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# FUNGAL DIVERSITY ASSOCIATED WITH VERTICILLIUM WILT OF COTTON

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**Abstract**

The association of fungal diversity with Verticillium wilt is rarely known, which is important to know for the control of this detrimental disease. Our study is the preliminary attempt to find the associations of fungal diversity with Verticillium wilt and provides the baseline information for biological control. About 30 different fungi from soil and 23 from cotton plants were isolated and confirmed through molecular characterization. The colony forming unit (CFU)/g dry soil of fungi before and after planting cotton showed significant variation among all the fungi. The overall frequency of all fungi for soil after sowing was significantly higher than before sowing. *A. alternata*, *F. equiseti*, *F. concentricum, A. flavus*, *F. proliferatum,* and *Chaetomium* sp. associated with high resistance (Arcot-1) to *Verticillium* wilt, whereas, *V. dahliae*, *A. niger* and *Paecilomyces* sp., with high susceptible (Arcot-438) germplasm. However, *T. basicola, C. ramotenellum* and *G. intermedia* were isolated from both. Soil plating was comparatively easiest than soil dilution method for the determination of frequency percentage, however, later method is useful for the screening of single spore isolation. Most of the antagonistic species were screened from soil; nevertheless, *Paecilomyces* and *Chaetomium* spp. were screened from plant and soil. *In vitro* test of *T. longibrachiatum. T. atroviride, Paecilomyces* and *T. viride* showed the strongest efficacy against *V. dahliae.* These efficient bio-agents can be used as an effective tool for other future studies regarding to *Verticillium* wilt of cotton.

## Introduction

Cotton (*Gossypium* spp.) is the pillar of agricultural commodity in many regions of the world, and benefits a large rural population. It is an important raw material for the textile industries of China and plays a significant and irreplaceable role in the national economy (Anon., 2002).

The productivity rate of cotton is declined due to many reasons including various biotic and abiotic factors (Zafar, & Athar, 2013). Nevertheless, several pathogenic and antagonistic fungi and bacteria are also associated with cotton plant including *Verticillium dahliae* Kleb. It had been known that cotton crop suffers from more than 60 diseases caused by fungi, bacteria, nematodes, viruses and physiological disorders (Lyda & Watkins, 2001). The association of these fungi cause heavy losses and ultimately reduces the quality and production of cotton.

In addition to above mentioned diseases, Verticillium wilt caused by a fungus inhabits the soil, *Verticillium dahliae* Kleb, is one of the most devastating and widespread diseases, cause substantial yield losses in cotton worldwide (Agrios, 2005), including China (Jing *et al.,* 1999). The major factors that influence the severity of Verticillium wilt are inoculum density, virulence of the pathogen, behavior of germplasm, temperature, soil conditions and availability of biological antagonists (Bell, 1993). There have been reports of almost complete yield losses, with this disease causing epidemics in cotton, cauliflower, and sunflower (Bell, 1992).

Numerous management practices have been applied to prevent and/or reduce the loss of crops caused by Verticillium wilt. Examples of commonly used management practices for Verticillium wilt include cultural practices, soil solarisation (Melero-Vara *et al.,* 1995), organic amendments (Huang *et al.,* 2006) and fungicides (Niu *et al.,* 2006). However, while these practices may suppress the disease to some extent, they cannot completely control its severity. Hence, alternative biocontrol agents are also being developed (Zheng *et al.,* 2011). These comprise the use of fungal antagonists (Narisawa *et al.,* 2002) and antagonistic rhizobacteria (Berg *et al.,* 2001). However, the practical application of these bio-agents remained inadequate. The antagonistic effects of these bio-agents observed *In vitro* do not always correlate with the effect in the greenhouse and field (Berg *et al.,* 2000). This may be due to the association of various fungi with Verticillium wilt. Those need to be analyzed for sustainable management and biological control of this serious issue.

Currently there is no comprehensive knowledge available for the association of pathogenic and antagonistic fungi with Verticillium wilt of cotton, which is quite important for biological control. The objective of current study was to determine the associations of fungal diversity with Verticillium wilt of cotton plant and its rhizosphere to obtain baseline information of fungal communities in relation.

## Materials and Methods

**Collection of samples:** Soil samples were randomly collected from the experimental field of Anyang, Henan province, China one week before sowing and harvesting of cotton crops from 9 different locations at the depth/core of 0-10, 10-20, 20-30cm with three replicates. Samples were put into plastic bags and stored at 4°C for further analysis. Plant samples (branch，shoot and root) were collected before and after appearance of Verticillium wilt symptoms (BAWS & AAWS) from high resistance (Arcot-1) and sensitive (Arcot-438) germplasm. These experiments were conducted and repeated during two successive years, 2010-2011.

## Isolation of fungi

**Soil assay:** Samples were air dried at room temperature for 4 weeks and then analyzed by using soil dilution plate (Waksman, 1922) and soil dry plate (Warcup, 1950) methods.

**Soil dilution plate:** Soil samples of each core were separately mixed and thoroughly crushed. 10g of mixed soil was diluted in 90ml sterile distilled water (w:v) and then series of dilutions viz; $10^{-1}$, $10^{-2}$ and $10^{-3}$ were made. About 0.2ml volume was streaked for the final dilution over three different media viz; Potato Dextrose Agar (PDA), Potato Carrot Agar (PCA: potatoes=20g; Carrot=20g; agar=15g; distilled water=1L) and *Verticillium* medium (VM: Part-1: Pentachloronitrobenzene (75%)=0.015g; sucrose=7.5g; NaNO=2g; KC1=0.5 g; MgSO4 • 7H2O=0.5 g; K2HPO4=1g; FeSO4 • 7H20=0.01g; agar=15g; Part-2: streptomycin sulfate=0.1g; 95% ethanol=5 ml; distilled water=1L. Ingredients of part-1 were aseptically added after autoclaving (Ausher 1975) and then incubated at 25±1°C. The data on colony forming units (CFU) was observed after 4, and 7 days of inoculation. The data for CFU count in soil dilution plate technique is presented here is from $10^{-1}$ dilution and calculated by: CFU/g = Total No. of colonies/Volume*Dilution factor. All strains were purified by single spore isolation techniques and stored on PDA medium at 4°C for further analyses.

**Soil dry plate:** Soil samples were passed through different grading sieves from 20 to 200 (mesh/sq. in.). About 0.025g of dry soil (passed through 200 meshes) was gently scattered manually with the help of sterilized spatula on petri plates contained PDA, PCA and VM media and then incubated at 25±1°C. The CFU per gram was calculated by: CFU/g = Total No. of colonies/quantity of soil plated*40

**Cotton plant assay:** Standard tissue culture isolation method was followed to isolate the fungi from root, stem and branch tissues of both germplasm, with a great care under aseptic conditions. Fragments were surface-sterilized with 0.1% $HgCl_2$. All the plates incubated at 25±1°C and data was recorded after 3 and 7 days. The incidence percentage of fungi was recorded with:

$$\text{Incidence (\%)} = \frac{\text{Tissue colonized by fungi}}{\text{Total No. of tissues plated}} \text{ X } 100$$

**Identification of the fungi**

**Morphological examination:** The genera of cultivable filamentous fungi (CFF) isolated from soil and plant samples were indentified on the basis of their typical colony characteristics, mycelial growth and presence of conidia, with the help of light microscopic observations using standard diagnostic keys (Barnett & Hunter, 1986).

