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Biocontrol efficacy of local *Trichoderma* isolates against *Fusarium oxysporum* F. sp. *lycopersici* and *Fusarium oxysporum* F. sp. *ciceri*

Ved Ratan, Mukesh Srivastava, Supriya Dixit, Shubha Trivedi, DK Srivastava and Yatindra Kumar Srivastava

Abstract
Recently, the environmental contamination caused by excessive use of chemical pesticides increased the interests in integrated pest management, where chemical pesticides are substituted by bio-pesticides to control plant and soil diseases. *Trichoderma* species are well known potential fungal bio-control agents against a wide range of soil borne plant pathogens. A total 25 *Trichoderma* isolates were isolated from soil samples collected from Kanpur Nagar, Kanpur Dehat, Unnao and Lucknow districts of U.P. Based on cultural and morphological characters, six *Trichoderma* isolates found efficient and taken for further studies. All six isolates were sent to NFCCI & FIS, Pune for species level identification. Based on the identification report from NFCCI, 4 isolates were identified as *T. asperellum* (CST-02, CST-09, CST-21, CST-22), one each as *T. longibrachiatum* (CST-14) and *T. koningii* (CST-05). The *in vitro* antagonistic activity of the 06 *Trichoderma* isolates was assayed against both test pathogens. Our results showed that the highest inhibitory effect was achieved by isolate CST-14 on growth of FOC and FOL as 33.4 and 37.1 per cent, respectively. Isolate CST-21 showed significant inhibition in mycelial growth against FOC as 30.0 per cent while, isolate CST-22 showed significant inhibition as 32.8 per cent in case of FOL. In case of volatile assay CST-14 again showed highest inhibitory effect against test pathogens ranged from 29.3 - 39.4 percent respectively. Similarly in case of non-volatile assay, 100 percent inhibition in mycelial growth of test pathogens caused by CST-14 @ 50% concentration. Present investigations concluded that isolates CST-14 (*T. longibrachiatum)* showed significant inhibitory effect against FOL and FOC. It can be used in the form of bio formulation for effective management of soil borne pathogens.

Keywords: *Trichoderma* spp, *Fusarium* spp, antagonistic activity, inverse plate technique, volatile

Introduction
Various important and beneficial crops suffer from a number of diseases caused by microorganisms like fungi, bacteria, nematode, viruses and plant parasites (cal *et al*., 2004) [3]. Among fungi, *Fusarium* causes heavy loss to the crop (Keshwan and chaudhary, 1977) [7]. Wilt of tomato and chickpea is caused by *Fusarium oxysporum* species is one of the main limiting factors to successful cultivation to respective crops. The pathogen is responsible for severe losses of both tomato and chickpea crop (Srinon *et al*., 2006; Ramezani, 2009) [10, 9]. The pathogen is soil borne and for such pathogens, chemical control is recommended, which is uneconomical and causing groundwater pollution, loss of non-target beneficial flora and develop fungicidal resistant variants. Due to prolonged saprophytic survival ability of the pathogen, cultural methods are also not much effective. Use of resistant varieties is the best option but their availability is limited (Hayes and laws, 1991) [6].
In the recent era of agricultural biotechnology, biological control is considered as important approach for controlling many fungal plant and soil borne pathogens (deshmukh *et al*., 2010) [5]. *Trichoderma* species are free-living filamentous fungi that are highly interactive in root, soil and foliar environments. It has been known for many years that they produce a wide range of antibiotic substances and that they parasitize other fungi (Chet, 1987) [4]. Thus, this research study focuses to collect and isolate locally available biocontrol agent from different districts of Uttar Pradesh and find out the potent isolate for the management of disease against *Fusarium* species.

Materials and Methods
Collection of soil samples
Soil samples were collected from different ecological habitat of different crops of four districts of Uttar Pradesh (India) for the isolation of *Trichoderma* spp. and were brought to Biocontrol Lab of the Department of Plant pathology and stored at 4 ^{0}C until used.

Isolation, purification and identification of *Trichoderma* Isolates

In each district, randomly 15 soil samples from 5-6 cm depth were collected from the fields of villages adjoining to Block Headquarters. Each soil sample was kept in parchment paper bags covered with polythene bags and properly labeled with the name of district, block, village, name of the farmer, crop and date of the collection etc. Isolation was made from 1g soil dissolved in 10 ml sterilized distilled water.

Five-fold serial dilutions of each soil samples were prepared in sterilized distilled water and 0.5 ml diluted sample was poured on the surface of Trichoderma Specific Medium (TSM). Plates were incubated at 28 ± 1 ^{0}C for 96 hr. morphologically different colonies appeared on the plates were purified in the Potato Dextrose Agar (PDA) (HiMedia, India) and observed under microscope.

Cultural and morphological characterization of bio-agents

The isolated strains of the *Trichoderma* was characterized based on cultural, and morphological characters to study the growth pattern, characteristics of the mycelium/ spores, shape and size of phialids and conidiophores etc.

For visual observation, the isolates were grown on PDA agar for 3-5 days. The mode of mycelia growth, colour, odour and changes of medium colour for each isolate were examined. For morphological studies, a slide culture technique was used. Examination on the shape, size, arrangement and development of conidiophores and phialides provided a tentative identification of *Trichoderma* spp.

Phenotype characters of the fast growing *Trichoderma* isolates

Based on the growth of mycelia on PDA plates, 06 isolates were found as fast growing among 25 *trichoderma* isolates and were selected for further for detailed study. The morphological and cultural characteristics of 06 isolates of *Trichoderma* were studied in two different media viz., Potato Dextrose Agar (PDA) and Rose Bengal Agar (RBA). Mycelial discs (5 mm) of young growing culture of respective isolates of *Trichoderma* was inoculated in the center of the petri plates containing above said media and three replications were maintained for each isolate, incubated at 28 ± 2C for one week. Colony radius was measured at 24, 48 and 72 h.

