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Morphological and molecular identification of four isolates of the entomopathogenic fungal genus *Akanthomyces* and their effects against *Bemisia tabaci* on cucumber

Fereshteh Broumandnia, Ali Rajabpour, Mohamad Hamed Ghodoum Parizipour and Fatemeh Yarahmadi

Department of Plant Protection, Faculty of Agriculture, Agricultural Sciences and Natural Resources University of Khuzestan, Mollasani, Ahvaz, Iran

Research Paper

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Author for correspondence:
Ali Rajabpour,
Email: a_rajabpour2000@yahoo.com; rajabpour@asnrukh.ac.ir

Abstract

The cotton whitefly, *Bemisia tabaci* Gen. (Hem., Aleyrodidae), is a key pest of many vegetables. Entomopathogenic fungi are promising microbial control agents against *B. tabaci*, but limited information is available concerning indigenous Iranian isolates. In this study, three isolates of *Akanthomyces lecanii* (PAL6, PAL7, and PAL8) and one isolate of *A. muscarius* (AGM5) were obtained from citrus hemipteran pests, *Pulvinaria aurantii* Cock. and *Aphis gossypii* Glover, in Mazandaran province, northern Iran. The isolates were then morphologically and molecularly identified. The efficacies of five different agar media for vegetative growth and conidiation of each isolate were determined. Potato dextrose agar was the medium on which the fungal mycelia developed at a relatively high rate. However, the highest rate of conidiation was found on Sabouraud dextrose agar. To determine the effects of the isolates on *B. tabaci*, a dose–response bioassay was carried out to estimate lethal concentration (LC_{50}) and lethal time (LT_{50}) values of each fungal isolate to second instar nymphs. The mean LC_{50} values of *A. lecanii* isolates ranged from 4.22×10^6 to 7.35×10^{13} conidia ml^{-1} at 5 to 7 days after the treatment. For *A. muscarius*, the values varied from 9.2×10^4 to 8.7×10^{10} conidia ml^{-1} at 5 to 7 days after the treatment. The lowest and the highest mean LC_{50} values were observed for *A. mucarius* (AGM5) and *A. lecanii* (isolate PAL6), respectively. The mean LT_{50} values of *A. lecanii* and *A. muscarius* isolates were 7.1–9.0 and 4.9–7.2 days, respectively. The LT_{50} values of *A. muscarius* were significantly lower than the other isolates. Overall, all isolates, especially *A. muscarius* (AGM5), exhibited appropriate potential as a biological control agent against *B. tabaci*.

Introduction

The cotton whitefly, *Bemisia tabaci* Gen. (Hem., Aleyrodidae), is an important pest of many crops, including vegetables and ornamentals (De Barro *et al.*, 2011). This whitefly has more than 600 host plants causing economic damages in both greenhouse and field cucumber crops (Oliveira *et al.*, 2001). The damage is caused through (i) sucking the plant sap leading to plant growth and yield reductions; (ii) excreting honeydew resulting in the invasion of the sooty molds, potentially decreasing photosynthesis and reducing the yield quality and quantity, and (iii) virus transmission (Berlinger, 1986; Thompson, 2011).

The application of synthetic insecticides such as imidacloprid is the main strategy to control *B. tabaci* in many regions of the world (Palumbo *et al.*, 2001). Pesticide resistance, side effects on non-target organisms, pest resurgence, and secondary pest outbreak are some limitations of the chemical control (Pedigo, 2002; Safaei *et al.*, 2016; Shahbi and Rajabpour, 2017).

Microbial control agents can serve as environmentally friendly components of integrated pest management (IPM) programs due to their selectivity, safety, and compatibility with other natural enemies (Lacey and Shapiro-Ilan, 2008). Entomopathogenic fungi (EPF) are the most abundant group, ~60%, of insect pathogens (Liu *et al.*, 2009). EPF can directly penetrate through the arthropod cuticle, unlike bacteria and viruses which are ingested to induce pathogenicity (Ortiz-Urquiza and Keyhani, 2013). Therefore, EPF can serve as efficient biocontrol agents against sap-feeding pests, e.g. the whiteflies.

There are several EPF that have been reported as efficient biocontrol agents against *B. tabaci* (Osborne and Landa, 1992; Faria and Wraight, 2001; Cuthbertson and Walters, 2005; Cuthbertson *et al.*, 2008; Cuthbertson *et al.*, 2010). These agents have been systematically classified into phylum Ascomycota, order Hypocreales, and family Cordycipitaceae (Litwin *et al.*, 2020). The important species within Cordycipitaceae include *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (1912), *Cordyceps fumosorosea* (Wize) Kepler, B. Shrestha, and

