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Isolation, Evaluation and Characterization of Indigenous *Trichoderma* spp. for the Management of Wilt of Chickpea Caused by *Fusarium oxysporum* f. sp. *ciceris*

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ABSTRACT

Background: *Fusarium oxysporum* f. sp. *ciceris* causing chickpea wilt, a significant soil-borne disease that causes severe crop losses. *Trichoderma* spp. is a diverse fungal bio-control agent with the ability to limit plant disease growth through a variety of ways. The goal of this work is to assess the efficiency of indigenous *Trichoderma* spp. isolated from chickpea rhizosphere from different North Eastern districts of Karnataka against the wilt pathogen *in vitro*, as well as to investigate the cultural and morphological aspects of isolates.

Methods: Dual culture approach was used to investigate the antagonistic potential of *Trichoderma* isolates and inverted plate technique was used to examine the synthesis of volatile compounds from *Trichoderma* spp. The isolates were cultured on potato dextrose agar medium for cultural characterization. Under a microscope, morphological characters were observed.

Result: Twenty *Trichoderma* isolates were reported to be effective against *F. oxysporum* f. sp. *ciceris*, preventing mycelial growth by 48.52 to 91.85%. All the isolates produced significant amount of volatile compounds that inhibited *F. oxysporum* f. sp. *ciceris* growth by 2.59 to 72.22%. At 72 hours after incubation, the isolates had different radial mycelium growth and formed fluffy and elevated colony growth, with colony margins ranging from smooth to wavy.

Key words: *Fusarium oxysporum* f. sp. *ciceris*, *Trichoderma*, Volatile compounds.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an annual legume crop produced worldwide for its high quality protein (20-22%), fibre, carotene and minerals. It is one of the oldest cultivated legumes, with 7500-year-old remains discovered in the Middle East. It is grown in India, Australia, Myanmar, Pakistan and the United States. After the common bean, it is the world's second most significant pulse crop. It is also known to fix nitrogen from the atmosphere (40 kg N ha^{-1}) and hence reduce the demand for nitrogen fertilizers. With an output of 101.3 million tonnes from a land area of 94.4 million hectares and a productivity of 1073 kg per hectare, India is a big producer of chickpeas. Madhya Pradesh has provided 37 per cent of the total area and 46 per cent of total production, thereby ranking first in both area and production (Ministry of Agriculture and Farmers Welfare, GOI, 2019).

Diseases and insect pests cause major productivity losses in chickpea cultivation, ranging from 50-100 per cent in tropical areas to 5-10 per cent in temperate areas (Van-Emden *et al*., 1988). In total, roughly 172 pathogens have been identified as infecting chickpea in various parts of the world. Approximately 67 fungi, 3 bacteria, 22 viruses and 80 nematodes have been identified among these pathogens (Nene *et al*., 1996), although only a handful have the ability to destroy the crop.

Wilt, caused by an important soil borne pathogen *Fusarium oxysporum* f. sp. *ciceris* (Padwick), is considered one of the most dangerous and widespread diseases of chickpea throughout the world's growing areas (Haware, 1990; Jalali and Chand, 1992). Wilt is a vascular disease that causes xylem browning or blackening due to melanin pigment and disrupts water and nutrient transfer, causing the plant to wilt or die. Under extreme circumstances, the wilt can entirely destroy the crop, resulting in a 100% loss.

Several chemicals are used to treat wilt disease, but their use has negative consequences for the environment, such as residual effects, health risks for humans and animals, soil degradation and pathogen resistance development. Biological control, which is an environmentally benign method, plays a significant role in the management of this soil-borne illness in this area.

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Trichoderma is a filamentous fungus that reproduces asexually and has a sexual teleomorph in the genus *Hypocrea*. *Trichoderma* spp. is a flexible bio-control agent that may be utilized to effectively manage a variety of plant diseases. Mycoparasitism, antibiosis, competition for nutrients or space, tolerance to stress through improved root and plant development, solubilization and sequestration of inorganic nutrients, induced resistance and inactivation of pathogen enzymes are only a few of the strategies used by *Trichoderma* spp. (Lewis and Lumsden, 2001). It generates both volatile and non-volatile compounds that inhibit the growth of many fungi (Corley *et al*., 1994; Horvath *et al*., 1995). As a result, the goal of this study was to examine the indigenous fungal bio-control agent *Trichoderma* spp. against the wilt pathogen.

