C for 7 days. Genomic DNA was extracted using the CTAB method and stored at -20°C for subsequent molecular analysis (16). Specific primers and PCR cycles were employed to amplify the ITS-rDNA (ITS1 & ITS4) and 16S rDNA regions (27F & 1492R), following the standardized protocols (14, 17). Each PCR reaction had a total volume of 10 µl, which included 1 µl of each primer, 1 µl of genomic DNA, 2 µl of sterile Milli-Q water and 5 µl of Master Mix (Eurofin). The extracted genomic DNA was analyzed on a 0.8% agarose gel and visualized with ethidium bromide staining at 120V and 400 mA for 60 minutes. Subsequently, the ITS and 16S rDNA amplicons were sequenced by Biokart Pvt. Ltd. in Bangalore. The sequencing data were trimmed at the 5' and 3' ends, converted to FASTA format and subjected to BLAST analysis at NCBI for homologous sequence identification and phylogenetic analysis. The Neighbor-Joining method was utilized with 1000 replications for each bootstrap value, using MEGA 11.0 software.

In vitro evaluation of rhizosphere antagonists against S. rolfsii SR(D)-5

Dual plate assay: A preliminary screening study was conducted to identify potential antagonists for co-culture by evaluating the antagonistic potency of various *Bacillus* and *Trichoderma* isolates against the virulent *S. rolfsii* isolate, SR(D)-5, which causes collar rot disease in jasmine. Two 9 mm culture discs from seven-day-old isolates and the virulent *S. rolfsii* isolate were placed on opposite sides of the same diagonal line on PDA medium for the *Trichoderma* isolates. Plates without *Trichoderma* served as controls. In a similar setup, a 9 mm culture disc of the five-day-old *S. rolfsii* isolate was placed at one end of a PDA plate, and a bacterial isolate was vertically streaked at the opposite end. Three replications were maintained for each antagonist, with plates lacking bacterial isolates serving as controls. The petri plates were incubated at 28±2°C. The percentage inhibition of radial pathogen growth (PIRG) was calculated after 7 days using the formula

$$I = [(C - T)/C] \times 100 \qquad \text{(Eqn. 01)}$$

where C represents the radial growth measurement of the pathogen in the control plates. T represents the radial growth of the pathogen in the dual plates (18).

Paired plate assay: The paired petri dish technique was performed to assess the impact of volatile metabolites produced by various *Bacillus* and *Trichoderma* isolates on inhibiting pathogen growth. One-week-old *Bacillus and Trichoderma* isolates were inoculated at the center of PDA plates and incubated at 25±2°C for 48 hrs. Subsequently, the upper lids were replaced with lids containing PDA

inoculated with a virulent *S. rolfsii* isolate. The plates were then wrapped with parafilm to trap volatiles. Each treatment was replicated thrice, with plates containing only the pathogen isolate serving as controls. The Petri plates were kept at 28±2°C for 7 days. On the 7th day, the antagonist plates were removed and radial pathogen growth was measured. The results were represented as a percentage inhibition of radial growth (PIRG) over the control (18).

Co-culture of potential antagonists: Based on the *in vitro* results from the dual plate and paired plate assays, the potential *Bacillus* isolate *B. amyloliquefaciens* BC(M)-2 was cultured on NA medium at 28±2°C for 48 hrs. It was then transferred to Nutrient broth and incubated at room temperature (28°C) with constant shaking until a cell concentration of 4x10^8 CFU/ml was achieved (19). Similarly, *Trichoderma harzianum* TR(D)-4 was grown on PDA medium at 28±2°C for 5 days and the spores were harvested to reach a final spore concentration of 2x10^8 spores/ml (8). For co-culture, 1 ml of TR(D)-4 inoculum (10^6 spores/ml) was pre-cultured in 30 ml of YMC medium (composed of 20 g yeast powder, 20 g molasses, and 20 g corn flour per liter, pH 7) at 28°C for 2 days. After 48 hrs of incubation, 100 µl (1.0 OD at 600 nm) of BC(M)-2 was sequentially added to the pre-culture medium and maintained at 180 rpm at 28°C for 5 days. Following incubation, fungal growth was assessed on PDA with antibiotics, while bacterial growth was measured on NA containing cycloheximide and nystatin (7). The growth matrix of TR(D)-4 and BC(M)-2 in both mono and co-culture setups were evaluated by measuring the cell concentration (CFU/ml).

Scanning Electron Microscope (SEM) analysis: To investigate the growth conditions of antagonist microbes in monocultures and co-culture environments within their respective media, we conducted SEM analysis at various magnifications, including 2.0KX, 3.0KX and 4.0KX. Initially, the samples were filtered through a Miracloth (Millipore) filter using vacuum filtration. The filters were washed separately with gradually increasing ethanol concentrations: an overnight bath in 70% ethanol (with the ethanol changed three times), followed by two 30-minute baths in 90% ethanol and a final bath in 100% ethanol. The samples were then dried using the critical point drying method, replacing ethanol with carbon dioxide in an agar scientific chamber (20). After drying, the samples were coated with a 24 nm layer of gold and examined in high vacuum using a TESCAN VEGA3 SBH SEM. The hyphal width and bacterium morphology were observed and images were captured at a magnification of 2.0 KX (Fig. 1).

***In vitro* antagonism assays:** The antifungal effectiveness of monocultures and co-culture filtrates of TR(D)-4 and BC(M)2 in suppressing the growth of *S. rolfsii* SR(D)-5 was evaluated using the poison food technique. After a week of incubation, the fermented culture filtrates were passed through a 0.22µm filter unit and mixed with 100 mL of 10% PD broth. The PD broth lacked any culture filtrate and served as a control. A 9 mm culture disc of the virulent isolate SR(D)-5 was placed into the PD broth poisoned with 10% axenic and co-culture filtrates, respectively and kept at room temperature (28°C) for 7 days. On the 7th day, the wet and dry weights of *S. rolfsii* were measured.

