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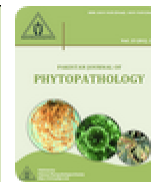

FIRST RECORD OF *TRICHODERMA LONGIBRACHIATUM* AS ENTOMOPATHOGENIC FUNGI AGAINST *BEMISIA TABACI* IN PAKISTAN

Waheed Anwar, Muhammad N. Subhani*, Muhammad S. Haider, Ahmad A. Shahid, Hamid Mushtaq, Muhammad Z.U. Rehman, Usman Hameed, Sidra Javed

Institute of Agricultural Sciences (IAGS), University of the Punjab, Lahore, Pakistan.

A B S T R A C T

The purpose of this research was to find indeginous insect associated fungi and their virulence against *Bemisia tabaci.* In present study, *Trichoderma longibrachiatum* was isolated from cotton mealybug and its pathogenicity test was conducted against *Bemisia tabaci* under controlled conditions. Entomopathogenic potential of *T. longibrachiatum* was accessed on nymphal and adult stages of *B. tabaci*. Entomopathogenic activity of *T. longibrachiatum* was severe on nymphal stage as compared to adult *B. tabaci*. Germination of conidia on outer surface of nymphal and adult stage of whitefly was also observed. The Results revealed that *T. longibrachiatum* is an entomopathogenic fungi against *B. tabaci*. *Trichoderma* is previously known as antagonistic organism against broad spectrum fungal as well as bacterial plant pathogens but it is not commonly found as insect pathogen.

Keywords: Biocontrol, Entomopathogens, Pathogenicity, Whitefly

INTRODUCTION

The *Bemisia tabaci* Gennadius belonging to order Hemiptera suborder Aleyrodidae is an economical important pest worldwide which cause losses in different crops (Xu *et al.,* 2012). *B. tabaci* feeds on phloem sap on the underside of plant leaf and produces huge quantity of sticky honeydew which retards the photosynthesis of leaf by blocking stomata. Although the use of chemical insecticides in different combinations are the key denominator in *B. tabaci* to control stretegies (Prabhaker *et al.,* 1998; Palumbo *et al.,* 2001). Chemical as inputs are increasing day by day and effects negatively by developing resistance to the pathogens and also harmful impacts on environment (Gaigole *et al.,* 2011; Gerhardson 2002). Biological control has emerged as one of the most important method for the management of soil-borne plant pathogens (Kamal *et al.,* 2012; Bal *et al.,* 2014). Among the natural enemies of insects different entomopathogenic fungi are mostly used as biological control of pest (Eilenberg *et al*., 2001). More than 20 different species of entomopathogenic fungi have been known to infect *B. tabaci* (Steenberg and Humber, 1999) but *Paecilomyces* , *Lecanicillium* and *B. bassiana* have been most widely studied and used to control pests (Poprawski *et al*., 2000; Cuthbertson *et al.,* 2012).

Many species of *Trichoderma* have evolved a synergistic association with plants, promoting the plants growth by providing nutrients and producing elicitors which stimulate the expression of the genes involved in plant defense mechanism (Harman, 2000). *Trichoderma* release a variety of compounds that induce localized or systemic resistance responses, and this explains their lack of pathogenicity to plants (Leelavathi *et al*., 2014). *T. longibrachiatum* has been previously reported from Cowpea aphid, *Aphis craccivora* Koch (Ibrahim, 2011) and its soil isolate showed entomopathogenic activity against *Leucinodes orbonalis* which is one of the major pests of brinjal (eggplant, *Solanum melongena*) (Gosh and Pal, 2016).

In present study, we isolated *T. longibrachiatum* from cotton mealy bug in different localities of Pakistan and bioassayed its pathogenicity against 4th instar nymphal and adult *B. tabaci.*

* Corresponding Author:
Email: nasirsubhani@hotmail.com
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MATERIALS AND METHODS

Survey and Sampling: Detailed survey of cotton fields for sample collection was conducted during July and August 2013 in Layyah from hot arid zone and Lahore from central mixed zone of Punjab, Pakistan. Naturally dead mealybug of family Coccidae, suborder Sternorrhyncha infected with fungal growth was collected from leaves, 3 to 4 feet above the soil level. Ten insect samples of each family from each field were collected, carried separately in sterilized jars and stored at 4°C for isolation of fungi associated with them.

