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Biological Control of Fungal Phytopathogens with *Trichoderma harzianum* and Its Fungicidal Compatibility.

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Abstract

Excessive synthetic fungicide use reduces soil's antagonistic population, allowing soil-borne plant pathogens to cause significant global losses. Despite high fungicide application, plant diseases persist, harming the ecosystem. *Trichoderma* spp, an eco-friendly plant symbiont, can serve as an alternative biocontrol agent. This study evaluated *Trichoderma harzianum*'s antagonistic effect against fungal pathogens and its compatibility with fungicides in *in-vitro* using a Completely Randomized Design with four replications per treatment. *Trichoderma harzianum* showed high antagonistic activity for *Alternaria brassicicola* (70.35%), *Fusarium solani* (70.82%), *Helminthosporium sorokinana* (66.55%), *Rhizoctonia solani* (78.58%), *Sclerotium rolfsii* (92.53%). Among the tested fungicides, Copper oxychloride and Mancoxeb at 400 ppm showed maximum compatibility with growth inhibition per cent (GIP) of 2.41% and 7.91%, respectively, after 60 hours of incubation. Fungicides viz., Carbendazim, Hexaconazole, and Carbendazim+Mancozeb at all concentrations aren't compatible with 100% GIP throughout the experiment, and for Metalaxyl+Mancozeb, high growth inhibition percent was observed ranging from 42.77% to 78.40% making it incompatible. In integrated disease management, compatible fungicides at recommended doses can be used in combination with *T. harzianum*.

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Trichoderma; Fungicides; Food Poisoned Technique; Integrated Disease Management; Antagonistic Effect.

Introduction

Prolonged and continuous application of chemical inputs for enhancing soil fertility and better disease management has resulted in unforeseen hazardous environmental effects. This has led to the reduction of bacterial and fungal antagonistic populations, as well as an increase in several soil-borne diseases. Despite the various noxious effects on the environment, the use of the total amount of fungicides and fertilisers has skyrocketed globally

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to control different plant pathogens and to fulfil the food demand of the growing world population through intensive agriculture (Vitousek et al., 1997; Frink et al., 1999). Thus, the main challenge in present agriculture is to increase productivity and control plant disease by reducing the use of harmful chemical fungicides. In this regard, it is imperative to use microbial inoculants such as plant growth-promoting rhizobacteria and *Trichoderma* spp. as an alternative (Bagwan, 2010; Alori and Babalola, 2018).

Trichoderma spp. is an omnipresent, asexually reproducing fungus found most frequently in the soil which can antagonise a wide range of soil-borne plant pathogens, induce both localised and systemic resistance in various plants with a substantial influence on plant immunity, growth, and development in field and greenhouse conditions (Enshasy et al., 2020; Tyśkiewicz et al., 2022). Since the 1920s, *Trichoderma* spp. has been known for its capability to function as a biocontrol agent (BCA) against various plant pathogens, and among its many strains, mainly *Trichoderma harzianum*, *Trichoderma viride*, and *Trichoderma virens* have been identified as promising strains having potential in biological controls because of its multiple modes of action such as mycoparasitism, antibiosis, endophytic survival on host and secretion of enzymes and metabolites (Monte & Llobell, 2003; Benítez et al., 2004; Inayati et al., 2020; Zaenab et al., 2020). Significant growth inhibition of *Phytophthora capsici* and *Fusarium oxysporum* (Das et al., 2019), *Alternaria solani* of tomato (Imran et al., 2022), *Plasmopara viticola* of *Vitis vinifera* (Perazzoli et al. 2012), *Rhizoctonia solani* in tobacco seedlings (Gveroska and Ziberoski, 2011) by *T. harzianum* had reported by other authors as well. Halifu et al., (2019) reported that applying *T. harzianum* E15 had increased seedling biomass and height, root structure index, soil nutrients, and soil enzyme activity.

