Egyptian Journal of Biological Pest Control

RESEARCH

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Identification and virulence of entomopathogenic fungi, *Isaria javanica* and *Purpureocillium lilacinum* isolated from the whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) in Malaysia

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Abstract

Background Three of entomopathogenic fungi, identified as *Isaria javanica* (Cjc-03 and Cjw-01) and *Purpureocillium lilacinum* (TS-01) were found naturally infecting the whiteflies, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) on chili and tomato plants. All the isolates were identified by morphological characterization and molecular identification (ITS region amplification). The virulence of the three isolates was evaluated against nymphs of *B. tabaci* at three different concentrations (1×10^6, 1×10^7, and 1×10^8 conidia/ml) under laboratory conditions and on adults of *B. tabaci* at one conidia concentration (1×10^7 conidia/ml) under laboratory and glasshouse conditions.

Results *Isaria javanica* showed the highest virulence against *B. tabaci* nymphs and adults in both laboratory and glasshouse conditions. *I. javanica* exhibited the highest mortality in the laboratory against *B. tabaci*, nymphs at the highest concentration (1×10^8 conidia/ml) 8 days post-inoculation. Likewise, in the laboratory bioassay, *B. tabaci,* adults, Cjc-03 isolate exhibited maximum mortality (80.0%), followed by Cjw-01 isolate (77.5%), and TS-01 isolate (65.0%) at the concentration of 1×10^7 conidia/ml at 7 days post-inoculation. The highest mortality rate (63.45%) was recorded by the Cjc-03 isolate and TS-01 (54.22%) isolate at 3 days post-inoculation in glasshouse bioassay using the concentration of 1×10^7 conidia/ml of each of the 2 isolates (Cjc-03 and TS-01) against *B. tabaci* adults.

Conclusion Overall, the findings showed that both isolates of *I. javanica* and *P. lilacinum* reduced the number of eggs, nymphs, and adults' emergence of *B. tabaci,* following the application of 1×10^7 conidia/ml on tomato leaves in the glasshouse. The newly isolated strains could be developed as a potential commercial biopesticide for managing *B. tabaci*.

Keywords *Bemisia tabaci*, Entomopathogenic fungi, *Isaria javanica*, *Purpureocillium lilacinum*, Virulence

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Background

Sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is one of the major pests of vegetable and ornamental crops in many tropical and subtropical regions around the world (Singh and Kaur 2020). Adults and nymphs of *B. tabaci* cause damage to the plant directly during feeding and oviposition and indirectly through the transmission of plant. Moreover, sooty mold colonizes on the surface of plants due to honeydew secreted by *B. tabaci* affecting photosynthetic activity in plants, resulting in a considerable decrease in agricultural product quality (Shah et al. 2020). Plant viruses transmitted by *B. tabaci*, such as begomoviruses are estimated to cause yield losses ranging from 20 to 100% and losses worth millions of dollars (Gangwar and Charu 2018). Insecticides have been widely used to control the whitefly, but this approach has several disadvantages, including the development of insecticide resistance and food safety concerns (Gangwar and Charu 2018).

In recent years, there has been increasing research on using biological control agents (BCA) as alternative strategies to control *B. tabaci*. Over the past few decades, entomopathogenic fungi (EPF) have become one of the major components of integrated pest management (IPM) that are being investigated for the control of several insect pests, and more than 20 species documented to be effective against *B. tabaci*, including *Beauveria bassiana, Isaria fumosoroseus, Metarhizium anisopliae, Ashersonia* spp.*, Isaria javanica, Purpureocillium lilacinum,* and *Verticillium lecanii* (Zafar et al. 2016). Previous findings showed that *B. tabaci* is susceptible to *I. javanica* and *P. lilacinum* infection under laboratory conditions (Du et al. 2021).

Native isolates or EPF strains from various hosts provided unique control programs to manage several indigenous pests because they are more effective to control pests of a particular environment (Sayed et al. 2018). Soil is also a natural habitat for EPF and acts as important reservoir of entomopathogens capable to survive for long time without the host insects. However, it is extremely challenging to isolate new species and strains of EPF from the soil in particular regions due to human activities that results in the frequent genetic connections among soil inhabitants (Dong et al. 2016). Moreover, the discovery of isolates from the insect host, the isolate characterization, and the virulence test are the first steps in producing novel EPF as BCA (Dayanti et al. 2018).

So far, three species of EPF; *B. Isaria,* and *Metarhizium* spp. have been discovered from infested insects in Peninsular Malaysia (Kin et al. 2017). Apart from *I. fumosorosea* documented by Eslamizadeh et al. (2013) in Malaysia, there are unaware other studies of EPF species or isolates isolated from *B. tabaci*. The effectiveness of this isolate *I. fumosorosea* isolate (Pf-UPM) was compared with the other nine indigenous *Isaria* isolates detected from the soil on *B. tabaci*, and the infectivity rate of Pf-UPM isolate for all life stages was significantly higher than the other nine isolates. Here, two new species of EPF, *I. javanica* and *P. lilacinum* from infected *B. tabaci* were successfully isolated. Therefore, the present study aimed to isolate and identify EPF infecting *B. tabaci* associated with vegetables as a contribution to the IPM of *B. tabaci.*

Methods

Isolation of entomopathogenic fungi from the whitefly samples

A survey for the collection of nymphal instars of whitefly was conducted in a glasshouse and field planted with vegetables in the Glasshouse and a research experimental station at the Faculty of Agriculture, UPM. Leaflets of five different types of vegetables (chili, eggplant, tomato, pepper, and okra) infested with nymphs were collected, place in a plastic container (15×9 cm) and brought to the laboratory for isolation of EPF. Nymphal instars were observed under a stereomicroscope (Leica Zoom 2000 Stereo Microscope, USA) for probable infection with EPF. To isolate fungal strains, infested leaves with second to fourth instars of *B. tabaci* were surface sterilized with 70% ethanol for 5 min and washed 5 times with sterile distilled water in a laminar flow. The treated leaves were air-dried on sterile tissue papers for 30 min. The surface-sterilized nymphs from the leaves were then picked using a surface sterile needle and placed directly onto the PDA media supplemented with 50 mg/ml of antibiotic, penicillin, streptomycin, and tetracycline. Two nymphs were placed in the opposite direction on each plate and ten plates were used for each vegetable infested with nymphs. The plates were incubated at 25 ± 2 °C for 12 days and the presence or absence of EPF on the plates inoculated with whitefly nymphs was observed daily until the fungal growth was observed.