MATERIALS AND METHODS

Isolation of pathogen *F. oxysporum* f. sp. *ciceris*

Chickpea plants with typical wilt symptoms were gathered from the field. The adhering dirt particles and other debris from the diseased stem area were thoroughly washed away with tap water. The infected stem section was chopped into small 1 cm pieces and surface sterilised by soaking for a minute in a 1% sodium hypochlorite solution. To remove traces of sodium hypochlorite, the bits were rinsed three times in sterile distilled water. The sterilized parts were inoculated onto Potato Dextrose Agar (PDA) medium and cultured for 5 to 7 days at 28±1°C (Rangaswami, 1972).

Collection of rhizosphre soil

During the cropping season, soil samples from healthy chickpea rhizosphere were obtained (*rabi* 2021). Six places in the field were chosen at random for soil sampling and a sample was taken from the root of each healthy plant from each spot. The top 2-3 cm of dirt was scraped away and the loose soil around the healthy root was collected. The six samples taken were thoroughly mixed to form a typical sample of that area, then placed in a polythene bag and appropriately labelled. A total of twenty samples were obtained from various taluks in North Eastern Karnataka.

Isolation of *Trichoderma* spp. from rhizosphere soil of chickpea

Trichoderma spp. were isolated from the collected samples at the Bio-input Entreprenureship Centre, College of Agriculture, University of Agricultural Sciences, Raichur. To obtain a 1:10 dilution, ten grams of soil sample were suspended in 90 ml of sterilised distilled water and vigorously mixed with vertex mixture (10^{-1}). To produce a 1:100 (10^{-2}) dilution, one ml of this was transferred to a test tube holding 9 ml of sterilised distilled water. Similarly, a 1:100000 dilution of the material was prepared (10^{-5}). Each sterile Petri plate was pipetted with one millilitre of a final dilution of each sample, followed by 15-20 ml of sterilized and melted *Trichoderma* Selective Medium (TSM) (Ingredients per lit: $MgSO_4$. $7H_2O$ - 0.2 g; K_2HPO_4 - 0.9 g; KCL - 0.15 g; NH_4NO_3 - 1 g; Glucose - 3 g; Rose Bengal - 0.15 g; p-dimethylamino benzene diazo sodium sulfonate - 0.3 g; Chloramphenicol - 0.25 g; Pentachloronitrobenzene- 0.2 g; Agar-15 g). The plates were incubated at 28±1°C for roughly 6-10 days after being filled and gently rotated for uniform mixing. The appearance of *Trichoderma* colonies on the Petri plates was monitored on a daily basis. The colonies started out white and eventually turned green. An actively developing colony of *Trichoderma* was picked from these plates among the various colonies and plated on PDA medium, with plates incubated at 28±1°C for four days.

Cultural characterization of native *Trichoderma* isolates

The isolates of *Trichoderma* spp. were described based on their cultural characteristics. Petri plates were inoculated with pure cultures of twenty *Trichoderma* spp. and incubated at 28±1°C for this experiment. After 72 h of incubation, the colonies were evaluated for colony diameter, colony growth type, colony margin and the first appearance of green conidia.

Morphological characterization of native *Trichoderma* isolates

The cultures were incubated at 20°C for morphological characterization of *Trichoderma* spp. Microscopic preparations included slides made in 3% KOH followed by lactophenol-cotton blue from pustules with white conidia within 7 days of incubation at 25±2°C. The slide was examined under the microscope for mycelium type, phialides arrangement, conidial shape and colour, production of chlamydospores and their position, after the cover slip was placed. Bisset (1991) provided morphological and taxonomic keys that were used to identify the species.

