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Fungal isolates of genus *Trichoderma* induce wilt resistance to pea caused by *Fusarium oxysporum* f. sp. *pisi* through competitive inhibition

Los aislados de hongos del género *Trichoderma* inducen la resistencia al marchitamiento del guisante causada por *Fusarium oxysporum* f. sp. *pisi* a través de la inhibición competitiva

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Abstract

Four different *Trichoderma* species were isolated from pea field soil rhizosphere of selected areas of Punjab, Pakistan. Based on morphological, cultural and moelcular characteristics, species were recognized as *T. harzianum*, *T. viridae*, *T. hamatum* and *T. koningii*. The isolated species were screened for the production of extracellular hydrolytic enzymes. The strains were found positive for chitinase and glucanase activities. *Fusarium oxysporum* f. sp. *pisi* (FOP) was isolated from roots of wilted pea plants. *in vitro* biocontrol potential of isolated *Trichoderma* species was assayed by dual culture technique against FOP. Maximum growth inhibition was observed by *T. viridae* (76.42%) followed by *T. harzianum* (74.29%), *T. koningii* (71.43%) and *T. hamatum* (69.64%), after 7 days of incubation. All four isolated species of *Trichoderma* were used in confrontational assay against FOP. A competition of *Trichoderma* sp. v/s FOP was evidente. All *Trichoderma* strains showed strong antagonistic activity, clearly observed on dual culture agar plates and further confirmed under scanning electron microscopy (SEM). Pot experiments also confirmed a very strong competitive inhibition of FOP evidently due to presence of glucanase and chitinases in *Trichoderma* spp. The primary screening and basic findings of present work will be helpful to obtain an efficient and novel biocontrol agent for further experimental trials on pea plants and may also enhance the chance of using *Trichoderma* species in integrated disease management (IDM) programs as an effective biological agent against several phyto-pathogens.

Keywords: *Trichoderma* Antagonistic effect, FOP, biocontrol potential, SEM.

Resumen

Se aislaron cuatro especies diferentes de *Trichoderma* de la rizosfera del suelo de un campo de guisantes de áreas seleccionadas de Punjab, Pakistán. Con base en las características morfológicas y culturales, se reconocieron especies como *T. harzianum*, *T. viridae*, *T. hamatum* y *T. koningii*. Las especies aisladas se cribaron para la producción de enzimas hidrolíticas extracelulares. Las cepas resultaron positivas para las actividades de quitinasa y glucanasa. *Fusarium oxysporum* f. sp. *pisi* se aisló de raíces de plantas de guisantes marchitas. El potencial de biocontrol in vitro de la especie aislada de *Trichoderma* se evaluó mediante una técnica de cultivo dual contra *Fusarium oxysporum* f. sp *pisi*. La inhibición máxima del crecimiento fue observada por *T. viridae* (76,42%) seguido de *T. harzianum* (74.29%), *T. koningii* (71.43%) y *T. hamatum* (69.64%), luego de 7 días de incubación. Las cuatro especies aisladas de *Trichoderma* se utilizaron en un ensayo de confrontación contra *Fusarium oxysporum pisi* (FOP). Una competición de *Trichoderma* sp. v / s FOP fue evidente, todas las cepas de *Trichoderma* mostraron una fuerte actividad antagonista que se observó claramente en placas de agar de cultivo dual y se confirmó adicionalmente con microscopía electrónica de barrido (SEM). Los experimentos en macetas también confirmaron una inhibición competitiva muy fuerte de FOP, evidentemente debido a la presencia de glucanasa y quitinasas en *Trichoderma* spp. El cribado primario y los hallazgos básicos del presente trabajo serán útiles para obtener un agente de control biológico eficaz y novedoso para ensayos experimentales adicionales en plantas de guisantes y también pueden mejorar la posibilidad de usar especies de *Trichoderma* en programas de manejo integrado de enfermedades (IDM) como un agente biológico eficaz contra varios fitopatógenos.

Palabras clave: *Trichoderma* Efecto antagonista, FOP, potencial de biocontrol, SEM.

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1 Introduction

Plant diseases, caused by several phytopathogens, are reason of huge productivity loss in economically important crops (Oerke 2006). The chemicals like fungicides and pesticides, used to treat phyto-pathogens, can accumulate dangerous and poisonous compounds which can be lethal to human beings and their atmosphere. These toxic compounds can be bio accumulated in microbes/ pathogen so they developed resistance against them. Hence some alternative methods may be employed to solve the problem. (Alamri *et al*., 2016). Bio-control based strategies has proved to be the method of choice to tackle the situation by using other microbes that interact with pathogens, thereby, can overcome the problem imposed by these chemicals and protect the plant from disease (Anand & Reddy, 2009). Some of these bio-control micro-organisms occurred naturally in ecosystem. One class of such useful microbes belonged to genus *Trichoderma*, usually a normal microbiota of soil and root ecosystem (Herath *et al*., 2015).

Trichoderma species are abundantly present in soil ecosystems and have gained immense importance during last few decades due to its remarkable ability to control many phyto-pathogens (Gerhardson, 2002). *Trichoderma* spp. represent a class of distinctive, facultative, anaerobic, and diverse fungi abundant in agricultural lands and in some other environment like decaying wood (Druzhinina and Kubicek, 2005). As a member of subdivision Deuteromycetes, which neither had or nor displayed a specific sexual state, they mostly adjusted to an asexual mode of life (Harman *et al*., 2004). *Trichoderma* spp. has been reported as effective against aerial, root and soil pathogens (Elad, 2000), discovered as an important bio-control agent that worked as antagonist to pathogenic fungi and minimized the instance of disease (Harman *et al*., 2004; Monte, 2001). *Trichoderma* spp. established as more efficient as compared to conventional fungicides and less prone towards development of resistance (Kullnig *et al*., 2001). They used a number of antagonistic mechanisms for that purpose like antibiosis and myco-parasitism. In myco-parasitism the bio-control agents attacked the pathogen and secrete many cell-wall hydrolyzing enzymes like glucanases, chitinases, cellulases and proteases (Almeida *et al*., 2007). These enzymes dissolved the components of pathogen's cell wall like cellulose, glucan, proteins, and chitin effectively; consequently, limit the development of pathogens thereby impart a great role in bio-control (Langner and Göhre, 2016).