Morphological identification of entomopathogenic fungi

The isolated fungal strains were transferred to PDA plates and cultured for 5 days at 25 °C. Pure cultures of the observed fungi were prepared using microscope glass slides. A sterile needle was used to pick fresh mycelia and placed in a single drop of sterile distilled water and/or lactophenol blue mounted on a clean glass slide. The identification of fungal isolates was based on the morphological characteristics as described by Watanabe (2010) and Humber (2012). Morphological features of the colony, conidial shape and size, and mycelia growth rate were studied using a light microscope (Olympus CX31 series, England), equipped with a digital Dinolite eyepiece camera (magnification 40×).

Molecular identification of entomopathogenic fungi

Direct PCR amplification was conducted according to the manufacturer's protocols for the KOD FX Neo PCR master mix (Toyobo Co. Ltd., Japan). Mycelium of 7 days fungal cultures was scraped, suspended in 100 µl TE buffer and vortexed for 30 s. The fungal ITS region was amplified using the primers ITS1 (5′-TCCGTAGGT GAACCTGCGG-3′ and ITS4 (5′-TCCTCCGCTTAT TGATATGC-3′) (White et al. 1990). The PCR reaction was performed in 25 µl consisting 2 µl mycelium suspension from each sample, 12.5 µl of 2 × PCR buffer, 5ul dNTP mixture, 1.5 µl of each forward and reverse primers, 0.5 µl of Kod FX Neo,) and ITS4 and 3 µl sterile water. PCR was performed using Thermocycler Biometra (T-Persona, Germany). The PCR reactions were amplified with the following conditions: An initial denaturation temperature of 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 53.7 °C for 1 min, and extension at 72 °C for 2 min; and a final elongation of 72 °C for 10 min. The PCR products were subjected to electrophoresis on a 1.5% agarose gel stained with GelRed™ and run at 90 V. PCR products were visualized under UV illumination and photographed. PCR products were sent to Apical Scientific Sdn. Bhd. Malaysia for purification and direct sequencing. The forward and reverse DNA sequences were manually analyzed to remove the low-quality and ambiguous bases using chromos software. The sequences were identified using the Basic Local Alignment Search Tool (BLAST)n search of GenBank. (http://www.ncbi.nlm.nih.gov). Trimmed Clustal W alignment of sequences, top hit sequences from GenBank, and *Metarhizium anisopliae* outgroup sequence were used to create a maximum likelihood phylogenetic tree with 1000 bootstrap replicates using MEGA (version 7.0), and whitefly species was identified. The phylogenetic tree for ITS sequences was constructed by the maximum likelihood method with 1000 bootstraps replicates using MEGA version 7 (Kumar and Stecher 2016).

Whitefly colonies and host plant

A stock culture of whiteflies (*B. tabaci*) which colonized tomato plants in the greenhouse was collected, reared, and maintained in the greenhouse, Ladang 15, Faculty of Agriculture, UPM. The *B. tabaci* was identified and confirmed by PCR using the mitochondrial DNA cytochrome oxidase I COI (mt COI) gene with the primers (C1-J-2195 and L2-N-3014) as described by Frohlich et al. (1999). The sequencing data were registered and assigned the accession number; OM638559 (GenBank). The infested leaves used for bioassay were obtained by placing whitefly-free plants (21 day-old) close to the adult-infested tomato seedlings for 72 h. Adult whiteflies were then removed, and plants infested with eggs were transferred to another glasshouse and placed in a cage for 12–15 days until the nymphs entered the second instar. This method resulted in more than 40 eggs per leaf (Mascarin et al. 2013). *B. tabaci* colonies were maintained on tomato seedlings, which planted in 300 l plastic pots held in meshed cages at greenhouse conditions (25–30 °C and 60–80% RH with a photoperiod of 12L:12D light: dark. Tomato plants were checked daily, and the damaged seedlings were replaced with new ones.

Growth of the fungal isolates and production of conidia

Mycelial growth and sporulation of each 3 EPF isolates were examined, following the method described by Ali et al. (2010). Agar plugs (7 × 7 mm) containing mycelia of each EPF pure culture (Cjc-03, Cjw-01, and TS-01) were taken from 12 day-old culture with a cork borer and aseptically inoculated at the center of the Petri plates (90 mm) containing PDA media and incubated at 25 ± 2 °C. Each isolate had four replicates. The mycelial growth of each EPF culture was measured at 3, 5, 7, 9, and 12 days after inoculation (DAI) by measuring the average of two perpendiculars measured diameters of each colony (D1 and D2). To estimate spore concentration, a 7 mm agar disc of actively growing culture was cut using a sterilized cork borer and was placed into a test tube containing 10 ml sterile distilled water. One ml suspension was diluted with 9 ml sterile distilled water using a serial dilution to obtain concentrations of 1×10^6, 1×10^7, and 1×10^8 conidia/ml. The spore suspensions were vortexed for 3 min to obtain a homogeneous solution. For each suspension, spores were counted using a Neubauer hemacytometer (Sumikarsih et al. 2019).

