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Morphological and molecular identification of four *Purpureocillium* isolates and evaluating their efficacy against the sweet potato whitefly, *Bemisia tabaci* (Genn.) (Hemiptera: Aleyrodidae)

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Abstract

Background: Entomopathogenic fungi are widely distributed and well described within the fungal kingdom. This study reports the isolation, characterization, and virulence of 4 *Purpureocillium lilacinum* isolates against the sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae).

Results: Four strains of *Purpureocillium lilacinum* (XI-1, XI-4, XI-5, and J27) were isolated from soil samples from different localities of China. The morphological studies observed that four strains showed essentially the same morphological characteristics. After 7 days of cultivation, the colonies were purple, round, and bulged. Conidia were single-celled, oval to spindle-shaped, chain-like, and the spore size was about 2.0–2.3 × 3.1–4.0 μm. The genome-based identification results showed that ITS sequences of XI-1 (GenBank accession # MW386433), XI-4 (GenBank accession # MW386434), XI-5 (GenBank accession # MW386435), and J27 (GenBank accession # MW386436) were similar to another *P. lilacinum*. The newly identified strains of *P. lilacinum* proved pathogenicity to *B. tabaci* under laboratory conditions. In addition, the *P. lilacinum* isolate XI-5 was the most virulent one against different nymphal instars of whitefly having median lethal concentration (LC_{50}) values of 4.99×10^6, 4.82×10^5, and 2.85×10^6 conidia/ml, respectively, 7 days post application.

Conclusion: The newly isolated strains of *P. lilacinum* can be developed as a potential biopesticide against the whitefly although extensive field bioassays as well as development of proper formulation are still required.

Keywords: Entomopathogenic fungi, *Purpureocillium lilacinum*, Isolation, *Bemisia tabaci*, Virulence

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Background

Sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae), is a key pest of different crops and vegetables worldwide along with serving as a vector of many plant viruses (Naranjo et al. 2010 and Stansly and Naranjo, 2010). *B. tabaci* Middle East-Asia Minor 1 (MEAM1) cryptic species (formerly "B biotype") exists in 31 provinces or municipalities of the People's Republic of China causing huge economic losses to different crops (Liu and Liu 2012). Management of heavy whitefly infestations is still mainly dependent upon broad spectrum conventional insecticides, which have led to the development of insecticide resistance by *B. tabaci* (Liang et al. 2012). Therefore, finding alternate pest control strategies such as use of entomopathogenic fungi (EPF) is necessary in order to overcome the above-mentioned problems (Cuthbertson et al. 2005).

Identification and characterization of EPF is an initial step of biopesticide development (Dunlap et al. 2017). During the past decade, the techniques employed for isolation and identification of EPF have evolved from morphological characterization to amplification of different DNA or RNA fragments through polymerase chain reactions improving the basic understanding about EPF (Canfora et al. 2016). In recent years, molecular techniques have become widely used for identification of EPF as the genotypic identification methods (such as random amplified polymorphic DNA-polymerase chain reaction) are more precise than the morphological character-based identification (Du et al. 2019).

The genus *Purpureocillium* Luangsa-ard (Hypocreales: Ophiocordycipitaceae), previously classified in genus *Paecilomyces*, consists of fungal species having pathogenicity against herbivore insects and different species of nematodes (Fan et al. 2013; Goffré and Folgarait, 2015).

P. lilacinum is a known species being used for biological control of aphids, thrips, whiteflies, fruit flies, beetles, mosquitoes, and plant parasitic nematodes (Amala et al. 2013; Goffré and Folgarait 2015). *P. lilacinum* isolates are sometimes misidentified to *Isaria* spp. as the anamorphs of both groups are similar (Yamamoto et al. 2020). Therefore, use of genotypic identification tools should prove an effective solution for the correct identification.

The present experiments reported morphological and genome-based identifications of 4 *P. lilacinum* isolated from soil samples collected from different localities of China along with their virulence against *B. tabaci* under laboratory conditions.

Methods

Insect culture

Whitefly cultures, reared (*Gossypium hirsutum* on 26 ± 1 °C, 70 ± 10% R.H. and 14:10 h light: dark photoperiod) at the Engineering Research Center of Biological Control, Ministry of Education, South China Agricultural University, were used during this study.