**Molecular identification:** Identification of fungal isolates from soil and cotton plants were confirmed by sequencing of the internal transcribed spacer (ITS) regions 4 and 5, including the 5.8 rDNA.

**DNA extraction.** The DNA of each fungus was extracted from the conidia and mycelium by following the standard protocol of the DNA-Out Kit (TIANDZ, Inc., China, Cat# 100303-50).

**Polymerase chain reaction (PCR) and sequencing.** DNA amplification of ITS region of rDNA was performed by PCR (Zhang *et al*., 2012) using universal primers ITS4 /5 (5'-TCCTCCGCTTATTGATATGC-3'/5-GGA AGT AAA AGT CGT AACAAGG-3') (White *et al.,* 1990) to generate nucleotides from the complete ITS, including 5.8S rDNA region. The amplification f target region was performed in a 50 µL reaction volume according to the protocol of Premix Ex Taq version 2.0(code: D332A) Takara Biotechnology (Dalian) Co., Ltd, China. PCR reaction for all the regions were performed as follows: initial denaturation at 94ºC for 3min, followed by 30 cycles of 94ºC for 1 min, 52ºC for 50s, 72ºC for 1min, and final extension of 72ºC for 10min. The PCR products were examined by gel electrophoresis in 1.5% (w/v) agrose gels, stained with ethidium bromide, and visualized with a UV transilluminator. PCR products were sequenced by company (Invitrogen, Shanghai). The resulting sequence of the ITS rDNAs were compared and confirmed with those available in GenBank using Basic Local Alignment Search Tool (BLAST) of Nucleotide Sequence Database of National Center for Biotechnology Information (NCBI) to determine their phylogenetic affiliation.

***In vitro* efficacy of antagonistic fungi:** The antagonistic potential of some selective fungi against *Verticillium dahliae* Kleb was conducted by dual culture techniques under laboratory condition as described by Morton & Strouble (1955). The experiment was conducted two times in completely randomized design with 7 Petri dishes (repeats) for each isolates. The data was recorded after every 2days and final observations were taken when any of the petri dish contained full growth of test fungi. The inhibition rate of mycelial growth of test fungi over control was calculated by using the formula suggested by Vincent (1947) as: I (%) =(C-T)/ C*100; where, I = Percent inhibition, C = Growth in control, T = Growth in treatment.

**Statistical analysis:** All the experiments were repeated twice and combine analysis of both trials are presented here. Data were statistically analysed according to standard procedures for analysis of variance, ANOVA (linear model), and mean separation (least significant difference, LSD) of all parameters (after calculating with corresponding formulaes) and interaction between the trials were calculated by using the computer software Statistix 8.1 (Analytical Software, 2005). All differences described in the text were significant at the 5% level of probability.

**Results**

**Identification of fungal diversity**

**Fungi associated with Verticillium wilt infested soil.** Diversity of fungi with lower and higher frequency and CFU/g dry soil associated with the soil and rhizosphere of *Verticillium* wilt affected cotton plants (Supporting Table 1). A total of 30 different fungal species belonging to different genera were screened out through soil plating and dilution methods before and after planting of cotton crop (Table 1). All the fungi were identified through their typical morphological characteristics with the help of light microscopic observations using standard diagnostic keys (Fig. 1). The specific genera and species of all isolates were confirmed through molecular characterization using ITS region of rDNA of fungi.

**Table 1. Fungal species isolated from soil and cotton plant infected with Verticillium wilt.**

| Fungal species | Gen Bank Accession | Present or absent | |
|---|---|---|---|
| | | Soil | Cotton plant |
| *Alternaria alternata* strain GL22 | GQ169728.1 | + | - |
| *Alternaria alternata* | - | + | + |
| *Aspergillus flavus* NRRL:6412 | HQ340109.1 | + | + |
| *Aspergillus niger* | - | + | + |
| *Cercospora physalidis* strain HAL 2319 F | FJ866504.1 | + | - |
| *Chaetomium* sp. ATT211 | HQ607887.1 | + | + |
| *Cladosporium ramotenellum* strain CPC18224 | JF499839.1 | - | + |
| *Epicocum* sp. | - | - | + |
| *Fusarium* cf. *equiseti* MY-2011 | JN038467.1 | - | + |
| *Fusarium concentricum* isolate F21 | HQ379647.1 | - | + |
| *Fusarium equiseti* isolate GGF2 | HM008677.1 | + | + |
| *Fusarium oxysporum* isolate FO-12 | AY928419.1 | + | - |
| *Fusarium proliferatum* isolate 14 | EU839366.1 | + | + |
| *Fusarium solani* isolate S-0900 | EU029589.1 | + | - |
| *Fusarium solani* strain MOD-5 | EU625405.1 | + | - |
| *Fusarium* sp. | - | - | + |
| *Gibberella intermedia* isolate F22 | HQ379695.1 | + | + |
| *Oidiodendron cerealis* isolate NG_p39 | HQ115707.1 | + | - |
| *Paecilomyces* sp. ALAS-1 | HM626196.1 | + | + |
| *Penicillium granulatum* isolate 732 | DQ681334.1 | + | - |
| *Penicillium verruculosum* isolate CY196 | HQ608025.1 | + | - |
| *Rhizopus* sp. | - | + | + |
| *Stemphylium solani* strain SS1 | AF203451.1 | + | - |
| *Trichoderma viride* | X-93981.1 | + | - |
| *Thielaviopsis basicola* | - | + | + |
| *Trichoderma atroviride* isolate MIAE00220 | HM176575.1 | + | - |
| *Trichoderma hamatum* strain HBJZ1001 | JQ040347.1 | + | - |
| *Trichoderma koningiopsis* strain CQSQ4004 | JQ040366.1 | + | - |
| *Trichoderma longibrachiatum* strain CIB T29 | FJ478089.1 | + | - |
| *Trichoderma virens strain* GL-20 | AF099007.1 | + | - |
| *Verticillium dahliae* isolate 566 | GU799602.1 | + | + |
| *Verticillium dahliae* strain R-B | GU799602.1 | - | + |
| **Total fungal species** | **32** | | |

Note: "+" indicated present fungus; "-" indicated absent fungus

**Fungi associated with Verticillium wilt of cotton:** Total of 23 (Supporting Table 2) different fungal species were isolated from Verticillium wilt affected high resistance (Arcot-1) and high susceptible (Arcot-438) germplasm (Table 1). All the fungi identified through their typical morphological characteristics and confirmed through molecular characterization as described before (Fig. 1).

