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Assessment of the biocontrol attributes of native *Trichoderma* isolates and their field evaluation against Fusarium wilt of vegetable cowpea

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Abstract
In this study, five novel isolates of *Trichoderma viz.*, TRMW-2, TRKR-2, TRPN-3, TRPN-11 and TRPN-17 from virgin forest soils of different agro-climatic zones of Kerala were evaluated for its potent antagonistic efficacy against Fusarium wilt of vegetable cowpea. Based on the *in vitro* studies the effective isolates TRKR-2, TRPN-17, TRPN-3, consortia of isolates TRKR-2 and TRPN-17 and consortia of isolates TRPN-3 and TRPN-17 were selected for the *in vivo* studies. In the present study, *Trichoderma* isolates TRKR-2, TRPN-3, and consortium of TRPN-3 and TRPN-17 were found effective against Fusarium wilt of vegetable cowpea under field conditions. The efficacy of the isolates against other soil-borne diseases of vegetable cowpea and species level identification need to be explored.

Keywords: Antogonistic efficacy, *Trichoderma*, Fusarium wilt, lytic enzymes

1. Introduction
Vegetable cowpea or yard long bean (*Vignaunguiculata* subsp. *sesquipedalis* (L.) Verdcourt) is one of the economically important legume crops widely cultivated in the wetland fallows of Kerala due to its favourable agro-climatic conditions as it fits well in the multi-farming system. Green pods and seeds are consumed as fresh vegetables and the plants can also be used as fodder, green manure, or cover crop. The tender pods are an inexpensive and rich source of digestible protein (28%), iron (2.5 mg/100 g), calcium (80 mg/100 g), phosphorus (74 mg/100 g), vitamin A (941 IU/100 g), vitamin C (13 mg/100 g), and dietary fiber (2 g/100 g), which makes it an outstanding vegetable crop (Singh *et al*, 2001) [32]. The crop has become very important in agriculture due to its ability to fix atmospheric nitrogen through its well-branched root structure. It reduces soil erosion by enhancing the soil binding effect.
Soil-borne diseases, such as Fusarium wilt, collar rot, web blight, etc., have a significant impact on the crop at various stages of its development. In recent years, Fusarium wilt has become one of the most devastating crop diseases in Kerala (Reghunath *et al*., 1995) [30]. The management of Fusarium wilt commercially relies on the use of fungicides. The necessity to find effective natural bio control agents in integrated crop management programs has arisen due to the negative impact of fungicides and market regulations. The use of bioagents as part of integrated disease management has also been investigated.
Although many other bio control strategies have been explored, one of the most promising ways to both decrease fungicide use and guarantee safe food production is to deploy more potent strains of *Trichoderma*. It is a predominantly used fungal biocontrol agent against soil-borne pathogens due to its capacity to effectively counteract soil-borne fungal infections. It inhibits plant pathogens through several mechanisms *viz.*, competition, enzyme release, hyphal contacts and mycoparasitism.
In addition, knowledge on bio control attributes like enzyme production etc. of *Trichoderma* isolates also helps in selecting the best candidate for effective disease management. Field evaluation of promising bioagents will help in carrying forward new strains to combat soil-borne diseases.

2. Materials and Methods
2.1 *Trichoderma* isolates
Five promising isolates of *Trichoderma* spp. *viz.*, TRMW-2, TRKR-2, TRPN-3, TRPN-11 and TRPN-17 from the previous study (Cyriac *et al*., 2021 [8]; Nair *et al* 2022 [26]), collected earlier

from virgin forest soils of Kerala and found effective in *in vitro* and pot culture experiments against *Fusarium* wilt of vegetable cowpea were used in the present study. In addition, *Trichoderma asperellum* (KAU strain) was also used. All six isolates were sub-cultured and maintained in Potato Dextrose Agar (PDA) medium.

2.2 Assay of activity of cell wall degrading enzymes of *Trichoderma* isolates

2.2.1 Preparation of culture filtrate for enzyme assay of *Trichoderma* spp. isolates

Culture filtrate was prepared by transferring the actively growing mycelial bits of the *Trichoderma* isolates from a three-day-old Petri plate to freshly prepared 100 ml Potato Dextrose Broth (PDB) in 250 ml conical flasks. One % of either chitin, casein or triolein was used for enzyme induction and incubated at room temperature (28 ± 2 ℃) for five days. Using Whatman filter paper No. 1 the contents in the conical flask were filtered and centrifuged at 9000 rpm at 4°C for 10 min for clarification. The supernatant was collected and 2X volume of ice-cold acetone was added for protein precipitation and incubated overnight at 4 °C. Then it was again centrifuged at 9000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet was air-dried. Then the pellet was dissolved in the appropriate buffer (sodium acetate buffer for chitinase; Tris HCL for lipase and protease) and the resulting solution was used as the culture filtrate for the enzyme assay.

