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Selection of *Lecanicillium* Strains with High Virulence against Developmental Stages of *Bemisia tabaci*

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Selection of fungal strains with high virulence against the developmental stages of *Bemisia tabaci* was performed using internal transcribed spacer regions. The growth rate of hyphae was measured and bioassay of each developmental stage of *B. tabaci* was conducted for seven days. All of the fungal strains tested were identified as *Lecanicillium* spp., with strain 4078 showing the fastest mycelium growth rate (colony diameter, 16.3 ± 0.9 mm) among the strains. Compared to strain 4075, which showed the slowest growth rate, the growth rate of strain 4078 was increased almost 2-fold after seven days. Strains 4078 and Btab01 were most virulent against the egg and larva stages, respectively. The virulence of fungal strains against the adult stage was high, except for strains 41185 and 3387. Based on the growth rate of mycelium and level of virulence, strains 4078 and Btab01 were selected as the best fungal strains for application to *B. tabaci,* regardless of developmental stage.

KEYWORDS : *Bemisia tabaci*, Developmental stage, Internal transcribed spacer, *Lecanicillium* sp., Virulence

Due to certain health and environmental problems caused by the use of chemical pesticides [1], entomopathogenic fungi are now considered as potential biological control agents. Insect pathogenic viruses, bacteria and protozoa are just a few entomopathogens that must be ingested by insect pests in order to initiate disease. On the other hand, entomopathogenic fungi can infect insect pests without ingestion. Entomopathogenic fungi invade the host's cuticle via contact and consequent penetration. The host is then killed due to a lack of soluble nutrients in its hemolymph and the release of toxins from the fungi. Therefore, entomopathogenic fungi are viable candidates to control insects such as *Bemisia tabaci*.

B. tabaci (whitefly) affects agricultural plants both indoors and outdoors via transfer of pathogenic viruses [2-4]. Control of *B. tabaci* is primarily accomplished through the pervasive use of insecticides, which has resulted in the development of resistant *B. tabaci* populations. Additionally, its negative environmental impact has encouraged the development of alternative pest management strategies involving the use of microbes [5].

Whiteflies feed by piercing the surface of plants and directly sucking out the sap. Consequently, entomopathogenic fungi are the only insect pathogens that infect their hosts by direct penetration of the cuticle [5]. Among the various entomopathogenic fungi, *Lecanicillium* spp. are widely known to control sucking insects such as whitefly and aphids; thus, much researches has been conducted using them [6-9]. These fungi have also been registered as microbial control agents for whitefly management [5]. The developmental stages of *B. tabaci* are divided into egg, larva and adult stages, each of which has individual features regarding appearance, state and period. For instance, the larva of *B. tabaci* has no mobility and constitutes the longest stage (almost 3 wk). Considering these features, effective control against *B. tabaci* may be possible.

In this study, we selected *Lecanicillium* sp. strains having high virulence against the developmental stages of *B. tabaci*.

Materials and Methods

Fungal strains. The fungal strains used in this study are shown in Table 1. They were provided by Agricultural Research Service's Collection of Entomopathogenic Fungi (ARSEF), Korean Agricultural Culture Collection (KACC) and National Institute of Agricultural Science and Technology (NIAST). Fungal strains were cultivated in a 250 mL flask containing 100 mL of potato dextrose broth in an orbital shaking incubator operated at 150 rpm at 25°C for 5 days.

B. tabaci. Adult stage *B. tabaci* were reared on paprika leaf (12~16 cm tall) in acryl cages (30 × 30 × 50 cm) maintained at 25°C, 50~60% relative humidity (RH) and under a diurnal day/night cycle of 16 hr : 8 hr.

