ORIGINAL ARTICLE

Fungicide-Specific Responses of *Trichoderma viride*: Implications for Integrated Disease Management

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Abstract

Background: Compatibility of chemical fungicides with beneficial soil fungi is an important factor in sustainable plant disease management. **Aims:** In this study, the impact of four commonly used fungicides, mancozeb, carbendazim, propiconazole, and hexaconazole, on conidial germination, radial growth, and fungicide tolerance of three *Trichoderma viride* strains (microbial type culture collection [MTCC] 793, MTCC 800, and a commercial biofungicide isolate, T1) was assessed. **Methodology:** Fungicides were tested at ×0.1, ×1, and ×10 concentrations of the recommended label dose. **Results:** Carbendazim inhibited germination and mycelial growth completely at all concentrations, while propiconazole and hexaconazole showed pronounced dose-dependent inhibition. Mancozeb was found to be less toxic, supporting growth and germination at all strains and concentrations. Strain-specific variations in tolerance were also noted, with the commercial isolate showing relatively higher tolerance. **Conclusion:** This study reveals the presence of pronounced fungicide-specific risks to beneficial *Trichoderma* populations and suggests mancozeb as the most compatible fungicide for integrated chemical-biological disease management approaches. This approach is aligned with the sustainable development goals (SDG) like zero hunger, responsible consumption and production and life on land corresponds to SDG 2, 12 and 15, respectively.

Key words: Conidial germination, fungicide compatibility, integrated disease management, radial growth, *Trichoderma viride*

INTRODUCTION

The growth of the chemical industry has resulted in the synthesis of tens of thousands of chemical compounds, of which fungicides are a prominent class that has been widely used in modern agriculture. Fungicides are biocidal chemical substances or biological organisms that are used to inhibit or reduce fungi and fungal spores, and they are an important tool in the control of plant diseases and the enhancement of crop productivity.[1] Fungicides are used as contact, translaminar, or systemic formulations based on their uptake and translocation in plants.[2]

However, fungicides inevitably find their way into the soil ecosystem by spray drift, runoff, leaching, and residue buildup. Phytopathogenic fungi are very hard to control due to the presence of resistant resting structures such as sclerotia, chlamydospores, and oospores, which require multiple applications of fungicides.[3] Despite the effectiveness of fungicides in managing pathogens, their impact on non-target soil microorganisms is not taken into account.[4,5] The persistence of fungicide residues in soil and water can cause harmful effects on soil microbial populations and ecosystem processes.[6,7]

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Soil microbes are very important in the sustainability of soil fertility and ecosystem equilibrium, particularly in carbon, nitrogen, and phosphorus cycles.[8] The disturbance of soil microbial populations by fungicides might affect these processes and reduce soil health.[9] Because fungicides are generally non-selective in their mechanism of action, they might affect beneficial fungi that share similar biochemical processes with phytopathogens.[10]

Among the useful soil fungi, the genus *Trichoderma* is of prime importance. *Trichoderma* species are ubiquitous filamentous fungi that are commonly found in agricultural soils, where they exist as plant symbionts, mycoparasites, and biocontrol agents.[11,12] *Trichoderma viride* has been known for its plant growth-promoting properties, phytopathogenic fungal and nematode inhibitory effects, soil fungal population control, and xenobiotic compound degradation.[13,14] However, the liberal and persistent use of chemical fungicides might decrease the natural antagonistic potential of *Trichoderm*.[15,16] Unlike many previous studies, which evaluated the compatibility of fungicides with a single strain of *Trichoderma* or a single biological parameter, the current study provides a comparative evaluation of four commonly used fungicides at different concentrations with three different strains of *T. viride*, including a biofungicide isolate that is formulated as a commercial biocontrol agent. Since the current study considers all three biological parameters together, it provides a more holistic understanding of the results. The current study will provide important information regarding the selection of fungicides that are compatible with *Trichoderma*-based biological control agents.

Effective and sustainable methods of crop protection must therefore achieve a balance between the control of pathogens and the conservation of beneficial soil microbiota. This strategy is directly in line with the United Nations' Sustainable Development Goals (SDG), specifically SDG-2: Zero Hunger, which stresses the importance of sustainable food production systems; SDG 12: Responsible Consumption and Production, which encourages environmentally responsible management of chemicals; and SDG 15: Life on Land, which urges the conservation of terrestrial ecosystems and soil biodiversity. The compatibility of chemical fungicides and biological control agents such as *T. viride* is important in the development of evidence-based strategies that will reduce ecological risks and damage while maintaining agricultural productivity.

