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In-vitro evaluation of *Trichoderma asperellum* as a bio-control agent against *Pythium aphanidermatum* causing rhizome rot in Turmeric

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Abstract
Turmeric (*Curcuma longa*) stands as a principal spice crop in India, with its economic significance primarily tied to the rhizome, a subterranean stem, utilized for diverse applications. However, the emergence of rhizome rot, a destructive ailment affecting turmeric crops, has led to substantial yield losses, courtesy of the soil-borne pathogen *Pythium aphanidermatum*. In this context, *Trichoderma*, recognized as a potent bio-agent, has gained widespread utilization for managing soil-borne diseases. This study aims to assess the effectiveness *of T. asperellum* against *P. aphanidermatum* under *in vitro* conditions. Ten isolates of *T. asperellum*, designated as Ta1 to Ta10, were subjected to evaluation. Among these isolates, Ta5 demonstrated the highest degree of mycelial inhibition, recording a substantial Percent reduction over the control (73.01%). Following closely, Ta6 exhibited a mycelial growth inhibition of 68.40%, while Ta8 demonstrated the least inhibition at 51.46%. Notably, the poison food technique at a 30% concentration yielded the highest percentage of inhibition, surpassing both 20% and 10% concentrations, respectively.

Keywords: Turmeric, rhizome, *Pythium aphanidermatum*, *Trichoderma asperellum*, *In vitro*

Introduction
Turmeric (*Curcuma longa* L.), a prominent spice crop cultivated in India since ancient times (Vinod, 2020) [31], holds significant economic potential due to its widespread use in the Ayurvedic industry (Rajalakshmi *et al*., 2016) [22]. Referred to as the "golden spice," it belongs to the Zingiberaceae family (Gupta *et al*., 2012) [15] and goes by various names such as 'hidden Lilly,' 'golden spice,' 'turmeric of commerce,' 'Indian saffron,' or 'Haldi' (Chavan *et al*., 2017) [5]. The rhizome of turmeric contains the active compound "curcumin," known for its diverse effects, including antioxidant, antitumor, antibacterial, antiviral, antiprotozoal, anti-fibrotic, antivenin, antiulcer, hypotensive, and hypocholesteremic activities (Gupta *et al*., 2012; Gujral *et al*., 1953; Kapoor, 1990; Araujo *et al*., 2001) [14, 15, 16, 4]. In turmeric-growing regions of India, rhizome rot caused by *Pythium sp.* poses a significant challenge (Rathiah, 1987; Nageshwar Rao, 1995; Ramarethinam and Rajagopal, 1999) [24, 20, 23]. This soil-borne disease is challenging to control with chemical applications, prompting the exploration of alternative methods. *Trichoderma*, identified as a potent biocontrol agent, is widely utilized for disease management. Experts recommend treating turmeric rhizomes with bioagents like *Trichoderma* spp. to effectively combat rhizome rot. *Trichoderma* spp. produces various antibiotics and lytic enzymes that prove effective against soil-borne pathogens, including its antagonistic activity against *P. aphanidermatum* and *P. myriotylum* (Ushamalini *et al*., 2008) [28].

Materials and Methods
Collection of soil samples
Soil specimens were systematically gathered from diverse turmeric cultivation regions within Namakkal, Salem, and Trichy districts of Tamil Nadu. These specimens were subsequently transported to the laboratory to facilitate the isolation process of *Trichoderma* spp.

Isolation and morphological identification of *Trichoderma* spp.
Soil samples were procured from an area cultivated with rhizomes on a farm to isolate *Trichoderma* spp. Subsequently, the samples were transported to the laboratory and refrigerated at 4 °C until analysis.

Serial dilutions, performed at a five-fold increment, were prepared for each soil sample in sterilized distilled water. A volume of 0.5 ml of the diluted sample was applied to the surface of *Trichoderma*-specific medium (TSM) as described. The plates were then incubated at 28±2 °C for 96 hours. Isolated colonies were subjected to further purification through single hyphal tip isolation and plating on Potato Dextrose Agar (PDA) medium. Morphological identification involved cultural characterization and microscopic observation in accordance with the method proposed by Savitha and Sriram (2015) [26]. Morphological characterization of the isolates included the assessment of colony morphology, conidial size, and conidial breadth (µm).

Identification of native *Trichoderma* spp.

Trichoderma spp. isolated in this study underwent identification using the taxonomic key proposed by Domsch *et al*. (1980) [9]. Pure cultures of the *Trichoderma* isolates were cultivated on Potato Dextrose Agar (PDA) and preserved at 4 °C in a refrigerator for subsequent applications. The identified isolates were designated as *T. asperellum* Ta1 to Ta10.

