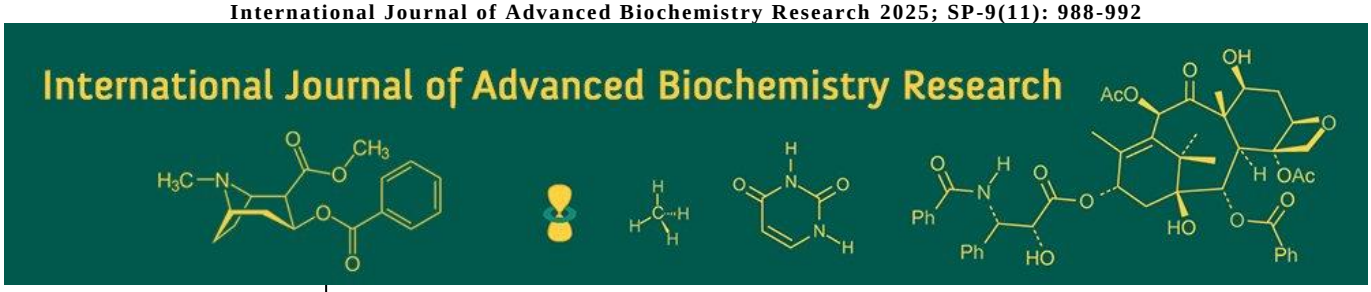

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Antifungal activity of non-volatile metabolites from entomopathogenic fungi against the foliar pathogens *Drechslera bicolour*

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Abstract
The present study investigated the antagonistic efficacy of non-volatile secondary metabolites produced by five fungal bioagents (*Beauveria bassiana*, *Nomuraea rileyi*, *Metarhizium anisopliae*, *Verticillium lecanii*, and *Trichoderma viride*) against the fungal foliar pathogen *Drechslera bicolor* in an *in-vitro* assay. Culture filtrates of the bioagents were incorporated into PDA medium at three concentrations: 10%, 25%, and 50%.

All effective treatments showed statistically significant and concentration-dependent inhibition of *D. bicolor* linear growth after seven days (168 hrs). *Beauveria bassiana* was the most potent antagonist across all dilutions, achieving the highest inhibition of 55.64% at the 50% concentration (39.93 mm growth). *N. rileyi* (38.82% inhibition) and *M. anisopliae* (37.07% inhibition) demonstrated moderate, comparable levels of antagonism at 50%. *T. viride* exhibited the lowest biological activity (16.18%). Crucially, *V. lecanii* was completely ineffective at all tested concentrations, recording 0.00% inhibition, performing at par with the uninhibited control (90.00 mm growth). These findings highlight the strong fungistatic potential of *B. bassiana* metabolites against *D. bicolor*, underscoring its promise in developing natural biofungicides.

Keywords: Biocontrol, entomopathogenic fungi, *Drechslera bicolor* and brinjal

Introduction
The pure cultures of the entomopathogenic fungi (EPF) *Beauveria bassiana*, *Nomuraea rileyi*, *Metarhizium anisopliae*, *Verticillium lecanii*, and *Trichoderma viride* were procured from the Entomology Section, College of Agriculture, Dhule, and maintained on Potato Dextrose Agar (PDA) slants. Disease samples exhibiting typical leaf spot symptoms on brinjal were collected from farmer's fields in Varkhedi village of Dhule district. The pathogen was isolated from the diseased tissue and maintained on PDA slants.

The pathogenicity of the isolated fungi was confirmed using Koch's Postulates. The reisolated fungi were compared to the original isolates and identified at the Agharkar Research Institute (ARI), Pune.

The bioagent were grown in Potato dextrose broth at 28±2 °C with intermittent shaking. The metabolites were collected after 12 days and filtered through filter paper. Then the culture filtrates of bioagents were added to PDA medium to make 10%, 25% and 50 % concentration and sterilized in autoclave at 121 °C for 15 minutes. The solidified agar plates in triplicates were inoculated at the center with 5 mm diameter mycelial disc of the foliar pathogen and incubated at 28±2 °C. The Plates without filtrate was served as control. The colony diameter of the test pathogen was measured when the control plate was fully gets covered by pathogen. The experimental detail was as follows.

