Biocontrol efficacy of *Trichoderma harzianum* and *Trichoderma virens* against different soil-borne fungal pathogens

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ABSTRACT

An experimental investigation was carried out to evaluate the biocontrol efficacy of two *Trichoderma* species on pathogenic fungi isolated from the rhizospheric soil. Experiments revealed the beneficial interactions between the test biocontrol agents against the isolated pathogenic fungi. Two *Trichoderma* species *viz. T. harzianum*, and *T. virens* were assessed for their biological control efficiency against ten different pathogenic fungi *viz. Alternaria alternata, Chaetomium cochliodes, Cladosporium cladosporoides, Colletotrichum fructicola, Curvularia lunata, Fusarium oxysporum, F. ventrichosum, F. proliferatum, F. solani* and *Mucor hiemalis*. *T. harzianum* exhibited the highest mycelial growth inhibition against *F. solani* (71%), followed by *C. cochliodes* and *C. lunata* (70%), *F. ventrichosum* (69%), and *C. fructicola* (68%). Moderate inhibition was recorded against *F. oxysporum, A. alternata*, *C. cladosporioides*, and *F. proliferatum* (58–65%), while the lowest inhibition (44%) was observed against *M. hiemalis*. Similarly, *T. virens* showed maximum mycelial growth inhibition against *C. fructicola* (80%), followed by *F. oxysporum* (75%), *C. cochliodes* and *C. lunata* (72%), and *F. proliferatum* (70%). Inhibition against *F. solani*, *A. alternata*, *F. ventrichosum* and *C. cladosporoides* ranged from 59–68%, while the lowest inhibition was observed against *M. hiemalis* (54%). The study also assessed parameters such as the time to colony contact, overgrowth, and plate coverage by the biocontrol agent, pathogen radial growth, resistance, and Pakdaman's Biological Control Index (PBCI). These attributes collectively also revealed the significant biocontrol efficacy of *T. virens* and *T. harzianum* against the tested soil-borne fungal pathogens.

Keywords: Dual culture; confrontation; *Trichoderma*; growth inhibition; pathogen resistance

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INTRODUCTION

Fungal pathogens pose a major threat to all agricultural crops, often leading to substantial environmental degradation and economic losses (Agrios, 2009). Over three to four decades, plant pathogens have caused tremendous loss of crops worldwide (Oerke, 2006). Most of the diseases in plants occurring in horticultural as well as agricultural crops are caused by pathogenic fungi (Agrios, 2009).

Plant fungal pathogens are generally managed by synthetic/chemical fungicides (Ul Haq *et al.*, 2020). However, excessive and improper use of these chemicals over decades has disrupted natural environment and resulted in development of pathogen resistance (Gianessi and Reigner, 2006; Nega, 2014; Patel *et al.*, 2014; Kourgialas, 2017; EFSA BIOHAZ Panel *et al.*, 2021; Pathak *et al.*, 2022; Salam *et al.*, 2023; Muñoz-Bautista *et al.*, 2025). With the increasing concerns about the ground water pollution, ecological contamination and ill health as a consequence of agricultural chemicals, there is a dire need of sustainable and environmentally friendly alternatives for fungal

disease management (Stenberg *et al.,* 2021). This paved the way to the use of biocontrol agents as a promising strategy to manage fungal infections (Singh *et al.,* 2020). Biological control harnesses the power of nature by utilizing beneficial organisms such as bacteria, fungi, viruses, and predators to suppress pathogenic fungi (Van Lenteren, 2018; Van den Bosch *et al.,* 2019; Hassan *et al.,* 2022). Fungal biocontrol agents have proven to be highly effective tools in management of fungal diseases of plants (Gawai, 2018; Jan *et al.*, 2022). The most widely utilized fungal biocontrol agents belong to the genus *Trichoderma*, which constitute a major group of rhizosphere microorganisms employed in biological control (Abdul-Halim *et al.,* 2023). Several *Trichoderma* species have also been developed and commercialized as biopesticides and biofertilizers (Ciancio *et al.,* 2019; Alfiky, 2021; Abdul-Halim *et al.*, 2023).