Laboratory bioassay of EPF against *B. tabaci* nymphs

Leaves infested with second instar nymphs of *B. tabaci* were excised and dipped into three concentrations: 1×10^6, 1×10^7, and 1×10^8, conidia/ml for 30 s. Nymphal instars were recognized, following Naranjo and Ellsworth, (2017). Third and fourth instars were removed from the leaves by needles, and the number of the second instar was counted and adjusted to 20–25 before dipping into different concentrations. The excised leaves were air-dried and placed on the abaxial surface up in a dishes (90 mm diameter) line with a skinny layer of 1.5% agarose gel (Shah et al. 2020). The control excised leaves were dipped in 0.01% Tween 80, and all the treatments were incubated at 25 ± 2 °C. To prevent saprophytic fungi from growing on whitefly honeydew, aeration was established daily by opening each plate for 30 min (Santiago-Alvarez et al. 2006). Three experiments were conducted evaluating four treatments: (Cjc-03, Cjw-01, TS-01, and 0.01% Tween 80). Dead nymphs (discoloured, and/or

desiccated, or developed symptoms with fungal growth or sporulation) were observed using a stereo microscope (Leica Zoom 2000 Stereo Microscope, USA) and mortality was recorded at 3, 5, and 8 days after inoculation. Each treatment consisted of three replicates. The entire experiment was conducted three times using a fresh conidial suspension and a fresh batch of nymphs. Treated whitefly was randomly chosen for confirming whether mortality was caused by the fungal grown on the dead whitefly. After eight days of inoculation, dead whitefly specimens were observed with a stereomicroscope (Leica Zoom 2000 Stereo Microscope, USA), and some of which were processed with a scanning electron microscope (SEM) procedure.

Laboratory bioassay of the EPF against adult whitefly

The experiment was conducted using the method of Mascarin et al. (2013) with minor modifications. Leaf disks (approximately 30 mm) were dipped into 1×10^7 conidia/ml of each fungal isolate and 0.01% tween 80 was served as control. Treated leaf disks were allowed to dry and placed on acrylic vials (77×38 mm) containing 10 ml of 1.5% agarose gel. Adult whiteflies ≤ 5 days old were exposed to treated leaf disks and confined using a mesh with a rubber band. The assessment for adult mortality was recorded at 7 DAT. The whole experiment was conducted two times with four treatments: (Cjc-03, Cjw-01, TS-01, and 0.01% Tween 80) and four replicates where each replicate consisted of one leaf disk.

Virulence of EPF against adult whitefly in greenhouse

Tomato seedlings, 30 day old were grown in an 800 l capacity pot. The substrate (sterilized peatmoss) from each pot was covered with aluminum foil to prevent the run-off of fungal suspension.

The seedlings were sprayed with 1×10^7 spore suspension until run-off and placed in a cage (an acrylic aquarium) (40×25 cm) held vertically, which serves as a bioassay cage. Control plants were sprayed with sterile distilled water having 0.01% Tween 80. One side of the cage was covered with fine mesh and treated seedlings were kept for 24 h. Following that, 50 to 60 adult whiteflies (≤ 5 days) were manually aspirated from the stock colony and released into the bioassay cage containing treated plants for 3 days. The EPF treatments and control were replicated six times within the greenhouse. Dead whiteflies were counted 3 days after the release of insects, and live insects were removed. To confirm if mycosis was the cause of mortality, all dead insects were placed on PDA plates, incubated for 5–7 days at room temperature, and fungal growth on the insect cadavers was observed.

Effect of EPF isolates on the number of eggs, nymphs, and adults' emergence of *B. tabaci* on tomato leaves

The number of eggs laid per one square centimeter of leaflets was counted from each treated plant. Three leaflets per leaf were sampled and two leaves in the midsection of each treated plant were assessed (Barra-Bucarei et al. 2020). Thereafter, the treated seedlings were moved to a new cage and placed in another glasshouse for subsequent development of immature stages and adult emergence. The number of nymphs (instar III and IV) per leaflet was counted at 35 DAT. Eight leaflets (approximately 40×20 mm) per treated plant were sampled from four leaves of each treated plant and evaluated in the laboratory. At 42 DAT, adult emergence was calculated by counting the exuviae left by adults' whitefly on the leaves**.** Ten leaflets (approximately 40×20 mm) per treated plant were sampled from three leaves of each treated plant and evaluated in the laboratory. This bioassay was conducted twice with three treatments: (Cjc-03, TS-01, and 0.01% Tween 80) and six replicates.

Data analysis

All data analyses were performed using R (version 3.6.1). Experimental design in the laboratory bioassay with the second instar nymphs was completely randomized, and responses (*B. tabaci* mortality) were analysed using a three-way (fungus, concentration, and time) factorial design using Fischer's least significant difference (LSD) test at $\alpha = 0.05$. The effect of these isolates on adults' mortality was analysed using one-way ANOVA under a randomized complete design (RCD). For the glasshouse study, adult mortality, the mean number of eggs, nymphs, and adults' emergence were subjected to an ANOVA test. Mortality data were corrected by using Abbott's formula (Abbott 1925). Comparisons of the mean treatment were performed using Fischer's least significant difference (LSD) test at $\alpha = 0.05$.

Results

Fungal identification of entomopathogenic fungi: morphological identification

A total of three fungal isolates of EPF were identified from whitefly infesting two vegetable crops (chili and tomato) in Glasshouse and Research Experimental Station at the Faculty of Agriculture, UPM. The pure culture isolates were grown in PDA and identified based on macro and micro-morphological features. The morphological identification showed that the two isolates were *I. javanica* and the other isolate was characterized as *P. lilacinum*.

Isaria javanica

Two fungal isolates (Cjw-01 and Cjc-03) had morphological features related to *I. javanica* based on colony, colour, sized and conidial shape. On PDA, fungal colony of Cjw-01 was pinkish white cottony, with dense mycelia in the centre, formation of light-yellow pigment with a concentric ring pattern. However, the Cjc-03 colony was white to whitish-yellow (Fig. 1A). The conidia of both isolates were oval to spindle in shape, with a length of about 6×2.2 µm (length × width) (Fig. 1D and E).

Purpureocillium lilacinum

The colony was round, white in the first three days, and gradually turned pink after 7 days (Fig. 1C). Conidia were oval and single-celled, and the size was 4.2×2.1 µm (Fig. 1F). During twelve days of incubation, the radial growth attained 41.15 ± 0.22 mm, 48.50 ± 0.38 mm, and 63.30 ± 0.18 mm for Cjw-01, Cjc-03, and TS-01 isolates respectively. The sporulation; 1.27×10^6, 1.38×10^6, and 3.99×10^6 conidia/ml were also recorded on Cjw-01, Cjc-03, and TS-01 isolates, respectively.

