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Evaluation of Antifungal Activities of *Trichoderma* spp. Against *Phytophthora* spp.: In-Vitro Bioassay

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Abstract

This study evaluated the antifungal potential of native *Trichoderma* spp. in inhibiting the growth of *Phytophthora* spp. *Trichoderma* spp. and *Phytophthora* spp. were isolated from rhizospheric soil and diseased chilli plants, respectively. The isolates of *Trichoderma* spp. and *Phytophthora* spp. were subjected to in-vitro laboratory bioassay under controlled environmental conditions following the dual culture method. The pathogenic *Phytophthora* spp. were isolated, purified using the hyphal tip method and identified based on key morphological features. The virulence of the *Phytophthora* spp. isolates was tested on detached leaves of the chilli plant. The *Trichoderma* spp. were isolated following serial dilution and characterized based on key morphology features. The in-vitro experiment to evaluate the antifungal properties of *Trichoderma* spp. against *Phytophthora* spp. was designed in randomized complete block design in three replicates. Three isolates of *Trichoderma* spp. namely, TCP1, TCP2 and TCP3 were tested for antagonistic activity against four different isolates of *Phytophthora* spp., namely, PP1, PP2, PP3 and PP4. Results revealed that TCP3 exhibited the highest inhibition, reaching 61.49% (PP1), 62.10% (PP2), 60.19% (PP3), and 36.11% (PP4). TCP1 also showed strong antagonism with inhibition percentages of 56.48% (PP1), 59.11% (PP2), 56.63% (PP3), and 34.07% (PP4). In contrast, TCP2 displayed lower inhibition values: 44.44% (PP1), 45.18% (PP2), 39.63% (PP3), and 32.41% (PP4). A two-way ANOVA revealed a statistically significant interaction between Trichoderma and Phytophthora isolates ($F(30, 82) = 7.261$, $p = 0.001$), confirming variation in inhibitory efficacy.

Keywords: **:** Antifungal, Biological, Chilli, In-vitro, *Phytophthora* spp, *Trichoderma* spp

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Introduction

Chilli (*Capsicum annuum* L.) is one of the most extensively grown commercial spices valued for its culinary, nutritional, and medicinal properties (Geetha & Selvarani, 2017; Jha & Prasad, 2011). In Bhutan, chilli is a vital and preferred income crop among vegetables for smallholder farmers. Chilli is cultivated across Bhutan, at elevations of up to 3,500 meters

above sea level. . It is grown as a rainfed dry-land monoculture crop in the higher elevations and mid-hills in early spring and as the first crop in paddy fields in rice-based farming systems in the lowlands (Rai & Guest, 2021). In 2021, the chilli harvest amounted to 5,864 metric tonnes (National Statistics Bureau, 2021). However, chilli blight caused by the soil-borne oomycete, *Phytophthora capsici* remains a major challenge, first reported in Lobesa, Punakha district, in 1995 (Wangdi et al., 2019).

Chilli blight is difficult to control owing to the pathogen's polycyclic disease cycle, broad host range, and fungicide resistance (Gevens et al., 2007; Lamour et al., 2012). While current management practices incorporating integrated disease management, including cultural practices, resistant varieties and use of fungicides have been explored, no single method ensures long-term success (Sanogo & Ji, 2012; Granke et al., 2012). The risks associated with chemical fungicides led to the exploration of biological control as an alternative approach to chemical control (Ayilara et al., 2023). Biological control agents (BCAs), particularly *Trichoderma* spp. have shown promise suppression against *Phytophthora capsici* through antibiosis, mycoparasitism, niche exclusion, and induced resistance (Bae et al., 2011; Vinale et al., 2008; Zembek et al., 2011). In the Bhutanese context, integrating BCA, particularly *Trichoderma* spp. into organic farming initiatives will be a crucial way forward (Hawkins & Fraaije, 2016; Tjamos et al., 2013).

