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ANTIFUNGAL POTENTIAL OF *TRICHODERMA* STRAINS ORIGINATED FROM NORTH WESTERN REGIONS OF PAKISTAN AGAINST THE PLANT PATHOGENS

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Abstract

Trichoderma spp. is an important genus of fungi because of its potential biocontrol and catalytic ability. Here, we isolated *Trichoderma* spp. from the North Western areas of Pakistan with the prime target to investigate their antagonistic potential. We tested them against different strains of phytopathogenic *Fusarium oxysporum* using different *In vitro* antagonistic assays. In total 22 strains out of 29 were evaluated against different *F. oxysporum* strains (FO. 866, FO. 899, FO. 1025) by dual culture, volatile inhibition, and culture filtrate inhibition tests. Moreover, the experimental set up also allowed us to suggest the probable mechanisms involved in the observed inhibition. Three strains (TMK22, TMK19, and TMK20) could completely inhibit the growth of *F. oxysporum* strains. The volatile metabolites and culture filtrates of the selected antagonistic *Trichoderma* strains were inhibitory. Twenty five percent concentration of the culture filtrates showed the maximum inhibition. More than 60% inhibition was achieved using 5% concentration of culture filtrates of *Trichoderma*. *Trichoderma* strains were identified based on morphology and sequence analysis of the internal transcribed spacer (ITS) region. All the strains belonged to only 3 species (*T. longibrachiatum, T. brevicompectum* and *T. virens)* although they were picked from spatially and climatically diverse areas. The present study, established the antagonistic abilities of the indigenous strains that can further be used to develop an efficient biocontrol system that may uphold in the prevalent environment.

Key words: Phytopathogens, Antagonistic potential, *Fusarium oxysporum,* Biodiversity, *Trichoderma longibrachiatum,* Ecology.

Introduction

Soil microorganisms influence ecosystems by contributing to plant nutrition (George *et al.*, 1995), plant health (Smith & Goodman, 1999), structure of soil (Wright & Upadhyaya, 1998) and soil fertility (Yao *et al.*, 2000). It has been widely recognized, mainly in the last two decades, that greater part of harsh environments are inhabited by astonishingly diverse microbial communities. Bacteria, archeae and fungi are three major groups of soil inhabiting microorganisms.

Trichoderma is an important ascomycete fungal genus present in soil and known for their ability to produce a wide range of antibiotic substances (Sivasithamparam & Ghisalberti, 1998) and for their activity as mycoparasites. *Trichoderma* species rapidly grow (Howell, 2002) and compete with soil microorganisms for nutrients and space (Elad, 1996). Furthermore, they inhibit or degrade pectinases and other enzymes that are essential for plant-pathogenic fungi, such as *Botrytis cinerea* to penetrate leaf surfaces (Zimand *et al.*, 1996). The biocontrol potential of *Trichoderma* spp., is based on a number of mechanisms including antibiosis, mycoparasitism, and the host induced systemic resistance (Anees *et al.*, 2010). Some members are known for plant growth stimulation (Windham *et al.*, 1986), regulation of fungal community structure (Papavizas, 1985), and degradation of xenobiotics (Smith, 1995). Despite all the above, biocontrol has never been a reliable control method against the plant diseases in the fields. This may be because 1) most of the research conducted so far was about relatively fewer strains and 2) the lack of adaptability of the exogenous biocontrol strains in the local environment compared with the already established microflora and pathogens.

The distribution of various species of *Trichoderma* across the world is a matter of interest because of its great economic importance (Druzhinina *et al.*, 2005). In Russia, Nepal and North India, *T. asperellum, T.atroviride, T. ghanense, T. hamatum, T. harzianum, T. virens* and *T. oblongisporum* were found (Kullnig *et al.*, 2000). In Tunisia, *T. longibrachiatum* were predominant in northern forest soils, while *T. harzianum* and *T. saturnisporum* were present in central forest soils (Sadfi-Zouaoui *et al.*, 2009). *Trichoderma atroviride* and *T. hamatum* were found in the cultivated fields of North-East Tunisia (Sadfi-Zouaoui *et al.*, 2009). *T. viridescens* was found in Peru at high elevation, and *T. neokoningii* in a tropical region of Peru (Jaklitsch *et al.*, 2006). Among other species, *T. scalesiae* was isolated as an endophyte from the trunk of daisy tree (*Scalesia pedunculata*) in the Galapagos Islands of Ecuador, *T. paucisporum* as a mycoparasite of *Moniliophthora roreri* on pods of *Theobroma cacao* in Ecuador, and *T. gamsii*, an apparently cosmopolitan species that has been found in France, Italy, Rwanda, South Africa, and Romania as well as Guatemala (Jaklitsch *et al.*, 2006; Anees *et al.*, 2010). Unfortunately, not much work has been done to explore its indigenous diversity in Pakistan on molecular basis especially in the North Western regions. Moreover, the region is hypothesized to be unique in its microbial ecology because of its rich mineral resources and diverse climates. As it is largely unexplored area, recently new

species of bacteria have been isolated from this region (Bangash *et al.*, 2015). We were also expecting some new strains worth checking for their antagonistic potential.

Fusarium oxysporum is a widely distributed soil inhabiting fungus that is a known plant pathogen and produces enzymes and toxins that degrade the plant cell wall components. *F. oxysporum* causes wilt, root rot and crown rot diseases on a wide variety of crops, often limiting crop production (Pereiro *et al.*, 2001). Synthetic fungicide is the only reliable way to control the plant pathogens so far, however, due to the their adverse effects, attention is rapidly being shifted to non-synthetic, safer alternatives of plant disease control (Akhtar *et al.*, 2017).

