DOI Link: https://doi.org/10.61586/2QjXR
Vol.43, Issue.5, Part.1, May 2025, PP. 120-142

BIOCONTROL POTENTIAL OF *BACILLUS PARAMYCOIDES* FOR MANAGEMENT OF GREEN MOLD OF AGARICUS *BISPORUS CAUSED BY TRICHODERMA SPP.*

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Received Jan 2025 Accepted April 2025 Published May 2025

ABSTRACT

Trichoderma spp. are significant fungal contaminants causing green mold in *Agaricus bisporus*, which reduce yield and quality. This study, conducted at the Prof. TNA Innovation Research Centre (TNAIRC), Jiblakpally, Greater Hyderabad from 2022 to 2024, aimed to identify an effective biocontrol agent for managing *Trichoderma*-induced green mold. In dual culture assay, out of 25 bacterial treatments (19 isolated from rhizospheric soil and six from the culture bank of TNAIRC), 11 cultures inhibited growth of at least one species of *Trichoderma* (2.7-73.3%). These 11 cultures reduced dry biomass of test fungi also ranging from 10.1 to 82.6%. Out of top 5 promising cultures were chosen for VOC production and field efficacy. In VOC studies, *Bacillus* isolate V37 was most effective in inhibiting spore germination of *Trichoderma* spp. (0.3-8.7%). In field efficacy studies, bags treated with *Bacillus* isolate V37 showed lowest incidence of green mold (2.4-4.6%), than control (16-25%). Isolate V37 was identified as *Bacillus paramycoides* PP757924.The intracellular extract of *B. paramycoides* demonstrated the highest antifungal activity against *T. asperellum* (21.69 mm), while the extracellular extract was most effective against *T. viride* (22.51 mm) and *T. harzianum* (19.96 mm). GC-MS analysis of intra- and extra-cellular extracts of *B. paramycoides* showed 19 and 27 secondary metabolites, respectively. As far as we are aware, this is the first publication to show that *B. paramycoides* has the ability to biocontrol *Trichoderma spp*.

KEYWORDS

Trichoderma spp. Green mold. *A. bisporus*. *Bacillus paramycoides*. Biocontrol.

INTRODUCTION

Trichoderma, is a free-living fungus which widely used for managing phytopathogens and insect-pests and also as a bio-stimulant (Tariq Javeed et al. 2021). However, some strains have been reported to cause green mold on mushrooms leading to yield and quality losses (Allaga et al. 2021). Over thirty *Trichoderma* species have been identified to infect mushroom substrates and fruiting bodies, causing green mold disease, alongside *Ganoderma lingzhi*, *Pleurotus ostreatus*, and *Lentinula edodes* (Altaf et al. 2022; Kredics et al. 2022; Colavolpe et al. 2014; Hatvani et al. 2007). *Bacillus* species, known for their antimicrobial properties, effectively reduce *Trichoderma* infestations (Rocha et al. 2017). For instance, *B. velezensis* inhibits *T. aggressiveum* growth by producing lipopetides, surfactin, and fengycin (Pandin et al. 2018). Similarly, *B. subtilis* inhibited growth of *T. pleuroti* by 54-62% (Potočnik et al. 2019).

Limited research has been conducted to evaluate potential biocontrol agents against *Trichoderma* spp. that infect mushrooms (Stanojević et al. 2019). To address this gap, 19 isolates of *Bacillus* (B4, B7, B21, B22, B28, B32, B35, B36, B37, B43, B53, B62, B65, B73, B79, B81, B85, B88, B98) with proven biocontrol potential (Jyothi et al. 2023), one isolate of *Bacillus* sp. V37, *B. subtilis*, *B. amyloliquefaciens*, *P. fluorescens*, *B. clausii*, and *B. polymyxa* they were evaluated for their ability to counteract infecting mushrooms.

MATERIALS AND METHODS

Culture source

The current study was carried out during 2022-2024 at the Prof. TNAIRC, Varsha Bioscience and Technology India Pvt Ltd., Jiblakpally, Greater Hyderabad, 508284, India. Bacterial strains were maintained on standard nutrient agar (NA) plates and the Potato Dextrose Agar was used for growing fungal strains (PDA). As mentioned earlier, 25 bacterial treatments were evaluated against *Trichoderma viride*, *T. harzianum*, and *T. asperellum*. Field research was done on the effects of VOCs on *Trichoderma* spp. conducted using *B. paramycoides* (V37), B4, B7, *B. subtilis*, and *B. amyloliquefaciens*, following the results from *in-vitro*, and co-cultivation studies.

Dual Culture in vitro assay

All bacterial cultures were tested for antagonistic activity against *Trichoderma* spp. (Kim et al. 2008). The growth inhibition was computed using the formula:

$$PI\ (\%) = \left(\frac{C - T}{C}\right) \times 100$$

where PI is percentage inhibition, C is growth of *Trichoderma* spp. in control, and T is growth of *Trichoderma* spp.

Co-cultivation assay

The bacterial culture was inoculated into 25 mL of NB, kept for 24 h at 28±2°C, centrifuged, re-suspended in sterile saline density was maintained to 10^7 cfu/mL.

Trichoderma species were grown in PD broth, centrifuged, and adjusted to 1×10^7 cfu/mL. The fungal and bacterial suspensions combined with 1:1 ratio, incubated in PDB for 7 days, then centrifuged and dried (Es-soufi et al. 2020).

The percentage inhibition was calculated as

% percent inhibition = 1-((Dry matter with antagonist)/ (Dry matter of control)) × 100

Production of volatile compounds (VOC) by bacterial strains

The effect of bacterial VOCs on *Trichoderma* CFU was assessed (Choub et al. 2022) with slight modifications. 100 µL *Trichoderma* suspension of spores (10^6 spores/mL) was inoculated onto PDA plates, and bacterial suspensions (10^5, 10^6, and 10^7 CFU/mL) were grown onto NA plates. Placement of the plates was done face-to-face, with *Bacillus* plates at the bottom and *Trichoderma* plates on top. Parafilm was used to seal the dishes, and incubated at 28°C for 48 h. Colony count was recorded at 24 and 48 h.

$$GR\ (\%) = ((C-T)/C)\ x100$$

where GR is the reduction in spore germination %, C is the percentage spore germination in control, and T is the percentage spore germination in the treatment.