Molecular identification

Polymerase chain reaction amplification and DNA sequencing results indicated that the rDNA-ITS gene of Cjw-01, Cjc-03, and TS-01 isolates were 594 bp (Fig. 2), following this the DNA sequence were submitted to GenBank; where the GenBank accession number MW857170, MW857169, and MW857173, respectively. BLASTn results in GenBank revealed that the ITS gene of Cjw-01 and Cjc-03 isolates were 99.81% homologous to the species *I. javanica* ex-type strain (GenBank accession number AY624186). Moreover, phylogenetic analysis showed that the isolate was closely clustered in the *I. javanica* clade (Fig. 3) this confirmed the results of the morphological identification that the Cjw-01 CJc-03 isolates were *I. javanica* strain. Sequencing of the ITS placed the TS-01 isolate in the species *P. lilacinum* and the ITS sequence showed 99% similarity with strain *P. lilacinum*

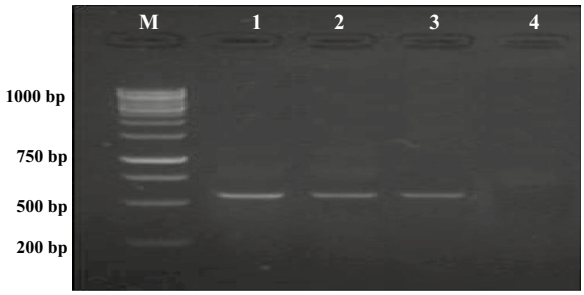

Fig. 2 PCR amplification from ITS region and amplification fragment showing approximately 594 bp. Lane M: 1 kb ladder; Lane 1–3: Cjw-01, Cjc-3, TS-01, Lane 4: Negative control (distilled water)

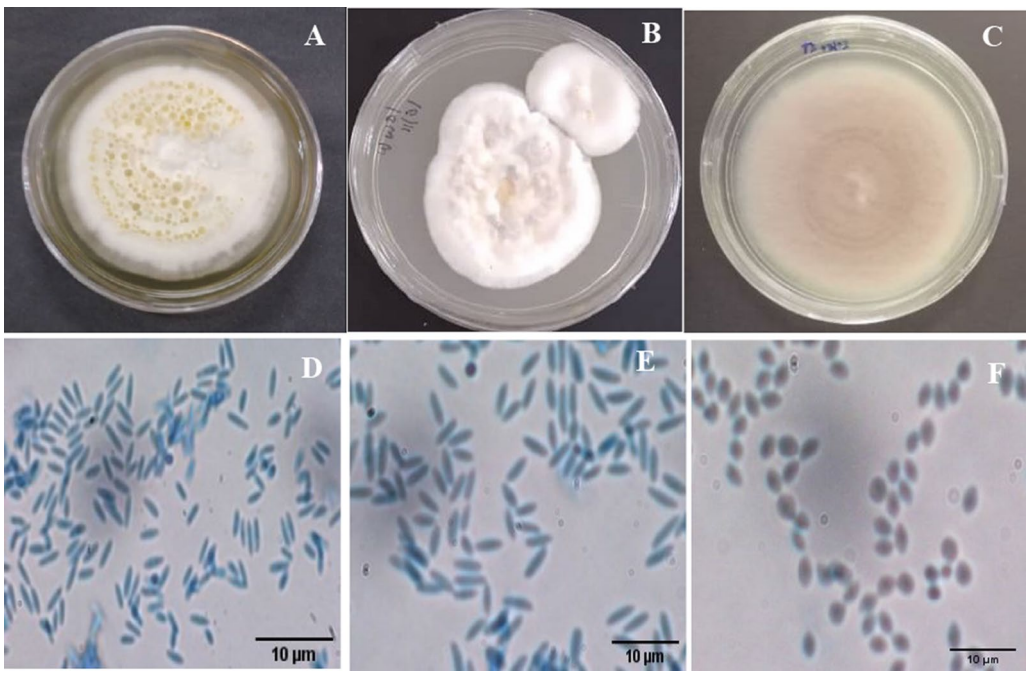

Fig. 1 Colony morphology of *Isaria javanica* and *Purpureocillium lilacinum* isolated from infested whitefly (*Bemisia tabaci*) on PDA media at 25 °C after 10 days of growth **A** Colony of *I. javanica* (Cjw-01) **B** Colony of *I. javanica* (Cjc-03) **C** Colony of *P. lilacinum* (TS-01) **D** Conidia of *I. javanica* (Cjw-01) **E** Conidia of *I. javanica* (Cjc-03) **F** Conidia of *P. lilacinum* (TS-01)

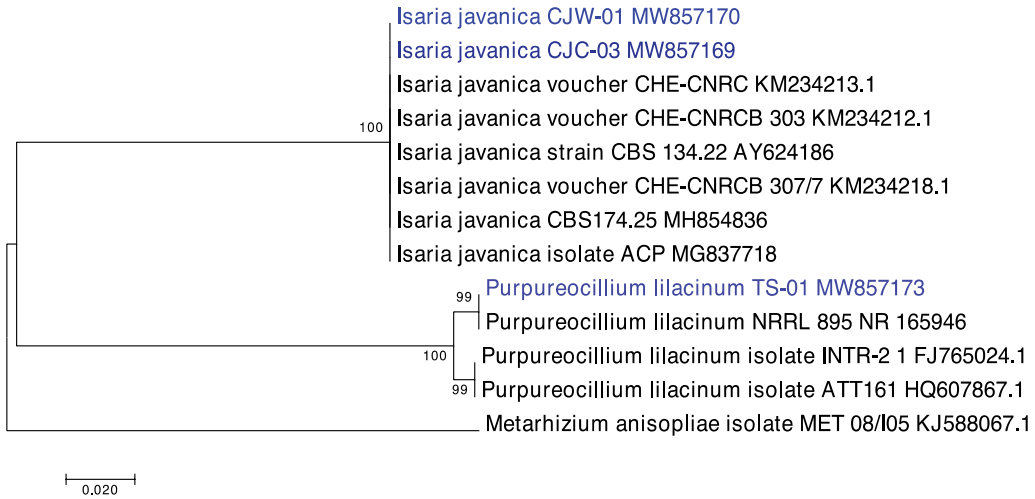

Fig. 3 Maximum likelihood (ML) tree of *Isaria javanica* isolates and *Purpureocillium lilacinum* isolate based on ITS region. *Metarhizium anisopliae* was used as an outgroup