2.2.2 Chitinase

The chitinase activity was determined using the method described by Gajera and Vakharia (2012) [13]. Colloidal chitin (0.5%) was prepared using 10 mM of sodium acetate buffer (pH 5.2). Two hundred µl of 0.5% colloidal chitin and 100 µl of culture filtrate were used to prepare the reaction mixture which was incubated at 50 °C for one hour. For a known volume of the reaction mixture, an equal volume of 120 mM of potassium borate buffer was added to the test tube and boiled vigorously in the water bath for three min. Then three ml p-dinitro methyl amino benzaldehyde (DMAB) reagent was added to each test tube and incubated at 38 °C for 20 min. Then the tubes were allowed to cool and absorbance was measured at 544 nm using a spectrophotometer. Standard was prepared using N-acetyl glucosamine in borate buffer and the above procedure was carried out. The amount of N-acetyl glucosamine was measured. A blank of 200 µl of water was used instead of the colloidal chitin.

2.2.3 Protease

The protease activity was calculated using the protocol given by Cupp-Enyard, (2008) [6]. A casein solution of 0.65% was prepared using 50 mM of potassium phosphate buffer at 85 °C for about 10 min of gentle stirring. Five ml of 0.65 percent was added to the test tube and incubated at 37 °C for 5 min using water bath. One ml of enzyme solution was added to the tubes except to the blank. It was then mixed properly and incubated at 37 °C for 10 min. After 10 min, a 5 ml of 110 mM trichloro acetic acid was added to each tube to stop the reaction and incubated at 37 °C for 30 min. The absorbance was read at 660 nm. The standard used was tyrosine. To varying concentration of 1.1 mm tyrosine, distilled water was added to make up the volume to one millilitre and a blank was maintained. Then it was incubated for 30 min. Using syringe filter of size 0.45 µm the samples were filtered. To the filtrate 5 ml of sodium carbonate was added followed by 1ml of 0.5 M Folins reagent to measure the activity of free protease by colour change and the drop in pH was adjusted using drops of sodium carbonate. The samples are then incubated at 37 °C for 30 min and then the absorbance for the standard was measured at 660 nm using a spectrophotometer.

2.2.4 Lipase

The lipase enzyme activity was assayed using the methodology given by Gupta *et al.* (2002) [15]. Ten ml of isopropanol was used to dissolve 30 mg of p-Nitro phenyl palmitate (p-NPP) to make the stock solution. Then the working sample was prepared by taking 0.2 mg $ml^{-1}$of the stock which was diluted with distilled water five times. The reaction mixture consisted of 100 µl of culture filtrate and 500 µl of the substrate, which was incubated at 23°C for 30 min. Four hundred µl of Tris HCl was added and the absorbance reading was taken at 410 nm with help of a spectrophotometer. Only Tris HCl without substrate served as a blank.

2.3 Dual culture assay of *Trichoderma* isolates against *Fusarium* spp.

In vitro screening of *Trichoderma* isolates against *Fusarium* spp. was performed based on the dual culture method described by Skidmore and Dickinson (1976) [33]. From the actively growing tip of the nine-day-old culture of *Fusarium* spp., a five-millimetre mycelial disc was cut using a cork borer and was placed at one end of the Petri plate at two cm away from the periphery. An actively growing three-day-old mycelial disc of *Trichoderma* was placed at the opposite end of the Petri plate at two cm from the periphery, on the fifth day of pathogen inoculation. The control was maintained with the monoculture of the pathogen placed at one end of the Petri plate. Triplicates were maintained for each treatment. Until the control plate showed full growth, the radial growth (cm) of the pathogen and antagonist was measured.

% inhibition (PI) between the growth of control and treatment plates was calculated using the formula given by Vincent (1927) [36].

$$PI = [C-T/C] \times 100$$

Where,

C = Radial growth of pathogen (cm) in the control plate

T= Radial growth of pathogen (cm) in the treatment plate

2.4 *In vivo* testing of the efficacy of selected isolates of *Trichoderma* spp. Against Fusarium wilt of vegetable cowpea

2.4.1 Mass multiplication of *Trichoderma* spp. isolates

2.4.1.1 Preparation of formulations of individual isolates

All five isolates of *Trichoderma* spp. *viz* TRMW-2, TRKR-2, TRPN-3, TRPN-11 and TRPN-17 were grown in 300 ml of PDB individually and incubated at room temperature (28 ± 2 °C) for seven days. It was thoroughly mixed with one kilogram of sterilized talc powder. The formulation was shade dried to maintain a moisture content ofseven to eight %. The population of *Trichoderma* in the formulations was confirmed to be a minimum of 2 x 10^6 cfu g^{-1} before use.

2.4.1.2 Preparation of consortial formulations of *Trichoderma* spp.

Mycelial bits of three-day-old *Trichoderma* isolates used in consortia *viz.,* TRKR-2, TRPN-3 and TRPN-17 were aseptically transferred to a conical flask containing pre-

sterilized 300 ml of PDB and incubated at room temperature (28 ± 2 ℃) for seven days. One kg of talc powder was autoclaved at 121 °C at 15 psi for 30 min for three consecutive days. The seven-day-old mycelial mat of one isolate from PDB was filtered using Whatman No.1 filter paper and kept. It was then mixed with seven-day-old growth of another isolate in 300 ml PDB (without filtration) which was in turn thoroughly mixed with one kilogram of autoclaved talc. The mixture was shade dried for five days. The mass-multiplied talc-based formulation was used for the seed treatment and soil drenching at specified intervals.

