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Genetic diversity of *Fusarium* Isolated from Members of *Sternorrhyncha* (Hemiptera): Entomopathogens against *Bemisia tabaci*

Waheed Anwar*, Muhammad S. Haider, Ahmad A. Shahid, Hamid Mushtaq, Usman Hameed, Muhammad Zia Ur Rehman and Muhammad Javed Iqbal

Institute of Agricultural Sciences, University of the Punjab, 54590, Lahore, Pakistan.

ABSTRACT

In the present study, fungal flora from families *Aleyrodidae*, *Aphididae* and *Coccidae* of the order Hemiptera, suborder *Sternorrhyncha,* were isolated from three different agroecological zones of Pakistan. *Fusarium equiseti, Fusarium solani*, *Fusarium incarnatum* and *Fusarium* sp. along with other fungi were isolated and characterized morphologically as well as genetically by amplifying internal transcribed spacer region (ITS). Variability among *Fusarium* species based on ITS showed that it is not enough to score diversity within species and isolates. Additionally pathogenicity of isolated *Fusarium* species was evaluated against nymph and adult of *Bemisia tabaci*. Under controlled conditions different species of *Fusarium* restrained the growth of *B. tabaci* as compared with control.

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Authors' Contributions
MSH and AAS conceived and designed the study. WA, HM and JI surveyed and collected the samples. WA isolated and identified the fungi. WA and MZR maintained *Bemisia tabaci* and analyzed the data. WA and UH performed bioassays and wrote the article.

Key words
Fungi associated with insect, ITS, *Bemisia tabaci*, Entomopathogens, Fungal diversity

INTRODUCTION

The sweet potato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) is a major pest of economically important crops worldwide (Xu *et al*., 2012). *B. tabaci* is a species complex composed of more than 35 morphologically indistinguishable cryptic species which exhibit a wide range of genetic variations and many species of this complex show a specific pattern of geographic distribution (Liu *et al*., 2012; Tay *et al*., 2012). Some members of this species complex are considered major pests for a wide range of agricultural crops causing damage directly through excessive sap feeding and, indirectly through transmission of plant pathogenic viruses that whitefly acquire when feeding on phloem sap. There are 288 species of begomoviruses (family Gemniviridae) which are known to be transmitted by *B. tabaci* (Brown *et al*., 2015).

Fusarium species are mainly soil borne fungi and frequently associated with plant roots as parasites causing serious diseases on many plants all over the world (Bockus *et al*., 2007). *Fusarium* related diseases include citrus decline, vascular wilts, crown rot and root rot, head blight on cereal grain and Bakanae disease on rice (Safdar *et al*., 2013; Saremi *et al*., 2007). On the other hand some of *Fusarium* species were reported as entomopathogens and may live as saprophyte on dead insects (Teetor-Barsch and Roberts, 1983; Sun, 2008). As entomopathogens, some *Fusarium* species have been reported to cause moderate to high levels of infection, principally against homopterous and dipterous insects. However, low to moderate levels of infections with these entomopathogens have been reported from insects of other orders (*e.g*., Coleoptera and Lepidoptera) (Teetor-Barsch and Roberts, 1983). Many species of entomopathogenic *Fusarium* may kill their host insects through the activity of toxins produced by penetrating hyphae (Gupta *et al*., 1991).

Here, we also observed the genetic diversity of *Fusarium* species isolated from insects on the bases of Internal transcribed spacer region (ITS). ITS genetic diversity has been reported within species and individuals from a diverse range of eukaryotes, including insects (Fairley *et al*., 2005; Li and Wilkerson, 2007), marine sponges (Vierna *et al*., 2010) and fungi (Smith *et al*., 2007; Horton, 2002). The ITS regions are located between the repeating array of nuclear 18S, 5.8S and 28S ribosomal RNA genes and have 100-200 copies/genome. These regions are rapidly evolving and thus have been routinely used in species-level phylogeny in a wide range of organisms (Blouin, 2002) and this region can be used for taxonomic evidence of fungi (Kolawole *et al*., 2015).