Identification of fungal strains. The identification of fungal strains was performed by following a modified

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Table 1. Fungal strains used in this study

Lecanicillium strain	Original host	Geographical origin	Source
4078	*Myzus persicae*	Denmark	ARSEF, USA
6541	*Aphis gossypii*	UK	ARSEF, USA
3387	*Myzus persicae*	USA	ARSEF, USA
4075	*Myzus persicae*	Denmark	ARSEF, USA
6543	*Myzus persicae*	UK	ARSEF, USA
Btab01	*Bemisia tabaci*	Korea	DAB-NIAST
41185	*Trialeurodes vaporariorum*	Korea	KACC

ARSEF, Agricultural Research Service's Collection of Entomopathogenic Fungi; DAB-NIAST, Department of Agricultural Biotechnology, National Institute of Agricultural Science and Technology; KACC, Korean Agricultural Culture Collection.

method of Henry *et al*. [10] and White *et al.* [11]. Mycelium was harvested by centrifugation at 14,000 rpm for 5 min. Genomic DNA was extracted using the protocol of TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH, USA). To perform lysis, 1 mL of TRI-REAGENT was added and left at room temperature for 5 min. Two hundred microliters of chloroform was then added and vigorously inverted for 15 sec. Tubes were left at room temperature for 2 min and centrifuged at 14,000 rpm for 15 min at 4°C. Next, 500 μL of back extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate, 1 M Tris) was added and vigorously inverted for 15 sec. Tubes were left for 10 min and centrifuged at 14,000 rpm for 15 min. After the supernatant was transferred to a new tube, 400 μL of isopropanol was added and vigorously inverted for 15 sec. Tubes were left for 5 min at room temperature and centrifuged at 14,000 rpm for 5 min at 4°C, after which the supernatant was removed. The resulting DNA pellets were washed with 1 mL of 75% ethanol and centrifuged at 14,000 rpm for 5 min. Supernatant was then discarded, and the DNA pellets were air-dried at room temperature for 5 min and dissolved in 30 μL of distilled water (DW). Amplification of the internal transcribed spacer (ITS) region was performed using the primer pairs ITS1 and ITS4 [11]. PCR reactions were performed in 50 μL reaction volumes containing 3 μL of template, 4 μL of each primer, 5 μL of 10 × PCR reaction buffer, 5 μL of dNTP mixture, 1 μL of Taq polymerase (INTRON Biotechnology, Seongnam, Korea) and 28 μL of DW. Amplification of the ITS region involved pre-heating for 2 min at 94°C and then 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, followed by a final extension cycle of 5 min at 72°C. PCR products were confirmed on 1.2% agarose gel and visualized by ethidium bromide staining, after which the PCR products were purified and sequenced. Sequences were analyzed by the DNA star program (Lasergene ver. 6.0; Madison, WI, USA) and a Blast search of National Center for Biotechnology Information (NCBI, USA).

Measurement of growth rate of mycelium. After liquid culture of the mycelia in potato dextrose agar (PDA) medium, a spore suspension of the fungal strains was obtained by filtering the spores and mycelia through a sterile cheesecloth. The spore suspension was counted using an improved Neubauer haemacytometer (Marienfeld, Germany), diluted to 1×10^6 spores/mL and then spread onto PDA medium using a spreader. After the fungal strain was cultivated at 25°C for 3 days, mycelia were bored using a cork borer (diameter, 1 mm) and transferred to new PDA medium. Mycelial growth was determined by measuring the diameters of the mycelia after cultivation on PDA at 25°C for seven days. The results were analyzed with error bars showing the standard deviation.

Bioassay of virulence against developmental stages of *B. tabaci*. A virulence test was conducted against the egg, 3~4 instar larvae and adult stages of *B. tabaci*. Thirty milliliters of the diluted spore suspension (1×10^6 spores/mL) was applied using a handheld sprayer onto paprika plant, whose leaves were infested with over 35 nymphs of 3~4 instar and over 30 eggs. After the leaves were air-dried for 5 min, 10 adults were transferred into a clip cage placed on the leaf. Control was treated with sterile 0.01% aqueous Tween 20. The potted plants were placed in acryl cages and maintained at 25.3 ± 1.5°C, 97% RH and under a diurnal day/night cycle of 16 hr : 8 hr. The mortality rate for whiteflies was measured after seven days and were analyzed with error bars showing the standard deviation of three samples. Only whiteflies covered with fungi or exhibiting fungal sporulation were considered to have died due to the fungi.