The present study was based on isolation and identification of *Trichoderma* app. from different regions of the North Western regions of Pakistan on basis of the phenotypic and molecular traits. We evaluated the antagonistic potential of the indigenous *Trichoderma* strains against the phytopathogenic *F. oxysporum* strains. Different antagonistic assays were also performed to assess the probable biocontrol mechanisms used by the *Trichoderma* strains.

Materials and Methods

Soil sampling: Our study involved sampling of small quantities of soils and did not involve endangered or protected species; therefore, no specific ethical considerations were required. Soil samples were collected from different tropical areas of Malakand and Karak divisions of the North western province of Khyber Pakhtunkhwa. Malakand is located at 34.57° North latitude, 71.93°East longitude and about 844 m altitude above the sea level. It is rich with deposits of chromite, iron, china clay and fuller earth. The average temperature is 0 to 10°C in winter with an occasional snowfall and 30 to 35°C in summer. The soil of Malakand is loamy and moist irrigated by the River Swat. Different samples were collected from rice, okra, pepper and tomato fields of different towns of Malakand district including Timergara, Munda, Balambat and Takwar. On the other hand, Karak is a district of the Khyber Pakhtunkhwa, Pakistan situated in the south of the province at 33.12° North latitude, 71.09° East longitude. It is about 582 m altitude above the sea level containing a series of high and low mountainous salt ranges. Soils of Karak are dry and sandy while the climate is hot during the summer with temperature in the range of 40-45°C with frequent sandy storms. The temperature varies from 5 to 15°C in winter. There is a lack of irrigation and rainfed wheat, maize, gram and peanuts are principally cultivated. The vegetables are also cultivated in areas where tube wells are installed.

Before collecting samples, 2-3 cm surface soil was removed and 1 Kg of soil was taken in plastic bags. Soil was cleaned by removing debris and sieved. The moisture contents of the samples were calculated and then air dried for storage in paper bags until further use. Before microbiological analyses, the stored samples were rewetted followed by incubation at room temperature for 3 days to reactivate the microflora.

Isolation of *Trichoderma* species: Malt extract agar medium (MEA) supplemented with Triton X100 (1 ppm) and *Trichoderma* selective medium (TSM; (Elad *et al.*, 1981)) were used for isolation of *Trichoderma* from soil samples. The media was supplemented with chloramphenicol (100 mg liter^{-1}) after autoclaving. Triton X100 was added in order to prevent the expanding of fungal colonies. Chloramphenicol was added to suppress the bacterial growth in samples. The soil serial dilutions were used for isolation of the fungal species (Anees *et al.*, 2010).The suspicious *Trichoderma* colonies were then purified by single spore isolation method.

Identification of *Trichoderma* isolates: *Trichoderma* isolates were identified based on molecular tools by extracting DNA, amplifying and sequencing the internal transcribed spacer (ITS) region (Anees *et al.*, 2010). For DNA extraction, *Trichoderma* isolates were cultured on MEA and the culture was collected using scalpel and shifted to 2ml microtubes. Seven hundred and fify milliliter of lysis buffer (0.1M Tris-HCl pH 8, 20 mM EDTA, 1.4M NaCl and 2% CTAB) were added; the mixture was vortexed and incubated in a water bath at 65°C. After 1 h, 500µl of phenol: chloroform: isoamlyl alcohol (25:24:1) were added, vortexed and centrifuged at 12000 g for 15min at 4°C. The supernatants were collected and the equal volume of isopropanol was added to precipitate DNA. The tubes were incubated for 1 h at 4°C and centrifuged at 12000 g for 15 min at 4°C to obtain the DNA pellet. The pellet was washed with 70% ethanol, dried and dissolved in 100 µl of TE buffer (10mMTris-HCl pH 8, 1mM EDTA).

The ITS region of the different isolates was amplified by PCR by mixing template DNA (2µl) with PCR master mix (48 µl) containing 10X *Taq* polymerase buffer (5 µl), 5U *Taq* polymerase enzyme (0.4 µl), 3.7 µl of 2 mMdNTPs, 2.5 µl of 10 mM ITS1F (3'CTTGGTCATTT AGAGGAATAA5') (Gardes & Bruns, 1993) and ITS4 (3'TCCTCCGCTTATTGATATGC5') (White *et al.*, 1990) as the forward and reverse primers respectively. PCR was programmed with an initial denaturation step at 94°C for 5min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min followed by a final extension step at 72°C for 30 min. Aliquots of 5 µl of PCR products were checked by electrophoresis in a 1.5% agarose gel at 120 V for 15 min. The ITS amplicons were then sequenced using primers ITS1F and ITS4 by Cogenics (Meylon France). The sequences from both strands were assembled using SeqMan 6.0 (DNA STAR Inc., 2004). Sequence identities were determined using the International Subcommission on *Trichoderma* and *Hypocrea* (ISTH, www.isth.info) *trich*OKEY v 2.0 based on an oligonucleotide barcode within the ITS1 and ITS2 sequences. Moreover, the morphology based identification of the selected isolates from Malakand and Karak was confirmed by using phenotypic key as previously described by Anees *et al.,* (2010).

The phenotypic identification was based on the standard procedures using *Trichoderma* interactive morphological key (www.isth.info; (Anees *et al.*, 2010)).