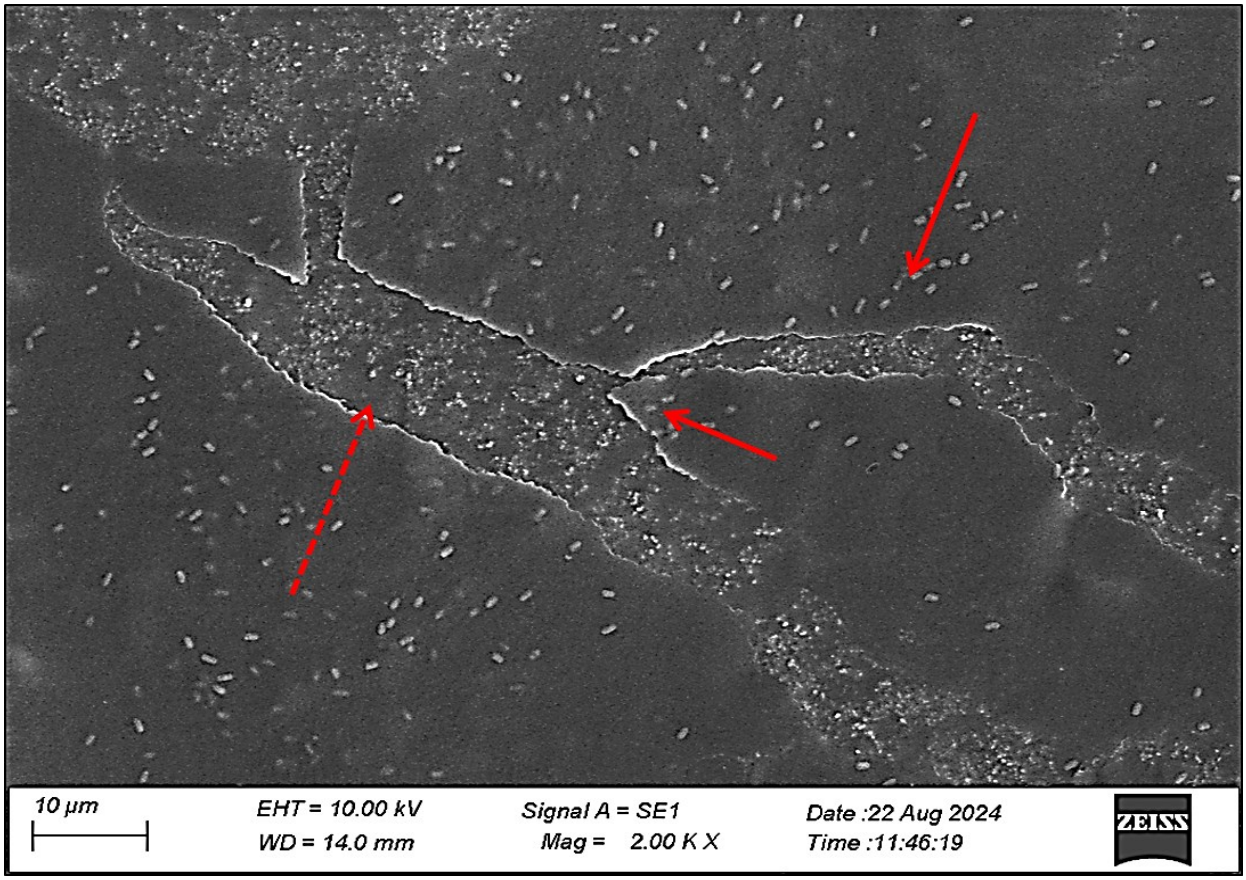

Fig.1. SEM image of BC(M)-2 (full line arrow) colonizing near the TR(D)- 4 mycelium (dotted line arrow) in a 6-day-old co-culture in YMC medium.

Preparation of crude extracts from monocultures and co-culture for GC-MS analysis: Three Conical flasks containing 100 ml of sterile NB and PDB were inoculated with monocultures of BC(M)-2 and TR(D)-4, respectively, and their co-culture in 100 ml of YMC broth. The inoculated flasks were incubated at 26±2°C for 7 days in an orbital shaker at 150 rpm (7). After fermentation, the broth was filtered and centrifuged at 10,000 rpm for 15 minutes. The filtrates were then extracted with ethyl acetate (EA) using a separation funnel in a 1:1 ratio. Subsequently, the extracts were purified using a rotary vacuum evaporator and the final crude extracts in ethyl acetate (EA) were resolved in 2mL of HPLC grade ethyl acetate for bioassay studies and GC-MS analysis.

Biological assay: The crude extracts from the monocultures and co-culture of TR(D)-4 and BC(M)-2 were formulated for secondary screening using a PDA well diffusion assay to confirm the presence of antifungal metabolites. A 9 mm mycelial disc of *S. rolfsii* was placed at the center of a Petri plate. Following this, 100µL of monoculture and co-culture filtrate extracts were added to agar wells positioned 1 cm from the edge at four equidistant points around the perimeter of each Petri plate. The plates were incubated at room temperature and monitored until the mycelium in the control plate, which received sterilized distilled water instead of crude extract, covered the entire surface. Radial growth measurements of the pathogen were taken once the mycelium completely covered the control petri dish.

Characterization of bioactive metabolites by GC-MS

The antifungal compounds present in the final ethyl acetate (EA) crude extracts of monocultures and co-cultures of *T. harzianum* TR(D)-4 and *B. amyloliquefaciens* BC(M)-2 were identified using a Shimadzu Nexis GC-2030 MS - TQ8040 NX system at TNAU, Tamil Nadu, India. The analysis was conducted under the following conditions: an HP-5MS capillary column (30 m x 0.25 mm, 0.25 µm film thickness) was employed. The injection volume was set at 1 µL with a split ratio of 10:1. The oven temperature was programmed to rise from 60°C (held for 2 minutes) to 300°C at a rate of 10° C/min, followed by a final hold for 5 minutes. Helium served as the carrier gas at a constant 1 mL/min flow rate. The ion source temperature was maintained at 230°C and the mass range was scanned from 50 to 550 m/z. Using a computer-driven algorithm, major constituents were identified based on their retention times, peak intensities and mass spectra. The mass spectra obtained from the analysis were compared to those in the National Institute of Standards and Technology (NIST) library (Version 2.0, 2005). The software utilized for Gas Chromatography-Mass Spectrometry (GC-MS) analysis was Turbo Mass 5.1 (21)

In planta biocontrol assay under protected greenhouse conditions

An *in planta* study was conducted on one-month-old Jasmine plants (var. Gundu Malli) using pre- and post-inoculation approaches in soil inoculated with the virulent *S. rolfsii* isolate SR(D)-5 to assess the antagonistic potential of monoculture and co-culture filtrates from *Bacillus amyloliquefaciens* BC(M)-2 and *Trichoderma harzianum* TR (D)-4. The study evaluated the efficacy of these treatments in suppressing *S. rolfsii,* either alone or in combination. The experiment was designed as a completely randomized design (CRD) with eight treatments, including BS, TS and TBS, for both pre and post-inoculation techniques. The details of the treatments are listed in Table 1. Three replications were maintained for each treatment, with five plants per replication.

Table 1. Details of experimental treatments followed in this study

Treatment code	Treatment details
BS	plants inoculated with BC(M)-2 and SR(D)-5
TS	plants inoculated with TR(D)-4 and SR(D)-5
TBS	plants inoculated with TR(D)-4 + BC(M)-2 and SR (D)-5
Inoculated control (S)	control plants inoculated with SR(D)-5 only
Uninoculated control (H)	Control plants without treating the pathogen and bioagents

Experimental design overview: Completely Randomized Design (CRD) with treatments replicated three times, each consisting of five plants per replication

For the experimental setup, the potting soil was prepared by mixing red soil, sand and farmyard manure in a weight ratio 2:1:1. This mixture was sterilized at 121°C and 15 psi for two hours on two consecutive days. To prepare the pathogen inoculum, 1900 g of autoclaved sand was combined with 100 g of maize powder (19:1 ratio), moistened with 400 ml of water per kg, and then packed into polypropylene bags. These bags were sterilized at 1.4 kg/cm^2 for two hours on alternate days. Each bag was inoculated with five 9 mm PDA culture discs of actively growing *Sclerotium rolfsii* SR(D)-5. The bags were then incubated at room temperature (28 ± 2°C) for 15 days to serve as the inoculum source. After this incubation period, the pathogen *S. rolfsii*, multiplied in the sand-maize medium, was mixed at 10 g per pot, excluding the healthy control (H). Bioagents were inoculated using spore suspensions of axenic and co-cultures grown in NB, PDB and YMC medium, respectively. These suspensions were diluted to 1x10^6 conidia/mL with sterile distilled water and applied evenly to the soil. The inoculated control group (S) consisted of plants treated only with the pathogen and sterile water.

Pre-inoculation approach

During transplantation, 30-day-old Jasmine seedlings were placed into pots filled with sterilized media combined with the pathogenic *S. rolfsii* SR(D)-5. Prior to planting, each pot was treated with 100 mL of a spore suspension (1 × 10^6 conidia/mL) derived from the monoculture and co-culture filtrates of BC(M)-2 and TR(D)-4, which were applied separately via soil drenching.

Post-inoculation approach

In the post-inoculation approach, 100 mL of spore suspension (1 × 10^{6}conidia/mL) derived from monocultures and co-culture filtrates of BC(M)-2 and TR(D)-4 were separately applied to the soil as a drench five days after the plants were transplanted into the infected pots.