Chitinases and Glucanases from *Trichoderma* spp. were found more effective as compared to synthetic fungicides and proved safe to plants even at high concentrations. There is no risk of resistance; therefore they became excellent source for strengthening plant defense hypersensitive reactions (Umapriyatharshini *et al*., 2009). The activity and expression of these hydrolytic enzymes vary in different strains of *Trichoderma*, leading to differences in the performance, when used as biocontrol agents (Alamri *et al*., 2016).

Fusarium spp. was considered as foremost prevailing fungi isolated from soil that caused severe damage and losses to wide variety of crops (Kamala and Indira, 2011). *Fusarium oxysporum* caused significant changes in physiology and morphology of plant. Some researchers reported behavior of vegetable crops by using *Trichoderma atroviride* along-with *Glomus intraradices* promoted growth, yield and nutrient uptake (Colla *et al*., 2015). Morphological characteristics were also influenced by the action of FOP. *Fusarium oxysporum pisi* revealed as one of the main devastating pathogens of pea (*Pisum sativum*) and a causative agent of wilt. Pea wilt prevailed almost in every pea cultivating areas of the world. The disease reported essentially as soil borne and posed great problem in management by employing traditional methodologies (Haglund & Kraft, 2001).

This work was aimed for the isolation and identification of various *Trichoderma* spp. from soil of pea fields and *Fusarium oxysporum pisi* from roots of wilted pea plants of selected areas of Punjab, Pakistan. Assessment was carried out for *in vitro* bio-control potential of antagonistic fungi *Trichoderma* spp. against pathogenic fungus *Fusarium oxysporum pisi*, a causative agent of pea wilt.

2 Materials and methods

The experiments were performed several times and only representative results are shown in this manuscript. The time period encompassed November 2016 to January 2020 for various experiments, data replication and data collection.

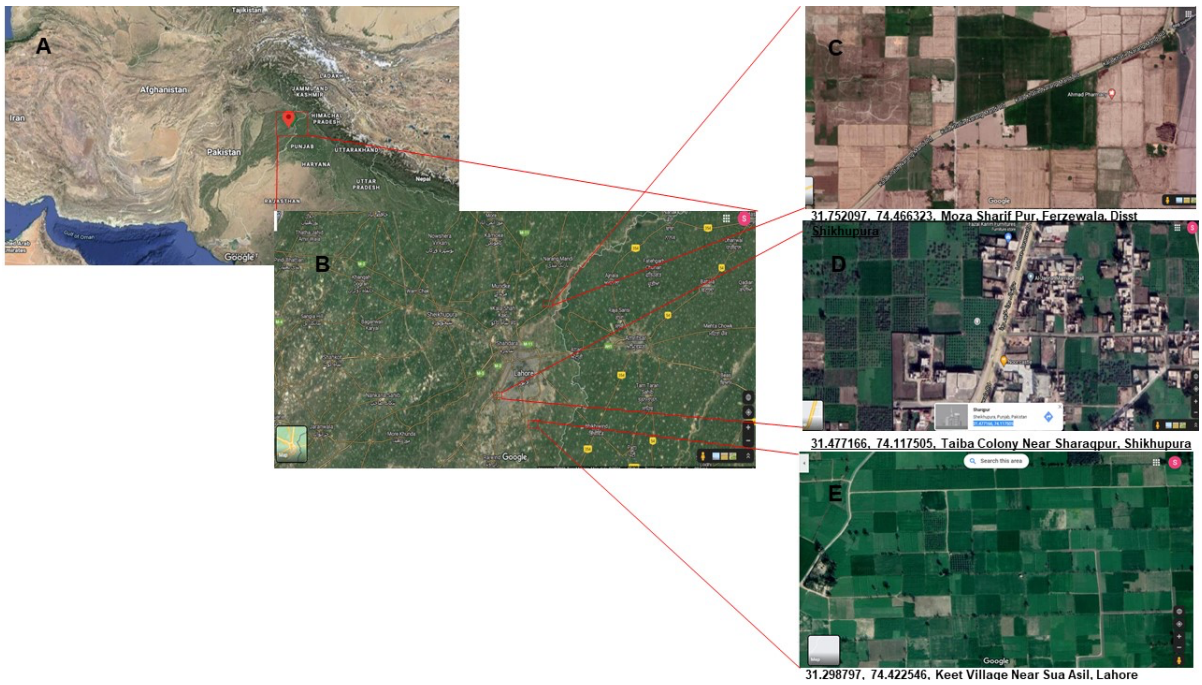


Figure 1. Site identification of soil (for *Trichoderma* spp. isolation) and wilted pea (*Fusarium* sp.) sampling. A. Map of Pakistan and neighboring countries. B. Map of areas selected for sampling. C. site of sampling at Moza Sharif Pur, Ferozewala, Distt. Sheikhupura, google coordinates (31.752097, 74.466323) D. Site of sampling at Taiba Colony Near Sharaqpur, Sheikhupura, peas were cultivated with guava orchards, google coordinates 31.477166, 74.117505. E. site of sampling at Keet Village, Near Sua Asil, Distt. Lahore, peas were cultivated with guava orchards, google coordinates 31.298797, 74.422546, (https://www.google.com/maps)

2.1 *Soil samples collection*

Soil samples were collected at depth of 2-3 cm from pea-field rhizosphere of different areas of Punjab, Pakistan (Figure 1). They were carried to Molecular Biotechnology Lab, PCSIR, Lahore, Pakistan for isolation and identification of *Trichoderma* spp. and for further study trials.

2.2 *Diseased plants samples collection*

Diseased plants showing clear symptoms of wilt along with soil were carefully collected in polyethylene bags and transported to lab for isolation of pathogen i.e., *Fusarium oxysporum* f. sp. *pisi* (FOP).

2.3 *Media preparation*

Potato Dextrose Agar-PDA (Merck, Germany) was used for the isolation of fungi. Media was prepared according to manufacturer's instructions (following standard protocol). About 39.0 gm of powdered PDA medium was dissolved in 1 liter of sterile distilled water and sterilized by autoclaving at 121 ºC for 15 minutes. The media was allowed to cool at 60 ± 2 ºC before pouring into sterile petri plates.