Dual culture technique

Effect of antagonist on the mycelial growth of *P. aphanidermatum*

The antagonistic efficacy of biocontrol agents (Ta1-Ta10) against *Pythium aphanidermatum* was assessed using the dual-culture technique as described by Dennis and Webster (1971). A 9 mm mycelial disc from a 7-day-old *Trichoderma asperellum* culture was placed in a petri plate containing 15 ml of solidified potato dextrose agar (PDA) media under aseptic conditions. Positioned approximately 75 mm away from the *Trichoderma* culture disc, a 9 mm mycelial disc from a seven-day-old culture of *P. aphanidermatum* was placed, and the Petri plate was incubated. A control group was established by inoculating *P. aphanidermatum* alone at one end of the Petri dish. The plates were then incubated at room temperature (28±2 °C) for a period of nine days.

$$\text{Percent inhibition (I)} = \frac{C-T}{T} \times 100$$

Where
I = Percent inhibition in growth of test pathogen
C = Radial growth (mm) in control
T = Radial growth (mm) in treatments

Preparation of the culture filtrates of *T. asperellum*

The *T. asperellum* isolates (Ta1-Ta10) were cultivated in Potato Dextrose Broth (PDB) within conical flasks and subjected to a 21-day incubation period in a BOD incubator set at 28±2 ºC. Subsequently, the liquid culture filtrate of *Trichoderma* was obtained after 21 days of incubation through filtration using Whatman filter paper No. 1 to eliminate mycelial material, and Seitz filter was employed for spore separation, as described by Alka *et al*. in 2017 [3].

Poison food technique (Groove and Moore 1962) [13].

A solution of TPDA medium was prepared in a 100 ml conical flask and subjected to autoclaving for sterilization. The culture filtrates obtained from antagonists of *T. asperellum* were introduced into sterile PDA medium at concentrations of 10%, 20%, and 30% using a sterile pipette. A control group consisted of PDA medium without the addition of culture filtrate. The modified media were then dispensed into sterile petri dishes at 15 ml each and allowed to solidify. Subsequently, a 7-day-old (9 mm) PDA culture disc of *P. aphanidermatum* was centrally inoculated onto each plate. Each treatment was replicated three times. The diameter of the mycelial growth (mm) of *P. aphanidermatum* was measured. As a comparative standard, the fungicide Metalaxyl was incorporated into the medium at a concentration of 0.1%.

$$\text{Percent inhibition (I)} = \frac{C-T}{T} \times 100$$

Where
I = Percent inhibition over control
C = Radial growth (mm) in control
T = Radial growth (mm) in treatment

Results and Discussion

Isolation and morphological characteristics of antagonistic fungi (*Trichoderma* sp.)

The ten *Trichoderma* strains were obtained from various turmeric cultivation regions in Tamil Nadu, as detailed in Table 1. Notably, Ta5 from Chinnasalem exhibited the highest level of dark green sporulation, featuring conidia with a length ranging from 2.93 to 3.98 µ and a breadth of 3.57 to 4.02 µ. Subsequently, Ta7 collected from Ramanayakampatti displayed green to bright green sporulation, with conidia measuring 2.84 to 3.61 µ in length and 2.61 to 3.69 µ in breadth. Ta4 from Veppur showed dark green sporulation, with conidia measuring 2.52 to 3.32 µ in length and 2.49 to 3.33 µ in breadth.

These *Trichoderma* strains exhibited white aerial mycelia, transitioning to green with yellow pigmentation in *T. koningii* and a dark green color in *T. hamatum*, as described by Parmar *et al*. (2015) [21]. Additionally, the genus *Trichoderma*, known for its rapid growth in culture medium, displayed the development of conidia with a green-yellow color, in accordance with findings by Chaverri *et al*. (2015) [6]. García-Núñez *et al*. (2017) [19] documented several *Trichoderma* isolates characterized by profuse fluffy mycelium, forming two to three well-defined concentric rings of mycelium (white) and conidia (green). Furthermore, Matas-baca *et al*. (2022) [19] observed that the microscopic examination of *Trichoderma* spp. isolates revealed dense conidia, branched conidiophores, ampulliform phialides, and slightly globose conidia with yellow-green pigmentation.

Efficacy of *Trichoderma asperellum* isolates against *P. aphanidermatum* (Dual culture)

The antagonistic potential of *T. asperellum* isolates collected from various turmeric cultivation regions in Tamil Nadu was assessed for their inhibitory effects against *P. aphanidermatum* using the dual culture technique (refer to Table 2). Among the tested isolates, *T. asperellum* (Ta5) exhibited the highest percentage of inhibition (73.01%), followed by *T. asperellum* (Ta6) with 68.40% inhibition, while *T. asperellum* (Ta8) demonstrated the least inhibition (51.46%) on the mycelial growth of *P. aphanidermatum*.