Thirty days after transplanting (DAT), phytostimulatory and biocontrol factors such as plant height (root and shoot lengths), plant weight (wet and dry weights) and disease incidence (%) were recorded. Disease incidence was calculated using the following formula:

Disease Incidence (%) = (Total number of infected plants / Total number of plants) × 100 (Eqn.02)

Statistical analysis

The experimental data were analyzed using Analysis of Variance (ANOVA) with SPSS version 17.0. A Completely Randomized Design (CRD) was employed for the *in-vitro* investigations. At significant levels of *P*=0.05, the data were subjected to ANOVA and means were compared using Duncan's Multiple Range Test (DMRT). Each treatment was replicated three times to ensure the consistency of the results.

Results

Survey and purification of Pathogen

A survey on the prevalence of collar rot was conducted in the main jasmine-growing districts of Tamil Nadu, India, revealing significant disease pressure. Data were recorded from four randomly marked plots in each field, with each plot covering an average area of ten square meters. In total, 40 fields were surveyed across all five districts, with 8 fields in each district selected based on specific criteria, including a history of disease incidence, geographical diversity and varying cultivation practices to ensure a representative sample across the regions. The infection rate in the surveyed area ranged from 28.00 to 64.00 % (Table 2, Fig. 2). Among the regions surveyed, Salaiputhur village in the Dindugal district recorded the highest incidence of collar rot disease with 64.33%. In comparison, Pudhupatti village in the Theni district reported the lowest incidence at 27.253%. In total, 10 isolates were obtained from the infected collar regions of jasmine plants and identified as *S. rolsii* based on cultural and sclerotial characteristics.

Cultural and sclerotial variability of the Pathogen

All 10 isolates exhibited a distinctive fan-like mycelium growth pattern, characterized by white cultures with either fluffy or compact mycelium with an upright growth habit. The sclerotial characteristics of the isolates included small, round, or spherical shapes with a dull-brown color and peripheral arrangements. Among the 10 isolates, 71.6% formed compact mycelium with dull-brown sclerotia, while 28.4% displayed fluffy mycelium with deep-brown sclerotia. The mycelial growth rates of the ten isolates were evaluated, with the virulent culture SR(D)-5 showing a significant growth rate of 24.00 mm per day, followed by SR(M)-2 at 20.00 mm per day (Fig. 3). The other isolates exhibited growth rates ranging from 16.60 to 18.00 mm daily. There was notable diversity in sclerotia and growth rates among the different surveyed areas.

Table 2. Occurrence of collar rot disease in major jasmine-growing districts of Tamil Nadu

Location	Districts	Isolate code	Geo co-ordinates		Collar rot Incidence* (%)
			Latitude	Longitude	
Thoppulanpatti	Madurai	SR(M)-1	10.41330°N	78.43965°E	51.873^{ab} (46.12)
Manjapatti	Madurai	SR(M)-2	10.06111°N	78.17696°E	39.340^{bcd} (38.84)
Pudhupatti	Theni	SR(T)-3	9.765236°N	77.30287°E	27.253^{d} (30.96)
Usilampatti	Theni	SR(T)-4	9.969382°N	77.78581°E	43.177^{bc} (41.05)
Salaiputhur	Dindugal	SR(D)-5	8.528606°N	77.85299°E	64.330^{a} (53.45)
Kodairoad	Dindugal	SR(D)-6	10.17566°N	77.90675°E	37.96^{bcd} (38.03)
Kariapatti	Virudhunagar	SR(V)-7	9.679759°N	78.10031°E	29.137^{cd} (32.59)
Kalvimadai	Virudhunagar	SR(V)-8	9.737841°N	78.21498°E	37.20^{bcd} (37.56)
Thangachimadam	Ramanathapuram	SR(R)-9	9.283024°N	79.24411°E	28.674^{d} (32.03)
Akkalmadam	Ramanathapuram	SR(R)-10	9.285945°N	79.24534°E	32.150^{cd} (34.54)
		CD (P=0.05)			**8.834**

*The average of five observations was analyzed using Duncan's Multiple Range Test (DMRT) to compare treatment means. The values in parentheses are arcsine transformed. Within each column, means followed by the same letter differ non-significantly at P ≤ 0.05 according to DMRT

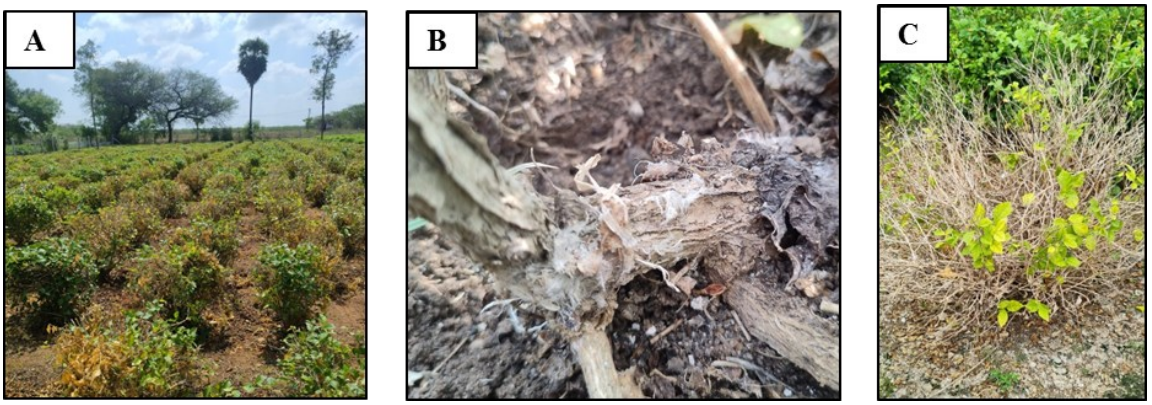

Fig.2. Survey on collar rot incidence in Jasmine: A) Overview of an infected field; B) Whitish mycelium with mustard-seed-like sclerotia on the collar; C) Infected plant showing wilting symptoms.

Isolation and purification of the native bioagents

A total of 20 native rhizospheric soil isolates of *Bacillus* and *Trichoderma* strains (10 of each) were obtained through serial dilution and purified on NA and PDA media, respectively. All *Bacillus* isolates displayed deep creamy white colonies with smooth to sharp edges. Comprehensive biochemical characterization of all 10 *Bacillus* isolates, following Bergey's Manual of Systematic It was observed that there is a positive reactions for growth at 45°C, citrate utilization, catalase activity and Gram staining (14). Similarly, microscopic examination identified all ten fungal antagonists as *Trichoderma* spp., characterized by branched, predominantly paired conidiophores, branched phialides and colored conidia that were slightly ovoidal to globose in shape. Based on an *in vitro* antagonism study, the effective isolate TR(D)-4, which exhibited high antagonistic activity, was identified as *Trichoderma harzianum*. The isolates displayed a tuft of mycelium characterized by branched, predominantly paired conidiophores, branched phialides and hyaline conidia that were slightly ovoid to globose in shape (Fig. 3).

Molecular confirmation of pathogen and antagonists

PCR amplification was performed solely for the virulent pathogen isolate and the potential antagonists to confirm their molecular identities using the ITS and 16S rDNA gene sequences, respectively. Amplification of the virulent isolate SR(D) -5's ITS region produced a 550bp amplicon, which was subsequently sequenced. Further, BLASTn analysis of the ITS sequence revealed a 98% similarity with *Agroatheli arolfsii* isolate IGFRI 1(MT026581) from India. The most effective *Bacillus* and *Trichoderma* isolates were selected and verified using the ITS and 16S rDNA gene sequences, respectively. The amplification of the ITS region of isolate TR(D)-4, produced a 630bp amplicon, which was also sequenced. BLASTn analysis of the ITS region of isolate TR(D)-4 revealed a 99% similarity, identifying it as *Trichoderma harzianum.* Similarly, 16S rDNA gene sequencing of the potential isolate BC(M)-2 was searched in the NCBI database, which showed a 97.50% sequence similarity, confirming it as *B. amyloliquefaciens.* The ITS and 16S gene sequences were registered in the NCBI database under the following accession numbers, *Trichoderma harzianum* TR(D)-4 (PQ305667) and *Bacillus amyloliquefaciens* BC(M)-2 (PQ118388).