2.4 *Isolation of Trichoderma spp*

A serial dilution technique was employed for isolation of *Trichoderma* spp. from soil, for this purpose, 1×10^4 dilution of the soil samples was prepared (Johnson and Curl, 1972). A stock suspension was prepared by adding 1 gram of soil samples to 9 ml of sterile distilled water using sterilized glass tube. One ml of dilution level 10^4 was dispersed in petri plate with 15-20 ml of sterilized molten Potato Dextrose Agar (PDA). The agar and inoculum were swirled softly and allowed to set. The plates were incubated at 25-28 ºC for 7 days. Individual colonies were isolated and transferred to freshly prepared PDA plates. Pure cultures were maintained on PDA slants and kept at 4 ºC for further use.

2.5 Identification of Trichoderma isolates

Macro and microscopic characteristics along with visual observations were used for the identification of fungi. The macroscopic features used for species identification were diameter of colony, color of conidia and mycelia, colony texture and shape etc. The confirmation of species level identification was done with support of plant pathology, Institute of Agricultural Sciences, University of the Punjab Lahore, Pakistan. Identified species were confirmed by expert mycologist and plant pathologists.

Lacto-phenol cotton blue staining procedure was used for microscopic examination of fungi. Slides were prepared and visualized from 2 weeks old cultures using Meiji MT5300H biological microscope (Meiji Techno Co. Ltd. Japan) studded with Meiji Infinity 1 camera using Infinity Analyze 6.5 software. Microscopic Characters for identification were mainly conidial head, shape and roughness (Rai *et al*, 2016). The molecular characterization of each *Trichoderma* spp. was also performed by ITS sequencing and published on NCBI, GenBank accession numbers are: MZ562720 (*Trichoderma koningii*), MZ562719 (*Trichoderma hamatum*), MZ562474 (*Trichoderma viridae*), MZ562345 (*Trichoderma harzianum*).

2.6 Isolation and identification of Fusarium oxysporum f.sp. pisi

The infected stems and roots were washed with sterilized distilled water and cut into small pieces. The surface of cut portions was topically sterilized by dipping them in 5% solution of Sodium Hypochlorite (NaOCl) for 2 minutes. These treated plant bits were further rinsed thrice with sterilized distilled water. The cut parts were placed on sterile blotting paper to remove excess of water. The sterilized plant tissues were placed on PDA plates and incubated for 7 days at 25-28 ºC. The pathogen was identified based on mycelia and sclerotia characters and maintained on PDA at 4 ºC for further use (Haglund & Kraft 2001). The molecular characterization performed by ITS sequencing for pea root isolate of *Fusarium oxysporum* revealed that isoalte was *Fusarium oxysporum* f. sp. *pisi*. The sequence was deposited to NCBI with GenBank accession number MZ562344. ITS sequencing for *Fusarium* sp. was also carried out by Ramos-Ibarra and co-workers (Ramos-Ibarra *et al*., 2017).

2.7 Screening of Trichoderma species for chitinase activity

Cell wall of phytopathogen consisted mainly of chitin with other macro molecules. *Trichoderma* degrade the pathogen cell wall by secretion of mycolytic enzymes, mainly chitinases and glucanases. In order to metaboliz the cell wall componants, especially chitin, antagonistic fungi synthesized lytic enzymes specially chitinases and glucanses (Osorio-Hernández *et al*., 2016), Therefore, the estimation / quantification of this enzyme is necessary to relate its extent of mycoparasitism displayed against pathogen. All four isolates of *Trichoderma* were screened for chitinolytic activity. Bromocresol purple (0.15 g/L) and colloidal chitin were added to chitinases detection medium. Chitinase detection medium (1 liter) was prepared by adding 4.5 gm of colloidal chitin, 0.3 gm of $MgSO_4$. $7H_2O$, 3.0 gm of $(NH_4)_2SO_4$, 2.0 gm of KH_2PO_4, 1 gm of citric acid monohydrate, 15 gm of Agar, 0.15gm of bromocresol purple (BCP) and 200 μl of tween-80. The prepared media had a sunny yellow color at pH 5.5 and autoclaved at 121 ºC for 15 minutes. The medium was cooled and poured in sterilized petri dishes and allowed to solidify. Seven days old culture plugs of *Trichoderma* were inoculated and incubated at 25-28 ºC for 48 hours (Ramaraju *et al*., 2017).

Trichoderma spp. had remarkable ability of chitin degradation to N-acetyl glucosamine due to presence of chitinases. This degradation resulted a color change of BCP from yellow to purple zone in surrounding areas of freshly inoculated plates. Activity of *Trichoderma* chitinases was estimated qualitatively by measuring diameter of purple colored zone after 3 days of incubation. Based on diameter of purple zone and color intensity chitinase activity of each *Trichoderma* spp. was determined (Almeida *et al*., 2007; Agrawal and Kotasthane, 2012). Chitinase production was also optimized in liquid medium using Erlenmeyer flasks. Various parameters including, time, pH, temperature, nitorgen source, carbon source, effect of chelating agents etc were optimized for chitinase production form four *Trichoderma* spp. followed by partial purification of enzymes (Nawaz *et al*., our unpublished data). A representative graph showing effect of time on chitinase activity is presented here only. The experiment was performed in quarduplicate and values of enzyme activity ± SD are presnted here. The chitinase activity was perfomed as described by Mayorga-Reyes and co-workers (Mayorga-Reyes *et al*., 2012)

2.8 Screening of Trichoderma species for glucanase activity

To determine glucanase activity, the *Trichoderma* strains were grown on carboxymethyl cellulose (CMC) medium (1% w/v) as sole carbon source, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.05% peptone and 2% agar. The plates were inoculated with a fungal mycelium disc of 1 cm diameter and incubated at 28°C for 48 h in darkness. After 48 hours petri plates were flooded with 0.1% Congo Red Dye (Sigma-Aldrich, Germany) and left for 30 minutes. After that excessive stain was drained out, plates were washed with 5 M NaCl for 10 minutes. Congo red is an organic, water-soluble, azo dye, yielding a red colloidal solution (Colonia and Chagas Junior 2014). Glucanase production was indicated by the appearance of the halo (hydrolysis zone) surrounding the colonies. Some researchcers has also reported production of endoglucanse and xylase from Fusarium spp. too (Ramos-Ibarra *et al*., 2017). Glucanse production was also optimized in liquid medium and various parameters including, incubation time, pH, temperature, Nitrogen source, Carbon source effect of chelating agents etc were optimized for glucanse production form four *Trichoderma* sp. (Nawaz *et al*., our unpublished data). A representative graph showing effect of time on glucanse activity is presented here only. The experiment was performed in quarduplicate and values of enzyme activity ± SD are presnted here. The glucanase activity was perfomed as described by Marco and Felix (Marco and Felix, 2007).