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Spatafora (2017) [Syn.: *Isaria fumosoroseus* Wize (1904)], *A. lecanii* (Zimmerman) Spatafora, Kepler and B. Shrestha (2017) [Syn.: *Lecanicillium lecanii* (Zimmerman) Zare and Gams (2001)], and *A. muscarius* (Petch) Spatafora, Kepler and B. Shrestha (2017) [Syn.: *Lecanicillium muscarium* Petch (1931)]. However, no study has been carried out to determine the pathogenicity of Iranian isolates of *Akanthomyces* spp. on whitefly. Iranian isolates can show significantly different potentials to control the pest due to the different climate of Iran in comparison with other climates in which the previous isolates were originated. Various entomopathogenic isolates with different geographic origins may indicate different pathogenicity to determine pests (Feng *et al.*, 1994; Sani *et al.*, 2020). Furthermore, *Lecanicillium* isolates originating from a single geographical region were reported to exhibit different mortality rates against the target insect (Zhu and Kim, 2011; Manfrino *et al.*, 2019; Xie *et al.*, 2019; Abdulle *et al.*, 2020). Although it was mostly demonstrated that, in comparison to *B. bassiana*, *L. lecanii* isolates are more pathogenic to *B. tabaci* (Abdel-Raheem and Al-Keridis, 2017; Espinosa *et al.*, 2019), a Russian isolate of *L. lecanii* was found to display relatively low virulence against the insect (Keerio *et al.*, 2020). Therefore, during the current study, the potentials of four Iran-originated isolates of *A. lecanii* and *A. muscarius* to control *B. tabaci* on cucumber were studied under laboratory conditions. In Iran, cucumber is one of the important hosts of the whitefly causing significant quantitative and qualitative losses every year. Moreover, vegetative growth and conidiation of the isolates on various media were investigated to find the optimum medium for vegetative growth and conidiation of the EPF.

Materials and methods

Insect and host plant cultures

Seeds of greenhouse cucumber (*Cucumis sativus* cv. Negin) were sown in pots containing perlite-cocopeat mix (1:1, v: v) and were daily moistened with Hoagland's nutrient solution. The pots were placed in the rearing cages (1 × 0.6 × 1.2 m) in a growth chamber at 25 ± 1°C, 65 ± 5% relative humidity (RH), and 16:8 h (light:dark) photoperiod (Mohammadi *et al.*, 2015).

Adults of the whitefly, *B. tabaci* biotype B, were collected using an aspirator from a commercial cucumber greenhouse in Shushtar district, Khuzestan province, southwest of Iran (32°05′30.5″N 48° 45′25.2″E(. After their identification by Martin (1987) identification key, the whiteflies were introduced to the rearing cages. The cages were kept inside an air-conditioned room at a temperature of 25 ± 2°C, RH of 65 ± 5%, and a photoperiod of 14:10 h (light: dark). The plants were replaced biweekly. The colony was maintained until the end of the bioassay trials.

Isolation and identification of the fungi

Isolation

The EPF were isolated from the orange Pulvinaria scale, *Pulvinaria aurantii* Cock. (Hem., Coccidae), and cotton aphid, *Aphis gossypii* Glover (Hem., Aphididae), in citrus orchards of Citrus and Subtropical Fruits Research Center of Iran, Ramsar district, Mazandaran province, northern Iran (36°54′24.2″N 50° 39′26.7″E). The fungus isolation was carried out using the method described by Kumar *et al.* (2015) with some modifications. Briefly, dead individual of each species was superficially sterilized using 10% sodium hypochlorite for 3 s and washed twice with distilled water. The specimens were then air-dried and transferred to Petri dishes lined with wet Whatman paper (No. 1) and incubated at 25°C for 48 h without light. Cadavers exhibiting fungal growth were selected and placed onto Sabouraud dextrose agar (SDA) medium (Merck, Germany) and incubated at 25°C. The single spore method was applied to obtain purified cultures of each fungal isolate.

Morphological identification

The fungal isolates were morphologically identified using classical taxonomy following general and specific identification keys (Petch, 1925; Humber, 1997; Zare and Gams, 2001; Zare and Gams, 2004). Fungal stocks were prepared by sub-culturing the pure culture of the isolates into glass tubes containing potato dextrose agar (PDA) medium and incubating at 4°C for 72 h and then stored at room temperature. Microscope slides were prepared from each fungal isolate and morphological features including conidia, conidiophores, and phialides were photographed using a digital camera-equipped microscope (Olympus, Tokyo, Japan) at 400× magnification.

DNA extraction

The fungal DNA was extracted using cetyl-trimethyl-ammonium bromide (CTAB) solution as described by Gawel and Jarret. Fungal isolates were grown in potato sucrose broth (PS) for 7 days at room temperature. Then, the liquid medium was removed and mycelia were ground to a fine powder under liquid nitrogen. Pre-heated [60°C] extraction buffer (1.4 M NaCl, 20 mM EDTA, 2.5% CTAB, 2% 2-mercaptoethanol, 10 mM Tris-HCl, pH 8) was added and the mixture was incubated at 65°C for 30 min. The DNA was extracted with an equal volume of chloroform/isoamyl alcohol (24:1), precipitated with one volume of ice-cold isopropanol, washed for two times with 70% ethanol, and re-suspended in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The resulting DNA was stored at −20°C.