Two techniques, visual observation on petri dishes and micro-morphological studies in slide culture, were adopted for identification of *Trichoderma* species. For visual observation, the isolates were grown on PDA agar for 3-5 days. The mode of mycelia growth, colour and changes of medium colour for each isolate were examined every day.

For micro-morphological studies, a slide culture technique was used. Examination of the shape, size, arrangement and development of conidiophores or phialides provided a tentative identification of *Trichoderma* spp. Samples were compared to a taxonomic key for the genus *Trichoderma* and further sent to NFCCI & FIS, Agharkar Research Institute, Pune for species level identification and Accession Number.

In vitro effect of *Trichoderma* antagonists against FOL and FOC pathogen

For determination of efficacy of *Trichoderma* isolates against phytopathogens dual culture technique was used. A 5 mm mycelial disc of fifteen days old fungal pathogen culture was placed on PDA medium away from the edge of the plate, separately and 5 mm mycelial disc of *trichoderma* isolates was placed at opposite side of the petri plate. Three replicated plates for each treatment was maintained and incubated at 25±3 ^{o}C. Per cent inhibition over control was calculated as per the formulae

PI = C-T/C*100

Where, PI = Per cent inhibition over control
C = Growth of test pathogen with absence of antagonist (mm)
T = Growth of test pathogen with antagonist (mm)

Production of volatile compounds by *Trichoderma* isolates

The production of volatile compounds by *Trichoderma* isolates was determined by inverse plate technique. A 5 mm mycelial disc of *Trichoderma* isolates and test pathogens were placed in the centre of two separate bottom portions of petri plates containing PDA, and one of the plates was placed in an inverted position over the other. The plates were sealed with parafilm tape and incubated at 28 ^{0}C for 8 days. Observation was recorded after one week and proportion of inhibition was calculated.

Production of non- volatile compounds by *Trichoderma* isolates

The production of non-volatile compounds was determined by food poison technique. *Trichoderma* isolates were inoculated in 100 ml sterilized potato dextrose broth and incubated at 25±1 ^{0}C for 12-14 days. *Trichoderma* mycelium was collected after 12-14 days and filtered through what man filter paper. Different volumes of filtrates were added to the PDA medium at 40±3 °C to obtain final concentrations of 5, 10 and 15. The medium placed into petri plate was inoculated with 5 mm mycelial disc of pathogen at the centre. The plates were incubated at 25±1 °C for 7 days. Plates without culture filtrate were maintained which served as a control.

Result and Discussion

Collection of bio-agents

A total of 25 *Trichoderma* isolates were isolated from soil samples collected from 4 districts of Uttar Pradesh (Table 1). From Kanpur Nagar 6 *Trichoderma* isolates were collected. Similarly from Kanpur Dehat, Unnao and Lucknow districts of U.P. 6, 7 and 6 *Trichoderma* isolates respectively were isolated from soil samples collected from these districts.

Table 1: Bio-agents isolated from soil samples collected from four districts of Uttar Pradesh

District	Blocks	Bio-agents isolated
Kanpur Nagar	Bidhanu	*Trichoderma* spp.(+)
	Chaubeypur	*Trichoderma* spp. (+)
	Ghatampur	*Trichoderma* spp. (+)
	Kalyanpur	*Trichoderma* spp. (+)
	Patara	*Trichoderma* spp. (+)
	Sarsaul	*Trichoderma* spp. (+)
Kanpur Dehat	Akbarpur	*Trichoderma* spp. (+)
	Amraudha	*Trichoderma* spp. (+)

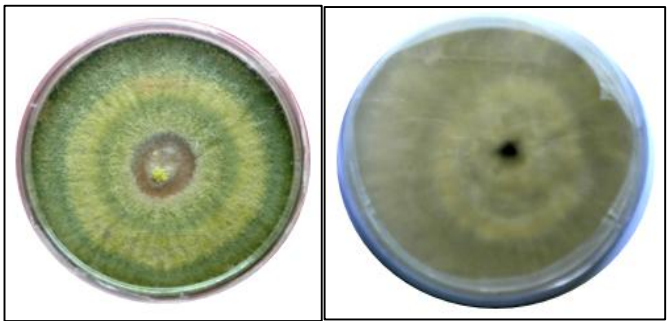
	Derapur	*Trichoderma* spp. (+)
	Jhinjhak	*Trichoderma* spp. (+)
	Maitha	*Trichoderma* spp. (+)
	Rasulabad	*Trichoderma* spp. (+)
Unnao	Bangarmau	*Trichoderma* spp. (+)
	Bighapur	*Trichoderma* spp. (+)
	Fatehpur Chaurasi	*Trichoderma* spp. (+)
	Hasanganj	*Trichoderma* spp. (+)
	Nawabganj	*Trichoderma* spp. (+)
	Safipur	*Trichoderma* spp. (+)
	Sumerpur	*Trichoderma* spp. (+)
Lucknow	Bakshi Ka Talab	*Trichoderma* spp. (+)
	Chinhat	*Trichoderma* spp. (+)
	Gosainganj	*Trichoderma* spp. (+)
	Kakori	*Trichoderma* spp. (+)
	Mal	*Trichoderma* spp. (+)
	Mohanlal ganj	*Trichoderma* spp. (+)

Cultural characteristics of bioagents

Growth rate of 25 *Trichoderma* isolates was studied on PDA for 120 h at 25±1^{0}C. Based on the growth 10 isolates were found as slow growing exhibited 6-7 cm growth while 8 isolates as medium growing exhibited 7-8 cm growth, while 7 isolates were found as fast growing with 8-9 cm growth at 120 h (Table 2 and Figure 1). In case of colony colour and growth patterns most of the isolates were light bice green to pale yellow green in colour and exhibited floccose to arachnoid growth pattern.