Design: CRD
Replications: 3
Treatments: 6

Sr. No.	Treatment No.	Treatment Details
1	T_1	*Beauveria bassiana*
2	T_2	*Nomuraea rileyi*
3	T_3	*Metarhizium anisopliae*
4	T_4	*Verticillium lecanii*
5	T_5	*Trichoderma viride*
6	T_6	Control

All the linear measurements were measured with the help of Magnus Magvision software. The HD photographs were taken from a fixed distance and the software was calibrated for the number of pixels per unit length. After calibration, the linear growth was measured with the help of software.

The data obtained in all the experiments were statistically analyzed to compare different numerical observations. The results obtained were compared statistically by using the standard statistical method given by Panse and Sukhatme (1995).

Results and Discussion

The non-volatile compounds produced by all five bioagents showed variable effect on the linear growth of the target pathogens (*D. bicolor*) at 10%, 25% and 50% concentration of culture filtrates. At 168 hrs (7^{th} day), all the bioagent treatments at 10%, 25% and 50% concentration of culture filtrates showed a statistically significant effect on linear growth of *D. bicolor* (from brinjal).

The data presented in Table 1 shows that all the treatments significantly inhibited the linear growth of *D. bicolor* at 10%, 25% and 50% concentration of culture filtrates (Plate 1). At 10%, 25% and 50% concentration of culture filtrates, the treatment T_6 (90.00 mm) recorded the highest linear mycelia growth of the test pathogen *D. bicolor*.

At 10% concentration of culture filtrates, among all the bioagents treatments, the treatment T_4 recorded the highest linear growth of the test pathogen *D. bicolor* (90.00 mm) followed by the treatment T_5 (79.16 mm) but treatment T_4 (90.00 mm) was at par with control treatment T_6 (90.00 mm) while the lowest linear growth of the test pathogen *D. bicolor* was recorded in treatment T_1 (60.26 mm) followed by the treatment T_2 (69.89 mm) but treatment T_2 (69.89 mm) was at par with treatment T_3 (73.43 mm). At 10% concentration of culture filtrates, among all the treatments, the treatment T_1 recorded the highest inhibition of the test pathogen *D. bicolor* (33.05%) followed by the treatment T_2 (22.35%) but treatment T_2 (22.35%) was at par with treatment T_3 (18.41%) while treatment T_4 (0.00%) was totally ineffective in inhibition of the test pathogen *D. bicolor* followed by the treatment T_5 (12.04%).

At 25% concentration of culture filtrates, among all the bioagents treatments, the treatment T_4 recorded the highest linear growth of the test pathogen *D. bicolor* (90.00 mm) followed by the treatment T_5 (78.25 mm) but treatment T_4 (90.00 mm) was at par with control treatment T_6 (90.00 mm) while the lowest linear growth of the test pathogen *D. bicolor* was recorded in treatment T_1 (54.45 mm) followed by the treatment T_2 (62.26 mm) and T_3 (70.35 mm). At 25% concentration of culture filtrates, among all the treatments, the treatment T_1 recorded the highest inhibition of the test pathogen *D. bicolor* (39.50%) followed by the treatment T_2 (30.82%) and T_3 (21.83%) while treatment T_4 (0.00%) was totally ineffective in inhibition of the test pathogen *D. bicolor* followed by the treatment T_5 (13.06%).

At 50% concentration of culture filtrates, among all the bioagents treatments, the treatment T_4 recorded the highest linear growth of the test pathogen *D. bicolor* (90.00 mm) but was at par with control treatment T_6 (90.00 mm) while the lowest linear growth of the test pathogen *D. bicolor* was recorded in treatment T_1 (39.93 mm) followed by the treatment T_2 (55.06 mm) but treatment T_2 (55.06 mm) was at par with treatment T_3 (56.63 mm). At 25% concentration of culture filtrates, among all the treatments, the treatment T_1 recorded the highest inhibition of the test pathogen *D. bicolor* (55.64%) followed by the treatment T_2 (38.82%) but treatment T_2 (38.82%) was at par with treatment T_3 (37.07%) while treatment T_4 (0.00%) was totally ineffective in inhibition of the test pathogen *D. bicolor* followed by the treatment T_5 (16.18%).