Results and Discussion

Identification of fungal strains using the ITS region. According to the sequence of the ITS region, all fungal strains used in this study were identified as *Lecaniicillium* spp. Strain 4078 (accession no. EU284721) showed 100% similarity with *L. fusisporum*, whereas strains 4075 (accession no. EU284717), Btab01 (accession no. EU284713) and 41185 (accession no. EU284716) exhibited higher than 98% similarity with *L. attenuatum*. Strains 6543 (accession no. EU284720) and 6541 (accession no. EU284719)

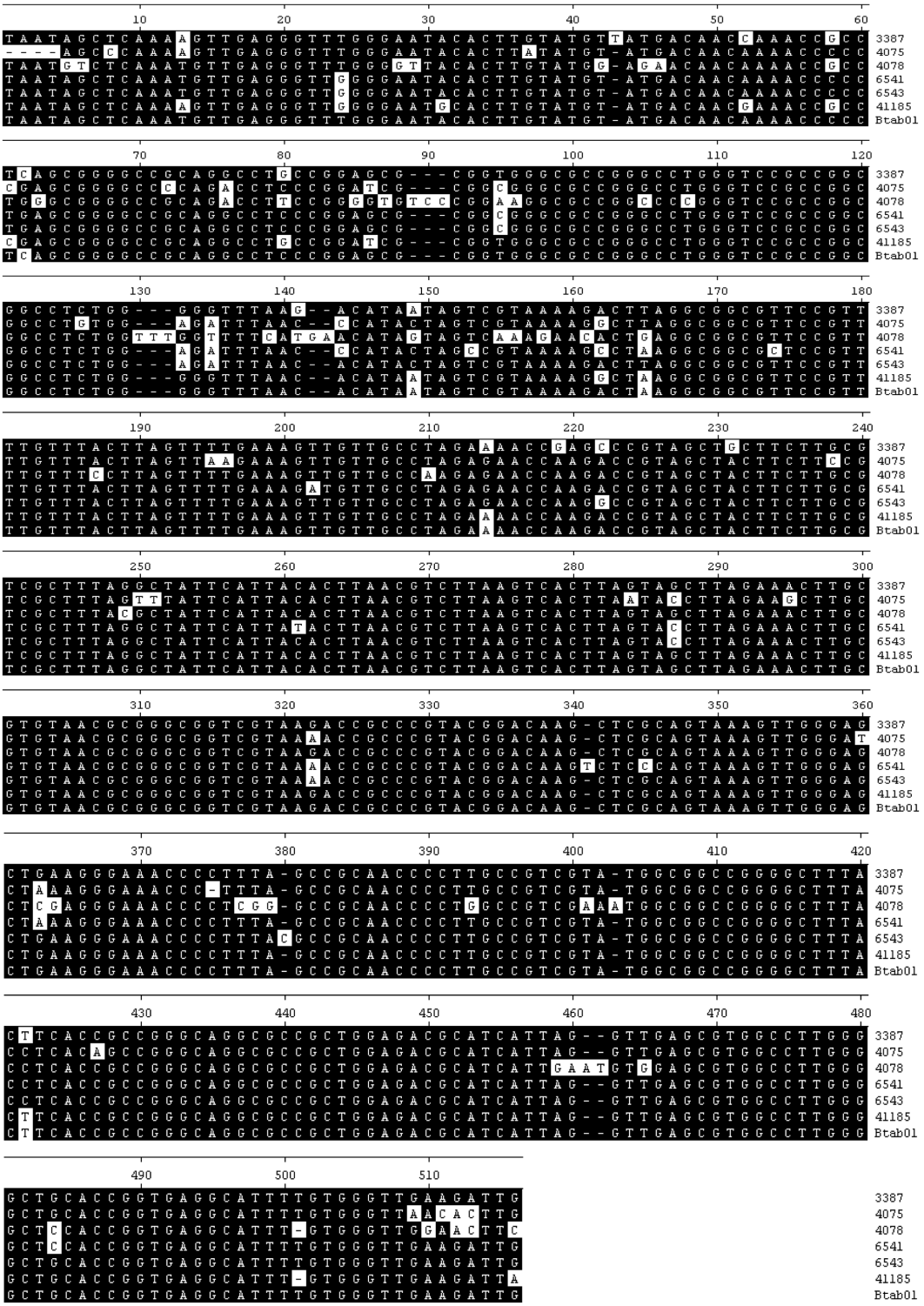

Fig. 1. Internal transcribed spacer sequences of fungal strains used in this study. Sequences deleted and inserted are marked with white color.