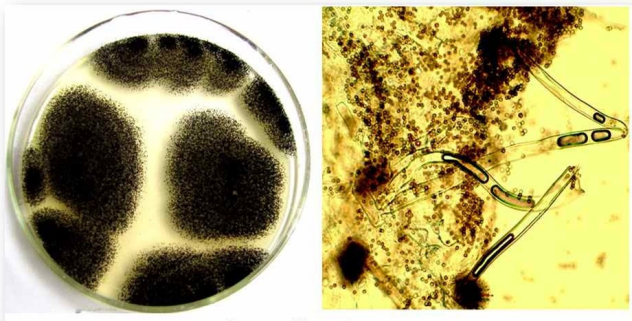
**Comparison of fungal species associated with soil and cotton plant:** Table 1 shows the list of total 32 different fungal species associated with soil and cotton plant. This indicates that all fungi found in the soil and/or in the rhizosphere of cotton were not associated with cotton plants. These fungal species have specific preference of host existence (Table 1). Almost all antagonistic species such as *Trichoderma virens, T. viride, T. koningiopsis, T. hamatum, T. koningiopsis, T. atroviride, T. longibrachiatum, Paecilomyces* sp., and *Chaetomium* sp. were screened out from soil, however, their association with wilt affected cotton plant under field conditions were not exist. Only *Paecilomyces* sp. and *Chaetomium* sp. associated with cotton rhizophere as well as with wilt affected cotton plants (Table 1 and Fig. 1).

**Frequency of fungi**

**Soil plating:** Fig. 2 indicates the CFU/g dry soil of different fungi at three different depth/cores of soil. Significantly highest CFU/g dry soil of *Alternaria alternata* was recorded through soil plating method from three different cores and media followed by *F. equiseti, T. virens, V. dahliae, Fusarium solani* and *T. viride*. However, *Asprgillus niger, A. flavus, Penecillium granulatum, V. dahliaeR, Fusarium solani, Paecilomyces* sp, *Cladosprium physalid, P. verrucul, Rhizopus* sp, *Epicocum* sp, *O. cerealis,* and *T. basicola* were obtained with lower CFU/g dry soil with no significant difference (Fig. 2, Supporting Table 3).

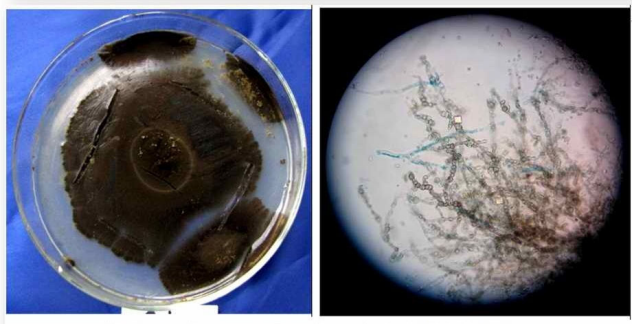
There was no significant difference among three soil cores of all fungi for CFU/g dry soil (Tables 2, Supporting Table 3). However, *A. alternata* revealed the highest CFU/g dry soil at the upper (10cm), on the contrary, *S. solani* and *Paecilomyces* sp. were recorded with higher CFU/g dry soil at lower (30cm) layers. *F. equiseti* and *V. dahliae* showed higher CFU/g dry soil at middle and lower (20 & 30cm), and *T. virens* at middle (20cm) layers. No significant difference was observed for *F. solani* and *T. viride* among the three soil cores (Fig. 2).

*Alternaria alternata* **strain GL22**



*Aspergillus niger*

*Aspergillus flavus* **NRRL:6412**



*Cladosporium ramotenellum* strain CPC 18224

*Cercospora physalidis* strain HAL 2319 F

***Chaetomium* sp. ATT211**

*Epicocum* sp.

*Fusarium concentricum* isolate F21

**Fig. 1a**

*Fusarium equiseti* isolate GGF2

*Fusarium oxysporum* isolate FO-12

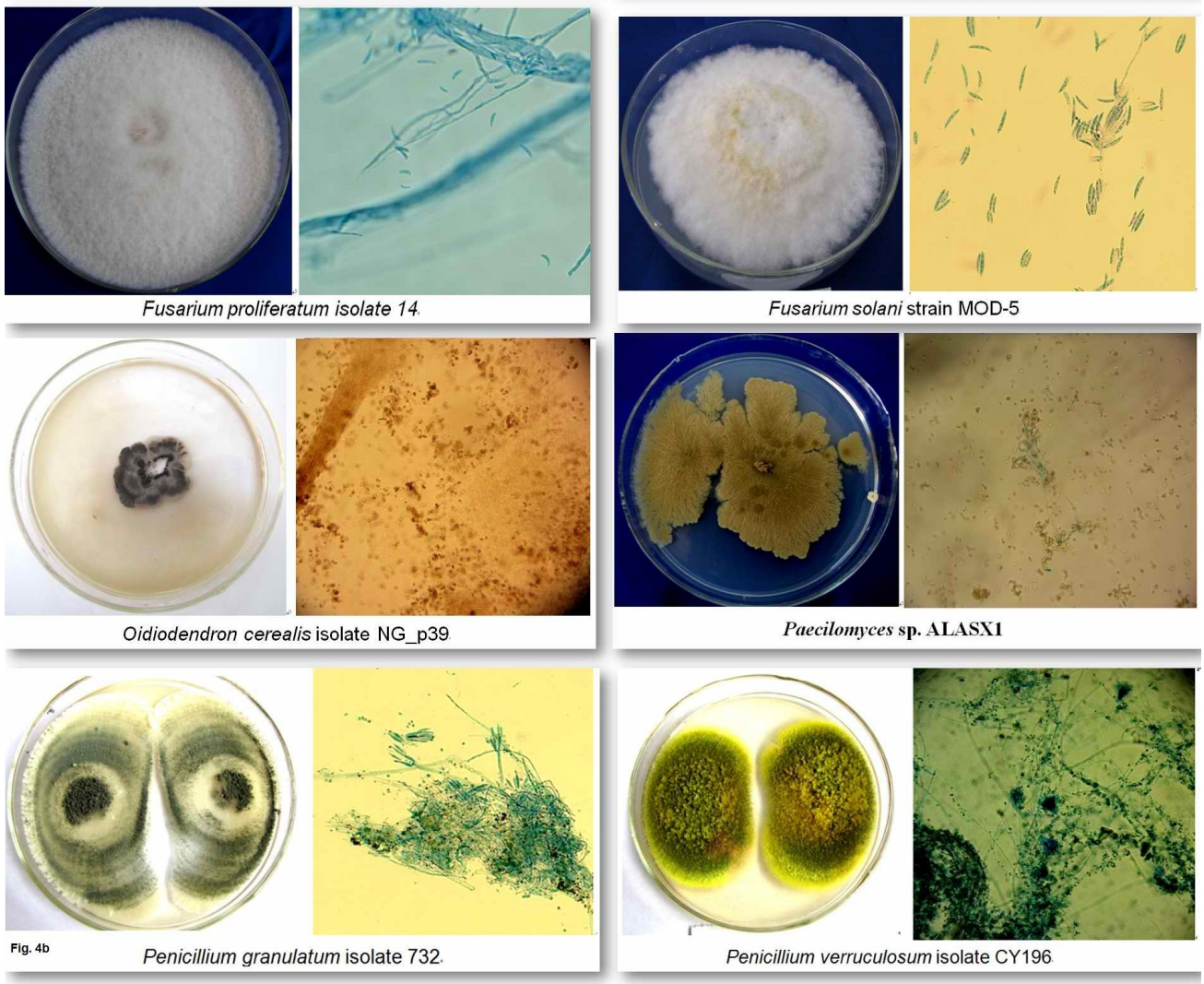
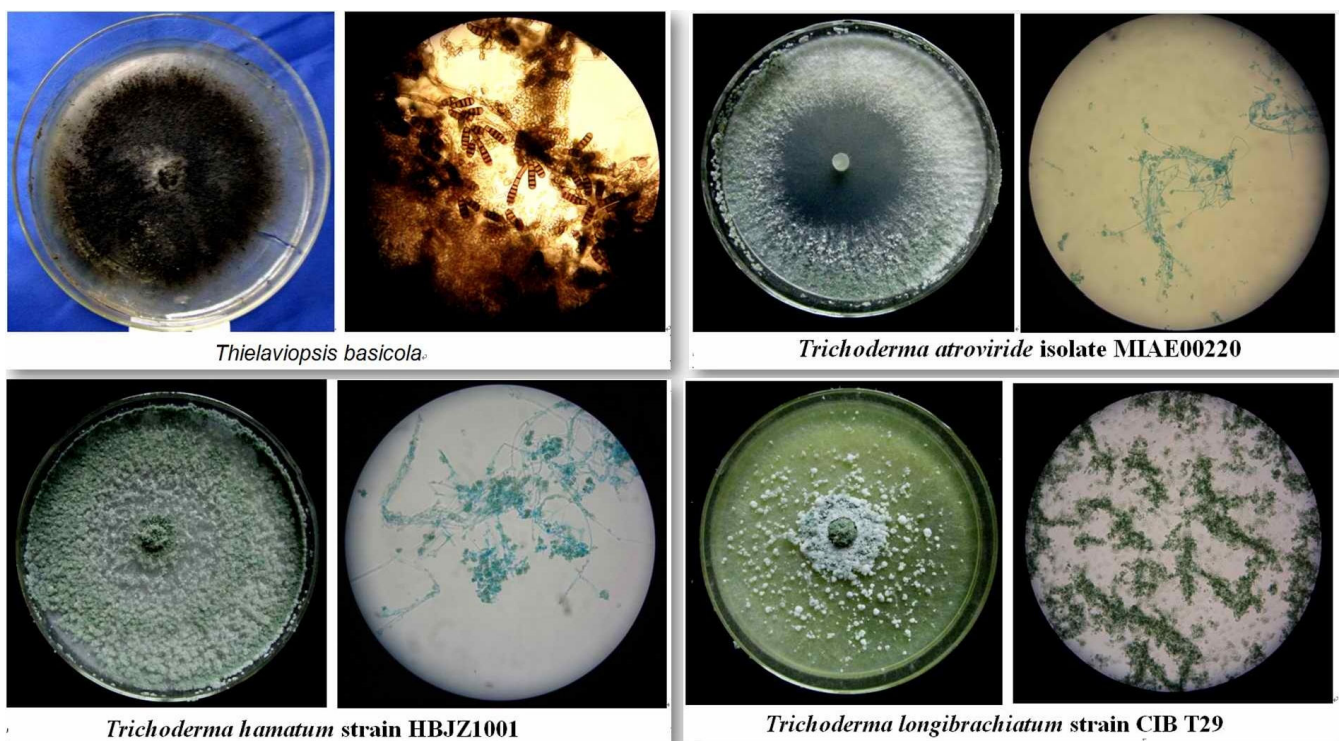