In vitro efficacy of various rhizosphere antagonists against SR(5)-D

Dual plate assay: Using dual culture assays, ten rhizosphere antagonist isolates were evaluated against *S. rolfsii* SR(D)-5. The results indicated that all *Bacillus* and *Trichoderma* isolates significantly inhibited radial growth of *S. rolfsii*, with inhibition levels ranging from 35.00% to 72.00% for *Bacillus* isolates and 47.00% to 86.00% for *Trichoderma* isolates (Fig. 3). Among the *Bacillus* isolates, BC(M)-2 demonstrated the highest inhibition at 71.34%, followed by BC(D)-4 at 64.53%. Similarly, *Trichoderma* isolate TR(D)-4 exhibited the strongest inhibition at 86.35%, with TR(M)-2 following at 74.56% (Fig. 4a and Fig. 4b)

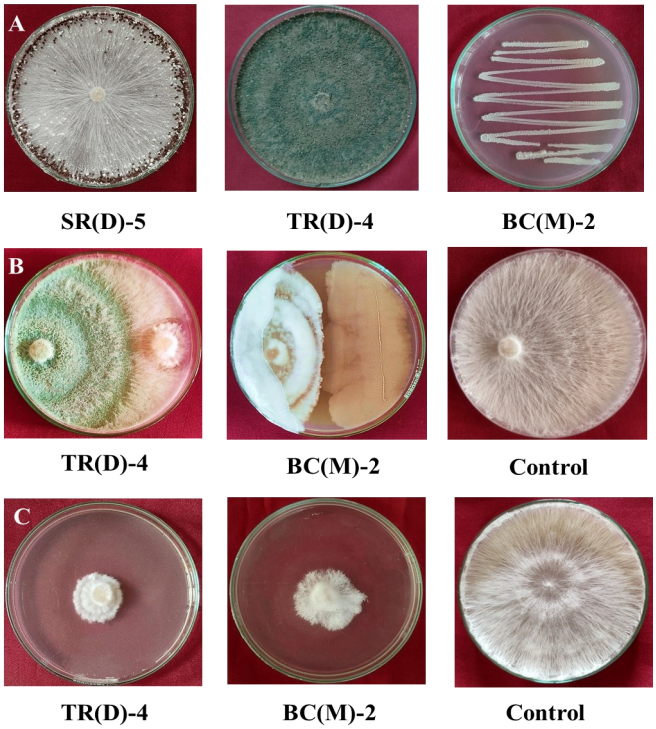

Fig.3. Antifungal efficacy of TR(D)-4 and BC(M)-2 against *S. rolfsii* SR(D)-5: A) Culture plate; B) Dual culture assay; C) Paired plate assay.

Paired plate assay: This study also assessed the ability of Volatile Organic Compounds (VOCs) produced by rhizospheric antagonists to inhibit the radial growth of S.*rolfsii* SR(D)-5 directly using a paired plate assay. The PIRG values indicated that VOCs from all microbial isolates significantly suppressed pathogen growth and induced abnormalities in both mycelial and sclerotial structures (Fig. 3). Among the ten *Bacillus* isolates tested, BC(M)-2 showed the highest inhibition at 78.27%, followed by BC(D)-4 with a 68.36% reduction compared to the control. Among the fungal antagonists, *Trichoderma* isolate TR(D)-4 demonstrated the highest inhibition, with a 75.43% reduction in pathogen radial growth, followed by TR(M)-2 at 68.56%, relative to the control (Fig. 4a and Fig. 4b).

Quantification of *Trichoderma* and *Bacillus* growth in co-culture: Table 3 illustrates the growth dynamics of *Trichoderma harzianum* TR(D)-4 and *Bacillus amyloliquefaciens* BC(M)-2 at various time points (48, 72, 96 and 120 hours) under monoculture and co-culture conditions in their respective media. Optimal cell concentrations of BC (M)-2 and TR(D)-4 were recorded periodically after 24 hours of inoculation in the co-culture setup. Colony counts of *Trichoderma* and *Bacillus* increased steadily within the first 96 hours, reaching concentrations of 7 x 10^8 and 8 x 10^9 cells/ mL, respectively. By the fifth day, *Trichoderma* and *Bacillus* cell concentrations in co-culture rose tenfold, reaching 7 x 10^9 and 2 x 10^11 cells/mL, respectively. Compared to monoculture, *Trichoderma* and *Bacillus* cell concentrations in co-culture showed increases of over 35-fold and 50-fold, respectively. The synergistic effects observed in co-culture facilitated balanced growth, allowing both microorganisms to mutually benefit from shared resources and metabolic exchanges, leading to stabilized and potentially enhanced growth rates.

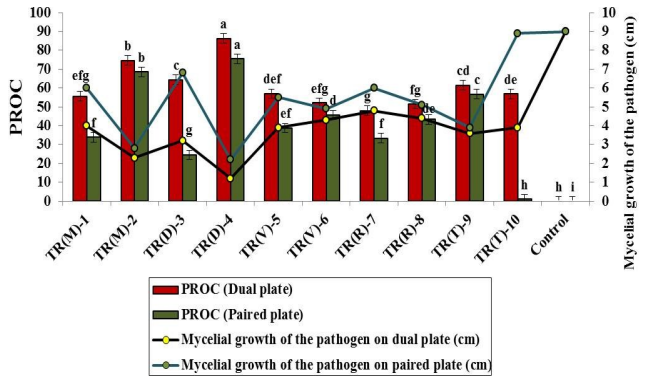

Fig.4a. Antifungal potency of rhizosphere *Trichoderma* spp. against *S. rolfsii* SR(D)-5 via dual and paired plate assays. Values are the mean of three replicates with standard deviation. Treatment means were compared at the 5% significance level using Duncan's Multiple Range Test. PROC indicates a Percentage Reduction Over Control.

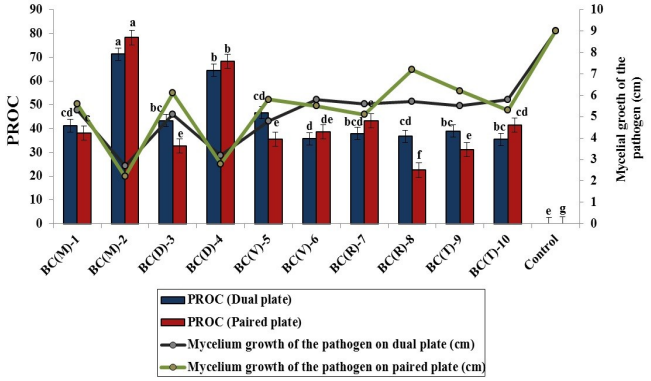

Fig.4b. Antifungal Inhibitory Activity of Rhizospheric *Bacillus* spp. against *S. rolfsii* SR(D)-5 via Dual Plate and Paired Plate Assays. Values represent the mean of three replicates (± SD). Treatment means were compared at the 5% significance level using Duncan's Multiple Range Test. PROC indicates a Percentage Reduction Over Control.