Isolation and Characterization of *T. longibrachiatum:* For the isolation of *T. longibrachiatum*, sabouraud dextrose peptone yeast extract agar (SDAY) media plates with one quarter strength were used. 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 (Gindin *et al.,* 2000). Plates were incubated at 25±1°C for 7 days. The fungi were sub-cultured to obtain pure cultures and stored at 4°C as pure culture. Pure cultures were cultured on SDAY media plates in order to conduct bioassay.

Characterization of Fungi: Morphological characterization of isolated *T. longibrachiatum* was studied using different dichotomous keys (Choi *et al*., 2003) and submitted to First Fungal Culture Bank of Pakistan (FCBP) for the issuance of accessions numbers.

Identification based on morphological characterization was complimented with amplification and sequencing of internal transcribed spacer (ITS) region. Fungal DNA was extracted by modified CTAB method (Stenglein and Balatti, 2006). DNA was extracted by sub culturing fungi in SDAY broth media and 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 uL 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 DNA at 72°C for 10 min. The amplified PCR products were resolved on 1% agarose gel. Desired 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.

Phylogenetic analysis: Sequences with similar region were collected from NCBI. 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 6.0. 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 *B. tabaci:* For maintaining *B. tabaci*, seeds of cotton plants were sown in pots containing sterilized soil in growth chamber under control condition of temperature (26± 2 °C), Humidity (60 ± 5%) with 12 hours day and night light conditions (Sharaf *et al*., 1985). After 20 days plants attain sufficient growth (having 6-7 leaves each). Plants were shifted to the glass cages and *B. tabaci* were maintained on these plants. After 15 days, the plants leaves were observed for presence of *B. tabaci* eggs, instars and nymphs. 4th instar nymphal and adult whitefly were separated on different leaves while all other stages were removed from leaves by camel hair brush.

For pathogenicity bioassay, clip cages were fixed on leaves containing nymph and adult whitefly separately (5 nymph and 5 adult in each clip cage) before the application of spore suspension. For these bioassays, two conidial concentrations 4×10^8 / mL & 4×10^4 / mL of *T. longibrachiatum* was sprayed in clip cages for nymph and adult *B. tabaci* respectively under controlled environmental conditions. Total spore suspension was applied on whole leaf before nymph and adult whitefly were allowed to feed on it. Data regarding mortality rate in nymphs and adults of *B. tabaci* was observed after 24 hours intervals and is determined by modified Abbott formula (Flemings and Ratnakaran, 1985).

$$\text{Mortality (\%)} = 1 - \left[\frac{\text{Post-treated nymph/adult population in treatment}}{\text{Pre-treated nymph/adult population in treatment}} \times \frac{\text{pre-treated nymph/adult population in control}}{\text{post-treated nymph/adult population in control}} \right] \times 100$$

Data Analysis: Microsoft Excel 2010 was used to compute the standard errors of means of five replicates. All the results were subjected to ANOVA followed by mean separation through Tukey's test (P≤0.05) (Steel and Torrie, 1980) using computer software CO-STAT.

RESULTS

T. longibrachiatum (FCBP- EPF- 1491) was isolated from 2 mealybug samples collected from Layyah and 3 samples from Lahore. It was observed under compound microscope that conidiophores of *T. longbrachiatum* are typically in tree branched shape, the phialides are bowling pin, lageniform in shape having average 11 µm length and 3 µm width, the conidia are oblong and a little bit ellipsoidal having average 4.5 µm length and 3 µm width respectively (Figure 1).