2.4.2 Mass multiplication of *Fusarium* spp.

Sand-maize mixture was used for the mass multiplication of *Fusarium* by the modified method given by Lewis and Papa*v*izas (1984). Sand and maize were mixed thoroughly in a ratio of 9:1 and were moistened sufficiently to increase the fungal growth. The mixture was autoclaved in a conical flask. Then actively growing mycelial bits of *Fusarium* were transferred to it in an aseptic condition. Then it was incubated at room temperature (28 ± 2 ℃) for 15 days.

2.4.3 Pot culture experiment

A pot culture experiment was carried out at the Department of Plant Pathology, College of Agriculture, Vellayani to evaluate the efficacy of the five *Trichoderma* isolates against the Fusarium wilt of vegetable cowpea. The experiment consisted of eight treatments and three replications in a Completely Randomized Design (CRD) and the variety used was Githika.

The details of the treatment were as follows:

T_1: *Trichoderma* isolate TRKR2
T_2: *Trichoderma* isolate TRPN17
T_3: *Trichoderma* isolate TRPN3
T_4: Consortia of *Trichoderma* isolates TRPN 17 and TRKR 2
T_5: Consortia of *Trichoderma* isolates TRPN 17 and TRPN 3
T_6: KAU strain of *Trichoderma* sp.
T_7: Standard check (fungicide)
T_8: Control

Sand, soil and cow dung were mixed in a ratio of 1:1:1 as a potting mixture and it was fumigated with five % formaldehyde. The potting mixture was filled into medium-sized pots. The cowpea seeds (Var. Githika) after seed treatment were sown in the pots. The package of practice was adopted as per the Package of Practice Recommendations: Crops (KAU, 2011)

2.4.3.1 Method of application

Pathogen inoculation: Pathogen multiplied in the sand-maize mixture (9:1) was applied to the root zone and incorporated to soil @ 0.2% (w/w) in pots at 20 days after sowing (DAS) (Sreeja, 2014) [35].

***Trichoderma* spp.:** Seed treatment of talc-based formulation @ 20 g kg^{-1} of seed followed by soil drenching @ 2% at 20, 40 and 60 DAS.

Standard check: Seed treatment with carbendazim @ 2 g kg^{-1} of seed followed by soil drenching @ 0.2% at 20 and 40 DAS.

Biometric observations like the number of plants infected, days for flowering, number of pods per plant, length of the pod, number of seeds per pod and yield were recorded. Disease incidence was calculated using the formula.

$$\text{Disease incidence} = \frac{\text{Total number of plants infected x 100}}{\text{Total number of plants observed}}$$

The total microbial population of *Trichoderma* spp. was enumerated at 30, 60 and 90 DAS by serial dilution technique (Jhonson and Curl, 1972) [18]. A sample of 10 g of soil was collected from each treatment and mixed individually with 90 ml of sterilized distilled water. One ml of the aliquot from this suspension was mixed with nine ml of sterilized distilled water in a fresh test tube making 10^{-3} dilutions and continued up to 10^{-6} dilutions. From 10^{-3} and 10^{-4} dilutions, one ml of the suspension was plated into three Petri plates containing TSM media by pour plate method and swirled for uniform mixing of the suspension and the media. It was incubated at room temperature (28 ± 2 ℃) and the colonies were counted after three to four days.

2.4.4. Field evaluation of *Trichoderma* isolates against Fusarium wilt of vegetable cowpea

A field experiment was conducted to determine the efficacy of the selected isolates of *Trichoderma* spp. in the management of Fusarium wilt. The experiment was laid out in Randomized Block Design (RBD) with eight treatments and three replications and the variety used was Githika.

The details of the treatment were as follows:

T_1: *Trichoderma* isolate TRKR2
T_2: *Trichoderma* isolate TRPN17
T_3: *Trichoderma* isolate TRPN3
T_4: Consortia of *Trichoderma* isolates TRPN 17 and TRKR 2
T_5: Consortia of *Trichoderma* isolates TRPN 17 and TRPN 3
T_6: KAU strain of *Trichoderma* sp.
T_7: Standard check (fungicide)
T_8: Control

2.4.4.1. Method of application

***Trichoderma* spp.:** Seed treatment of talc-based formulation @ 20 g kg^{-1} of seed followed by soil drenching @ 2% at 20, 40 and 60 DAS.

Standard check: Seed treatment with carbendazim @ 2 g kg^{-1} of seed followed by soil drenching @ 0.2% at 20 and 40 DAS.

Liming was done at the time of land preparation for all the treatments. The other package of practices was adopted as per the standard and recommended Package of Practices (KAU, 2011). Then the biometric observations were recorded as mentioned earlier in 3.3.3.1

2.5. Statistical analysis

The data obtained from the experiments was used to calculate Analysis of variance (ANOVA) and Critical Difference (CD) at a five % level of significance to compare the means of the treatments. The analysis was performed using KAU GRAPES 1.0.0 and standard error as well as standard deviation was also calculated for all the treatment means.