MATERIALS AND METHODS

Fungal strains

Three strains of *T. viride* (microbial type culture collection [MTCC] 793, MTCC800 and T1) were used in the study. *T. viride* MTCC793 and MTCC800 were obtained from the MTCC, Institute of Microbial Technology, Chandigarh, India. The T1 *T. viride* strain was recovered from a commercial biofungicide formulation (Agro Derma; Sun Agro Biosystem Private Limited, Chennai, India) through repeated subculturing on potato dextrose agar (PDA; HiMedia, India) and was designated as T1. All strains were maintained on PDA slants at 4°C and subcultured periodically. Cultures were incubated on PDA at 32 ± 1°C for routine growth and experimentation. Details of the fungal strains used in the study are provided in Table 1.

Fungicides

Four fungicides mancozeb, propiconazole, hexaconazole, and carbendazim were used in this study. Recommended rates were selected based on manufacturer label instructions expressed as active ingredient equivalents. The fungicides were tested at three concentrations: One-tenth of the manufacturer's recommended rate (×0.1 Rr), the recommended rate (×1 Rr), and ten times the recommended rate (×10 Rr). The stock solutions were prepared using sterile distilled water, and the desired concentrations were obtained by appropriate dilutions. The concentrations used for each fungicide are presented in [Table 2]. The concentrations used enable the evaluation of Trichoderma responses to real and stressful conditions, which include sub-lethal exposure (×0.1), field-recommended concentrations (×1), and potential over-exposure (×10) levels.

Effect of fungicides on conidial germination

Preparation of conidial suspension

Conidia were collected from 10-day-old cultures grown on PDA supplemented with 1% yeast extract at 32 ± 1°C. Ten milliliters of sterile distilled water containing 0.01% Tween 80 were added to each culture plate, and the agar surface was carefully scraped using a sterile loop. The conidial suspension was filtered through two layers of sterile muslin cloth to remove mycelial fragments and then vortexed for 15 min

Table 1: Details of the *Trichoderma viride* strains used in the study

Fungi	Strain number	Substrate/source	Geographical location
Trichoderma viride	MTTC 793	Soil	Pantnagar, Uttar Pradesh, India
	MTCC 800	Soil	Pantnagar, Uttar Pradesh, India
	T1	AGRO DERMA[a]	N/A

MTCC: Microbial type culture collection, N/A: No data available, [a]Products from Product from Sun Agro Biosystem Private Limited; AGRO DERMA[a] were designated as T1

Table 2: Different concentration of fungicides used in the study

Fungicides	Fungicides concentration		
	×0.1 Rr	×1 Rr	×10 Rr
Mancozeb	0.2 mg/mL	2 mg/mL	20 mg/ml
Propiconazole	0.025 µL/mL	0.25 µL/mL	2.5 µL/mL
Carbendazim	0.0001 µg/mL	0.001 µg/mL	0.01 µg/mL
Hexaconazole	0.05 µL/mL	0.5 µL/mL	5 µL/mL

to get a uniform conidial suspension. The concentration of conidia was measured using an improved Neubauer hemocytometer under a compound microscope at ×40 magnification and made up to 1×10^7 conidia per milliliter.

Germination assay

PDA was then prepared, autoclaved at 121°C for 15 min, and cooled to around 50°C. Fungicide solutions with varying concentrations were well mixed with melted PDA to achieve the desired concentrations. The plates without fungicides were the control. The medium was then poured into sterile 90 mm Petri dishes and left to solidify at room temperature. Each plate was inoculated with 0.5 mL of conidial suspension (1×10^7 conidia mL^{-1}) and spread evenly using a sterile glass spreader. Plates were incubated at 32 ± 1°C for 24 h. Following incubation, conidia were stained with lactophenol cotton blue, and four agar sections (10 mm^2 each) were examined microscopically. Germination percentage was determined by counting 100 conidia per sample; a conidium was considered germinated when the germ tube length exceeded the width of the conidium. Each treatment was replicated three times. The germination rate was expressed in percentage.[17]

Effect of fungicides on radial growth of the T. viride

The effect of fungicides on mycelial growth was assessed using the poisoned food technique. PDA amended with different fungicide concentrations was poured into 90 mm Petri dishes. Mycelial discs (5 mm diameter) obtained from the margins of 5-day-old, actively growing cultures were placed at the center of each plate with the mycelium facing downward. Control plates contained PDA without fungicides.

Plates were sealed with parafilm and incubated in darkness at 32°C. Radial growth was measured every 24 h along two perpendicular diameters until the control plates were fully covered or for a maximum of 10 days. Mycelial growth rate was expressed as mm day^{-1}. Five replicates were maintained for each treatment.[18]

Antifungal activity (tolerance test)

Fungicide tolerance of *T. viride* strains was evaluated using the agar well diffusion method. PDA plates were uniformly inoculated with 1×10^7 conidia mL^{-1} using a sterile cotton swab. After 1 h, four wells (5 mm diameter) were punched equidistantly using a sterile cork- borer. Wells were filled with 25 µL of sterile distilled water (control) or fungicide solutions at ×0.1, ×1, and ×10 Rr. Plates were incubated at 32C° for 48 h, and zones of inhibition were measured in millimeters. Each treatment was replicated 3 times.[19] The agar well diffusion assay was included to complement radial growth measurements by providing an additional qualitative and quantitative assessment of fungicide diffusion effects and strain-specific tolerance under direct exposure conditions.