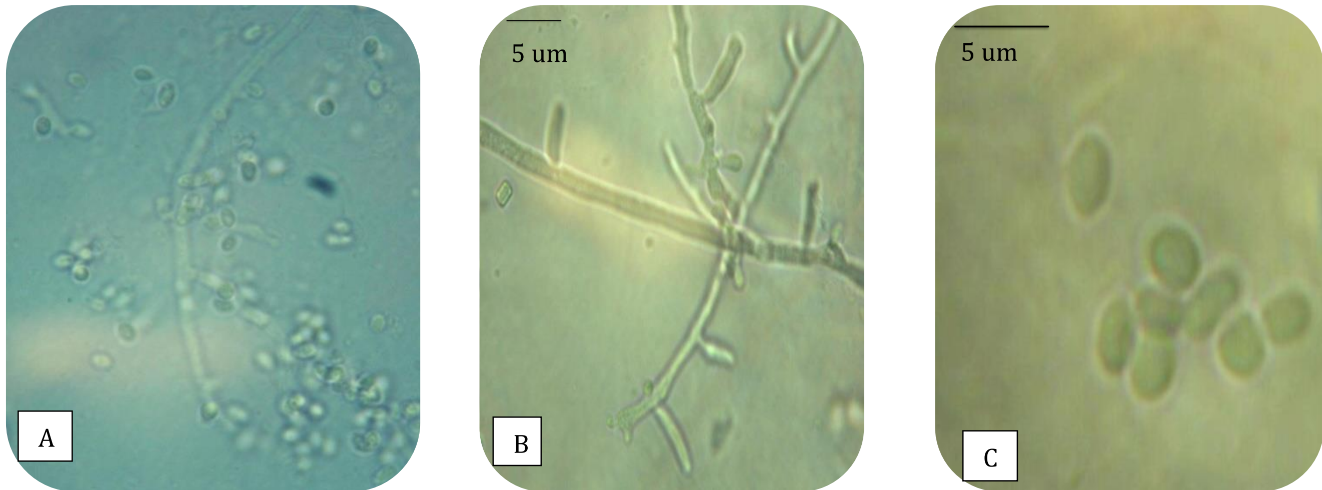

Figure 1. Typical conidiophores (A), phialides (B) and conidia (C) of *T. longibrachiatum* grown on SDA/4 culture media and observed under the stereomicroscope at magnification 100x.

Genetic Variations among Isolates of *T. longibrachiatum*: Genetic variations among internal transcribed spacer region of *T. longibrachiatum* isolated in this study were compared with the other isolates of same region taken from NCBI. A phylogenetic tree was constructed and it was observed that *T. longibrachiatum* (LT159847) isolated from insect could not be categorized in the same cluster while all other isolates show showed higher homology with each other as compare to insect isolates. This genetic variation of insect isolate made it unique as compare to all other isolates taken from data base (Figure 2).