Table 3. Growth rates of BC(M)-2 and TR(D)-4under monoculture and co-culture setup

Culture	Days After Inoculation (DAI)							
	Trichoderma (CFU)				Bacillus (CFU)			
	48 h	72 h	96 h	120 h	48 h	72 h	96 h	120 h
BC(M)-2	-	-	-	-	1×10^5	4×10^6	2×10^7	3.5×10^8
TR(D)-4	2×10^6	3.5×10^6	4×10^7	2×10^8	-	-	-	-
BC(M)-2+ TR(D)-4	12×10^6	8×10^7	7×10^8	7×10^9	2×10^6	5×10^7	8×10^9	2×10^{11}

Value are means of three replicates for each experiments; CFU - Colony Forming Units

Co-culture of T. harzianum TR(D)-4 and B. amyloliquefaciens BC(M)-2 showed improved antifungal activity

The antifungal potency of culture filtrates from monocultures and a co-culture of *Bacillus amyloliquefaciens* BC(M)-2 and *Trichoderma harzianum* TR(D)-4 was assessed against the virulent isolate *S.rolfsii* SR(D)-5 using the poisoned food method. The co-culture filtrate of BC(M)-2 and TR(D)-4 significantly reduced the wet mycelial weight to 1.02 g and the dry mycelial weight to 0.11 g. Conversely, the monoculture filtrate of TR(D)-4 resulted in wet and dry mycelial weights of 2.55 g and 0.43 g, respectively. The highest mycelial wet weight (4.86 g) and dry weight (0.75 g) were recorded in the control (Fig. 5). The enhanced antifungal activity observed in the co-culture filtrate is attributed to the synergistic interactions between *Trichoderma* and *Bacillus* species. In co-culture, these microorganisms generate a wider array of antimicrobial substances, encompassing volatile organic compounds (VOCs) and non-volatile metabolites, via metabolic crosstalk. This collaboration stimulates the production of secondary metabolites not expressed in monoculture, thereby increasing the spectrum and antagonistic efficacy of these biocontrol agents.

Secondary screening of antifungal potency

The antifungal potency of the ethyl acetate (EA) fraction from both monocultures and co-cultures was further evaluated against the virulent *S. rolfsii* isolate SR(D)-5 using the PDA well diffusion method to confirm the antagonistic activity of bioactive metabolites. The EA filtrate from the co-culture achieved the highest inhibition of *S. rolfsii* at 86%. In comparison, the monoculture fractions from TR(D)-4 and BC (M)-2 showed inhibition rates of 72.22% and 66.45%, respectively, relative to the control. These findings confirm the presence of unique secondary metabolites in the co-culture EA fraction that were either absent or present in lower concentrations in the monoculture EA fractions. This enhanced production and diversity of metabolites likely explains the increased antifungal activity observed in the co-culture EA fraction, which was further analyzed through GC-MS.

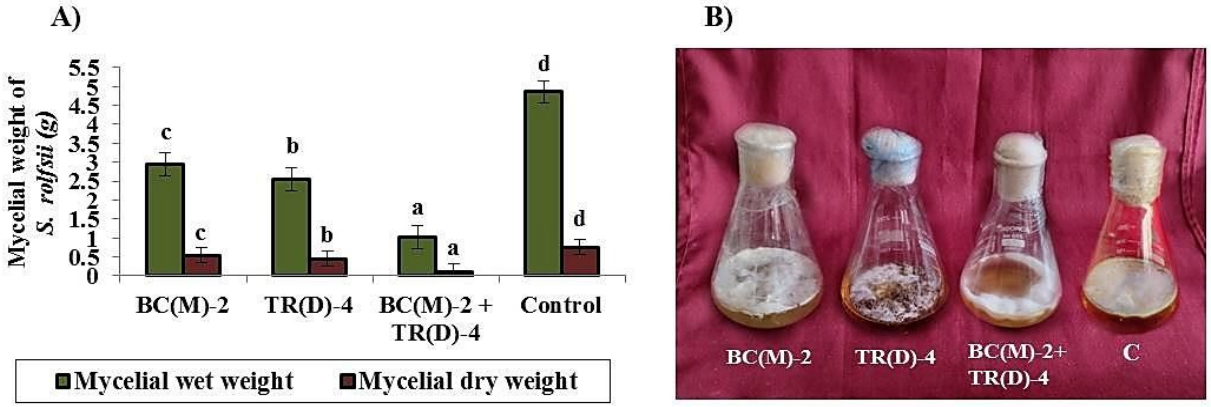

Fig.5. Antifungal activity of co-culture filtrates against *S. rolfsii* SR(D)-5 via poison food assay. A) Mean values from three replicates (± SD); analyzed at 5% significance using Duncan's Test. B) Comparison of axenic and co-culture filtrates of BC(M)-2 and TR(D)-4, with mycelial weights recorded after one week. C) Control.

Comparative analysis of differential compounds produced under monoculture and co-culture environment

GC-MS analysis suggested significant differences in the release of secondary metabolites between monoculture and co-culture setups (Supplementary Fig. 1). The crude EA extracts from axenic cultures of *B. amyloliquefaciens* BC(M)-2 and *T. harzianum* TR(D)-4 contained a range of predominant antifungal compounds. The antagonistic properties of these extracts may be attributed to various chemical classes, including alcohols, ketones, aromatic hydrocarbons, amines, alkenes, alkanes, peptides and fatty acids. The enriched metabolic pathways associated with these compounds, identified via GC-MS analysis, were mapped using MetaboAnalyst software 6.0.

In the crude extracts from axenic cultures of TR(D)-4 and BC(M)-2, 25 and 30 major antimicrobial compounds were identified, respectively, based on their retention time (RT) values and peak area percentages, as detailed in Supplementary Table 1. Both axenic cultures showed a notable presence of hydrocarbons and acids, including dodecane, hexadecane, eicosane, octadecane, heneicosane, tetradecane, docosane, heptadecane, hexacosane, pentadecane, octadecenoic acid and pentadecanoic acid. The GC-MS analysis of *Bacillus amyloliquefaciens* BC(M)-2 revealed metabolic pathways linked to glycerolipid metabolism, fatty acid biosynthesis and tryptophan (Supplementary Fig. 2). Similarly, metabolic pathways associated with secondary metabolites identified in *Trichoderma harzianum* TR(D)-4 include fatty acid biosynthesis, glycerolipid metabolism, cysteine metabolism, fatty acid metabolism, pyruvate metabolism and amino sugar metabolism (Supplementary Fig. 3).

Interestingly, several antimicrobial metabolites from various functional groups-including alcohols, ketones, terpenes, and acids-were identified exclusively in the co-culture setup and not in the monocultures. These unique compounds include 2-bromotetradecane, dimethyl disulfide, isopropyl myristate, Bis (2-ethylhexyl) phthalate, carbonic acid, eicosyl vinyl ester, tridecanol, 2,3-butanediol, 3-methyl butanoic acid, oxalic acid, α-bisabolol, hentriacontane, tetratriacontane, harziandione and 1-naphthalene sulfonic acid (Supplementary Table 1). Among these, notable compounds with significant antifungal activity include alpha-bisabolol (AB), bis(2-ethylhexyl) phthalate and harziandione, which were exclusively present in the co-culture setup. These compounds exhibit strong antifungal properties, each contributing uniquely to the inhibition of fungal cell membranes by disrupting ergosterol biosynthesis and interfering with the metabolic processes of various plant pathogens. Exclusive metabolic pathways related to these specific compounds in the co-culture of BC(M)-2 and TR(D)-4 include glycerolipid metabolism, tryptophan metabolism, glutamate metabolism, pyrimidine metabolism, pyruvate metabolism and fatty acid metabolism (Fig. 6). The heat map clearly illustrates the presence, absence, up-regulation and down-regulation of these metabolites (Fig. 7).