Statistical analysis

Data were tested for normality and homogeneity of variance before analysis. One-way analysis of variance (ANOVA) was performed separately for each strain and fungicide concentration using Minitab® 16 software. Mean comparisons were conducted using Tukey's honestly significant difference test at $P \leq 0.05$. Results are expressed as mean ± standard deviation.

RESULTS

Effects of fungicides on the germination of *T. viride*

All tested fungicides significantly affected the conidial germination of *T. viride* in a concentration-dependent manner across all strains (MTCC 793, MTCC 800, and T1). Carbendazim caused complete inhibition of conidial germination at all tested concentrations (×0.1, ×1, and ×10 recommended rate) in all strains. No germinated conidia were observed in any carbendazim-treated plates.

Propiconazole and hexaconazole demonstrated partial inhibition of conidial germination at lower concentrations, while complete inhibition was noticed at higher concentrations. However, the extent of inhibition increased with the increase in fungicide concentration in all strains. Mancozeb demonstrated the lowest level of inhibition of conidial germination. Germination was noticed in all strains at all concentrations of mancozeb tested, although there was a decrease in the percentage of germination with the increase in concentration. Among the three strains, the commercial isolate (T1) demonstrated relatively higher percentages of germination than the MTCC strains when exposed to the fungicides. The microscopic examination revealed normal germ tube formation in control and mancozeb-treated conidia, while decreased or absent germination was noticed in treatments containing propiconazole, hexaconazole, and carbendazim [Figures 1 and 2].

Effects of fungicides on the radial growth of *T. viride*

Radial growth assays revealed similar patterns to those in conidial germination. Carbendazim completely inhibited

mycelial growth in all strains of *T. viride* at all concentrations tested, with no visible growth of the colonies being detected during the entire incubation period. Propiconazole and hexaconazole concentration dependently inhibited mycelial growth. There was partial inhibition of growth at ×0.1 and ×1 recommended concentrations, while near-complete or complete inhibition was noticed at the highest concentration (×10 recommended concentration). Growth inhibition was consistent in all strains, although the extent of inhibition slightly varied among the strains.

Mancozeb moderately inhibited radial growth compared to other fungicides. All strains grew at all concentrations of mancozeb tested, although growth rates progressively decreased with increasing concentrations of fungicides. Among the strains tested, the commercial isolate (T1) grew relatively faster than the MTCC strains in the presence of fungicides [Figures 3 and Tables 3-6].

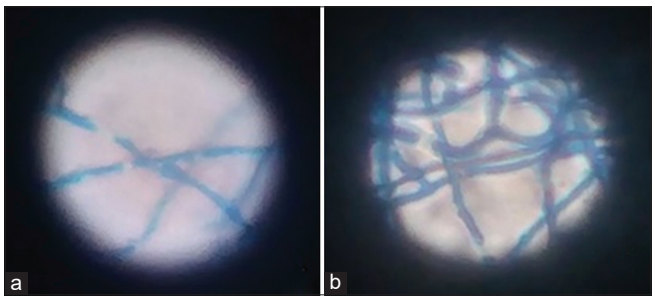

Figure 1: Germination of *Trichoderma viride* microbial type culture collection 800 treated with Mancozeb (a) Germination of conidia with ×10 Rr concentration (b) Germination of conidia with ×0.1 Rr concentration

All fungicides significantly reduced conidial germination and radial growth of *T. viride* in a concentration-dependent manner. Carbendazim caused complete inhibition across all strains and concentrations. Statistical analysis was performed using one-way ANOVA ($P \leq 0.05$).

Fungicide tolerance and strain-specific responses

Fungicide tolerance assays also supported the results from the germination and radial growth experiments. Carbendazim had the largest inhibition zones, followed by propiconazole and hexaconazole. Mancozeb had the smallest inhibition zones for all concentrations. There were clear differences among the strains. The field isolate (T1) always had smaller inhibition zones than MTCC 793 and MTCC 800 for the same concentrations of the respective fungicides, which indicated higher tolerance. The MTCC strains were relatively more sensitive, especially to carbendazim and propiconazole. Fungicide tolerance was affected by fungicide type, concentration, and fungal strain [Figures 4-6]. The ANOVA revealed that the type and concentration of fungicide significantly affected conidial germination, radial growth,