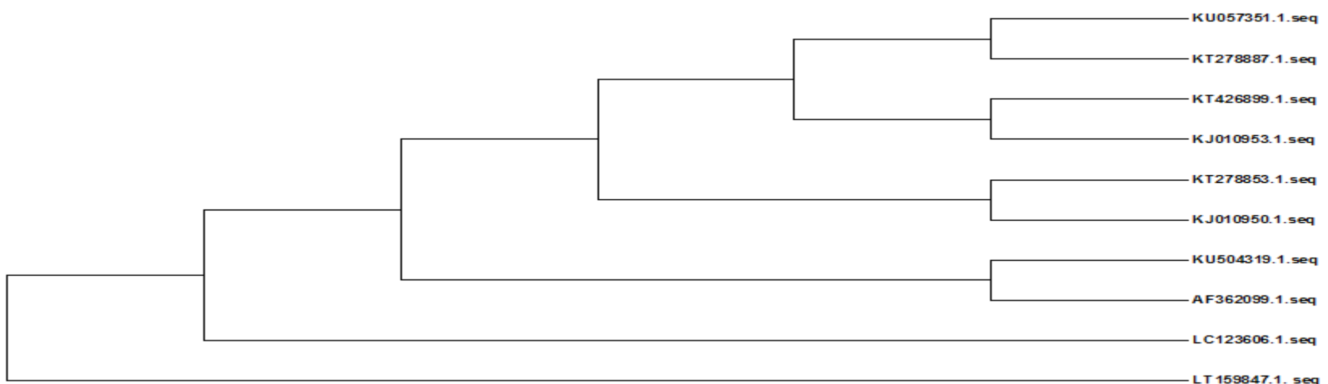

Figure 2. *T. longibrachaitum* maximum likelihood tree formed Using Mega 6 Software Pathogenicity Bioassay against *B. tabaci*

Pathogenicity of *T. longibrachiatum* was tested on nymph and adult whitefly to check mortality. Mortality of *B. tabaci* at Nymphal and Adult stages were observed after 24 hour intervals. Percentage mortality of nymphs treated with 4 x 10 ^{4} / mL conidia of *T. longibrachiatum* was 20, 24, 36 and 40% after 48, 72, 96, 72, 96, 120 and 144 hours respectively. There was no significant difference in mortality after 120 and 144 hours while there were significant difference in mortality after 24, 48, 72 and 96 hours (Figure 3).

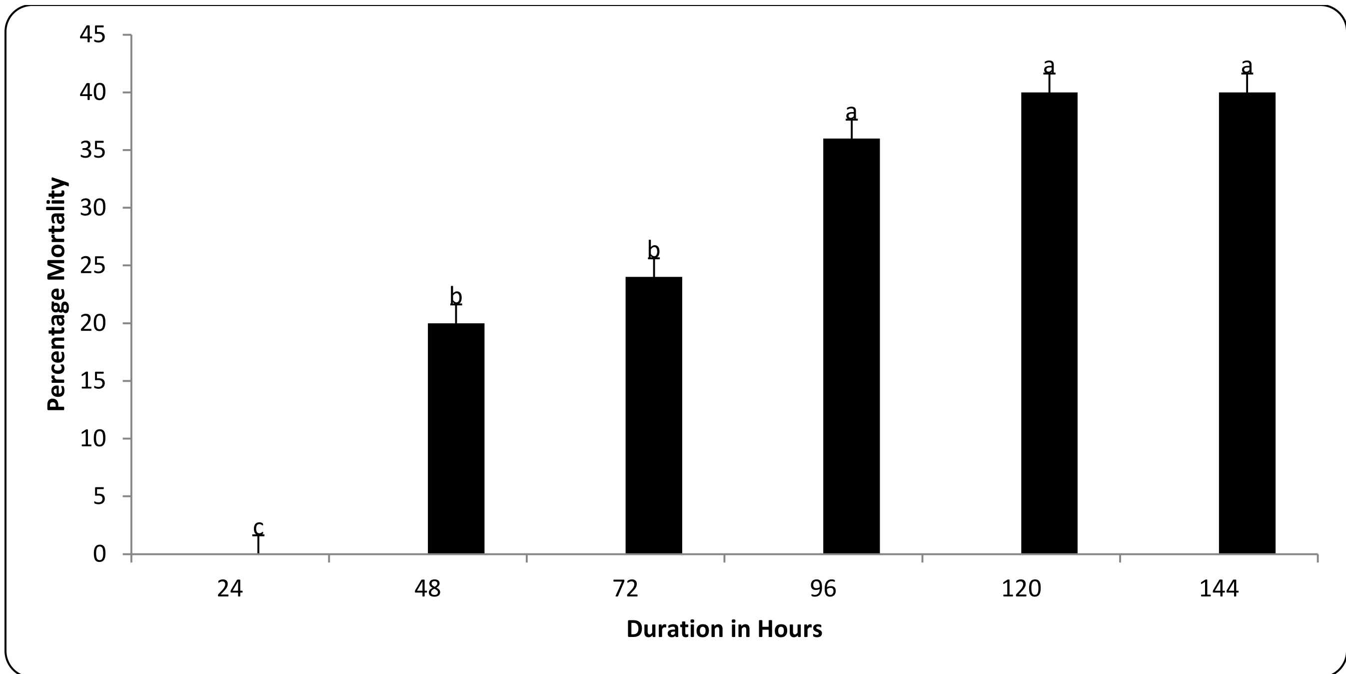

Figure 3. Mortality of Nmphal stage of *B. tabaci* with 4×10^4 spore concentration of *Trichoderma longibrachiatum.*