Assessment of antagonistic activity using *In vitro* tests: *Trichoderma* strains were evaluated for their potential to antagonize the phytopathogenic fungus *Fusarium oxysporum In vitro* using three different tests. Three strains of pathogenic fungus *F. oxysporum* (FO866, FO899, FO1025) obtained from the Fungal Culture Bank, University of Punjab, Lahore, Pakistan were used. For all the *In vitro* tests, discs (5 mm) from the edge of growing fungal colonies were used to inoculate MEA medium in sterile plastic round 9 cm Petri dishes. The plates were incubated in the dark at 25°C.

Dual culture technique: Fresh culture agar plugs of *Trichoderma* were grown in dual culture along with the different strains of *F. oxysporum* 6 cm apart on the same plate and incubated at 20°C. The radii of colony of *F. oxysporum* approaching and not approaching the colony of *Trichoderma* isolate were measured twice a day for 3 to 4 days. Experiments were performed in triplicate. Inhibition of growth rate of *F. oxysporum* was calculated as percentage of difference of the radius not approaching and the radius approaching *Trichoderma,* over the radius not approaching and anlyzed by analysis of variance (ANOVA).

Effect of the volatile metabolites: The MEA plates were cultured at center with agar disks of *Trichoderma* isolates and the lid of each dish was replaced by a bottom dish containing MEA newly inoculated with *F. oxysporum*. The two dishes were then taped together with adhesive tape. The growth of *F. oxysporum* was recorded after 24, 48 and 72 h. In the control, *F. oxysporum* was cultured in the same way but without *Trichoderma* isolates. For volatile inhibitors tests, the percentage of inhibition was calculated dividing the difference between the radial growth of control and antagonized culture of *F. oxysporum* by the radial growth of the control and multiplied by 100. The experiments were performed in triplicate and results were analyzed by ANOVA as above.

Effect of extracellular metabolites using cell free culture filtrates: Flasks containing malt broth were inoculated with 5 mm agar discs of each of the *Trichoderma* species from the margins of actively growing *Trichoderma* colonies. Inoculated flasks were incubated under static conditions at 28°C for 4 weeks. After 4 weeks the cultures were filtered through sterile Whatman filter paper no. 1. The filtrate was added to pre cooled potato dextrose agar (PDA) medium at final concentration of 5 and 25% (v/v) before pouring into Petri plates. Each plate was inoculated with 5 mm mycelia disc of the pathogen that was previously cultured on PDA. The inoculated plates were then incubated at 27±10°C. The colony diameter in each concentration was recorded. The pathogen inoculated on PDA medium without any culture filtrate served as control.

Results

Isolation and identification of *Trichoderma* spp.: A total of 29 *Trichoderma* isolates were picked and initially identified at the generic level using microscopy. The isolates were observed under the 40X microscope, phialides were found constricted at the base, more or less swollen near the middle and abruptly near the apex into short subcylindric neck (Fig. 1). The strains were then identified based on molecular techniques and their NCBI accession numbers were obtained (Table 1). A subset of 13 strains was characterized based on morphology (Table 2). The morphological and molecular identifications of the isolates were found in agreement. Overall three species could be identified out of 29 strains after sequence analysis i.e. *T. brevicompectum, T. virens* and *T. longibrachiatum. Trichoderma longibrachiatum* was the most prevalent species identified from different parts of Pakistan. All the strains were also submitted to Microorganisms of Interest for Agriculture and Environment (MIAE), INRA, Dijon, France.

Antagonistic screening in dual culture: Among 22 *Trichoderma* strains tested in dual culture, three strains TMK22, TMK19, TMK20 completely inhibited the growth of *F. oxysporum* strain 866 (FO866; Fig. 2). The least inhibition in the growth of the pathogen was shown by the TMK17 that inhibited FO866 by 57%.

The antagonistic effect of *Trichoderma* strains against *F. oxysporum* strain 899 (FO899) were in the range of 56 - 93% (Fig. 3). TMK20 showed the highest inhibitory effect (92%), followed by TMK22 (89%), TMK19 (88%) and TMK9 (78%). The lowest inhibitory effect recorded was by TMK7 (56%).

The highest inhibitory effect on growth of *F. oxysporum* strain 1025 (FO1025) was observed by TMK20 (95%) followed by TMK19 (95%), TMK22 (91%) and TMK6 (79%). TMK7 showed the lowest inhibitory effect against FO1025 (54%; Fig. 4).

