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Research Article

Virulence of Entomopathogenic Fungi *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* for the Microbial Control of *Spodoptera exigua*

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Abstract The beet armyworm *Spodoptera exigua* (Lepidoptera: Noctuidae) is difficult to control using chemical insecticides because of the development of insecticide resistance. Several pest control agents are used to control the beet armyworm. Entomopathogenic fungi are one of the candidates for eco-friendly pest control instead of chemical control agents. In this study, among various entomopathogenic fungal strains isolated from soil two isolates were selected as high virulence pathogens against larva of beet armyworm. Control efficacy of fungal conidia was influenced by conidia concentration, temperature, and relative humidity (RH). The isolates *Metarhizium anisopliae* FT83 showed 100% cumulative mortality against second instar larvae of *S. exigua* 3 days after treatment at 1×10^7 conidia/mL and *Paecilomyces fumosoroseus* FG340 caused 100% mortality 6 days after treatment at 1×10^4 conidia/mL. Both *M. anisopliae* FT83 and *P. fumosoroseus* FG340 effectively controlled the moth at 20~30°C. *M. anisopliae* FT83 was significantly affected mortality by RH: mortality was 86.7% at 85% RH and 13.4% at 45% RH. *P. fumosoroseus* FG340 showed high mortality as 90% at 45% RH and 100% at 75% RH 6 days after conidia treatments. These results suggest that *P. fumosoroseus* FG340 and *M. anisopliae* FT83 have high potential to develop as a biocontrol agent against the beet armyworm.

Keywords Beet armyworm, Entomopathogenic fungi, *Metarhizium anisopliae*, *Paecilomyces fumosoroseus*, *Spodoptera exigua*

The beet armyworm (*Spodoptera exigua* Hüber) is a widely distributed polyphagous pest for many economically important crops, such as cotton, tomato, celery, lettuce, cabbage, alfalfa and so on [1, 2]. In Korea, beet armyworm occurs 4~5 generations annually in open fields. In greenhouses, this pest can survive throughout the year and causes year-round damage to crops across the country [3].

The first and second instar larvae of beet armyworm are gregarious and devour plant leaves. Early stage of *S. exigua* is difficult to control because it feeds in hidden parts of plants, for example, inside of welsh onion and the heart of Chinese cabbage which may be less exposed to insecticides [4]. The late larvae of beet armyworm could not be controlled with chemical insecticides because it develops resistance towards insecticides such as spinosad, chlorinated hydrocarbon, organophosphates, carbamates, pyrethroids, and benzoylphenylureas, etc. [5-11].

Studies on the biocontrol of *S. exigua* have mainly focused on nuclear polyhedrosis viruses (NPV) [12, 13] and *Bacillus thuringiensis* which are now commercially available many countries. However, *S. exigua* have developed *B. thuringiensis* resistance [14]. Mass production of NPV required much time and cost and productivity is so low. Therefore, it is important to develop alternative biocontrol agents to control *S. exigua*.

There are more than 700 species of fungi belonging to 90 genera which isolated from various insect species [15, 16]. At least 12 species or subspecies of fungi have been used as active ingredients for mycoinsecticides. One hundred seventy-six mycopesticides were developed in several countries using *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosorosea*, and *B. brongniartii* to control several agricultural pests. Among these only ten mycopesticides (seven *B. bassiana*, two *M. anisopliae* and one mixture of two or more species) have been used to control Noctuidae [17].

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Several fungal isolates including *B. brongniartii*, *Nomuraea rileyi*, *I. fumosorosea*, and *B. bassiana* were reported the pathogenicity to *S. litura* in China [18] and *M. anisopliae* and *I. fumosorosea* in Pakistan [19]. But there are few studies against *S. exigua* [20].

Therefore we studied and selected two fungal isolates having high virulence to control beet armyworm [21]. In this study, we conducted several tests at various conidial concentrations, temperatures and relative humidities (RHs) to find effective control condition of the two selected isolates for commercialization.

MATERIALS AND METHODS

Beet armyworm rearing. Beet armyworms were obtained from the Crop Protection Division, National Academy of Agricultural Science, Rural Development Administration in South Korea. Larvae were reared on artificial diet (#F9219B, mixing direction; Bio-Serv, San Diego, CA, USA) and maintained at 25 ± 1°C with a 14 : 10 h (L : D) photoperiod. Newly molted second instar larva (7 days after hatching) were used for bioassays.