Antagonistic potential of *Trichoderma* spp. against *F. oxysporum* f. sp. *ciceris*

Using a dual culture approach, all 20 isolates of *Trichoderma* spp. were tested for antagonistic capability against the pathogen. The vigorously growing culture of *F. oxysporum* f. sp. *ciceris* and *Trichoderma* spp. was sliced into 5 mm dia. mycelial discs and deposited on fresh PDA media on either side of the Petri plate. Control plates were inoculated with *F. oxysporum* f. sp. *ciceris* but not with *Trichoderma* spp. Each treatment was tested three times. Plates were cultured for 10 days at 28±2°C, until the pathogen completely covered the control plate. The degree of antagonism was assessed by comparing the pathogen's radial development with that of a control *Trichoderma* culture. The per cent inhibition was calculated in comparison to the control.

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

Where;

I= Per cent inhibition.

C= Radial growth of pathogen in control.

T= Radial growth of pathogen in treatment.

Volatile compounds production by *Trichoderma* spp

The inverted plate technique was used to examine isolates of *Trichoderma* spp. for the generation of inhibitory volatile chemicals (Dennis and Webster, 1971). PDA medium was put into sterilised Petri plates and allowed to solidify. The pathogen *F. oxysporum* f. sp. *ciceris* and the mycelial disc (5 mm) of *Trichoderma* spp. were infected in the centre of Petri plates. Both *Trichoderma* spp. and pathogen inoculated plates had their upper lids removed. The pathogen-containing plate was flipped over the *Trichoderma* spp. containing plate and the two were sealed with adhesive tape (parafilm), with *Trichoderma* spp. on the lower side and pathogen on the upper side. The pathogen-bearing Petri plate was inverted over the Petri plate containing PDA media as a control. Each treatment was repeated three times and the plates were incubated for five days at 28±1°C. The per cent inhibition was calculated by using formula of Vincent (1947).

Statistical analysis

The data obtained in the present investigations for various parameters in the experiments were subjected ANOVA for a completely randomized design (CRD) for *in vitro* studies by using OPSTAT programme.

RESULTS AND DISCUSSION

Symptoms of wilt

Initially, indications such as yellowing of older leaves followed by drooping and drying, were noted in adult plants. Later, partial wilting, drying and death of the plant were observed. The entire plant wilted under extreme conditions. When the stem was longitudinally split apart, there were no evidence of rotting on the outside, but brown to black discoloration of internal tissue was visible (Fig 1) (Sageera *et al*. 2012; Kandoliya and Vakharia, 2013).

Isolation of pathogen *F. oxysporum* f. sp. *Ciceris*

At seven days after incubation, a whitish colony of fungus with fuzzy profuse mycelium was seen. It eventually turned to pink. The pathogen was identified using standard mycological criteria based on mycelial and conidial features (Barnett and Hunter, 1972). The fungus generated a large number of spindle-shaped, curved macroconidia with three to five septa, whereas microconidia were fusiform with rounded apex and no septa. The chlamydospores were globose to oval in shape, had a thick wall and appeared terminally or intercalarily (Fig 2). Nelson (1981) and Di-Pietro *et al*. (2003) made similar results, reporting that