Field bio-efficacy in the mushroom production unit

Based on co-cultivation studies, five cultures *viz.*, B4, B7, V37, *B. subtilis*, and *B. amyloliquefaciens* were evaluated for their field efficacy at Quality Button Mushroom Pvt. Ltd., Manik Chand Road, Nacharam, Hyderabad, 500076 during 2022-2023. The population of each culture was adjusted to 1×10^7 CFU/mL and sprayed on the mushroom mycelial mat in the 14-16-day-old bags. For each treatment, 100 bags were evaluated and experiment was repeated 5 times. The number of green-mold infected bags and mushroom yield were recorded periodically until the final harvest.

Molecular identification

DNA from *Bacillus* isolate V-37 was extracted from an exponentially growing culture using the Qiagen kit (Italy). Primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′AAGGAGGTGWTCCARCC-3′) were used to amplify the 16S rRNA gene under the following PCR conditions.: denaturation at 94°C (30–60 s), Primers are annealed at 62°C for 30 seconds and extended at 72°C for 60 seconds. for 28 cycles, and final extension at 72°C (240 s). The resulting amplicons were purified and sequenced using a BDT v3.1 kit from Applied Biosystems and the ABI 3500xl Genetic Analyzer. MEGA v7 was used to align the sequences, and the Neighbour-Joining methodology was used to create a phylogenetic tree, after performing a BLAST search on the NCBI GenBank database.

In vitro antifungal activity assay

Secondary metabolites of *B. paramycoides* were extracted following the method described by Abdel-Nasser et al. (2023). For intracellular extraction, the *B. paramycoides* pellet was mixed with same proportion of ethylaetate. The resulting mixture was subjected to ultra-sonication and vortexed for 1 h. After phase separation, at 40°C, a rotary evaporator was used to evaporate the organic phase. The residual extract was weighed and re-dissolved in phosphate buffer (pH 7.0) to solubilize the volatile compounds. For extracellular extraction, 100 mL of *B. paramycoides* broth supernatant was combined with an equal volume of ethyl acetate in a separatory funnel, left undisturbed for 5 min, and the organic phase was collected.

Antifungal activity was evaluated using the agar well diffusion method (Omar et al. 2006) against *T. viride*, *T. asperellum*, and *T. harzianum*. A PDA plate was covered with a 50 µL suspension of fungal spores (10^6 spores/mL). 50 µL of intra- and extracellular extracts (treatment control), DMSO (negative control), and 100ppm carbendazim (positive control) were added into the wells. Plates were sealed and stored at 28±1°C for 4 days. After incubation, inhibition zones were measured to assess antifungal activity using the formula:

$$FI = D - d$$

Where, FI = Fungal inhibition (mm), D= diameter of the inhibition zone appearing on the agar plate (mm), and d= diameter of the agar well (mm).

Evaluation of secondary metabolites

The organic phase was collected and analyzed in a GC-MS (Agilent 7890A).Two µL sample was injected onto a DB-5 MS column (30 cm x 0.25 mm; ID x 0.25 µm)and the oven temperature was programmed at 50°C for 1 min, raised to 210°C @ 30°C/min, then to 310°C @ 5°C/min, and held at 310°C for 3 min. Helium was used as the carrier gas @ 1 mL/min, with the injector and auxiliary heaters preserved at 280°C.MS was performed over 50-550 m/z for compound identification using the NIST-2017 Mass Spectral Library (Rakhmawatie et al. 2023).

Statistical Analysis

A completely randomized design (CRD) was employed for all experimental setups with a significance level of $p < 0.05$. Wasp 2.0 was used to conduct the statistical assessment of the experimental data.

RESULTS AND DISCUSSION

The results of the dual-culture *in vitro* assay evaluating the antifungal activity of the tested bacterial treatments against *Trichoderma viride*, *T. harzianum*, and *T. asperellum* are presented in Table 1. Of the 19 bacterial isolates and other six bacterial spp. tested, 8 showed antagonistic activity against *T. viride*, with inhibition rates ranging from 10.3% to 73.3%. V37 showed the highest antagonism (73.3%), followed by *B. amyloliquefaciens* (33.7%). *B. amyloliquefaciens* also inhibited growth of *T. harzianum* (24.2%) and *T. asperellum* (24.4%). But, 14 treatments (21, 22, 32, 36, 37, 43, 53, 62, 81, 85, 98),

P. fluorescens, B. clausii, and B. polymyxa did not effective against *Trichoderma* spp. The results align with the findings of Stanojević et al. (2019) and Shrivastava et al. (2017), communicated a similar bio-efficacy of *Streptomyces* spp. and *Pseudomonas aeruginosa* against mushroom green mold pathogens.

Table 1 *In vitro* antagonistic effects of bacteria against *Trichoderma spp.*

Treatments	% growth inhibition		
	T. viride	*T. harzianum*	*T. asperellum*
B. amyloliquefaciens	33.7^{b} (35.3)	24.2^{a} (29.5)	24.4^{a} (29.6)
B. subtilis	21.1^{c} (26.9)	19.6^{b} (26.0)	0.0^{f} (0.2)
V37	73.3^{a} (58.9)	20^{ab} (26.4)	16.7^{b} (24.1)
B7	13.3^{de} (21.3)	10^{d} (18.3)	12.6^{c} (20.6)
B4	20^{cd} (26.2)	0.0^{f} (0.2)	6.7^{e} (14.9)
B28	13.3^{de} (21.2)	10.7^{d} (19.0)	11.1^{c} (19.4)
B35	0.0^{f} (0.2)	2.7^{f} (8.7)	0.0^{f} (0.2)
B65	22.2^{c} (27.9)	17.4^{b} (24.5)	8.9^{d} (17.2)
B73	0.0^{f} (0.2)	15.5^{bc} (23.1)	0.0^{f} (0.2)
B79	0.0^{f} (0.2)	4.8^{e} (12.4)	0.0^{f} (0.2)
B88	10.3^{e} (17.1)	12.9^{cd} (20.8)	15.2^{b} (22.8)
CV	30.75	19.48	15.19
CD (0.05)	5.50	3.46	1.74

B21, B22, B32, B36, B37, B43, B53, B62, B81, B85, B98, *P. fluorescens, B. clausii,* and *B. polymyxa* did not exhibits antagonistic effects against *Trichoderma* spp. values indicate CRD values. Means with different superscripts are significantly different ($p < 0.05$).

