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Influence of native microbial biocontrol agent on radial growth of *Fusarium oxysporum* f. sp. *ciceri* isolates.

Influencia del agente de biocontrol microbiano nativo sobre el crecimiento radial de *Fusarium oxysporum* f. sp. aislados de *ciceri*.

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ABSTRACT

Microbial biocontrol agents (MBCA) play an important role in managing various plant pathogenic diseases. Several bacteria and fungi are known to act as MBCA and one of the fungi, the *Trichoderma* spp. is considered as most effective MBCA against numerous fungal plant pathogens. Earlier reports revealed that it acts through different mechanisms and inhibit the plant pathogens. *Fusarium oxysporum* f.sp *ciceri* (FOC) is one of the soil born plant pathogen which can survive in soil for a longer period and it largely infect the highly nutritious chickpea pulse crop causing chickpea wilt disease. In present study, the effects of secondary metabolites released from the native MBCA *i.e. T. harzianum* was investigated by using series of experiment on seventeen representative FOC isolates of Bundelkhand region of India. Findings of present study indicated that the secondary metabolites released from native *T. harzianum* were significantly found effective against seventeen representative FOC isolates. In Dual culture technique it significantly inhibits radial growth of MPFOC42 (65.19%). In addition secondary metabolites released in the form of volatile compounds at 2 DAI to 4 DAI show higher percent inhibition in MPFOC 37 (from Mudara village of Tikamgarh district) *i.e.* 45.45% at 2DAI and 35.29 % at 4DAI, and as well as through non-volatile secondary metabolites highest radial growth inhibition was observed in MPFOC21 (isolated from Sonagiri village, district Datia) *i.e*. 71.11, 78.52 and 83.70% at different concentration of culture filtrate *i.e.* 10%, 15%, 20% respectively. These findings indicate high efficiency of native MBCA *i.e T. harzianum*, which significantly inhibits radial growth of FOC isolates of Bundelkhand region.

Key words: Chickpea, Dual culture, FOC, Microbial control agents, secondary metabolites

RESUMEN

Los agentes de biocontrol microbiano (MBCA) juegan un papel importante en el manejo de diversas enfermedades patogénicas de las plantas. Se sabe que varias bacterias y hongos actúan como MBCA y uno de los hongos, el Trichoderma spp. se considera como el MBCA más efectivo contra numerosos patógenos fúngicos de

plantas. Informes anteriores revelaron que actúa a través de diferentes mecanismos e inhibe los patógenos de las plantas. Fusarium oxysporum f.sp *ciceri* (FOC) es uno de los patógenos de plantas nacidos en el suelo que puede sobrevivir en el suelo durante un período más largo e infecta en gran medida el cultivo de leguminosas de garbanzo altamente nutritivo que causa la enfermedad del marchitamiento del garbanzo. En el presente estudio, se investigaron los efectos de los metabolitos secundarios liberados del MBCA nativo, es decir, *T. harzianum*, mediante el uso de una serie de experimentos en diecisiete aislamientos FOC representativos de la región de Bundelkhand en la India. Los hallazgos del presente estudio indicaron que los metabolitos secundarios liberados de la *T. harzianum* nativa se encontraron significativamente efectivos contra diecisiete aislados de FOC representativos. En técnica de cultivo Dual inhibe significativamente el crecimiento radial de MPFOC42 (65,19%). Además, los metabolitos secundarios liberados en forma de compuestos volátiles a los 2 DAI a 4 DAI muestran un mayor porcentaje de inhibición en MPFOC 37 (del pueblo de Mudara del distrito de Tikamgarh), es decir, 45,45 % a 2 DAI y 35,29 % a 4 DAI, y también a través de no- La mayor inhibición del crecimiento radial de metabolitos secundarios volátiles se observó en MPFOC21 (aislado de la aldea de Sonagiri, distrito de Datia), es decir, 71,11, 78,52 y 83,70 % a diferentes concentraciones de filtrado de cultivo, es decir, 10 %, 15 % y 20 %, respectivamente. Estos hallazgos indican una alta eficiencia de MBCA nativo, es decir, *T. harzianum*, que inhibe significativamente el crecimiento radial de los aislamientos FOC de la región de Bundelkhand.

Palabras clave: garbanzo, cultivo dual, FOC, agentes de control microbiano, metabolitos secundarios

INTRODUCTION

According to agro-climate zone data, Bundelkhand region (a part of central India -consists of six district of Madhya Pradesh and seven district of Uttar Pradesh), shares most of the land with major pulses (44.5%), followed by the central plain zone (20.5%) (Kumar et al. 2022; Pathak et al. 2005).

Chickpea (*Cicer arietinum* L) also known as Gram is the main pulse crop in the Bundelkhand region followed by urad, lentil, pea and mung bean (Kumar et al. 2022). Chickpea is one of the highly cultivated *Rabi* season legume pulse crops in India and the third most major pulse crop in the world. Chickpea wilt disease, also known as Fusarium wilt, is one of the most destructive plant diseases, caused by wilt pathogen which is known as *Fusarium oxysporum* f. sp. *ciceri* (FOC). Worldwide it is majorly responsible for the low production of chickpea which causes production loss up to 10-15% every year. Indeed, in severe epidemics scenario this loss may increase up to 60-70% on annual basis (Anuragi and Sharma, 2016).

Wilt pathogen generally damages vascular part of the plant by entering through root parts and degrading the cell wall and block the vascular part of the plant. Subsequently discoloration of vascular part, yellowing & wilting of the aerial parts finally results in mortality of the plant (Thambugala et al. 2020).