Table 2: Categorization of *Trichoderma* isolates based on radial growth.

S.No.	Group	Growth (in cm)	No. of Isolates
1.	Slow growing	6-7	10
2.	Medium growing	7-8	09
3.	Fast growing	8-9	06



(a) **(b)**

Fig 1: (a) Cultural growth of *Trichoderma* spp. on PDA **(b)** Reverse

Morphological characteristics of bioagents

Microscopic examinations on morphological characters of all *Trichoderma* isolates revealed that the asexual states of all isolates have typical *T. harzianum*- like morphology. Phialides arise in whorls at the tips of secondary branches and from the tip of the main axis. The average dimensions of phialides ranged between 4.3-11.6 x 2.1-3.7 (Figure 2). Longest phialides were found in 17 isolates while shortest in eight *Trichoderma* isolates. Conidia did not vary in shape and most were globose to subglobose or broadly ovoidal.

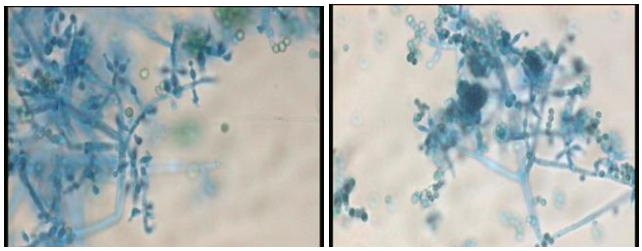
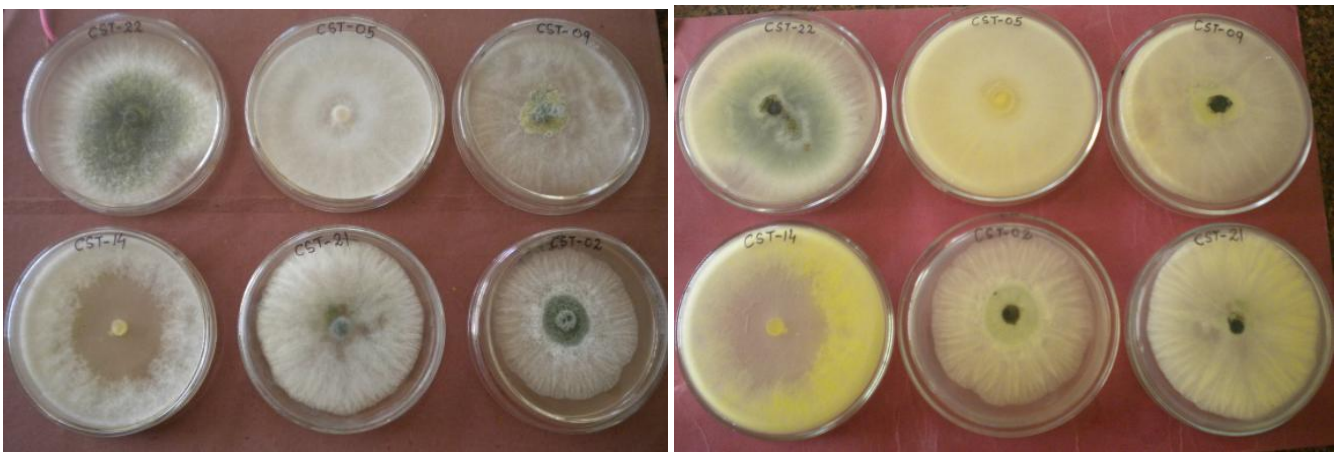


Fig 2: Mycelium, conidiophores, phialids, and Phialospores of *Trichoderma*

Phenotype characters of the fast growing *Trichoderma* isolates

The fastest growing *Trichoderma* isolates were sent to NFCCI & FIS, Agharkar Research Institute, Pune for species level identification. Based on the identification report from NFCCI *Trichoderma* isolates were identified as *T. asperellum* (CST-02, CST-09, CST-21, and CST-22) (Table 3 and Figure 4), *T. koningii* (CST-05) (Table 4 and Figure 5) and *T. longibrachiatum* (CST-14) (Table 5and Figure 6). For characterization of *Trichoderma* sp. were studied for their cultural and morphological characters. All 03 *Trichoderma* sp. were grown separately on PDA plates for 5 days in an incubator at 25± 1°C. Light microscope was used to study colony growth rate, colony texture and colour, reverse colony colour, colony edge, mycelial form and colour. Growth-rate trials were done in PDA poured petri-plates inoculated with *Trichoderma* sp. after 24 and 72 hrs at 25^{0}C to measure colony radius.

Cultural and morphological studies were carried out in order to observe radial growth pattern of *Trichoderma* isolates on solid culture media (Figure 3).