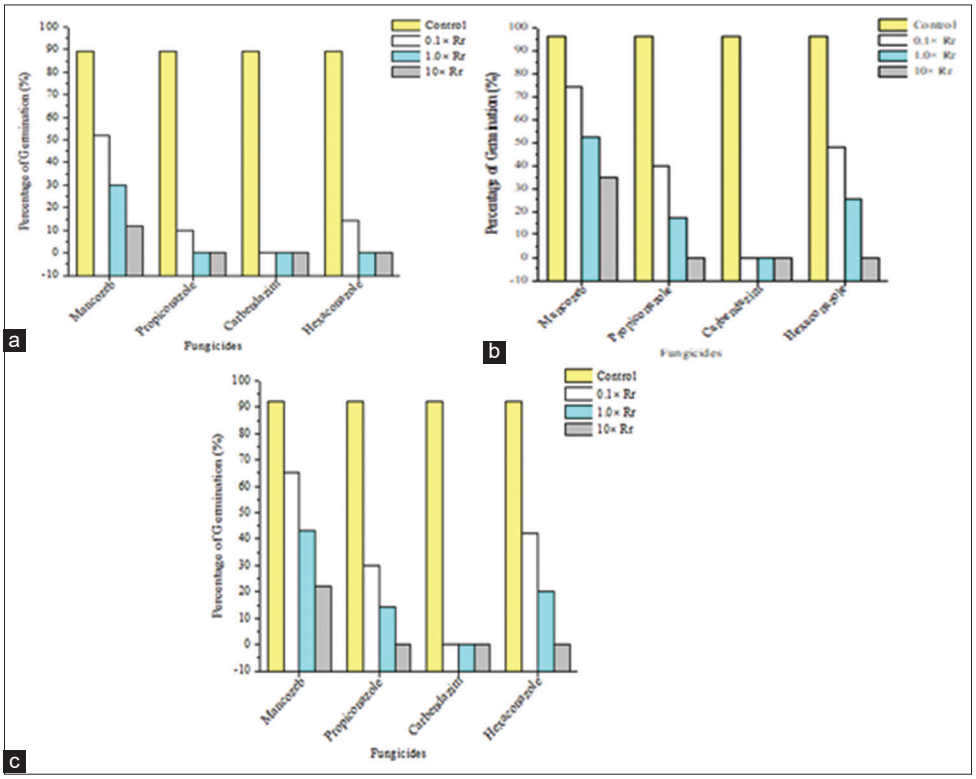

Figure 2: Effect of different concentration of fungicides on the germination of (a) *Trichoderma viride* microbial type culture collection (MTCC) 793 (b) *T. viride* MTCC 800 (c) T1 incubated at 32°C for 20 h

Table 3: Effects of different concentration of fungicides on the Radial growth (mm/day) of the *Trichoderma viride* MTCC 793 in *in vitro* after 10 days incubation at 32°C

Fungal strain	Fungicides	Fungicides concentration		
		×0.1 Rr	×1 Rr	×10 Rr
			mm/day	
MTCC 79 (*Trichoderma viride*)	Control	26.6±2.07	26.6±2.07	26.6±2.07
	Mancozeb	12.14±2.92	8.50±2.34	5.20±1.63
	Propiconazole	2.85±0.80	0.00±0.00	0.00±0.00
	Carbendazim	0.00±0.00	0.00±0.00	0.00±0.00
	Hexaconazole	4.20±1.30	1.6±1.14	0.00±0.00

MTCC: Microbial type culture collection

Table 4: Effects of different concentrations of fungicides on the radial growth (mm/day) of the *Trichoderma viride* MTCC 800 *in vitro* after 10 days incubation at 32°C

Fungal strains	Fungicides	Fungicides concentration		
		×0.1 Rr	×1 Rr	×10 Rr
			mm/day	
MTCC 800 (*Trichoderma viride*)	Control	30.1±0.74	30.1±0.74	30.1±0.74
	Mancozeb	16.0±3.65	10.2±1.87	7.50±2.36
	Propiconazole	4.33±2.11	1.56±0.82	0.00±0.00
	Carbendazim	0.00±0.00	0.00±0.00	0.00±0.00
	Hexaconazole	6.0±2.23	2.7±1.92	0.00±0.00

MTCC: Microbial type culture collection

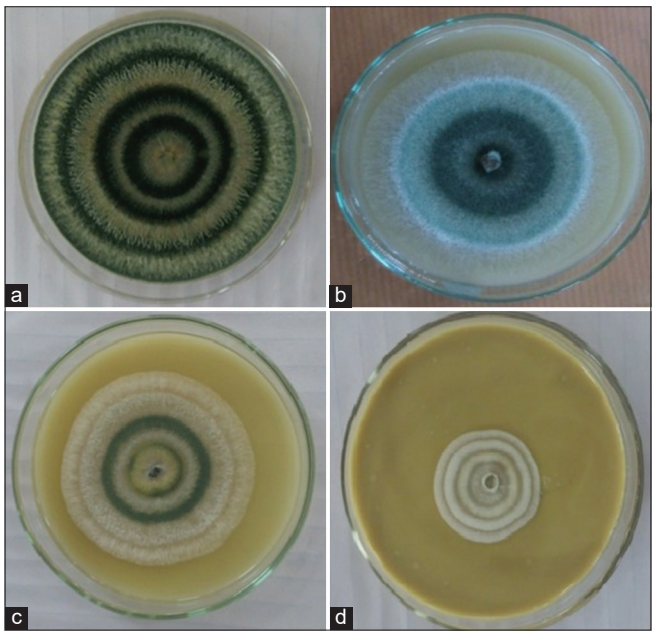

Figure 3: Radial growth of *Trichoderma viride* T1 on potato dextrose agar media treated with mancozeb at the concentration of (a) Control (b) ×0.1 Rr Concentration (c) 1× Rr Concentration (d) ×10 Rr Concentration