Plant-pathogenic disease management through chemical based fungicides or pesticides is a costly approach, which is associated with lots of adverse effects on both, the soil as well as on environment.

Now a day's application of microbial biocontrol agents (MBCA) is one of the most effective approaches being used in controlling plant diseases and their agro-economical management. Microbial biocontrol agents are generally micro-organisms including bacteria and fungi, isolated from plant microbiome including phyllosphere, endosphere or rhizosphere, play an important role in controlling various plant diseases caused by plant pathogens. MBCA act with plant pathogen through several mechanisms. Indirect interaction with pathogens include competition for resources and available space and nutrients between organism (Spadaro and Droby 2016) or directly through antibiosis or hyper parasitism, induced resistance of plant defence response against plant pathogens and some of them reduced the effect/ level of pathogenic infection either via endogenous mechanisms or via action of naturally occurring/ introduced antagonists. Indeed, MBCA are reported to affect the micro-environment in such a way that it favours the activity of antagonists (Thambugala et al. 2020). Another direct and substantial route of action is the production of secondary metabolites having antibacterial properties that inhibit microorganisms (Raaijmakers and Mazzola 2012).

Application of fungi as biological control agent against plant pathogens is most popular over bacteria or any other microorganism and it has largely increased, as fungi are having high reproductive rate both, the sexual as well as asexual, taking the short generation time and being target specific. In addition, fungi can survive in extreme environment by shifting their mode of living *e.g.* parasitism to saprotrophism, thus maintaining their sustainability. Interestingly, many fungal species possess the mechanisms which efficiently protect plants from pathogenic diseases caused by other pathogenic fungi. Various fungi including *Trichoderma, Gliocladium, Candida, Ampelomyces and Coniothyrium* have been identified as fungal antagonist beside their promising role against number of plant pathogens (Sankar et al. 2018). Among these, *Trichoderma* has been recognised as most potential biocontrol agent against many plant-fungal diseases.

Therefore, people across the world have adopting the use of Microbial biocontrol agents (MBCA) for protection of crop from various plant-pathogenic diseases as well as improve crop yield.

In present study, native MBCA *Trichoderma* spp. has been investigated by using a series of experiments, which include dual culture method, the effects of secondary metabolites released from MBCA through volatile and non-volatile methods on seventeen FOC isolates of Bundelkhand region of India.

MATERIALS AND METHODS

In the present study, two districts (Datia and Tikamgarh) of Bundelkhand region, from Madhya Pradesh, India, were selected for the isolation of wilt pathogen during *Rabi* season (December, 2017 to February 2018). Three villages from each district *(*Sonagiri, Ganghari, Rajpura from Datia district and Newari, Prithvipur, Mudara from Tikamgarh district) were surveyed and selected for collection of diseased chickpea plant and native soil sample from three to five fields (Table 1 and Fig. 1).

Table 1: Sample collected from different locations of Bundelkhand region

District	Village	FOC Code
Datia	Ganghari	MPFOC1
		MPFOC3
	Rajpura	MPFOC7
		MPFOC8
		MPFOC13
		MPFOC14
	Sonagiri	MPFOC18
		MPFOC21
Tikamgarh	Niwari	MPFOC23
		MPFOC24
		MPFOC25
		MPFOC26
	Mudara	MPFOC33
		MPFOC36
		MPFOC37
	Prathivipur	MPFOC42
		MPFOC43

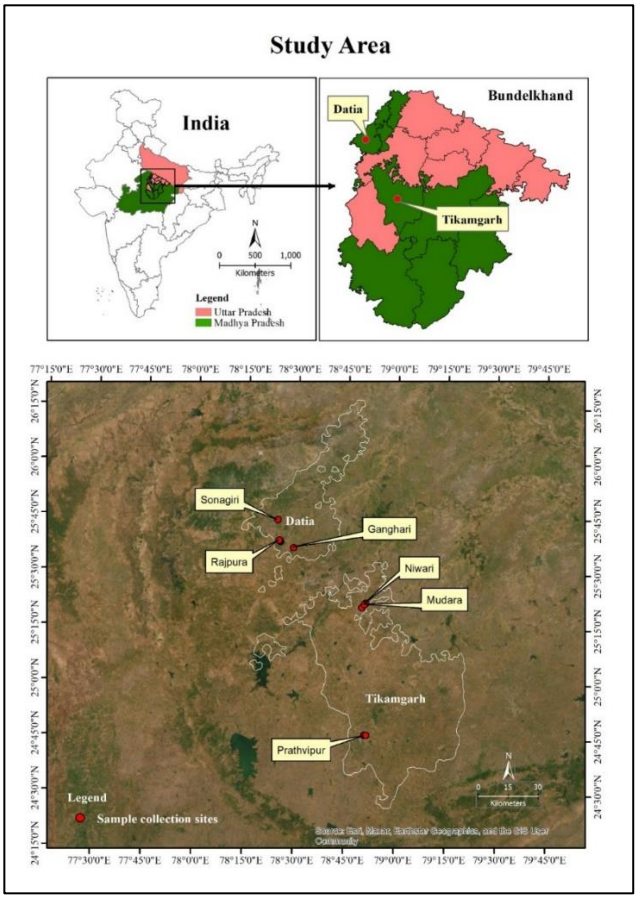

Fig. 1: Geographical location of study area

Chickpea plant samples showing wilt disease symptoms, were collected along with native soil samples (for native *Trichoderma* spp*.* isolation). Further, the confirmation of wilt pathogen as *Fusarium oxysporum* f. sp. *ciceri* (FOC) isolates was done by morphological identification, and kotch postulates (pathological characteristics). Isolation and identification of FOC isolates was carried out by standard procedure as described below. Further these purified samples evaluated with native isolated *Trichoderma* spp. This research work was carried out at Department of Microbiology, Bundelkhand University, Jhansi, U.P. and IGFRI, ICAR, Jhansi, U.P.