The present findings demonstrate that the non-volatile compounds contained within the culture filtrates of the five tested bioagents have a variable and concentration-dependent antagonistic effect on the mycelial growth of the phytopathogen, *D. bicolor*. This aligns with the well-established mechanism of antibiosis, where microbial antagonists produce inhibitory secondary metabolites that diffuse into the medium and suppress the growth of other organisms (Wirth & Wolf, 2008; Vinale *et al.*, 2008) [27, 25]. The observed statistically significant effect at all tested concentrations (10%, 25%, and 50%) after 168 hours indicates that the growth inhibition is directly attributable to the bioagents' metabolites and not random variation, a finding consistent with similar *in vitro* assays across numerous fungal systems (Chaudhary *et al.*, 2021; Yassin *et al.*, 2021) [4, 28, 29].

The results clearly establish that *B. bassiana* provided the highest inhibition of *D. bicolor* at all concentrations (33.05% at 10%, 39.50% at 25%, and 55.64% at 50%). This superior efficacy is demonstrated by the lowest linear growth recorded in T_1 across all dilutions. This finding is strongly supported by literature reporting the significant antifungal capabilities of *B. bassiana* culture filtrates against a diverse range of plant pathogenic fungi. *B. bassiana* is known to produce potent secondary metabolites, most notably beauvericin (a cyclic depsipeptide) and various bassianolides, which exhibit broad-spectrum antifungal properties (Wang & St. Leger, 2007; Cheio *et al.*, 2023) [26, 5]. For instance, studies have shown that metabolites from *B. bassiana* can effectively suppress the growth of pathogens like *Fusarium oxysporum* and *Rhizoctonia solani* with high inhibition percentages, mirroring the potent activity observed against *D. bicolor* here (Ownley *et al.*, 2008; Ghadge *et al.*, 2022) [18, 11]. The concentration-dependent increase in inhibition by *B. bassiana* from 33.05% to 55.64% as the filtrate concentration rose from 10% to 50% further confirms that the inhibitory mechanism is tied to the metabolite concentration in the growth medium (Feng *et al.*, 2021) [9].

The EPF, *N. rileyi* and *M. anisopliae* exhibited moderate but significant levels of inhibition, with *N. rileyi* generally showing higher activity than *M. anisopliae*. This difference in inhibitory potential is evident across the concentrations, where *N. rileyi* consistently showed lower mycelial growth than *M. anisopliae*, yet both were significantly better than the control. The antagonistic capacity of *N. rileyi* and *M. anisopliae*, both being entomopathogenic fungi, is attributed to their production of secondary metabolites such as destruxins and cyclosporin-like compounds by *M. anisopliae*, and various diketopiperazines and other bioactive compounds by *N. rileyi* (Karthick *et al.*, 2021;

Edelstein *et al.*, 2017) [14, 8]. Several researchers have noted the antifungal activity of *Metarhizium* culture filtrates against phytopathogens like *Curvularia clavata* and *Fusarium oxysporum*, albeit with varying degrees of success depending on the strain and target pathogen, which explains the moderate efficacy observed in the present study (Ravindran *et al.*, 2016; Sasan & Bidochka, 2013) [21, 22]. Similarly, while *N. rileyi* is primarily recognized as a biological insecticide, its metabolites have been reported to possess some antifungal capabilities, justifying the observed inhibition by *N. rileyi* (Mohamed *et al.*, 2006) [17].

The bioagent *T. viride* showed inhibition against *D. bicolor* (e.g., 16.18% at 50% concentration), but its efficacy was lower compared to *B. bassiana*, *N. rileyi* and *M. anisopliae*. This is noteworthy because *Trichoderma* species, particularly *T. viride*, are widely documented as highly effective biological control agents due to the production of non-volatile antibiotics such as gliotoxin, viridin, and various peptaibols (Harman *et al.*, 2004; Benitez *et al.*, 2004) [13, 3]. Literature frequently shows *T. viride* culture filtrates causing high levels of inhibition (>50%) against pathogens like *Rhizoctonia solani* and other *Drechslera* species (e.g., *D. oryzae*), suggesting that the specific strain used in this experiment or the concentration may have been less effective against this particular *D. bicolor* isolate, or that the active metabolites were not sufficiently produced under the given culture conditions (Singh *et al.*, 2017; Yassin *et al.*, 2021) [23, 28]. Nonetheless, the observed inhibition is a clear sign of antibiosis, which remains a key mechanism of this fungal species (Vinale *et al.*, 2006) [24].