Table 1 GenBank sequences data of entomopathogenic fungi isolated from the whitefly

Isolate	Species	GenBank accession	GenBank accession no	Host	Location	Percent identity (%)
Cjc-03	*Isaria javanica*	MW857170	AY624186	*Bemisia tabaci*	USA	99.81
Cjw-01	*Isaria javanica*	MW857169	AY624186	*Bemisia tabaci*	Mexico	99.81
TS-01	*Purpureocillium lilacinum*	MW857173	MH483727.1	Soil	China	99.80

in the GenBank (Table 1). However, phylogenetic analysis revealed that the two isolates (Cjc-03 and Cjw-01) and one isolate (TS-01) were closely clustered in the *I. javanica* and *P. lilacinum* clade, respectively, confirming the morphological identification of the *I. javanica* and *P. lilacinum* isolates.

The tree was constructed by the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980).

Virulence of EPF isolates against second nymphal instar of *B. tabaci* in the laboratory

The result of the present study showed that all the three EPF strains exhibited a significant effect on the second instar nymphs of *B. tabaci* ($F=686.40$, $p<0.01$). However, the factorial analysis of variance yields significant effects of fungal concentration, time interval, and their interaction on the mean mortality of *B. tabaci* ($F=.99.15$, $p>0.001$; $F=382.70$, $p>0.001$; $F=4.60$, $p>0.05$) (Table 2).

At 8 DAT, the highest mortality recorded in the highest concentration, 1×10^8 conidia/ml, was 91.1% in Cjc-03 isolate, 76.5% in Cjw-01 isolate and 62.7% in TS-01 isolate, and low mortality observed at the lowest concentration 1×10^6 conidia/ml were 67.7%, 55.6% and 42.89% for Cjc-03, Cjw-01 and TS-01, respectively. Accordingly, at the 5 DAT, the maximum mortality recorded (84.4, 61.3 and 55.6%) at the highest concentration 1×10^8 conidia/ml, while the mortality recorded at

Table 2 Factorial Analysis of Variance of the virulence of two isolates of *Isaria javanica* and one isolate of *Purpureocillium lilacinum* against the second instar of *Bemisia tabaci*

Source	Df	SS	MS	*F* value	pr (>*F*)
Fungal	3	39,493	13,164.4	686.40	< 0.001
Day	2	14,679	7339.6	382.70	< 0.001
Concentration	2	3803	1901.6	99.15	< 0.001
Fungal × day	6	2533	422.1	22.0088	< 0.001
Fungal × concentration	6	2400	399.9	20.8525	< 0.001
Day × concentration	4	353	88.2	4.60	0.0023
Fungal × day × concentration	12	281	23.4	1.2209	0.28
Error	72				
Total	107	63,542			

DF Degree of freedom, *SS* Sum of squares, *MS* Mean sum of squares, *F F*-statistic, $p<0.001$ (highly significant) and $p<0.05$ (significant); three-way factorial analysis of variance (ANOVA) at $\alpha=0.05$

the lowest concentration, 1×10^6 conidia/ml (51.1, 35.6 and 35.36%) for Cjc-03, Cjw-01 and TS-01, respectively. On the other hand, the maximum mortality observed at the 3 DAT in the highest concentration 1×10^8 conidia/ml was 47.7% for Cjc-03 isolate, 39.4% for Cjw-01 isolate, and 33.3% for TS-01 isolate, and minimum mortality was recorded at the lowest concentration 1×10^6 conidia/ml Cjc-03 isolate (33.5%), Cjw-01 isolate (26.7%) and TS-01 isolate (14.8%) (Fig. 4). In all treatments, *I. javanica* isolates shown to be the most virulent against *B. tabaci* at 3rd, 5th and 8th day after infection.

Based on the scanning electron microscope (SEM) analysis of the dead whitefly, it was shown that the conidia are attached and the mycelium penetrated to whitefly's body. Scanning electron microscopy of *I. javanica* isolates showed dense mycelium growing on the integument and on the cuticle surface of whitefly (Fig. 5). Furthermore, a thickening of the margins of the germ tubes was observed during fungal penetration, indicating the formation of appressoria (an infective structure) (Fig. 5D). The infection of healthy *B. tabaci* second instar nymphs treated with *I. javanica* and *P. lilacinum* were also observed under a stereomicroscope before and after the application (Fig. 6).

Virulence of EPF isolates against adult *B. tabaci* in the laboratory

Each one of the two isolates of *I. javanica*, (Cjc-03 and Cjw-01) and one isolate of *P. lilacinum* (TS-01) tested at a single conidia concentration showed pathogenicity to adults of *B. tabaci*, caused more than 50% mortality, following the 7th day of infection. There was non-significant difference among the three isolates (Cjc-03, Cjw-01, and TS-01), but significantly differed than control treatment ($F = 22.56$ $p < 0.001$) (Fig. 7). The highest mortality (80.0%) was recorded for Cjc-03 isolate