In the co-cultivation study, the dry biomass of *Trichoderma* spp, was significantly lower than the control, except when co-cultured with B65 (Table 2). *T. viride* biomass ranged from 1.3-3.0 g/L when co-cultured with *B. amyloliquefaciens, B. subtilis,* and B7, B4, B28, B35, B79, and B88 compared to the control (6.2 g/L). Similarly, *T. harzianum* biomass ranged from 1.2-1.8 g/L with *B.*

amyloliquefaciens, B. subtilis, V37, and B7, and B4 as against control (6.9 g/L). *T. asperellum* (1.5-4.2 g/L) when co-cultured with *B. amyloliquefaciens*, *B. subtilis,* and B7, B28, B35, B79, and B4 as compared to control (6.7 g/L). Thus, V37 was significantly superior against all the three *Trichoderma* spp. These outcomes are consistent with those of Fifani et al. (2022); Wu et al. (2018). The inhibition was reported to be due to antifungal secondary metabolites (Li et al. 2020). *Trichoderma* biomass was not adversely affected when co-cultured with *Bacillus* isolate 65, similar to the observations recorded in the co-cultivation of *T. asperellum* with *B. amyloliquefaciens* (Karuppiah et al. 2019).

Table 2 Dry biomass of *Trichoderma* spp. during co-cultivation with *Bacillus* spp.

Treatments	Dry biomass (g/L)		
	T. viride	*T. harzianum*	*T. asperellum*
B. amyloliquefaciens	1.3^{a} (1.3)	1.8^{abc} (1.8)	2.0^{abc} (2.0)
B. subtilis	1.8^{a} (1.8)	1.6^{abc} (1.6)	2.5^{c} (2.5)
V37	1.5^{a} (1.5)	1.2^{ab} (1.2)	1.5^{a} (1.5)
B7	1.8^{a} (1.8)	1.6^{abc} (1.6)	1.6^{ab} (1.6)
B4	2.1^{a} (2.1)	1.8^{abc} (1.8)	2.4^{bcd} (2.4)
B28	3.0^{d} (3.0)	2.2^{cd} (2.2)	2.1^{abc} (2.1)
B35	4.6^{c} (4.6)	2.0^{b} (2.0)	1.8^{abc} (1.8)
B65	5.6^{de} (5.6)	6.2^{gh} (6.2)	7.5^{g} (7.5)
B73	3.1^{b} (3.1)	2.9^{de} (2.9)	1.9^{a} (1.9)
B79	1.9^{a} (1.9)	4.0^{f} (4.0)	4.2^{e} (4.2)
B88	2.1^{a} (2.1)	3.3^{ef} (3.3)	3.0^{d} (3.0)
Control	6.2^{e} (6.2)	6.9^{h} (6.9)	6.7^{fg} (6.7)
CV	20.5	18.3	16.1
CD (0.05)	0.941	0.879	0.837

Values indicate CRD values. Means with different superscripts are significantly different ($p < 0.05$).

The volatile study (Table 3) showed that exposure to *Bacillus* spp. at 10^5–10^7CFU/ml significantly impacted *Trichoderma* spore germination. V37 notably reduced spore germination of *T. harzianum*, *T. viride*, and *T. asperellum* and reduction was inversely correlated to bacterial CFU. Both *B. amyloliquefaciens*, and V37 were antagonistic (37.82%-99.28%) against *Trichoderma* spp. (Fig. 4-6). The colony colour was changed *viz*., *T. viride* (white), *T. harzianum (*pale green-white), and *T. asperellum* (dark green-white) when exposed to the volatiles of *B. subtilis*, *B. amyloliquefaciens*, V37, and B7, and B4 (Fig. 1-3, Table 4). Massawe et al. (2018) recorded that mycelium texture of *Sclerotinia sclerotiorum* turned from hard to soft when exposed to *Bacillus* VOCs for 48 h. Conidial and chlamydospore lysis in *T. harzianum* was observed due to iturin production by *B. subtilis* (Li et al. 2005). Interestingly, in the present study, dose-dependent reduction in *Trichoderma* spore germination was not observed as reported by Tyskiewicz et al. (2022) and Singh et al. (2016). This could probably due to the early VOC release at 10^5 CFU/ml by *Bacillus* isolates, thus not aligning with *Trichoderma* spore germination (14 h after the inoculation). Short-term exposure to VOCs at low concentrations may have significantly reduced *Trichoderma* CFU.

Table 3 Impact of *Bacillus* spp. volatile compounds on CFU of *Trichoderma* spp. Data represent the average of three replications; values indicate CRD values. Major variations ($p < 0.05$) emerge among means with various superscripts.