Trichoderma, a member of the ascomycetous fungi is ubiquitous fungal genus found in diverse soil types as plant symbiont, saprotroph, and acts mycoparasite helps to mitigate unfavourable growth conditions and improve plant growth (Alfiky, 2021). Many *Trichoderma* species have been identified as beneficial, non-pathogenic partners for various plants, forming endophytic, mutualistic, symbiotic and saprotrophic relationships (Harman *et al.,* 2004; Alfiky, 2021). Moreover, they exhibit biocontrol capabilities against plant fungal pathogens, compete and parasitize them (mycoparasitism), showcasing their potential as effective biocontrol agents which has been extensively investigated and are currently utilized in agriculture for plant protection (Pfordt *et al.,* 2020; Poveda, 2021; Hassan *et al.,* 2022). *Trichoderma* species also exhibit a property to stimulate development and growth in plants (Abdul-Halim *et al.,* 2022). *Trichoderma* species exhibit strong ability to readily colonise in the rhizospheric zone of plants, and can extend their persistence for longer periods even up to several months (Tyśkiewicz *et al.,* 2022). This potential is a key attribute that also enhances their effectiveness as biocontrol agents. The persistent and aggressive behaviour of *Trichoderma* makes them highly competitive and suppressive toward other soil microorganisms, thereby enhancing their biocontrol efficacy (Vinale *et al.,* 2008). *Trichoderma* species have several biocontrol mechanisms which include parasitism, antibiosis, host-plant resistance induction, and competition for nutrition and space while confrontation with fungal pathogens (Howell, 2003; Mayo, 2015). *Trichoderma* species contain a wide range of metabolites which enable them to use diverse and complex nutrient sources especially the Carbon and Nitrogen sources which prevent competing pathogenic fungi from further spreading and growing in the surrounding environment. (Guzmán-Guzmán *et al.,* 2023).

Owing to the importance of *Trichoderma*, the quantification of their impact on the growth of pathogenic fungi becomes very important in the local temperate soil types with abundant moisture content. Although *Trichoderma* species are well-documented for their antagonistic potential against various phytopathogens, comparative studies focusing on the detailed biocontrol performance of *T. harzianum* and *T. virens* against multiple soil-borne fungal pathogens under uniform experimental conditions are limited. Hence, there exists a gap in understanding the comprehensive biocontrol dynamics and relative efficacy of these *Trichoderma* species. Furthermore, insufficient information exists on their specific interaction patterns and degree of antagonism against locally prevalent pathogenic fungi, creating a need for detailed investigation. Keeping these aspects in view, confrontation experiments were carried out to extensively analyse the biocontrol ability of *Trichoderma*. In the current study, two *Trichoderma* species *viz. T. harzianum*, and *T. virens* were evaluated for their biological control efficiency against ten different pathogenic fungi *viz. Alternaria alternata, Chaetomium cochliodes, Cladosporium cladosporoides, Colletotrichum fructicola, Curvularia lunata, Fusarium oxysporum, F. ventrichosum, F. proliferatum, F. solani* and *Mucor hiemalis*.

MATERIALS AND METHODS

In the current study, confrontation study was carried out between two *Trichoderma* species against ten pathogens to analyse the biocontrol efficacy of *Trichoderma* species.

Fungal Cultures

Fungal isolates of soil-borne pathogens like *A. alternata, C. cochliodes, C. cladosporoides, C. fructicola, C. lunata, F. oxysporum, F. ventrichosum, F. proliferatum, F. solani* and *M. hiemalis* were isolated from the rhizospheric soil samples of *Berberis lycium* collected from three locations *viz.* Sinthan Pass (33°34′47.84″ N, 75°30′39.34″ E; 3277 masl), Aharbal (33°39′13.33″ N, 74°47′35.45″ E; 2231 masl), and Gulmarg (34°02′54.13″ N, 74°22′49.72″ E; 2745 masl) in Kashmir Himalaya. Isolates of *Trichoderma* species *viz*. *T. harzianum*, and *T. virens* were isolated from the rhizospheric soil of *Bergenia ciliata.* The fungal pathogens as well as *Trichoderma* species were cultured on Potato dextrose Agar (PDA) medium and kept at 4°C for further use.