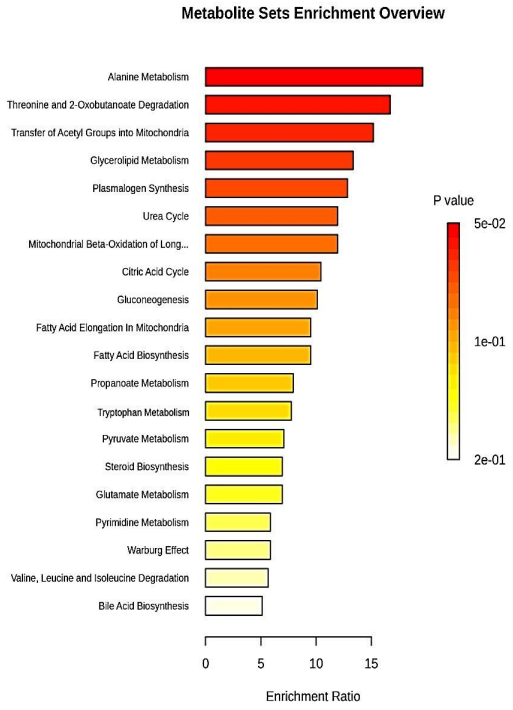

Fig.6. Metabolic pathways identified from the GC-MS analysis of the co-culture of TR(D)-4 and BC(M)-2.

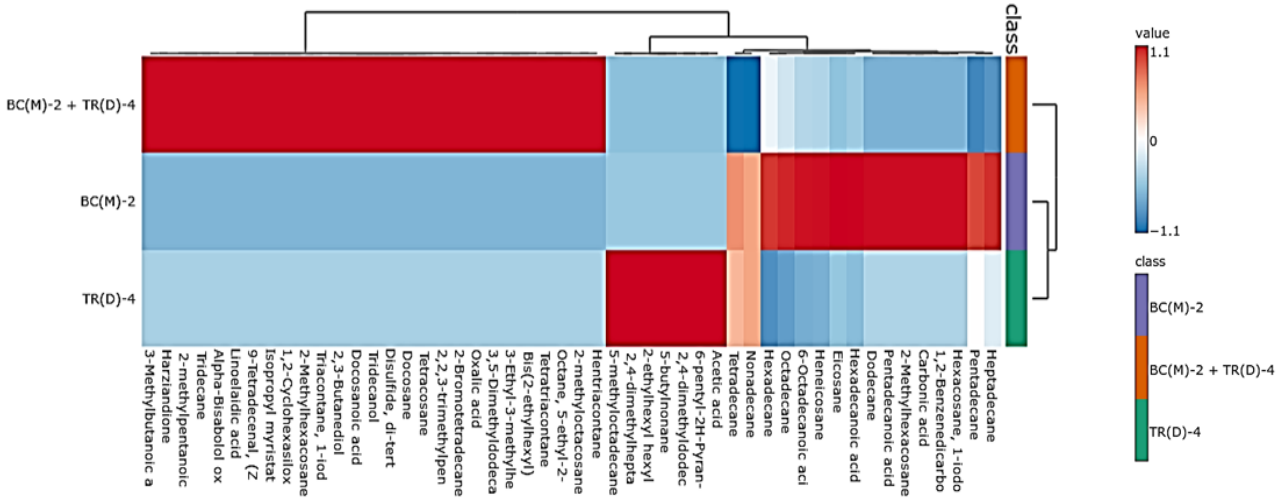

Fig.7. Heatmap analysis of metabolites produced under monoculture and co-culture conditions. The heatmap illustrates the presence and absence of secondary metabolites from the interaction between TR(D)-4 and BC(M)-2, generated using Metabo Analyst software.

In planta assay under greenhouse conditions

A greenhouse pot assay was conducted to evaluate the biocontrol efficacy of *B. amyloliquefaciens* BC(M)-2 and *T. harzianum* TR(D)-4, both as monocultures and co-cultures, against the jasmine collar rot pathogen. The experiment followed a completely randomized design, with disease incidence (%) recorded for pre-and post-inoculation treatments. Results indicated that the TBS treatment group (co-culture) significantly reduced *S. rolfsii* disease incidence compared to the TS and BS treatment groups (monocultures) during pre- and post-inoculation treatments.

In the pre-inoculation approach, disease incidence in the BS and TS monoculture treatment groups showed the highest rates at 25.65% and 34.65%, respectively. By contrast, the co-culture treatment group (TBS) with both antagonistic agents demonstrated a lower disease incidence of 12.01%, with fewer symptoms than the pathogen-only inoculated control group (S). Similar results were observed in the post-inoculation phase using monoculture and co-culture filtrates against *S. rolfsii*. The BS and TS monoculture treatment groups demonstrated the highest disease incidences at 29.70% and 38.32%, respectively, while the co-culture treatment group (TBS) incorporating biocontrol agents showed a diminished disease occurrence of 18.32%, alongside fewer symptoms relative to the pathogen-inoculated control group (Table 4 and Fig. 8).

In addition to disease control, the phytostimulatory effects of both monoculture and co-culture treatments were evaluated by measuring plant growth parameters, including root and shoot lengths and plant weight (wet & dry weights). The co-culture treatment (TBS) exhibited superior growth results, with a shoot length of 38.48 cm and a root length of 17.43 cm, significantly higher than those observed in the monoculture treatments (BS and TS). Similarly, the wet and dry weights of plants in the co-culture treatment were 235g and 63g, respectively, indicating better performance than the monoculture groups (Table 4 and Fig. 8).

The low disease occurrence recorded in the TBS treatment group may be attributed to the synergistic effect of BC(M)-2 and TR(D)-4, which enhanced the production of potent secondary metabolites effective against *S. rolfsii*. This treatment group also showed the highest plant weight and height in jasmine, likely due to the novel growth-promoting metabolites produced through their synergistic interaction.

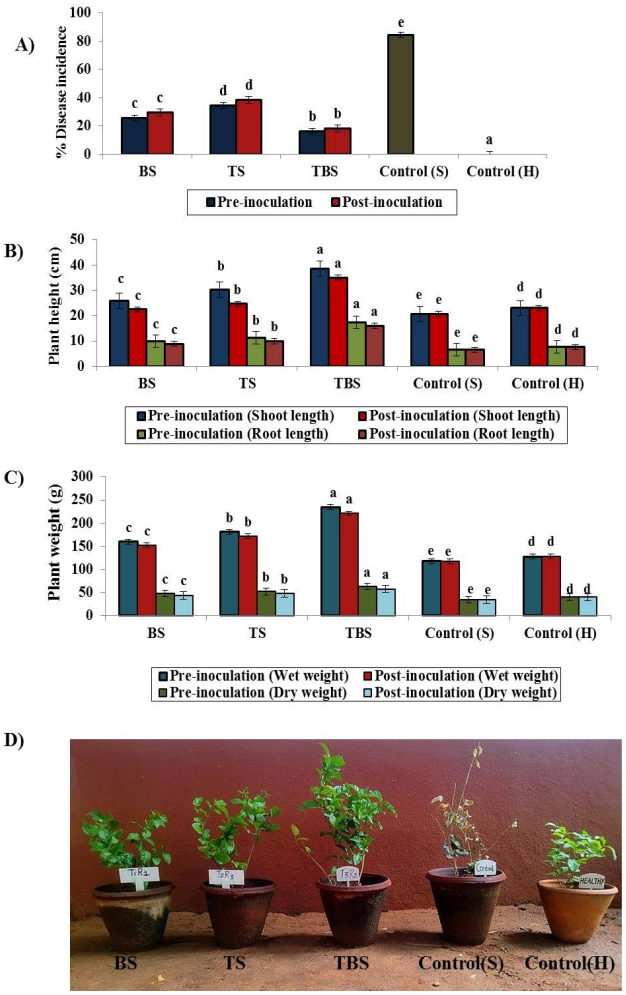

Fig.8. Impact of monocultures and co-culture of TR(D)-4 and BC(M)-2 on plant growth and biological control of *S. rolfsii* in a protected greenhouse environment using pre- and post-inoculation approaches. (A) Disease incidence of *S. rolfsii* in control and treated groups. (B) Shoot and root lengths. (C) Wet and dry weights of jasmine plants. (D) Effects of treatments: BS - BC(M)-2 + SR(D)-5, TS - TR(D)-4 + SR(D)-5, TBS - TR(D)-4 + BC(M)-2 + SR(D)-5, S (inoculated control), H (uninoculated control). Values are means of three replicates; means followed by the same letter are not significantly different at P ≤ 0.05 (DMRT).