Preparation of FOC isolates: Isolation of wilt pathogen was carried out from the infected root samples and the lower part of the stem as well. The diseased/infected part of samples were cut into small pieces of 2-3 mm followed by surface sterilization using 1% NaOCl (Sodium hypochlorite solution) for 60 to 80 seconds and washing four time using distilled sterilized water for 60 sec. Subsequently, surface sterilized infected plant parts were inoculated by placing them on Potato Dextrose Agar (PDA) petri plates aseptically and kept in incubator for 4-5 days on 26^0C. Further, speculative cultures of *Fusarium oxysporum* f. sp. *ciceri* (FOC) were purified by using single spore isolation method, and maintained by the periodical transfer on PDA slants and allowed to grow for 7 to 8 days at 26^0C. Finally, these slants were preserved in a refrigerator at 5^0C and revived once in every 30 days (Abou-Zeid, Halila, and Khalil 2002; Aneja 2005). Purified isolates were observed under stereoscopic (Model: Leica) and compound microscope (Model: LEICA DM 2500 LED) for desired pathogen and characterized based on microscopic observations *i.e.* shape and size of the spore, culture morphology and colony characteristics. On the basis of culture characterisation wilt pathogen also verified through kotch postulates. Total 17 FOC isolates were selected as representative FOC isolates and further studied to for dual culture and effect of secondary metabolites released by native MBCA *Trichoderma* spp. on these FOC isolates.

Influence of secondary metabolites on radial growth of wilt pathogen: It has been reported in literature that due to the production of ROS, lytic enzymes, and secondary metabolites, numerous species of *Trichoderma* have been found to have considerable antagonistic action against soil-borne pathogens such as *F. oxysporum, F. solani*, etc (Singh, Ratan, and Singh 2009). In present study native microbial biocontrol agent (MBCA) *Trichoderma* spp*.* on representative FOC isolates was assessed by performing a series of experiments, which include dual culture method, Effect of secondary metabolites released by volatile method and non-volatile method.

Isolation of the native Microbial Biocontrol Agent (MBCA): A microbial biocontrol agent *i.e. Trichoderma* spp, was isolated from native soil of area of interest. Soil samples were taken from rhizospheric region of infected chickpea plants at a depth ranging from 5 to 6 inch and kept at room temperature. The isolation of *Trichoderma* spp*.* from soil samples was done by using serial dilution method and samples were serially diluted from 10^{-3} to 10^{-5} dilutions in sterile distilled water followed by 1ml plating on Potato Dextrose Agar (PDA) plates separately. Speculative *Trichoderma* spp. were picked from PDA plates and again purified on Trichoderma specific media (TSM). Further, the picked colonies were screened/ identified based on morphological and cultural characteristics and maintained on PDA media to see the effect of released secondary metabolites on wilt pathogen (Chandra and Sonkar 2017).

Dual Culture Technique: *In vitro* bio-efficacy of native MBCA was tested by using zone of inhibition assay (dual culture technique) against representative *Fusarium oxysporum* f. sp. *ciceri* (FOC) isolates. A 5 mm dia discs from actively growing (7 days old) cultures of native *Trichoderma* spp. was transferred to one side of petri plates (1 cm away from the edge) containing PDA medium, whereas on opposite side of petri plate (1 cm away from the edge), the representative FOC isolate was transferred on same petri plates. Similar mycelial bits of respected FOC was also incubate at the centre of another PDA containing petri plate which is used as a Control. Experiment was conducted in three replications for each isolate. These petri plates were incubated at 27°C and allowed to grow for appropriate time, the radial growth of FOC mycelium (in mm) was recorded till the mycelium completely covered the control plate. The percentage inhibition of the mycelium was calculated as per the method earlier described by Kumar et.al 2019 (Kumar et al. 2019).

$$\mathrm{L} = \frac{\mathrm{C} - \mathrm{T}}{\mathrm{T}} X100$$

Where;

L = Percentage inhibition of pathogen mycelium;

C = Redial growth in Control plate;

T = Redial growth in Treated plate

Effect of secondary metabolites: Volatile compounds: In this study, the effect of volatile compounds (metabolites) produced by native *Trichoderma* spp. on the radial growth of the representative seventeen FOC isolates was investigated by using inverted plate technique as described by Dennis and Webster et al.(Dennis and Webster 1971a). The 5 mm mycelial disk of native *Trichoderma* spp. excised from a 7 days old actively growing culture, was inoculated at the centre of a PDA petri plate. Top of each petri plates was replaced by the bottom of another PDA petri plate containing a 5 mm mycelial disk of *Fusarium* isolates so as to expose the test pathogens directly to antagonistic environment created by *Trichoderma*. Subsequently, the bottom of two petri plates were paired and sealed together with parafilm and incubated at 28°C in the dark.

The assembly which serves as control, contains the respective test pathogens on the lid of the plate whereas the bottom plate is kept blank. Each treatment group replicates three times. Colony growth in the assembled petri plates was recorded up to the day when FOC isolates in control plates full covered and the radial growth of the test pathogen was measured in each treated plate as well as control (Sumana and Devaki 2002), Percentage inhibition of pathogen by secondary metabolites was calculated, compared with control, by the formula Kumar et al (Kumar et al. 2019).

$$\mathrm{L} = \frac{\mathrm{C} - \mathrm{T}}{\mathrm{T}} X100$$

Where:

L – Percent inhibition of radial mycelial growth;

C – The radial growth measurement of the pathogen in the control;

T – The radial growth measurement of the pathogen in the presence of antagonists.