Various *T. harzianum* strains, alone or in combination with fungicides, have been playing a pivotal role in the management of various seed and soil-borne diseases in Integrated Disease Management (IDM), and these strain's application not only suppresses diseases but also have comprehensive advantages of improved seed germination, increased plant size, promote crop precocity, augmented leaf area and weight (Samuels, 1996; Atomare et al., 1999; Banjac et al., 2021; Yassin et al., 2021). Biocontrol agents, along with a combination of chemical fungicides, would also provide almost similar levels of disease suppression as achieved with higher fungicides (Monte, 2001). However, several chemicals harm the growth of *Trichoderma* spp. In Nepal, the data suggest that there has been an indiscriminate use of chemicals, especially in vegetable farming (Bhandari et al., 2018). This helps to develop fungicide-resistant strains of pathogens, even demanding more toxic chemicals in the coming days. So, it is mandatory to seek compatible fungicides to be used with *Trichoderma* spp. in IDM. Our long-term goal is to adopt environmental-friendly approaches for disease management, hinder the development of resistance in pathogens to chemicals, build up bacterial and fungal antagonistic populations in the soil, and seek cost-effective methods in disease management. Hence, this study aims to evaluate the compatibility of *T. harzianum* with chemical fungicides for the control of plant pathogens, to develop environmentally friendly and cost-effective disease management strategies. This study has the potential to

offer valuable insights into the selection of fungicides that do not harm the growth and effectiveness of *T. harzianum*, enabling the development of integrated strategies that enhance disease suppression while decreasing chemical inputs.

Materials and Methods

The experiment was conducted at the central laboratory of the Institute of Agriculture and Animal Science, Lamjung Campus, and Paklihawa Campus, Nepal, in 2020 and 2021.

Isolation of Pathogens

Two fungal phytopathogens: *Helminthosporium sorokiniana* and *Alternaria brassicicola* were isolated from the diseased leaf of wheat (Agronomy farm of IAAS, Phaklihawa) and cabbage (Horticulture farm of IAAS, Lamjung). For isolation, diseased samples were cut into pieces of 1 cm and surface sterilised in 1% sodium hypochlorite solution for 1 minute, followed by three consecutive washes in distilled water. Samples were then placed on sterilised blotter paper and air-dried inside laminar flow. They were subsequently plated on water agar plates and incubated at 27±1°C. After 48 hours, the growth of the pathogen was observed, and the pathogen was confirmed based on its microscopic characteristics, as in Figure 1. The identified mycelia were later transferred to potato dextrose agar plates to obtain pure cultures of the pathogens. Pure cultures of *Fusarium solani*, *Rhizoctonia solani*, and *Sclerotium rolfsii* were obtained from the Nepal Agriculture Research Council (NARC).

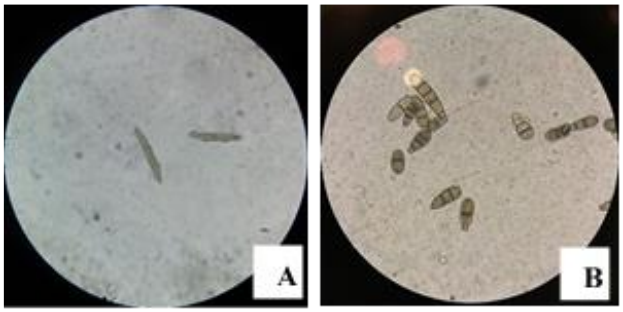

Figure 1. Conidia – (A) *Helminthosporium sorokiniana*, (B) *Alternaria brassicicola* Food-Poisoned Technique

A pure culture of *T. harzianum* was obtained from NARC, and an *in-vitro* compatibility test was conducted using six commonly used chemical fungicides by Nepalese farmers (listed in Table 1) in a completely randomised design by food poisoned technique described by Nene and Thapliyal (1993).

Table 1. Chemical Fungicides Used In An Experiment.