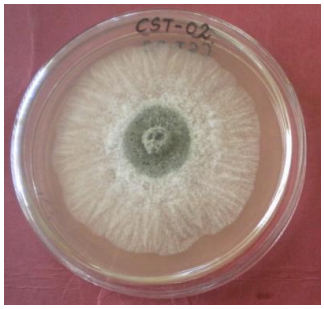
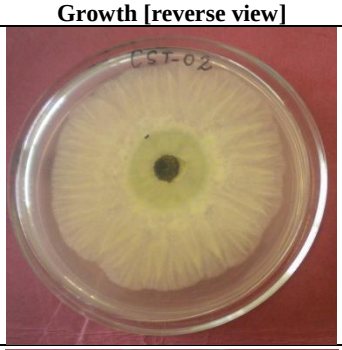
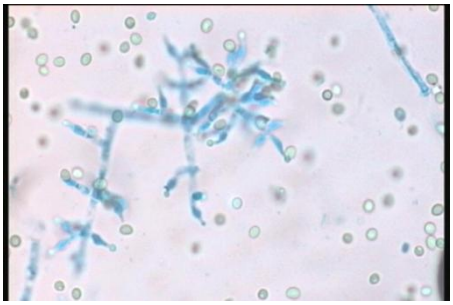
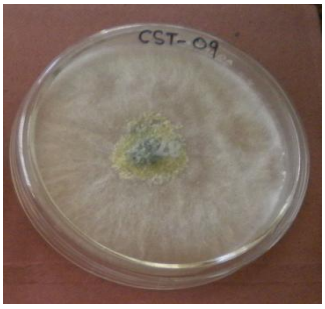
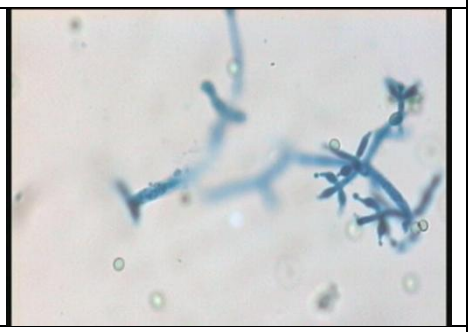
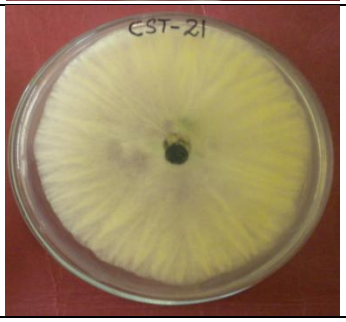
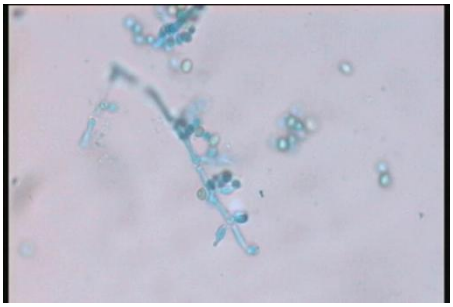


(A) **(B)**

Fig 3: (A) Radial growth pattern of all six isolates (CST-02, CST-05, CST-09, CST-14, CST-21 and CST-22) on PDA **(B)** Growth (reverse view)

Table 3: Cultural characteristic of *Trichoderma asperellum*

Characters	CST-02	CST-09	CST-21	CST-22
Colony growth rate	75-79 mm	90 mm	83-90 mm	87-90 mm
Colony colour	Green in center and white	Light green in center and white	Cottony white	White
Reverse colony colour	Off white	White	Off white	Yellow
Colony edge	Smooth	Smooth	Smooth & floccose	Smooth & floccose
Mycelial form	Smooth	Smooth & upressed	Smooth & fluffy	Smooth & upressed
Mycelial colour	White	Cottony white	Cottony white	White

Isolate Code	Growth on PDA medium	Growth [reverse view]	Light micrograph (viewed at 40x)
CST-02			
CST-09			
CST-21			

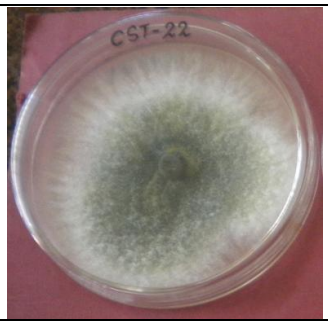
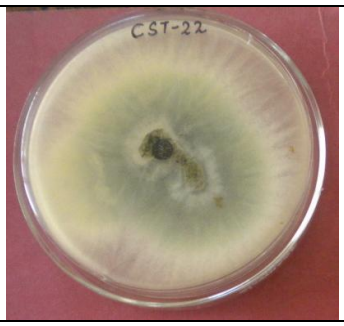
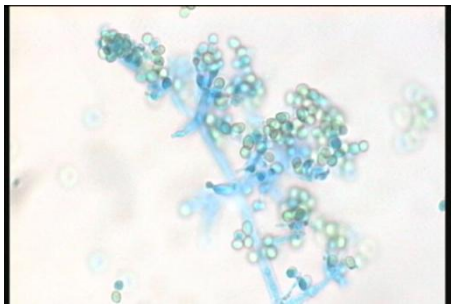
CST-22			

Fig 4: Growth on PDA medium, reverse view and microscopic examination of *Trichoderma asperellum*

Table 4: Cultural characteristics *Trichoderma koningii*

Characters	CST-05
Colony growth rate	90 mm
Colony colour	Cottony white
Reverse colony colour	Off white
Colony edge	Smooth
Mycelial form	Smooth and upressed
Mycelial colour	Off white

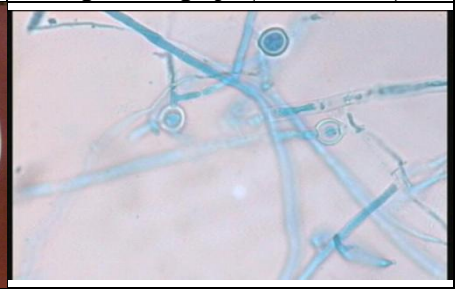
Isolate Code	Growth on PDA medium	Growth [reverse view]	Light micrograph (viewed at 40x)
CST-05			

Fig 5: Growth on PDA medium, reverse view and microscopic examination of *Trichoderma koningii*

Table 5: Cultural characteristics of *Trichoderma longibrachiatum*

Characters	CST-14
Colony growth rate	90 mm
Colony colour	white
Reverse colony colour	Yellow
Colony edge	Smooth and floccose
Mycelial form	Smooth
Mycelial colour	White