Collection of soil samples and isolation of fungi

Samples of soil for fungal isolation were collected from different provinces of the People's Republic of China during 2015–2017. The samples were collected from 10 cm below the soil surface and placed in plastic bags. Collected samples were stored at 4 °C. Fungi were isolated by the methods of Imoulan et al. (2011) and Du et al. (2019). Briefly, a mixture of soil sample (3 g) and 30 ml sterilized 0.05% Tween-80 solution in ddH$_2$O was prepared by stirring for 15 min. The prepared suspension (1 ml) was inoculated on PDA plates, followed by incubation at 25 ± 1 °C and 80 ±
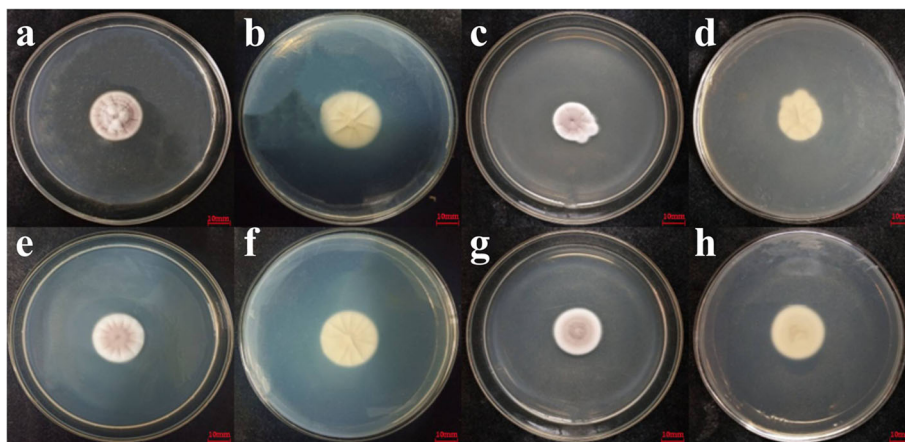

Fig. 1 Colony morphology of different *Purpureocillium lilacinum* isolates after 7 days of growth. **a** Frontal view of isolate Xl-1 colony. **b** Rear view of isolate Xl-1 colony. **c** Frontal view of isolate Xl-4 colony. **d** Rear view of isolate Xl-4 colony Xl-4. **e** Frontal view of isolate Xl-5 colony. **f** Rear view of isolate Xl-5 colony Xl-5. **g** Frontal view of isolate J-27 colony. **h** Rear view of isolate J-27 colony
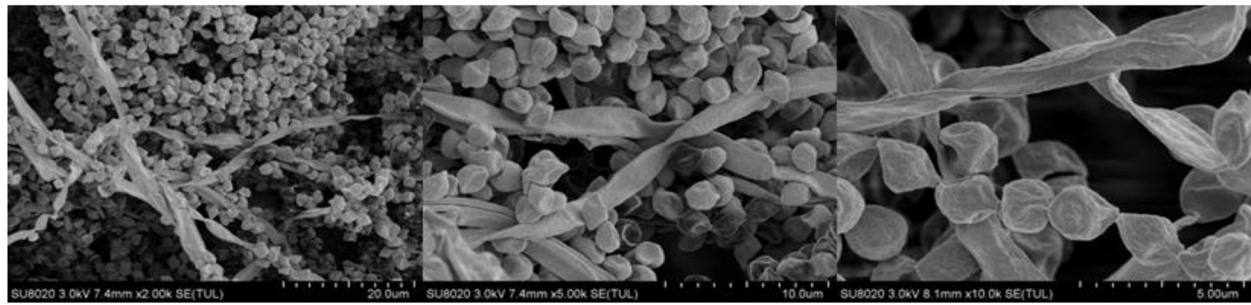
Fig. 2 Scanning electron micrograph of conidia and conidiophores of strain *Purpureocillium lilacinum*

5% R.H, 16:8 h (light/dark photoperiod). The PDA plates were observed after 7 days of inoculation, followed by re-inoculation of grown fungi to fresh PDA plates. The inoculation process was repeated until pure fungal culture was obtained. According to the phenotypic characteristics and fungal morphology (Saito and Brownbridge 2016), the cultured fungi were purified until there was only one fungus colony on the PDA plate.

Morphological characterization
Morphological characters of 4 fungal isolates (XI-1, XI-4, XI-5, and J27) were studied through inoculating fungal mycelial plug (1 cm Ø) observed by culturing small fungal mycelia PDA block draped by a cover slip for 10 days (Imoulan et al. 2011; Du et al. 2019). The slides were stained by lactophenol cotton blue and were observed under a phase-contact microscope (×100). Conidial images were taken through Axio Cam HRc camera (Carl Zeiss) having the AxionVision SE64 Release 4.9.1 software.