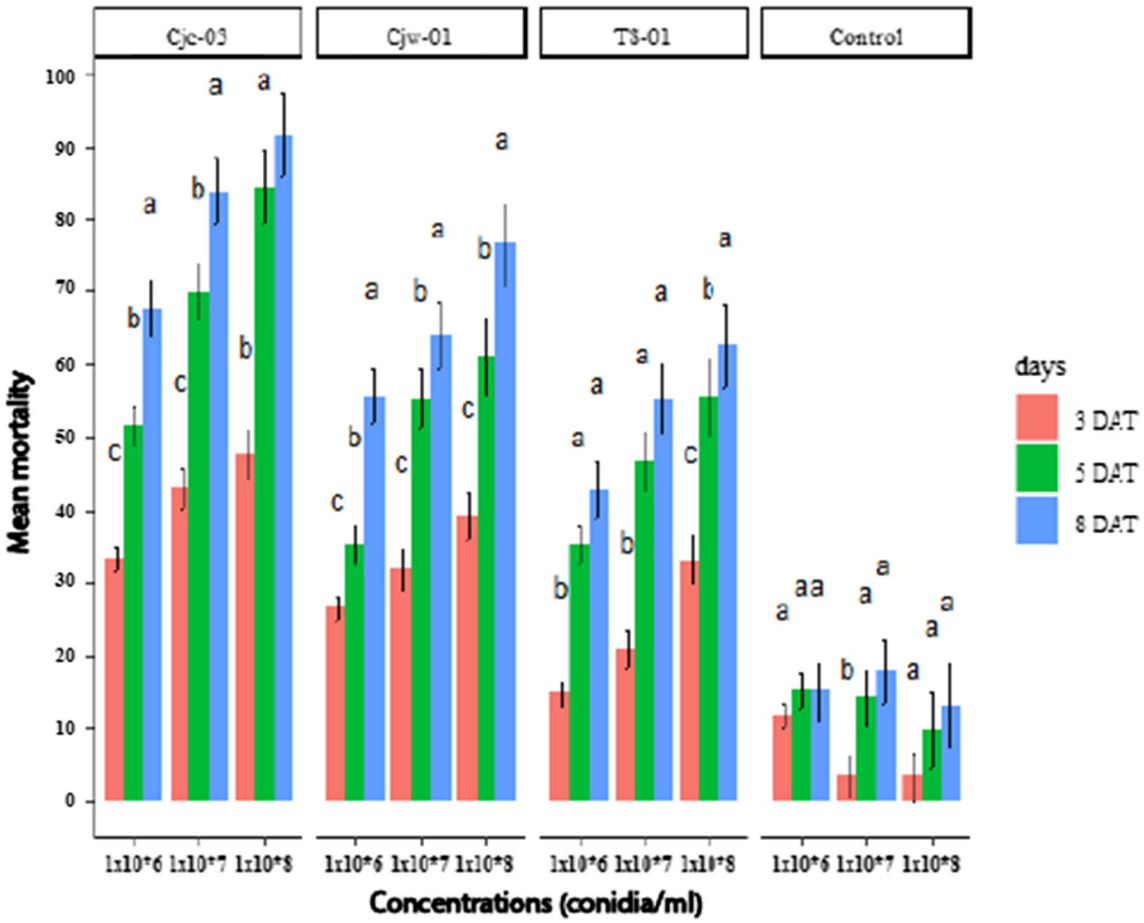

Fig. 4 Mortality (%) of *Bemisia tabaci* at different time intervals (3, 5, and 8 DAT) with 3 fungal isolates *Isaria javanica*, (Cjc-03, Cjw-01), and *Purpureocillium lilacinum* (TS-01) at three concentrations of each fungal isolates and control treatment. The column shows the % mortality. Treatment columns with different alphabets are significantly different from other treatments (LSD test at $p = 0.05$)

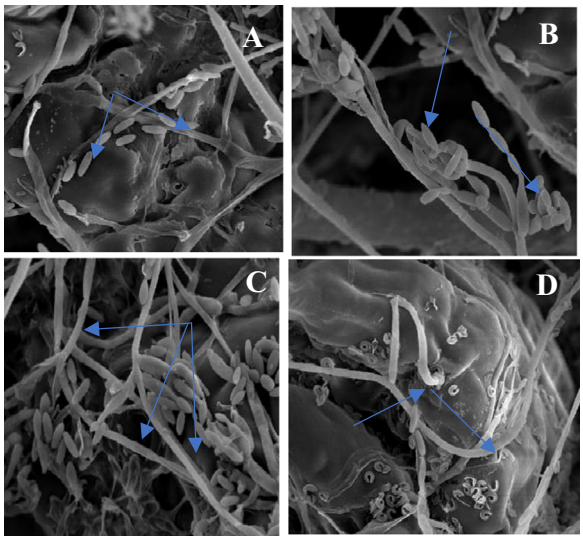

Fig. 5 Scanning electron microscopy (SEM) of the nymph of *Bemisia tabaci* infected with *Isaria javanica* at 8 days post-inoculation **A** Conidia of *I. javanica* attached to the body of *B. tabaci* **B** Germinated conidia of *I. javanica* **C** Mycelium growing on the integument of infected nymph **D** Hyphal penetration of *I. javanica* into the integument of an infected nymph of *B. tabaci*

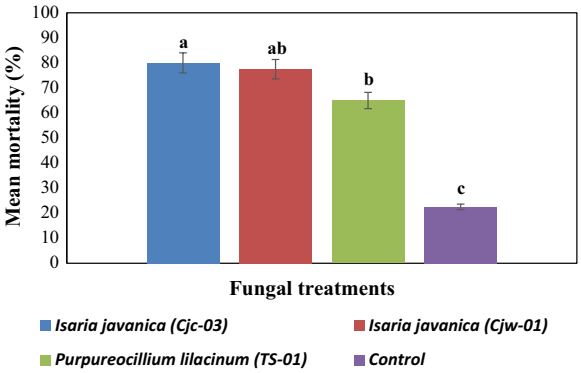

Fig. 7 Mortality of adult *Bemisia tabaci* at 7 DAT when treated with *Isaria javanica* (Cjc-03, Cjw-01) and *Purpureocillium lilacinum* (TS-01) at 1×10^7 conidia/ml concentration. Different letters above the bars indicate statistical significance ($p < 0.05$, LSD test)

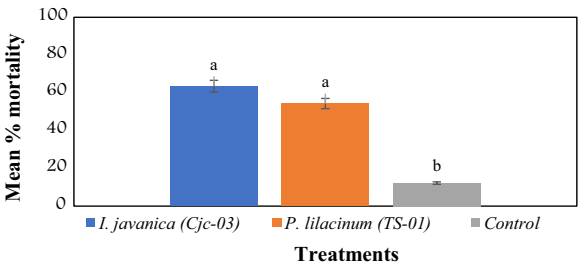

Fig. 8 Mortality (%) of *Bemisia tabaci* at 3 DAT when treated with 2 isolates (Cjc-03 and TS-01) at 1×10^7 conidia/ml concentration. Different letters above the bars indicate statistical significance ($p < 0.05$)

(Fig. 7) and *I. javanica* was the most effective against adults of *B. tabaci.*

Virulence of EPF isolates against *B. tabaci* adults on tomato leaves at 3 days after treatment in the glasshouse

In the glasshouse, the virulence of isolated fungi against adult whitefly showed that the two isolates, *I. javanica*, (Cjc-03) and *P. lilacinum* (TS-01) had significantly suppressed the adults of *B. tabaci* with a single conidia concentration (i.e., 1×10^7) ($F = 27.22$ $p < 0.001$). When *B. tabaci* adults infected with *I. javanica* and *P. lilacinum*, the mortality rates were greater than 50% on the 3rd day after treatment (Fig. 8). Results revealed that the infection and pathogenicity of *I. javanica* isolate to *B. tabaci*, in glasshouse conditions, was higher than those with *P. lilacinum*.