Dual Culture (Confrontation) Experiments

T. virens, and *T. harzianum* were assessed for their effectiveness against fungal pathogens using the dual culture confrontation technique, as defined by Sonnenbichler *et al*., in 1983. During the present experiment, 9 cm Petri plates were filled with 20 ml of sterile PDA. These Petri plates (9 cm) were then inoculated with a 5mm mycelial disc of one week old pure culture of both the biocontrol agent (*Trichoderma* sp.) and the pathogenic fungi. A mycelial disc of both antagonist and pathogen was positioned at opposite ends of the plates containing PDA and placed in BOD incubator at a temperature of 25±1°C for 3,5 and 7 days. After incubation the growth of the pathogen colony was measured on the third, fifth, and seventh day. Similar set of plates containing PDA inoculated with the pathogens only and the other set of plates containing antagonist only served as control. Each treatment was having three replicates.

Radial mycelial growth inhibition percentage of pathogenic fungi was calculated. Four other parameters were also determined per interaction i.e., the time period in days post-inoculation till the colonies of biocontrol agent and pathogen came in contact (C); till the biocontrol agent fully overlap a pathogen colony (Z); till the fungal biocontrol agent colony fill the whole plate (M) and the growth of radial pathogenic colony distance (in mm) between the margin of pathogen inoculation disc and the marginal point of the colony radiating towards the center of inoculation disc of biocontrol agent in the same plate (P).

A parameter for pathogen resistance (R) to the biocontrol agent was determined based on time periods required for the full fungal biocontrol agent growth in the presence (Z) and absence (M) of pathogenic fungi. R (without dimensions) was defined as the ratio as $R = \frac{Z}{\mathrm{M}}$ by Pakdaman *et al.* (2013). With the increase of pathogen resistance as well as pathogen colony growth, the success of biological control decreases. Therefore, a new index known as Pakdaman's biological control index was also determined to check the biocontrol efficacy of antagonists against different fungal pathogens. Pakdaman's biological control index (PBCI) was defined as combining temporal parameters of biocontrol agents and growth parameters of pathogens. PCBI was calculated using the equation, $\mathrm{PBCI} = \frac{\mathrm{M}}{Z.P}$(Pakdaman *et al.*, 2013). The dimension of the PBCI is L^{-1} (with the unit of mm^{-1})

Data analysis

Least Significant Difference (LSD) test was carried out to determine whether the observed difference between the means of the growth inhibition due to the two *Trichoderma* species is statistically significant.

RESULTS

The results (Table 1) in dual culture confrontation technique revealed that growth inhibition of the test soil-borne pathogens *viz. A. alternata, C. cochliodes, C. cladosporoides, C. lunata, C. fructicola, F. oxysporum, F. ventrichosum, F. solani, F. proliferatum,* and *M. hiemalis* by treating with two *Trichoderma* species showed significant percentage inhibition in mycelial

Table 1. Percentage growth inhibition of all test pathogenic fungi by *T. harzianum* and *T. virens* **149**

Pathogens	Control			*T. harzianum*				*T. virens*			
	Growth (cm)			Growth (cm)			Growth Inhibition (%) (7 day)	Growth (cm)			Growth Inhibition (%) (7 day)
	3 day	5 day	7 day	3 day	5 day	7 day		3 day	5 day	7 day	
Chaetomium cochliodes	1.80	3.00	4.20	2.06	2.33	1.27	70.00 ± 1.67	2.07	2.33	1.17	72.00 ± 0.99
Fusarium ventrichosum	1.20	4.90	5.90	1.10	2.43	1.80	69.00 ± 1.33	1.23	2.00	2.00	66.00 ± 1.03
Fusarium proliferatum	1.80	4.80	6.00	0.87	2.47	2.50	58.00 ± 1.27	1.10	1.80	1.80	70.00 ± 1.55
Curvularia lunata	2.70	3.40	5.50	2.00	2.07	1.67	70.00 ± 1.62	1.83	1.77	1.57	72.00 ± 1.63
Fusarium oxysporum	3.00	4.50	5.70	1.97	2.13	2.00	65.00 ± 0.59	1.97	2.00	1.40	75.00 ± 1.06
Fusarium solani	2.70	4.20	5.60	1.97	2.13	1.63	71.00 ± 1.33	1.83	2.40	1.80	68.00 ± 0.77
Colletotrichum fructicola	2.50	3.60	5.00	1.90	2.53	1.60	68.00 ± 0.91	1.23	1.10	1.00	80.00 ± 0.95
Alterneria alternata	2.30	5.50	6.30	2.00	2.37	2.47	61.00 ± 1.23	1.93	2.13	2.07	67.00 ± 0.95
Mucor hiemalis	4.80	5.90	7.20	3.30	3.40	4.07	44.00 ± 1.06	3.70	3.83	2.93	54.00 ± 1.12
Cladosporium cladosporoides	2.60	3.40	3.80	2.07	1.80	1.57	59.00 ± 1.23	2.00	2.03	1.77	60.00 ± 0.95