Effect of secondary metabolite: Non-volatile compounds: The effects of non-volatile secondary metabolites released by culture filtrate of native *Trichoderma spp.* was studied on the representative test pathogens (Dennis and Webster 1971b).

Preparation of the culture filtrate: For the preparation of the culture filtrate, a 50 ml of Potato Dextrose Broth (PDB) was prepared in 250 ml Erlenmeyer conical flask and sterilized by autoclaving. Each conical flask was inoculated with a 5mm mycelial disc of the native *Trichoderma* spp. (antagonist) excised by using a cork borer from the margin of actively growing 7 day old culture and incubated at 27ºC for 15 days with constant shaking in an orbital shaker. The culture filtrate was harvested by passing it through whatman filter paper No. 1, and the residue filtrate was collected in a sterilized conical flask and again sterilized by passing through a cellulose membrane filter (Millipore) after centrifuging it at 6000 rpm for 15 min. This filtrate was mixed with PDA in different ratio *i.e.* 10, 15, and 20 % and poured in petri plates. The 5 mm mycelial disc of the test pathogen was inoculated in the centre of the petri plate containing 10, 15 and 20 % of mixture of the PDA and culture filtrate and incubated at 27^{o}C, till the pathogen fully covered the control plate. PDA plates without culture filtrate inoculated with the test pathogen used as control. Each treatment was replicated three times. A periodic observations on the radial growth of the test pathogen were recorded (Narsimha Reddy, Venkata Saritha, and Hindumathi 2014). The percentage inhibition of the mycelial growth of the pathogen by non-volatile compounds (metabolite) was calculated as per the formula by Garcia et al. (Garcia et al. 1994).

$$L = \frac{C - T}{C} \times 100$$

Where:

L – Percent inhibition of radial mycelial growth;

C = Radial growth of pathogen in control;

T = Radial growth of pathogen in treatment.

Statistical Analysis: The research experiment was laid out in Completely Randomized Design (CRD) and three independent observation of each sample for each test were taken and mean of these observation was used for the analysis. Data were analysed using online OPSTAT software, a Statistical Software Package for Agricultural Research Workers (Sheoran et al. 1998).

RESULTS AND DISCUSSION

Isolation of Microbial Biocontrol Agent *i.e.* native *Trichoderma spp.*: In current study, the *Trichoderma* spp. was isolated from native soil of study area by using serial dilution technique. Speculative *Trichoderma* spp. was picked for morphological identification and on the basis of morphological and cultural characteristics, they identified as *T. harzianum, T. viride and T. asperellum* .Out of which potential Trichoderma species *i.e T. harzianum* was

selected and further exploited to investigate the effect of released secondary metabolites on representative FOC isolates (Fig. 2).

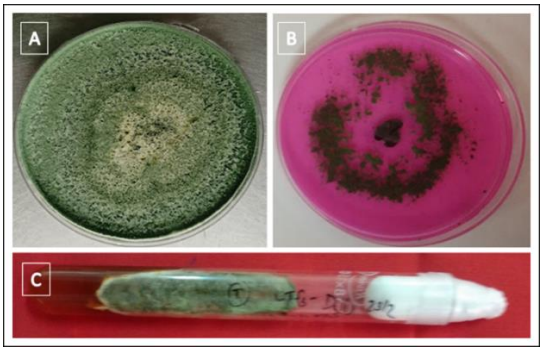

Fig. 2: Pure culture of isolated native *T. harzianum* (A) in PDA; (B) in TSM; (C) PDA Slant

Dual culture Technique: The antagonistic effect of native *T. harzianum* isolate against representative *F. oxysporum* f. sp *ciceri* (FOC) isolates was investigated using dual plate (poison food tests) methods (Fig. 3). The observations from dual plate culture method indicated that the native *T. harzianum* significantly reduced the radial growth of FOC isolates as shown in (Table 2 and Fig. 4).

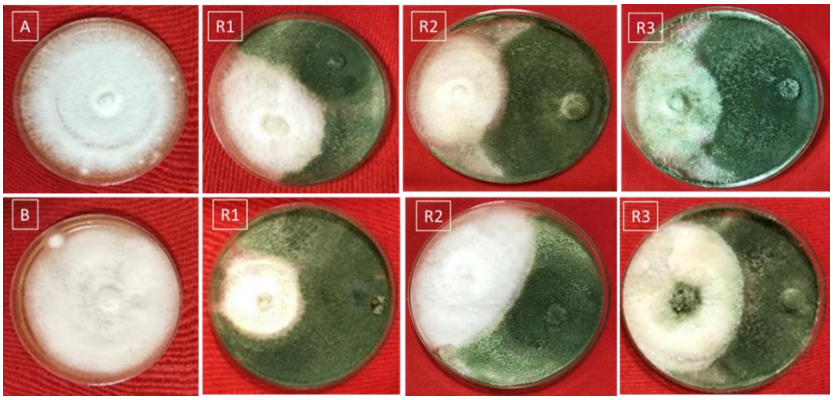

Fig. 3: Efficacy of native MBCA assessed against representative *F. oxysporum* f.sp *ciceri* isolates using Dual Culture Technique (A. MPFOC21; B. MPFOC23).