Fungal growth and conidia production
Expansion rates of fungal colonies and conidia production by different *P. lilacinum* isolates were observed, following Ali et al. (2009). Fungal mycelial plugs (1 cm Ø) were cultured on PDA plates, followed by incubation at 25 ± 1 °C and 80 ± 5% R.H and 16: 8 h (light/dark photoperiod) for 10 days. Colony growth was measured every day, and conidia were scrapped from PDA plates 10 days post inoculation.

Table 1 Colony expansion (per day) and conidia produced by different fungal *Purpureocillium lilacinum* isolates after 7 days

Isolate	Radial growth (mm/day)	Conidial yield (conidia/mL)
XI-1	3.64 ± 0.04 a	$3.78 \times 10^6 \pm 0.12$ a
XI-4	3.09 ± 0.02 b	$2.87 \times 10^6 \pm 0.07$ b
XI-5	3.62 ± 0.06 a	$0.88 \times 10^6 \pm 0.07$ d
J27	3.67 ± 0.05 c	$1.22 \times 10^6 \pm 0.06$ c
F; d.f; P	36.77; 3,8; < 0.001	271.26; 3,8; < 0.001

Conidia were added into 100 ml of 0.05% Tween-80. Muslin cloth was used to filter the conidial suspension. Total conidial yield was calculated by counting the conidia in a hemocytometer under a phase-contrast microscope at × 40 (Ali et al. 2009).

Observation of spores of strains by scanning electron microscopy
Conidia were harvested from a PDA plate, and the conidial suspensions at the 1×10^7 conidia/ml were prepared by using deionized water containing 0.05% Tween-80 solution. Conidial suspensions (8 ml) were inoculated into 100 ml SDA medium (1% peptone, 4% glucose), and the inoculated medium was cultured at 26 °C, 180 rpm for 5 days. The hyphae were obtained through a vacuum suction pump and were fixed with 2.5% glutaraldehyde at 4 °C for 24 h, rinsed with phosphate buffer solution, dehydrated, and dried with alcohol, and the sample was fixed on the sample stage with a conductive adhesive and photographed with a scanning electron microscope (SU8020, Hitachi, Japan).

DNA extraction, PCR amplification, and sequence analysis
Genomic DNA of newly isolated fungi was obtained by using a fungal DNA isolation kit (Ezup, Sangon Biotech, Shanghai, China). The obtained DNA served as a template for amplification of ITS (internal transcribed spacer) fragment through PCR. Reaction mixture (50 μl) was used to perform all PCR reactions. The ITS primers (ITS4F: TCCTCCGCTTATTGATATGC; and ITS5R: GGAA GTAAAAGTCGTAACAAGG) reported by White et al. (1990) were used to amplify ITS regions. The PCR cycling and conditions of Du et al. (2019) were used for amplification of ITS regions. The purity of PCR products was observed through agarose gel electrophoresis, followed by GenGreen (TianGen Biotech, Beijing, China) staining. Pure PCR products were sequenced by Shanghai Majorbio Bio-pharm Technology (Shanghai, China). Geneious version 9.1.4 was used to assemble the sequences, followed by GenBank blast. MEGA version 7.0 was used to compare the sequences whereas Kimura-2-parameter (K2P)

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Table 2 Details of GeneBank sequences used in this study

Isolate	GenBank accession	ITS Species	Accession number	Strain No.	Host	Location	Query coverage	E value	Percentage nucleotide identity
XI-1	MW386433	*Purpureocillium lilacinum*	FJ765024.1	INTR-2	Soil	China	99%	0.0	99.81%
XI-4	MW386434	*Purpureocillium lilacinum*	HQ607867.1	ATT161	Ant	Brazil	100%	0.0	99.45%
XI-5	MW386435	*Purpureocillium lilacinum*	GU980033.1	NRRL22958	/	Thailand	100%	0.0	99.81%
J27	MW386436	*Purpureocillium lilacinum*	MK361144.1	B1	Soil	France	100%	0.0	100.00%
		Beauveria bassiana	AF430703.1	Bb235	Curculionidae	UK	/	/	/
		Beauveria bassiana	AJ560668	IMI 386701	Storage pests	UK	/	/	/

was used to build maximum likelihood (ML) tree (Kimura, 1980; Goloboff and Catalano, 2016).