Similarly the pathogenicity bioassay of *T. longibrachiatum* against adult *B. tabaci* was studied. Percentage mortality of adults treated with 4 x 10 ^{4} / mL conidia were 5, 10 and 20% after 24, 96, 120 and 144 hours (whereas mortality started after 96 hr). There was significant difference in morality between 96, 120 and 144 hours (Figure 4).

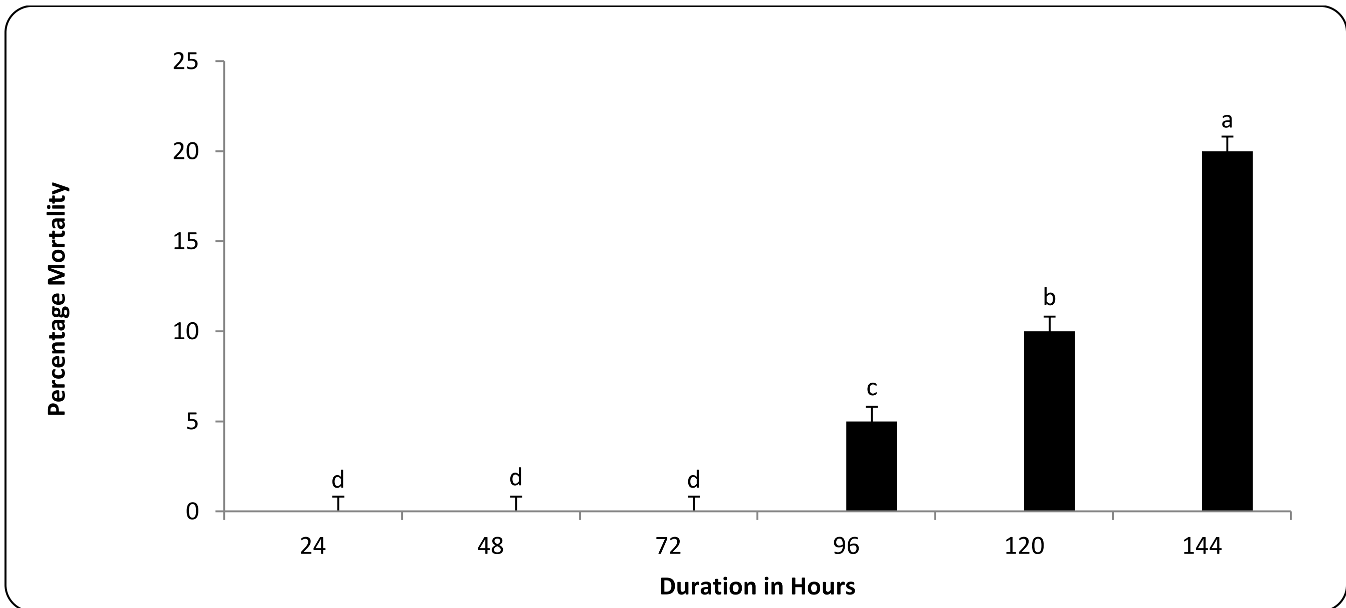

Figure 4: Mortality of adult *B. tabaci* with 4×10^4 spore concentration of *Trichoderma longibrachiatum*

It was observed that with increase in spore concentration, the mortality rate was also increased in nymph as well as adult *B. tabaci.* Percentage mortality of nymphs treated with 4 x 10^8 / mL conidia of *T. longibrachiatum* was 35, 40, 65 and 73 % (improper way) after 48, 72, 96,120 and 144 hours respectively. There was no significant difference in mortality after 120 and 144 hours while there were significant difference in mortality after 24, 48, 72 and 96 hours (Figure 5).