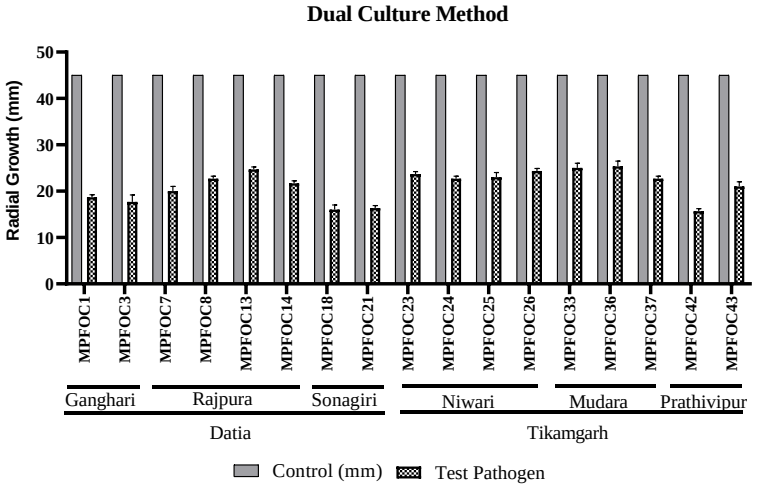

Fig. 4: Radial growth of representative FOC isolates in Dual Culture

Table 2: Percent inhibition and mean radial growth of representative FOC isolates in Dual Culture technique

District	Village	Code	Mean	SE	% Inhibition
Datia	Ganghari	MPFOC1	18.67	0.33	58.52%
		MPFOC3	17.67	0.88	60.74%
	Rajpura	MPFOC7	20.00	0.58	55.56%
		MPFOC8	22.67	0.33	49.63%
		MPFOC13	24.67	0.33	45.19%
		MPFOC14	21.67	0.33	51.85%
	Sonagiri	MPFOC18	16.00	0.58	64.44%
		MPFOC21	16.33	0.33	63.70%
Tikamgarh	Niwari	MPFOC23	23.67	0.33	47.40%
		MPFOC24	22.67	0.33	49.62%
		MPFOC25	23.00	0.58	48.89%
		MPFOC26	24.33	0.33	45.93%
	Mudara	MPFOC33	25.00	0.58	44.44%
		MPFOC36	25.33	0.67	43.70%
		MPFOC37	22.67	0.33	49.63%
	Prathivipur	MPFOC42	15.67	0.33	65.19%
		MPFOC43	21.00	0.58	53.33%
	C.D.		1.400		
	SE(m)		0.485		
	SE(d)		0.686		
	C.V.		3.956		

The Maximum radial growth inhibition of FOC was observed in MPFOC42 (65.19%) which was isolated from Prithvipur village of Tikamgarh district and MPFOC18 (64.44%), isolated from Sonagiri village of Datia district as compared to Control. Which was followed by MPFOC21 and MPFOC3 (isolated from Sonagiri and Ganghari village of Datia district respectively) with percent inhibition of FOC isolates by 63.70% and 60.74% respectively.

The FOC isolates MPFOC33 and MPFOC36 (isolated from Mudara village of Newari district) shows relatively lesser inhibition *i.e.* 44.44% and 43.70% respectively. A higher inhibition of radial growth indicates the high efficacy (antagonist effect) of native *T. harzianum* and the effective control of radial growth (in vitro) of wilt pathogen. Whereas lesser inhibition of radial growth indicates significant confrontation of FOC against antagonist.

The outcomes of present findings showed that the native antagonists impressively slowed the growth of *F. oxysporum* f.sp. *ciceri* and also displayed inhibition zones. Earlier study by Lakshman Prasad (2016) revealed that in-vitro dual culture studies of *F. oxysporum* and *T. harzianum*, together led to a variety of interactions. *F. oxysporum* growth was generally inhibited by the *T. harzianum*. The *F. oxysporum* hyphae lysed on dual culture media and were intensely parasitized by *T. harzianum* (Prasad et al. 2016).

In Earlier study Hossain et al. (2013) also found that, a *T. harzianum* (T75) isolate totally prevented *F. oxysporum* f. sp. *ciceris* from growing its mycelium in a dual culture experiment (Hossain et al. 2013). Another previous study reported that the application of microbial biological control agent is considered as promising tool for increased agriculture production, which is associated with reduced incidence of disease. Indeed, a previous study reported that one of the distinctive strains of *T. harzianum* (SVPUTh91) was found to be potential candidate for developing as microbial biological control agent. Due to the diverse mechanism, the fungus *T. harzianum* found to be significantly inhibiting the growth of other fungi. Without a doubt, the application of *T. harzianum* (SVPUTh91) in production as well as development of biocontrol agent, to regulate the fusarium wilt diseases is apparently a beneficial approach rather could be an exemplary environment friendly biocontrol agent (Prasad et al. 2016). The findings from present study are in line to the results from those studies as mentioned above.

Generally, the mycelial growth inhibition was assessed by using the lysis of *Fusarium* mycelium, which could be due to the nutrient reduction or production of toxic substances like enzymes, metabolites, antibiotics, volatile and non-volatile elements released by the antagonist. Indeed, several studies on phytopathogenic *fungi* have reported the same findings (Askew and Laing 1994; Kay and Stewart 1994). Due to enzymatic actions, the pathogen mycelium gets split. In biocontrol agent *Trichoderma spp.,* the role of chitinolytic and/ glucanases enzymes has been well established. Indeed, the *T. harzianum* was found to be superior over *T. viride* in terms of antagonist activity against *F. oxysporum,* and the synthesis of glucanase and chitinase as well (Metcalf and and Wilson 2001; Ojha and Chatterjee 2011). These enzymes found to act through disaggregation of polysaccharides, chitin, and damaging the integrity of fungal cell wall, and finally preventing the pathogen from spreading in host system (Siameto et al. 2011).