In the present study, different strains of *Fusarium* infecting families in the suborder Sternorrhyncha were isolated, identified on morphological and molecular

* Corresponding author: waheedanwar.iags@pu.edu.pk
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bases. Further the pathogenicity of isolated *Fusarium* species were tested against *B. tabaci.* This study provides information about a potential use of fungus as biological control agent against whitefly.

MATERIALS AND METHODS

Collection of insect samples

Detailed survey of cotton fields for sample collection was conducted in different localities of three agroecological zones of Punjab, Pakistan. These regions include: (1) Hot arid Zone (Layyah) (2) Cotton Zone (Bahawalpur) (3) Central mixed zone (Lahore). Naturally dead *Aphis gossypii* from family *Aphididae*, whitefly from family *Aleyrodidae* and mealybug from family *Coccidae* from suborder *Sternorrhyncha* infected with fungi were collected from upper leaves from 3 to 4 feet above the soil level. Ten insect samples of each species from each field were collected, carried out separately in sterilized jars and stored at 4°C for isolation of total fungi associated with them.

Isolation of fungi

For the isolation of fungi, sabouraud dextrose peptone yeast extract agar (SDAY) plates with one quarter strength was used (1/4). Insect samples were surface sterilized with 1% sodium hypochloride solution for 1 min followed by washing with sterilized distilled water and transferred to SDAY media plates. Plates were incubated at 25±1°C for 7 days. Total isolated fungi from each insect were purified before being stored at 4°C as pure culture. Pure cultures were sub-cultured on SDAY media plates in order to conduct bioassay.

Characterization of fungi

Morphological characterizations of isolated fungi were studied using different dichotomous keys (John *et al*., 2006; Domsch *et al*., 1980; Seifert, 1996) and submitted to First Fungal Culture Bank of Pakistan (FCBP) for accessions numbers.

Identification based on morphological characterization was complimented with amplification and sequencing of internal transcribed spacer (ITS) region. For this purpose, total DNA of isolated fungi was extracted by modified CTAB method (Stenglein and Balatti, 2006). DNA was extracted by sub culturing fungi in SDAY broth media and it was incubated at 26 C for 7 days at 100 rpm. The extracted DNA was analyzed on 1% agarose gel. Internal transcribed spacer region was amplified using ITS1 and ITS4 primers (White *et al*., 1990). PCR amplification was carried out in 25 µL reaction volumes containing 20–100 ng genomic DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 200 uM of each dNTP, 0.4 mM of each primer and 1 uL Taq polymerase. The PCR amplification reactions were carried under following conditions ; one cycle of denaturation for 3 min at 94°C followed by 30 cycles of denaturation for 1 min at 94°C, annealing at 50°C for 1 min, elongation at 72°C for 1 min and final extension of 72°C at 10 min. The amplified PCR products were resolved on 1% agarose gel. Amplified PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, United States). Purified PCR products were sequenced from 1st BASE Malaysia using above mentioned primer based on procedure described by Sanger *et al*. (1977). Sequences were submitted to the NCBI database for the assignment of Genbank accession numbers.

Sequence and statistical analysis

Alignment and determination of consensus sequences were carried out using BioEdit (Hall, 1999). Homologous sequences in the databases were searched using the Basic Local Alignment Search Tool (Altschul *et al*., 1997), while phylogenetic tree was constructed using MEGA software version 7.0. The statistical procedure for phylogenetic tree construction used was the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) while 10000 bootstrap replications were carried out.

Pathogenicity bioassay against Bemisia tabaci

The *Fusarium* isolates sub-cultured on Dextrose Peptone yeast extract Agar (SDAY) medium were utilized for preparation of stock solutions and tested for their pathogenicity against *B. tabaci.* The healthy nymph and adult *B. tabaci* were used for all screening bioassay procedure. The stock spore suspension of fungal concentration of 4.0 x 10^8 / mL was prepared with the help of haemocytometer and further diluted up to concentration of 4.0 x 10^4 /mL. Pathogenicity test was conducted on the test by spraying different spore suspension on healthy nymph and adult *B. tabaci* as compare to control treated with sterilized water.