Treatments	Dilution factor	Mean colony numbers per plate		
		T. viride	*T. harzianum*	*T. asperellum*
B7	10^5	12^c	4.0^c	13.7^c
	10^6	6.0^b	1.3^a	12.3^b
	10^7	4.0^b	0.3^a	6.0^b
B4	10^5	10^c	1.3^{ab}	11.3^{bc}
	10^6	4.7^{ab}	0.7^a	6.7^a
	10^7	2.3^{ab}	1.3^a	4.3^{ab}
B. subtilis	10^5	10.3^c	3.0^{bc}	13.0^c
	10^6	6.3^b	1.3^a	8.0^{ab}
	10^7	3.0^{ab}	0.3^a	5.3^b
B. amyloliquefaciens	10^5	3.3^a	1.3^{ab}	8.3^{bc}
	10^6	2.7^a	0.3^a	5.0^a
	10^7	0.7^a	0.0^a	2.0^a
V37	10^5	7^b	0.7^a	4.0^a
	10^6	3.7^a	0.3^a	8.7^{ab}
	10^7	1.0^a	0.3^a	6.7^b
Control	10^5	19.3^d	15^d	53.8^d
	10^6	19.3^c	15^b	53.8^c
	10^7	19.3^c	15^b	53.8^c
CD (0.05)	10^5	2.8	1.80	3.23
	10^6	2.16	1.72	4.7
	10^7	2.3	1.44	2.5
CV	10^5	14.9	23.6	10.3
	10^6	16.8	29.9	14.2
	10^7	24.9	26.5	10.8

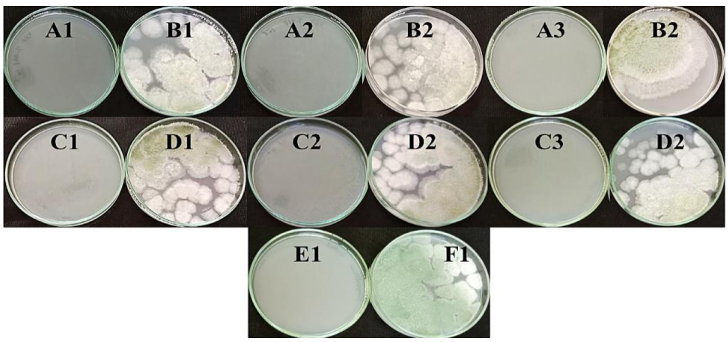

Fig. 1 Effect of volatile compounds on the spore germination of *T. asperellum*. A1- *B. paramycoides* (10^5), B1- *T. asperellum* (10^5), C1- *B. amyloliquefaciens* (10^5), D1- *T. asperellum* (10^5), A2- *B. paramycoides* (10^6); B2- *T. asperellum* (10^6), C2- *B. amyloliquefaciens* (10^6), D2- *T. asperellum* (10^6), A3- *B. paramycoides* (10^7), B3- *T. asperellum* (10^7), C3- *B. amyloliquefaciens* (10^7), D3- *T. asperellum* (10^7); E1- Nutrient broth and F2- *T. asperellum* control.

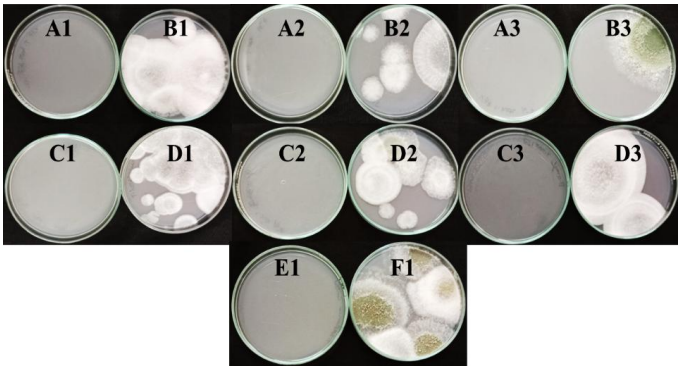

Fig. 2 Effect of volatile compounds on the spore production of *T. harzianum.* A1- *B. paramycoides* (10^5); B1- *T. harzianum* (10^5), C1- *B. amyloliquefaciens* (10^5), D1- *T. harzianum* (10^5); A2- *B. paramycoides* (10^6); B2- *T. harzianum* (10^6), C2- *B. amyloliquefaciens* (10^6), D2- *T. harzianum* (10^6), A3- *B. paramycoides* (10^7); B3- *T. harzianum* (10^7); C3- *B. amyloliquefaciens* (10^7), D3- *T. harzianum* (10^7), E1 - Nutrient broth and F1 - *T. asperellum* control.

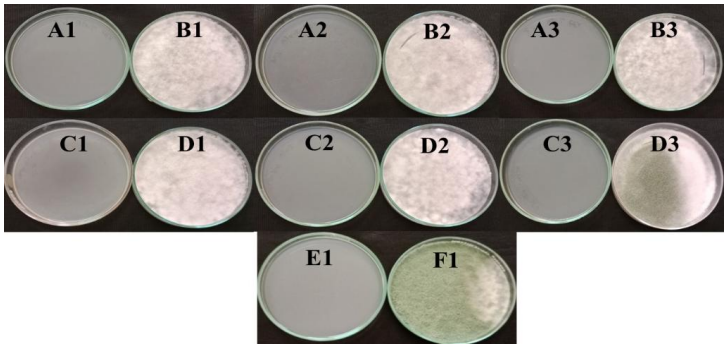

Fig. 3 Effect of volatile compounds on spore production from *T. viride.* A1- *B. paramycoides* (10^5), B1- *T. viride* (10^5), C1- *B. amyloliquefaciens* (10^5); D1- *T. viride* (10^5), A2- *B. paramycoides* (10^6), B2- *T. viride* (10^6), C2- *B. amyloliquefaciens* (10^6), D2- *T. viride* (10^6), A3- *B. paramycoides* (10^7), B3- *T. viride* (10^7), C3- *B. amyloliquefaciens* (10^7), D3- *T. viride* (10^7); E1 - Nutrient broth and F1 - *T. asperellum* control.