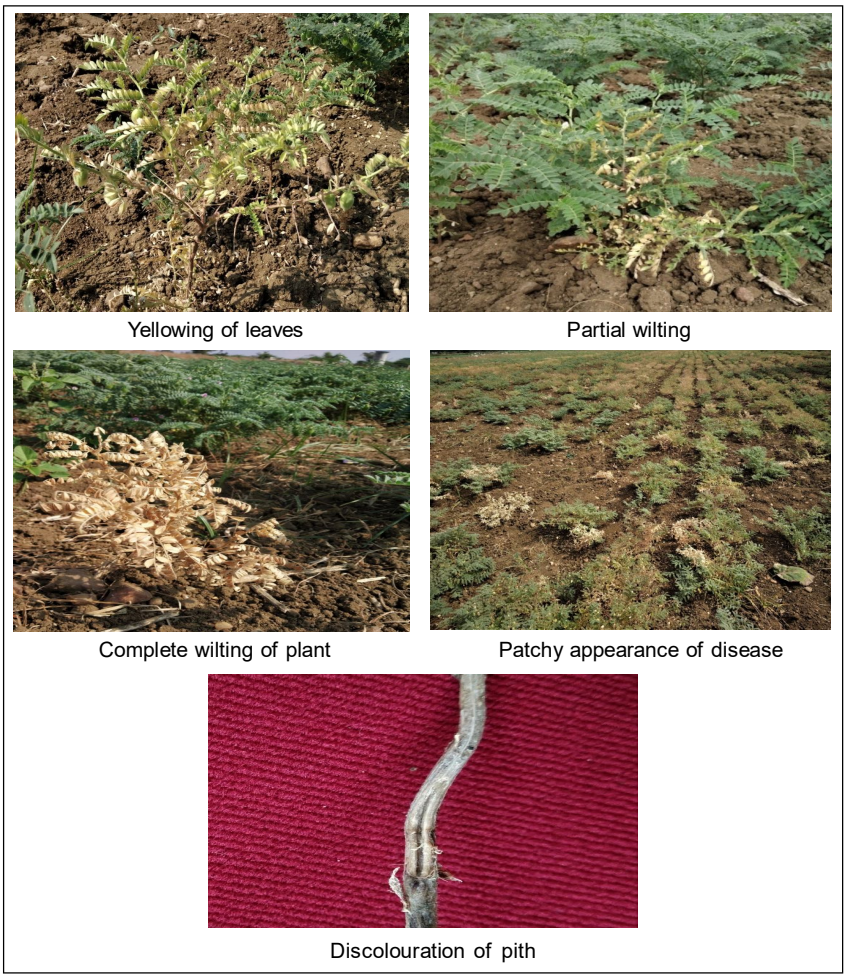

Fig 1: Symtomatology of chickpea wilt.

F. oxysporum develops colourless to pale mycelium that turns pink or purple with age when produced in culture with ovoid microconidia and spindle-shaped macroconidia with septa. Chlamydospores with one or two cells might be terminal or intercalary.

Isolation of *Trichoderma* spp. from rhizosphere soil of chickpea

Following incubation, all 20 isolates developed a typical greenish colony on TSM as well as features that were identical to *Trichoderma* under the microscope.