growth of all the tested fungal pathogens. Both the *Trichoderma* species used in the present study were found to be effective against all the test pathogenic fungi.

The results of the dual culture confrontation experiments (Table 1) revealed that both the antagonistic fungal species significantly inhibited mycelial growth of the tested fungal pathogens. After 3, 5 and 7 days of incubation results revealed that *T. harzianum* caused maximum mycelial growth inhibition of *F. solani* followed by *C. cochliodes*, and *C. lunata*, *F. ventrichosum*, and *C. fructicola*. However, *T. harzianum* caused least mycelial growth inhibition (44%) in *Mucor hiemalis*. Similarly, observations after 3, 5 and 7 days of incubation revealed that *T. virens* caused maximum growth inhibition of *C. fructicola* (80%) followed by *F. oxysporum* (75%), *C. cochliodes* (72%), *C. lunata* and *F. proliferatum* (70%). Mycelial growth inhibition of other fungal pathogens *viz. F. solani*, *A. alternata*, *F. ventrichosum* and *C. cladosporoides* was *68%*, 67%, 66% and 59% respectively. *T. virens* was also found to be least effective against *Mucor hiemalis* where mycelial growth inhibition was only 54%. On comparing *T. virens* and *T. harzianum*, it was revealed from current results that *T. virens* show more inhibition against all the tested pathogenic fungi except for *F. ventrichosum* and *F. solani* than *T. harzianum* (Table 1).

In addition to the mycelial growth inhibition and the antagonistic efficacy of these by biocontrol fungi against different fungal pathogens was further determined by calculating the biological control index (Table 2).

On comparing the C values (Table 2) it was found that both *T. harzianum* and *T. virens* isolates grow with equal speed in towards the pathogens. However, the Z values (Table 2) indicate that *T. virens* grows over pathogen in lesser time periods than *T. harzianum*. Similarly, P values (Table 2) also indicate that treatments containing *T. virens* as antagonist show more growth inhibition (smaller pathogen colonies) than the treatments containing *T. harzianum*. M values (Table 2) also show the faster growth of *T. virens* than *T. harzianum*. R=1 (calculated as R=Z/M) indicate the same ability of

Table 2. Effect of *T. harzianum* and *T. virens* on different parameters of pathogenic fungi

Pathogens	*T. harzianum*						*T. virens*					
	C*	Z*	P(mm)*	M*	R*	PBCI*	C*	Z*	P(mm)*	M*	R*	PBCI
Chaetomium cochliodes	3.00	7.00	6.50	7.00	1.00	0.15	3.00	5.00	5.00	6.00	0.83	0.24
Fusarium ventrichosum	3.00	5.00	11.50	6.00	0.83	0.10	3.00	5.00	7.50	7.00	0.71	0.19
Fusarium proliferatum	4.00	9.00	16.00	9.00	1.00	0.06	3.00	7.00	9.00	7.00	1.00	0.11
Curvularia lunata	5.00	8.00	8.00	8.00	1.00	0.13	5.00	7.00	7.50	7.00	1.00	0.13
Fusarium oxysporum	2.00	6.00	9.50	6.00	1.00	0.11	2.00	6.00	10.00	6.00	1.00	0.10
Fusarium solani	3.00	7.00	10.00	7.00	1.00	0.10	3.00	7.00	8.50	7.00	1.00	0.12
Colletotrichum fructicola	3.00	7.00	8.00	7.00	1.00	0.13	3.00	7.00	5.00	7.00	1.00	0.20
Alternaria alternata	5.00	7.00	12.50	7.00	1.00	0.08	5.00	7.00	10.00	7.00	1.00	0.10
Mucor hiemalis	3.00	10.00	14.00	6.00	1.67	0.04	3.00	9.00	14.00	6.00	1.50	0.05
Cladosporium cladosporoides	4.00	7.00	7.00	7.00	1.00	0.14	4.00	7.00	8.00	7.00	1.00	0.13