2.9 Antagonistic effect of Trichoderma species by dual culture technique

Dual culture technique was performed for the estimation of antagonistic effect of *Trichodema* spp. against plant pathogen *F. oxysporum pisi* (FOP) following the method reported by different researchers (Tariq *et al*., 2010; Kamala and Indira 2011). For this purpose fresh cultures of pathogen and biocontrol agents were used. A mycelial disc (5mm) of test pathogen was placed at one end of petri plate having PDA medium. The antagonist disc was placed just opposite to the pathogen. The pathogen plate without antagonist serves as a control. All tests were performed in quarduplicate and were maintained along with control. The plates were incubated at 28 ± 2 oC for 3-7 days. Plates were observed after 24 hrs for the antagonistic potential of biocontrol agents. Antagonistic effect of test fungi was estimated by measuring their radial growth in comparison to the control plates by following the formula described by Fokkema and Meulen (Fokkema and Meulen, 1976).

$$\text{Antagonistic effect} : \frac{A-B}{A} \times 100 \qquad (1)$$

Where A is diameter of mycelial growth of pathogen in control and B is the diameter of mycelial growth of pathogenic fungus with antagonistic fungus.

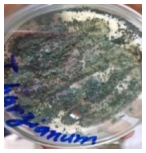
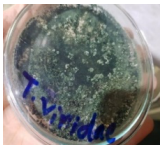
2.10 Antagonistic effect by scanning electron microscopy (SEM)

Antagonism of *Trichoderma* spp. against FOP was studied by Scanning Electron Microscope Model S-3700N (Hitachi High-Technologies Corporation, Tokyo, Japan) following the procedure of Inch and Gilbert (Inch and Gilbert, 2011). To obtain the interaction sites with pathogen, *Trichoderma* and FOP were grown on PDA at 28 ºC for 5 days. The biocontrol and the pathogen grew towards each other and their hyphae interact with each other. After incubation the interaction sites were first observed under light microscope before subjecting to SEM analysis. The interaction sites were marked on 1 cm^2 agar blocks, carefully cut, placed on stub and the interaction of pathogen with antagonist were visualized under SEM. Each duo was observed at 300x, 1500x, 2000x, 3000x and / or 5000x and sites of interaction were observed at the resolution of 100 μm, 30 μm, 20 μm and 10 μm respectively. The conidia of *Trichoderma* spp. were marked alongwith ruptured cell wall of pathogens.

2.11 Pot experiments

Selected pot experiments were carried out as described by Kaur and coworkers (Kaur *et al*., 2019) to study biocontrol and recovery from FOP induced wilt through *T. viridae* and crude glucanase and chitinase on 10 different pea varieties (data regarding all studied varieties not shown in this manuscript and are part of our unpublished data), one famous variety, Azad P4 (kindly gifted by NIAB seed bank, NIAB, Faisalabad, Pakistan) was grown in 16.4 cm pots using10 replicates. Soil was first mixed with natural leaves and organic manure compost and sterilized. The pots containing sterile soil were first treated with FOP followed by respective biocontrol agents, *T. viridae* or enzymes (after 15 days), except blank.

Table 1. Comparison of microscopic characteristics of *Trichoderma* species grown on PDA.

Parameters	**Characteristic features of *Trichoderma harzianum***	**Characteristic features of *Trichoderma viridae***	**Characteristic features of *Trichoderma hamatum***	**Characteristic features *of Trichoderma koningii***
Colony color	concentric rings with whitish green-bright green color	*T. viridae* appears to be a bit granular on PDA, watery white bluish green	T. hamatum seems partly dark green	Colony color mostly appears green color
Colony mycelia	Compact	Floccose, white	Floccose, compact mycelia	Floccose or with compact mycelia
Conidia shape	Smooth, subglobose	Oval to elongate	Conidia smooth walled, phialids	Conidia ellipsoidal oblong, often appearing angular
Conidial color	Yellow-pale green	Green conidia distributed throughout	Yellowish, bright, dull to dark green,	Yellowish, bright, dull to dark green
GenBank Accession number (ITS sequences)	MZ562345	MZ562474	MZ562719	MZ562720
Culture appearance on plate				

The seeds were sown after another 15 days in early November, kept in open atmosphere and harvested after 18 weeks of sowing and basic parameters were recorded. Inoculum of both strains were maintained to 1×10^9 spores/ml using hemocytometer and used as mentioned elsewhere following standard conditions.