Table 1. *In vitro* activity of chitinase, protease and lipase by isolates of *Trichoderma* spp. grown in potato dextrose broth

Sl. No.	Isolate	Chitinase (U ml^{-1})*	Protease (U ml^{-1})*	Lipase (U ml^{-1})*
1	TRMW-2	61.82 ± 0.18^{d}	64.43 ± 0.23^{d}	2.69 ± 0.006^{e}
2	TRKR-2	71.06 ± 0.40^{c}	48.68 ± 0.12^{e}	4.20 ± 0.010^{b}
3	TRPN-3	76.75 ± 0.32^{b}	74.80 ± 0.14^{c}	4.86 ± 0.008^{a}
4	TRPN-11	55.68 ± 0.11^{e}	165.86 ± 0.19^{a}	3.14 ± 0.010^{c}
5	TRPN-17	88.39 ± 0.18^{a}	92.90 ± 0.14^{b}	2.83 ± 0.005^{d}
	SE(m)	0.157	0.096	0.005
	CD (0.05)	0.40	0.28	0.01

Table 2. *In vitro* efficacy of selected isolates of *Trichoderma* sp. against *F. oxysporum*

Sr. No.	Isolate	Radial growth of pathogen (cm)*	Inhibition (%) **
1.	TRMW-2	2.60 ± 0.26^{bc}	$57.10\ (49.10)^{bc}$
2.	TRKR-2	2.50 ± 0.00^{bc}	$58.76\ (50.05)^{bc}$
3.	TRPN-3	2.53 ± 0.15^{bc}	$58.21\ (49.73)^{bc}$
4.	TRPN-11	2.63 ± 0.15^{bc}	$56.56\ (48.78)^{bc}$
5.	TRPN-17	2.20 ± 0.00^{c}	$63.71\ (52.96)^{b}$
6.	*Trichoderma* sp. (KAU strain)	1.60 ± 0.59^{d}	$73.04\ (58.94)^{a}$
7.	*T. harzianum* (NBAIR strain)	2.80 ± 0.05^{b}	$53.26\ (46.87)^{c}$
8.	Control	6.07 ± 0.21^{a}	$0\ (0)^{d}$
	SE (m)	0.14	1.50
	CD (0.05)	0.43	4.52

*Mean ± SD of three replication ** Values in the parenthesis are arcsine transformed; in a column, means followed by Common letters are not significantly different from each other.

Table 3. Population of *Trichoderma* spp. in soil from different treatments of the pot culture experiment

Sl. No.	Treatment	Population in CFU g^{-1}at 10^{-4}dilution*		
		30 DAS	60 DAS	90 DAS
1	T1- (TRKR-2)	$6.33\ (0.83)^{ab}$	$8.33\ (0.94)^{abc}$	$3.33\ (0.57)^{bc}$
2	T2- (TRPN-17)	$3.67\ (0.58)^{b}$	$6.33\ (0.83)^{bc}$	$1.33\ (0.14)^{cd}$
3	T3- (TRPN-3)	$6.67\ (0.85)^{ab}$	$11.67\ (1.08)^{a}$	$7.00\ (0.86)^{a}$
4	T4- (TRKR-2 + TRPN-17)	$5.00\ (0.64)^{ab}$	$6.33\ (0.81)^{c}$	$4.33\ (0.67)^{ab}$
5	T5- (TRPN-3 + TRPN-17)	$8.33\ (0.93)^{a}$	$9.00\ (0.97)^{abc}$	$4.00\ (0.61)^{bc}$
6	T6- (KAU)	$6.00\ (0.78)^{ab}$	$9.67\ (0.99)^{ab}$	$3.67\ (0.60)^{bc}$
7	T7- (Carbendazim)	$0.00\ (-0.30)^{c}$	$0.00\ (-0.30)^{d}$	$0.00\ (-0.30)^{d}$
8	T8- (Control)	$0.00\ (-0.30)^{c}$	$0.00\ (-0.30)^{d}$	$0.33\ (-0.14)^{d}$
	SE(m)	1.26	1.18	0.91
	CD (0.05)	0.33	0.30	0.38

Mean value of three replication; *Values in parentheses are log transformed; In a column, means followed by common letters are not significantly different from each other.

Table 4. Efficacy of *Trichoderma* isolates against Fusarium wilt of vegetable cowpea in pot culture

Sl. No.	Treatment	DI (%) **	Days for flowering*	No. of pods / plant*	Pod length (cm)*	No. of seeds/ pod*	Yield (g/plant)*
1	T1- (TRKR-2)	$0.00\ (4.05)^{c}$	33.33 ± 0.58^{b}	12.33 ± 1.53^{c}	43.55 ± 4.18^{a}	20.57 ± 1.74	261.60 ± 42.35^{bc}
2	T2- (TRPN-17)	$25.00(4.92)^{b}$	33.00 ± 1.00^{b}	11.33 ± 1.53^{cd}	42.37 ± 0.60^{a}	20.39 ± 0.18	186.35 ± 21.69^{d}
3	T3- (TRPN-3)	$0.00\ (4.05)^{c}$	31.33 ± 1.16^{c}	19.00 ± 1.00^{a}	43.61 ± 2.23^{a}	21.05 ± 0.66	369.57 ± 21.53^{a}
4	T4- (TRKR-2 + TRPN-17)	$8.33\ (4.35)^{bc}$	33.67 ± 0.58^{b}	9.67 ± 1.53^{d}	43.85 ± 2.59^{a}	20.88 ± 1.13	218.27 ± 15.47^{cd}
5	T5- (TRPN-3 + TRPN-17)	$0.00\ (4.05)^{c}$	33.67 ± 0.58^{b}	11.33 ± 1.16^{cd}	43.35 ± 1.58^{a}	20.36 ± 1.01	344.52 ± 41.39^{a}
6	T6- (KAU)	$0.00\ (4.05)^{c}$	33.67 ± 0.58^{b}	12.00 ± 1.00^{c}	40.13 ± 1.91^{a}	20.37 ± 1.30	246.79 ± 33.28^{bcd}
7	T7- (Carbendazim)	$0.00\ (4.05)^{c}$	33.33 ± 0.58^{b}	15.33 ± 1.16^{b}	40.69 ± 4.59^{a}	19.71 ± 1.79	310.99 ± 75.80^{ab}
8	T8- (Control)	$100(7.03)^{a}$	35.33 ± 0.58^{a}	5.67 ± 1.53^{e}	35.29 ± 0.78^{b}	18.20 ± 0.51	105.57 ± 42.83^{e}
	SE (m)	0.204	0.43	0.76	1.54	0.67	23.6
	CD (0.05)	0.61	1.27	2.29	4.63	NS	16.00