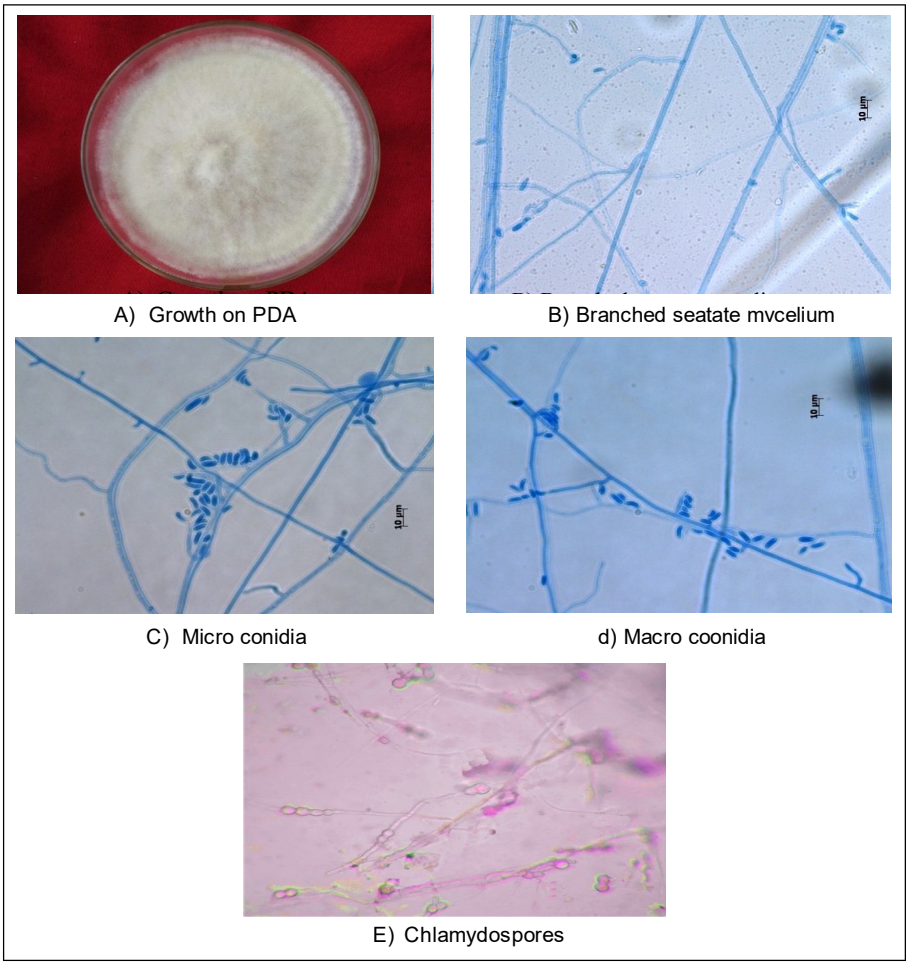

Fig 2: Culture and morphological characters of *Fusarium oxysporum* f. sp. *Ciceris*.

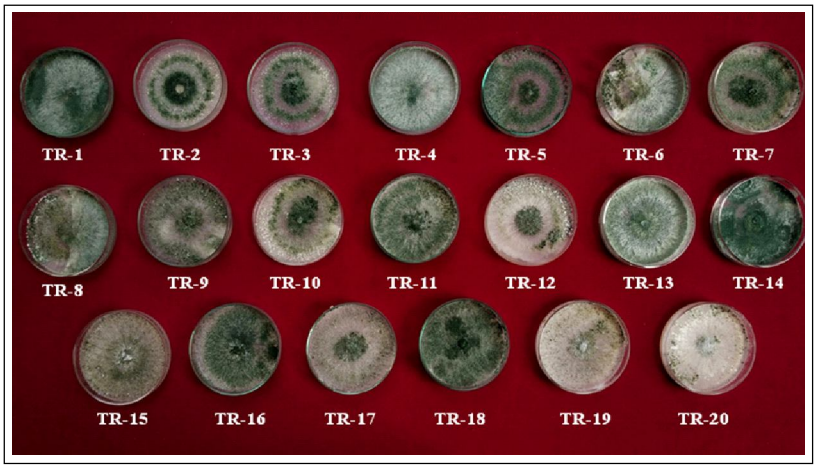

Fig 3: Cultural variability of native isolates of *Trichoderma* spp.

Cultural characterization of native *Trichoderma* isolates

At 72 h after incubation, all of the isolates had different radial mycelium growth. When compared to other isolates, TR-1, TR-13, TR-14 and TR-18 grew to a maximum of 90.00 mm after 72 h of incubation. Colony development was fluffy and elevated in all isolates and colony margins ranged from smooth to wavy. Green conidia production, on the other hand, was began 36 h after incubation and measured from the colony's edge after 72 h. The results are presented in Table 1 and Fig 3. Sharma and Singh (2014) additionally looked at the 30 *Trichoderma* isolates' cultural features and growth rates. All of the isolates grew quickly and generated

Table 1: Cultural characteristics of native *Trichoderma* spp. isolated from chickpea rhizosphere.