Insect bioassays

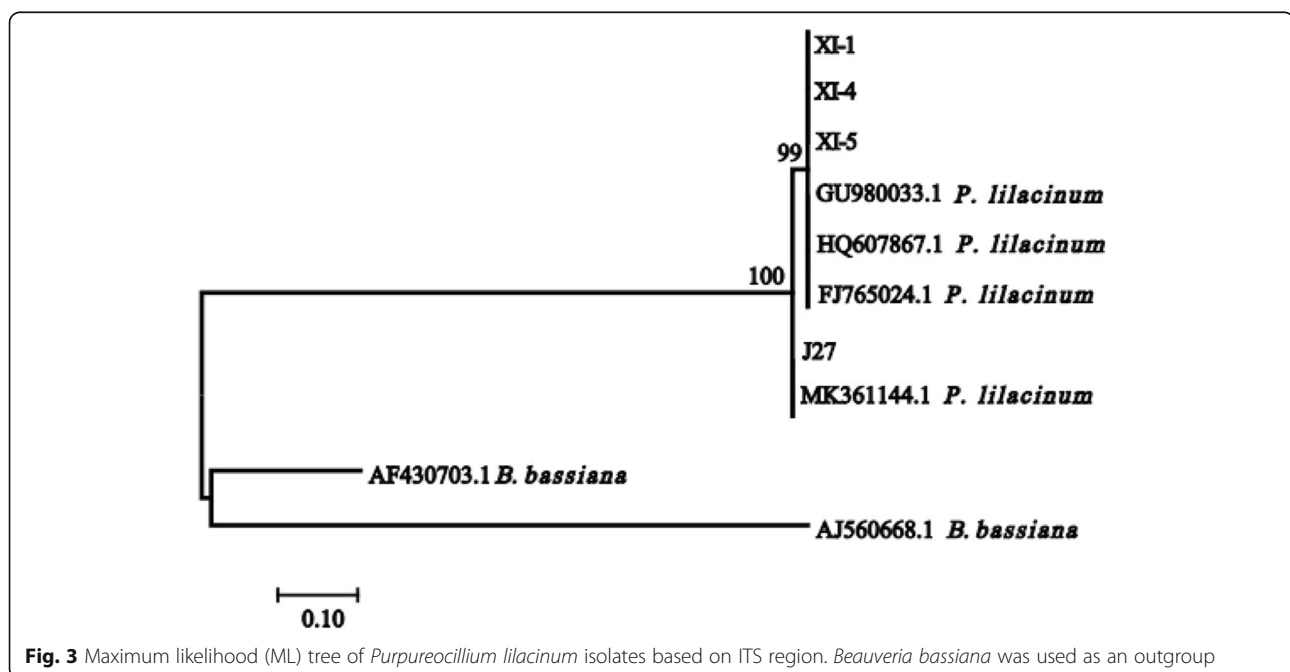#### Pathogenicity of isolated fungi against B. tabaci

The newly isolated *P. lilacinum* strains (XI-1, XI-4, XI-5, and J27) grown on PDA were used to prepare conidial suspension (1×10^8 conidia/ml), following Ali et al. (2009). The leaf dip bioassay method of Zhang et al. (2017) was used to carry out the pathogenicity studies. Cotton leaves having 2nd instar *B. tabaci* nymphs were dipped in the fungal suspension for 30 s. The leaves were air dried and placed in ø 9 cm having a moist filter paper for moisture maintenance. *B. tabaci* nymphs, dipped in 0.01% Tween 80, served as control. Four cotton leaves having 100 *B. tabaci* nymphs/leaf were used in each treatment. The whole experimental setup was placed at 25 ± 1 °C and 80 ± 5% R.H and 16:8 h (light/dark photoperiod). The whole experiment was performed thrice (with fresh batch of insects and fresh conidial
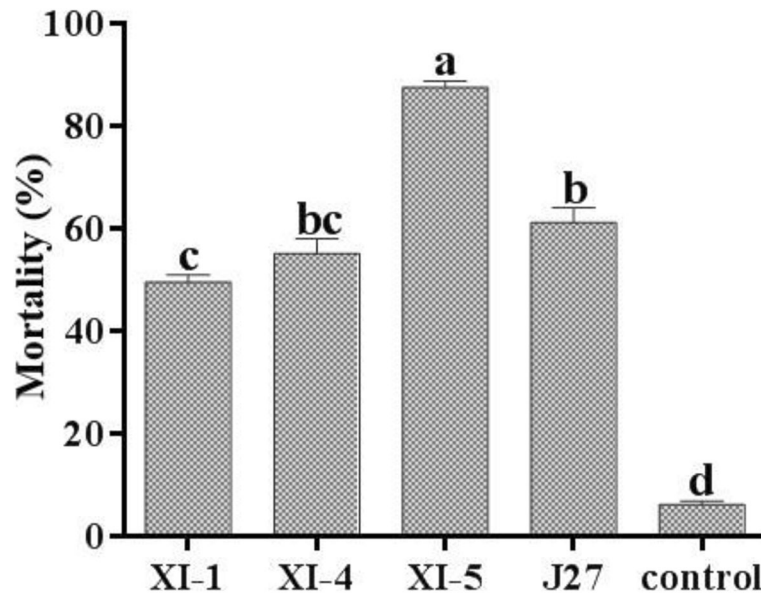
suspension). Insect mortality was recorded on daily basis until 7 days post treatment.