DNA amplification

PCR was performed using two primer pairs designed to amplify two regions of the fungal genome including internal transcribed spacer (ITS: ITS4/ITS5) and mitochondrial DNA (mtDNA: NMS1/NMS2) (White *et al.*, 1990). PCR was performed in a volume of 25 µl containing 10–20 ng of fungal DNA template, 1 µM of each primer, 12.5 µl of *Taq* DNA Polymerase Master Mix (Ampliqon, Denmark), and 9.5 µl of PCR-grade H_2O. The mixture was heated for 3 min at 94°C and subjected to a 30 cycle-PCR program of 1 min at 96°C, 30 min at 55°C, and 1 min at 72°C. The final cycle was followed by 5 min incubation at 72°C. PCR products were excised from the gel and purified using a PCR clean-up kit (Denazist, Iran). Purified DNAs were then subjected to Sanger sequencing at BIONEER Corporation (South Korea).

Phylogenetic analysis

Base-calling was performed using the DNAStar Lasergene suite (DNAStar, Madison, WI, USA). Nucleotide sequences of the ITS region from the fungal isolates were deposited on the NCBI database under the accession numbers of MT130430, MT130431, MT130432, and MT130433. The resulting sequences were compared to those sequences whose information is available in the GenBank database. A total of 35 ITS sequences from EPF within Cordycipitaceae, including four sequences determined in this study and 31 sequences from different sources and diverse

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regions previously deposited on GenBank were used to carry out a phylogenetic analysis (Supplemental material, table S1). Multiple alignments of the sequences were performed using CLC Main Workbench software (ver. 7.6.2), and the maximum likelihood approach using General Time Reversible model test (Tavaré, 1986) with 100 bootstrap replicates was used to construct a phylogenetic tree. One corresponding sequence from *Lecanicillium psalliotae* was included in the analysis as an outgroup.

Fungal vegetative growth and conidiation

To find the optimum medium for vegetative growth and conidiation of the EPF, the fungal isolates (PAL6, PAL7, PAL8, and AGM5) were grown on different media including PDA (Himedia, India), SDA (Merck, Germany), SDA + yeast extract (SDA + Y) (Merck, Germany), PCA (Himedia, India), and malt agar (MA) (Merck, Germany). Three-mm discs were excised from the peripheral mycelium of 5-day-old cultures and were separately placed upside down in the center of 9-cm plates containing the aforementioned media. The cultured media were kept in a growth chamber under the conditions of 23 ± 1°C, 16/8 photoperiod (light/dark), and 70 ± 5% RH. The growth of fungal isolates was determined by measuring the colony diameter (in millimeter) using a ruler at 7-days post-incubation. Moreover, the conidial concentration (conidia ml^{-1}) of each isolate cultured in different media was determined using a hemocytometer (HGB, Germany) after 7 days (see below). Each treatment was replicated three times.

Bioassay trials

Second instar nymphs of *B. tabaci* (2 days old) were used for the experiments due to their susceptibility to the EPF (Cuthbertson *et al.*, 2005). The developmental stage was obtained according to the method described by Banihashemi *et al.* (2017). Males and females of the whitefly (about 50 pairs) were maintained together for mating for 48 h. After this period, the mated females were collected using an aspirator and introduced into the rearing cages. Nine days after introducing the mated adults to cucumber plants (at 25 ± 2°C, 60 ± 5% RH, and a photoperiod of 16 h light:8 h dark), the second instar nymphs were obtained for the experiments.

The protocol described by Wraight *et al.* (1998) was used for the bioassay test. For each fungal isolate, five concentrations of conidial suspensions (10^4, 10^5, 10^6, 10^7, and 10^8 conidia ml^{-1}) were prepared. Briefly, 12 ml of sterile distilled water + 0.05% Tween-80 (Merck, Germany) were added to the 15-day old PDA cultures and conidia and mycelium of each isolate was harvested using a sterile glass rod. To obtain the conidia, the mixture was filtered through four layers of cheesecloth. The conidia concentration (as conidia ml^{-1}) was estimated using a hemocytometer (HGB, Germany). Three replications were considered for each counting. Two to three days before the bioassay, conidial viabilities of the EPF were checked using the method described by Castillo *et al.* (2000). Briefly, 0.1 ml of 10^7 conidia ml^{-1} concentration was spread onto PDA media and incubated at 23 ± 1°C for 18 h. The germination of 50 conidia was randomly checked under a light microscope. A cucumber leaf infested by 10-s instar nymphs was sprayed once with each concentration using a handle sprayer from ~5 cm distance. In this situation, the preliminary test indicated that about 1 ml of the suspension was uniformly spread on the leaf surface in each spray. The treated leaf was immediately transferred to a Petri dish, 10 cm diameter, placed upside down on a 20 ml layer of water agar (5%) (Merck, Germany). The dishes were enclosed using plastic bags and incubated at 23 ± 1°C, 70 ± 5% RH, and a photoperiod of 16:8 h (light:dark) for 6 h. Subsequently, the dishes were removed from the bags, allowed to air dry, covered with ventilated lids (lids with an 8-cm diameter hole covered with a fine screen), and returned to the incubator. The treated leaves were incubated ventral surface up to prevent entrapment of humid air at the leaf surface. Mortalities of the nymphs were recorded at intervals of 24 h for 8 days. Mortality was defined by the presence of the fungal mycelia (Cuthbertson *et al.*, 2005). Seven replications, treated leaf, were used for each conidial concentration.