Data regarding mortality rate in nymphs and adults of *B. tabaci* was observed after 24 h intervals by using modified Abbott's formula (Fleming and Ratnakaran, 1985).

$$\text{Mortality (\%)} = 1 - \frac{\text{PoTN/APT}}{\text{PrTN/APT}} \times \frac{\text{PrTN/APC}}{\text{PoTN/APC}} \times 100$$

Where; PoTN, post-treated nymph; PrTN, pre-treated nymph; APT, adult population in treatment; APC, adult population in control.

Excel was used to compute the standard errors of means of five replicates. All the results were subjected to ANOVA followed by mean separation through Duncan's

Table I.- Different species of *Fusarium* isolated from Order Hemiptera suborder *Sternorrhyncha,* families *Aleyrodidae*, *Aphididae* and *Coccidae.*

FCBP acc. No.	Name of fungi	Source of isolation	Genbank No (ITS)
FCBP-EPF-1299	*Fusarium equiseti*	*Aphis gossypii* of Cotton [Field, IAGS], Central Mixed Zone	LN827599
FCBP-EPF-1301	*Fusarium incarnatum*	*Bemisia tabaci*, [IAGS field], Central Mixed Zone	LN827601
FCBP-EPF-1302	*Fusarium equiseti*	Mealybug, [IAGS field], Central Mixed Zone	LN827600
FCBP-EPF-1304	*Fusarium oxysporum*	*Aphis gossypii*, cotton field, [Bahawalpur], Cotton Zone	N/A
FCBP-EPF-1376	*Fusarium* sp.	Mealybug of cotton field, IAGS, [Lahore], Central Mixed Zone	LN827602
FCBP-EPF-1389	*Fusarium equiseti*	*Bemisia tabaci* of cotton Field, IAGS, Central Mixed Zone	LN827603
FCBP-EPF-1420	*Fusarium solani*	*Bemisia tabaci*, [IAGS field], Central Mixed Zone	LT159846

multiple range test (P≤0.05) (Steel and Torrie, 1980) using computer software CO-STAT.

RESULTS AND DISCUSSION

Different fungi from various genera were isolated from different agroecological zones along with five species of *Fusarium* from families *Aleyrodidae*, *Aphididae* and *Coccidae* of order Hemiptera and suborder *Sternorrhyncha,*. Different species of *Fusarium* were isolated from Central mixed zone, Cotton zone and hot arid zone with many isolates. Details of *Fusarium* isolates are given in (Table I).

Genetic variations among isolates of fusarium species

Genetic variations among internal transcribed spacer region of *Fusarium* species isolated in this study were compared with the other isolates taken from NCBI. A phylogenetic tree was constructed and it was observed that *Fusarium equiseti* (LN827600) isolated from insect could not be categorized in the same group in which 2 other isolates (LN827599 and LN827603) formed a cluster with other isolates of *F. equiseti* taken from data base. *Fusarium* sp. (LN827602) and *F. incarnatum* (LN827601) both isolates of insects were classified in the same main cluster and having different sub clusters as mentioned in (Fig. 1). Whereas, *F. solani* (Sample X) isolated from insect was placed in the same cluster along with the other isolates of data base.

Pathogenicity bioassay against Bemisia tabaci

Pathogenicity of different isolated *Fusarium* species and their isolates were tested upon healthy nymph and adult whitefly to check mortality. It was observed that no isolate showed mortality till 48 h when 4.0 x 10^4 / mL spore suspension of different isolates of *Fusarium* were applied on nymph stage of *B. tabaci*. After 72 h, *F. equiseti* FCBP-EPF-1299 and FCBP-EPF-1389 showed maximum mortality of 26.67% while *F. incarnatum* FCBP-EPF-1301 showed minimum mortality of 13.33%.