Fusarium proliferatum isolate 14
Fusarium solani strain MOD-5
Oidiodendron cerealis isolate NG_p39
Paecilomyces sp. ALASX1
Fig. 4b
Penicillium granulatum isolate 732
Penicillium verruculosum isolate CY196

**Fig. 1b**


Thielaviopsis basicola
Trichoderma atroviride isolate MIAE00220
Trichoderma hamatum strain HBJZ1001
Trichoderma longibrachiatum strain CIB T29

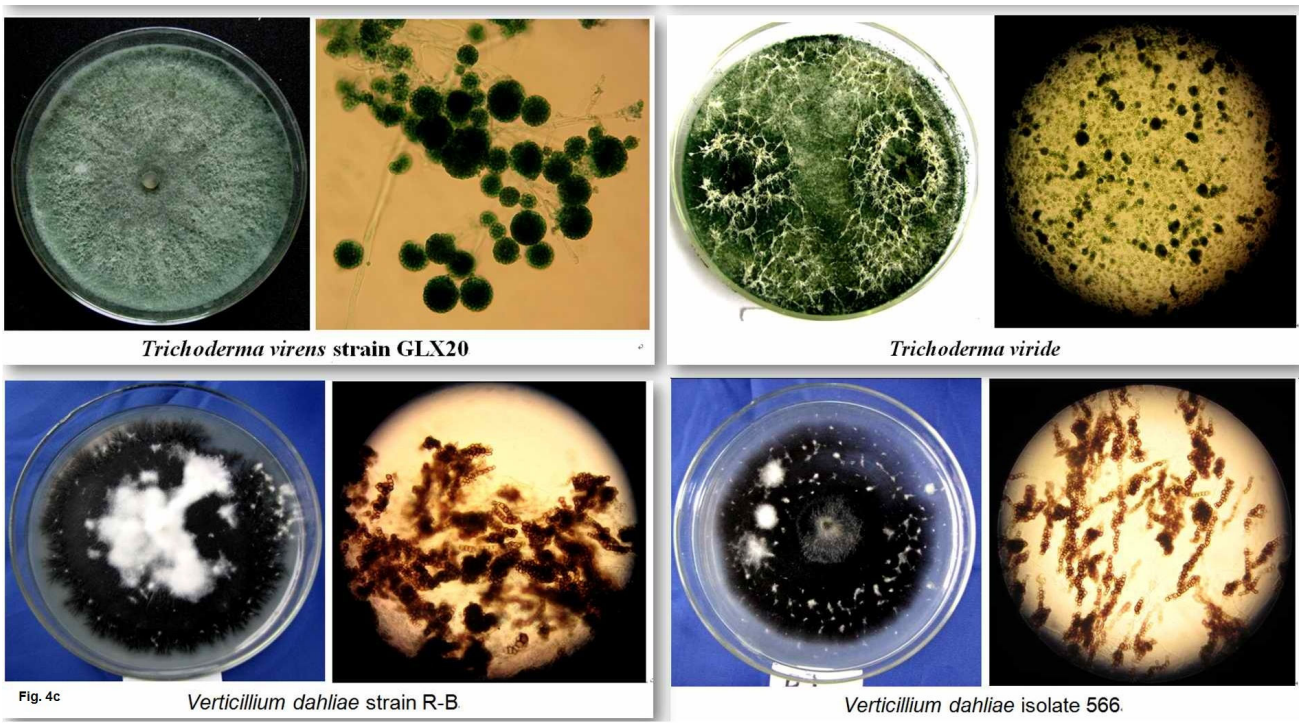



**Fig. 1c**

Fig. 1a, b and c. Morphological characteristics of fungal species isolated from Verticillium wilt infested soil and cotton plant.