Currently, Bhutanese farmers rely on cultural practices such as crop rotation, elevated beds and the use of chemical fungicides as a last resort to control chilli blight. However, the increasing reports of chilli blight indicate the need for sustainable alternatives (National Plant Protection Centre, 2017). This study was conducted to isolate and identify native *Phytophthora* spp. and *Trichoderma* spp., assess the pathogenicity of *Phytophthora* spp. on detached chilli leaves and evaluate the antagonistic activity of native isolates of *Trichoderma* spp .against *Phytophthora* spp. Findings from this study would suggest the potential of developing native *Trichoderma* spp. as biofungicides, contributing to the nation's efforts to promote organic and sustainable disease management practices.

Materials and Methods

Sample collection

Soil and diseased chilli plant samples were collected from Wokuna (27°39'12.24'' N, 89°46'27.79'' E) and Kabjisa Gewog, Punakha District (1,300-1,800 masl), a major chilli-growing area where chilli blight is prevalent. Sampling followed a diagonal pattern within fields and the soil samples were taken at a 15 cm depth using a soil auger. For *Phytophthora* spp., diseased chilli plant parts with wilting and brown lesions were collected while soil samples were taken for *Trichoderma* spp. Ten plant samples for each fungus were collected in sterilized polyethene bags, labelled, transported in a cool box to the College of Natural Resources Biotechnology Laboratory and stored at 4°C to maintain viability for isolates.

Isolation of Trichoderma spp.

Trichoderma spp. was isolated using the serial dilution technique following the method described by Kumar et al. (2012) with slight modifications. 0.1 mL of 1×10^4 and 10^6 dilutions were plated on PDA plates amended with 0.1 g of strepto-penicillin (Dicrysticin-s, Zydus AH). The PDA plates were incubated at

28°C for 96 to 168 hours. Morphologically distinct colonies were iteratively subcultured using the hyphal tip isolation technique for the purification and the purified cultures were maintained on agar slants for further identification and biological assays.

Identification of Trichoderma spp.

Trichoderma spp. were identified both on macroscopic characteristics. The colony growth rate and pattern of the isolates were observed for macroscopic characteristics. The mycelia of each isolate were stained with lactophenol cotton blue and examined under a light microscope (Nikon Eclipse, E100) at 40X and 100X magnification. Characters of conidiophore branching and chlamydospores were recorded and compared to the key characteristics outlined by Samuels et al. (2002).

Isolation of Phytophthora spp.

The infected plant parts were rinsed under running tap water, blotted dry and surface sterilized with 70% ethanol and sodium hypochlorite for 10 seconds each. Further, infected plant parts were rinsed with distilled water and cut into 1 cm x 1 cm pieces. These were placed on PDA plates amended with 0.1 g of strepto-penicillin (Dicrysticin-s, Zydus AH) and incubated at 25°C in the dark for 96 hours (Sid et al., 2003). The fungal cultures were purified using the hyphal tip culture technique and maintained on PDA slants for further identification

Identification of Phytophthora spp.

Phytophthora spp. were identified both on macroscopic features (growth rate and colony patterns) and microscopic characteristics (mycelial structure and asexual spores) as described by Schmitthenner and Bhat (1994), which were examined under a light microscope (Nikon, Eclipse, E100) using 40X and 100X magnification. The asexual spores were induced by keeping the isolates under fluorescent light for 24 hours.

Pure culture preservation

Pure cultures of *Phytophthora* spp. and *Trichoderma* spp. were preserved in agar slants for short-term preservation. Pure cultures of both fungal cultures were stored in sterile distilled water for long-term preservation.

Pathogenicity testing

To prove Koch's postulate, a pathogenicity bioassay was carried out using one-month-old, detached leaves of a chilli variety *Sha Ema* grown in sterile conditions. Surface sterilized leaves were inoculated with 4 mm agar discs from 7 days-old culture isolates of *Phytophthora* spp. For the control, a sterile agar disc was positioned. The plates were sprayed with sterile distilled water to maintain humidity and incubated in the dark at 20–30ºC. The plates were replicated five times for each isolate. Infection severity was rated on a 0–3 scale, where 0 = healthy plant/leaf, 1 = 0.1 to 2 cm of infection length, 2 = 2.1 to 4 cm of infection and 3 = more than 4 cm of infection (Van der Waals et al., 2004).