In prior research work, the *T. harzianum* was grown over *F. oxysporum*, and as a result the sporulation of *T. harzianum* occured as in dark green colour however the pathogen's mycelium was in light yellow hyphae. The observation indicated 40% of inhibition due to MBCA. In the beginning of incubation, *T. harzianum* developed as in multiple coils around the pathogen's hyphae and looped around as a thick mycelial rope, resulting in two distinct green zones, the pathogen and *T. harzianum's* hyphae (Morsy, Abdel-Kawi, and Khalil 2009; Upadhayay and Mukhopadhyay 1986; Windham, Elad, and Baker 1986). The observations from our finding were in line to the observation from other researchers as discussed above in which *T. harzianum* isolated from native place was significantly inhibited the FOC isolates of Bundelkhand region.

Effect of volatile metabolites on radial growth of FOC isolates: In the present study, the ability of released volatile metabolites from native *Trichoderma spp.* was tested for growth reduction (% inhibition) of selected FOC isolates was assessed. The in-vitro growth reduction of FOC isolates by volatile substance of native *Trichoderma spp.* was observed at 2DAI to 4DAI (Fig. 5). Graphical presentation of radial growth (mm) depicted in fig. 6 and radial growth including % inhibition shows in table 3. The observation indicated that MPFOC 37 (from Mudara village of Tikamgarh district) had higher percent inhibition *i.e.* 45.45% at 2DAI and 35.29 % at 4DAI. Which is followed by MPFOC 3 (from Ganghari village of Datia district) showed 39.39% at 2DAI, 36.84 % at 4DAI and MPFOC 7 (from Rajpura village of Datia district) 33.33% at 2DAI, 30.77% at 4DAI. However other FOC isolates showed moderate to

low percent inhibition. Above findings of present investigation indicate that the volatile substance released by native *T. harzianum* must be playing a significant role in growth inhibition of FOC isolates.

Table 3: Percent inhibition and mean radial growth (at 2DAI and 4DAI) of representative FOC isolates in volatile method

District	Village	FOC Code	Radial Growth (mm)				% Inhibition		SE	
			Control		Test Pathogen					
			Day 2	Day 4	Day 2	Day 4	Day 2	Day 4	Day 2	Day 4
Datia	Ganghari	MPFOC1	7.00	10.00	6.00	8.00	14.29	20.00	0.00	0.00
		MPFOC3	11.00	19.00	7.50	11.17	31.82	41.23	0.29	0.17
	Rajpura	MPFOC7	9.00	13.00	6.50	9.00	27.78	30.77	0.29	0.00
		MPFOC8	8.00	13.00	6.67	10.00	16.67	23.08	0.17	0.00
		MPFOC13	8.00	15.00	7.17	10.50	10.42	30.00	0.17	0.29
		MPFOC14	8.00	14.00	6.50	10.00	18.75	28.57	0.29	0.00
	Sonagiri	MPFOC18	7.00	10.00	6.00	8.00	14.29	20.00	0.00	0.00
		MPFOC21	9.00	13.00	6.50	10.00	27.78	23.08	0.29	0.29
Tikamgarh	Niwari	MPFOC23	9.00	18.00	6.00	11.67	33.33	35.19	0.00	0.33
		MPFOC24	8.00	14.00	6.00	10.17	25.00	27.38	0.00	0.17
		MPFOC25	10.00	17.00	6.00	10.33	40.00	39.22	0.00	0.33
		MPFOC26	9.00	19.00	6.50	12.33	27.78	35.09	0.29	0.33
	Mudara	MPFOC33	10.00	18.00	8.00	14.67	20.00	18.52	0.00	0.33
		MPFOC36	10.00	19.00	7.00	13.67	30.00	28.07	0.00	0.17
		MPFOC37	11.00	17.00	6.00	11.00	45.45	35.29	0.00	0.58
	Prathivipur	MPFOC42	8.00	11.00	6.00	9.00	25.00	18.18	0.00	0.00
		MPFOC43	7.00	10.00	6.00	8.00	14.29	20.00	0.00	0.00
	C.D.				0.481	0.710				
	SE(m)				0.167	0.246				
	SE(d)				0.236	0.348				
	C.V.				4.448	4.079				

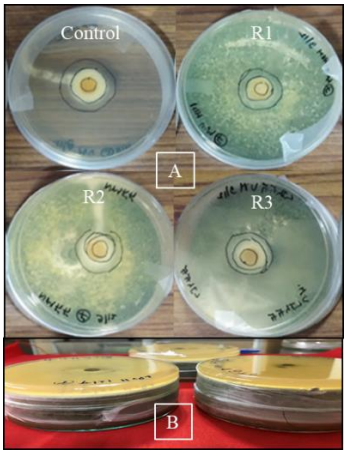

Fig. 5: Effect of volatile metabolites; Inverted plate technique (Assembly): upper view (A) & side view (B)