Pathogenicity of P. lilacinum isolate XI-5 against immature instars of B. tabaci

Different conidial concentrations (1×10^8, 1×10^7, 1×10^6, 1×10^5, and 1×10^4 conidia/ml) of *P. lilacinum* isolate XI-5 were prepared following Ali et al. (2009). Cotton leaves having *B. tabaci* nymphs (2nd, 3rd, or 4th instars) were dipped in each conidial concentration for 30 s. The leaves were air dried and placed in ø 9 cm having a moist filter paper for moisture maintenance. *B. tabaci* nymphs, dipped in 0.01% Tween 80, served as control. Four cotton leaves having 100 *B. tabaci* nymphs/leaf were used in each treatment. The whole experimental setup was placed at 25 ± 1 °C and 80 ± 5% R.H and 16:8 h (light/dark photoperiod). The whole experiment was performed thrice (with fresh batch of insects and fresh conidial

Fig. 3 Maximum likelihood (ML) tree of *Purpureocillium lilacinum* isolates based on ITS region. *Beauveria bassiana* was used as an outgroup

Fig. 4 *Bemisia tabaci* mortality in response to treatment with conidial suspension of (1×10^8 conidia/mL) of different *Purpureocillium lilacinum* isolates after 7 days. Bars indicate standard error of means. Bars having different letters show significant difference between means (Tukey's, $P \leq 0.05$)

suspension). Insect mortality was recorded on daily basis until 7 days post-treatment.

Transmission electron microscopy

Second instar *B. tabaci* nymphs treated with *P. lilacinum* isolate XI-5 (1.0×10^8 conidia/ml) were observed for external as well as internal anatomical changes after different 3, 5, and 7 days post-treatment. The insects' samples were treated and observed following Du et al. (2019).

Statistical analysis

Data regarding colony expansion rate and conidial yield were analyzed through ANOVA-1, and significant differences among means were calculated through Tukey's HSD test ($P \leq 0.05$). Insect mortality values were percent-transformed, followed by probit analysis (Finney et al. 1971). The SAS 9.2 software was used for all data analysis (SAS et al. 2000).

Results

Morphological identification of fungal isolates

The four strains showed essentially the same morphological characteristics. The morphological characteristic is reported below. First, it grows white colonies, such as the edges of the colonies were white. The later growth gradually turned purple. After 7 days of cultivation, the colonies were purple, round, and bulged. The texture was tightly cotton-like, and there was no secretion on the surface (Fig. 1). Conidia are single-celled, oval to spindle-shaped, chain-like, and the spore size is about 2.0–2.3 × 3.1–4.0 μm microns (Fig. 2). The radial growth/day of fungal isolates showed significant differences after 7 days of growth ($F_{3,16} = 36.77$, $P < 0.001$). The highest colony expansion rate (3.67 mm/day) was observed for *P. lilacinum* isolate J27, whereas the lowest minimum colony expansion rate was recorded for *P. lilacinum* isolate XI-4, having a mean value of 3.09 mm/day (Table 1).

Table 3 Median lethal concentration (LC$_{50}$) of *Purpureocillium lilacinum* isolates against *B. tabaci* nymphs 7 days post fungal application

Strains	LC$_{50}$ (conidia/ml)	95% confidence limit
XI-1	3.44×10^8 a	(8.44×10^7–3.67×10^9)
XI-4	3.09×10^7 b	(9.53×10^6–1.99×10^8)
XI-5	4.82×10^5 c	(1.05×10^5–1.80×10^6)
J27	1.76×10^7 b	(5.22×10^6–1.22×10^8)

Table 4 Median lethal time (LT$_{50}$) values for 1×10^8 conidia/mL of *Purpureocillium lilacinum* against *B. tabaci*

Strains	LT$_{50}$ (days)	95% confidence limit
XI-1	7.13 a	(6.20–8.66)
XI-4	5.23 b	(4.75–5.86)
XI-5	2.54 c	(1.93–3.10)
J27	5.45 b	(4.89–6.23)

Means followed by lowercase letters in the same columns are significantly different from each other (Tukey's $P \leq 0.05$); ± indicate the standard error of means based on five replicates ($n = 5$)

The number of conidia produced by different fungal isolates showed significant differences after 7 days of growth ($F_{3,16} = 271.26$, $P < 0.001$). The highest conidial yield (3.78×10^6 conidia/ml) was observed for *P. lilacinum* isolate XI-1, whereas the lowest conidial yield was obtained from *P. lilacinum* isolate XI-5 having a mean value of 8.8×10^5 conidia/ml (Table 1). The conidial polymorphism found within these isolates is probably due to their genetic variability (Varela and Morales, 1996).