S.N.	Trade Name	Active Ingredient	Mode of Action
1	Uthane M-45	Mancozeb 75%WP	Contact
2	Blitox	Copper Oxychloride 50%WP	Contact
3	Saaf	Carbendazim 12% + Mancozeb 63% WP	Systemic + Contact
4	Kriloxyl Gold	Metalaxyl 8% + Mancozeb 64% WP	Systemic + Contact
5	Navistin	Carbendazim 50% WP	Systemic
6	Hexa	Hexaconazole 5% EC	Systemic

Under dark conditions, pure culture was incubated for four days in a bacteriological incubator at 25±2˚C. Further, a subculture was made, and a 5-day-old culture with green mycelia was used for an experiment. For the evaluation of compatibility of chemical fungicides, stock solutions of five fungicides were prepared by dissolving 1 gm of each fungicide in 10 ml of distilled water to make 75,000 mg/l of Mancozeb 75% WP and Carbendazim 12% + Mancozeb 63% WP, 72,000 mg/l of Metalaxyl 8% + Mancozeb 64% WP and 50,000 mg/l of Copper oxychloride 50% WP and Carbenazim 50% WP. In the case of Hexaconazole, it's available 5% EC solution was taken as the original stock solution. 80 µl, 160 µl, and 240 µl stock solutions of each Mancozeb and Carbendazim + Mancozeb, 83.3 µl, 166.6 µl, and 249.9 µl stock solutions of Metalaxyl + Mancozeb, 120 µl, 240 µl, and 360 µl stock solutions of each Copper oxychloride, Carbendazim and Hexaconazole were mixed in lukewarm molten PDA to prepare 100 mg/l, 200 mg/l and 300 mg//l of 60 ml amended media. Then in each 90 mm sterilised Petri plate, 20 ml of poisoned media with different fungicides was poured and allowed to solidify. The poisoned PDA plates amended with fungicides were inoculated aseptically in laminar flow with a 5mm mycelial disc of *T. harzianum* grown on sub-cultured plates. For each treatment, four replications were made, and an unamended PDA media served as a control, and all the treated plates along with the control were incubated in a bacteriological incubator at 25±2˚C. Measurement of mycelial growth (mm) was measured using a Verniercalliperr scale after 24, 36, 48, and 60 hrs of incubation, and growth inhibition per cent (GIP) was calculated. GIP was calculated using the following formula given by Vincent (1947).

Per cent growth inhibition (%) = A–B/A × 100

Where, A = Mycelium growth of the *T. harzianum* on the control plate

B= Mycelium growth of the *T. harzianum* on a treated plate

After each observation, all the data were entered in Ms Excel (2013), an analysis of variance was done using RStat software (version 3.5.3), and a mean comparison was made using the Fisher-LSD test at a 0.05 level of significance.

Dual Culture

Five days old cultures of *T. harzianum* and a week of the culture of each fungal pathogen were taken for the study. A 7 mm disc of pathogens and *T. harzianum* were placed on solidified PDA medium on the opposite ends of the plate at equidistance from the

periphery. The experiment followed a completely randomised design with three replications for each treatment. Plates incubated with the pathogen alone were used as a control. After seven days of incubation, the radial mycelial growth of *T. harzianum* and the pathogens was measured, and the growth inhibition of fungal pathogens by *T. harzianum* was calculated using the formula given by Vincent (1947).

Results and Discussion

In Vitro Screening of *T. harzianum* for Compatibility with Different Fungicides

All screened fungicides significantly inhibited the radial mycelium growth of *T. harzianum* over the control, as demonstrated in Table 2 and Figure 2.

Table 2. Per cent growth inhibition of *Trichoderma harzianum* at various concentrations of different chemical fungicides under *in-vitro* at different time intervals.