3 Results and discussion

3.1 Isolation of Trichoderma spp

Four species of *Trichoderma* were isolated from soil samples. Initial identification of *Trichoderma* isolates was carried out based on the appearance and pigmentation of the respective colonies and growth pattern of mycelium on PDA plates. White colonies later changed in green color mycelium with characteristic clear concentric rings. The microscopic characteristics such as branching patterns of conidiophores, the arrangement of phialides, and the shapes and sizes of conidia, as described previously by Kamala and Indira 2011, were used for species-level identification of isolates observed on PDA plates (Table 1; Figure 2). Main branches of conidiophores produce many lateral branches. Sometimes phialides arise directly from axis tip. They were enlarged in middle, bottle shaped long or cylindrical. All types of branches arose 90º respect to

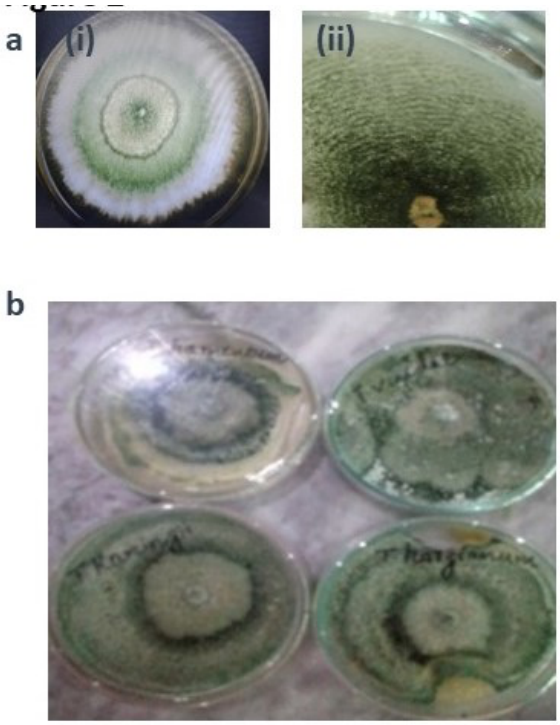


Figure 2: Trichoderma isolates purified from soil. a. Representative *Trichoderma harzianum* culture showing characteristic features of concentric rings (i), finger print like impression (ii). b. Purified *Trichoderma* sp. showing concentric ringed colony morphology at earlier stage, top right: *T. viridae*; top left: *T. hamatum*; bottom right: *T. harzianum*; bottom left: *T. koningii* were identified.

main axis. Four *Trichoderma* spp. identified were *T. harzianum*, *T. viridae*, *T. hamatum* and *T. koningii*, further confirmed by ITS sequencing, GenBank accession numbers and individual characteristics of each species observed are summarized in Table 1.

3.2 Isolation of pathogen

Soil borne pathogens induce problems to crops and thereby cause major financial losses (Oerke 2006). In this study soil borne pathogens infecting pea plants were collected from selected areas of Punjab Pakistan (Figure 3). Among various isolated fungi from roots of infected pea plants, *Fusarium oxysporum pisi* (causative agent of pea's wilt) was identified based on light microscopy and SEM viualization (Figure 4) alongwith morphological characteristics and ITS sequencing. Purified cultures were maintained on PDA slants for further use.

Figure 3. a. Representative pea field picture clearly depicting signs of wilt. b-f. Infected pea plant samples collected from different areas during two pea growing seasons.

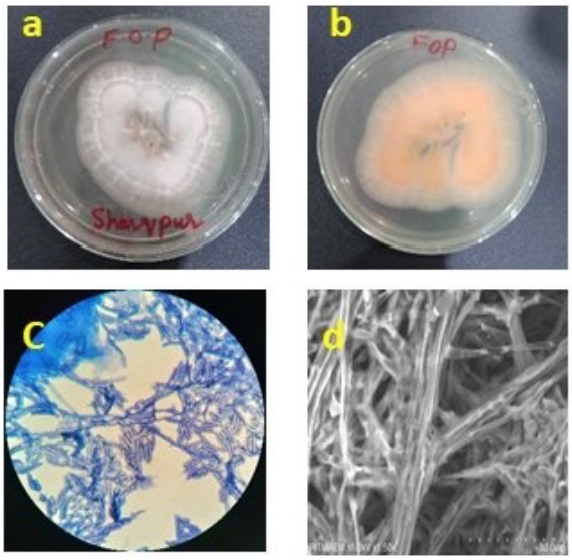


Figure 4. Purified cultures of *F. oxysporum pisi* (FOP), *Fusarium oxysporum* identified as whitish, cottony, fluffy mycelium on PDA plate (a), while reverse side of plate was observed as slight pinkish with irregular margins (b). Under light microscope elongated conidia were observed (c). SEM micrograph of FOP observed at 1500X at a resolution of 30 μm, branching pattern is clearly visible (d).

3.3 Screening of chitinases production

All isolates of *Trichoderma* were screened for chitinase production. Remarkable chitinolytic potential was observed in all four isolated *Trichoderma* variants, colloidal chitin was used as substrate. When *Trichoderma* spp. inoculated in media containing colloidal chitin and BCP,

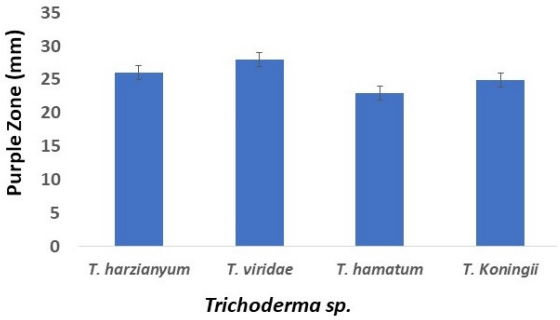


Figure 5: Comaprison of chitinoloytic activities of *Trichoderma* strains depicted by purple color zones (mm) data presented as mean±SD (n=3).

chitinase in it readily converted the chitin into N-acetylglucosamine and resulted in pH change from acidic to more alkaline and appeared as change in color of indicator dye (BCP) from yellow to purple (Agrawal and Kotasthane, 2012). All isolated species of *Trichoderma* efficiently converted yellow color media to purple with slight variations. Color zone formation was measured (in mm) after 48 hours of incubation, maximum zone for chitinase production was formed by *T. viridae* (28mm) followed by *T. harzianum* (26mm), *T. koningii* (25mm) and *T. hamatum* (23mm), respectively (Figures 5 & 6a (i-iv)). Benitez and coworkers concluded that *Trichoderma* isolates that over produce chitinases were found to be more effective biocontrol agent against several pathogens (Benitez *et al*., 2004). Chitinase production was also optimized in liquid medium from four *Trichoderma* spp. A representative graph showing effect of time on chitinase activity is presented here. The experiment was performed in quarduplicate and values of enzyme activity ± SD are presnted here. All four iolates exhibited time dependent chitinase production with maximum chaitinase production after 72 hours of incubation (Figure 6b).