Molecular analyses and BLASTN comparisons

Partial ITS sequences were obtained through PCR amplification of purified DNA. The sequence length of ITS fragment of XI-1 was 535 bp, XI-4 was 543 bp, XI-5 was 536 bp, and J27 was 540 bp. The results showed that ITS sequence of XI-1 (GenBank accession # MW386433) was 99.81% similar to another *P. lilacinum* GenBank sequence FJ765024.1. The ITS XI-4 sequence (GenBank accession # MW386434) showed 99.45% similarity with *P. lilacinum* sequences HQ607867.1, while ITS sequence of XI-5 (GenBank accession # MW386435) was 99.81% similar to *P. lilacinum* sequence GU980033.1. The ITS sequence of *P. lilacinum* isolate J27 (GenBank accession # MW386436) was 100% similar to *P. lilacinum* strain sequences MK361144.1. The details of

GenBank sequences used are shown in Table 2. Multiple comparisons were performed by Clustal W, and phylogenetic trees were constructed separately according to the maximum likelihood method, and *B. bassiana* was used as an outgroup. The results are shown in Fig. 3.

Pathogenicity of *P. lilacinum* against *B. tabaci*

Preliminary bioassays of different fungal isolates against *B. tabaci*

The mortalities of *B. tabaci* nymphs, following treatment with *P. lilacinum* isolates (XI-1, XI-4, XI-5, and J27), were significantly different at the end of experimental period. The highest corrected *B. tabaci* mortality (86.81%) was caused by *P. lilacinum* isolate XI-5. *P. lilacinum* isolate XI-1 proved the least virulent having corrected mortality value of 46.24% (Fig. 4). The median lethal concentration (LC_{50}) values after 7 days of application were significantly different among different isolates. The highest LC_{50} value (3.44×10^8 conidia/ml) was observed at *P. lilacinum* isolate XI-1, while the lowest LC_{50} value (4.82×10^5 conidia/ml) was observed at *P. lilacinum* isolate XI-5 (Table 3).

Means followed by lowercase letters in the same columns are significantly different from each other (Tukey's $P \leq 0.05$)

The median lethal time (LT_{50}) values of different *P. lilacinum* isolates against *B. tabaci*, when applied at a rate of 1×10^8 conidia/ml, showed significant differences. The highest LT_{50} value (7.13 days) was observed
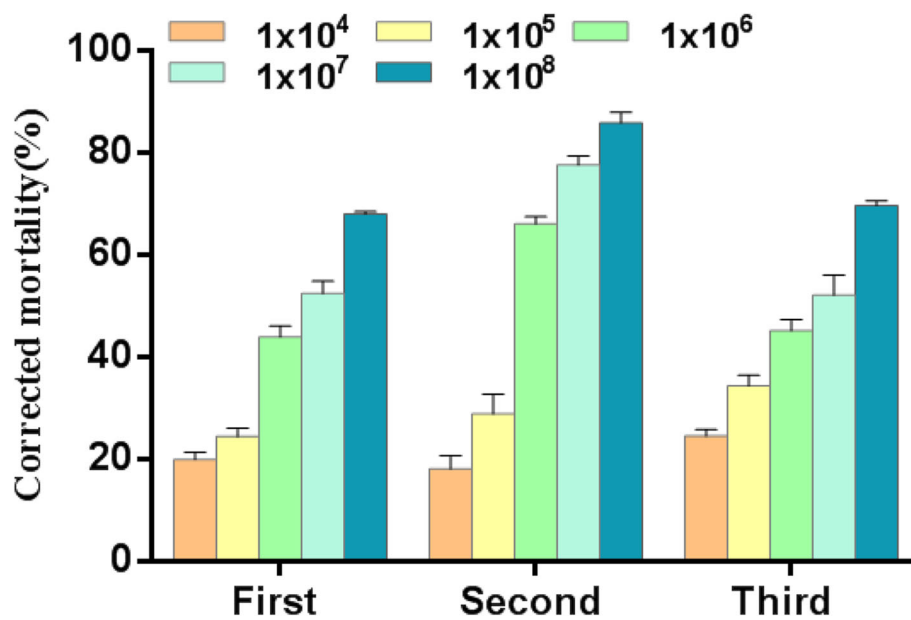