S. N.	Treatments	Conc (ppm)	Mycelial diameter(mm)				Per cent Growth Inhibition (%)			
			24 hrs	36 hrs	48 hrs	60 hrs	24 hrs	36 hrs	48 hrs	60 hrs
1	Control		52.53	66.76	78.59	83	0.00	0.00	0.00	0.00
2	Mancozeb	400	32.66	46.12	67.81	76.44	37.81^{f}	30.92^{fg}	13.71^{h}	7.91^{h}
		500	24.02	40.03	57.40	70.63	54.27^{de}	40.03^{e}	26.96^{f}	14.91^{fg}
		600	20.4	28.98	51.38	64.54	61.16^{d}	56.59^{c}	34.62^{e}	22.25^{e}
3	Carbendazim	400	0.00	0.00	0.00	0.00	100^{a}	100^{a}	100^{a}	100a
		500	0.00	0.00	0.00	0.00	100^{a}	100^{a}	100^{a}	100^{a}
		600	0.00	0.00	0.00	0.00	100^{a}	100^{a}	100^{a}	100^{a}
4	Hexaconazole	400	0.00	0.00	0.00	0.00	100^{a}	100^{a}	100^{a}	100^{a}
		500	0.00	0.00	0.00	0.00	100^{a}	100^{a}	100^{a}	100^{a}
		600	0.00	0.00	0.00	0.00	100^{a}	100^{a}	100^{a}	100^{a}
5	Carbendazi+ Mancozeb	400	0.00	0.00	0.00	0.00	100^{a}	100^{a}	100^{a}	100^{a}
		500	0.00	0.00	0.00	0.00	100^{a}	100^{a}	100^{a}	100^{a}
		600	0.00	0.00	0.00	0.00	100^{a}	100^{a}	100^{a}	100^{a}
6	Metalaxyl + Mancozeb	400	21.49	34.52	40.65	47.50	59.09^{d}	48.30^{d}	48.28^{d}	42.77^{d}
		500	16.37	26.50	32.88	40.78	68.83^{c}	60.30^{c}	58.17^{c}	50.87^{c}
		600	11.34	16.98	25.48	34.57	78.40^{b}	74.57^{b}	67.58^{b}	58.35^{b}
7	Copper oxychloride	400	39.09	56.10	71.25	81.00	25.57^{g}	15.96^{h}	9.34^{h}	2.41^{i}
		500	33.14	49.08	63.75	73.93	36.92^{f}	26.48^{g}	18.89^{g}	10.93^{gh}
		600	26.87	43.87	54.50	68.75	48.84^{e}	34.29^{ef}	30.65^{ef}	17.17^{f}
	Grand Mean						76.16	71.52	67.12	62.64
	CV (%)						4.26	3.64	3.87	3.28
	LSD (p≤0.05)						5.37	4.31	4.29	3.40

Conc= Concentration, CV= Coefficient of variation, hrs= hours, LSD= Least significance difference, mm= Millimetre, ppm= Parts per million