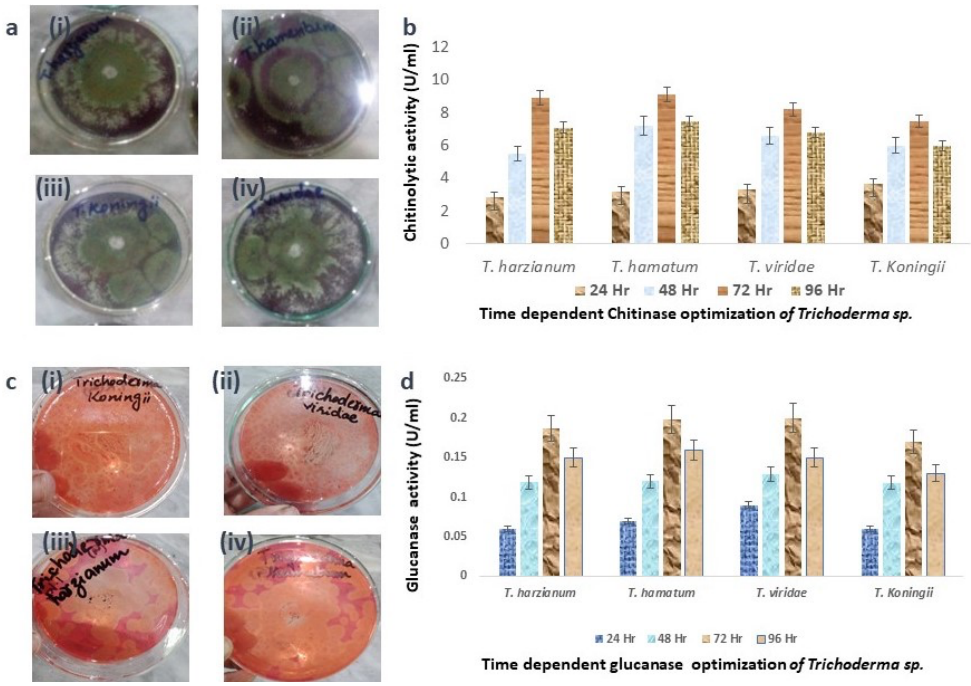


Figure 6. Screening of *Trichoderma* isolates for Chitinases and Glucanses. Characteristic chitinase activity of, a (i): *T. Koningii*; a (ii): *T. viridae*; a (iii): *T. hamatum*; a (iv): *T. harzianum*. All four isolates showed characteristic chitinase activity, a change of color for yellow to puprple is visible. b. Effect of time on chitinase production in liquid medium, values of enzyme activity ± SD are presented here. Screening of *Trichoderma* isolates for presence of glucanases corresponding to, c (i): *T. viridae*; c (ii): *T. Koningii*; c (iii): *T. hamatum*; c (iv): *T. harzianum*. All four isolates showed characteristic glucanase activity, a change of clear zone v/s Red colonies is visible. d. Effect of time on glucanase production in liquid medium, values of enzyme activity ± SD are presented here.

Table 2. Percentage growth inhibition of *F. oxysporum* against *Trichoderma* spp.

Trichoderma species	Day 3	Day 4	Day 5	Day 6	Day 7
T. viridae	30 .0 %	55.55%	64.08%	69.00%	76.42%
T. harzianum	26.66%	53.33%	61.27%	68.00%	74.29%
T. hamatum	20.00%	50.00%	59.99%	67.00%	71.43%
T. koningii	16.66%	47.77%	59.15%	66.50%	69.64%

3.4 Screening of glucanase production

The isolates of *Trichoderma* spp. produced significant amounts of glucanases, clearly observed by presence of opaque areas in plates having Congo Red dye, while areas with no gowth of fungus remained red. *T. harzianum* and *T. hamatum* showed comparatively bigger transparent patches while *T. viridae* and *T. koningii* indicated scattered and diffused but regular small areas (Figure 6 c i-iv). Several researchers used Congo red dye as chromogenic indicator for detection of extracellular enzymes such as glucanases (Jo *et al*., 2011). Meddeb-Mouelhi and colleaguees reported this method as a simple, fast and cost efficient technique for *in vitro* selection of polysaccharides degrading micro-organism (Meddeb-Mouelhi *et al*., 2014). Glucanase production was also optimized in liquid medium from four *Trichoderma* spp. Representative data showing effect of time on glucanse activity (± SD) from *Trichoderma* isolates is presented here. All four isolates exhibited time dependent chitinase production while maximum activity was observed after 72 hours of incubation (Figure 6d).

3.5 Trichoderma spp. v/s FOP antagonism

Antagonistic effect of *Trichoderma* isolates on *F. oxysporum* f. *pisi* clearly indicated growth reduction of the pathogen. In dual culture experiment, *T. viridae* exhibited highest inhibition of 76.42 % followed by *T. harzianum* (74.29 %), *T. koningii* (71.43%) and *T. hamatum* (69.64%) at 7th day of dual culture incubation (Figure 7; Table 2). Our study indicated that *T. viridae* and *T. harzianum* depicted better growth retardation efficiency as comapred to *T. koningii* and *T. hamatum*. Some researchers concluded that *T. viridae* and *T. harzianum* significantly suppressed the growth of *F. oxysporum* (Alwathnani *et al*, 2012). Chitinase and Glucanase of *Trichoderma* species degrade chitin and glucans responsible for rigidity of pathogenic fungi cell wall, hence destroy cell wall integrity and limit the growth of fungus (Kumar *et al*., 2012). *Trichoderma* spp. are reported