Crucially, the results indicate that the culture filtrate of *V. lecanii* was totally ineffective in inhibiting the pathogen, recording 0.00% inhibition and a linear growth of 90.00 mm (at par with the control) at all concentrations. This result is contrary to some reports that demonstrate the potential of *V. lecanii* (now often referred to as *Lecanicillium lecanii*) to produce metabolites with antifungal activity, especially due to its production of cell-wall degrading enzymes like chitinases and beta-1,3-glucanases, which have been shown to inhibit fungi like *Oidium* spp. (Gao *et al.*, 2011; Barranco *et al.*, 2009) [10, 2]. However, its primary role is generally in insect control, and its effectiveness as a direct antagonist against plant pathogenic fungi via culture filtrate is highly strain-specific and target-pathogen-specific (Devi *et al.*, 2014; Ownley *et al.*, 2004) [6, 19]. It is possible that the specific metabolites produced by the *V. lecanii* strain, or the particular components of the *D. bicolor* cell wall, rendered its non-volatile compounds ineffective under the tested conditions. This highlights the importance of screening for the *fungistatic* or *fungicidal* potential of individual strains for effective biocontrol (Akutse *et al.*, 2013; Li *et al.*, 2021) [1, 16].

A clear concentration-dependent effect was observed for the effective bioagents (*B. bassiana, N. rileyi, M. anisopliae* and *T. viride*), where increasing the culture filtrate concentration from 10% to 50% resulted in a proportional increase in the percentage of inhibition. This strongly suggests that the concentration of the inhibitory secondary metabolites in the culture filtrate is the limiting factor for the antagonism, confirming the general principle of dose-response relationships in microbial interactions (Dubey *et al.*, 2012) [7]. The overall efficacy order in the current study was: *B. bassiana* > *N. rileyi* approx *M. anisopliae* > *T. viride* > *V. lecanii* approx Control (at 50% concentration). This differential response between bioagents against *D. bicolor* illustrates the biochemical diversity in metabolite production among different fungal species, an observation consistently reported in studies on fungal biocontrol (Prapagdee *et al.*, 2007; Lahlali *et al.*, 2017) [20, 15]. This research confirms the potent fungistatic activity of *B. bassiana* non-volatile metabolites against *D. bicolor*, indicating its high potential for use in a formulation as a natural fungicide for managing this pathogen, while simultaneously underscoring the necessity of strain and concentration optimization for all bioagents (Gupta *et al.*, 2017; Zhang *et al.*, 2017) [12, 30].

Table 1: The effect of non-volatile compounds produced by entomopathogenic fungi on *Drechslera bicolor* at 7th day

Sr. No.	Treatment	*Linear growth (mm) of *Drechslera bicolor* at 7th day			**Percent inhibition of *Drechslera bicolor* at 7th day		
		Culture filtrates of entomopathogenic fungi			Culture filtrates of entomopathogenic fungi		
		10%	25%	50%	10%	25%	50%
T_1	*Beauveria bassiana*	60.26 (7.76)	54.45 (7.38)	39.93 (6.32)	33.05 (35.09)	39.50 (38.94)	55.64 (48.24)
T_2	*Nomuraea rileyi*	69.89 (8.36)	62.26 (7.89)	55.06 (7.42)	22.35 (28.21)	30.82 (33.72)	38.82 (38.51)
T_3	*Metarhizium anisopliae*	73.43 (8.57)	70.35 (8.39)	56.63 (7.51)	18.41 (25.41)	21.83 (27.84)	37.07 (37.39)
T_4	*Verticillium lecanii*	90.00 (9.49)	90.00 (9.49)	90.00 (9.49)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T_5	*Trichoderma viride*	79.16 (8.90)	78.25 (8.84)	75.44 (8.69)	12.04 (20.06)	13.06 (20.98)	16.18 (23.71)
T_6	Control	90.00 (9.49)	90.00 (9.49)	90.00 (9.49)	---	---	---
	S.E.d	0.08	0.09	0.23	1.56	1.56	2.54
	C.D. @ 1%	0.25	0.27	0.71	4.94	4.96	8.06