*Time to colony contact of pathogen and biocontrol agent, C(days); Time till the biocontrol agent fully overlap a pathogen colony, Z(days); Pathogen radial growth in presence of biocontrol agent, P(mm); Plate coverage by the biocontrol agent in absence of pathogen, M(days); Pathogen resistance, R and Pakdaman's Biological Control Index, PBCI (mm^{-1}) values of all the tested fungi using *T. virens* and *T. harzianum*

antagonist growth in the absence of the fungal pathogen as well as in the presence of pathogen on PDA. R < 1 (Table 2) as in case of *F. ventrichosum* indicate more ability of both the antagonists i.e. *T. harzianum* and *T. virens* in presence of a fungal pathogen in comparison to its growth on a medium in absence of fungal pathogen and because of growth encouraging effect of a pathogen could be used as an easy target to control the pathogen. R > 1 in case of *Mucor hiemalis* indicate week ability of both the antagonists i.e. *T. harzianum* and *T. virens* in presence of a pathogen in comparison to its growth on a medium in absence of pathogen and because of growth inhibiting effect of a pathogen could not be used as an easy target to control the pathogen. Comparison of PBCI values also indicate that *T. virens* is more effective than *T. harzianum* for management of fungal pathogens like *Chaetomium cochliodes, Fusarium ventrichosum, F. proliferatum, F. solani, Colletotrichum fructicola* and *Alternaria alternata.* In the LSD test, the difference of mean i.e. 0.48 was found to be lower that the *LSD* value of 0.74 at the 5% level of significance ($p < 0.05$). Hence it can be deduced that the difference between the biocontrol efficacy of *T. harzianium* and *T. virens* is statistically insignificant which means that both the species of *Trichoderma* used in the present study are almost equally effective against the fungal pathogens.

DISCUSSION

The antagonistic potential of *Trichoderma harzianum* and *T. virens* was evaluated through dual culture assays against ten soil-borne pathogenic fungi under in vitro conditions. Their effects were measured in terms of mycelial growth inhibition and Pakdaman's biocontrol index. The

findings indicated that both *Trichoderma* species exhibited strong and significant inhibitory effects against all the tested fungal pathogens. *Trichoderma* species employ multiple defense strategies, including mycoparasitism and antibiosis, to protect plants from phytopathogenic fungi. They achieve this protection by producing a wide range of secondary metabolites and by triggering plant resistance and defense responses (Sood *et al.,* 2020; Yassin *et al.,* 2022). In addition to synthesizing potent antibiotics, *Trichoderma* spp. are known to produce mycotoxins and over a hundred bioactive metabolites with antimicrobial properties (Sood *et al.,* 2020; Hamrouni *et al.,* 2021). Certain *Trichoderma* species produce specific enzymes that enable them to effectively target particular plant pathogens (Bastakoti *et al.,* 2017). Considering overall mycelial growth inhibition by test fungal biological control agents, *T. virens* was found to be more effective against *C. fructicola* causing 80% mycelial growth inhibition and least effectiveness against *M. hiemalis* causing only 54% mycelial growth inhibition. Similar results of antagonism using *T. virens* as biocontrol agent were found against different fungal pathogens like *F. oxysporum, C. gloeosporioides* and *Lasiodiplodia theobromae* (Buensanteai and Athinuwat, 2012; Dissanayak *et al.,* 2021). *T. virens* produces gliotoxin, an antibiotic that effectively suppresses various plant pathogens (Maurya, 2020). The inhibition mechanism of *T. virens* also involves the secretion of lytic enzymes such as β-1,3-glucanase, chitinase, which degrade the cell wall components (mainly β-1,3-glucan) of pathogenic fungi (Ghasemi *et al.,* 2020). This enzymatic hydrolysis weakens and lyses the pathogen's cell wall, leading to its growth inhibition and eventual death (Buensanteai and Athinuwat, 2012). Similarly, *T. harzianum* caused 71% mycelia growth inhibition in *Fusarium solani* and only 44% mycelial growth inhibition in *Mucor heimalis*. Our studies are in conformity with Gveroska and Ziberoski (2012) and Bunbury-Blanchette and Walker (2019) who also reported more efficacy of *T. harzianium* against *Alternaria* and *Fusarium*. The inhibition of these pathogens by *T. harzianum* is primarily due to its rapid growth, competition for space and nutrients, and production of diffusible and volatile antifungal metabolites. Additionally, mechanisms such as mycoparasitism, antibiosis, and secretion of hydrolytic enzymes (e.g., chitinase) contribute to hyphal deformation, reduced sporulation, and suppression of pathogen germination, leading to strong antagonistic activity (Gveroska and Ziberoski, 2012).