and inhibition zone formation in all strains [Figures 7] at $P \leq 0.05$ [Table 7].

DISCUSSION

The current study clearly shows that the commonly used fungicides are highly variable in their compatibility with *T. viride*, and the effect is largely dependent on the type of fungicide, concentration, and *Trichoderma* strain. The germination of conidia and mycelial growth were found to be highly sensitive to the fungicides, thus underlining the susceptibility of the early stages of *Trichoderma* development to chemical stress. The sensitivity of fungal propagules to chemical fungicides has been documented earlier.[11,16]

The absence of conidial germination and mycelial growth of *T. viride* due to carbendazim at all concentrations and strains clearly shows its high incompatibility with *T. viride*. This finding is consistent with the mode of action of benzimidazole fungicides, which inhibit fungal mitosis by interfering with spindle formation, thereby causing fungicidal effects.[20] This finding clearly shows that carbendazim is a potential danger to *T. viride* survival when used in combination with biocontrol agents.

Propiconazole and hexaconazole demonstrated strong inhibitory effects against *T. viride*, with a concentration-dependent increase in inhibition. These fungicides are demethylation inhibitors (DMI), which target sterol

Table 5: Effects of different concentrations of fungicides on the radial growth (mm/day) of the *Trichoderma viride* T1 *in vitro* after 10 days incubation at 32°C

Fungal strains	Fungicides	Fungicides concentration		
		×0.1 Rr	×1 Rr	×10 Rr
			mm/day	
T1 (*Trichoderma viride*)	Control	28.3±3.64	28.3±3.64	28.3±3.64
	Mancozeb	13.3±2.30	9.1±1.92	5.5±2.00
	Propiconazole	3.57±1.87	1.42±0.76	0.00±0.00
	Carbendazim	0.00±0.00	0.00±0.00	0.00±0.00
	Hexaconazole	5.60±1.20	2.1±0.94	0.00±0.00

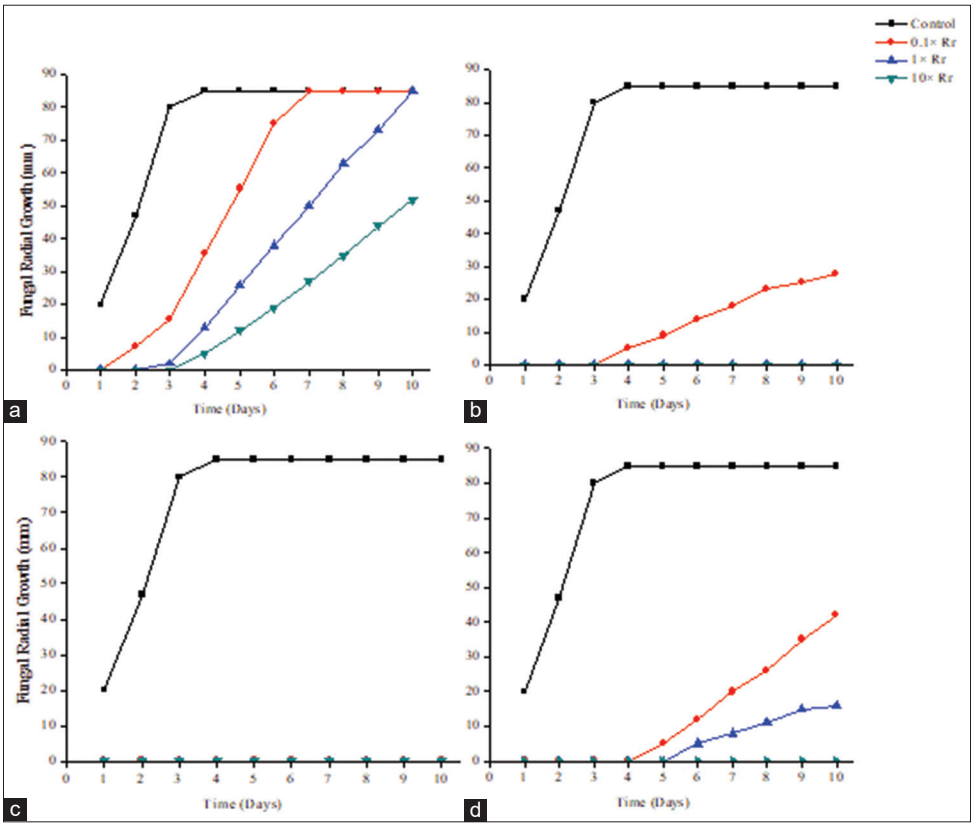

Figure 4: Growth rate of *Trichoderma viride* microbial type culture collection 793 strain exposed to three different concentrations (×0.1 Rr; ×1 Rr; ×10 Rr) of fungicides (a) Mancozeb (b) Propiconazole (c) Carbendazim (d) Hexaconazole