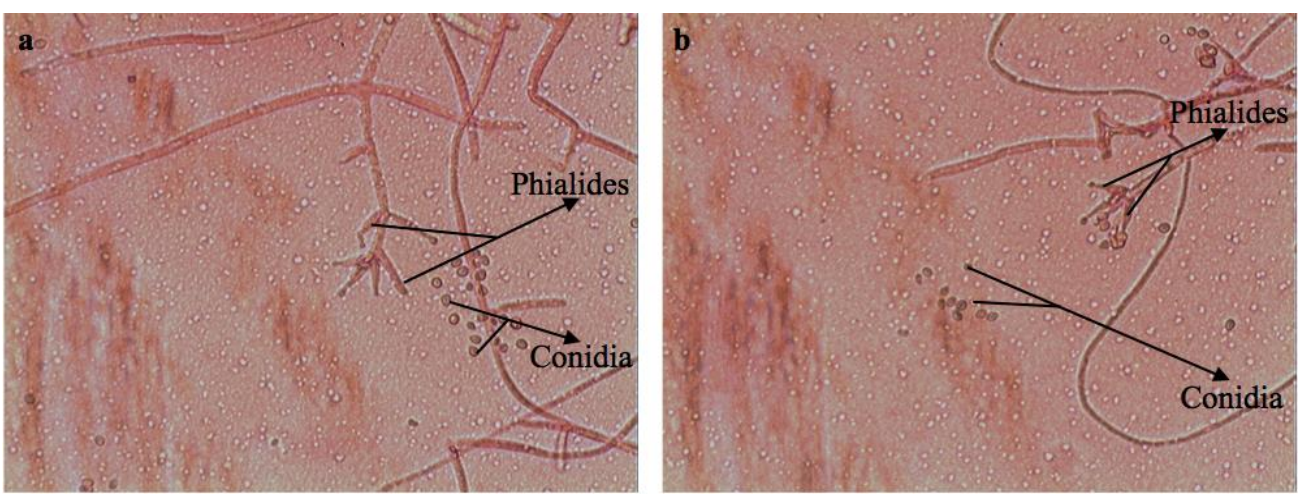

Fig. 1. Microscopic observation of *Trichoderma* isolates under 400X (a) TMK01 (b) TMK02.

Table 1. Molecular identification of *Trichoderma* spp. based on amplification and sequencing of internal transcribed spacer region using the International Subcommission on *Trichoderma* and *Hypocrea* (ISTH, www.isth.info) *trich* OKEY v 2.0.

Strain id	MIAE* id	Soilorigin	Species identification	NCBI Accession
TKK01	MIAE00801	Sandy wheat field Shnawa, Karak	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225882
TKK03	MIAE00803	Clayey maize field Shnawa, Karak	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225883
TKK04	MIAE00804	Rocks particulate, Zarkinasrati, Karak	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225884
TKK06	MIAE00806	Sandy field, Shnawa, Karak	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225885
TKK07	MIAE00807	Dry clayey field, Zarkinasrati, Karak	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225886
TKK08	MIAE00808	Dry clayey field, Zarkinasrati, Karak	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225887
TKK09	MIAE00809	Sandy field, Shnawa, Karak	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225888
TMK01	MIAE00810	Clayey chilli field Balamabat, Malakand	*Trichoderma brevicompactum*	KM225889
TMK02	MIAE00811	Clayey chilli field, Balamabat, Malakand	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225890
TMK03	MIAE00812	Clayey tomato field, Takwar, Malakand	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225891
TMK04	MIAE00813	Clayey chilli field Balamabat, Malakand	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225892
TMK05	MIAE00814	Clayey tomato field Takwar, Malakand	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225893
TMK06	MIAE00815	Clayey tomato field Takwar, Malakand	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225894
TMK08	MIAE00817	Clayey tomato field Takwar, Malakand	*Trichoderma virens/ Hypocreavirens*	KM225895
TMK09	MIAE00818	Clayey rice field Timergara, Malakand	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225896
TMK10	MIAE00819	Clayey rice field, Timergara, Malakand	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225897
TMK11	MIAE00820	Clayey rice field, Timergara, Malakand	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225898
TMK12	MIAE00821	Clayey ladyfinger field, Munda, Malakand	*Trichoderma brevicompactum*	KM225899
TMK13	MIAE00822	Clayey ladyfinger field Munda, Malakand	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225900
TMK14	MIAE00823	Clayey tomato field, Takwar, Malakand	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225901
TMK15	MIAE00824	Takwar, Malakand	*Trichodermabrevicompactum*	KM225902
TMK16	MIAE00825	Clayey ladyfinger field, Munda, Malakand	*Trichodermabrevicompactum*	KM225903
TMK17	MIAE00826	Clayey chilli field, Balamabat, Malakand	*Trichodermabrevicompactum*	KM225904
TMK18	MIAE00827	Clayey chilli field, Balamabat, Malakand	*Trichodermabrevicompactum*	KM225905
TMK19	MIAE00828	Clayey rice field, Timergara, Malakand	*Trichoderma longibrachiatum /Hypocreaorientalis*	KM225906
TMK20	MIAE00829	Clayey rice field, Timergara, Malakand	*Trichoderma longibrachiatum /Hypocreaorientalis*	KM225907
TMK21	MIAE00830	Clayey chilli field Balamabat, Malakand	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225908
TMK22	MIAE00831	Clayey tomato field Takwar, Malakand	*Trichoderma longibrachiatum/ Hypocreaorientalis*	KM225909

*Collection MIAE: Microorganisms of interest for agriculture and environment (INRA, Dijon, France)