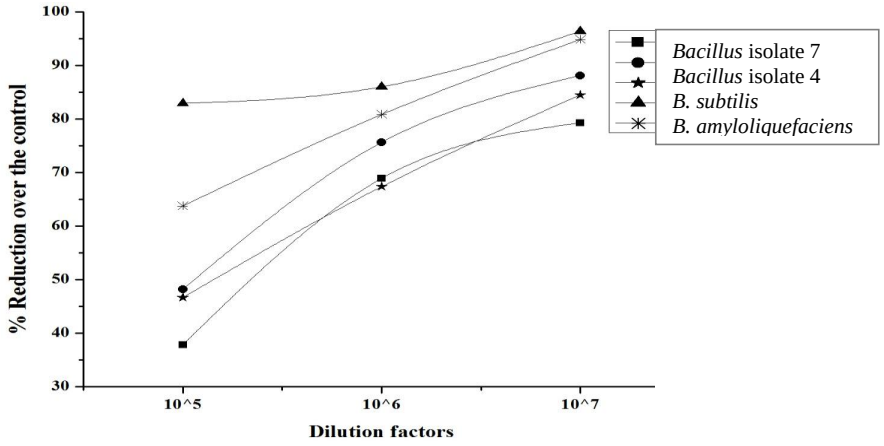

Fig. 4 *T. viride* spore production under co-cultivation of bacterial strains

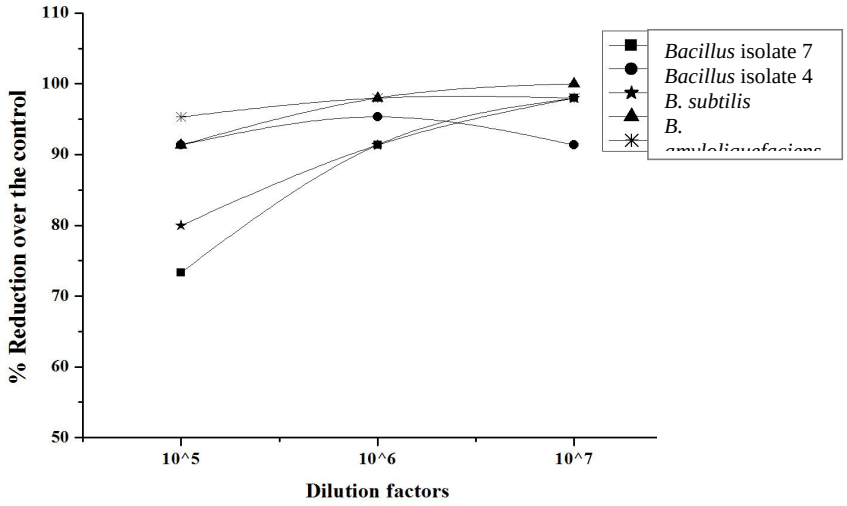

Fig. 5 *T. harzianum* spore production under co-cultivation of bacterial strains

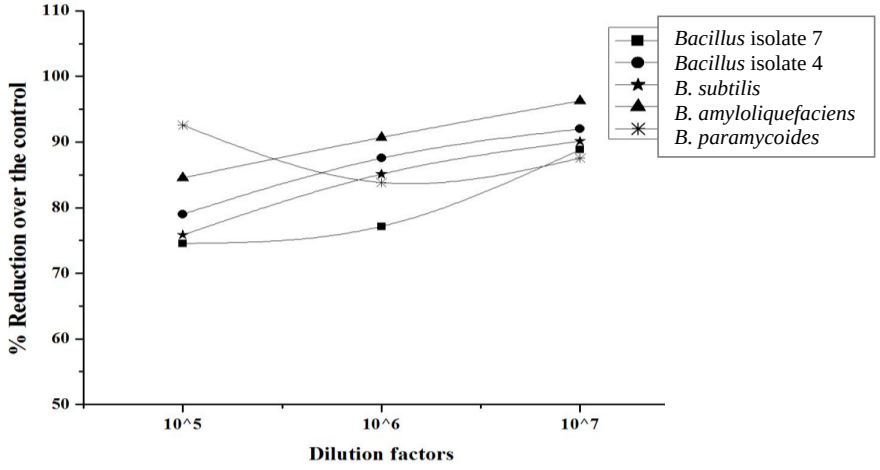

Fig. 6 *T. asperellum* spore production under co-cultivation of bacterial strains

Table 4 *In vitro* morphological observation of *Trichoderma* mycelia production.

Treatments	Variation in mycelial colour								
	T. viride			*T. harzianum*			*T. asperellum*		
	10^5	10^6	10^7	10^5	10^6	10^7	10^5	10^6	10^7
B7	1	1	1	1	2	1	1	1	1
B4	1	1	1	3	4	4	5	5	1
B. subtilis	1	1	1	1	3	1	1	4	4
B. amyloliquefaciens	1	1	2	1	6	6	3	3	1
V37	1	1	1	1	6	2	1	1	4
Control	2	2	2	6	6	6	5	5	5

1- White; 2- Green; 3- Brownish green with white margin; 4- Green with white margin; 5- Dark green with white margin; 6- pale green with white margin.

Field bio-efficacy of V37, *B. subtilis*, *B. amyloliquefaciens*, and B7, B4 against green mold in the mushroom production industry during 2022-2023 (Table 5, Table 6). The highest mushroom yield of 231.2 kg was recorded withV37, followed by *B. amyloliquefaciens* (222.8 kg) thus showing the superiority of *B. paramycoides* against *Trichoderma* spp. for button mushroom management. Studies by Pandin et al. (2018) demonstrated that *B. velezensis* QST713 effectively controlled *T. aggressivum* and prevented production losses in *A. bisporus*. Kumari and Narain (2021), Kim et al. (2008) demonstrated the ability of *Bacillus* isolates, M27 and RM29 to inhibit sporulation in *T. harzianum*, *T. koningii*, and *T. viridescens*. This current study *B. paramycoides* has exhibited higher than previous reports of 221.71 kg by Sharma et al. (2018).