biosynthesis, an important process for fungal cell membrane integrity. The toxicity of DMI fungicides to *Trichoderma* spp. and other non-target soil fungi has been extensively documented.[21-23] On the other hand, mancozeb demonstrated relatively low toxicity to *T. viride*, which permitted both conidial germination and mycelial growth of all strains and concentrations. Although growth and germination were reduced at higher concentrations, no complete inhibition was observed, suggesting a fungistatic mode of action. Similar findings of relatively higher compatibility between contact fungicides and *Trichoderma* spp. have been observed by various researchers.[24-26]

Differences among strains were apparent in all tests, with the commercial isolate (T1) always showing higher tolerance to fungicide exposure compared to the MTCC reference strains. Higher tolerance in commercial strains has been explained on the basis of adaptation abilities developed during the selection, formulation, or repeated exposure during large-scale production.[27] The results emphasize the need for individual strain screening instead of relying on species-level data.

The results of the fungicide tolerance assays also supported the above findings, and the inhibition zone formation showed significant differences depending on the type, concentration, and strain of the fungicide. Similar findings have been reported in previous studies on the compatibility of *Trichoderma* with chemical fungicides.[28,29] It is pertinent to mention here that the above study was conducted in an *in vitro* environment, which

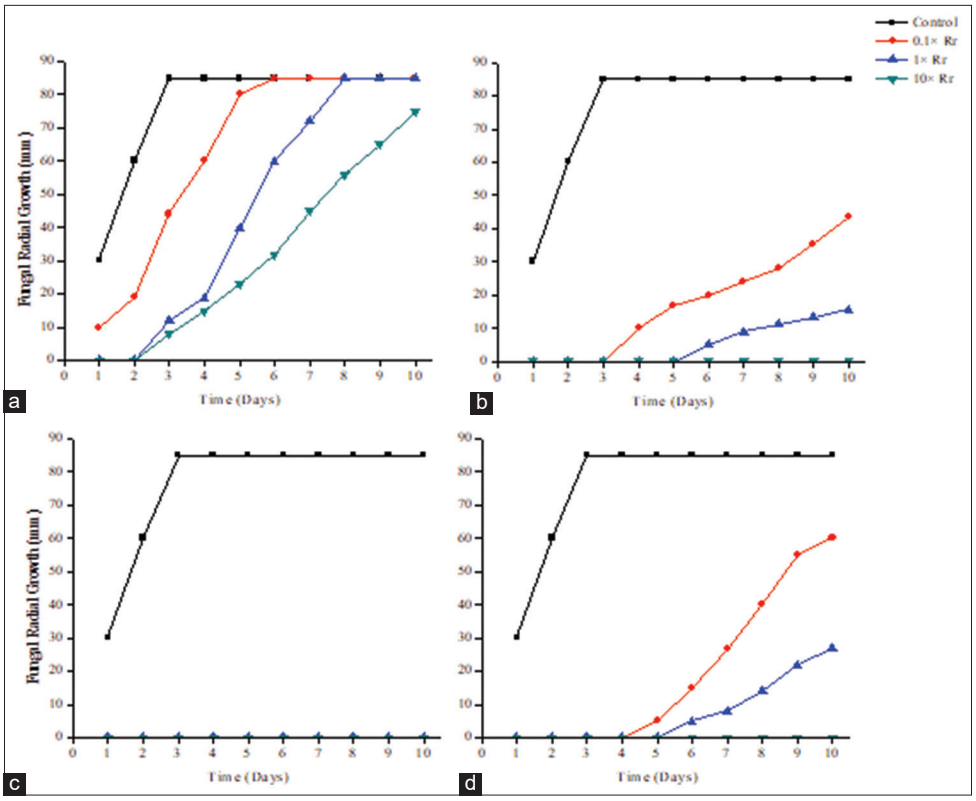

Figure 5: Growth rate of *Trichoderma viride* microbial type culture collection 800 strain exposed to three different concentrations (×0.1 Rr; ×1 Rr; ×10 Rr) of fungicides (a) Mancozeb (b) Propiconazole (c) Carbendazim (d) Hexaconazole