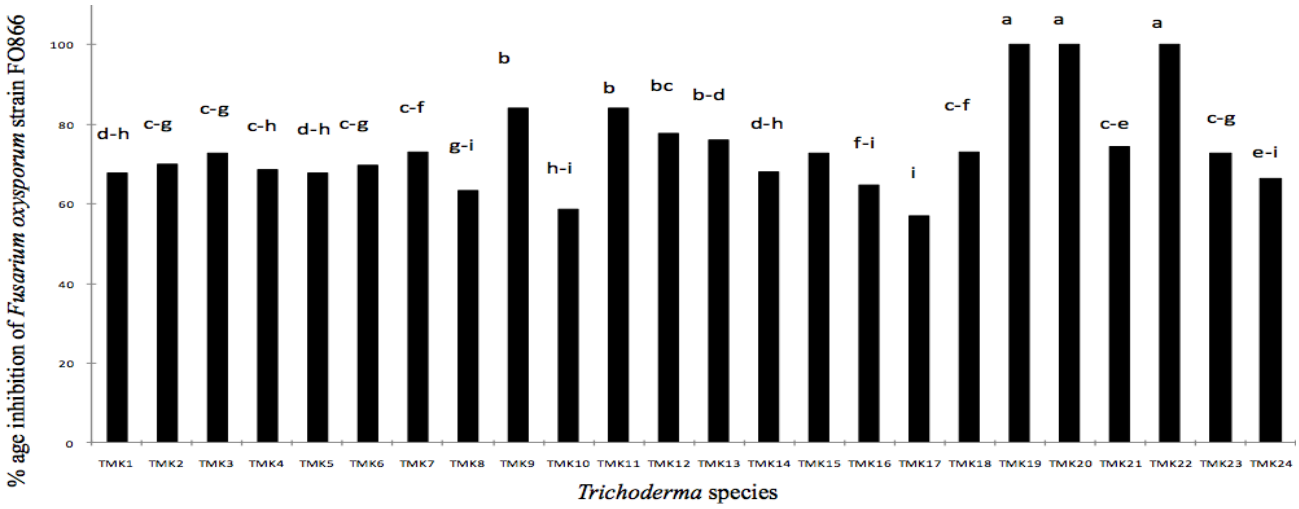

Fig. 2. The percentage growth inhibition of *Fusarium oxysporum* FO866 in dual culture by different *Trichoderma* strains isolated in the present study. Bars represent the mean values obtained for three independent repeats. ANOVA and LSD tests were performed and bars designated by a different small letter(s) are significantly different, $p<0.05$.

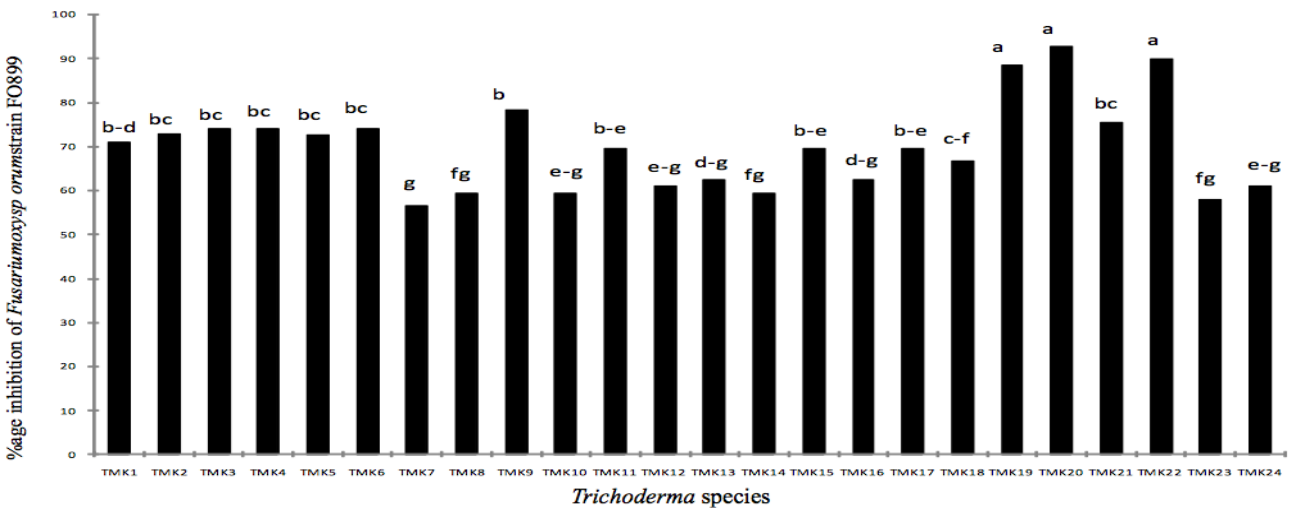

Fig 3. The percentage growth inhibition of *Fusarium oxysporum* FO899 in dual culture by different *Trichoderma* strains isolated in the present study. Bars represent the mean values obtained for three independent repeats. ANOVA and LSD tests were performed and bars designated by a different small letter(s) are significantly different, $p<0.05$.

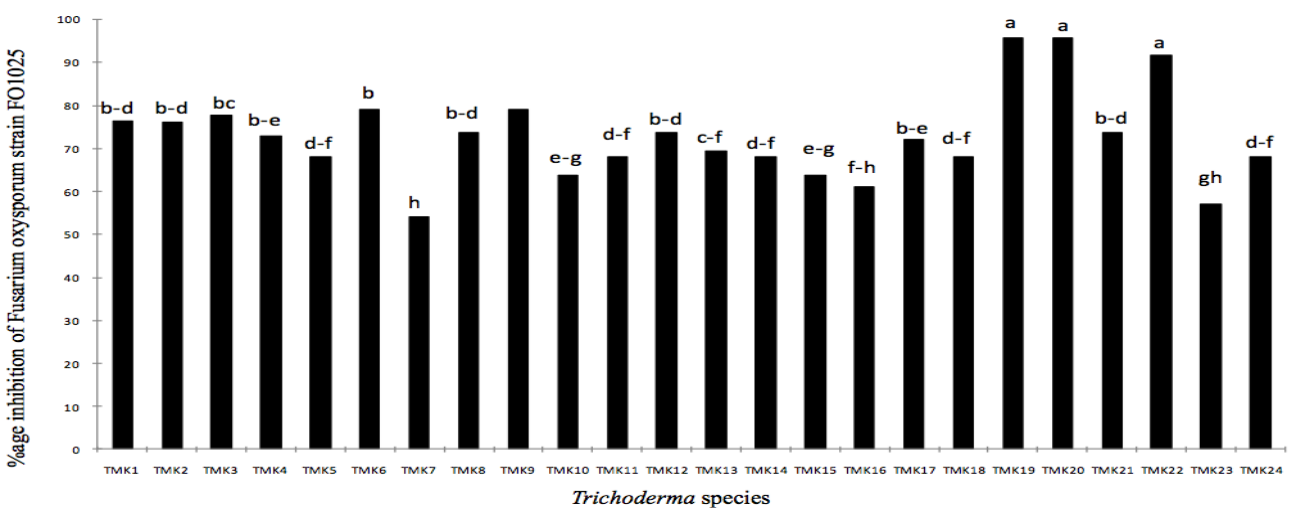

Fig. 4. The percentage growth inhibition of *Fusarium oxysporum* FO1025 in dual culture by different *Trichoderma* strains isolated in the present study. Bars represent the mean values obtained for three independent repeats. ANOVA and LSD tests were performed and bars designated by a different small letter(s) are significantly different, $p<0.05$.