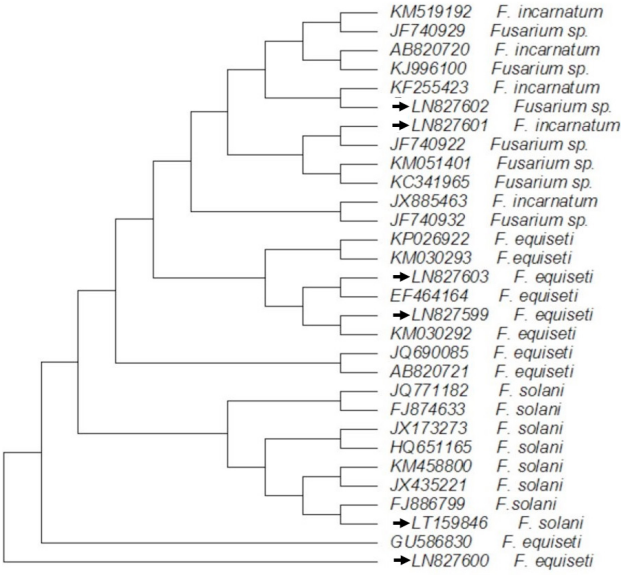

Fig. 1. Maximum likelihood tree formed Using Mega 7 Software. → Fusarium isolates of recent study.

Similarly after 96 and 120 h, maximum mortality was observed in isolates of *F. equiseti* followed by *F. solani* and *F. oxysporum* while minimum mortality was observed for *F. incarnatum*. After 144 h, all the isolates showed 100% mortality (Fig. 2A). Infection of *Fusarium* on nymph and adult *B. tabaci* were shown in Figure 4. When 4.0x10^8 / mL spore suspension of different isolates of *Fusarium* were applied on nymph stage of *B. tabaci*, it was observed that except *F. incarnatum* and *F. oxysporum* all isolates showed mortality after 48 h. After 72, 96 and 120 h, maximum mortality was observed in isolates of *F. equiseti* followed by *F. solani* and *F. oxysporum* while minimum mortality was noticed by F. *incarnatum*. After 144 h, all isolates of *Fusarium* showed 100% mortality (Fig. 2B).

A significant difference was observed in mortality rate of *B. tabaci* Nymph by isolates of *F. equiseti* as compared with other species. After different time durations a

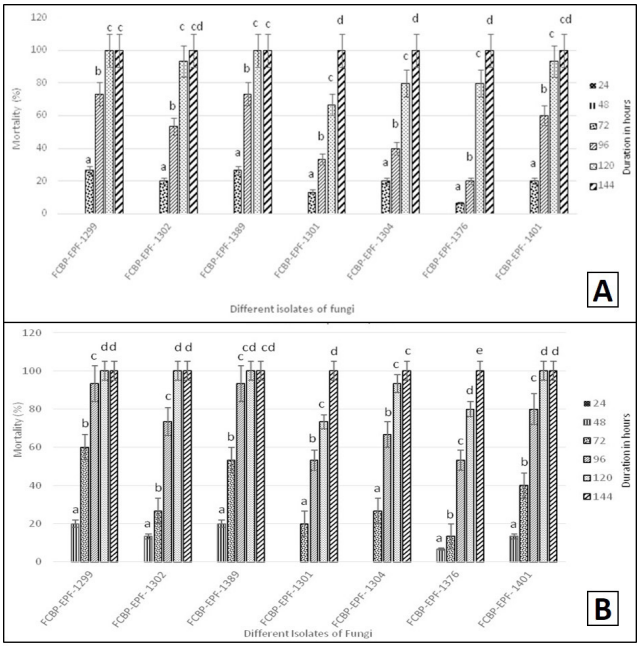

Fig. 2. Mortality of *B. tabaci* nymph with different isolates of *Fusarium* at the concentration of 4.0 x 10^4 spores/ mL. (A) and 4.0 x 10^8 spores/ mL (B).