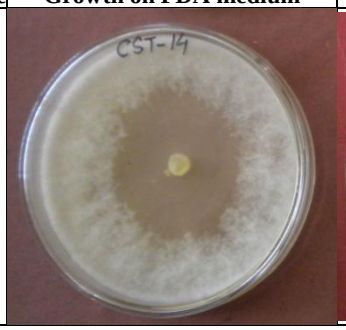
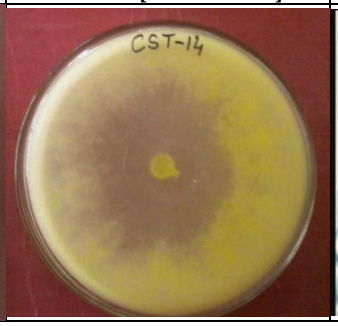
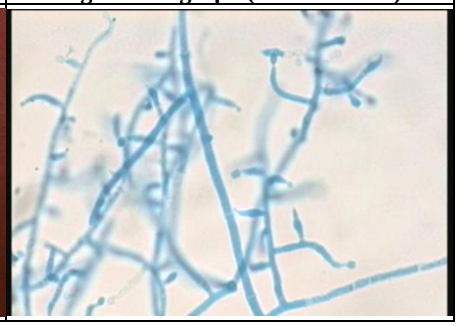
Isolate Code	Growth on PDA medium	Growth [reverse view]	Light micrograph (viewed at 40x)
CST-14			

Fig 6: Growth on PDA medium, reverse view and microscopic examination of *Trichoderma longibrachiatum*

Dual Culture

The choice of active *Trichoderma* isolate is important to design effective and safe bio-control mechanism, because many *Trichoderma* sp. have multiple activities for fungal antagonism and indirect effects on plant health or plant growth promotion. Some species are potent antibiotic producer and their suitability for use in bio-control systems must be assessed carefully.

Therefore, an attempt has been made to cultivate 03 *Trichoderma* sp. (*T. asperellum*, *T. koningii* and *T. longibrachiatum*) against two soil borne phytopathogens (*Fusarium oxysporum* f. sp. *lycopersici and Fusarium oxysporum* f. sp. *ciceri*). The antagonistic potentiality of *Trichoderma* sp. was determined by dual culture technique described by Morton and Stroube (Table 6 and Figure 7-9).

Three *Trichoderma* species screened to evaluate the efficient isolates for antagonistic activity by dual culture technique, the highest inhibitory effect was achieved by *Trichoderma longibrachiatum* on growth of both phytopathogens i.e. FOC and FOL as 37.6 and 40.8 per cent, respectively. *Trichoderma asperellum* also showed significant inhibition percentage of mycelial growth against FOC and FOL as 39.5 and 43.6 per cent, respectively.

Table 6: Mycelial inhibition percentage of *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *ciceri* and *Fusarium oxysporum* f. sp. *lycopersici*

Isolate Name	*Fusarium oxysporum* f. sp. *ciceri*		*Fusarium oxysporum* f. sp. *lycopersici*	
	Average Growth	%inhibition	Average Growth	% inhibition
CST-02	48.17	14.8	49.17	24.3
CST-05	41.67	26.3	46.67	28.2
CST-09	41.17	27.2	45.00	30.7
CST-14	37.67	33.4	40.83	37.1
CST-21	38.17	30.0	48.33	25.6
CST-22	42.33	25.1	43.67	32.8
CONTROL	57.33	-	65.00	-
CD@ 5%	3.73448		4.2468	
SD (d)	1.74119		1.98006	

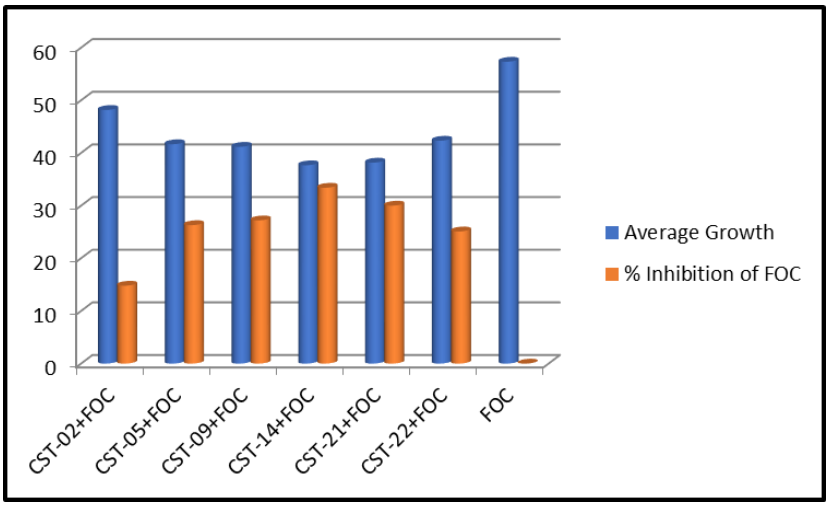


Fig 7: Graphical representation of mycelial inhibition percentage of *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *ciceri*