Quantifying the confrontation assay was essential to obtain measurable data for comparing two different fungal biocontrol isolates. Without such numerical data, accurate analysis would not be possible. Therefore, a new biological control index i.e., PBCI was used, based on the radial growth of the pathogen in the presence of a biocontrol agent (P), serving as a parameter to assess the pathogen's resistance to the applied antagonist. Time was also incorporated into the concept of R, representing the level of resistance exhibited by the phytopathogenic fungus against the biological control mechanism. Comparing the results C, P, and Z values with the result values observed of PBCI, it is revealed that none of those C, P, and Z parameters can accurately signify the biocontrol efficacy level. However, PBCI and R values can accurately signify the biocontrol efficacy level. Our results show that PBCI can be considered as a proper criterion useful in confrontation tests. R values were calculated to check the resistance of pathogen, wherein R value more than 1 indicate pathogen resistance to biocontrol mechanisms used by a biocontrol agent in battle with a fungal pathogen. R =1indicates that pathogenic fungi does not have any resistance against biocontrol mechanism. $R < 1$ does not only indicate that a pathogen has no resistance, but also shows that it promotes the development and growth of a biocontrol agent. In our present study, R values less than 1 were observed in *Fusarium ventrichosum* which indicate growth promoting effect of a pathogen on both the antagonists i.e. *T. harzianum* and *T. virens* and therefore could be used as an easy target to control this pathogen. R values more than 1 as in case of *Mucor hiemalis* indicate growth inhibiting effect of a pathogen on

antagonists i.e. *T. harzianum* and *T. virens* and therefore cannot be recommended for the management of this pathogen.

The antagonistic fungi act differentially against different plant pathogenic fungi and limited the pathogen colony growth. *T. virens* showed slightly more effectiveness than *T. harzianum* against all the test fungi except for *Fusarium ventrichosum* and *Cladosporium cladosporoides.* Comparison of PBCI values also indicate that *T. virens* is slightly more effective than *T. harzianum* in the biocontrol of most of the tested pathogens. However, in the Least Significant Difference (LSD) test, the difference of mean i.e. 0.48 was found to be lower that the LSD value of 0.74. Hence, it can be inferred from this statistical analysis that the difference between the biocontrol efficacy of *T. harzianium* and *T. virens* is statistically insignificant which means that both the species of *Trichoderma* used in the present study are almost equally effective against the tested fungal pathogens.

Author Contributions

MJ conceptualised the study. MJ and ZS devised the methodology, carried out experimental work and analysed the results. MJ, AHW and MYB contributed to writing the manuscript. All authors have reviewed and accepted to publish the manuscript.

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Declarations

Conflict of Interest: The authors declare that there is no conflict of interest and also declare that the manuscript does not infringe any personal or other copyright or property rights.

Consent to Publication: All authors have significantly contributed to the study and have given their approval for publication.

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