Data analysis

For vegetative growth and conidiation trials, the data were analyzed as a 5 (media) by 4 (EPF isolates) factorial experiment with three replications, based on a completely randomized design using the generalized linear model (GLM) procedure of SAS software (version 9.2) (SAS Institute, Inc., Cary, NC). Shapiro-Wilk tests were done to check the data normality assumption. The least significant difference (LSD) test was used as a post-hoc test for means comparisons.

Probit analysis was used for estimating the median lethal concentrations (LC_{50}) (Finney, 1971). Relative median potencies and their 95% confidence intervals were calculated for different treatments when their slopes did not differ significantly (Finney, 1971). Moreover, probit analysis was conducted to estimate the time for killing 50% of the insects (LT_{50}) (Throne *et al.*, 1995). All analyses were done using SAS software ver. 9.1 (SAS Institute, Cary, NC). Estimated LC_{50} or LT_{50} values were not considered statistically different when their confidence limits (95%) overlapped (Robertson *et al.*, 2007).

Results

Morphological identification

Morphological characteristics of the fungal colonies and conidia of the isolates identified two species of *Akanthomyces*, *A. lecanii* and *A. muscarium*. The isolates of *A. lecanii* were characterized by forming yellowish-white, fluffy, branched mycelium in a centered cycle pattern (data not shown). Moreover, typically short-ellipsoidal conidia (2.5–3.5 × 1–1.5 µm) were produced on conidiogenous cells (phialides) of short conidiophores (Zare and Gams, 2001). However, *A. muscarium* formed relatively compact, with reverse cream to pale yellow or colorless mycelium. It produced phialides generally longer than those of *A. lecanii* and ellipsoid to subcylindrical conidia (2.5–5.5 × 1–1.5 µm) more irregular in size and shape, longer, and narrower than in *A. lecanii* (Zare and Gams, 2001).

Phylogenetic analysis

Four ITS sequences from morphologically-identified *Akanthomyces* isolates including AGM5, PAL6, PAL7, and PAL8 were obtained. The isolate AGM5 showed the highest nucleotide identity (96.55%) to a French isolate (ARSEF 2323) of *A. muscarius* (Syn.: *L. muscarium* [EF513017]). The isolates PAL6, PAL7, and PAL8 exhibited the highest nucleotide identity (92.32, 92.12, and 91.94%, respectively) to a Turkish isolate (IMI 079606) of *A. lecanii*

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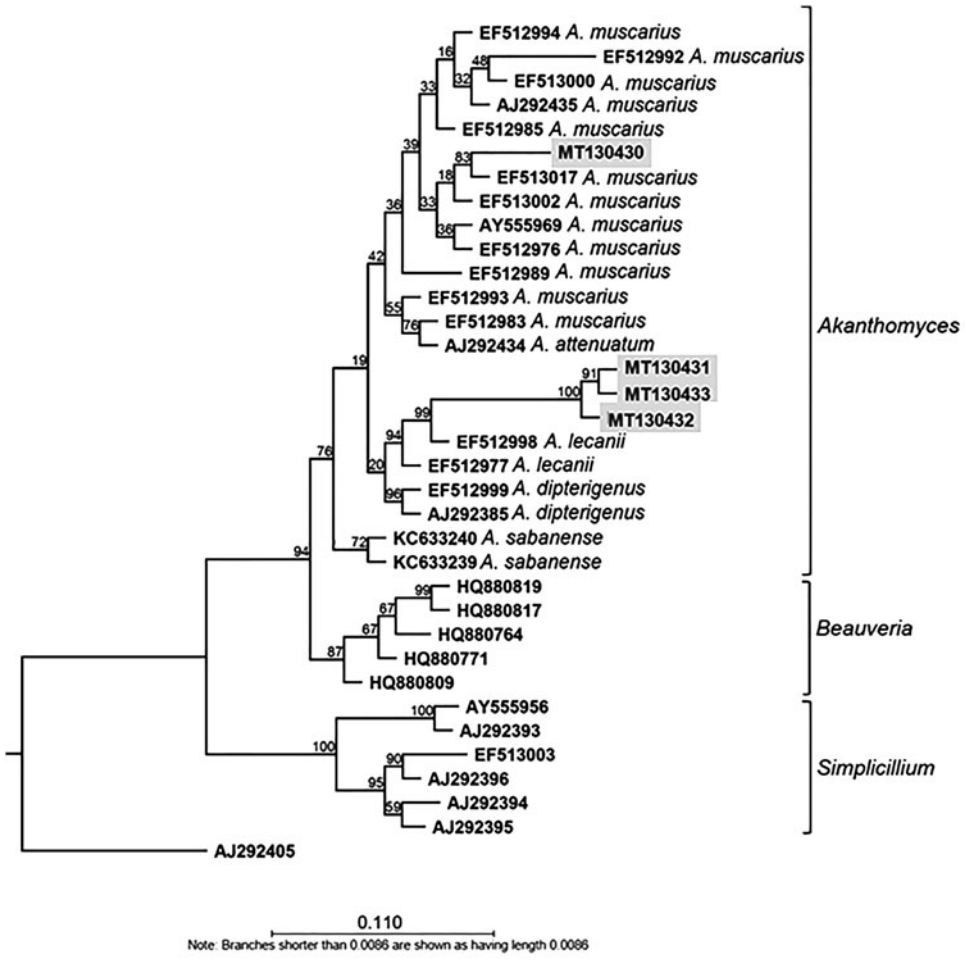