Antagonistic effect of Trichoderma spp. *against Phytophthora* spp.

The antagonistic effect of each *Trichoderma* spp. isolate against *Phytophthora* spp. isolates was evaluated using a dual culture assay based on the method described by Zhang and Wang (2012), with slight modifications. The experiment was laid out in a Completely Randomized Design (CRD) with three replications per treatment.

Three *Trichoderma* spp. isolates (TCP1, TCP2, and TCP3) were tested against four *Phytophthora* spp. isolates (PP1, PP2, PP3, and PP4). Each treatment combination was replicated three times, and the control consisted of *Phytophthora* spp. isolates grown alone.

Both *Trichoderma* spp. and *Phytophthora* spp. isolates were pre-cultured on Potato Dextrose Agar (PDA) plates at 28°C for 72 hours. For each assay, a 4 mm agar plug from the margin of an actively growing *Trichoderma* spp. culture and a *Phytophthora* spp. culture was placed 3 cm apart on the same PDA plate. The control plates contained only a plug of the respective *Phytophthora* spp. isolate. All plates were incubated at 28°C for 120 hours.

The inhibition of Phytophthora mycelial growth was assessed by measuring the radial growth of the pathogen towards the *Trichoderma* spp. colony and compared to the control. The percentage inhibition (I%) was calculated using the formula of Mirmajlessi et al. (2016).:

$$Growth\ Inhibition\ (I\%) = (C - P)/C * 100$$

Where, C is the average radius of the *Phytophthora* spp. in control plates and P is the average radius of *Phytophthora* spp. in dual culture plates.

Data analysis

The International Business Machines Corporation-Statistical Package for Social Science (IBM SPSS statistics version. 23) was used for the analysis of radial mycelial growth of the isolates. The general linear model multivariate procedure was adopted for mean analysis between and within treatments. For the pathogenicity assay, the effect of treatments on virulence rating was analysed using one-way ANOVA and the means among the pathogen isolates were compared using the least significant difference (LSD) test ($p = 0.05$).

Results and Discussion

Isolation of Trichoderma spp. and Phytophthora spp.

A total of seven fungal isolates were isolated (Table 1). Three isolates of *Trichoderma* spp. (TCP1, TCP2 and TCP3) from the rhizosphere soil of chilli and four isolates of *Phytophthora* spp. (PP1, PP2, PP3 and PP4) from different plant parts of wilted chilli plants.

Morphological identification of Trichoderma isolates

The *Trichoderma* isolates showed distinct macroscopic and microscopic characteristics (Table 2). The microscopic features of paired branching patterns (Figure 1b) of conidiophores (Figure 1a) helped in identification as a

Table 1: Details of *Trichoderma* and *Phytophthora* spp. isolates

Isolate code	Fungus type	Sample type
TCP1	*Trichoderma* sp.	Soil
TCP2	*Trichoderma* sp.	Soil
TCP3	*Trichoderma* sp.	Soil
PP1	*Phytophthora* sp.	Stem
PP2	*Phytophthora* sp.	Leaves
PP3	*Phytophthora* sp.	Stem
PP4	*Phytophthora* sp.	Leaves

Table 2: Morphological characteristics of *Trichoderma* isolates

Isolate ID	Colony colour	Colony reverse colour	Colony pattern	Phialide	Conidia shape
TCP1	Greyish white	Yellowish	White cottony	Semi-papillate	Globose to subglobose
TCP2	White	White	White cottony	Semi-papillate	Sub-globose
TCP3	Green	Green	White cottony	Semi-papillate	Globose to subglobose