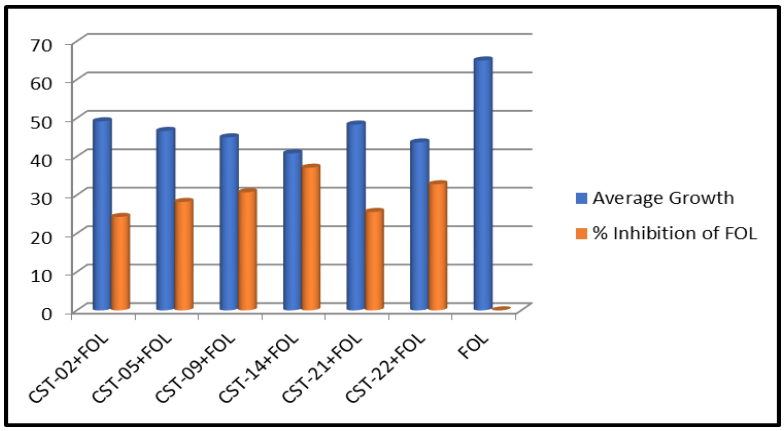
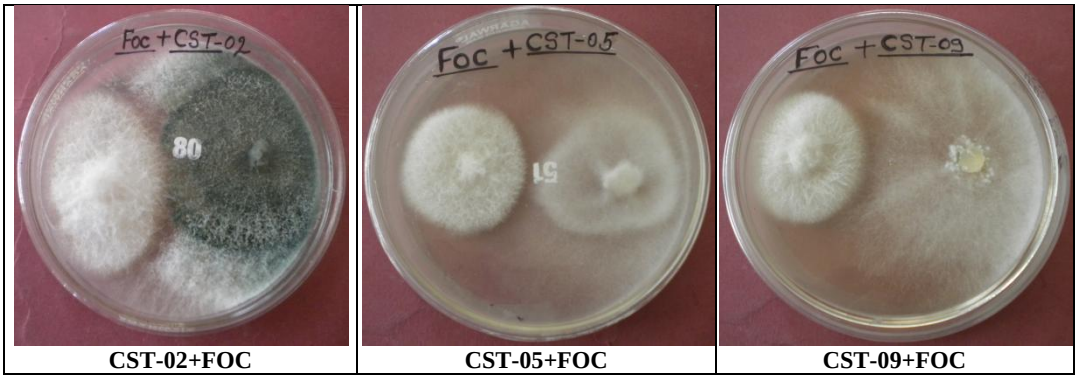


Fig 8: Graphical representation of mycelial inhibition percentage of *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *lycopersici*



CST-02+FOC | **CST-05+FOC** | **CST-09+FOC**

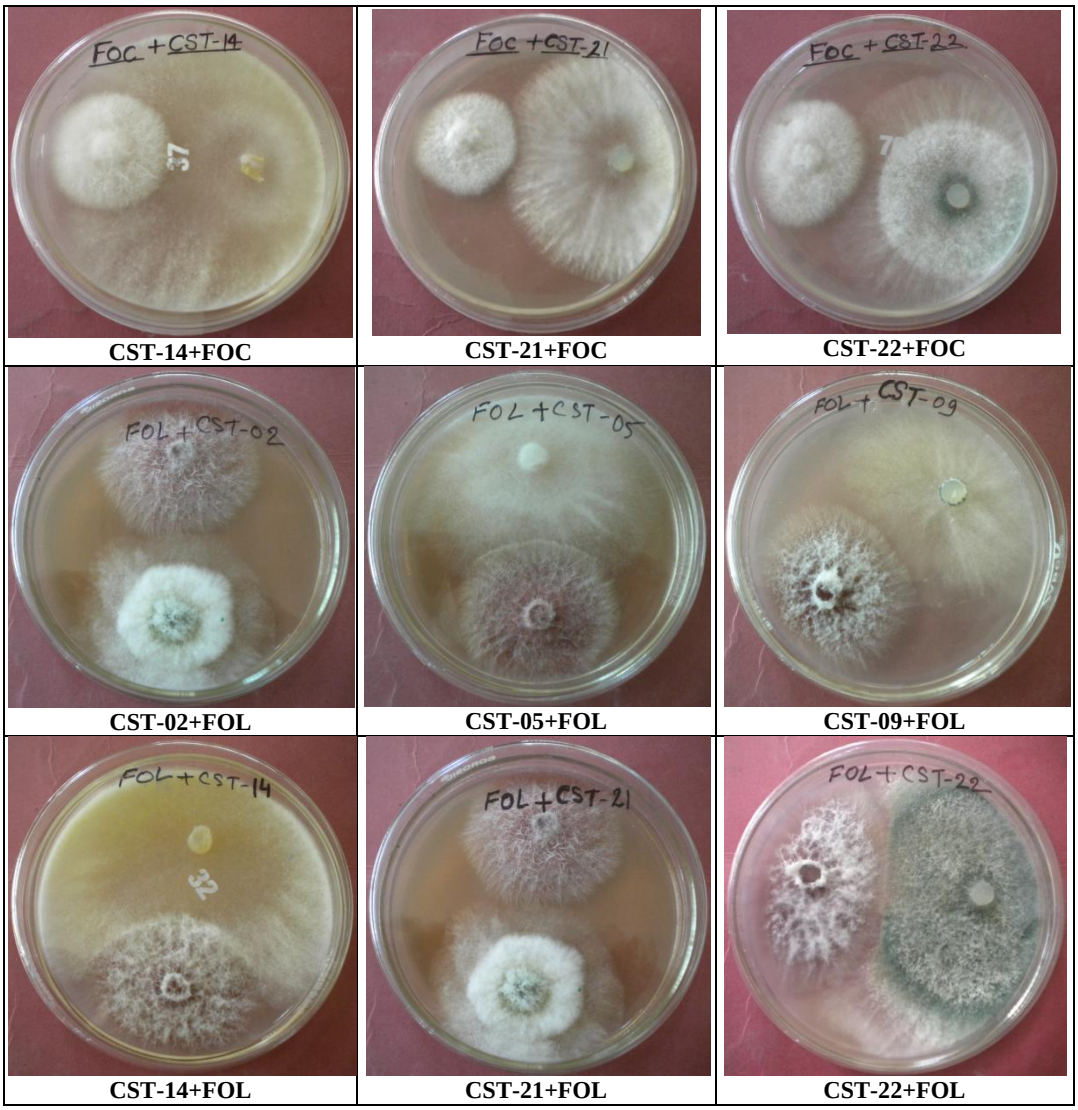


Fig 9: Antagonistic activity of trichoderma isolates against FOC and FOL

Inverse plate technique

In order to check the efficacy of volatile metabolites produced by *Trichoderma* sp. against soil borne phytopathogens inverse plate technique was used. Evaluation of volatile metabolite production by *T. asperellum*, *T. koningii* and *T. longibrachiatum* against two phytopathogens *Fusarium oxysporum* F. sp. *ciceri* and *Fusarium oxysporum* f. sp. *lycopersici* was done (Table 7 and Figure 10-11).