were higher than 98% similar with *L. lecanii*, and strain 3387 (accession no. EU 284718) showed 97% similarity with *L. lecanii.*

The ITS sequences of the fungal strains were 497~513 bp in length (Fig. 1). This size variation was also described in another report in which a 20 bp size discrepancy was related to insertion or deletion [12].

Growth rate of mycelium. The mycelial growth of various fungal strains was measured, and the results are

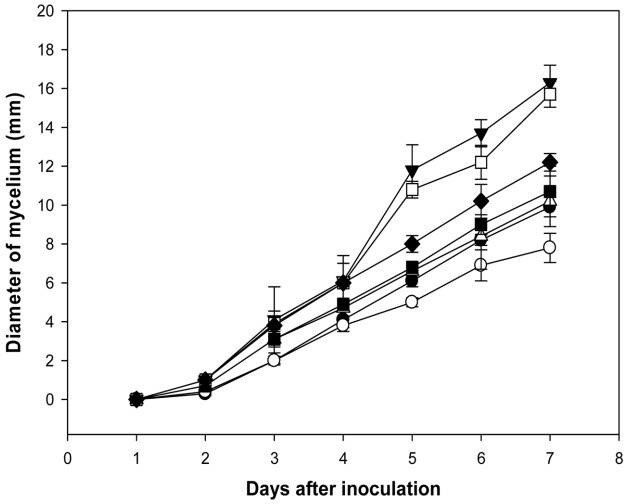

Fig. 2. Growth rate of mycelia of various fungal strains used in this study. ▼, 4078; □, Btab01; ◆, 41185; ■, 6543; △, 6541; ●, 3387; ○, 4075.

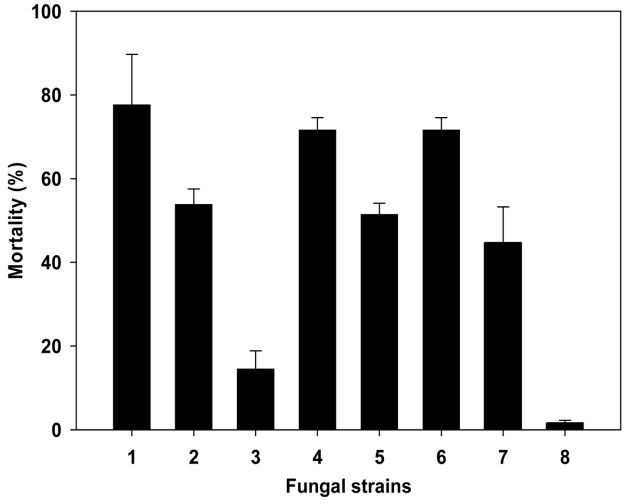

Fig. 3. Mortality of fungal strains applied to *B. tabaci* in the egg stage. 1, 4078; 2, 6541; 3, 3387; 4, 4075; 5, 6543; 6, Btab01; 7, 41185; 8, control.

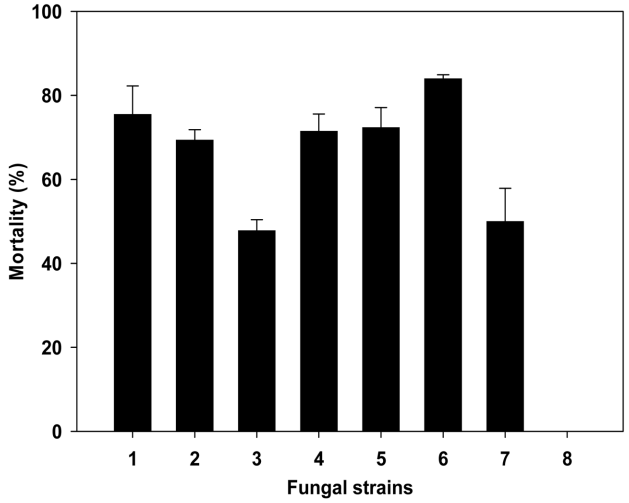

Fig. 4. Mortality of fungal strains applied to *B. tabaci* in the larva stage. 1, 4078; 2, 6541; 3, 3387; 4, 4075; 5, 6543; 6, Btab01; 7, 41185; 8, control.