Table 2. Morphological characterization of *Trichoderma* spp. isolated in the present study. The measurements of cultures on potato dextrose agar (PDA) and synthetic nutrient agar (SNA) media represent the mean of three independent values measured after 72 h in the dark. All the isolates showed green pigmentation of spores. Strains were identified using the morphological key (http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm).

Strain id	MIAE id*	Conidia			Phialides			Coconut odor	Culture on PDA and SNA (mm)				Species identified
		Shape	length (µm)	width (µm)	Length (µm)	Width at middle (µm)	Width at base (µm)		PDA 30°C	PDA 35°C	SNA 35°C	PDA 40°C	
TKK01	MIAE00801	Ellipsoidal	4.5	3	9	3	2.5	Absent	43	58	64	12	*T. longibrachiatum*
TKK03	MIAE00803	Ellipsoidal	4.5	2.5	9	2.9	2	Absent	42	60	64	9	*T. longibrachiatum*
TKK04	MIAE00804	Ellipsoidal	5	3	10	3	2.5	Absent	45	58	66	11	*T. longibrachiatum*
TKK06	MIAE00806	Ellipsoidal	4	3	8	3	2.5	Absent	44	57	66	9	*T. longibrachiatum*
TKK07	MIAE00807	Ellipsoidal	5	3.5	10	3.5	2.5	Absent	44	59	64	10	*T. longibrachiatum*
TKK08	MIAE00808	Ellipsoidal	4.5	3	9.5	2.8	2.2	Absent	42	60	65	9	*T. longibrachiatum*
TMK01	MIAE00810	subglobose	3.3	3	10	3.7	2.5	Absent	47	13	9	No growth	*T. brevicompectum*
TMK02	MIAE00811	Ellipsoidal	4	3	9	2.9	2	Absent	50	42	42	9	*T. longibrachiatum*
TMK03	MIAE00812	Ellipsoidal	3.7	2.8	8.5	2.6	2.5	Absent	49	40	40	6	*T. longibrachiatum*
TMK04	MIAE00813	Ellipsoidal	4.5	2.5	10	2.5	2.4	Absent	51	44	41	9	*T. longibrachiatum*
TMK05	MIAE00814	Ellipsoidal	5	3	9.5	2.9	2.5	Absent	52	41	43	10	*T. longibrachiatum*
TMK06	MIAE00815	Ellipsoidal	4.5	3	9.8	3	2	Absent	50	43	40	7	*T. longibrachiatum*
TMK08	MIAE00817	Ellipsoidal	5	3.8	13	4	3	Absent	52	45	No growth	No growth	*T. virens*

*Collection MIAE: Microorganisms of interest for agriculture and environment (INRA, Dijon, France)

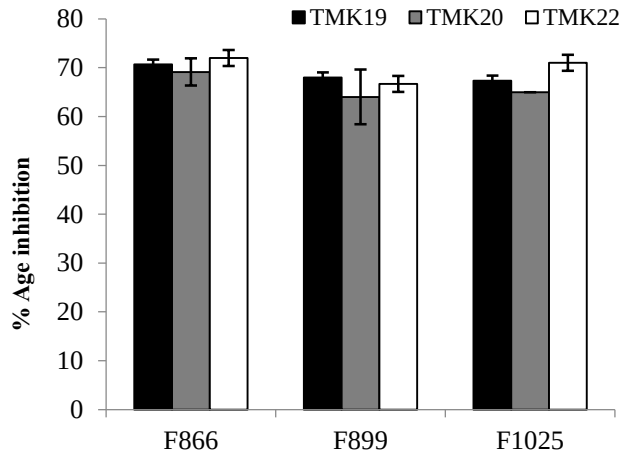

Fig. 5. The percentage inhibition of radial growth of *Fusarium oxysporum* strains (F866, F899, and F1025) by volatile compounds produced by *Trichoderma* strains (TMK19, TMK20, and TMK22). Bars represent the mean values obtained for three independent repeats. Error bars represent SD.

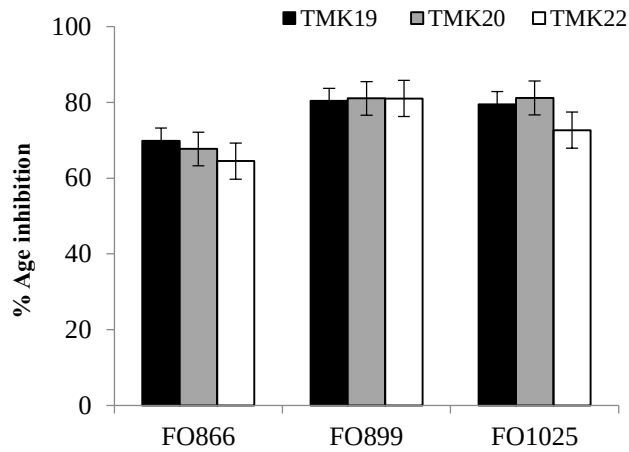

Fig. 6. The percentage inhibition of radial growth of *Fusarium oxysporum* strains (FO866, FO899, and FO1025) by 5% culture filtrates of *Trichoderma* strains (TMK19, TMK20, and TMK22). Bars represent the mean values obtained for three independent repeats. Error bars represent SD.