## Supplementary material

**Supporting Table 1. Fungal species isolated from soil and rhizosphere of cotton plant before and after planting.**

| Soil isolates | Gen Bank Accession | Species |
|---|---|---|
| S-001 | DQ681334.1 | *Penicillium granulatum isolate 732* |
| S-002 | - | *Alternaria alternata* |
| S-003 | HQ608025.1 | *Penicillium verruculosum isolate CY196* |
| S-004 | GU799602.1 | *Verticillium dahliae isolate 566* |
| S-005 | AF099007.1 | *Trichoderma virens strain GL-20* |
| S-006 | HQ340109.1 | *Aspergillus flavus culture-collection NRRL:6412* |
| S-007 | JQ040366.1 | *Trichoderma koningiopsis strain CQSQ4004* |
| S-008 | X-93981.1 | *Trichoderma viride* |
| S-009 | - | *Aspergillus niger* |
| S-0010 | HQ379695.1 | *Gibberella intermedia isolate F22* |
| | HQ379635.1 | *Fusarium concentricum isolate F7* |
| S-0011 | FJ866504.1 | *Cercospora physalidis strain HAL 2319 F* |
| S-0012 | AF203451.1 | *Stemphylium solani strain SS1* |
| S-0013 | HQ607887.1 | *Chaetomium sp. ATT211* |
| S-0014 | EU029589.1 | *Fusarium solani isolate S-0900* |
| S-0015 | EU625405.1 | *Fusarium solani strain MOD-5* |
| S-0016 | HM008677.1 | *Fusarium equiseti isolate GGF2* |
| S-0017 | GU799602.1 | *Verticillium dahliae isolate 566* |
| S-0018 | AY433814.1 | *Alternaria alternata* |
| S-0019 | JQ040347.1 | *Trichoderma hamatum strain HBJZ1001* |
| S-0020 | HQ115707.1 | *Oidiodendron cerealis isolate NG_p39* |
| S-0021 | - | *Thielaviopsis basicola* |
| S-0022 | - | *Un-identified* |
| S-0023 | GQ169728.1 | *Alternaria alternata strain GL22* |
| S-0024 | HM626196.1 | *Paecilomyces sp. ALAS-1* |
| S-0025 | AY928419.1 | *Fusarium o-ysporum isolate FO-12* |
| S-0026 | JQ040366.1 | *Trichoderma koningiopsis strain CQSQ4004* |
| S-0027 | AF099007.1 | *Trichoderma virens strain GL-20* |
| S-0028 | EU839366.1 | *Fusarium proliferatum isolate 14* |
| S-0029 | HM176575.1 | *Trichoderma atroviride isolate MIAE00220* |
| S-0030 | FJ478089.1 | *Trichoderma longibrachiatum strain CIB* |

**Supporting Table 2. Fungal species isolated from cotton plant before and after appearance of Verticillium wilt symptoms.**

| Plant isolates | Gen Bank Accession | Species |
|---|---|---|
| P-001 | GU799602.1 | *Verticillium dahliae isolate 566* |
| P-002 | HM008677.1 | *Fusarium equiseti isolate GGF2* |
| P-003 | - | *Thielaviopsis basicola* |
| P-004 | JF499839.1 | *Cladosporium ramotenellum strain CPC 18224* |
| P-005 | GU799602.1 | *Verticillium dahliae strain R-B* |
| P-006 | HM008677.1 | *Fusarium equiseti isolate GGF2* |
| P-007 | HQ379695.1 | *Gibberella intermedia isolate F22* |
| P-008 | HQ379695.1 | *Gibberella intermedia isolate F22* |
| P-009 | - | *Alternaria alternata* |
| P-0010 | HQ340109.1 | *Aspergillus flavus culture-collection NRRL:6412* |
| P-0011 | HQ379695.1 | *Gibberella intermedia isolate F22* |
| P-0012 | HQ379647.1 | *Fusarium concentricum isolate F21* |
| P-0013 | | *Fusarium sp* |
| P-0014 | HQ607887.1 | *Chaetomium sp. ATT211* |
| P-0015 | - | *Aspergillus niger* |
| P-0016 | HQ379695.1 | *Gibberella intermedia isolate F22* |
| P-0017 | AY433814.1 | *Alternaria alternata* |
| P-0018 | HM008677.1 | *Fusarium equiseti isolate GGF2* |
| P-0019 | JN038467.1 | *Fusarium cf. equiseti MY-2011 isolate AM-26* |
| P-0020 | - | *Fusarium sp* |
| P-0021 | HM626196.1 | *Paecilomyces sp. ALAS-1* |
| | EU037060.1 | *Talaromyces spectabilis strain CBS 121583* |
| P-0022 | EU839366.1 | *Fusarium proliferatum isolate 14* |
| P-0022 | - | *Epicocum sp.* |
| P-0030 | - | *Alternaria alternata* |

**Table 2. Frequency (CFU/g) of all fungi isolated for three soil cores on three different media.**

| Isolation methods | Soil core | | Frequency percentage | |
|---|---|---|---|---|
| | | | Media | |
| Soil plating* | 10cm Soil | 46.667 a | PDA | 57.161 a |
| | 20cm Soil | 39.877 a | PCA | 56.914 a |
| | 30cm Soil | 39.383 a | VM | 11.852 b |
| | SE | 5.2248 | SE | 5.2248 |
| | CV | 10.256 | CV | 10.256 |
| Soil dilution** | 10cm Soil | 5.05e-04 a | PDA | 7.61e-04 a |
| | 20cm Soil | 5.62e-04 a | PCA | 7.37e-04 a |
| | 30cm Soil | 5.33e-04 a | VM | 1.02e-04 b |
| | SE | 4.298e-05 | SE | 4.298e-05 |
| | CV | 8.431e-05 | CV | 8.431e-05 |

*CFU/g = Total No. of colonies/Volume*Dilution factor
**CFU/g = Total No. of colonies/quantity of soil plated*40

**Supporting Table 3. Analysis of variance for soil plating methods.**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Fungi | 17 | 1.430E+07 | 841633 | 167.17 | 0.0000 |
| Core | 2 | 6786.83 | 3393 | 0.67 | 0.5098 |
| Media | 2 | 1099147 | 549574 | 109.16 | 0.0000 |
| Time | 1 | 104309 | 104309 | 20.72 | 0.0000 |
| Replication | 5 | 38238.7 | 7648 | 1.52 | 0.1806 |
| Fungi*Core | 34 | 437124 | 12857 | 2.55 | 0.0000 |
| Fungi*Media | 34 | 6735697 | 198109 | 39.35 | 0.0000 |
| Fungi*Time | 17 | 526624 | 30978 | 6.15 | 0.0000 |
| Fungi*Core*Media | 72 | 453067 | 6293 | 1.25 | 0.0794 |
| Error | 1759 | 8855895 | 5035 | - | - |
| Total | 1943 | 3.256E+07 | - | - | - |