Table 4. Impact of co- cultivation of BC(M)-2 and TR(D)-4 on the height, weight and disease incidence of jasmine plants, under protected pot experiments

Pre-inoculation approach					
Treatments	**Disease incidence (%)**	**shoot length (cm)**	**root length (cm)**	**wet weight (g)**	**dry weight (g)**
BS	25.65 ± 1.00^{c}	27.92 ± 0.72^{c}	9.83 ± 0.17^{c}	160 ± 2.00^{c}	48 ± 1.00^{c}
TS	34.65 ± 0.57^{d}	31.18 ± 1.04^{b}	11.22 ± 0.75^{b}	181 ± 2.00^{b}	52 ± 1.52^{b}
TBS	12.01 ± 0.32^{b}	38.48 ± 0.611^{a}	17.43 ± 0.58^{a}	235 ± 3.50^{a}	63 ± 1.00^{a}
Post-inoculation approach					
Treatments	**Disease incidence (%)**	**shoot length (cm)**	**root length (cm)**	**wet weight (g)**	**dry weight (g)**
BS	29.65 ± 1.49^{c}	25.48 ± 1.11^{c}	8.88 ± 0.92^{c}	152 ± 2.25^{c}	44 ± 0.80^{c}
TS	38.32 ± 0.95^{d}	28.68 ± 1.13^{b}	9.51 ± 0.61^{b}	172 ± 2.00^{b}	48 ± 1.50^{b}
TBS	18.32 ± 0.96^{b}	35.06 ± 0.74^{a}	15.98 ± 0.39^{a}	221 ± 2.50^{a}	58 ± 1.00^{a}
Inoculated control (S)	84.42 ± 1.42^{e}	20.65 ± 1.00^{e}	6.55 ± 0.33^{e}	118 ± 5.68^{e}	35 ± 3.45^{e}
Uninoculated control (H)	0.00^{a}	23.01 ± 0.89^{d}	7.60 ± 0.70^{d}	128 ± 2.00^{d}	40 ± 0.90^{d}

Results represent the means of three replicates for each treatment, with the values indicating the standard error of the mean. According to ANOVA, superscripts that differ within the same column signify significant differences (P < 0.05).

Discussion

Co-culture methods are widely employed to investigate microbial interactions. Numerous studies have demonstrated that co-inoculations of *Trichoderma* and bacteria can significantly reduce disease incidence, achieving up to 97% reductions against bacterial pathogens (22) and 70-90% against fungal and oomycete pathogens (23). This approach can also enhance the production of novel antimicrobial metabolites by activating previously dormant genes (24). Previous research on the co-culture of the fungal antagonist *T. asperellum* and the bacterial antagonist *B. amyloliquefaciens* has shown effective inhibition of *Botrytis cinerea* and *Fusarium graminearum* (7, 8). Therefore, in this study, we conducted co-cultures of potential rhizosphere biocontrol strains to evaluate their synergistic interactions in producing novel bioactive metabolites. Additionally, we assessed the effects of these interactions on phytostimulation and biocontrol efficacy under in planta conditions.

In the present study, ten *Sclerotium rolfsii* isolates were purified using a PDA medium. Additionally, morphological and sclerotial characteristics were examined, confirming the isolates as *S. rolfsii*. This pathogen is known to produce either white fluffy or compact mycelium, with variations in sclerotial production, size and shape (25). The morphological variability of the jasmine collar rot pathogen observed in this study aligns with a previous report which noted similar variability in *S. rolfsii* isolates. Their study described *S. rolfsii* as producing silky white to pure white mycelium, accompanied by a peripheral arrangement of brown, round to spherical sclerotia (26).

Conventional morphological methods alone may not always yield accurate identification of fungal isolates. Therefore, integrating morphological techniques with molecular methods is essential for more precise identification (27). The current study confirmed the virulent isolate SR(D)-5 as *Sclerotium rolfsii* through molecular analysis using ITS1-ITS4 primers. These results are consistent with previous research, which confirmed various isolates of *S. rolfsii* based on culture and sclerotial characteristics, as well as ITS sequence-based homology with GenBank isolates (28). Furthermore, *S. rolfsii* was confirmed through a combined phylogenetic evolutionary analysis of ITS-1 sequences from worldwide isolates (26). Additionally, PCR amplification of the potential *Trichoderma* isolate TR(D)-4 and the *Bacillus* isolate BC(M)-2, targeting the ITS and 16S rDNA regions, confirmed their identities as *T. harzianum* and *B. amyloliquefaciens*, respectively, through sequencing and BLAST analysis. This approach aligns with the findings of another study which confirmed the morphological identification of *T. asperellum* by amplifying the ITS region to produce a 600 bp fragment (29). Similarly, molecular tools were employed to identify *Bacillus* isolates, confirming *B. amyloliquefaciens* using the 16S rDNA region with primers 27F and 1492R (30).

In dual plate assay, the antagonistic potency of *T. harzianum TR(D)-4* and *B. amyloliquifencis* BC(M)-2 against the virulent isolate of *S. rolfsii* SR(D)-5 was confirmed, with maximum mycelial inhibition percentages of 86.35 % and 71.34 %, respectively, compared to the control. These findings correlate well with those of other research groups. The mycoparasitism mechanism of *T. koningii* exhibited high antifungal activity achieving an 85% inhibition rate against *S. rolfsii*, followed by *T. harzianum* with 72%, effectively reducing sclerotia germination and production compared to the control (31). Similarly, a *Trichoderma* isolate (TR03), closely related to *T. harzianum*, demonstrated maximum antifungal activity, showcasing a growth inhibition of 63.35% against *S. rolfsii* (32).

Additionally, the antifungal potency of volatile compounds was demonstrated in an in vitro study, where TR (D)-4 and BC(M)-2 showed reductions of 75.43% and 78.27%, respectively. Our observations revealed that as the incubation period extended, pathogen growth normalized due to a decrease in the release of volatile compounds. Exposure of *S. rolfsii* to TR(D)-4 and BC(M)-2 resulted in inhibition and the production of smaller sclerotia compared to those on control plates. However, once the mVOCs were removed, the sclerotia regained their germination ability, indicating that the mVOCs function as fungistatic agents rather than fungicidal. These results align with findings of a previous study which reported that *T. reesei*, *T. longibrachiatum* and *T. harzianum* significantly inhibited the establishment of sclerotia from two isolates of *S. rolfsii* (33). Similarly, another study found that the *B. amyloliquefaciens* HSE-12 strain effectively inhibited the radial growth of *S. rolfsii* by releasing volatile compounds (34). These findings indicate that the observed reduction in pathogen growth and sclerotial size is due to the antifungal properties of the volatile compounds produced by various *Trichoderma* species, which likely induce mycolysis and protoplasm loss. This weakens the cell walls by thinning and removing melanin, making the fungi more susceptible to cell wall lysis enzymes. This disruption of cell wall structure contributes to the observed atypical growth patterns (35).