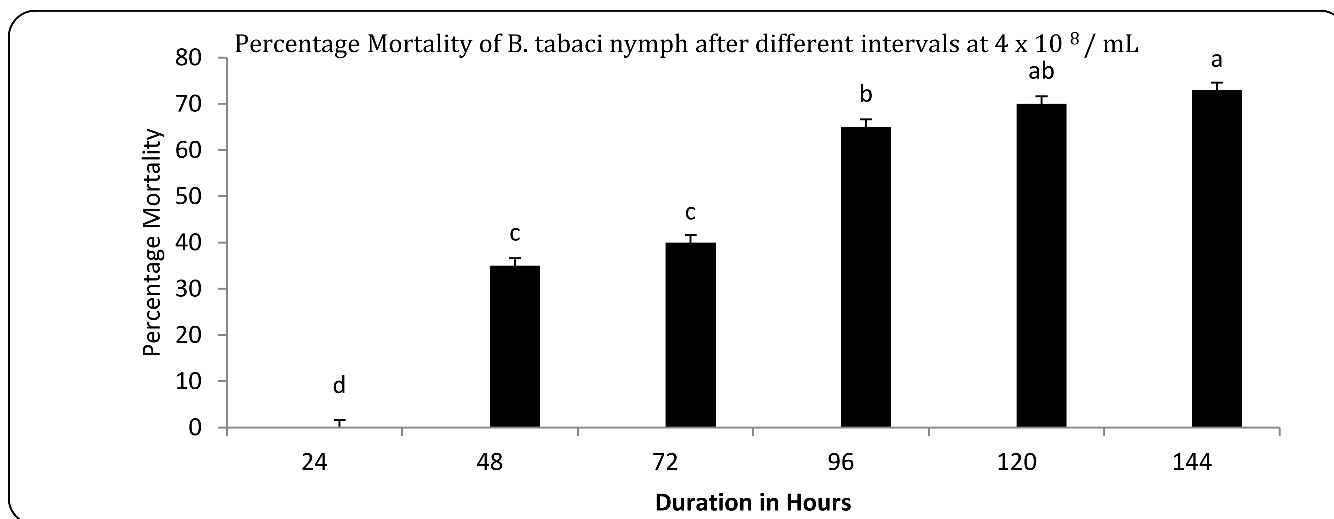

Figure 5. Mortality of Nymphal *B. tabaci* with 4×10^8 spore concentration of *Trichoderma longibrachiatum.*

Whereas, the percentage mortality of adults treated with 4 x 10 ^{8} / mL conidia were 18, 25 and 40% after 96, 120 and 144 hours (whereas mortality is shown after 96 hr).There were significant difference in morality between 24, 48, 72 and 96, 120, 144 hours treatments (Figure 6).