Table 5 Field bio-efficacy in button mushroom production factory

Bio-control agents	Impact of bio-control agents on yield& infestation of *Trichoderma* spp.					
	Feb-Mar 2022		Jun-July 2022		Aug-Sep 2022	
	No. of bags infested*	Yield/100 bags (Kg)	No. of bags infested*	Yield/100 bags (Kg)	No. of bags infested*	Yield/100 bags (Kg)
V37	2.6^{a}	231.2^{a}	3^{a}	211.0^{a}	2.4^{a}	223.8^{a}
B7	9.2^{b}	215.8^{b}	12.6^{bc}	202.2^{b}	10.4^{c}	205.0^{b}
B4	11.4^{b}	210.4^{b}	7.8^{ab}	190.0^{b}	10.0^{c}	207.0^{b}
B. subtilis	7.4^{ab}	220.0^{ab}	7.2^{a}	201.0^{b}	7.2^{bc}	213.2^{ab}
B. amyloliquefaciens	6.2^{ab}	222.8^{ab}	6.0^{a}	204.4^{a}	4.6^{ab}	218.8^{a}
Control	17.2^{c}	196.4^{c}	16^{c}	183.6^{b}	21.4^{d}	180.0^{c}
CD (0.05)	5.2	12.5	4.8	10.1	4.7	10.7

*Mean of five consecutive batches, each consisting of 100 bags. Values indicate CRD values. Means with various superscripts are notable dissimilar ($p < 0.05$).

Table 6 Field bio-efficacy in button mushroom production factory

Bio-control agents	Impact of bio-control agents on yield& infestation of *Trichoderma* spp.					
	Oct-Nov 2022		Jan-Feb 2023		Mar-Apr 2023	
	No. of bags infested*	Yield/100 bags (Kg))	No. of bags infested*	No. of bags infested*	Yield/100 bags (Kg))	No. of bags infested*
V37	3.2^{a}	211.0^{a}	3.8^{a}	3.2^{a}	211.0^{a}	3.8^{a}
B7	11.8^{b}	192.4^{b}	9.6^{b}	11.8^{b}	192.4^{b}	9.6^{b}
B4	11.0^{b}	193.8^{b}	11.6^{b}	11.0^{b}	193.8^{b}	11.6^{b}
B. subtilis	5.6^{a}	205.0^{a}	10.8^{b}	5.6^{a}	205.0^{a}	10.8^{b}
B. amyloliquefaciens	4.0^{a}	209.2^{a}	5.0^{a}	4.0^{a}	209.2^{a}	5.0^{a}
Control	24.4^{c}	164.2^{c}	26.6^{c}	24.4^{c}	164.2^{c}	26.6^{c}
CD (0.05)	4.4	10.5	3.4	4.4	10.5	3.4

*Mean of five consecutive batches, each consisting of 100 bags. Values indicate CRD values. Means with various superscripts are notable dissimilar ($p < 0.05$).

Bacillus isolate V37 genetic identity was determined by 16S rRNA gene sequencing, BLAST analysis, and phylogenetic tree construction was established as *Bacillus paramycoides* with 99.34% similarity (Fig. 7). The sequence is registered with NCBI GenBank with accession number PP757924.

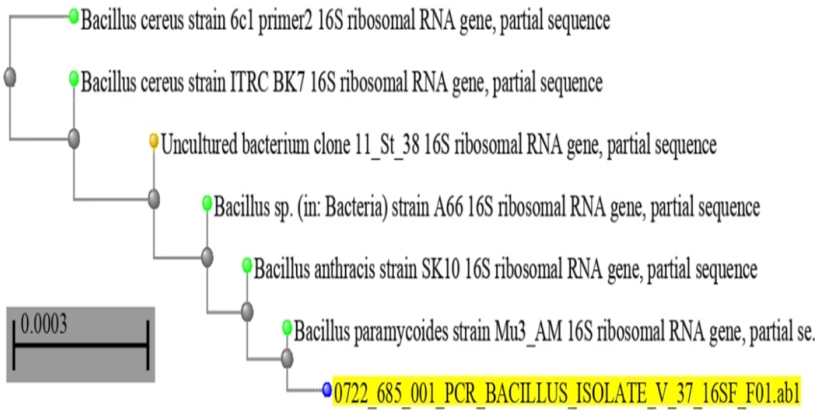

Fig. 7 Phylogenetic tree of *B. paramycoides*

Overall, the extracellular extract of *B. paramycoides* showed higher antifungal activity against *T. viride* (22.51 mm) and *T. harzianum* (19.96 mm). In contrast, the intracellular extract of *B. paramycoides* was most effective against *T. asperellum* (21.69 mm). In comparison, carbendazim (100ppm) demonstrated an antifungal activity of 21.69 mm against *T. viride*, followed by *T. asperellum* (20.84 mm) and *T. harzianum* (19.66 mm). The negative control (DMSO) did not inhibit any of the *Trichoderma* spp. (Table 7, Fig. 8).

Table 7 Antifungal effects of *B. paramycoides* crude extracts against *Trichoderma* spp.

Treatments	Average antifungal activity (mm) against		
	B. paramycoides (Intracellular)	*B. paramycoides* (Extracellular)	Carbendazim
T. viride	19.65^{b} (11.3)	22.51^{a} (14.6)	21.69^{a} (13.3)
T. harzianum	18.73^{b} (10.3)	19.96^{b} (11.6)	19.66^{b} (11.4)
T. asperellum	21.69^{a} (13.6)	20.25^{b} (12.0)	20.84^{a} (13.2)
CV%	4.624	3.109	2.417
CD (0.05)	1.85	1.29	1.00

The negative control, DMSO did not show any inhibitory effect against *Trichoderma* spp. Values indicate CRD values. Means with different superscripts are significantly different ($p < 0.05$).

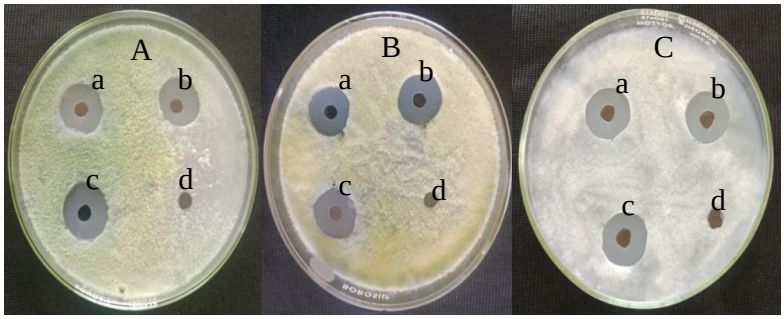

Fig. 8 A. Antifungal activity against *T. viride*; **B.** Antifungal activity against *T. asperellum*; **C.** Antifungal activity against *T. harzianum*; a- Intracellular crude extract of *B. paramycoides*; b- Extracellular crude extract of *B. paramycoides*; c- Carbendazim (positive control); d- DMSO (negative control).