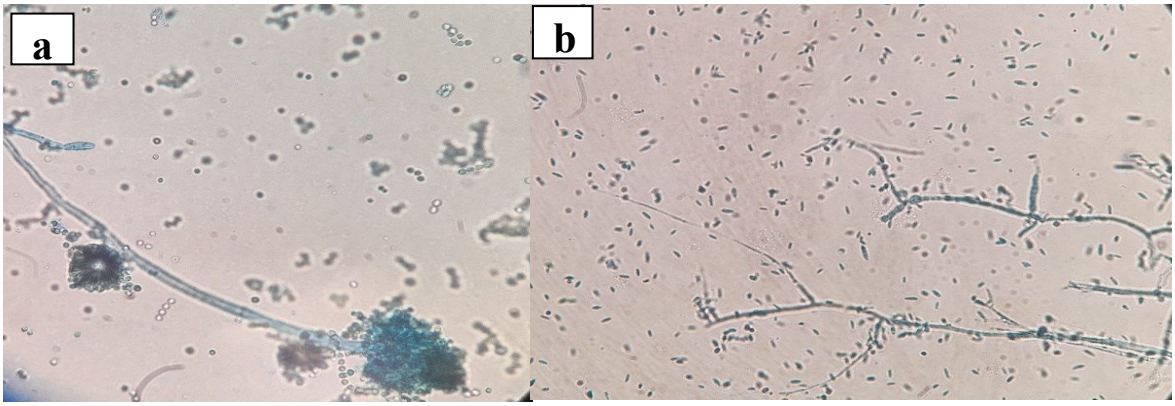

Figure 1: (a) Conidiophores of *Trichoderma* isolates (b) Paired primary branches

Morphological identification of Phytophthora isolates

Macroscopic observations of all four *Phytophthora* isolates exhibited different growth patterns on PDA but grew well at 28°C (Table 3). Microscopic observation of four isolates of *Phytophthora* spp. included the shape of sporangia which originated from an unbranched sporangiophore (Figures 2a and 2b).

The identification of *Phytophthora* spp. was carried out based on both macroscopic and microscopic characteristics. Morphological features such as colony appearance, growth temperature, and sporangial structures, including

Figure 2: (a)Unbranched sporangiophore bearing sporangia (b) zoospores under the microscope

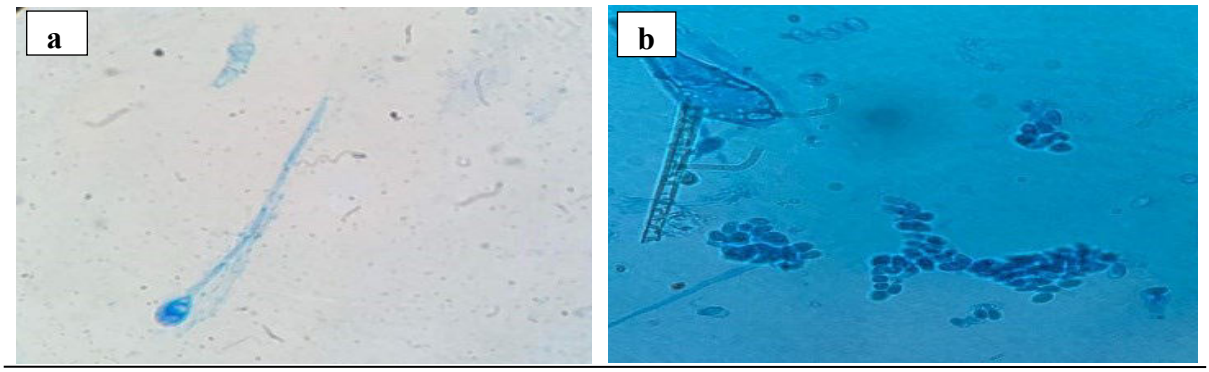

Table 3: Morphological characters of *Phytophthora* isolates

Isolate ID	Growth Tempera-ture	Colony character	Sporangia	Hyphae
PP1	21-31	Stellate White cottony appearance	Papillate	Swelled, globose
PP2	21-31	Stellate White cottony appearance	Papillate	Swelled, globose
PP3	21-31	No distinct pattern White cottony appearance	Papillate	Swelled, globose
PP4	21-31	No distinct pattern White cottony appearance	Papillate	Swelled, globose

branching patterns and sporangia morphology were key criteria, following the guidelines outlined by Abad et al. (2023). The isolates produced papillate, lemon-shaped sporangia,

consistent with descriptions reported by Ho (1981), Waterhouse et al. (1983), and Erwin and Ribeiro (1996). Additionally, identification incorporated observations of growth patterns, types of sporangia-bearing structures, degree of papillation, and spore types, as emphasized by Watanabe (2002). These combined traits provided reliable taxonomic evidence for the identification of *Phytophthora* spp.