CV= 143.92

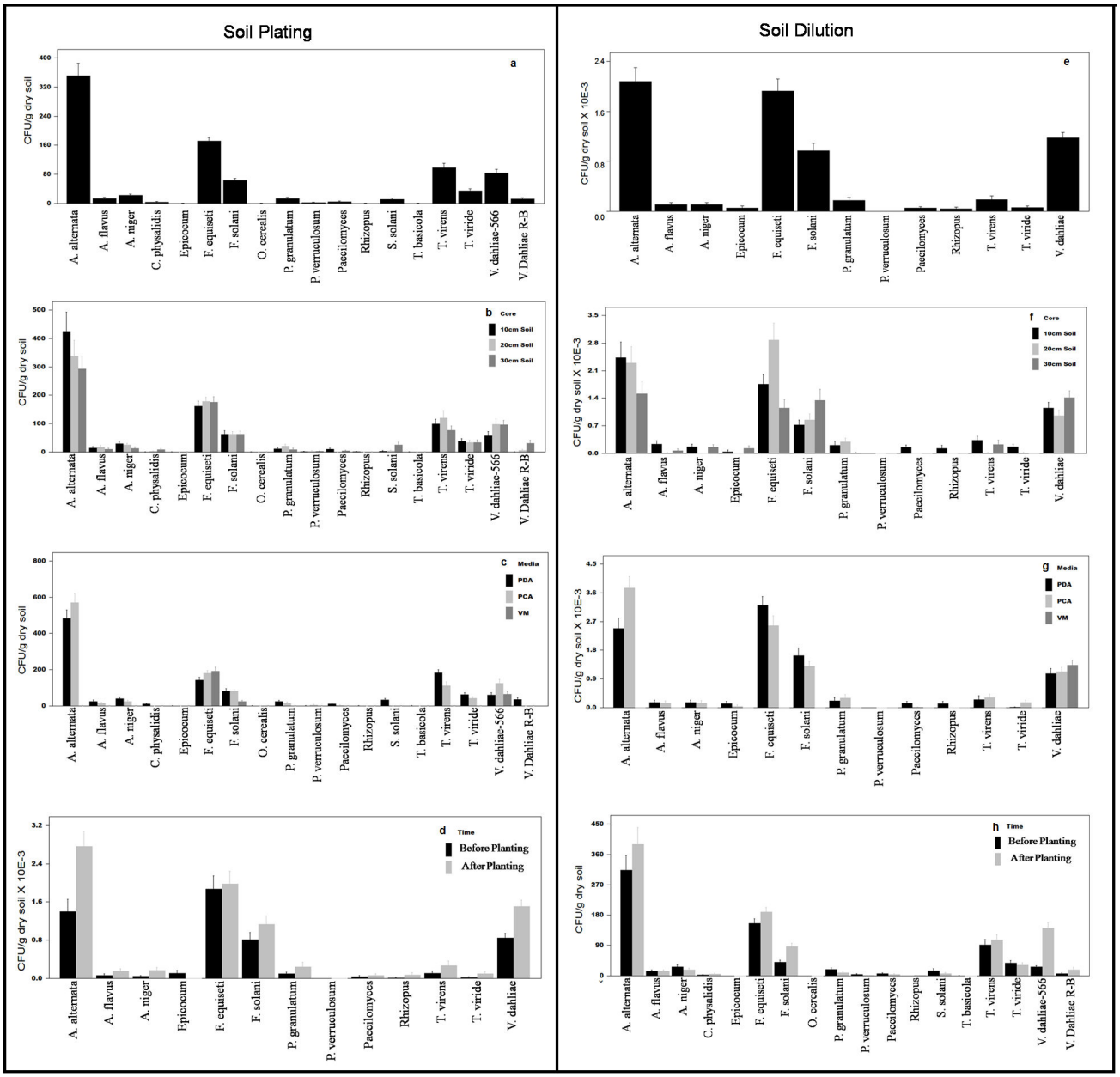



Fig. 2. CFU/g dry soil of fungal species through soil plating (A: fugal frequency; B: soil cores; C: media; D: before and after plating) and soil dilution methods (E: fugal frequency; F: soil cores; G: media; H: before and after plating).

Similarly, PDA and PCA media also favored the development of *A. alternate* as compared to other fungi. *A. alternate* produced maximum CFU/g dry soil in PCA followed by PDA media (Fig. 2). PCA media also supported the growth of *F. equiseti* and *V. dahliae*. Although, no significant difference in the CFU/g dry soil of *V. dahliae* was noted in VM and PDA media but VM was remained the best for purification of *V. dahliae* strains as compared to PDA and PCA. VM also support the development of *F. equiseti* and *F. solani* along with *V. dahliae* strains (Fig. 2). The overall frequency of all fungi significantly varied between PDA and PCA, and VM media, however, no significant for PDA and PCA media was observed in the present study (Tables 2, Supporting Table 3).

Fig. 2 also presents the CFU/g dry soil of fungi before and after planting the cotton crop that showing significantly greatest variation among all the fungi. The overall frequency of fungi after sowing was significantly higher than before sowing (Supporting Table 3).

**Soil dilution:** The frequency of fungi was recorded for three different dilutions ($10^{-1}$, $10^{-2}$, $10^{-3}$) indicating the similar progression from lower to higher CFU/g dry soil. The third dilution ($10^{-3}$) showed lower CFU/g dry soil of each fungus as compared to first dilution (10-1). The data presented in Fig. 2 was obtained only from one dilution (10-1). Similar to soil plating, the CFU/g dry soil of different fungi significantly varied (Supporting Table 4). The response of different media was also remained similar (Supporting Table 4). The CFU/g dry soil of fungi before and after planting for soil dilution was also significantly varied. The frequency of fungi after sowing was significantly higher than before sowing (Fig. 2, Supporting Table 4).