In a study by Thamarai Selvi *et al*. (2019) [27], *T. viride* emerged as the most effective antagonist against *P. aphanidermatum*, displaying the highest mycelial inhibition rate at 85.24%. This was followed by *T. hamatum* (80.64%)

and *T. harzianum* (73.13%). The observed reduction in the mycelial growth of *P. aphanidermatum* was attributed to a variety of mechanisms, including mycoparasitism, antibiosis, lysis, and hyphal interference.

Table 1: Isolation and morphological characteristics of antagonistic fungi (*Trichoderma* spp.)

S. No	Isolates	Locality	Colony characters	Conidia size	
				Length	Breath
1.	Ta_1	Sivapuri	Green to Dark green sporulation	2.63-3.41	2.11-3.12
2.	Ta_2	Vilambar	Dark green sporulation	2.49-3.33	2.27-3.14
3.	Ta_3	Pattanam	Green to bright green sporulation	2.22-3.12	1.92-3.04
4.	Ta_4	Veppur	Dark green sporulation	2.52-3.32	2.49-3.33
5.	Ta_5	Chinna Salem	Dark green sporulation	2.93-3.98	3.57-4.02
6.	Ta_6	Alanganatham	Whitish green to dull green sporulation	2.82-3.14	2.51-3.14
7.	Ta_7	Ramanayakampatti	Green to bright green sporulation	2.84-3.61	2.61-3.69
8.	Ta_8	Namagiripettai	Whitish green to dull green sporulation	2.14-2.99	1.75-2.92
9.	Ta_9	Gangavalli	Dark green sporulation	2.62-3.04	2.42-3.21
10.	Ta_{10}	Thalaivasal	Green to Dark green sporulation	2.54-3.69	2.39-3.09

Table 2: Efficacy of *Trichoderma asperellum* isolates against *P. aphanidermatum* (Dual culture)

S. No	Isolates	Mycelial growth (mm)		Percent reduction over Control (%)
		Trichoderma asperellum	*Pythium aphanidermatum*	
1.	Ta_1	59.14^{d}	30.86	65.71 (54.15)
2.	Ta_2	51.32^{h}	38.68	57.02 (49.03)
3.	Ta_3	48.26^{i}	41.74	53.62 (47.07)
4.	Ta_4	60.14^{c}	29.86	66.82 (54.82)
5.	Ta_5	65.71^{a}	24.29	73.01 (58.70)
6.	Ta_6	61.56^{b}	28.44	68.40 (55.79)
7.	Ta_7	53.93^{f}	36.07	59.92 (50.72)
8.	Ta_8	46.32^{j}	43.68	51.46 (45.83)
9.	Ta_9	52.81^{g}	37.19	58.67 (49.99)
10.	Ta_{10}	55.59^{e}	34.41	61.76 (51.80)
11.	Control	-	90.00	-

*Mean of three replications

*In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)

Effect of non-volatile compounds produced by *Trichoderma asperellum* isolate on the mycelial growth of *Pythium aphanidermatum* (Poison food technique)

The study observed a reduction in the mycelial growth of *P. aphanidermatum* as the concentration of culture filtrates from various isolates of *T. asperellum* increased. Notably, isolate Ta5 exhibited the greatest inhibitory effect with mycelial growth reductions of 25.01mm, 16.36mm, and 2.36mm at 10%, 20%, and 30% concentrations, respectively. The corresponding percent reductions were 72.21%, 81.82%, and 7.37%. In comparison, the control group without culture filtrate had a maximum mycelial growth of 90 mm.

Similar findings were reported by Alka *et al*. (2017) [3], who studied the impact of *Trichoderma* spp. culture filtrates on R. solani, revealing *T. asperellum* and *T. viride* as more effective inhibitors, while *T. virens* exhibited the least efficacy. Additionally, Misba Majeed *et al*. (2018) [18] identified *Trichoderma harzianum* as particularly effective against *Pythium aphanidermatum* due to the production of volatile metabolites. Elshahawy *et al*. (2018) [11] also reported a significant inhibitory effect of *Trichoderma virens* culture filtrates (TVS1, TVS2, TVS3) against *Pythium aphanidermatum*.