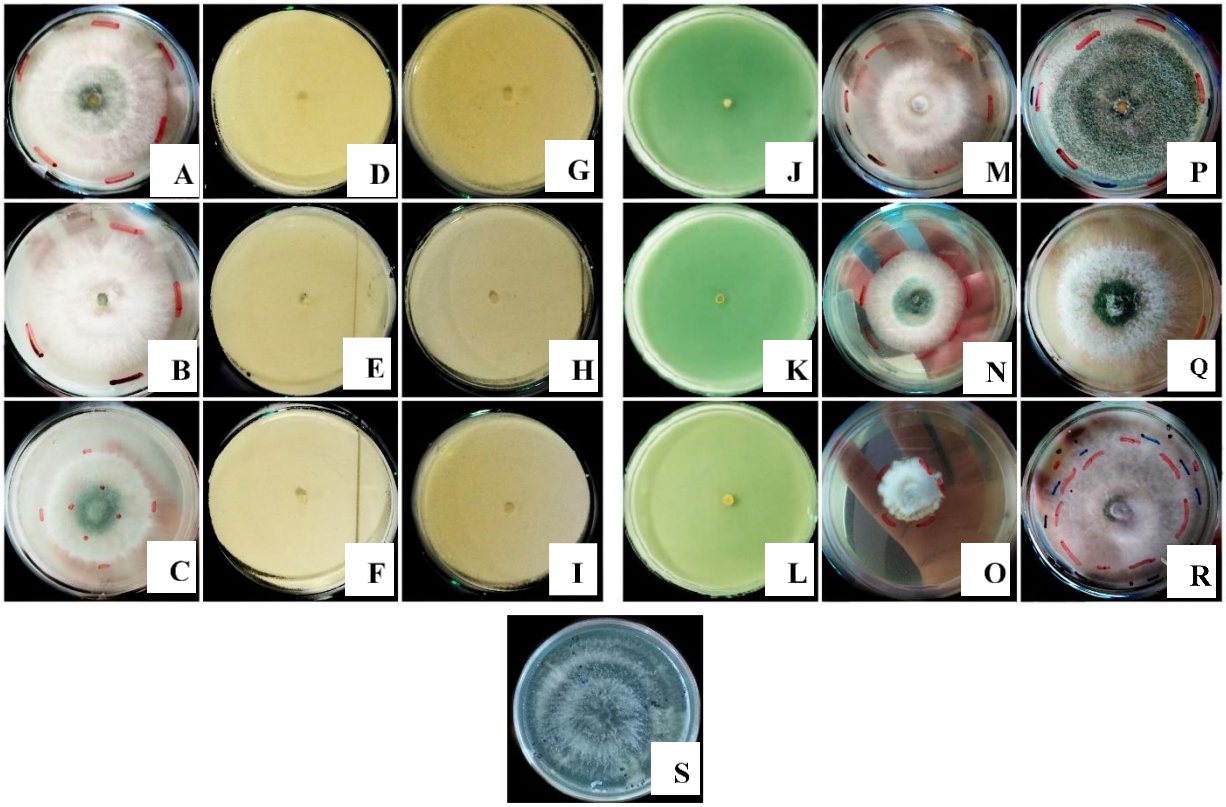

Figure 2. Mycelial growth diameter of *Trichoderma harzianum* after 60 hours of inoculation in media amended with different concentrations of various chemical fungicides- (A) Mancozeb 400 ppm, (B) Mancozeb 500 ppm, (C) Mancozeb 600 ppm, (D) Carbendazim 400 ppm, (E) Carbendazim 500 ppm, (F) Carbendazim 600 ppm, (G) Hexaconazole 400 ppm, (H) Hexaconazole 500 ppm, (I) Hexaconazole 600 ppm, (J) Carbendazium+Mancozeb 400 ppm, (K) Carbendazium+Mancozeb 500 ppm, (L) Carbendazium+Mancozeb 600 ppm, (M) Metalaxyl + Mancozeb 400 ppm, (N) Metalaxyl + Mancozeb 500 ppm, (O) Metalaxyl + Mancozeb 600 ppm, (P) Copper oxychloride 400 ppm, (Q) Copper oxychloride 500 ppm, (R) Copper oxychloride 600 ppm, (S) Control

Copper oxychloride at 400 ppm exhibited the highest compatibility with mycelial growth, resulting in diameters of 39.09 mm, 56.10 mm, 71.25 mm, and 81.00 mm, and GIP of 25.57%, 15.96%, 9.34%, and 2.41% after 24, 36, 48, and 60 hours of incubation, respectively, among the six chemical fungicides. Its 500 ppm concentration also showed promising results and was statistically at par ($p \leq 0.05$) with 400 ppm of Mancozeb after 24, 36, and 60 hours of incubation. On the other hand, Carbendazium, Hexaconazole, and Carbendazium+Mancozeb proved to be extremely lethal, with a GIP of 100% throughout the experiment. The fungicide Metalaxyl+Mancozeb had a varying GIP, ranging from 42.77% to 78.40%, and showed a deleterious effect on the mycelial diameter of *T. harzianum*. Overall, the results indicate that Copper oxychloride and Mancozeb are highly compatible with *T. harzianum* at all tested concentrations, suggesting that they can be used effectively in integrated disease

management strategies to control soil-borne plant pathogens while maintaining a healthy soil environment.

For fungicides viz., Mancozeb, Metalaxyl+Mancozeb, and Copper oxychloride, a significant increase ($p \leq 0.05$) was observed in per cent inhibition as the concentration increased, and with time, the GIP of these fungicides decreased gradually. This may be, up to our understanding, due to the decreased efficacy of these fungicides and the increased ability of the *T. harzianum* to degrade these chemicals with time. For carbendazium, Hexaconazole, and Carbendazium+Mancozeb, there was 100% growth inhibition at all the tested concentrations with no gradual decrease in GIP with time. Gowdar et al., (2006) and Manandhar et al., (2020) also reported that the percentage inhibition of the *T. harzainum* increased with an increase in concentration and decreased with an increase in the incubation period.