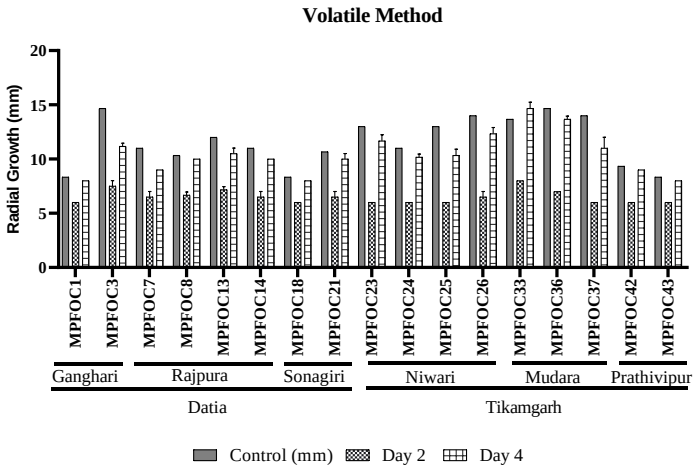

Fig. 6: Graphical representation of Mean radial growth (2DAI, 4DAI) of representative FOC isolates in volatile method

Earlier study revealed that, *Trichoderma species* are capable of producing a wide range of volatile secondary metabolites including the ethylene, hydrogen cyanide, aldehydes and ketones, which are very critical in suppressing a large number of plant diseases (Chen et al. 2015; Faheem et al. 2010; Siddiquee et al. 2012). In previous study volatile chemicals released by *T. harzianum* showed the highest growth inhibition against isolates of *Fusarium oxysporum* f.sp. *ciceri*.

Furthermore, In several studies it is evident that volatile secondary metabolites, produced by *Trichoderma harzianum* can extensively inhibit the growth of *Aspergillus flavus* and *Fusarium moniliforme, P.ultimum* and *R. solani* other than mycoparasitism process (Calistru, McLean, and Berjak 1997; Raut et al. 2014; Srivastava et al. 2011). A major chickpea disease known as "Chickpea wilt complex" was found to be successfully managed through *Trichoderma harzianum* and its combination with fungicides (Kaur and Mukhopadhyay 1992). The observations from present finding were in line to the observation from other reserachers, as discussed above in which volatile substances released from native *T. harzianum* significantly inhibited the FOC isolates of Bundelkhand region.

Effect of non-volatile metabolites: The observations from non-volatile method indicated the inhibitory effects of culture filtrate of native *T. harzianum* on the linear growth of FOC isolates at various doses. Present findings indicate the inhibitory effect of the culture filtrate was significantly enhanced by increasing the concentration of culture filtrate. Results of table 4 clearly indicates that among all FOC isolates, the MPFOC21 isolate from Sonagiri village, district Datia was found to be highly inhibited *i.e.* 71.11, 78.52 and 83.70% at different concentration of culture filtrate *i.e.* 10%, 15%, 20% respectively (Fig. 7 & 8).

Table 4: Percentage inhibition of wilt pathogen by non-volatile compound

District	Village	FOC Code	Control	Antagonist (Culture Filtrate)								
				10%			15%			20%		
				Mean	SE	% Inhibition	Mean	SE	% Inhibition	Mean	SE	% Inhibition
Datia	Ganghari	MPFOC1	45.00	18.67	0.33	58.52%	12.33	0.33	72.59%	9.33	0.33	79.26%
		MPFOC3	45.00	19.67	0.33	56.30%	13.67	0.17	69.63%	9.83	0.33	78.15%
	Rajpura	MPFOC7	45.00	15.33	0.33	65.93%	10.33	0.44	77.04%	7.67	0.33	82.96%
		MPFOC8	45.00	17.67	0.88	60.74%	14.33	0.17	68.15%	10.67	0.67	76.30%
		MPFOC13	45.00	19.33	0.67	57.04%	16.33	0.17	63.70%	11.67	0.33	74.07%
		MPFOC14	45.00	20.33	0.33	54.81%	17.33	0.88	61.48%	11.67	0.17	74.07%
	Sonagiri	MPFOC18	45.00	16.33	0.17	63.70%	14.67	0.88	67.41%	10.33	0.88	77.04%
		MPFOC21	45.00	13.00	0.58	71.11%	9.67	0.67	78.52%	7.33	0.33	83.70%
Tikamgarh	Niwari	MPFOC23	45.00	19.67	0.33	56.30%	17.33	0.33	61.48%	12.67	0.33	71.85%
		MPFOC24	45.00	18.67	0.33	58.52%	16.33	0.33	63.70%	12.00	1.00	73.33%
		MPFOC25	45.00	20.33	0.33	54.81%	18.33	0.67	59.26%	12.33	0.33	72.59%
		MPFOC26	45.00	21.33	0.33	52.59%	18.67	0.33	58.52%	13.33	0.33	70.37%
	Mudara	MPFOC33	45.00	20.00	0.58	55.56%	15.00	0.58	66.67%	10.00	0.58	77.78%
		MPFOC36	45.00	18.33	0.33	59.26%	13.67	0.33	69.63%	9.33	0.67	79.26%
		MPFOC37	45.00	19.00	0.58	57.78%	13.33	0.33	70.37%	9.33	0.33	79.26%
	Prathivipur	MPFOC42	45.00	17.00	0.58	62.22%	13.00	0.58	71.11%	10.33	0.67	77.04%
		MPFOC43	45.00	29.00	1.16	35.56%	22.00	0.58	51.11%	10.00	0.58	77.78%
	C.D.			1.552			1.467			1.535		
	SE(m)			0.538			0.508			0.532		
	SE(d)			0.761			0.719			0.752		
	C.V.			4.892			5.837			8.803		