Table 3: Effect of non-volatile compounds produced by *Trichoderma asperellum* isolate on the mycelial growth of *Pythium aphanidermatum* (Poison food technique)

S. No	Isolates	Mycelial growth					
		10%	Percent inhibition over control	20%	Percent inhibition over control	30%	Percent inhibition over control
1	Ta_1	32.15^{d}	64.27	23.14^{d}	74.28	8.95^{d}	90.05
2	Ta_2	43.54^{g}	51.62	33.03^{h}	63.30	14.00^{g}	84.44
3	Ta_3	45.18^{h}	49.83	35.42^{i}	60.64	16.34^{h}	81.84
4	Ta_4	29.54^{c}	67.17	21.49^{c}	76.12	6.93^{c}	92.30
5	Ta_5	25.01^{a}	72.21	16.36^{a}	81.82	2.36^{a}	97.37
6	Ta_6	28.21^{b}	68.65	19.11^{b}	78.76	4.94^{b}	94.51
7	Ta_7	37.16^{e}	58.71	29.32^{f}	67.42	10.17^{e}	88.70
8	Ta_8	47.20^{i}	47.55	37.21^{j}	58.65	18.79^{i}	79.12
9	Ta_9	40.87^{f}	54.58	31.01^{g}	65.54	12.34^{f}	86.28
10	Ta_{10}	34.51^{e}	61.65	27.17^{e}	69.81	9.89^{e}	89.01

*Mean of three replications

* In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)

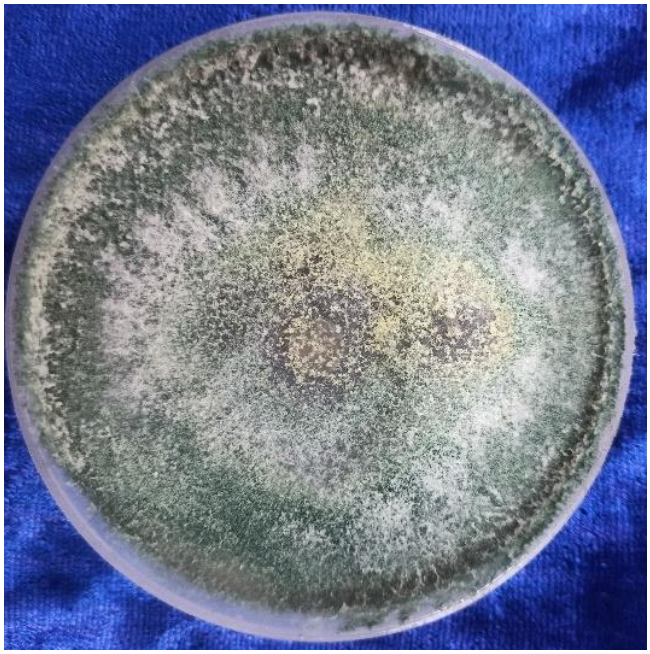

Fig 1: Axenic culture of antagonistic fungi (*T. asperellum*)

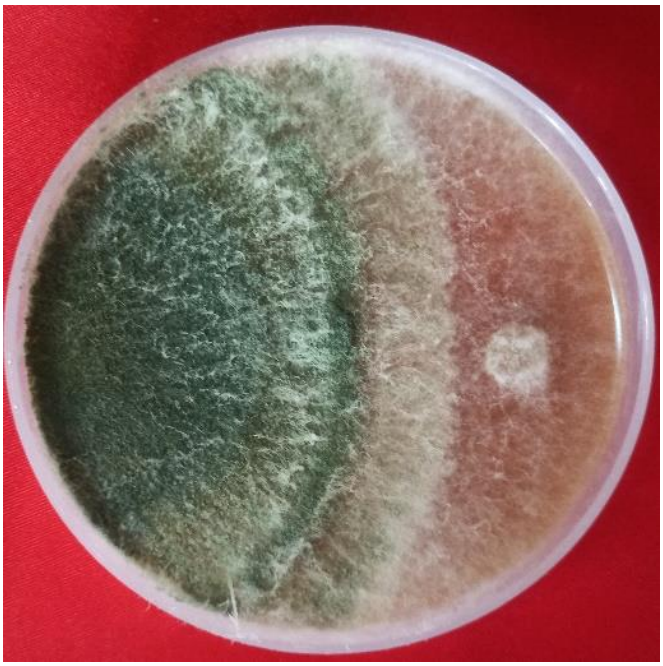

Fig 2: *In vitro* studies of *T. asperellum* and *P. aphanidermatum* (Dual culture) *T. asperellum* (Ta_5) against *P. aphanidermatum* (Pa_1)

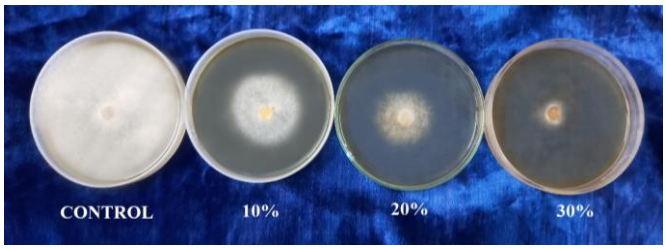

Fig 3: *In vitro* studies of bioagent and *P. aphanidermatum* (Poison food tech) Culture filtrate of *T. asperellum* with *P. aphanidermatum*.

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