*Mean ± SD of three replication; **Values in the parenthesis are arcsine transformed; NS = Not significant; In a column, means followed by common letters are not significantly different from each other

Table 5. Efficacy of *Trichoderma* isolates against Fusarium wilt of vegetable cowpea under field conditions

Sl. No.	Treatment	DI (%)	Days for flowering	No. of pods/ plant	Pod length (cm)	No. of seeds/ pod	Yield / plant (g)
1	T1- (TRKR-2)	$0.00\ (4.05)^{c}$	33.00 ± 0.00^{c}	6.11 ± 1.39^{d}	46.19 ± 0.41^{abc}	19.79 ± 0.63^{c}	352.53 ± 108.71^{d}
2	T2- (TRPN-17)	$44.44(5.54)^{b}$	34.00 ± 0.00^{b}	5.89 ± 0.70^{d}	44.58 ± 2.26^{bc}	20.28 ± 0.74^{bc}	301.44 ± 29.90^{d}
3	T3- (TRPN-3)	$0.00\ (4.05)^{c}$	31.00 ± 0.00^{e}	19.00 ± 0.88^{a}	47.53 ± 0.52^{ab}	20.85 ± 0.60^{abc}	1172.63 ± 59.86^{a}
4	T4- (TRKR-2 + TRPN-17)	$0.00\ (4.05)^{c}$	33.00 ± 0.00^{c}	6.11 ± 1.71^{d}	45.61 ± 2.17^{abc}	20.16 ± 0.61^{c}	358.94 ± 76.63^{d}

5	T5- (TRPN-3 + TRPN-17)	11.11(4.44)^{c}	33.00±0.00^{c}	10.11±0.84^{c}	48.16±3.26^{ab}	21.73±0.94^{a}	658.88±45.84^{c}
6	T6- (KAU strain)	0.00 (4.05)^{c}	33.00±0.00^{c}	11.11±0.51^{c}	43.45±2.33^{cd}	20.67±0.37^{abc}	653.08±33.20^{c}
7	T7- (Carbendazim)	0.00 (4.05)^{c}	32.33±0.57^{d}	14.22±1.35^{b}	48.90±1.37^{a}	21.34±0.66^{ab}	929.06±38.20^{b}
8	T8- (Control)	88.88(6.70)^{a}	34.67±0.57^{a}	4.56±0.51^{d}	39.78±3.20^{d}	18.51±1.17^{d}	260.98±24.75^{d}
	SE(m)	0.21	0.17	0.63	1.24	0.35	33.65
	CD (0.05)	0.65	0.54	1.92	3.77	1.07	102.09

Mean value of three replication; *Values in parentheses are log-transformed; in a column, means followed by common letters are not significantly different from each other.

Table 6: Population of *Trichoderma* spp. in soil from different treatments of the field experiment

Sl. No.	Treatment	Population in cfu g^{-1}at 10^{-4}dilution		
		30 DAS	60 DAS	90 DAS
1	T_1 - (TRKR-2)	5.00 (0.72)^{b}	7.33 (0.88)^{ab}	1.67 (0.30)^{abc}
2	T_2 - (TRPN-17)	5.00 (0.74)^{b}	6.33 (0.82)^{b}	1.33 (0.14)^{bc}
3	T_3 - (TRPN-3)	7.67 (0.91)^{ab}	9.0 (0.97) ^{ab}	4.33 (0.64)^{a}
4	T_4 - (TRKR-2 + TRPN-17)	5.33 (0.71) ^{b}	6.67 (0.84)^{b}	2.00 (0.26)^{abc}
5	T_5 - (TRPN-3 + TRPN-17)	9.00 (0.97)^{a}	10.67 (1.04)^{a}	3.67 (0.60)^{ab}
6	T_6 - (KAU strain)	6.67 (0.83)^{ab}	8.67 (0.95)^{ab}	4.00 (0.62)^{ab}
7	T_7 - (Carbendazim)	0.00 (-0.30)^{c}	0.00 (-0.30)^{c}	0.33 (-0.14)^{c}
8	T_8 - (Control)	0.33 (-0.14)^{c}	0.00 (-0.30)^{c}	0.00 (-0.30)^{c}
	SE(m)	1.18	1.15	0.90
	CD (0.05)	0.31	0.18	0.52

Mean value of three replication; *Values in parentheses are log transformed; in a column, means followed by common letters are not significantly different from each other