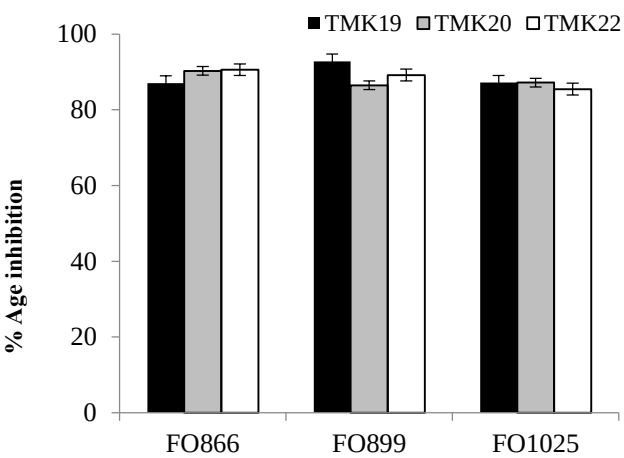

Fig. 7. The percentage inhibition of radial growth of *Fusarium oxysporum* strains (FO866, FO899, and FO1025) by 25% culture filtrates of *Trichoderma* strains (TMK19, TMK20, and TMK22). Bars represent the mean values obtained for three independent repeats. Error bars represent SD.

Effect of volatile metabolites in inhibiting growth of *F. oxysporum* strains: The most efficient *Trichoderma* strains observed in dual culture (TMK19, TMK 20, and TMK22) were selected for their ability to produce volatile metabolites that were effective in controlling radial growth of *F. oxysporum* strains. The results revealed that volatile compounds produced by *Trichoderma* spp. caused a significant inhibition of growth after incubation for 4 days (Fig. 5).The growth inhibition of FO866 ranged from 69 to 72%. The highest inhibitory effect on growth of FO866 was recorded by TMK22 that inhibited growth up to 72% followed by TMK19 (70%) and TMK20 (69%).Volatile metabolites were also found effective against FO899. Growth inhibition was in the range of 64-68% with maximum inhibition recorded by TMK19 that has inhibited 68% followed by TMK22 (66.66%) and TMK20 (64%). The highest inhibition of F1025 was due to culture filtrates of TMK22 (71%) followed by TMK19 (67.33%) and TMK20 (65%).

Growth inhibition of *F. oxysporum* by using culture filtrates of *Trichoderma* strains: The growth was inhibited with increase in the concentration of culture filtrates of the selected *Trichoderma* spp. The 5% culture filtrate of the selected strains was effective in inhibiting growth of the tested pathogens (Fig. 6). Maximum growth inhibition of FO866 was observed by TMK19 (69%), followed by TMK20 (67%) and TMK22 (64%). Similarly, growth of FO899 was inhibited by TMK20 (81%), TMK22 (81%), followed by TMK19 (80%). Culture filtrates of all the three *Trichoderma* strains were effective against FO1025 with maximum growth inhibition by TMK20 (81%) followed by TMK19 (79%) and TMK22 (72%).

The 25% culture filtrates showed the highest percentage of growth inhibition of *F. oxysporum* (Fig. 7). Out of three strains of *Trichoderma* species, the maximum inhibition of the radial growth of FO866 was observed by the culture filtrate of TMK22 (91%), which was followed by TMK20 (90%) and TMK19 (74%). Similarly, the growth of FO899 was inhibited by TMK19 (93%), TMK22 (89%) and TMK20 (86%). The maximum growth inhibition of FO1025 was due to culture filtrates of TMK19 and TMK20 (87%) followed by TMK22 (85%).

Discussion

Our work was focused on the isolation, identification and antagonistic potential of the indigenous strains of *Trichoderma* from the North Western regions of Pakistan. Biological control is the best alternative, especially against soil borne pathogens such as *Fusarium* spp. Moreover, biocontrol techniques may be environment friendly and cost effective because they may persist in soil for more than one season (Gohel *et al.*, 2007). *Trichoderma* species are common saprophytic fungi found in almost any soil and rhizosphere microflora, and have been investigated as potential biocontrol agents because of their ability to reduce the incidence of diseases caused by plant pathogenic fungi, particularly many common soil borne pathogens (Ashrafizadeh *et al.*, 2005; Dubey *et al.*, 2007).

Out of 29 isolates, only 3 species could be identified i.e. *T. longibrachiatum, T. brevicompectum* and *T. virens.* Gherbawy *et al.,* (2004) showed the occurrence of only two *Trichoderma* species in a total of twenty three soils, which was the lowest number of taxa reported from a comparable number of soils so far. Kullnig *et al.,* (2000) isolated 76 isolates from Russia, Nepal and North India, and reported seven species (*T. asperellum, T.atroviride, T. ghanense, T. hamatum, T. harzianum, T. virens* and *T. oblongisporum*) along with five new taxa. Similarly, Migheli *et al.,* (2009) isolated 482 isolates and only 14 species could be identified from 15 different soils from the island of Sardinia.