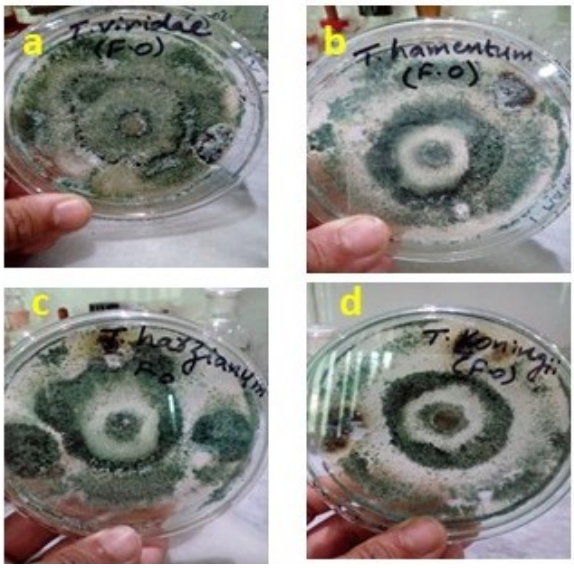


Figure 7. Antagonistic effect of *Trichoderma* isolates over FOP observed by dual culture technique. All four isolates of *Trichoderma* i.e. *T. viridae* (a), *T. hamatum* (b), *T. harzianum* (c) and *T. Koningii* (d) appeared as antagonists to FOP.

to utilize various mechanisms like hyperparasitism, antibiosis and inhibition in its bio control activity. Our results were in accordance with works reported by Almeida and coworkers and that of Agrawal & Kotasthane who reported that all the screened species of *Trichoderma* were effective against *F. oxysporum* with slight variations (Almeida *et al*., 2007; Agrawal and Kotasthane, 2012). *T. harzianum* and *T. viridae* were reported by several researchers as best antagonists for growth inhibition of many phytopathogens by 60% to 80%. (Rahman *et al*., 2009; Kumar *et al*., 2012). Our resualts are also in line with finding of Soliman and co-workers who reported, consortium of *T. harzianum* and *T. viride*, in combination with yeast showed strong antagonism against *Fusarium oxysporum* in pea (Soliman *et al*., 2017).

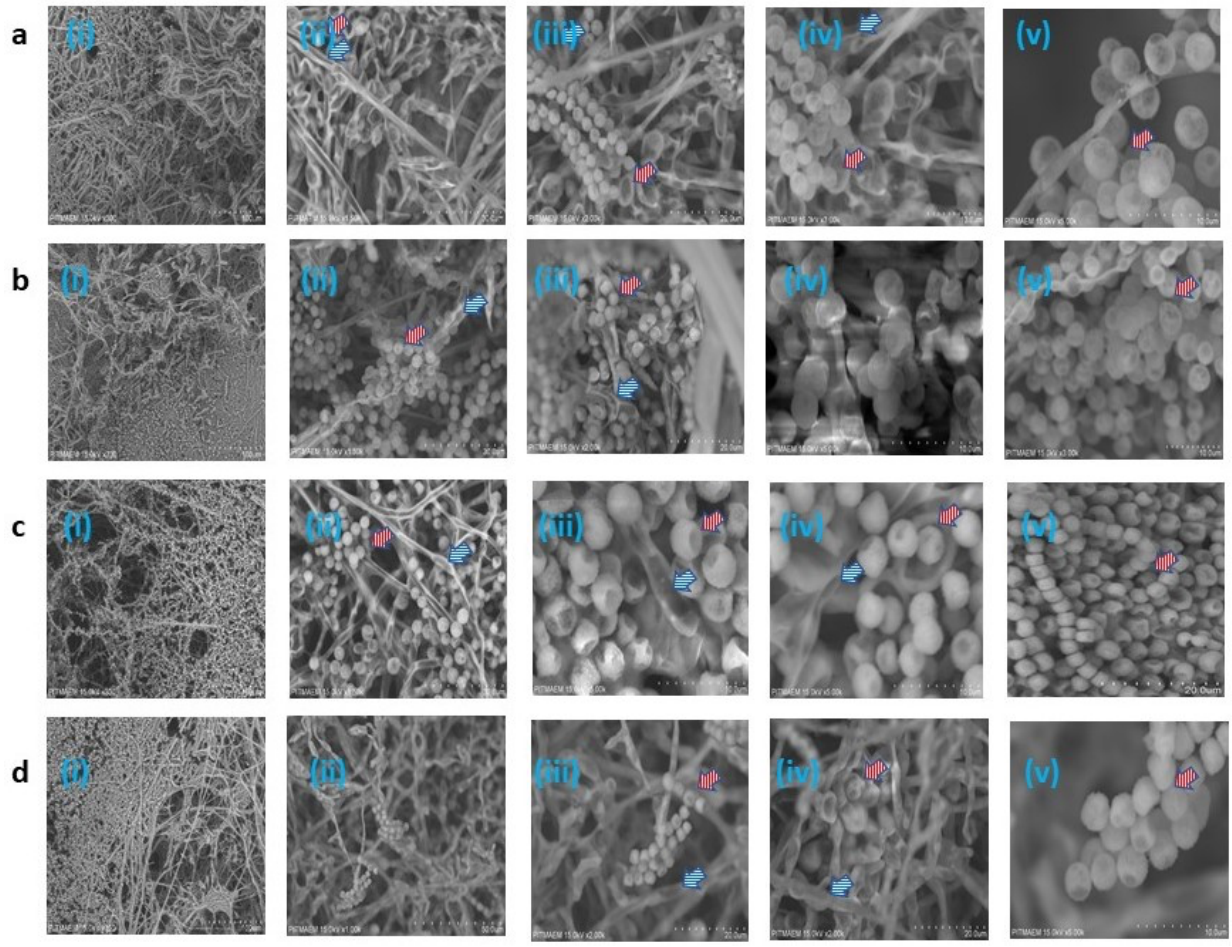


Figure 8. Scanning electron micrographs indicating the antagonistic activity of *Trichoderma* spp. against *Fusarium oxysporum pisi*. The interaction sites were marked on 1 cm^2 agar blocks placed on stub and the interaction of pathogen and antagonists were visualized under SEM. Arrows with vertical red lines ( ) depict conidia of *Trichoderma sp and* arrows with horizontal blue lines ( ) depict degraded cell wall of FOP. Each duo was observed (left to right) at 300x, 1500x, 2000x and 3000x or 5000 x and sites of interaction were observed at the resolution of 100 µm, 30 µm, 20 µm and 10 µm respectively. **a. (i-iv)** *T. Harzianum* v/s FOP, **a. (v)** control image of *T. harzianum* at 5000x and resolution of 10 µm; **b**. **(i-iv)** *T. Hamatum* v/s FOP, **b. (v)** control image of *T. Hamatum* at 3000x and resolution of 10 µm; **c**. **(i-iv)** *T. viridae* v/s FOP, **c. (v)** control image of *T. viridae* at 2000x and resolution of 20 µm; **d. (i-iv)** *T. koningii* v/s FOP, **d. (v)** control image of *T. koningii* at 5000x and resolution of 10 µm.