3. Results and Discussion

Management of Fusarium wilt is primarily dependent on the use of fungicides. The usage of such chemicals has a detrimental effect on human health and other living beings (Carvalho 2017 [5]; Kohl *et al*. 2019 [21]). In addition, the high expense of developing, producing, and registering new synthetic pesticides as well as the strong development of pathogen resistance has sparked interest in developing alternative disease management strategies (Matson *et al*. 2015 [24]; Bedine *et al.* 2022 [4]). The amendment of the soil with biocontrol agents is an alternative to manage soil-borne plant pathogens through parasitism, the synthesis of antagonistic compounds, competition for hosts and the development of resistance in plants against the phyto pathogens. The mycoparasitic nature and the ability to secrete a huge quantity of fungi-toxic enzymes make *Trichoderma* an effective biocontrol agent against several foliar and soil-borne plant diseases (Shafique *et al*. 2016 [31]; Panth *et al.* 2020 [27]).

3.1 Assessment of the biocontrol attributes of *Trichoderma* isolates.

To effectively lyse the cell wall of pathogens, antagonists secrete enzymes that hydrolyse the chitin, glucan, lipids and proteins that make up the skeleton of fungal cells (Khatri *et al*., 2017) [20].

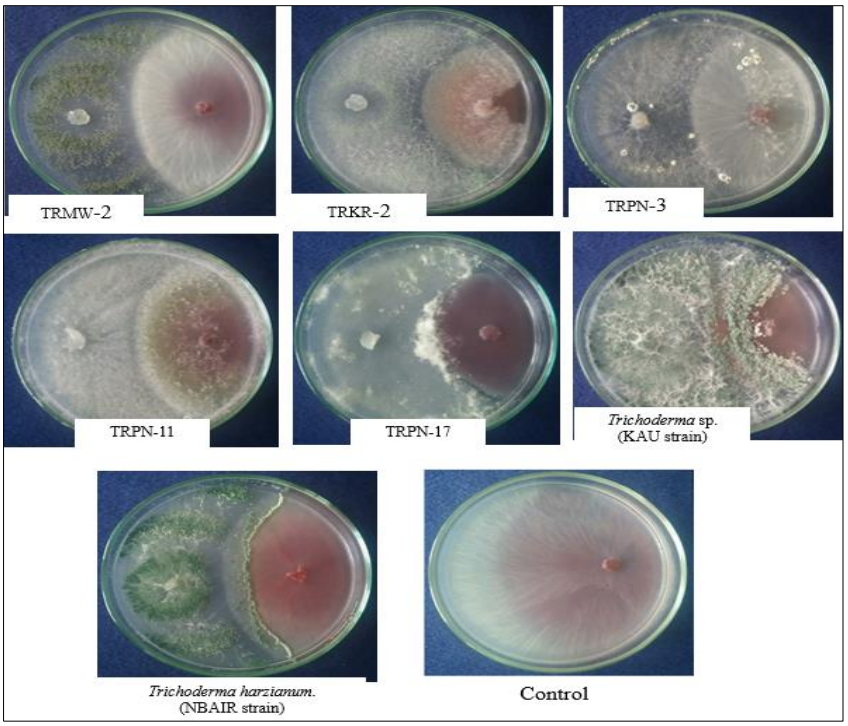

Plate 1: Dual culture assay of *Trichoderma* isolates against *F. oxysporum*

Trichoderma isolates

In the present study, the cell wall degrading enzyme *viz*., chitinase, protease and lipase were evaluated (Table 1) and found that the chitinase enzyme activity of the isolate TRPN-17 was observed to be the highest (88.39 U ml-1) followed by the isolates TRPN-3 (76.75 U ml-1) (Fig. 1) and TRKR-2 (71.06 U ml-1) while the highest protease activity was recorded by the isolate TRPN-11 (165.86 U ml-1) followed by TRPN-17 (92.90 U ml-1) and TRPN-3 (74.80 U ml-1)(Fig. 2). The isolate TRPN-3 had the highest (4.86 U ml-1) lipase enzyme activity followed by the isolates TRKR-2 (4.20 U ml-1) and TRPN-11 (3.14 U ml-1) (Fig. 3).

Asad *et al*. (2105) [1] reported that after 96 h of culture at 25 °C, T. *asperellum* reached its maximum chitinolytic activity (173 U ml-1), which was the highest of all of the isolates tested; after 72 h of culture at the same temperature, T. harzianum reached 117 U ml-1. Chitinase from several Trichoderma species has distinct properties and antifungal action. Two strains of S. rolfsii and Colletotrichum sp. are both inhibited by T. *asperellum* PQ34 chitinase (Loc *et al*., 2019). Chitin and glucan are the two major constituents of fungal cell walls, which are vulnerable to chitinase, N-acetyl-β-glucosaminidase, and β-glucosidase (Karlicic *et al*., 2021). Using RAPD analysis, Gajera and Vakharia (2010) [12] correlated the biocontrol efficacy of 12 Trichoderma isolates against *Aspergillus niger* to the production of cell wall-degrading enzymes such chitinase, β-1, 3 glucanase, and others during mycoparasitism. In terms of increased production of extracellular enzymes including chitinase, β-1,3 glucanases, protease, cellulose, and pectinase, Trichoderma isolates (UNT68, NAT70, UNT38, UNS63, UNT13, UNS30, and DET02) displayed a mycotrophic mode of antagonism that may be effective against *Fusarium oxysporum* f. sp. lycopersici, Alternaria alternate, Colletotrichum gloeosporoides and Rhizoctonia solani (Rai, 2017) [29] According to Solanki *et al*. (2011) [34], the superior chitinase manufacturers may not always show a higher level of β -1,3 glucanases and vice versa. This provides evidence for the possibility that each given metabolite or biomolecule is strain or species-specific in its production.