T. longibrachiatum (CST-14) showed maximum growth inhibition against both FOC and FOL as 29.3 and 17.7 percent, respectively. In case of FOC, *T. asperellum* (CST-21) showed better growth inhibition (17.7%) whereas, CST-22 showed better growth inhibition against FOL (34.6%). Mishra *et al.*, (2017) [13] studied effect of volatile metabolites released by *Trichoderma* species on fungal pathogens *Colletotrichum capsici* and *Fusarium oxysporum* f. sp. *capsici* etc.

Table 7: Per cent mycelial growth inhibition by different *Trichoderma* isolates producing volatile metabolites against 2 phytopathogens

Isolate Name	*Fusarium oxysporum* f. sp. *ciceri*		*Fusarium oxysporum* f. sp. *lycopersici*	
	Average Growth	%inhibition	Average Growth	% inhibition
CST-02	49.00	16.9	33.00	32.6
CST-05	50.67	14.1	39.00	20.4
CST-09	51.00	13.5	32.33	34.0
CST-14	41.67	29.3	29.67	39.4
CST-21	48.50	17.7	37.67	23.1
CST-22	52.00	11.8	32.00	34.6
CONTROL	59.00	-	49.00	-
CD@ 5%	2.68183		2.47659	
SD (d)	1.2504		1.1547	

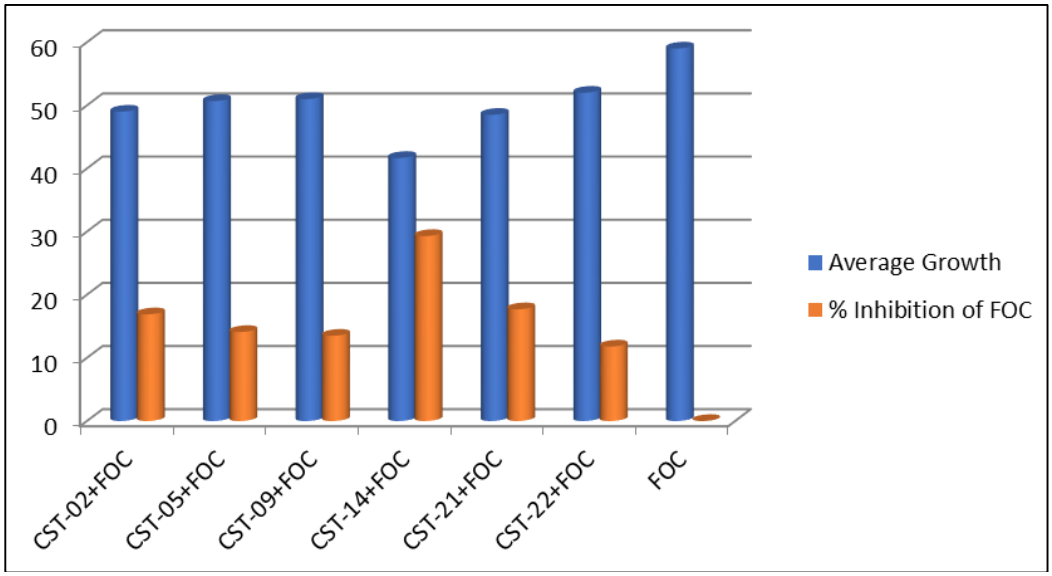


Fig 10: Graphical representation of mycelial inhibition percentage of *Trichoderma* isolates producing volatile metabolites against *Fusarium oxysporum* f. sp. *Ciceri*

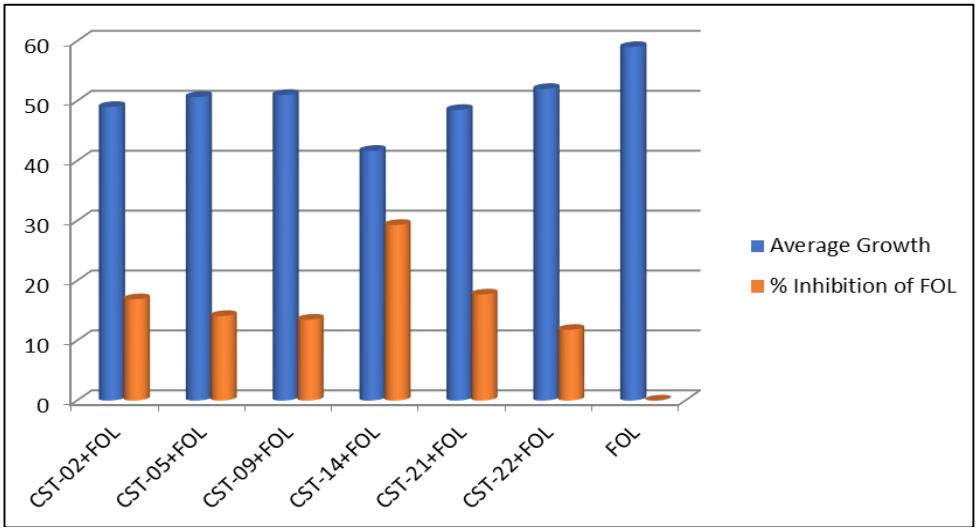


Fig 11: Graphical representation of mycelial inhibition percentage of *Trichoderma* isolates producing volatile metabolites against *Fusarium oxysporum* f. sp. *lycopersici*

Food poison technique

This experiment was carried out to investigate the inhibitory effect of culture filtrate of CST-14, CST-21 and CST-22 isolates at different concentrations on radial growth of FOC and FOL. Increasing the concentration significantly increased the inhibitory effect of the cultures filtrate. Data of the antagonistic effect of non-volatile compounds of CST-14, CST-21 and CST-22 against the mycelial growth of FOC and FOL *in vitro* are shown in (Table 8 and Figure 12-13). All the tested culture filtrates (non-volatile compound) of CST-14, CST-21 and CST-22 isolates at 50% concentration significantly reduced the mycelial growth as 83.8%, 100% and 73.0% for FOC and in case of FOL 100%, 100% and 49.2%, respectively. Similarly Trivedi *et al.*, (2013) [12] studied the effect of non-volatile metabolites (1000 ppm) of *Trichoderma* sp. on growth of pigeonpea wilt pathogen *Fusarium udum* and observed that the non-volatile compounds released by *Trichoderma* sp. significantly reduced the growth of the both pathogen.