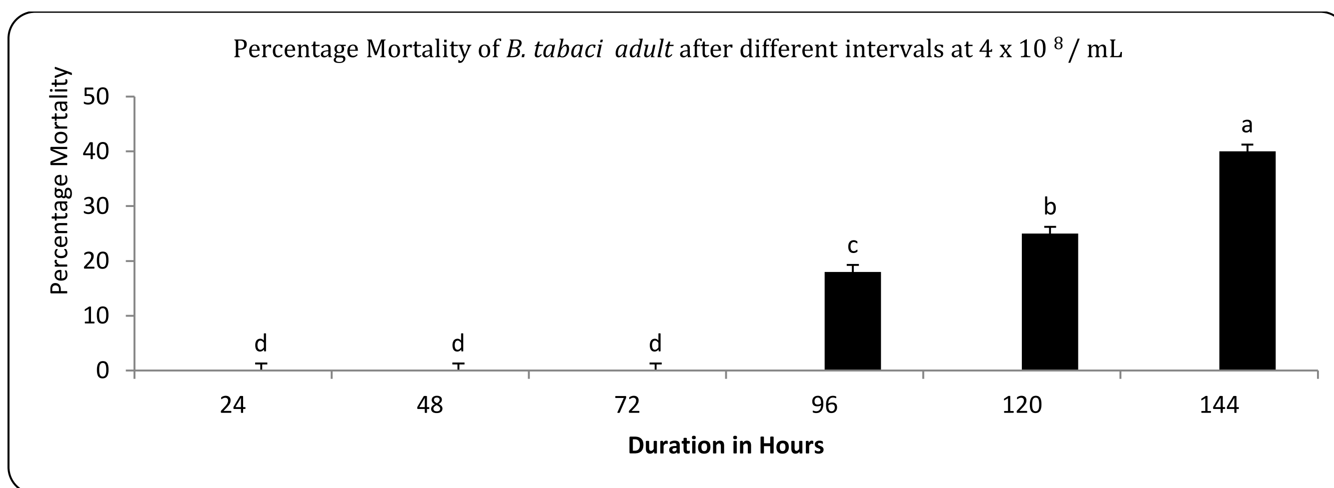

Figure 6: Mortality of adult *B. tabaci* with 4×10^8 spore concentration of *Trichoderma longibrachiatum*

It was also observed under microscope that fungal hyphae and conidia were present on dead nymph and adult *B. tabaci* while there were no such hyphae and conidia on dead nymph and adult in control (Figure 7). These results showed that *T. longibrachiatum* isolated from cotton mealybug shows entomopathogenic activity against *B. tabaci* similarly like other species of *Trichoderma* that have antagonistic activity against different bacterial (Leelavathi *et al*., 2014) and fungal pathogens (Nashw *et al*., 2008, Jegathambigai *et al*., 2009).

Table 1. Tukey HSD All-Pair wise Comparisons Test. Where Alpha = 0.05

Sr. No	Treatments	DF	F value	P value
1.	Nymph 4×10^4	5	181	0.0000
2.	Adult 4×10^4	5	193	0.0000
3.	Nymph 4×10^8	5	590	0.0000
4.	Adult 4×10^8	5	360	0.0000