Chu et al. (2022) highlighted *B. velezensis* HN-2's ability to produce antimicrobial metabolites inhibiting *Erysiphe quercicola*, the fungus responsible for oak tree powdery mildew. Similarly, Jan et al. (2023) showed that crude extracts from *Bacillus* strains inhibited the growth of *F. oxysporum*, *A. niger*, *A. flavus*, and *R. oryzae* by 56-92%. In another study, Soliman et al. (2022) found significant inhibition against *Alternaria* and *Helmintho sporium* species. Bulgasem et al. (2016) reported a 25 mm inhibition zone for *L. plantarum* supernatant against *C. albicans*, while *B. subtilis* showed a 30 mm zone, surpassing the fungicide itraconazole (13.67 mm) (Gharieb et al. 2024). This study emphasizes the antifungal capabilities of *B. paramycoides* crude extracts for controlling *Trichoderma* species in mushrooms.

GC-MS analysis of *B. paramycoides* showed 19 and 27 secondary metabolites in the intracellular and extracellular extracts, respectively (Fig. 9, Table 8, Table 9). Several metabolites, including acetamide, benzaldehyde, and phenyl acetaldehyde, are known for their fungistatic effects, suggesting their potential for biocontrol, especially against *Trichoderma* spp. Massawe et al. (2018), and Zhao et al. (2022) have reported compounds such as 1-decanol, 2-hexyl, 2-piperidinone, and tetradecane, while extracellular metabolites included phenol, 2,5-bis(1,1-dimethylethyl), and disulfide di-tert-dodecyl in *Bacillus* spp. *B. subtilis* and *B. amyloliquefaciens* have been shown to produce VOCs like acetic acid, benzaldehyde, and 1-octadecene, which possess antifungal properties (Awan et al. 2023; Tahir et al. 2017). 4-chloro-3-methylphenol and azulene from *B. velezensis* and *B. subtilis* also exhibited antifungal effects. This study highlights the potential of *B. paramycoides* as a source of antifungal metabolites for managing fungal pathogens such as *Trichoderma* spp.

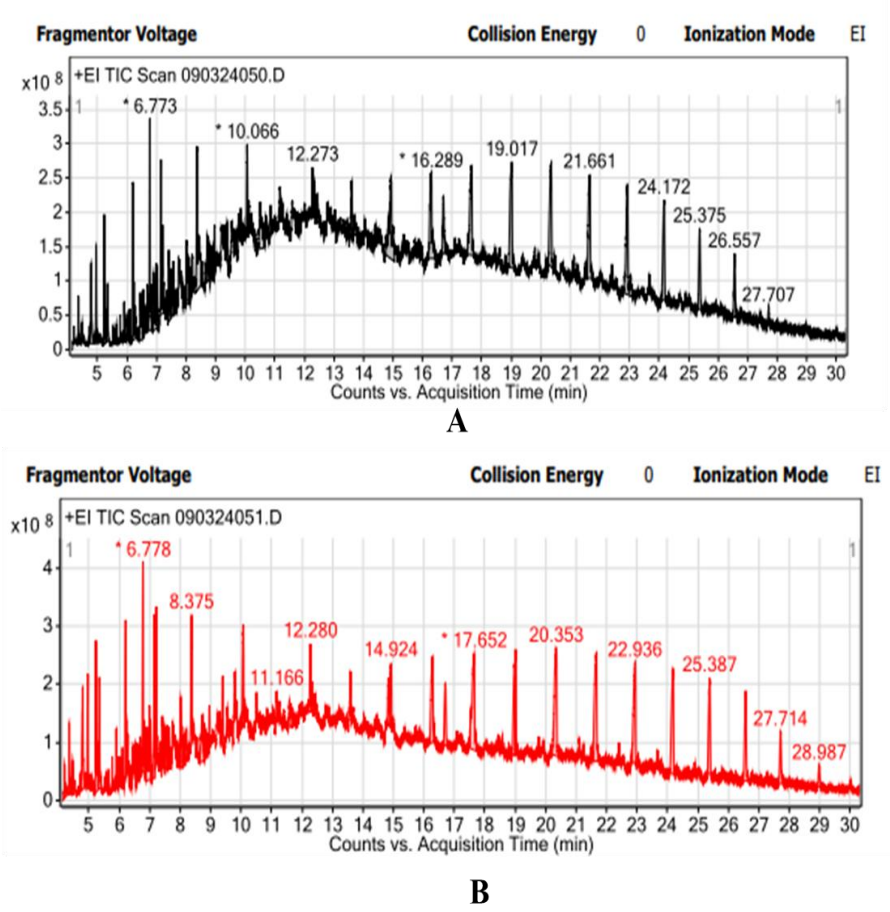

Fig. 9 *B. paramycoides* GC-MS chromatogram profile (A) intracellular crude

(B) extracellular crude

Table 8 GC-MS analysis of the volatile compounds identified in the intracellular crude extract of *B. paramycoides*