Table 8: Growth inhibition of FOC and FOL by non-volatile compounds produce by CST-14, CST-21 and CST-22 isolates

Name of pathogens	Concentration of CST-14			
	5%	10%	25%	50%
FOC	24.6	33.07	36.15	83.84
FOL	27.5	37.5	40	100
Concentration of CST-21				
FOC	44.20	50.76	56.92	100
FOL	42.02	49.27	53.62	100
Concentration of CST-22				
FOC	46.92	47.69	50	73.07
FOL	37.68	42.02	45.65	49.27

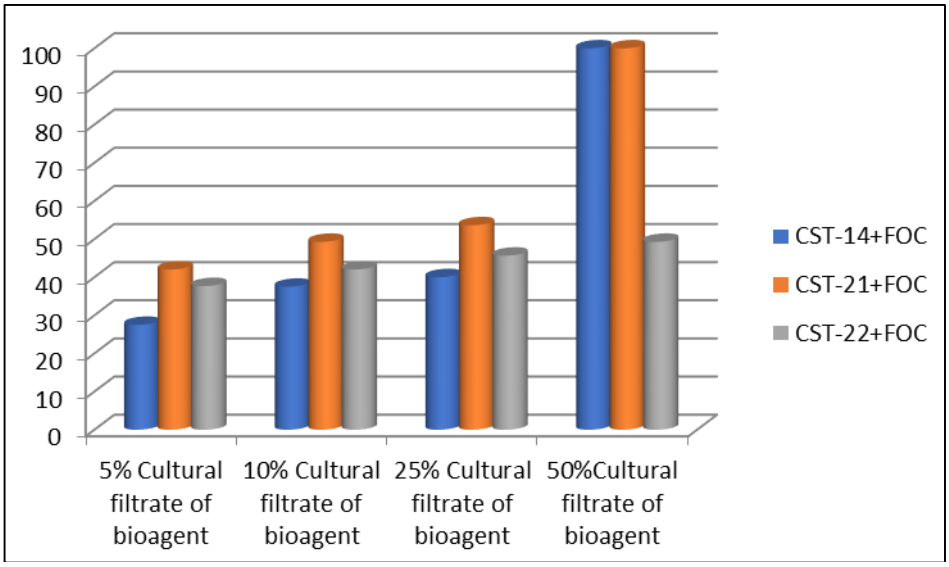


Fig 12: Graphical representation of mycelial inhibition percentage of *Trichoderma* isolates producing non-volatile metabolites against *Fusarium oxysporum* f. sp. *ciceri*

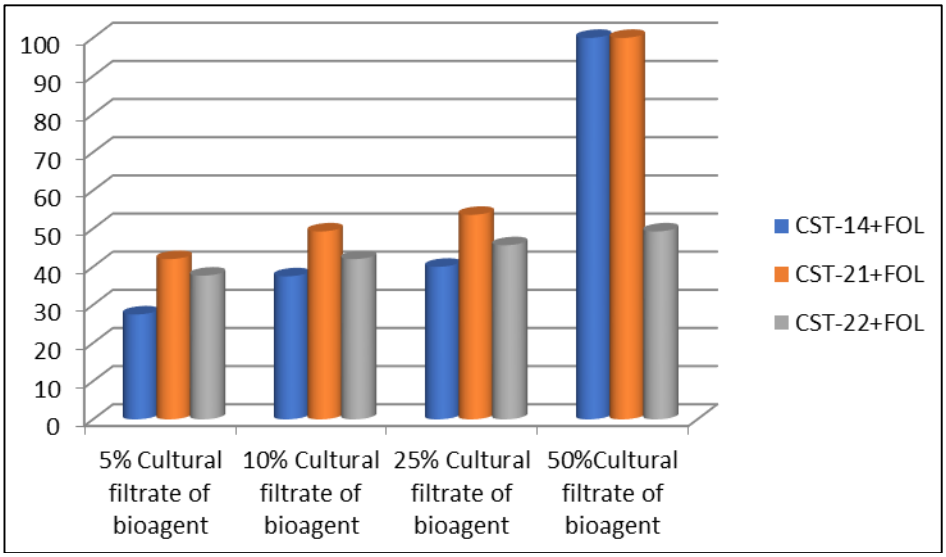


Fig 13: Graphical representation of mycelial inhibition percentage of *Trichoderma* isolates producing non-volatile metabolites against *Fusarium oxysporum* f. sp. *lycopersici*

Conclusion

The study conclude that out of 25 *Trichoderma* isolates, 06 isolates namely CST-02, CST-05, CST-09, CST-14, CST-21 and CST-22 were identified as *T. asperellum, T. longibrachiatum and T. koningii* on the basis of morphological characteristics confirmed by NFCCI & FIS, pune. The present study clearly showed the effect of the three antagonistic *Trichoderma* isolate namely CST-14, CST-21 and CST-22 against FOC and FOL. Volatile and non-volatiles compounds produced by Trichoderma isolates significantly reduced the mycelium growth of FOC and FOL. Based on the present investigation, a new strategy will be developed for in order to find out most promising *Trichoderma* sp. for controlling *Fusarium oxysporum* f. sp. *lycopersici* and *Fusarium oxysporum* f. sp. *ciceri.*

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Conflict of interest

Authors declare that they have no conflict of interest.

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