3.1.2. Dual culture assay of Trichoderma isolates against Fusarium spp.

In the present study, the efficacy of Trichoderma isolates against Fusarium spp. was tested by the dual culture method (Table 2) (Plate 1). Among the tested isolates, TRPN-17 caused the highest % inhibition (63.71%) of the mycelial growth and differed significantly from other isolates. It was followed by TRKR-2 (58.76%), TRPN-3 (58.21%), TRMW-2 (57.10%) and TRPN-11 (56.56%) with no significant difference. The reference strains T. *asperellum* (KAU strain) and T. harzianum (NBAIR strain) had the % inhibition of 73.04% and 53.26% respectively (Fig. 4). Based on the *in vitro* studies the effective isolates TRKR-2, TRPN-17, TRPN-3, consortia of isolates TRKR-2 and TRPN-17 and consortia of isolates TRPN-3 and TRPN-17 were selected for the *in vivo* evaluation against Fusarium wilt of vegetable cowpea.

The findings concur with Fan *et al*., (2020) [10] who reported that the T. citrinoviride strain Snef1910 suppressed the growth of F. graminearum by 60.76%, F. oxysporum by 49.28%, *F. moniliforme* by 21.73%, and *F. roseum* by 25.20%. Bastakoti *et al*., (2017) [3] reported that Trichoderma isolate TS 215 showed % inhibition of more than 60% against F. solani. T. harzianum effectively inhibited the mycelial growth of Fusarium spp. (Gwa and Nwankiti, 2017 [16]; Yassin *et al*., 2021 [37]. The isolates of Trichoderma *viz*., TRSN-1, TRMW-2, TRKR-2, TRPN-3, TRPN-7, TRPN-11, TRPN-14, TRPN-15, TRPN-17, TRPN-18 and TRML-1 were tested *in vitro* for their ability to inhibit the growth of two pathogens, Pythium aphanidermatum and F. oxysporum and were found to exhibit % inhibition of more than 57% and 45%, respectively (Nair, 2022) [25].

Awad-Allah *et al*., (2022) [2] investigated the antagonistic activity of Trichoderma isolates *viz*., *T. viride* and T. harzianum against F. solani pathogen that causes the Fusarium wilt disease in cherry tomatoes. They found that 10 days post-inoculation, T. harzianum suppressed F. solani mycelial growth by 78.0%, while *T. viride* inhibited it by 61.2%. Scanning electron microscopy (SEM) analysis also showed that Trichoderma isolates coiled themselves around F. solani hyphae, degraded and deformed pathogen hyphae, and grew in concordance with the Fusarium wilt pathogen. These results suggest that the antagonistic Trichoderma isolates studied here could provide a source of novel biological fungicides that avoid the harmful effects of chemical fungicides, particularly against the Fusarium wilt pathogen *F. solani*.