Figure 1. Maximum likelihood phylogenetic tree obtained from multiple alignments of internal transcribed spacer (ITS) sequence from worldwide isolates within the family Cordycipitaceae (Ascomycota, Hypocreales). The isolates obtained in the present study were highlighted. One isolate of *L. psalliotae* was used as an outgroup reference. The bar represents the estimated nucleotide substitutions per site.

Table 1. GLM parameters of main effects and their interaction for five different media and four isolates on colony diameters of the entomopathogenic fungi at 7-days after incubation

Source	df	Type III sum of square	Mean of square	*F*	*P*-value
Media	3	9.5	3.1	12	<0.0001
Isolates	4	0.9	0.3	2.1	0.0903
Media × isolates	12	0.7	0.3	1.1	0.3
Error	40	1.1	2.5		

Table 2. Mean of conidial concentrations (conidia ml^{-1}) ± SE ($n = 3$) of the entomopathogenic fungal isolates grown in five media at 7-days after incubation

	Fungus (isolate)			
Media	*A. muscarius* (AGM5)	*A. lecanii* (PAL6)	*A. lecanii* (PAL7)	*A. lecanii* (PAL8)
SDA	$3.8 \times 10^6 \pm 1.2 \times 10^6$b	$3.8 \times 10^6 \pm 1.5 \times 10^6$b	$1 \times 10^5 \pm 3.5 \times 10^4$a	$1.2 \times 10^6 \pm 3.6 \times 10^5$a
SDA + Y	$1.2 \times 10^6 \pm 4.4 \times 10^5$a	$2.6 \times 10^6 \pm 7.3 \times 10^5$ab	$5 \times 10^4 \pm 4 \times 10^3$a	$9.6 \times 10^5 \pm 5.8 \times 10^4$a
PDA	$4.6 \times 10^5 \pm 6.8 \times 10^4$a	$1.6 \times 10^6 \pm 1.1 \times 10^5$ab	$2.6 \times 10^4 \pm 3.3 \times 10^3$a	$1.4 \times 10^7 \pm 3.4 \times 10^6$b
PCA	$2.2 \times 10^5 \pm 8.8 \times 10^3$a	$4.3 \times 10^4 \pm 3.3 \times 10^3$a	$1 \times 10^4 \pm 1.5 \times 10^3$a	$1.9 \times 10^5 \pm 7.8 \times 10^4$a
MA	$3.1 \times 10^5 \pm 9.2 \times 10^4$a	$4.1 \times 10^5 \pm 4.6 \times 10^4$a	$9 \times 10^4 \pm 6.5 \times 10^3$a	$6.1 \times 10^5 \pm 2.6 \times 10^5$a

SDA, Sabouraud dextrose agar; SDAY, SDA + yeast extract; PDA: potato dextrose agar, PCA, potato carrot agar; MA, malt agar.
The same letters in each column indicate a non-significant difference (LSD test).

(Syn.: *Lecanicillium lecanii* [EF512998]), respectively. Phylogenetic analysis of the 35 nucleotide sequences of ITS showed 3 clades with 76–100% bootstrap support consisting of *Akanthomyces* ($n = 23$), *Beauveria* ($n = 5$) and *Simplicillium* ($n = 6$) isolates with *Lecanicillium psalliotae* as an outgroup (fig. 1). Sequences from the four EPF isolates obtained in this study all clustered within the *Akanthomyces* clade. These results confirmed the morphological identification of the fungal isolates.