Pathogenicity assay

Among the four isolates of the *P. capsica*, three of the isolates, namely, PP2, PP3 and PP3 showed the ability to cause disease on detached leaves of chilli with some visible distinct brown lesion. However, PP1 did not show any lesion failing to infect the leaves. There was variation in the disease severity score. The isolates PP2, PP3 and PP3 had an average virulence score of 1 (31–60 %) except for PP1 and control that had virulence score of 0. A one-way ANOVA test was used to test the virulence differences among the four isolates, namely, PP1, PP2, PP3 and PP4 of *Phytophthora capsici.*The result revealed that there was a statistically significant difference between the four isolates of *Phytophthora capsici*, ($F(4, 20) = 5.00$, $p = .006$). Post-Hoc test revealed significant difference between PP1 and PP2 (p=0.009), PP1 and PP3 (p=0.009), PP3 (p=0.009), PP1 and PP4 (p=0.009). However, there was no significant difference between PP1 and control (p=1.000).

A variation in the response to isolates by cultivars of pepper was also reported by Byung-Soo et al. (2010). The resistance in pepper cultivars depends on the inoculum concentration and virulence of the isolate (Jo et al., 2014). According to a time-course experiment carried out to investigate the interaction between *P. capsici* strain LT1534 and tomato, in the early stages of the infection, *P. capsici* ingress has two phases: a biotrophic phase in which the host tissues appear healthy

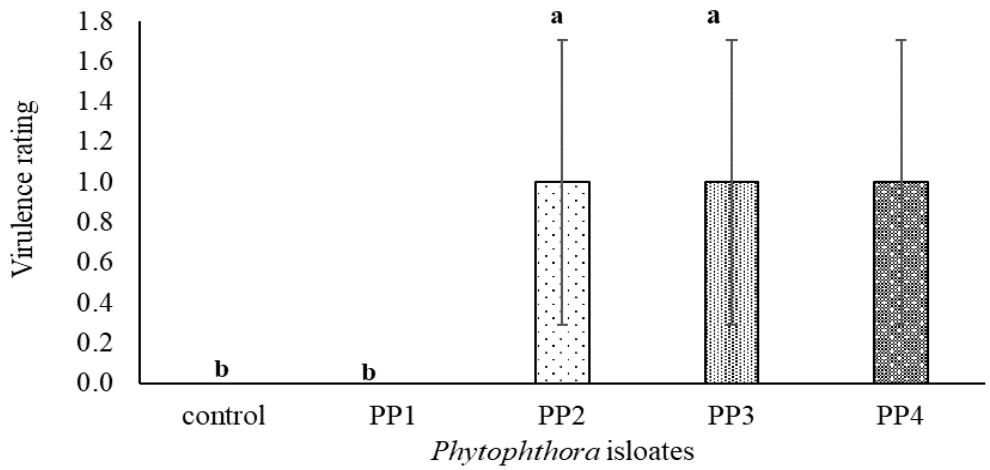

Figure 3: Virulence rating of four isolates of *Phytophthora* spp. on detached leaves under laboratory conditions. Each bar represents the mean virulence rating from three replicates, and the error bars indicate the standard deviation (SD). Bars labeled with the same letter are not significantly different at $p < .05$ based on one-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) test.

and unharmed, and a necrotrophic phase in which the host tissues collapse. Multiple inoculation trials revealed different phenotypic alterations in the later phases of infection, including water-soaked host tissue, cell death, and tissue collapse (Jupe & et al., 2013). *Phytophthora* pathogens secrete plenty of effectors that influence host processes to produce a favourable environment for pathogen colonization. Fan et al. (2018), reported that PcAvr3a12, a member of the Avr3a effector family and a *Phytophthora capsici* RXLR effector, lowers plant immunity by targeting and inhibiting host plant peptidyl-prolyl cis-trans isomerase (PPIase). Therefore, variation could be attributed to inoculation amount and leaves immunity.