Figure 9. Representative pot experiments on pea variety AZAD P4 depicting biocontrol and recovery from FOP induced wilt through *T. viridae* along-with glucanase and chitinase. a. Pea seeds grown normally in pre-sterilized soil without inoculation of either of fungal strains. b. Seeds sown with 5ml of 1×10^9 spores of FOP inoculated soil. c. Seeds sown with 5ml of 1×10^9 spores of FOP inoculated soil followed by inoculation of 5ml of 1×10^8 spores of Trichoderma viridae. d. Seeds sown with 5ml of 1×10^9 spores of FOP followed by addition of 5 ml crude glucanase from T. viridae. e. Seeds sown with 5ml of 1×10^9 spores of FOP followed by addition of 5 ml crude T. viridae chitinase. f. Seeds sown with 5ml of 1×10^9 spores of FOP followed by addition of 5 ml each of crude chitinase and glucanase isolated from *T. viridae*.

3.6 Mycoparasitism of Trichoderma spp. against FOP through SEM

The mycoparasitic effect and coiling of antagonist against pathogen was more clearly observed by scanning electron microscopy (SEM). All four *Trichoderma* isolates were grown on PDA plates with FOP. After 7 days of incubation the interaction sites were visualized under SEM which indicated complete colonization of *F. oxysporum pisi* with *Trichoderma* spp. isolates. SEM micrographs revealed the *Trichoderma* spp. coiling structures and condia formation alongwith degaraded cell wall of FOP (Figure 8 a-d). Some other researchers have investigated interaction sites of two microorganims by using SEM analysis (Contreras-López *et al*., 2021). The cell wall degaradtion of FOP by *Trichoderma* spp. reflected that the antagonistic effect imposed by *Trichoderma* spp. was mainly due to secretion of lytic enzymes (Agrawal and Kotasthane, 2012)

and coiling around each other (Almeida *et al* 2007). The production of lytic enzymes lead to degradation of pathogen's cell wall. Alamri and coworkers reported that cell wall of pathogen was degraded by production of lytic enzymes especially due to high concentration of chitinases which were produced by *Trichoderma* spp. (Alamri *et al*., 2016). Some other researchers reported antagonistic activity of *Trichoderma harzianum* from Saudi Arabia and concluded that after 3 days of incubation of pathogen and biocontrol agent the sites visualized under SEM showed partial degradation of pathogen's cell wall and the antagonistic effect was mainly because of lytic enzymes production (Mazrou *et al*., 2020).

3.7 In vivo pot experiments

Limited trials of pot experiments were performed in open atmosphere using 17 pea varities, however, results of one reprentative pea variety namely, Azad P4, clearly depicted recovery from induced wilt. Blank pea seeds neither inoculated with FOP nor *T. viridae* achieved shoot length of 11.5±0.7 inches (n=10). Pea seeds grown in the presence of pathogenic wilt causing fungus FOP, achieved length of 7.3±0.4 inches (n=10). Peas first treated with FOP to induce wilt followed by treatment of known quantity of *T. viridae* achieved length of 13.4±0.8 inches (n=10), clearly showing recovery from pathogenesis as well as approximately 15% growth enhancement as compared to blank experiments (Figure 9 a-c). As a measure to check the effect of *Trichoderma* glucanases and chitinases on recovery of FOP induced wilt, crude enzymes were isolated from *T. viridae* and added in soil after 15 days of post FOP treatment and peas were grown. It was observed that pea plants treated with glucanases, achieved shoot length of 8.2±0.6 inches (n=10). In the case of chitinase treatment the pea achieved shoot length of 8.4±0.4 inches (n=10). While on addition of combination of both glucanases and chitinases to FOP treated peas, the shoot length was 11.0 ± 0.3 inches (n=10) (Figure 9 d-e). Similar trend was observed in case of other *Trichoderma* strains and enzymes extracted and applied on limited pot experiments (our unpublished data not shown in this manuscript). Similar findings had also been reported by other researchers, who reported simultaneous biocontrol coupled with plant growth. They reported that actinobacterium MR14 depicting simultaneous antifungal and plant growth promoting activities (Kaur *et al*., 2019).

Conclusions

In present study, *Trichoderma* species and *Fusarium oxysporum pisi* were isolated from pea fields of Punjab, Pakistan. Isolation of four *Trichoderma* spp. from soil samples indicates diversity of *Trichoderma* spp. in cultivated pea fields. The findings revealed that qualitative and quantitiative methods proved valid and extremely important in selection of biocontrol agent. All the four isolated strains of *Trichoderma* produced significant amount of chitinases and glucanases. Our study further concludes that *Trichoderma* spp. secretes different amount of cell wall lytic enzymes in the presence of pathogenic fungi. The maximum growth inhibition was observed by *T. viridae* (76.42%) followed by *T. harzianum* (74.29%), *T. koningii* (71.43%) and *T. hamatum* (69.64%), after 7 days of incubation. All four isolated species of *Trichoderma* were used in confrontational assay against *Fusarium oxysporum pisi* (FOP). A competition of *Trichoderma* sp. v/s FOP was evident, all *Trichoderma* strains showed strong antagonistic activity that was clearly observed on dual culture agar plates and further confirmed under scanning electron microscopy (SEM). A very strong competitive inhibition of FOP was observed evidently due to presence of glucanase and chitinases in *Trichoderma* spp. Differential variation in producing different enzymes by *Trichoderma* spp. possibly due to the gene expression of certain enzymes at various time. The ability of *T. harzianum* and *T. viridae* to inhibit growth of *F. oxysporum pisi* by *in vitro* experiments was found greater than other species studied here. Both species or their enzymes individually can be considered for field applications in biocontrol of soil borne phytopathogens. SEM results also revealed that various *Trichoderma* spp. can be used as soil conditioning microorganism and as potent biological control agents in integrated agricultural management.

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