Vegetative growth and conidiation

There was a significant effect of media on the conidial concentrations produced after 7 days incubation, while there was no significant effect of fungal isolate nor the interaction between media and isolate (table 1). The conidial concentration of each isolate cultured on different media is shown in table 2. For *A. muscarium* (AGM5) and *A. lecanii* (PAL6), the highest condial concentrations were obtained on SDA. No significant difference was

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Table 3. GLM parameters of main effects and their interaction for five different media and four isolates on the conidial concentration of the entomopathogenic fungi 7-days after incubation

Source	df	Sum of square	Mean of square	*F*	*P*-value
Media	3	10.5	3.2	13.5	<0.0001
Isolates	4	0.8	0.3	1.5	0.2314
Media × isolates	12	0.7	0.3	1.1	0.5416
Error	40	1.1	2.8		

Table 4. Mean of colony diameter (mm) ± SE ($n = 3$) of the entomopathogenic fungal isolates cultured in five media at 7-days after incubation

Media	Fungus (isolate)			
	A. muscarius (AGM5)	*A. lecanii* (PAL6)	*A. lecanii* (PAL7)	*A. lecanii* (PAL8)
SDA	21.6 ± 0.3a	20.3 ± 1.2b	26 ± 2.1b	27.3 ± 0.8b
SDA + Y	28.6 ± 1.2b	20 ± 1.1b	27.3 ± 2.6b	36 ± 2.5d
PDA	43 ± 1d	30.6 ± 0.6c	24 ± 1b	32.3 ± 1.3d
PCA	22.3 ± 0.3a	14.1 ± 0.4a	14.6 ± 0.3a	15 ± 0a
MA	39.3 ± 0.3c	15.1 ± 0.4a	29.3 ± 0.6b	30 ± 0.5c

SDA, Sabouraud dextrose agar; SDAY, SDA + yeast extract; PDA, potato dextrose agar; PCA, potato carrot agar; MA, malt agar.
The same letters in each column indicate a non-significant difference (LSD test).

observed between the conidial concentration of *A. lecanii* (PAL7) grown on the various media. However, *A. lecanii* (PAL8) grown on the PDA medium exhibited the highest conidial concentration compared to other media (table 2).

The different media significantly affected the fungal colony diameters of each isolate (table 3). For PAL6, PAL8, and AGM5 isolates, the largest value of colony diameter was observed in fungal isolates grown on PDA (table 4).

Bioassay trials

The LC_{50} values of the four isolates at 5–7 days after treatment (DAT) are shown in table 5. There was not a significant difference between the estimated LC_{50} values of the EPF at each DAT. However, the LC_{50} value of AGM5 at 7 DAT is significantly lower than other isolates. For *A. lecanii* isolates (PAL6, PAL7, and PAL8), no significant differences were observed between the LC_{50} values at 5–7 DAT. Although, the LC_{50} values of *A. muscarius* isolate (AGM5) were significantly reduced at days 5–7. Totally, the highest and the lowest LC_{50} values were 7.3×10^{13} and 9.2×10^{4} (conidia ml^{-1}) which were observed in *A. muscarius* at 7 DAT and *A. lecanii* (isolate PAL6) at 5 DAT, respectively.

The LT_{50} values of the EPF in different conidial concentrations are presented in table 6. For all conidial concentrations, the LT_{50} value of *A. muscarius* (isolate AGM5) was significantly lower than other isolates. The LT_{50} values were decreased by increasing conidial concentrations of each isolate. Totally, the highest and the lowest LT_{50} values were 9.0 and 5.0 days, which was observed in *A. lecanii* (isolate PAL6) in the concentration 10^{4} conidia ml^{-1} and *A. muscarius* (isolate AGM5) in the concentration 10^{8} conidia ml^{-1}, respectively. The bioassay viabilities of *A. lecanii* and *A. muscarius* conidia were found as 98 and 99%, respectively.

Discussion

EPF have been included among microbial biocontrol agents which adversely affect the pest population without any hazardous influence on human and the environment (Butt, 2002; Thomas and Read, 2007). Due to their pathogenicity process and wide host range, they have been considered as key factors in IPM programs (Khan *et al.*, 2012). The two most important and widespread species of EPF, *A. lecanii* and *A. muscarius*, have been found as effective biocontrol agents against *B. tabaci* (Osborne and Landa, 1992; Faria and Wraight, 2001; Cuthbertson and Walters, 2005; Park and Kim, 2010; Ren *et al.*, 2010). Here we morphologically identified two species of *Akanthomyces* as the first Iran-originated entomopathogenic species. Phylogenetic analysis based on the ITS region has been extensively applied as a molecular marker to classify fungal species (Hillis and Dixon, 1991; Salazar *et al.*, 1999; Arenal *et al.*, 2000). Particularly, *Akanthomyces* spp. have been subjected to a comprehensive phylogenetic analysis according to which *A. lecanii* and *A. muscarius* formed separate clusters in the phylogenetic tree (Kouvelis *et al.*, 2008). Similarly, three isolates of *A. lecanii* and a single isolate of *A. muscarius* which had been obtained in this study clustered in two separate clades in the phylogenetic tree. Also, the fungal isolates from *Simplicillium* spp. were clustered together which was consistent with the results of Kouvelis *et al.* (2008).