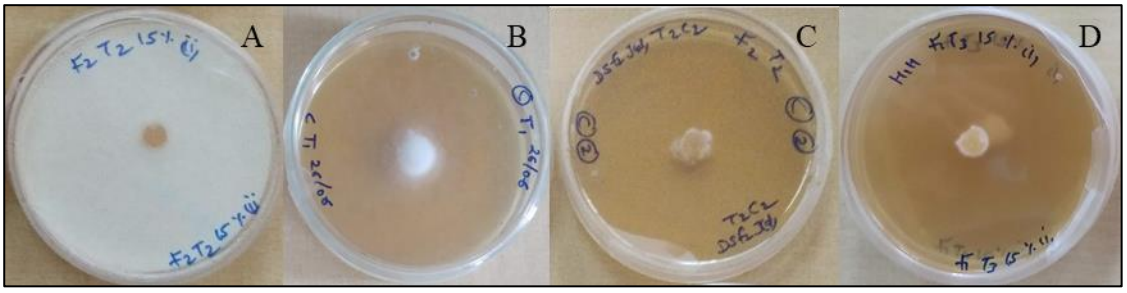

Fig. 7: Effect of non-volatile compounds; A. Control; B. 10%; C. 15%; D. 20%

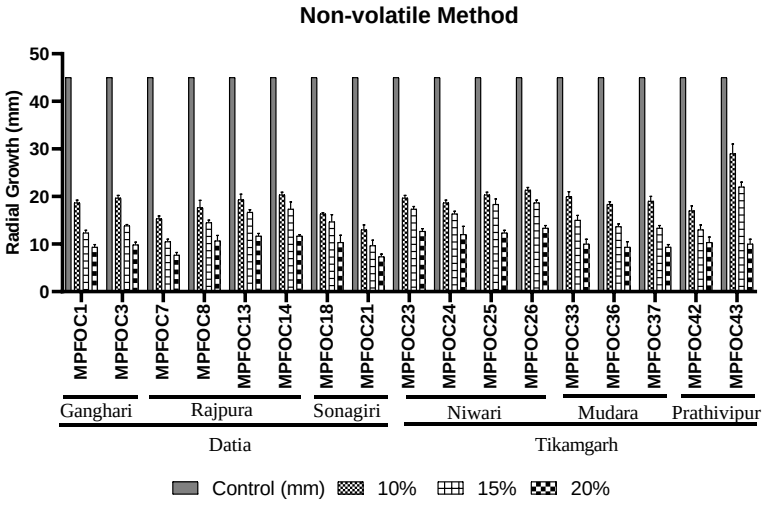

Fig. 8: Effect of non-volatile compounds on seventeen representative FOC isolates

These findings clearly depict that the percent inhibition of radial growth of FOC isolates at various doses enhances by increasing concentration of culture filtrate. At 10% concentration of culture filtrate, the % inhibition was observed ranges 35 to 71% while at 15% it ranges from 51.11 to 78.52% and 70.37 to 82% at 20%. The non-volatile metabolites released by native *T. harziunum*, significantly reduced the mycelial growth of FOC isolates as compared to the control treatment.

Above findings found to be in line with the observation from an earlier research reported by Barakat et al (2014) in which a significant decline was observed in the linear growth of *B. fabae* (Nubaria isolate) *i.e*. 66.58 and 71.50 mm, in comparison with Control treatment with the culture filtrates of *T. album* and *T. harzianum* at the different concentrations respectively (Barakat et al. 2014). Also, in another study, Kumar *et al* 2019 reported that *Trichoderma harzianum spp* was found be most effective in reducing the mycelial growth of *Fusarium* isolates by 76.9%, and least with *T. viride* and *T. koningi* i.e. 70.1% 58.1%. From several such studies, it is quite evident that the *Trichoderma harzianum*, one of the three species of *Trichoderma*, has significant potential for controlling disease-causing soil-borne pathogens (Kumar et al. 2019).

Additionally, Khan et al in 2014 also reported that in presence of non-volatile secondary metabolite, the carbendazim was found not efficient in reducing the severity of wilt and root-rot disease while applied to soil with *T. hamatum, T. harzianum* and *T. viride* (Khan et al. 2014). These *Trichoderma spp.* Are reported as vigorous soil colonisers (Akrami et al. 2009) and also produces cell wall-degrading enzymes, antibiotics including trichodermin, gliotoxins and viridin as well as biologically active heat constant metabolites like ethyl acetate (Mohiddin, Khan, and Khan 2010). Such enzymes, antibiotics and metabolites might contribute in stopping the soil-borne pathogens from causing plant diseases (Chet and Baker 1981; Khan, Anwer, and Shahid 2011; Khan, Khan, and Mohiddin 2004).

The study can be concluded that the existence of potential Microbial Biocontrol Agent (MBCA) in native soil and can be used to manage the chickpea wilt disease effectively. It is suggested that the native strain of *T. harzianum* could be a potential approach for biological control of plant diseases. This study suggests that the native isolate of

T. harzianum can be exploited for the production and development of native *T. harzianum* based biocontrol formulation development to control Fusarium wilt diseases of chickpea plants, and serve as a tool for Eco friendly microbial biocontrol agent. Current study partially demonstrated the protective effects of native *T. harzianum* antagonistic strains against wilt pathogen. Also, the native *Trichoderma spp.* indirectly *f*ound to produce, the volatile and non-volatile secondary metabolite that significantly slowed the wilt pathogen's in vitro mycelium growth. A more research is required to be done in this direction and new methods as well for preventing from chickpea wilt disease in-vivo.

CONFLICT OF INTEREST

The authors asserted no conflict of interest. This research received no external funding.

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