Fig. 6 The infection of *Bemisia tabaci* second instar nymphs treated with *Isaria javanica* and *Purpureocillium lilacinum* observed under a stereomicroscope **A** Healthy whitefly nymph before application **B** *B. tabaci* nymph after 8 days of infection by *I. javanica*; C: *B. tabaci* nymph after 8 days of infection by *P. lilacinum*

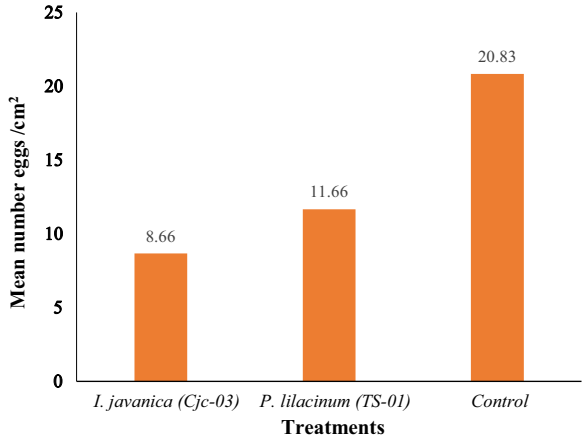

Fig. 9 Mean numbers (± SE) of *Bemisia tabaci* eggs on tomato leaves when infected with *Isaria javanica* and *Purpureocillium lilacinum* at 3 DAT

Effect of EPF isolates on the number of eggs, nymphs, and adults' emergence on tomato leaves in the glasshouse

The effect of *I. javanica* (Cjc-03) and *P. lilacinum* (TS-01) on the number of eggs, nymphs, and adults' emergence were examined on the tomato leaves following application on adult *B. tabaci*. There were significant differences in the number of eggs observed in the leaves from the treated plant and control leaves at 3 DAT ($F=17.76$ $p=0.0001$) (Fig. 9). The seedlings treated with Cjc-03 and TS-01 exhibited the least number of eggs with means of (8.66 and 11.66 eggs) (N cm^2 leaflet), respectively, when compared to seedlings of the control treatment 20.83 eggs (Fig. 9).

As for the number of the nymphs, it was observed that tomato plants treated with a single conidia concentration (i.e., 1×10^7 conidia/ml) reduced the numbers of nymphs of *B. tabaci* on the leaves after six weeks of treatment as compared to the control. A significantly high mean number was recorded in the control treatment 6.5 nymphs in comparison to the plants treated with Cjc-03 and TS-0 with means of 1.33 and 2.5 nymphs (N cm^2 leaflet), respectively ($F=36.05$ $p<0.001$) (Fig. 10). The adult emergence rate was also recorded from the exuviae left on the tomato leaves (leaflet 4×2 cm) of the treated plant at 42 days after application of single conidia concentration on adult *B. tabaci*. A significantly high mean number of adults emerged was recorded in the control treatment 18.66 adults in comparison to the plants treated with Cjc-03 and TS-0 with means of 8.33 and 11.83 adults (4×2 leaflet), respectively ($F=4.52$ $p=0.029$) (Fig. 10).

Discussion

In the present study, three EPF isolates, *I. javanica* (Cjw-01 and Cjc-03), and *P. lilacinum* (TS-01) were isolated and identified from immature *B. tabaci* individuals. Previous findings revealed that *I. javanica* and *P. lilacinum* could be isolated from infected *B. tabaci* and other insect pests and also potentially control many vegetable insect pests (Du et al. 2021). However, these two species were not previously isolated from insects in Malaysia and neighbouring countries. The first step in developing mycoinsecticides for the control of insect pests is to investigate them from a specific host. The isolation, identification, and virulence test of the fungal isolates are the important steps in developing EPF as BCA (Dayanti et al. 2018). Shah et al. (2020) had been previously isolated strains of *I. javanica* from the cadaver of *B. tabaci* sampled from the field of eggplant in Oman. Moreover, *I. javanica* had been isolated from several agricultural insect pests such as Asian citrus psyllid, *Diaphorina citri* Kuwayama, (Ou et al. 2019); Sunn pests (*Eurygaster* spp.); (Gül et al. 2021). *P. lilacinum* is well known to infect nematodes and little is known about its effect on

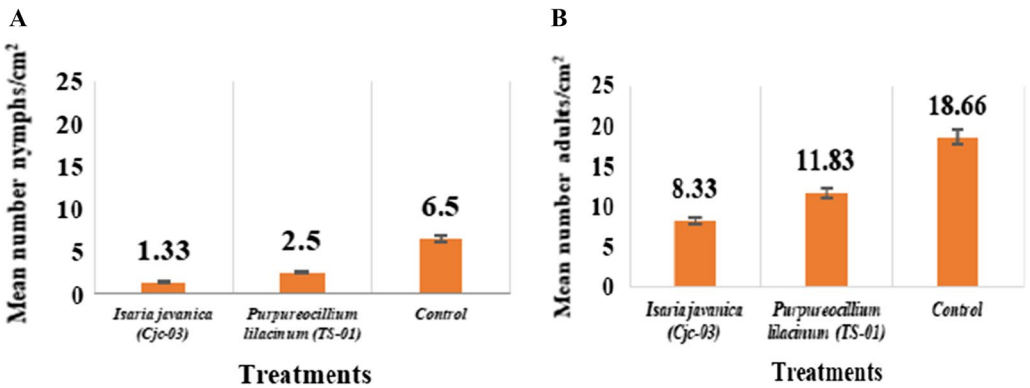

Fig. 10 Effect of *Isaria javanica* (Cjc-03) and *Purpureocillium lilacinum* (TS-01) on the mean number of nymphs and adults of *Bemisia tabaci* **A**: nymphs (III and IV) **B**: adult emergence of *B. tabaci* on tomato leaves at 42 DAT