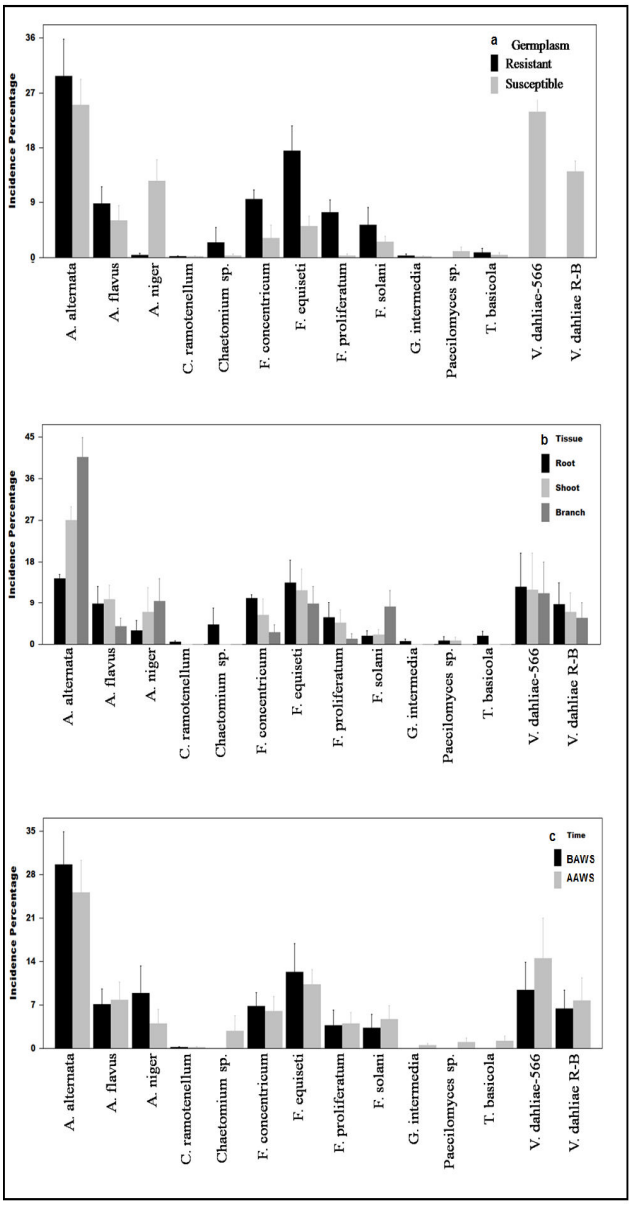



Fig. 3. Incidence percentage of fungal species isolated from Verticillium wilt affected germplasm.
(Note: A: Resistant (Arcot-1) and Susceptible (Arcot-438); B: tissues; C: BAWS means before appearance of wilt symptoms; AAWS means after appearance of wilt symptoms)

**Direct isolation of cotton plant:** Both resistance and susceptible germplasm showed varied response in the association of fungi. The highest incidence percentage of *A. alternata, F. equiseti, F. concentricum, A. flavus, F. proliferatum, Fusarium* sp. and *Chaetomium* sp. was recorded with resistance germplasm, whereas, *V. dahliae* strain-566, *V. dahliae* stain R-B, *A. niger* and *Paecilomyces* sp were observed with susceptible germplasm. No significant difference was observed in incidence percentage of *T. basicola, C. ramotenellum* and *G. intermedia* from resistance and susceptible germplasm (Fig. 3, Supporting Table 5). *A. alternata* followed by *Fusarium* sp and *A. niger* showed the highest incidence percentage in the branch of plant as compared to shoot and root. Whereas, *V. dahliae* strain-566, *V.dahliae* stain R-B, *F. equiseti, F. concentricum, F. proliferatum* and *A. flavus* were observed with significantly highest incidence percentage from root and shoot as compared to branches (Fig. 3).

Fig. 3 presenting the incidence percentage of fungi which were associated with cotton plant before and after appearance of wilt symptoms (BAWS & AAWS), showed the greatest variation among the fungal frequency especially in *V. dahliae* strain-566 and *V. dahliae* stain R-B. The incidence percentage of *A. alternata, F. equiseti, A.niger, A. flavus* and *F. concentricum* were remained higher BAWS as compared to AAWS. On the contrary the incidence percentage of *V. dahliae* strain-566, *V. dahliae* stain R-B, *Fusarium* sp., *F. proliferatum, Chaetomium* sp., *T. basicola, Paecilomyces* sp. and *G. intermedia* was higher AAWS (Fig. 3).

***In vitro* efficacy of antagonistic fungi:** Different antagonistic fungi from isolated from soil as well as Verticillium wilt affected cotton plants were tested *In vitro*, showed the greatest inhibition of *V. dahliae*. Significantly maximum inhibition percentage of colony diameter of *V. dahliae* was observed when treated with *T. longibrachiatum* (41.038%) followed by *T. atroviride, Paecilomyces* sp. and *T. viride* as compared to control. However, there was no significant difference found among these four fungi for the inhibition percentage. *T. longibrachiatum* also produced chemicals during the growth period which decolourised the media as compared to other treatments, indicates their strongest efficacy (Figs. 4 & 5, Supporting Table 6).

**Supporting Table 4. Analysis of variance for soil dilution method.**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Fungi | 12 | 7.298E-04 | 6.082E-05 | 140.73 | 0.0000 |
| Core | 2 | 3.571E-06 | 1.785E-06 | 4.13 | 0.0163 |
| Media | 2 | 1.305E-04 | 6.524E-05 | 150.96 | 0.0000 |
| Time | 1 | 1.892E-05 | 1.892E-05 | 43.79 | 0.0000 |
| Replication | 5 | 5.170E-06 | 1.034E-06 | 2.39 | 0.0359 |
| Fungi*Core | 24 | 8.753E-05 | 3.647E-06 | 8.44 | 0.0000 |
| Fungi*Media | 24 | 4.004E-04 | 1.668E-05 | 38.60 | 0.0000 |
| Fungi*Time | 12 | 4.853E-05 | 4.044E-06 | 9.36 | 0.0000 |
| Fungi*Core*Media | 52 | 9.908E-05 | 1.905E-06 | 4.41 | 0.0000 |
| Error | 1269 | 5.484E-04 | 4.322E-07 | - | - |
| Total | 1403 | 0.00207 | - | - | - |