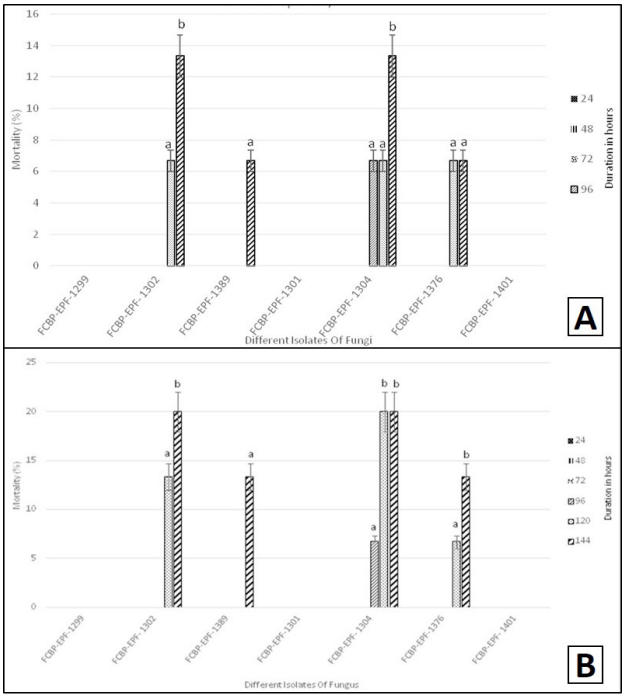

Fig. 3. Mortality of *B. tabaci* adult with different isolates of *Fusarium* at the concentration of 4.0 x 10^4 spores/ mL (A) and 4.0 x 10^8 spores/ mL (B).

significant difference was also observed by single isolate. When 4.0x10^4 /mL spore suspension of different isolates of *Fusarium* were applied on adult *B. tabaci*, it was observed that only *F. oxysporum*, *Fusarium* sp. and *F. equiseti* (FCBP-EPF-1302) showed 6.67% mortality up till 120 h. After 144 h, *F. equiseti* (FCBP-EPF-1302) and *F. oxysporum* showed 13.33% mortality while *F. equiseti* (FCBP-EPF-1389) and *Fusarium* sp. showed 6.67% mortality only (Fig. 3A).

When 4.0 x 10^8 / mL spore suspension of different *Fusarium* isolates were applied on adult *B. tabaci,* only *F. oxysporum* showed mortality after 96 h. After 120 h, *F. equiseti* (FCBP-EPF-1302), *F. oxysporum* and *Fusarium* sp. showed mortality. Maximum mortality of 20% was shown by *F. equiseti* (FCBP-EPF-1302) and *F. oxysporum* after 144 h (Fig. 3B).

There was no significant difference in mortality of adult *B. tabaci* between *F. equiseti* (FCBP-EPF-1302) and *F. oxysporum* (FCBP-EPF-1304) while significant difference in both was found when compared with other isolates used in this study.

In this study, we reported different species of *Fusarium* isolated from mealybug, aphid and whitefly. Most of the species were isolated first time from these insects and these fungi show mortality against *Bemisia tabaci*. Similarly, Jouda *et al*. (2010) isolated *Fusarium sacchari* and *Fusarium semitectum* from aphid and also showed Pathogenicity against it. Pelizza *et al*. (2011) reported *F. verticillioides* from grasshoppers. *Fusarium equiseti* (Corda) Sacc. was reported as a fungal parasite of the Brinjal mealybug, *Coccidohystrix insolita* (Green), in 1982 (Gopinathan *et al*., 1982). *F. incarnatum-equiseti* complex were also reported from a brown soft scale (Fan *et al*., 2014). These all studies showed that *Fusarium* is entomopathogenic in nature and cause Pathogenicity against different insects. According to our knowledge, isolates *Fusarium equiseti* are reported first time in this study from aphid, mealybug and *Bemisia tabaci* and it is obvious from the present study that among all the *Fusarium* species, *F. equiseti* (FCBP-EPF-1304) and *F. oxysporum* FCBP-1304 were most virulent and showed significant rate of mortality on nymph and adult *B. tabaci*. Further studies are required for best optimization of conidial suspension of these *Fusarium* species for their effective use in future.