Antagonistic effect of Trichoderma isolates against Phytophthora spp.

The antagonistic activity of three *Trichoderma* isolates (TCP1, TCP2 and TCP3) was evaluated against four *Phytopthora* spp. Isolates (PP1 -PP4) using a dual culture method over five days (Figure 4). The percentage inhibition of mycelial growth varied significantly based on both the *Trichoderma* and *Phytopthora* isolates.

Across all *Phytopthora* spp. isolates, TCP2 consistently showed the lowest inhibition, particularly during the early time points (72–96 hours), suggesting weaker antagonistic potential. In contrast, TCP1 and TCP3 were more vigorous, exhibiting faster growth and significantly higher inhibition percentages. For instance, TCP3 recorded the highest inhibition at 168 hours across PP1 (61.49%), PP2 (62.10%) and PP3 (60.19%), while still achieving moderate inhibition against PP4 (36.11%). TCP1 showed comparable trends, reaching 59.11% (PP2), 56.63% (PP3), 56.48% (PP1) and 34.07% (PP4). Meanwhile, TCP2 was the least effective, showing maximum inhibition of 45.18% (PP2), 44.44% (PP1), 39.63% (PP3), and 31.41% (PP4) by 168 hours. These results confirm that TCP3

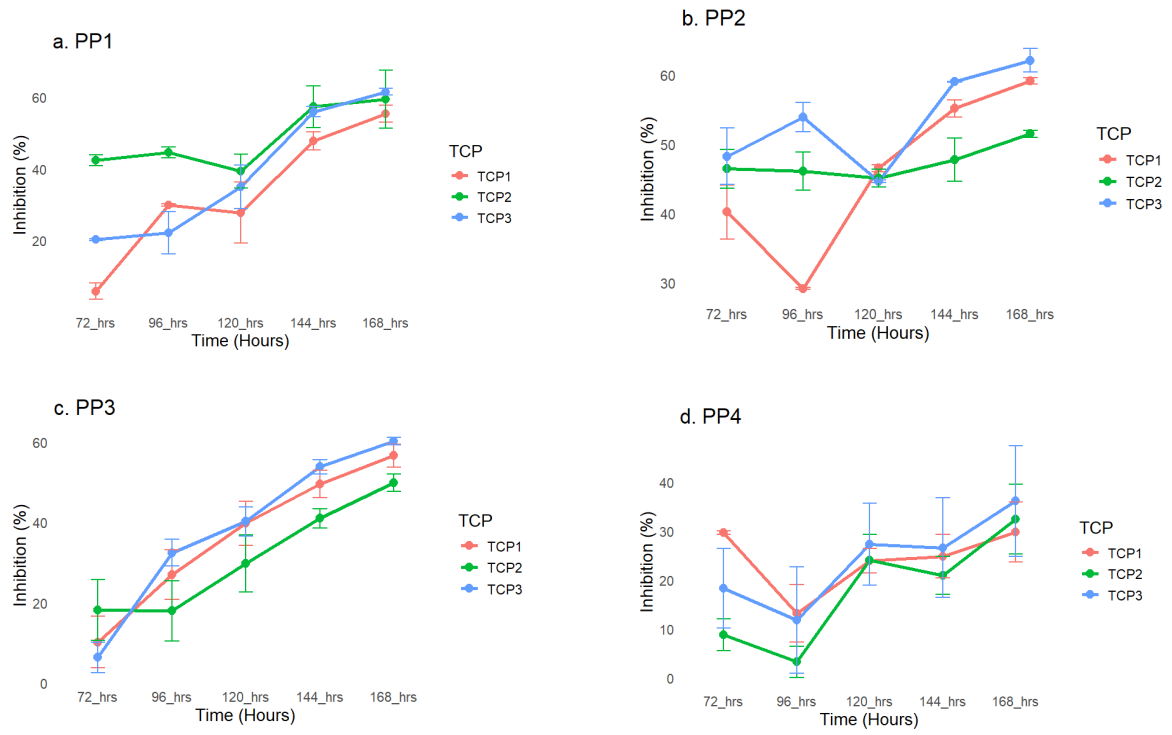

Figure 4: Inhibition of *Phytopthora* spp. by *Trichoderma* spp. isolates by dual culture assay. Figure 4a-d, Percentage inhibition (I%) of radial mycelial growth of four *Phytophthora* spp. isolates (PP1, PP2, PP3, and PP4) by three *Trichoderma* isolates (TCP1, TCP2, and TCP3) at 72, 96, and 168 hours post-inoculation. Each data point represents the mean of triplicate plates. Error bars represent the standard deviation of the

had the most robust and consistent antagonistic performance across the majority of *Phytopthora* spp. isolates.

A two-way ANOVA showed statistically significant interaction between *Trichoderma* isolates and *Phytopthora* spp. isolates ($F(30,82) = 7.261$, $p = .0001$), suggesting that the antagonistic effects were dependent on the specific isolate combinations.

These findings are in line with earlier studies. For instance, Ommati and Zaker (2012) evaluated the antagonistic capacities of different strains of *Trichoderma* spp. against *F. oxysporum* and concluded that *Trichoderma* spp. inhibited *Fusarium solani* with strong inhibition percentage. Anees et al. (2010) also reported similar inhibition of pathogen *R. solani* by *Trichoderma.* Timila and Manandhar (2019) proved in their study that *Trichoderma* spp. have potential biocontrol characteristics against *P. capsici* when subjected to a dual culture, bio-assay both with masking effects and with formation of zone of inhibition However, unlike previous studies that used lab-maintained isolates, this study utilized native isolates, which are better adapted to local environmental conditions—enhancing their potential for use in field-level biocontrol strategies.