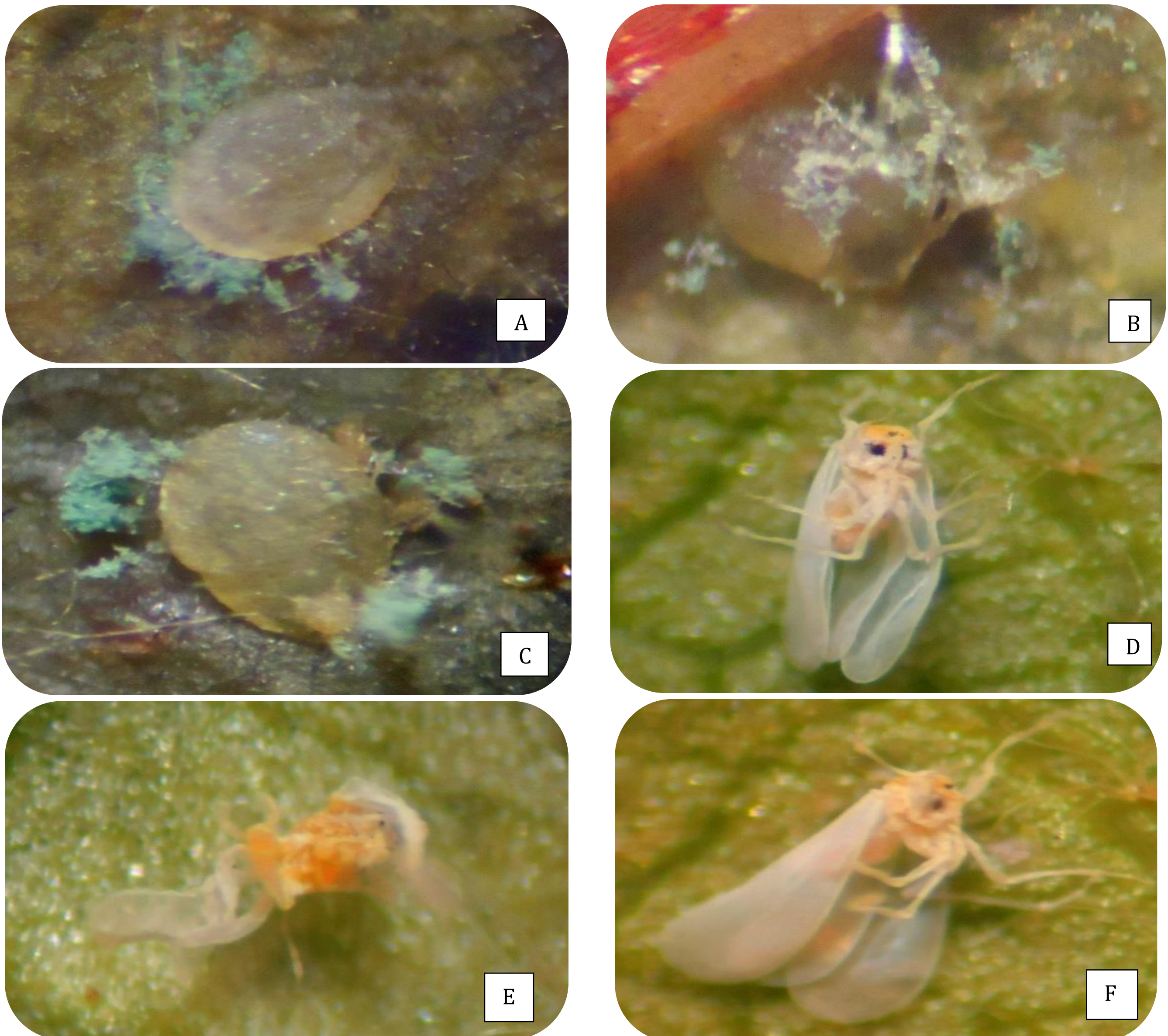

Figure 7. The infection of *T. longibrachiatum* in *B. tabaci* nymph (A, B and C) and the infection of *T. longibrachiatum* in *B.tabaci* adult (D, E and F).

DISCUSSION

T. longibrachiatum is known as soil fungi and mainly present in warm climate areas throughout the world. *T. longibrachiatum* commonly present on decaying plant material whereas it's ecological importance sortout from purely saprotrophs to parasite of other saprotrophic fungi (Samuels *et al*., 2012). The entomopathogenic effect of this species has been reported few years ago on naturally infected cowpea aphid (*Aphis craccivora*) (Ibrahim *et al*., 2011). Similarly according to Gosh and Pal 2016, when conidial spore suspension of *T. longibrachiatum* was used against *L. orbonalis* the minimum inhibitory effect (20 %) was obtained with a fungal spore dose of 10^3 spores/ mL, and the maximum inhibition (80 %) was obtained with a dose of $1x10^8$ spores/mL. But its entomopathogenic activity against *B. tabaci* was not reported previously. In this study, maximum mortality 73 % was observed at the concentration of 4×10^8 / mL when conidial spore suspension of *T. longibrachiatum* was used against *B. tabaci* nymph. According to our knowledge, this is first report of *T. longibrachaitum* as entomopathogenic fungi on *B. tabaci* from Pakistan but further studies are required to know the exact infection mechanism of *T.*

longibrachiatum against different insects including whitefly.

CONCLUSION

Entomopathogenic fungi are alternative to insecticides. They are important because they are environment friendly and persistent for a longer period in the field. Addition of *T. longibrachiatum* as entomopathogenic fungi against whitefly will open new control strategies, individually or in combination with other entomopathogenic fungi and various other biocontrol agents.

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