Isolates	Colony growth (mm)	Colony colour	Colony shape	Type of growth	Type of margin	Initiation of sporulation (h)
TR-1	90.00	Whitish green	Circular	Fluffy and raised	Smooth	48
TR-2	81.67	Whitish green	Circular	Fluffy and raised	Wavy	72
TR-3	75.00	Whitish green	Irregular	Fluffy and raised	Wavy	72
TR-4	90.00	Whitish green	Circular	Fluffy and raised	Smooth	48
TR-5	86.67	Green	Irregular	Fluffy and raised	Wavy	36
TR-6	78.33	Whitish green	Irregular	Fluffy and raised	Wavy	48
TR-7	81.67	Light green	Irregular	Fluffy and raised	Wavy	72
TR-8	83.33	Whitish green	Irregular	Fluffy and raised	Smooth	72
TR-9	85.00	Whitish green	Irregular	Fluffy and raised	Wavy	48
TR-10	88.00	Whitish green	Irregular	Fluffy and raised	Wavy	72
TR-11	90.00	Green	Irregular	Fluffy and raised	Wavy	48
TR-12	86.67	Whitish green	Irregular	Fluffy and raised	Wavy	72
TR-13	90.00	Light green	Circular	Fluffy and raised	Smooth	36
TR-14	90.00	Green	Irregular	Fluffy	Smooth	36
TR-15	90.00	White	Circular	Fluffy and raised	Wavy	72
TR-16	87.33	Whitish green	Irregular	Fluffy and raised	Wavy	48
TR-17	86.67	White	Irregular	Fluffy and raised	Wavy	72
TR-18	90.00	Whitish green	Irregular	Fluffy and raised	Wavy	48
TR-19	90.00	White	Irregular	Fluffy and raised	Smooth	72
TR-20	88.33	White	Irregular	Fluffy and raised	Wavy	72

Note: Observations were taken at 72 h.

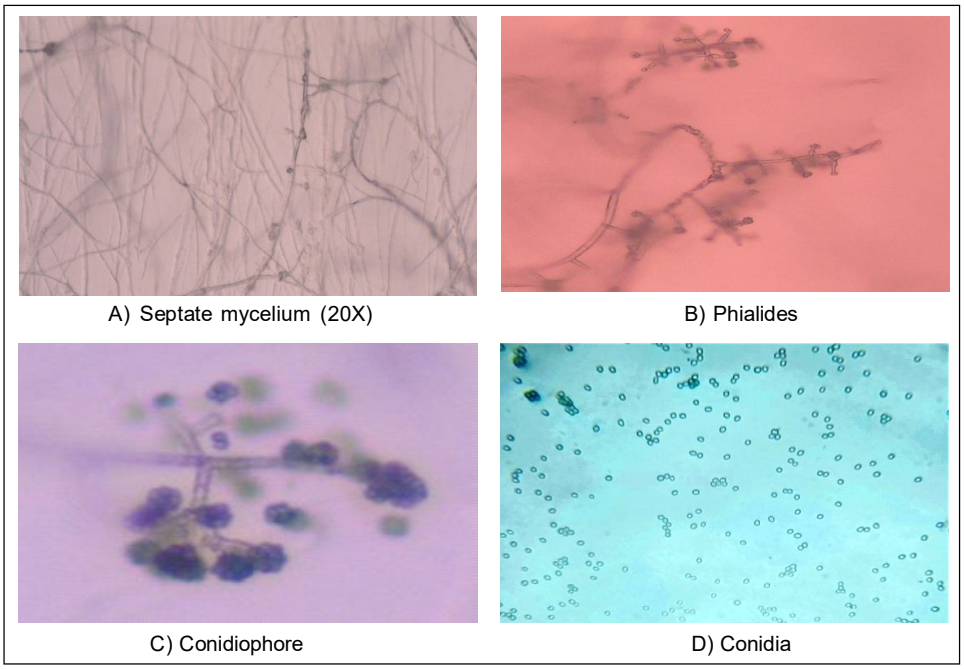

Fig 4: Morphological characters of *Trichoderma* spp.

abnormally compact colonies with uneven margins, as well as a shift in conidial colour from white to various hues of green. Divya *et al*. (2015) investigated the cultural features of 44 *Trichoderma* spp. isolates, finding that all of the isolates grew quickly and covered the full Petri plate within 72 hours. There was some variation among the isolates in rate of growth, margin, colour of mycelia and sporulation.