It has been shown that culture medium can significantly influence the growth and conidiation of *A. lecanii* (Romero and de Romero, 1986; Sun *et al.*, 2009; Prasad and Pal, 2014; Gao, 2018). Based on our results, PDA was found to be the optimal medium for growth and conidiation of *A. lecanii*. In the case of *A. muscarius*, however, the highest conidia concentration was observed on the SDA medium. Although there is no specific study on the selection of culture media for vegetative growth and conidiation of *A. muscarius*, the SDA medium has been used by some researchers (Marshall *et al.*, 2003; Lazreg *et al.*, 2009; Luz *et al.*, 2010; Mohammadipour *et al.*, 2010). In addition to the culture medium, other environmental factors such as pH, water potential, temperature, and light can affect fungal vegetative growth and/or conidiation (Gao *et al.*, 2009). Therefore, it is highly recommended that these factors be specifically determined to find an optimum condition for the growth and conidiation of the species of EPF.

The results of the infection bioassays showed that the EPF obtained in this study are pathogenic to second instar nymphs of *B. tabaci* among which *A. muscarius* (isolate AGM5) exhibited the highest efficiency as a biocontrol agent of the whitefly.

The LC_{50} values reported in the present study are relatively lower than the estimated LC_{50} values of four isolates of *A. muscarius* (V20, V26, V07, and V17) to *B. tabaci* nymphs, 1.07×10^{6}–5.08×10^{8} conidia ml^{-1} (Ren *et al.*, 2010). The LC_{50} values of six Canadian isolates of *A. lecanii*, (V3450, Vp28, V16063, V0175, V342, and V341) to third instar nymphs of *B. tabaci* ranged from 2.57×10^{5} to 6.03×10^{5} conidia ml^{-1} (Wang *et al.*, 2004) which are relatively lower values than for the isolates of our study. The lower LC_{50} values may be related to different susceptibility of *B. tabaci* life stages, second and third instar nymphs. The second instar nymph of *B. tabaci* has been reported as the most susceptible life stage of the insect (Cuthbertson *et al.*, 2005). In our study, progressive mortality of the whitefly nymphs was observed

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Table 5. The median lethal concentration (LC_{50}) values of five isolates of the entomopathogenic fungi (EPF) to the second instar nymph of *B. tabaci*

EPF	Day after the treatment	LC_{50} (conidia ml^{-1})	Confidence limits	χ^2 (df = 34)	*P*-value	Slope ± SE	Intercept ± SE
A. lecanii (PAL6)	5	7.3×10^{13}	5.7×10^{8}–2.1×10^{18}	70.2	<0.001	79.0 ± 21.0	−2.2 ± 0.5
	6	6.2×10^{8}	4.3×10^{7}–4×10^{12}	66.6	<0.001	07.0 ± 23.0	−2.1 ± 0.4
	7	2.1×10^{8}	2.9×10^{7}–8.8×10^{12}	69.7	<0.001	16.0 ± 42.0	−3.5 ± 1.2
A. lecanii (PAL7)	5	1.7×10^{11}	5.3×10^{8}–9.6×10^{13}	77.2	0.001	0.7 ± 0.16	−1.4 ± 0.4
	6	4.2×10^{6}	4.3×10^{7}–9×10^{10}	98.8	<0.001	0.8 ± 0.3	−1.8 ± 0.5
	7	1.1×10^{8}	1.1×10^{7}–6.4×10^{12}	94.8	<0.001	0.3 ± 0.1	−2.5 ± 0.8
A. lecanii (PAL8)	5	8.7×10^{10}	8.9×10^{8}–9.1×10^{11}	69.3	0.001	0.21 ± 0.05	−2.1 ± 0.5
	6	5.4×10^{6}	3.2×10^{5}–1.2×10^{8}	77.3	<0.001	0.35 ± 0.05	−2.6 ± 0.1
	7	7.2×10^{7}	2.9×10^{7}–8.8×10^{12}	94.8	<0.001	0.4 ± 0.2	−1.9 ± 0.5
A. muscarius (AGM5)	5	9.7×10^{8}	7.4×10^{7}–1.8×10^{12}	89.0	0.001	0.27 ± 0.07	−2.4 ± 0.4
	6	1.2×10^{6}	1.5×10^{5}–1.1×10^{7}	103.1	<0.001	0.26 ± 0.06	−1.8 ± 0.4
	7	9.2×10^{4}	6.3×10^{3}–1.3×10^{5}	89.4	<0.001	0.21 ± 0.06	−1.1 ± 0.3

Table 6. The median lethal time (LT_{50}) values of various isolates of the entomopathogenic fungi (EPF) to second instar nymph of *B. tabaci*