Antagonistic Effect of *Trichoderma Harzianum* Against Pathogen

After incubating for seven days, *T. harzianum* significantly inhibited the mycelial growth of several fungal phytopathogens, such as *Alternaria brassicicola* (by 70.35%), *Fusarium solani* (by 70.82%), *Helminthosporium sorokinana* (by 66.55%), *Rhizoctonia solani* (by 78.58%), and *Sclerotium rolfsii* (by 92.53%), as shown in Figure 3 and Figure 4.

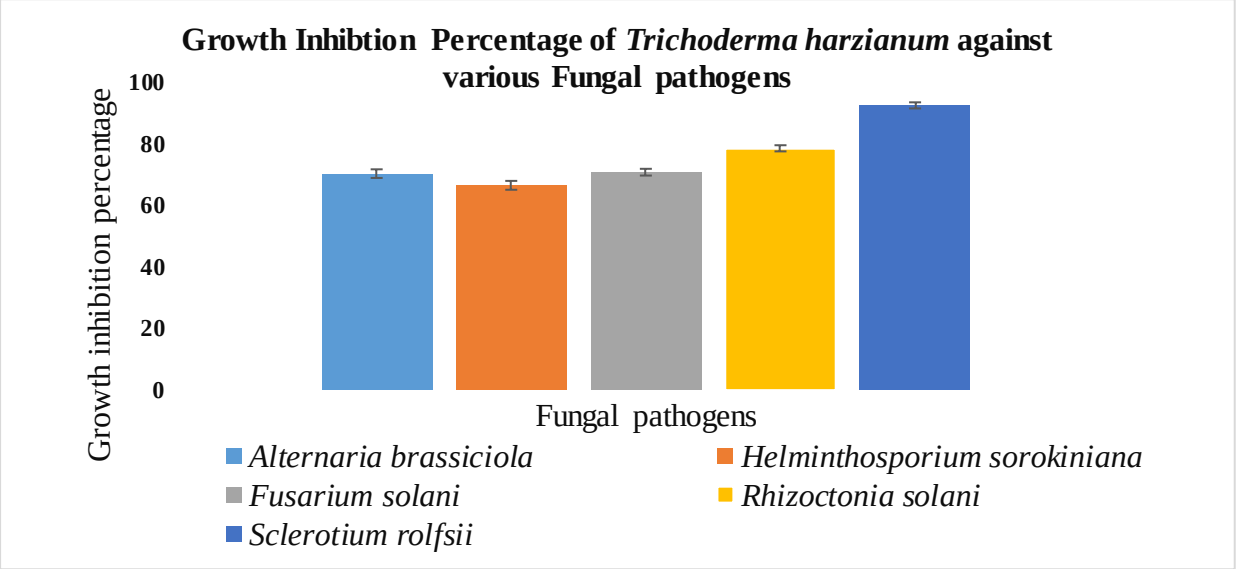

Figure 3. Growth Inhibition of Different Fungal Pathogens by *Trichoderma harzianum*

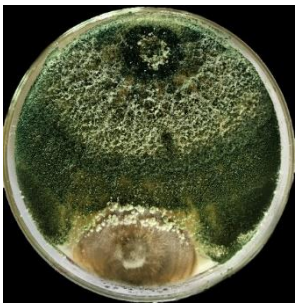

Figure 4: Growth Inhibition of *Helminthosporium sorokinana* by *Trichoderma harzianum*