insects pests (Toledo-Hernández et al. 2019). It was previously detected from mycoses instar of some certain insect pests such as *Antiteuchus innocens* Englemand & Rolston (Hemiptera: Pentatomidae) (Toledo-Hernández et al. 2019). To our knowledge, this is the new isolate of *P. lilacinum* isolated from field infested by *B. tabaci*. This is the fact that *Purpureocillium* spp., was a new divergent genus that was established in 2011 (Luangsa-Ard et al. 2011), and was more frequent and commonly isolated from soil samples as has been observed in several previous studies (Sun et al. 2021).

The growth rate of the three isolates was studied under laboratory conditions. Insect biocontrol depends on the fungal strain with good growth characteristics (Meng et al. 2017). All the three isolates grew optimally and produced spores that are similar and better than those previously reported (Du et al. 2021). Several studies have demonstrated the effectiveness of *I. javanica* and *P. lilacinum* for the control of *B. tabaci* (Sun et al. 2021). In Malaysia, the application of EPF as biological control of pests is currently in its early phase of evaluation. The study has been mainly focused on *M. anisopliae*. Despite the potentials of *I. javanica* and *P. lilacinum* as a BCA for a variety of insect pests (Sun et al. 2021), there has been little or no information on their use for the control of insect pests in Malaysia, compared to *M. anisopliae* and other species of EPF.

Based on the result of the laboratory study, both nymphs and adults *B. tabaci* were susceptible to all the three isolates (Cjw-01, Cjc-03, and TS-01). It is discernible from this study that second nymphal instar of *B. tabaci* were the most susceptible to infection than adults' stage, confirming previous reports that the second instar of *B. tabaci* was highly susceptible to EPF infection (Sain et al. 2019). The mortality rate of the second nymphal instar varied depending on the concentration of spores and period of application. It was noted that mortality increased by increasing the concentration of conidia and the time interval between applications. This result is in accordance with Abdulle et al. (2020) findings who demonstrated the effectiveness of three isolates of *L. lecanii* against *B. tabaci* under laboratory conditions and caused the highest mortality up to 93% in the highest concentration (1×10^7 conidia/ml) at 7 days post inoculation (dpi). Accordingly, Wu et al. (2020) reported the significant mortality of *P. xylostella* upon application of newly isolated *Isaria* spp. A significant effect of temperature, conidia concentration, and period of exposure in causing mortality of *B. tabaci* by *B. bassiana* and *L. lecanii* was observed by Keerio et al. (2020).

With the same conidia concentration and exposure time, it was observed that second instar mortality rate was higher in case of the *I. javanica* treatment than in the *P. lilacinum* treatment. This might be due to the variation in mechanical damage and production of toxins resulting in insect death (Singh et al. 2017). For example, Wu et al. (2021), conducted experiment to determine the virulence of *I. javanica* and other strains of *B. bassiana* and *M. anisopliae* against the 4th nymphal instar of *B. tabaci*. A strain of *I. javanica* was found to exhibit high virulence among all the strains tested.

Moreover, the single application of conidial concentration (1×10^7 conidia/ml) on adult *B. tabaci* resulted in more than 50% mortality for seven days post inoculation from all three isolates. A similar trend was also observed in previous research with *P. lilacinum* application. Glasshouse experiments are essential to confirm laboratory findings before moving on with full-scale commercial development (Cuthbertson et al. 2007). In this study, the glasshouse bioassay of one isolate each of *I. javanica* and *P. lilacinum* (Cjw-03 and TS-01) with single conidia concentration (1×10^7 conidia/ml) against adults *B. tabaci* caused mortality 63.5 and 54.2% over a period of 72 h, respectively.

The fungi evaluated significantly decreased the number of eggs and nymphs than control treatment. The conidial concentration of the EPF sprayed on the leaves was discovered to colonize a host plant completely, and retrieved from different parts (roots and shoots), demonstrating their potential to protect the entire plant from insect pests with a single application (Bamisile et al. 2019).

Furthermore, the adult emergence rate observed at six weeks after treatment was significantly lower in the seedlings treated with Cjc-03 and TS-01 isolates than to the control treatment. Other studies, *e.g.* Zhu and Kim (2011) reported that the application of fungal isolates, *Beauveria. bassiana*, *I. fumosorosea,* and *L. lecanii* against *B. tabaci* on eggplant significantly reduced the adults' emergence of *B. tabaci*.

Conclusion

Three EPF isolates, *I. javanica*, (Cjw-01 and Cjc-03), and *P. lilacinum* (TS-01) were isolated and identified from immature *B. tabaci* individuals. All three EPF strains showed pathogenicity on the adults and immature stages of *B. tabaci* where the reduction numbers of eggs, nymphs and adults' emergence were recorded. The locally isolated strains of *I. javanica* and *P. lilacinum* could be developed as potential biopesticides against *B. tabaci* although the development of proper formulation is still required.

Acknowledgements

The authors would like to express their gratitude to the Tertiary Education Trust Fund (TETFund), Nigeria for providing Ph.D. scholarship and Universiti Putra Malaysia (UPM) for the general support during the study.

Author contributions
IS analyzed and interpreted the data regarding the virulence of entomopathogenic fungi, *Isaria javanica* and *Purpureocillium lilacinum* and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Funding
This study was funded by the Fundamental Research Grant Scheme (FRGS) from the Ministry of Higher Education Malaysia (Grant No. 5540212) (FRGS/1/2019/WAB01/UPM/02/36).

Availability of data and materials
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 24 August 2022 Accepted: 2 February 2023
Published online: 12 February 2023

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