EPF	Concentrations (conidia ml^{-1})	LT_{50} (days)	Confidence limits	χ^2 (df = 34)	*P*-value	Slope ± SE	Intercept ± SE
A. lecanii (PAL6)	10^4	9.0	8.3–10.7	72.7	<0.001	2.6 ± 9.3	−92.8 ± 2.3
	10^5	8.6	8.0–9.8	69.2	<0.001	2.5 ± 9.0	−49.8 ± 2.0
	10^6	8.6	7.3–11.4	61.6	0.003	4.4 ± 11.4	−74.1 ± 4.1
	10^7	8.0	7.1–9.2	57.5	0.007	3.6 ± 10.3	−37.9 ± 3.0
	10^8	7.1	6.6–7.6	66.8	<0.001	1.5 ± 8.6	−41.7 ± 1.3
A. lecanii (PAL7)	10^4	8.0	7.2–8.4	73.1	<0.001	2.6 ± 10.7	−6.9 ± 2.3
	10^5	7.4	6.4–8.2	64.6	0.0012	4.3 ± 12.6	−15.1 ± 3.8
	10^6	7.1	6.5–7.5	40.5	0.0204	1.5 ± 9.1	−69.7 ± 1.3
	10^7	7.5	7.6–12.5	68.5	<0.001	4.7 ± 11.4	−66.1 ± 4.3
	10^8	7.8	7.1–9.4	66.3	<0.001	3.1 ± 9.5	−55.8 ± 2.7
A. lecanii (PAL8)	10^4	8.8	8.8–9.1	63.8	<0.001	3.4 ± 8.8	−74.8 ± 3.1
	10^5	9.5	8.7–13.6	64.6	<0.001	3.2 ± 9.5	−33.9 ± 2.9
	10^6	8.6	7.9–10.1	67.9	<0.001	2.0 ± 7.8	−34.7 ± 1.8
	10^7	6.9	6.5–7.3	63.7	<0.001	1.7 ± 10.5	−9.8 ± 1.4
	10^8	7.5	7.0–8.1	61.6	<0.001	1.6 ± 8.3	−31.7 ± 1.4
A. muscarius (AGM5)	10^4	6.1	5.2–6.9	69.8	<0.001	1.9 ± 7.3	−78.5 ± 1.5
	10^5	7.1	6.4–8.3	47.2	0.023	3.1 ± 10.1	−64.8 ± 2.6
	10^6	5.6	5.0–6.2	50.2	0.0118	1.7 ± 8.5	−42.6 ± 1.3
	10^7	4.9	4.0–5.5	66.9	<0.001	3.1 ± 11.5	−04.8 ± 2.4
	10^8	5.0	4.5–5.3	66.0	<0.001	2.4 ± 13.2	−23.9 ± 1.8

during a time similar to the finding reported by Saito and Sugiyama (2005).

The LT_{50} values of *A. lecanii* and *A. muscarius* isolates were 6.1–9.5 and 4.9–7.1 days, respectively. The values were relatively higher than those reported for three Chinese isolates of *A. lecanii* (L22, L14, and L18) on *B. tabaci* biotype Q, which were 3.4–4.6 days (Zhu and Kim, 2011). The different responses may be related to many factors including the different fungal isolate, different biotypes (genetic difference of the whiteflies), and different spray methods. Similar to our finding, the LT_{50} values of an Iranian isolate of *A. muscarius*, isolated from *Zeuzera pyrina* L. (Lep., Cossidae), at the concentrations 10^5, 10^6, 10^7, and 10^8 conidia ml^{-1} to *Trialeurodes vaporariorum* Westwood, were 8, 6, 6, and 4 days, respectively (Tabadkani *et al.*, 2010).

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In our study, the mortality of the infected nymphs started on day 3 after treatment. Similarly, the first mortality of third instar nymphs by other isolates of *A. lecanii* (V3450, Vp28, Vl6063, V0175, V342, and V341) began at day 3 after treatment (Wang *et al.*, 2004). Liu *et al.* (2009) investigated in detail the infection process and histopathological changes of Japanese wax scale, *Ceroplastes japonicus* Green (Hem., Coccidae) by the *A. lecanii*. They demonstrated that the death of infected insects occurred 6-days post-inoculation. This variation among infection and death times reported from different studies might be due to several factors, including insect host, environmental conditions, fungal isolate, host plant, etc. (Shah and Pell, 2003).

Totally, the fungal isolates, especially *A. muscarius* (AGM5), have appropriate potentials as a biological control agent to control *B. tabaci* in cucumber. Some isolates of *Akanthomyces* spp. have been applied as commercial microbial biocontrol agents to control various insect pests. Among them, two commercial isolates of *A. muscarius*, Mycotal®, and Verticillin®, were previously recommended to control whiteflies, especially under greenhouse conditions (Goettel *et al.*, 2008). The efficacy of the EPF can be enhanced in greenhouse conditions. However, microbial insecticides may have some limitations including slow effect and high costs (Cuthbertson and Walters, 2005).

Conclusion

Our finding showed that the fungi *A. lecanii* (PAL6, PAL7, and PAL8) and *A. muscarius* (AGM5), isolated from citrus hemipteran pests in the north of Iran, have appropriate potentials for applying as microbial control agents against *B. tabaci* on cucumber, especially in greenhouse condition. Among the fungi, *A. muscarius* (AGM5) was the most promising isolate according to its relatively low LC_{50} and LT_{50} values. However, further greenhouse and field experiments are required to better understand the EPF–whitefly interactions before the practical use of *A. muscarius* against *B. tabaci* in cucumber production can be implemented.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485321000298

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