Morphological characterization

Microscopic features such as mycelium, phialides organization, conidial shape and colour and chlamydospore development and location were observed. All of the isolates had hyaline, septate and branching mycelium, as well as narrow, pin and broad phialides. With terminal, intercalary and terminal to intercalary chlamydospores, pale to light green coloured conidia with globose to subglobose and oval shaped conidia were generated (Table 2 and Fig 4). *Trichoderma harzianum* (AF1), *T. viride* (AF2) and *T. virens* (AF3) were isolated from coconut rhizosphere soil samples by Ranjana *et al*. (2013). They observed that the branching of conidiophores, the shape of phialides, the emergence of phialospores and the shape of phialospores were used to make the identification.

Table 2: Morphological characteristics of native *Trichoderma* spp. isolated from chickpea rhizosphere.

Isolate code	Mycelium arrangement	Phialide arrangement	Conidial shape	Conidial colour	Chlamydospores
TR-1	Hyaline, septate and branched	Narrow at base pointed at tip	Globose to oval	Pale green	Intercalary
TR-2	Hyaline, septate and branched	Narrow at base pointed at tip	Oval	Green	Intercalary and terminal
TR-3	Hyaline, septate and branched	Narrow at base pointed at tip	Oval	Green	Terminal
TR-4	Hyaline, septate and branched	Pin shaped	Oval	Pale green	Intercalary and terminal
TR-5	Hyaline, septate and branched	Narrow at base pointed at tip	Oval	Light green	Intercalary and terminal
TR-6	Hyaline, septate and branched	Narrow at base pointed at tip	Globose	Light green	Intercalary and terminal
TR-7	Hyaline, septate and branched	Broad base and narrower at tip	Sub globose	Light green	Intercalary
TR-8	Hyaline, septate and branched	Broad base and narrower at tip	Sub globose	Light green	Terminal
TR-9	Hyaline, septate and branched	Narrow at base pointed at tip	Oval	Light green	Intercalary and terminal
TR-10	Hyaline, septate and branched	Narrow at base pointed at tip	Oval	Green	Intercalary and terminal
TR-11	Hyaline, septate and branched	Narrow at base pointed at tip	globose	Light green	Intercalary and terminal
TR-12	Hyaline, septate and branched	Narrow at base pointed at tip	Globose to oval	Light green	Intercalary and terminal
TR-13	Hyaline, septate and branched	Narrow at base pointed at tip	Globose	Light green	Intercalary and terminal
TR-14	Hyaline, septate and branched	Narrow at base pointed at tip	Oval	Green	Intercalary
TR-15	Hyaline, septate and branched	Narrow at base pointed at tip	Globose	Light green	Intercalary and terminal
TR-16	Hyaline, septate and branched	Broad base and narrower at tip	Sub globose	Light green	Terminal
TR-17	Hyaline, septate and branched	Pin shaped	Oval	Pale green	Intercalary and terminal
TR-18	Hyaline, septate and branched	Narrow at base pointed at tip	globose	Light green	Intercalary and terminal
TR-19	Hyaline, septate and branched	Broad base and narrower at tip	Sub globose	Light green	Terminal
TR-20	Hyaline, septate and branched	Narrow at base pointed at tip	globose	Light green	Intercalary and terminal

Table 3: Antagonistic potential of native *Trichoderma* spp. isolates against *F. oxysporum* f. sp. *ciceris*.

Isolate code	Colony growth* (mm)	Per cent mycelial inhibition*
TR-1	36.67	59.26 (50.33)
TR-2	30.00	66.67 (54.73)
TR-3	30.67	65.93 (54.28)
TR-4	40.67	54.81 (47.76)
TR-5	41.67	53.70 (47.12)
TR-6	41.33	54.07 (47.33)
TR-7	31.67	64.81(53.62)
TR-8	32.33	64.07 (53.18)
TR-9	21.67	75.93 (60.61)
TR-10	15.00	83.33 (65.90)
TR-11	36.33	59.63 (50.55)
TR-12	29.67	67.04 (54.95)
TR-13	40.00	55.56 (48.18)
TR-14	07.33	91.85 (73.40)
TR-15	42.33	52.96 (46.69)
TR-16	46.33	48.52 (44.15)
TR-17	40.00	55.56 (48.18)
TR-18	30.00	66.67 (54.73)
TR-19	37.00	58.89 (50.11)
TR-20	42.00	53.33 (46.91)
Control	90.00	0.00 (0.00)
S. Em. ±	-	0.73
CD at 1%	-	2.79

*Mean of three replications, figures in the parenthesis are arcsine transformed values.