Fig. 5 Corrected mortality of *Bemisia tabaci* nymphs in response to treatment with different conidial concentrations of different *Purpureocillium lilacinum* isolate XI-5 after 7 days. Bars indicate standard error of means

Table 5 Median lethal concentration (LC$_{50}$) of *P. lilacinum* isolate XI-5 against *B. tabaci* nymphs 7 days post fungal application

Nymphal instar	LC$_{50}$ (conidia/ml)	95% confidence limit
First instar	4.99×10^6 a	(2.25×10^6–1.32×10^7)
Second instar	4.82×10^5 b	(1.05×10^5–1.80×10^6)
Third instar	2.85×10^6 c	(1.14×10^6–8.42×10^6)

for *P. lilacinum* isolate XI-1, while the lowest LT$_{50}$ value (2.54 days) was observed for *P. lilacinum* isolate XI-5 (Table 4).

Means followed by lowercase letters in the same columns are significantly different from each other (Tukey's $P \leq 0.05$)

Concentration mortality response of *P. lilacinum* isolate XI-5 against different nymphal instars of *B. tabaci*

Mortality rates of *B. tabaci* nymphs (1st, 2nd, and 3rd instars), following treatment with *P. lilacinum* isolate XI-5 increased with increase of fungal concentrations (Fig. 5). LC$_{50}$ of *P. lilacinum* isolates XI-5 against different nymphal instars of *B. tabaci* were obtained through probit analysis. The LC$_{50}$ of *P. lilacinum* isolates XI-5 were 4.99×10^6, 4.82×10^5, and 2.85×10^6 conidia/ml against 1st, 2nd, and 3rd instar nymphs, respectively (Table 5).

Means followed by lowercase letters in the same columns are significantly different from each other (Tukey's $P \leq 0.05$)

Microscopic examination of *B. tabaci* infection

B. tabaci nymphs infected with *P. lilacinum* XI-5 showed significant morphological changes at different infection periods. The physical appearance of *B. tabaci* did not change much after 1 day of fungal application. On the 3rd day of infection, the fungal hyphae started to grow from the edges of the insect's body. On the 5th day of post-fungal application, the hypha covered most of the insect's body and turned yellowish brown. On the 7th day was post-fungal application, the insects were covered with hyphae (Fig. 6). Transmission electron microscope images clearly showed that the hyphae proliferated massively and occupied the whole haemocoel at 72 h post-inoculation (Fig. 7). Obtained findings revealed that *B. tabaci* were penetrated and killed by *P. lilacinum* XI-5 (Fig. 7).

Discussion

The correct understanding of taxonomy/identification as well as pathogenic potential of microbial control agents is a pre-requisite for their development as an alternative to synthetic chemicals (Du et al. 2019). During this study, 4 fungal isolates of *P. lilacinum* were identified on phenotypic as well as their genotypic basis. All of the identified isolates tested for their pathogenicity against 2nd instar *B. tabaci* nymphs showed considerable pathogenic potential. The concentration response relationship studies confirmed the pathogenic ability of *P. lilacinum* isolate X1-5 against different immature life stages of *B. tabaci*.
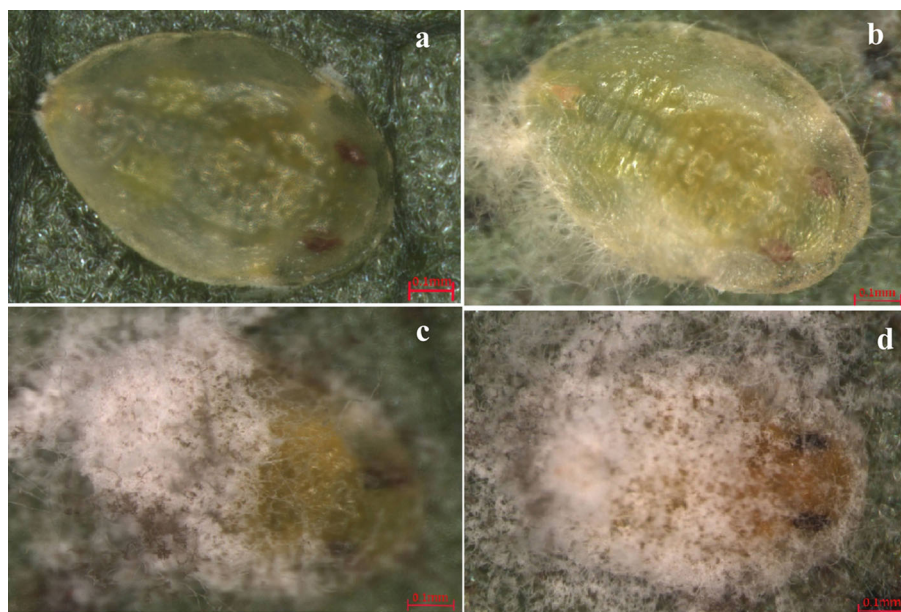