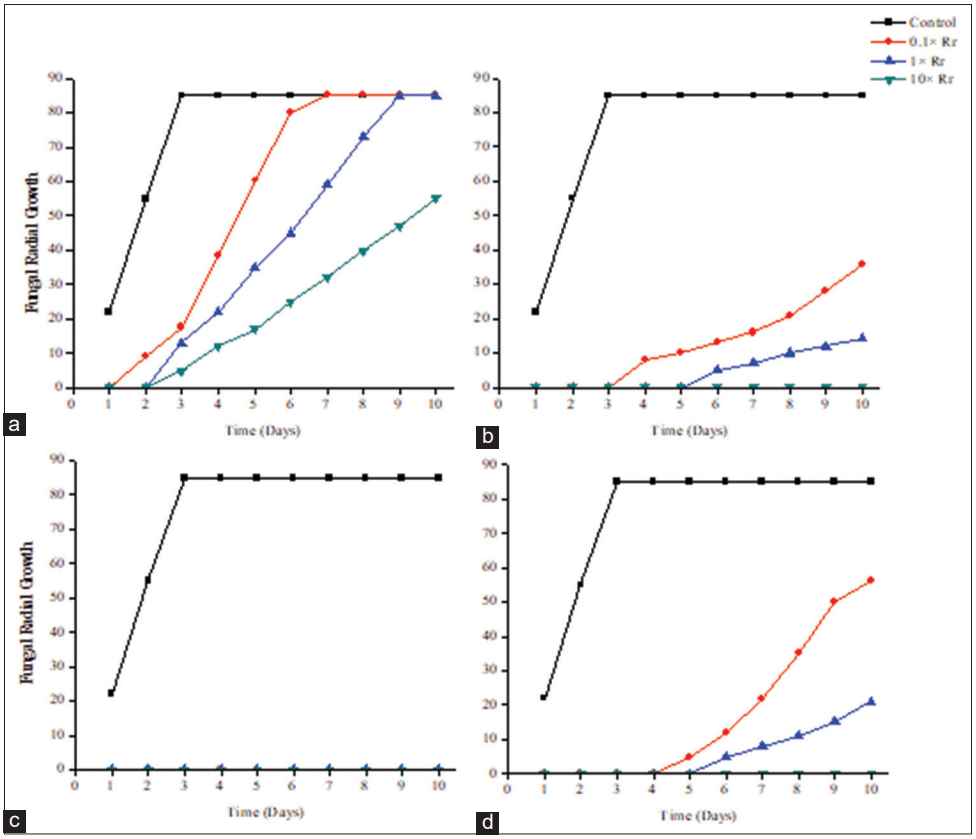

Figure 6: Growth rate of *Trichoderma viride* T1 strain exposed to three different concentrations (×0.1 Rr; ×1 Rr; ×10 Rr) of fungicides (a) Mancozeb (b) Propiconazole (c) Carbendazim (d) Hexaconazole

Table 6: Effect of fungicide concentration on conidial germination and radial growth

Fungicide concentration	Strain	df	F-value	*P*-value	Effect on conidial germination	Effect on radial growth
×0.1 recommended rate	MTCC 793	4	164.44	<0.000	Significant reduction	Significant reduction
	MTCC 800	4	279.24	<0.000	Significant reduction	Significant reduction
	T1 (Commercial isolate)	4	136.50	<0.000	Significant reduction	Significant reduction
×1 recommended rate	MTCC 793	4	152.12	<0.000	Significant reduction	Significant reduction
	MTCC 800	4	198.12	<0.000	Significant reduction	Significant reduction
	T1 (Commercial isolate)	4	187.64	<0.000	Significant reduction	Significant reduction
×10 recommended rate	MTCC 793	4	104.32	<0.000	Significant reduction	Significant reduction
	MTCC 800	4	78.64	<0.000	Significant reduction	Significant reduction
	T1 (Commercial isolate)	4	83.30	<0.000	Significant reduction	Significant reduction
Overall observation	All strains	—	—	—	Complete inhibition by carbendazim	Complete inhibition by carbendazim