Table 4: Effect of volatile compounds produced by native *Trichoderma* spp. isolates on mycelial inhibition of *F. oxysporum* f. sp. *ciceris*.

Isolate code	Colony growth* (mm)	Per cent mycelial inhibition*
TR-1	70.00	22.22 (28.12)
TR-2	60.00	33.33 (35.26)
TR-3	35.00	61.11 (51.41)
TR-4	82.00	8.89 (17.28)
TR-5	40.00	55.56 (48.18)
TR-6	41.67	53.70 (47.12)
TR-7	50.00	44.44 (41.80)
TR-8	50.70	43.70 (41.38)
TR-9	30.67	65.93 (54.28)
TR-10	85.00	5.56 (13.63)
TR-11	65.00	27.78 (31.80)
TR-12	31.00	65.56 (54.06)
TR-13	43.33	51.85 (46.06)
TR-14	25.00	72.22 (58.19)
TR-15	75.00	16.67 (24.09)
TR-16	40.00	55.56 (48.18)
TR-17	40.67	54.81 (47.76)
TR-18	87.67	2.59 (9.26)
TR-19	30.00	66.67 (54.73)
TR-20	85.67	4.81 (12.67)
Control	90.00	0.00 (0.00)
S. Em. ±	-	1.02
CD at 1%	-	3.91

*Mean of three replications, figures in the parenthesis are arcsine transformed value.

Antagonistic potential of native *Trichoderma* spp. against *F. oxysporum* f. sp. *ciceris*

The findings demonstrated that all twenty *Trichoderma* spp. isolates were antagonistic to *F. oxysporum* f. sp. *ciceris*, with mycelium inhibition ranging from 48.52 to 91.85 per cent. TR-14 had the highest percent inhibition of 91.85 per cent, followed by TR-10 (83.33%) and TR-9 (75.93%). TR-16 showed the least inhibition (48.52%), followed by TR-15 (48.52%). (52.96%) (Table 3). *T. asperellum* was used by Nayak and Pandey (2017) to fight *F. oxysporum* f. sp. *ciceris*. Thaware *et al*. (2017) also tested *Trichoderma* spp. against *F. oxysporum* f. sp. *ciceris* and found that *T. viride* and *T. harzianum* inhibited the test pathogen's mycelial growth by 75.55 and 73.77 per cent, respectively.

Volatile compounds production by native *Trichoderma* spp.

The findings revealed that each isolate produced a significant amount of volatile compounds, which differed between isolates. TR-14 (72.22%) produced the highest concentration of volatile compounds, followed by TR-19 (66.67%), TR-9 (65.93%) and TR-18 (65.93%). TR-18 produced the lowest concentration of volatile compounds (2.59%). When compared to the control, all of the isolates showed a substantial difference in mycelial growth inhibition (Table 4). Nagamani *et al*. (2017) also discovered that volatile metabolites produced by *T. asperellum* were the most effective in suppressing *F. oxysporum* f. sp. *ciceris* mycelial growth by 86.70 per cent. According to Mohit *et al*. (2019), volatile chemicals produced by *T. harzianum* had a strong inhibitory effect on the mycelial growth of *F. oxysporum* f. sp. *ciceris* (79.25%), followed by *T. viride* (79.25%).

CONCLUSION

A total of twenty native isolates *Trichoderma* spp. were shown to have antagonistic activity against *F. oxysporum* f. sp. *ciceris* and produced volatile chemicals that effectively reduced *F. oxysporum* f. sp. *ciceris* growth. Within twenty isolates of *Trichoderma* spp., there was heterogeneity in culture parameters such as colony diameter, type of colony growth, colony margin and initial appearance of green conidia. *Trichoderma* spp. are effective in the treatment of wilt disease.

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Conflict of interest

All authors declared that there is no conflict of interest.

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