The study found that *T. harzianum*, a soil antagonistic fungus, is compatible with Copper oxychloride and Mancozeb but not with Carbendazim 50 % WP, Hexaconazole 5 %, or Carbendazim 12 % + Mancozeb 63 % at all the tested concentrations. Several earlier reports had also reported good growth of *Trichoderma* spp. at low and moderate levels of different fungicides (Srinivasulu et al., 2005; Elshahawy et al., 2016; Shashikumar et al., 2019). This information holds significance in developing integrated disease management strategies, as soil-borne plant pathogens can be difficult to control with a single method. By using biocontrol agents such as *T. harzianum* in combination with compatible agrochemicals, farmers can more effectively eradicate plant diseases while also being environmentally conscious. Additionally, *T. harzianum* was found to significantly inhibit the growth of various fungal pathogens, suggesting its potential as a biocontrol agent for crop protection . Sonavane and Venkataravanappa (2017) found 0% mycelial inhibition of *T. harzianum* with Mancozeb 75WP and Copper oxychloride 50 WP even at higher concentrations of 1500ppm. Also, they found that Carbendazim 50 % WP at 250 ppm, Hexaconazole 5 % at 500 ppm, and Carbendazim 12 % + Mancozeb 63 % WP at 500 ppm completely suppressed the growth of *T. harzianum* with 100% mycelial inhibition revealing no compatibility with antagonistic soil fungus, *Trichoderma*. Manandhar et al. (2020) also reported Mancozeb, Copper oxychloride, and Metalaxyl + Mancozeb as compatible fungicides and Hexaconazole, Carbendazium, and Carbendazium + Mancozeb as incompatible fungicides when tested at 50 and 100 ppm. The high inhibition of benzimidazole compounds, i.e. Carbendazim is due to its binding with β-tubulin of fungal pathogen and causes inhibition of microtubule assembly which ultimately hinders cell division and may lead to cell death (Zhou et al., 2016). Moreover, it inhibits DNA synthesis and blocks nuclear division (Clemons & Sisler, 1971; Davidse, 1973; Hammerschlag & Sisler, 1973). Similarly, Hexaconazole's high inhibition capability is due to the presence of systematic demethylation inhibitors. These inhibitors primarily target the vegetative stage of fungi, disrupting the development of mycelium both internally and externally within the host plant (Khalfallaha et al., 1998). Saaf, combined fungicide, is a mixture of Carbendazim (12%) and Mancozeb (63%), so it has a collective effect of systematic and contact fungicides resulting in high mycelium inhibition.

T. harzianum significantly suppressed the growth of all the fungal pathogens that were tested. Chamoli et al. (2020) also recorded a high GIP of 67.22% for *Alternaria brassicicola* by *T. harzianum* and 73.55% by *T. asperellum*. Singh et al. (2018) reported high growth inhibition per cent of 73.07%, 63.84%, and 69.22% for *Bipolaris sorokiniana* by *Trichoderma viride*, *Trichoderma harzianum*, and *Trichoderma virens*, respectively. Bastakoti et al. (2017) reported high growth inhibition of *Sclerotium rolfsii* (100%), *Rhizoctonia solani* (62%), and *Fusarium solani* (68%) by *Trichoderma* spp. Similarly, Manandhar et al. (2019) also reported more than 80% growth suppression of *Fusarium solani* and *Rhizoctonia solani* by *Trichoderma* isolates. The high growth inhibition of *phytopathogens* by *T. harzianum* is due to the rupture of the cell wall of the host fungus by multiple enzymes, mainly glucanolytic and chitinolytic enzymes (Monte, 2001; Sood et al., 2020).

The study on the biocontrol potential of *Trichoderma harzianum* against soil-borne phytopathogens has promising implications for sustainable agriculture. However, there are some limitations to be considered, such as the specific strains of *T. harzianum* used in this study may have different interactions with various agrochemicals and fungal pathogens, and their efficacy may vary across different agroecological zones. Additionally, this study tested the efficacy of *T. harzianum* with a limited number of fungal pathogens, and other soil-borne plant pathogens may respond differently to biocontrol agents. These limitations highlight the importance of conducting further research to investigate the potential of *T. harzianum* to mitigate different soil-borne phytopathogens and their compatibility with a wider range of chemicals.

Conclusion

The finding reveals that *T. harzianum* poses high growth inhibition for *Alternaria brassicicola*, *Bipolaris sorokiniana*, *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Fusarium solani*. Among the fungicides tested, Mancozeb and Copper oxychloride are compatible with *T. harzianum* while Hexaconazole, Carbendazium, and Carbendazi+ Mancozeb are extremely lethal with 100 % GIP throughout the experiment. Metalaxyl + Mancozeb also poses high GIP throughout the experiment. Fungicides with lower GIP can be used in combination with *T. harzianum* in IDM to control different fungal phytopathogens. Future research should focus on field trials to evaluate the effectiveness of *T. harzianum*, either independently or in conjunction with compatible chemicals identified in our study, for managing soil-borne diseases in agriculture.

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