Fig 1: Absorbance value of chitinase activity of *Trichoderma* isolates

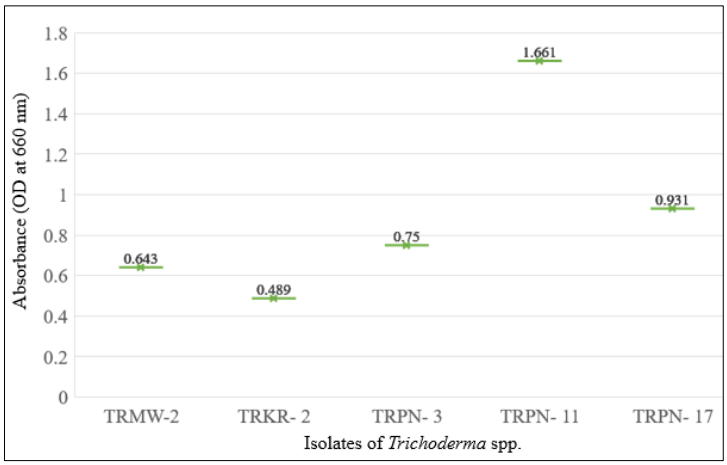

Fig 2: Absorbance value of protease activity of *Trichoderma* isolates

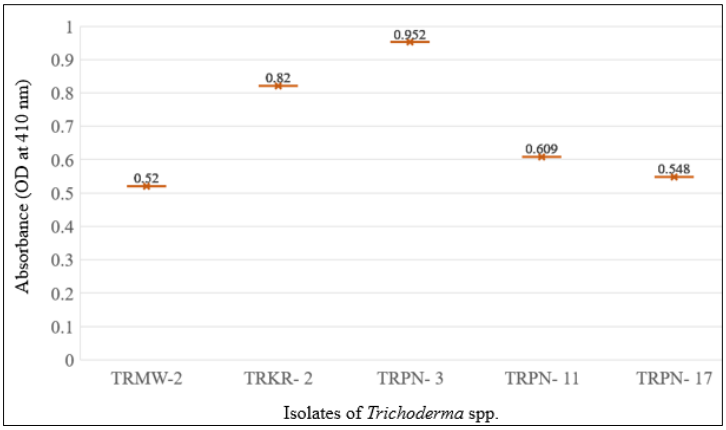

Fig 3: Absorbance value of lipase activity of *Trichoderma* isolates

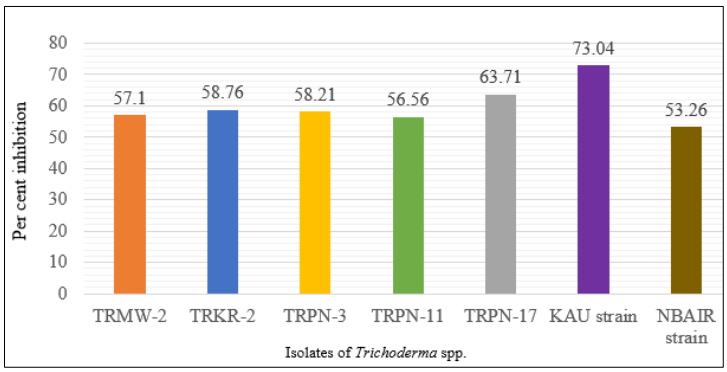

Fig 4: % inhibition of selected isolates of Trichoderma sp. against Fusariu

3.2. Efficacy of Trichoderma spp. against Fusarium wilt of vegetable cowpea

In the present study, Application of talc-based formulations of the isolates TRKR-2, TRPN-3, KAU strain and consortium of TRPN-3 and TRPN-17 as seed treatment @ of 20 g kg-1 of seed followed by soil drenching @ 2% at 20, 40 and 60 DAS effectively reduced the incidence of Fusarium wilt of vegetable cowpea in pot culture study (Table 4). The application of isolate TRPN-3 produced highest pod number (19) in pot culture experiment and also yield per plant (369.57 g) (Plate 2) on par with the yield per plant produced by consortia application of TRPN-3 and TRPN-17 (344.52 g). Under field conditions (Table 5), the disease was most effectively reduced by isolates TRKR-2, TRPN-3, KAU strain and consortia of TRKR-2 and TRPN-17. Lengthy pods (48.16 cm) and highest number of seeds per pod (21.73) were recorded by the application of consortium of TRPN-3 and TRPN-17 whereas application of TRPN-3 resulted in highest yield (1172.63 g/plant) (Plate 3), number of pods per plant (19) and reduced days of flowering. Enumeration of population of Trichoderma spp. from soil in different treatments at 90 DAS revealed that, highest population was in treatment with isolate TRPN 3 under pot culture (7 ×104 cfu g-1) (Table 4) and field conditions (4 ×104 cfu g-1) (Table 6). These findings are in agreement with Pradhan *et al*. (2022) [28] investigated the *in vivo* bio-potential of five *T. viride* strains (ITCC 6889, ITCC 7204, ITCC 7764, ITCC 7847, and ITCC 8276) for the control of Fusarium wilt of chickpea. When compared to seeds treated with carbendazim (80.33%) and a talc-based *T. viride* formulation (83.33%), seeds treated with

a dustable powder formulation comprising spores of the antagonist suppressed the growth and development of the pathogen, resulting in an increased germination percentage of 93.33%. The wilting percentage of plants treated with the dustable *T. viride* powder formulation was much lower than that of plants treated with carbendazim and talc-based formulations. Our findings are consistent with those of Fang-Fang *et al*. (2016) [11] who also found that Trichoderma increases growth and biomass accumulation in plants. The use of Trichoderma strains *viz*., T. harzianum and *T. viride* against the wilt pathogen *Fusarium oxysporum* f. sp. lycopersici led to a decrease in disease severity, an increase in fresh and dry weights, plant length, and yield in the field through the production of cell wall degrading enzymes and improved physiological activity (Jamil, 2021) [17].

Excellent control against Fusarium wilt of banana induced by *Fusarium oxysporum* f. sp. cubense tropical race 4 (Foc TR4) was observed using a formulation of the T. reesei isolate CSR-T-3, as reported by Damodaran *et al*. (2020) [9] in both *in vitro* and field conditions. Enhanced production of antifungal compounds and higher activity of defensive enzymes such β-1,3-glucanase, peroxidase, chitinase, polyphenol oxidase, and phenylalanine ammonia lyase reduce disease severity. In the field, plants that were given the treatment had a high phenological growth rate and yield, and they only had a disease severity score of 1.14.

4. Conclusion

In the present study it can be concluded that all the isolates of Trichoderma recorded a notable reduction in the disease incidence under both pot and field conditions. Trichoderma isolates TRKR-2, TRPN-3, and consortium of TRPN-3 and TRPN-17 were found effective against Fusarium wilt of vegetable cowpea under field conditions. Trichoderma acts as a biocontrol agent that is both effective and environmentally safe, in addition to being a beneficial symbiotic organism for plants in the management of soil-borne plant diseases. The field level efficacy of these isolates against other soil-borne pathogens of vegetable cowpea also need to be explored for their successful use by the farming community.

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