Fig. 6 Images of *B. tabaci* nymphs infected with strain *Purpureocillium lilacinum* XI-5, observed through a dissecting microscope. **a**, **b**, **c**, and **d** were XI-5-infected *B. tabaci* nymphs at 1 day, 3 days, 5 days, and 7 days post-infected
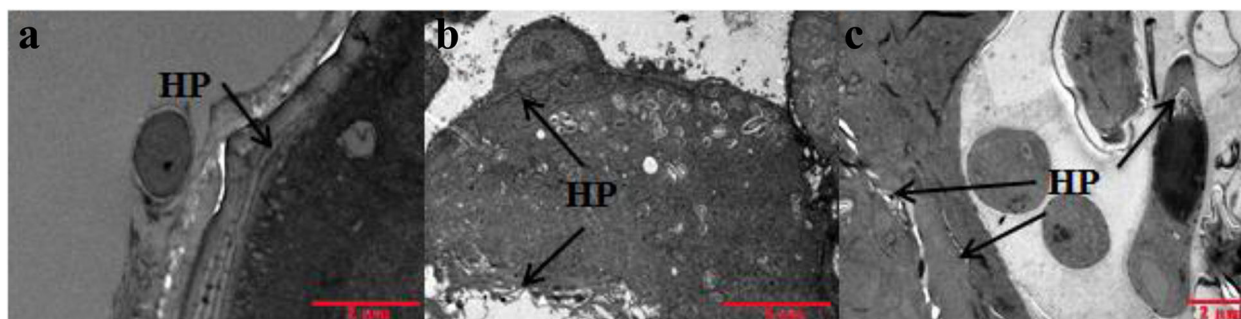
Fig. 7 Transmission electron microscope images of the cross sections of *Bemisia tabaci* bodies showing the growth of *Purpureocillium lilacinum* in *B. tabaci*. **a**, **b**, and **c** are XI-5-infected *B. tabaci* nymphs at 3 days, 5 days, and 7 days post-infected. HP, hyphae

Hypocrealean fungi, being a large and complex group, are not easy to classify based on morphological characters, which has led to the use of genomic data for identification of these fungi (Lu et al. 2015; Wang et al. 2017). The ITS rDNA fragment is a commonly used gene to classify morphologically similar species of fungi (Lu et al. 2015). The pairwise comparison of ITS sequence data showed that strains XI-1, XI-4, XI-5, and J27 had 99–100% similarity with different previously described strains of *P. lilacinum* (GenBank # FJ765024.1, HQ607867.1, GU980033.1, and MK361144.1).

The present studies revealed pathogenic capacities of the 4 *P. lilacinum* isolates (XI-1, XI-4, XI-5, and J27) against *B. tabaci*. Intraspecific variation was observed within different isolates during pathogenicity studies. The calculation of LT_{50} for different isolates revealed that isolate XI-5 seemed to be the most virulent one, when compared to other isolates, which is in line with the idea of pathogenicity as the ability of a pathogen to induce disease in target host described by Steinhaus and Martignoni (1970). The highest pathogenicity of *P. lilacinum* isolate XI-5 compared to other isolates can be attributed to variation in production of infection-related proteins or toxins (Khan et al. 2004). The variations might be related to different target insect species or size of the insect host (Jandricic et al. 2014). Based on these preliminary findings, *P. lilacinum* isolate XI-5 can be used for development of biopesticides against *B. tabaci*.

Conclusions

The newly identified strains of *P. lilacinum* (XI-1, XI-4, XI-5, and J27) showed good growth characteristics and caused considerable mortality to *B. tabaci* nymphs. *P. lilacinum* isolate XI-5 was the most virulent one. Based on these preliminary findings, *P. lilacinum* isolate XI-5 could be suggested for development of a biopesticide against *B. tabaci*.

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