RT	Area	Area %	Name of the compounds	DB Formula
4.354	88328448	10.6	Benzene, 1,3-dichloro-	$C_6H_4Cl_2$
4.786	264649411.9	31.77	Benzene, nitro-	$C_6H_5NO_2$
4.956	201013813	24.13	2-Cyclohexen-1-one, 3,5,5- trimethyl-	$C_9H_{14}O$
5.229	277480455.7	33.31	1-Octanol, 2-butyl-	$C_{12}H_{26}O$
5.344	143591293	17.24	Azulene	$C_{10}H_8$
5.905	107581149.5	12.91	Bicyclo[4.4.1]undeca- 1,3,5,7,9-pentaene	$C_{11}H_{10}$
6.101	80621645.04	9.68	Dodecane, 2-methyl-	$C_{13}H_{28}$
6.203	157610437.1	18.92	1-Tetradecene	$C_{14}H_{28}$
6.553	335407477.2	40.27	Tetradecane, 2,6,10-trimethyl-	$C_{17}H_{36}$
6.773	334242237.9	40.13	Geranylisovalerate	$C_{15}H_{26}O_2$
6.989	643194600.8	77.21	Disulfide, di-tert-dodecyl	$C_{24}H_{50}S_2$
7.146	465648647.9	55.90	2-Piperidinone, N-[4-bromo-n- butyl]-	$C_9H_{16}BrNO$
7.413	584644621.6	70.19	1-Decanol, 2-hexyl-	$C_{16}H_{34}O$
10.509	417214879	50.09	2-Dodecen-1-yl(-)succinic anhydride	$C_{16}H_{26}O_3$
13.599	215184883.9	25.83	erythro-7,8- Bromochlorodisparlure	$C_{19}H_{38}BrCl$
14.936	832994288.2	100	tert-Hexadecanethiol	$C_{16}H_{34}S$
16.703	276861868.5	33.24	tert-Hexadecanethiol	$C_{16}H_{34}S$
17.661	724179737.4	86.94	2,3,3-Trimethyl-2 (4- methylpentanoyl)-cyclopentanone	$C_{14}H_{24}O_2$
19.017	773818245	92.90	Heptacosane	$C_{27}H_{56}$
27.707	63180114.37	7.58	Octatriacontylpentafluoropropionate	$C_{41}H_{77}F_5O_2$

Table 9 GC-MS analysis of the volatile compounds identified in the extracellular crude extract of *B. paramycoides*

RT	Area	Area %	Name of the compounds	DB Formula
4.203	108975727.5	10.44	Benzene,1-ethyl-2-methyl-	C_9H_{12}
4.354	183760119.2	17.60	Benzene,1,3-dichloro-	$C_6H_4Cl_2$
4.471	112384493.6	10.76	Benzeneethanol, beta- ethenyl-alpha-phenyl-	$C_{16}H_{16}O$
4.799	418239882.2	40.06	Benzene,nitro-	$C_6H_5NO_2$
4.965	319700229.8	30.62	2-Cyclohexen-1-one,3,5,5- trimethyl-	$C_9H_{14}O$
5.232	490773873.3	47.01	1-Octanol,2-butyl-	$C_{12}H_{26}O$
5.343	334374693.2	32.03	Azulene	$C_{10}H_8$
5.904	136242819.2	13.05	Bicyclo[4.4.1]undeca- 1,3,5,7,9-pentaene	$C_{11}H_{10}$
6.208	85820103.87	8.22	1-Tetradecanol	$C_{14}H_{30}O$
6.778	360758369.3	34.55	Phenol,2,5-bis(1,1- dimethylethyl)-	$C_{14}H_{22}O$
6.991	675801289.7	64.73	Disulfide,di-tert-dodecyl	$C_{24}H_{50}S_2$
7.156	582524392	55.80	1-Octadecanol	$C_{18}H_{38}O$
7.217	580485687.2	55.60	DiethylPhthalate	$C_{12}H_{14}O_4$
7.413	146376840.9	14.02	2-Piperidinone,N-[4-bromo-n- butyl]-	$C_9H_{16}BrNO$
7.633	138326028.3	13.25	2-Dodecen-1-yl(-)succinic anhydride	$C_{16}H_{26}O_3$
8.375	482230791.3	46.19	1-Octadecene	$C_{18}H_{36}$
9.398	236301889.4	22.63	7,9-Di-tert-butyl-1- oxaspiro (4,5)-deca-6,9-diene- 2,8-dione	$C_{17}H_{24}O_3$
9.8	119058515.1	11.40	2-Dodecen-1-yl(-)succinic anhydride	$C_{16}H_{26}O_3$
10.07	417347938.4	39.97	Acetic acid, 3,7,11,15- tetramethyl-hexadecyl ester	$C_{22}H_{44}O_2$
12.28	307254151.9	29.43	Cyclohexane,1,2,3,4,5,6- hexaethyl-	$C_{18}H_{36}$
12.34	110984871.2	10.63	2,3,3-Trimethyl-2-(4- methylpentanoyl)-cyclopentanone	$C_{14}H_{24}O_2$

14.924	348902904.8	33.42	Pentacosane	$C_{25}H_{52}$
16.709	369358625.1	35.38	2-Methyl-2-(4-methyl-3- oxopentyl)-cyclohexane	$C_{13}H_{22}O_2$
17.652	1033321884	98.97	1-Hexadecanesulfonyl chloride	$C_{16}H_{33}ClO_2S$
18.996	583902679.9	55.93	Heptacosane	$C_{27}H_{56}$
19.016	371211576.8	35.56	Tetracosane	$C_{24}H_{50}$
21.109	81572398.12	7.81	Cyclohexane,1,2,3,5- tetraisopropyl-	$C_{18}H_{36}$
23.672	89728210.94	8.59	2-Dodecen-1-yl(-)succinic anhydride	$C_{16}H_{26}O_3$

Among all the test cultures *B. paramycoides* was identified as most effective biocontrol agent, which significantly reduced green mold infestation and improved yield of *A. bisporus*. The next best treatments were *B. amyloliquefaciens* and *B. subtilis*. The novelty of this study is that for the first time, *B. paramycoides* has been identified as a potential biocontrol agent against *Trichoderma* spp. for the management of *A. bisporus*. Further research may be conducted to ensure bio-safety of *B. paramycoides* and formulation of consortia of *B. paramycoides*, *B. amyloliquefaciens* and *B. subtilis* to explore possible synergistic effects for effective management of green mold in *A. bisporus*.

ACKNOWLEDGEMENTS

The authors extend their gratitude towards to Dr. Suseelendra Desai (Vice President at M/s Varsha Bioscience and Technology India Pvt Ltd.), and Dr. T.V. Rao (Director of Research at M/s Varsha Bioscience and Technology India Pvt Ltd.) for review of the manuscript.

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