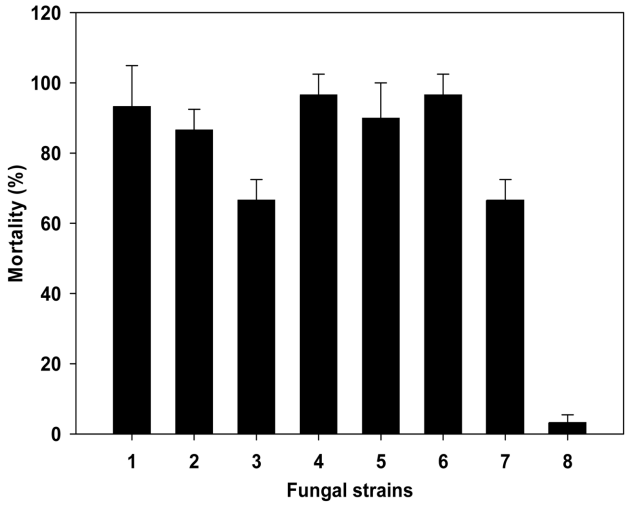

Fig. 5. Mortality of fungal strains applied to *B. tabaci* in the adult stage. 1, 4078; 2, 6541; 3, 3387; 4, 4075; 5, 6543; 6, Btab01; 7, 41185; 8, control.

shown in Fig. 2. Diameters of mycelia of strain 4078 and Btab01 were 16.3 ± 0.9 and 15.7 ± 0.67 mm, respectively, after seven days, and were larger than those of other strains. Since rapid growth of hyphae could result in fast mycelial penetration into insect body, it was suggested that strains 4078 and Btab01 may be more virulent than other fungal strains. As shown in Fig. 2, strain 4078 had the fastest growth rate; compared to strain 4075, which showed the slowest growth rate, the growth rate of strain 4078 after seven days of culture was almost 2-fold higher.

Entomopathogenic fungi are characterized by their ability to attach to and penetrate the host cuticle via release and replication of enzymes such as chitinase, protease and lipase from their hyphae. Nutrients in the hemolymph become depleted due to rapid hyphael growth, resulting in death of the host. Additionally, the fungal hyphae may invade and destroy other tissues or release toxin [13]. For this reason, the growth rate of fungal mycelia may be closely correlated with virulence. Therefore, considering hyphael growth rate only, strain 4078 may be the best candidate for controlling *B. tabaci*.

Bioassay against developmental stages of *B. tabaci*. To confirm the virulence of fungal strains against each developmental stage of *B. tabaci*, all of the experiments were conducted under identical conditions. The virulence of strain 4078 against the egg, larva and adult stages was 77.68 ± 12.01, 75.55 ± 6.68 and 93.33 ± 11.55%, respec-

tively, and was the most virulent strain against the egg stage. The virulence of strain Btab01 against the egg, larva and adult stages was 71.67 ± 2.89, 84.02 ± 0.87 and 96.67 ± 5.77%, respectively, and was the most virulent strain against the larva and adult stages. Strains 41185 and 3387 showed low virulence against all stages compared to the other fungal strains tested (Figs. 3, 4 and 5). Therefore, according to the above results, strains 4078 and Btab01 can be considered as candidates for biological control. Unlike the egg and larva, the adult is mobile and easily exposed to entomopathogenic fungal spores, which seems to explain the higher virulence against the adult stage (Fig. 5).

In conclusion, considering both high growth rate of hyphae and high virulence, strains 4078 and Btab01 were selected as the most effective fungal strains against *B. tabaci* regardless of the developmental stage.

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