CV =123.23

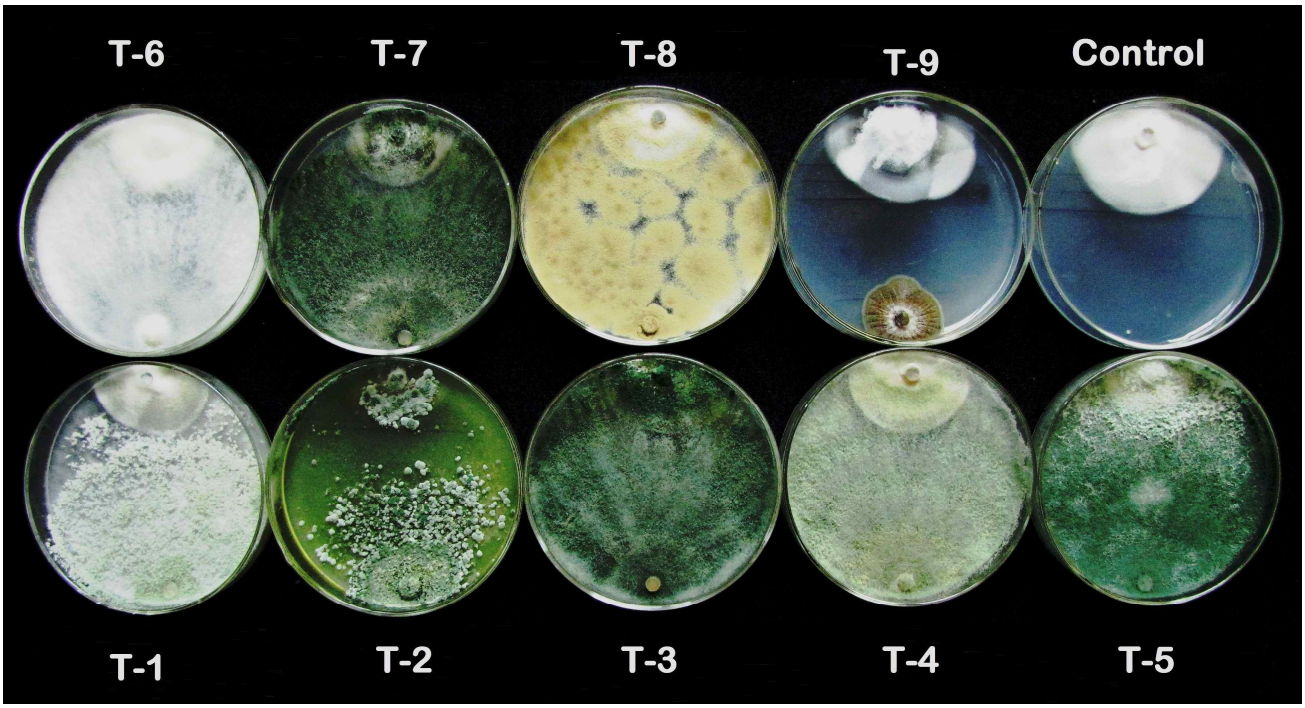



Fig. 5. Antagonistic potential of some fungi under *In vitro* condition against *Verticillium dahliae* Kleb.
(T-1 = *Trichoderma hamatum*; T-2 = *T.longibrachiatum*; T-3 = *T.virens*; T-4= *T.koningiopsis*; T-5 = *T.viride*; T-6 = *T. atroviride*; T-7 = *T.virens*; T-8 = *Paecilomyces* sp; T-9 = *Chaetomium* sp)
**Note:** Upper polar is *Verticillium dahliae;* Lower polar is tested fungus

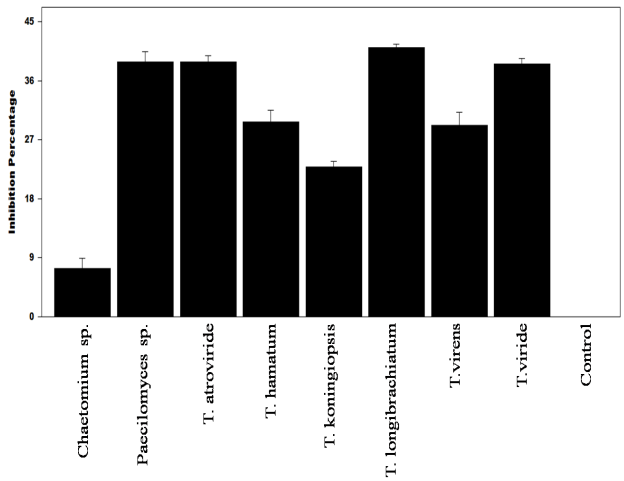



Fig. 4. Efficacy of antagonistic fungi under *In vitro* condition against *Verticillium dahliae* Kleb.

## Discussion

The diversity of microbes exists in the soil, which may or may not be beneficial for the main host/crop. Several studies had already been conducted pertaining to diagnosis of specific disease, however, rarely evident are available for the association of other fungi with Verticillium wilt of cotton except *V. dahliae* Kleb. It is also important to know that the management of any plant disease needs complete information and/or history of the crop. In addition, the knowledge about association and interaction of other micro-organism is also important step for designing of management guidelines and tools. Our study is the preliminary attempt to find the associations of pathogenic and antagonistic fungi with Verticillium wilt of cotton. It indicates that the diversity of fungi with lower and higher frequency is associated with the soil and rhizosphere of Verticillium wilt of cotton. It is also obvious from present study that there is variability in the frequency of pathogenic and antagonistic fungi in the cotton germplasm. Though some antagonistic fungi are present with higher frequency in comparison to pathogenic but their association with Verticillium wilt is not quite effective under field conditions. This may be due to adverse effect of environmental conditions, preference of host plant and genetic potential of germplasm. Almost all antagonistic species are screened out from soil, however; only *Paecilomyces* sp. and *Chaetomium* sp. have been associated with cotton rhizosphere as well as with wilt affected cotton plants. These fungi need to be studied in details for biological control. Our study is inconsistent with Zheng *et al.,* (2011) who screened fungal isolates from the endorhiza, rhizosphere, and bulk soil of field-grown cotton plants and demonstrated that the endorhiza of field-grown cotton plants may be a richer source of potential BCAs against Verticillium wilt than the rhizosphere and bulk soil. The present study suggested that the rhizosphere of the cotton plant have the maximum frequency of antagonist than the cotton plant.

Present studies also determine the association of fungi with cotton plant and found the specific association regarding to germplasm and plant parts including root, shoot and branches. Both resistance and susceptible germplasm have different response in the association of fungi. Some fungi are associated with resistant germplasm others with susceptible germplasm. However, some fungi are associated with both resistance and susceptible

germplasm. This association indicates that some fungi have more interaction with Verticillium wilt affected plants. Though, some of these such as *Paecilomyces* and *Chaetomium* species are reported as bio-agents but not remained quite effective for the suppression of *V. dahliae* strain under field conditions. However, there is no specific reference available pertaining to association of fungi with Verticillium wilt of cotton for comparison. The *In vitro* test of some fungi screened as best bio-control agents against *V. dahliae*. Due to the influence of various factors such as temperature, humidity and preference of host plant their antagonistic potential is not quite effective under field condition. Similarly, Zheng *et al.,* (2011) also screened some other effective bio-agents against Verticillium wilt. But our study screened the different efficient bio-agents than the previous study.

It is obvious from our results that isolation method has varied response pertaining to frequency of fungi. Soil plating method favored the development of maximum number of fungi compare to soil dilution. But the fungi grown faster have the influence over the development of slow growing fungi through soil plating. Soil dilution scattered the propagules of fungi due to series of dilution and is useful for purification of fungi, however, it may also inhibit the growth of some fungi which can be affected/inactivated through high content of water. Our study is in agreement with Czaban & Wroblewska (2006).

The present study is the preliminary attempt and provides the baseline information about fungal diversity associated with Verticillium wilt disease. The efficient bio-agents of the current study may also be used for future studies to overcome the Verticillium wilt of cotton. This information hopefully will be helpful in designing the management practices especially for biological control of this serious threat.

**Supporting Table 5. Analysis of variance for incidence percentage of fungi in cotton germplasm.**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Fungi | 13 | 8413.69 | 647.207 | 17.99 | 0.0000 |
| Germplasm | 1 | 33.76 | 33.763 | 0.94 | 0.3348 |
| Time | 1 | 0.91 | 0.909 | 0.03 | 0.8740 |
| Tissues | 2 | 3.55 | 1.777 | 0.05 | 0.9518 |
| Fungi*Germplasm | 13 | 4056.49 | 312.038 | 8.67 | 0.0000 |
| Fungi*Time | 13 | 278.46 | 21.420 | 0.60 | 0.8535 |
| Fungi*Tissue | 26 | 1993.69 | 76.681 | 2.13 | 0.0036 |
| Error | 110 | 3958.18 | 35.983 | - | - |
| Total | 179 | | - | - | - |

CV =94.72

**Supporting Table 6. Analysis of variance for inhibition percentage of antagonistic fungi.**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Antagonistic Fungi | 8 | 10525.5 | 1315.69 | 84.60 | 0.0000 |
| Replication | 5 | 143.8 | 28.76 | 1.85 | 0.1219 |
| Error | 46 | 715.4 | 15.55 | - | - |
| Total | 59 | - | - | - | - |

CV= 14.41

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