There were differences in inhibition rate

for different isolates in several studies conducted both in-vitro and in-vivo and the difference could be due to isolate's differing biological regulatory mechanisms and production of antagonistic genes at varying degree of efficiency (Anees et al., 2010; Scherm et al., 2008).

Singh and Islam (2010) reported that antagonistic strains of *Trichoderma* spp. inhibited mycelial growth in *Phytophthora nicotianae*, resulting in a 61% reduction in radial growth of pathogen development compared to control, indicating that there are physiological variations between these isolates and these variations are attributed to the mechanism involved in the antag-

onistic activity by differential secretion of antifungal substances.

Bae et al. (2016) reported that five isolates of *Trichoderma* showed inhibitory activity against five *Phytophthora* species. The growth of *P. capsici* and *P. nicotianae* was inhibited by 80% and 64% in response to the culture filtrates of antibiosis occurs during interactions with pathogens and low-molecular-weight diffusible compounds or antibiotics, which are produced by *Trichoderma* (Benítez et al., 2004).

Conclusion

The findings from this study demonstrate the potential of native ***Trichoderma* spp.** in inhibiting the growth of *Phytophthora* spp., a major pathogen responsible for **chilli blight** in Bhutan. The **in-vitro** bioassay highlighted the strong potential of **TCP2 and TCP3 isolates** which exhibited significant antifungal activity, effectively inhibiting the growth of ***Phytophthora* spp.** In a world where **environmental sustainability** is the keyway forward, the integration of ***Trichoderma* spp.** into **integrated disease management strategies** presents a promising and eco-friendly alternative to chemical fungicides. The findings of this study highlight the potential for further exploration and application of the native ***Trichoderma* spp. in organic and sustainable farming systems**, thereby supporting Bhutan's national policy on **organic agriculture**.

However, in-vivo and field trials are necessary to validate the efficacy and consistency of these *Trichoderma* isolates in real-world field scenarios. Future research should also focus on optimizing ideal growth conditions for the isolates and the **formulation** of **Trichoderma -based biopesticides**. Moreover, further research is needed to understand the synergistic effects of *Trichoderma* spp. when combined with other biological control agents and **organic amendments** to develop a more effective and **integrated strategy for** managing **chilli blight.**

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