At this point, only a limited number of studies have shown that co-cultures of *Trichoderma* and bacterial bioagents synergistically augment the synthesis of diffusible metabolites with biocontrol efficacy against significant agricultural diseases (8). In this study, the co-culture of *Bacillus amyloliquefaciens* BC(M)-2 and *Trichoderma harzianum* TR(D)-4 significantly enhanced antifungal activity, suggesting that the synergistic interactions between these microbes promote the production of novel antifungal compounds. This finding is supported by previous research, which showed that co-culture filtrates of *T. asperellum* and *B. amyloliquefaciens* demonstrated significant antagonistic effects against pathogens (7). Similarly, co-culture filtrates of the fungal antagonist *T. asperellum* and the bacterial antagonist *B. amyloliquefaciens* were reported to completely inhibit the radial growth of *B. cinerea* (8).

Volatile and diffusible metabolites primarily drive the interactions between *Trichoderma* species and other organisms. Notably, secondary bioactive metabolites play a crucial role in plant defense, with volatile compounds significantly contributing to antifungal activity (36). In our study, GC-MS analysis identified 73 compounds, revealing significant variations between axenic and co-cultured

conditions. A key volatile metabolite, 6-pentyl-2H-pyran-2-one (6-PP), recognized for its coconut-like odor, was found in monocultures and co-culture extracts of *T. harzianum* TR(D)-4. This compound has been shown to enhance plant growth and stimulate lateral root proliferation (37, 38). In addition, the sole identification of α-cuprenene, a precursor of sesquiterpene quinones, in the co-culture indicates its function in enhancing terpene production in that setting .

Prominent volatile metabolites, such as hexadecanoic acid and 6-octadecanoic acid, were detected in monoculture and co-culture systems of *B. amyloliquefaciens* BC(M)-2, indicating the release of volatile compounds. These compounds have been shown to suppress the growth of phytopathogens by forming complex compounds with active groups in fungal cell walls, explicitly reacting with the outer carboxyl group (COOH) rather than damaging the main chitin structure. Consequently, this partial reaction compromises the integrity of the fungal cell wall without being lethal, inhibiting fungal growth (39, 40). Consistent with these findings, our study identified several novel antifungal compounds in the co-culture system, including alcohols, alkanes, esters, alkenes, ketones, and terpenoids. Among the compounds exhibiting significant variability, those associated with biological control, such as alpha-bisabolol (AB), bis(2-ethylhexyl) phthalate and harziandione, are exclusively present in the co-culture setup.

Alpha-bisabolol (AB) is a sesquiterpene alcohol recognized for its antifungal properties, which include inhibiting fungal cell membranes by disrupting ergosterol biosynthesis (41). In the present GC-MS study, α-bisabolol was detected exclusively in the co-culture system, indicating its potential role in the observed antagonistic activity against *S. rolfsii*. Previous studies have demonstrated that alpha-bisabolol effectively inhibits the viability of conidia from various fungal species, including *Aspergillus terreus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium solani*, *F. oxysporum* and *F. verticillioides* in in vitro fungicidal tests. Similarly, a researcher identified alpha-bisabolol in *Bacillus amyloliquefaciens* through GC-MS analysis and reported its antifungal activity against *Phytophthora cinnamomi* (30).

Another potent antifungal compound, Bis (2-ethylhexyl) phthalate, abundantly present in our co-culture system, has been reported for its antimicrobial properties (42). Specifically, *in vitro* studies have shown that Bis(2-ethylhexyl) phthalate exhibits antimicrobial activity against gram-positive bacteria and pathogenic fungi (30). The presence of α -bisabolol and Bis(2-ethylhexyl) phthalate in the co-culture set-up likely enhanced the enhanced antagonistic activity of *B.amyloliquefaciens* when co-cultivated with *T. harzianum*.

Moreover, we identified harziandione, a unique tetracyclic diterpene, as a key antifungal metabolite produced exclusively during the co-culture of *T. harzianum* with *B. amyloliquefaciens*. Harziandione, a type of harziane diterpene, is known for its potent antagonistic activity against *Sclerotium rolfsii* (43, 44). To date, 44 harziane diterpenes have been identified, predominantly from *Trichoderma* species, except heteroscyphsic acid-A found in the Chinese liverwort. These diterpenes are recognized for their diverse biological activities, including phytotoxic, antibacterial and algicidal effects (45). The presence of harziandione in the co-culture setup likely enhances the enhanced antagonistic activity of *T. harzianum* when co-cultured with *B. amyloliquefaciens.*

In a co-culture condition, tryptophan, glutamate, and glycerolipid metabolism play crucial roles in biocontrol and phytostimulation activities, particularly against *S. rolfsii.* Glutamate is a precursor for defensive metabolites such as lytic enzymes and siderophores, which inhibit *S. rolfsii* growth by degrading its cell walls. Tryptophan metabolism produces auxin-related metabolites, including indole-3-acetic acid (IAA), which promote plant growth (46). Glycerolipid metabolism leads to the synthesis of lipopeptides and antibiotics like iturin and surfactin, both of which exhibit strong antifungal properties. Additionally, *Bacillus* species inhibit pathogen growth by producing purine and pyrimidine analogs that disrupt nucleic acid synthesis. Collectively, these metabolic pathways enhance the effectiveness of co-culture in combating *S. rolfsii*, underscoring their significance in biocontrol strategies for managing important agricultural diseases.

An *in planta* study using the jasmine variety Gundi malli assessed the antagonistic and phytostimulatory efficacy of mono- and co-culture treatments under greenhouse conditions. The co-culture treatment (TBS) significantly reduced disease incidence and enhanced plant growth compared to the monocultures (TS and BS) in pre-and post-inoculation approaches. These findings are consistent with a previous report which demonstrates that consortia of biocontrol microbes, such as *B. subtilis* and *T. harzianum*, can synergistically promote plant growth and yield across various crops (47). Similarly, co-culture filtrates of *T. asperellum* and *B. amyloliquefaciens* significantly enhanced phytostimulatory and antagonistic activities against *Fusarium oxysporum* and *Fusarium graminearum* (6). Furthermore, applying consortia of *T. atroviride* with *Bacillus* spp. and *Pseudomonas* spp. under greenhouse conditions increased banana plant biomass by 37%, outperforming individual treatments (48).

Overall, the increased plant growth and the observed decrease in disease occurrence in the co-culture treatment groups can be attributed to the synergistic actions of *B. amyloliquefaciens* BC(M)-2 and *T. harzianum* TR(D)-4, which may occur through both direct and indirect mechanisms. Directly, the application of the fungal biocontrol agent *T. harzianum* and the bacterial biocontrol agent *B. amyloliquefaciens* as cell suspensions resulted in a significant increase in plant growth. This enhancement was facilitated by the increased production of novel antifungal and phytostimulating metabolites, which collectively suppressed pathogen growth. Indirectly, the combined application of *Trichoderma* and *Bacillus* may alter the rhizospheric microbial community, enhancing the diversity and abundance of beneficial microorganisms that further suppress plant pathogens.

Conclusion

The present study underscores the significant impact of *S. rolfsii* on jasmine cultivation, highlighting variations in disease prevalence and severity. Native bioagents such as *B. amyloliquefaciens* BC(M)-2 and *T. harzianum*TR(D)-4 effectively suppressed the radial growth of the jasmine collar rot pathogen by 71.34% and 86.35 %, respectively. Their co-culture synergistically enhanced the production of novel antifungal and phytostiumlating metabolites. Furthermore, greenhouse studies confirmed that disease incidence was significantly reduced and plant growth was markedly improved in plants treated with co-culture spore suspensions. The increased production of antifungal metabolites and enhanced plant growth suggest that this co-culture approach has potential as a next-generation biofertilizer and biopesticide. Moreover, scaling up this technology in fermenters and conducting field trials will facilitate its adoption in the agricultural sector.

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Authors' contributions

AKA and YI conceived the idea and wrote the manuscript. YI gave the ideas and AKA designed the diagrams and tables. RN revised the manuscript. YI, RN, CP and MPKS finalized the manuscript. All authors read and approved the final manuscript.

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While preparing this manuscript, the authors used Grammarly to improve the language and readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the publication's content.

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