CONCLUSION

In this study, it is found that different species of *Fusarium* isolated from insects showed similarity on ITS region with the same species isolated from different sources. *Fusarium equiseti* and *Fusarium oxysporum* showed maximum

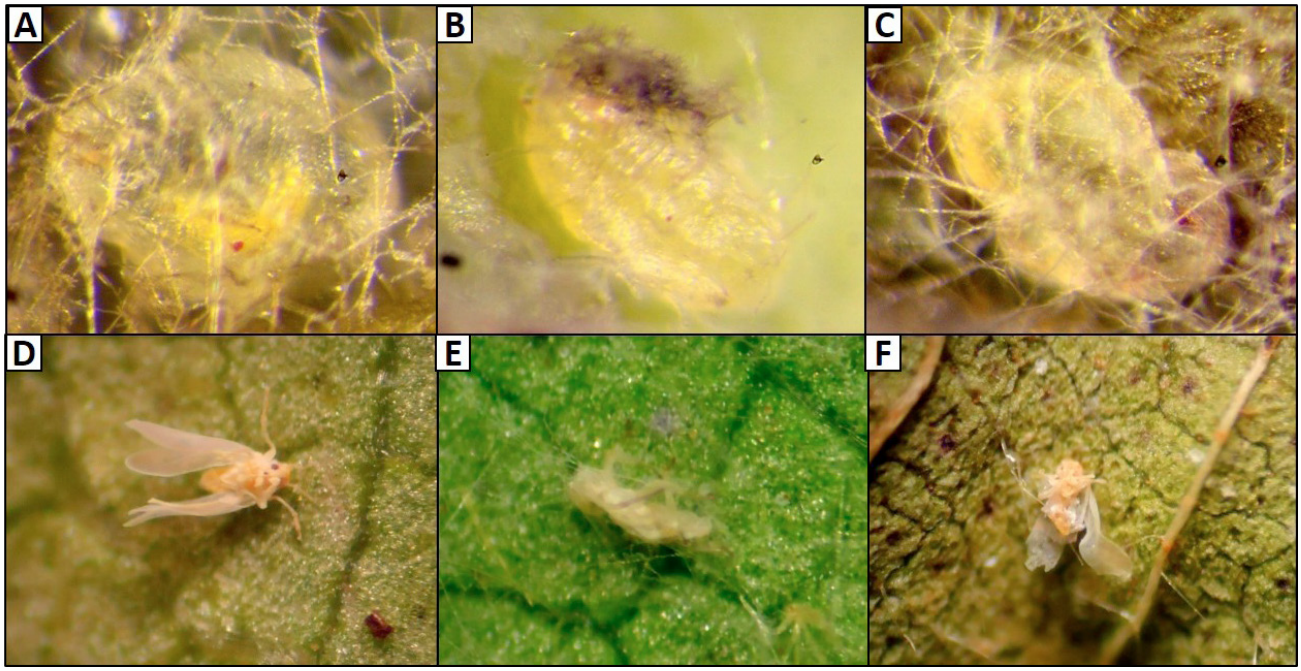

Fig. 4. Infection caused by different isolates of *Fusarium*. (A) Nymph infected by *F. oxysporum,* FCBP-1304. (B) Nymph infected by *F. equiseti*, FCBP-1302 (C) Nymph infected by *F. equiseti*, FCBP-1389 (D) Adult infected by *F. equiseti*, FCBP-1389 (E) Adult infected by *F. equiseti*, FCBP-1302. (F) Adult infected by *F. oxysporum*, FCBP-1304.

mortality as compare to others species against nymph and adult *B. tabaci* so these species can be used in conserved biological control strategies against *B. tabaci*. Therefore further studies are necessary to know the proper infection mechanism of *Fusarium* species against whitefly.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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