MTCC: Microbial type culture collection

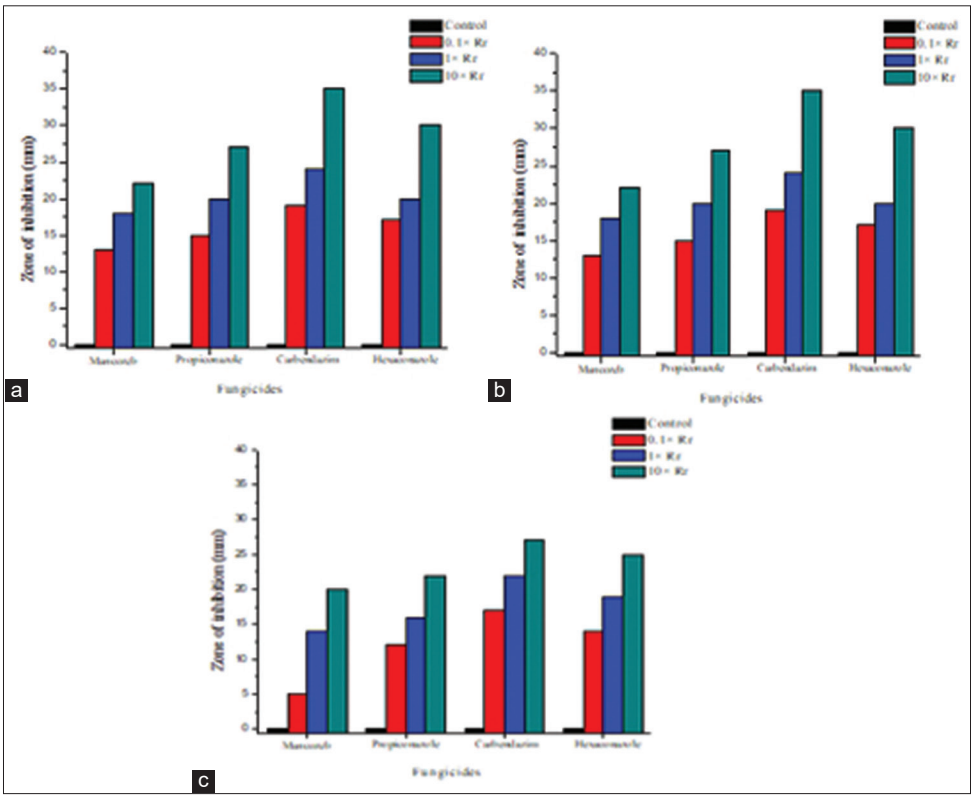

Figure 7: Antifungal activity (Tolerance Test) of four different fungicides to (a) microbial type culture collection (MTCC) 793 (b) MTCC 800 (c) T1

may not be entirely representative of the actual phenomenon that takes place in agricultural soil. The soil type, organic matter, microbial competition, adsorption, and degradation rates of fungicides, as well as *Trichoderma* survival, can be greatly influenced by environmental factors.[30] In terms of application, the strategies for the integrated management of diseases can be applied based on the results obtained in the present study. The compatibility of mancozeb with *T. viride* shown in the present study indicates that it can be used in integrated programs without affecting the efficiency of biological control. On the other hand, the present study's results indicate that the significant inhibitory potential of carbendazim, propiconazole, and hexaconazole suggests that these compounds should not be used in combination with *Trichoderma*-based biofungicides. The compatibility of fungicides and biocontrol agents has been highlighted by previous studies.[29,31]

Table 7: Effect of fungicide concentration on tolerance

Strain	Fungicide	df	F-value	*P*-value	Effect of increasing concentration	Relative tolerance
MTCC 793	Mancozeb	3	83.52	<0.000	Significant increase in inhibition zone	High
	Propiconazole	3	67.53	<0.000	Significant increase in inhibition zone	Moderate
	Carbendazim	3	120.34	<0.000	Significant increase in inhibition zone	Low
	Hexaconazole	3	62.16	<0.000	Significant increase in inhibition zone	Moderate
MTCC 800	Mancozeb	3	66.93	<0.000	Significant increase in inhibition zone	High
	Propiconazole	3	92.66	<0.000	Significant increase in inhibition zone	Moderate
	Carbendazim	3	140.44	<0.000	Significant increase in inhibition zone	Low
	Hexaconazole	3	22.94	<0.000	Significant increase in inhibition zone	Moderate
T1 (Commercial isolate)	Mancozeb	3	76.48	<0.000	Significant increase in inhibition zone	Highest
	Propiconazole	3	69.39	<0.000	Significant increase in inhibition zone	Moderate–high
	Carbendazim	3	145.91	<0.000	Significant increase in inhibition zone	Low
	Hexaconazole	4	29.14	<0.000	Significant increase in inhibition zone	Moderate

MTCC: Microbial type culture collection, ANOVA: Analysis of variance. Inhibition zone formation increased significantly with fungicide concentration in all strains. Carbendazim produced the largest inhibition zones, whereas mancozeb produced the smallest. The commercial isolate (T1) exhibited comparatively higher tolerance than MTCC strains. Statistical significance was determined using one-way ANOVA ($P \leq 0.05$)

CONCLUSION

Based on the present study, the compatibility of fungicides with *T. viride* is highly variable depending on the chemical groups and concentrations. Although carbendazim was found to be highly toxic and should not be used in combination with *Trichoderma* biofungicides, mancozeb was the most compatible fungicide, allowing germination and growth of all strains. The resistance of the strains to the fungicides, especially the commercial strain, underscores the importance of using resistant strains in integrated disease management. Although the present study was laboratory-based, it is recommended that the findings be validated in a field setting since they can offer important clues in the selection of fungicides.

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