T. longibrachiatum was overall the most prominent species isolated from different samples with 27 isolates out of 29 belonged to it. It may be because *T. longibrachiatum* can survive in extreme environments. Kullnig *et al.,* (2000) failed to find *T. longibrachiatum* in central Russia, Siberia and Himalayan mountains and concluded that *T. longibrachiatum* was less abundant or absent in the Himalayan soils. Our results showed that *T. longibrachiatum* was present in higher frequency in the North Western parts of Pakistan. Migheli *et al.,* (2009) found *T. harzianum* as the dominant species in the Island of Sardinia, a hot spot in the Mediterranean Sea near to the Western Europe. Turner *et al.,* (1997) hypothesized that the geographic occurrence of *T. citrinoviride* and *T. longibrachiatum* may be complementary. *T. longibrachiatum* was also prominent in province of Zhejiang, China (Kubicek *et al.*, 2003). The isolation of *T. longibrachiatum* from China was an important discovery (Kubicek *et al.*, 2003), along with the occurrence of *H. orientalis* in Yunnan, which was proposed as a potential teleomorph of *T. longibrachiatum* (Samuels *et al.*, 1998). *T. longibrachiatum* was found in forest soils of Tunisia (Sadfi-Zouaoui *et al.*, 2009). Abundance of *T. longibrachiatum* in the present study proposed that it may be the most prominent species in Pakistani soils. However, further investigation is needed on isolation and identification of *Trichoderma* spp. from various soils in various regions of Pakistan.

T. brevicompectum was isolated from samples taken from Malakand where soil was moist with humid atmosphere. *T. brevicompectum* was previously found abundant in North America where temperature was moderate (Kraus *et al.*, 2004). Based on our experiments, the optimum temperature for *T. brevicompectum* was recorded as 30°C which was complementary to previous findings (Kraus *et al.*, 2004). The optimum temperature for *T. virens* isolated from Malakand was 25°C and failed to grow on 35°C which indicated that *T. virens* belonged to cold environment. Our morphology based identification was supported by molecular identification although we could see some intra specific variability based on morphology and growth. However, further investigations may be required to confirm the low diversity of *Trichoderma* report in present study with more soil samples and more isolates per soil.

Selection of biocontrol agents and understanding the mechanisms involved in the antagonistic activity of *Trichoderma* spp. against plant pathogens are important in designing effective and safe biocontrol strategies. In the present study, different strains of *Trichoderma* spp. were evaluated for their antagonistic potential against different strains of phytopathogenic *F. oxysporum* strains using *In vitro* assays. The dual culture assays revealed the highest antagonistic potential of the indigenous *Trichoderma* strains. The colony growth inhibition observed may be because of production of some kind of antibiotics or toxic metabolites (Howell, 2002). The dual culture assay results were further reinforced by the culture filtrate assays in the present study. Culture filtrates were used in two different concentrations and demonstrated the possible role of water soluble fungal metabolites in the observed inhibition. In the present study, a dose dependent growth inhibition of *F. oxysporum* by the culture filtrates in media might be because of the increased concentration of the active compound(s). Growth inhibition of the phytopathogens by the *Trichoderma* metabolites was also previously reported (Ghisalberti & Sivasithamparam, 1991; Howell, 1998).

The nature of the antifungal metabolites in the present study may be antibiotic or hydrolytic (Upadhyay & Rai, 1987). Dennis & Webster, (1971) showed that culture filtrates produced by *Trichoderma* contained inhibitory substances against pathogenic microorganisms. The antibiotics produced by *T. harzianum* included 6-n-pentyl-2H-pyran-2-one, 6-n-pentenyl-2H-pyran-2-one, pyridine, anthraquinones, butenolides, isonitrin D and F, trichorzianines and furanone. Huang *et al.,* (1995) isolated peptaibols, named trichokonins from the culture broth of *T. koningii.* Calistru *et al.,* (1997) reported that the culture filtrates of *T. viride* and *T. harzianum* were inhibitory to *Fusarium moniliforme*. Kapil & Kapoor, (2005) reported that the culture filtrate of *T. viride* inhibited the mycelial growth of *Sclerotinia sclerotiorum* due to production of antibiotic-like substance. *Trichoderma* spp., are known to produce a number of antibiotics such as trichodernin, trichodermol, harzianum A and harzianolide (Kucuk & Kivanc, 2004) as well as cell wall degrading hydrolytic enzymes such as chitinases, glucanases that break down polysaccharides, chitins and glucans, thereby destroying cell wall integrity (Elad, 2000). These enzymes may also play a major role in mycoparasitism because of changes in cell wall integrity prior to penetration.

Another mechanism that was demonstrated in the present study was the effective role of volatile metabolites with significant control ability and inhibitory effect of *Trichoderma* strains on mycelial growth of *F. oxysporum*. The major volatile compound in *Trichoderma* species is 6-pentyl-α-pyrone (6-PAP), but *T. virens* produced a different spectrum of metabolites including viridin and viridol, and some strains produced gliovirin and heptelidic acid, whereas others produce gliotoxin (Howell *et al.*, 1993).

The present study demonstrated the antagonistic potential of the indigenous strains of *Trichoderma* and their possible mechanisms against the phytopathogenic *F. oxysporum* strains. Among the different strains tested in the present study, *T. longibrachiatum* strains TMK19, TMK20 and TMK22 showed the maximum inhibition in dual culture assays. The above results suggested that the antibiotics (non-volatile or volatile) possibly played an important role in suppressing the growth of the phytopathogen. The present study also suggested the predominant occurrence of *T. longibrachiatum* in this part of the world however that needs further study with more samples and isolates.

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