Note: *Figures in parenthesis are square root transformation (√n+1) values
**Figures in parenthesis are arc sin values

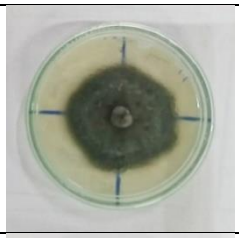
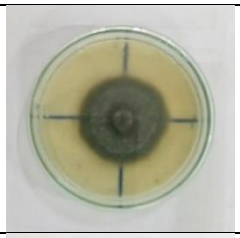
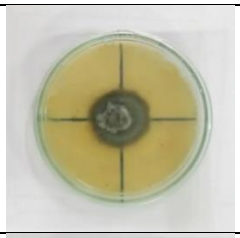
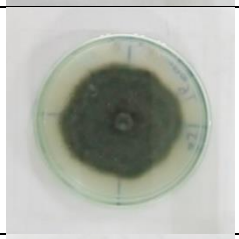
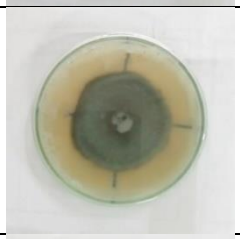
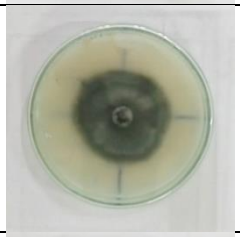
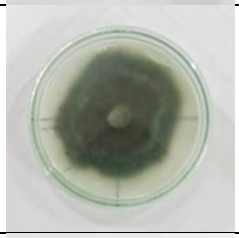
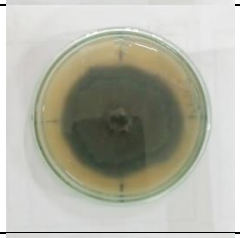
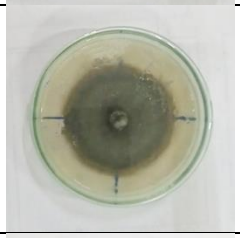
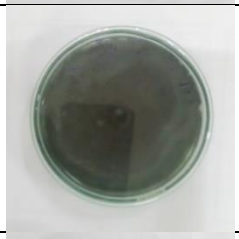
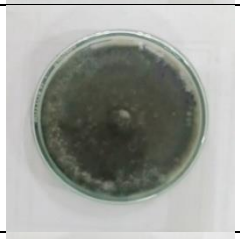
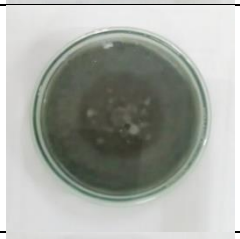
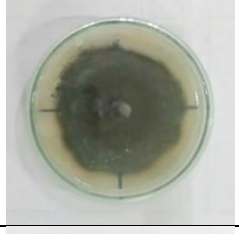
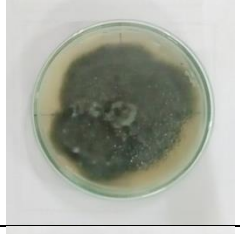
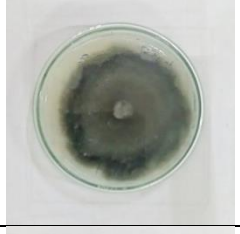
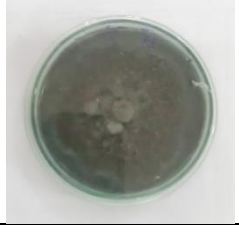
Treatments	Concentration of EPF culture filtrates		
	10%	25%	50%
T_1: *Beauveria bassiana*			
T_2: *Nomuraea rileyi*			
T_3: *Metarhizium anisopliae*			
T_4: *Verticillium lecanni*			
T_5: *Trichoderma viride*			
T_6: Control			

Plate 1: The effect of non-volatile compounds produced by entomopathogenic fungi on fungal foliar pathogen *Drechslera bicolor* at 10%, 25% and 50% concentration of culture filtrates

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