Fungal strains and preparation of conidial suspensions. Two fungal isolates, *M. anisopliae* FT83 [21] and *P. fumosoroseus* FG340 isolated from soil of agricultural fields using *Tenebrio molitor* and *Galleria mellonella* in Korea, were selected as high pathogenicity isolates to control beet armyworm. These fungi were cultivated at 25 ± 1°C on potato dextrose agar (PDA) medium for 14 days. Conidia were harvested by adding 5 mL of sterilized 0.05% Tween 80 and scraping with spreader. The suspensions were vortexed for 3 min and then filtered through four layers of sterilized cheese cloth. Conidial concentrations were measured using a hemacytometer. The suspensions were diluted to a range of concentrations for each bioassay.

Bioassay at various conidial concentrations, temperatures and RHs. Chinese cabbages for the bioassays were grown in a greenhouse for approximately 30 days. Leaf discs (9 cm diameter) were placed in a 90-mm-diameter insect-breeding dish. Six hundred microliters of conidial suspension at 6 different concentrations (1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 conidia/mL) of *M. anisopliae* FT83 and *P. fumosoroseus* FG340 was sprayed onto each sides of the leaf disc infested 10 second instar larvae of beet armyworm inside the breeding dishes using a Plexiglass spray box (90 × 90 × 90 cm). The spray box was installed a polyvinyl acetal cone nozzle (1.5 mm diameter) on the top layer and the nozzle was connected to a vacuum pump which was fixed at 100 kPA. To avoid cross contamination among the sprayed isolates, the sprayer head and line were rinsed with 1 mL of 70% ethyl alcohol and 0.05% Tween 80. After air drying, dishes containing the leaf discs + larva were incubated in a Plexiglass cage at 25 ± 1°C with > 90% RH and a 16L : 8D photoperiod. Mortality was recorded daily for six days, and dead insects were transferred to Petri dishes with dampened filter paper. Mycosis cadavers were counted daily for 1 wk. Spore viability was measured by inoculating 100 μL drops of spore suspension (10^6 conidia/mL) onto 1.5% water agar in 35-mm-diameter Petri dish, and incubating for 24-hr incubation at 25°C, as described by Goettel and Inglis [22]. The spore viabilities were 93.4% and 53.6% for *P. fumosoroseus* FG340 and *M. anisopliae* FT83, respectively. Each bioassay was conducted three different times. There are 3 replicate dishes with 30 larvae per treatment in each trial.

To study the effect of temperature and RH on insect mortality, dishes treated with *M. anisopliae* FT83 and *P. fumosoroseus* FG340 (1×10^8 conidia/mL) were incubated at different temperatures (15°C, 20°C, 25°C, 30°C, and 35°C) and different RH (45%, 75%, 85%, and 95%) at 25°C using the bioassay conditions described above. Constant humidities of 45%, 75%, 85%, and 95% were achieved with saturated solutions of potassium carbonate, sodium chloride, potassium chloride and potassium sulphate, respectively [22]. When the leaf disc of Chinese cabbage sprayed with the conidia suspension dried or were entirely consumed, the larvae were provided artificial diet.

Statistical analysis. Normally distributed data were compared using one-way ANOVA (Proc GLM; SAS ver. 9.2, 2010, SAS Institute Inc., Cary, NC, USA). Median lethal times (LT_{50}) were estimated using the LIFEREG procedure, and the data were fitted to a Weibull distribution (SAS ver. 9.2). Goodness of fit was estimated using the Pearson chi-squared test.

RESULTS

Infection symptoms. Cardaver infected with the isolate *M. anisopliae* FT83 was covered with white mycelia 3 days after treatment and changed to dark green color by conidia from creamy white mycelia 5 days after treatment. Infection with *P. fumosoroseus* FG340 caused noticeable hyphal growth on the surface of cuticle 3 days after treatment, and the cadavers covered by white to brown colored conidia 5 days after treatment (Fig. 1).

Virulence of entomopathogenic fungi. Larval mortality with *M. anisopliae* FT83 and *P. fumosoroseus* FG340 differed significantly at different conidial concentrations: the mortality caused by each fungus increased with conidial concentration. Mortality by *M. anisopliae* FT83 was 49.8%, 65.3%, 85.8%, 100%, 100%, and 100% at 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 conidia/mL, respectively ($F = 40.93$, $df = 6, 76$, $p < 0.0001$). The mortality caused by *P. fumosoroseus* FG340 was 100% all concentrations from 1×10^4 conidia/mL to 1×10^9 conidia/mL (F = Infty, $df = 6, 35$, $p < 0.0001$) (Table 1). *P. fumosoroseus* FG340 showed higher mortality (100% 6 days after treatment) than about 50% of *M. anisopliae* FT83 at 1×10^4 conidia/mL. The median lethal

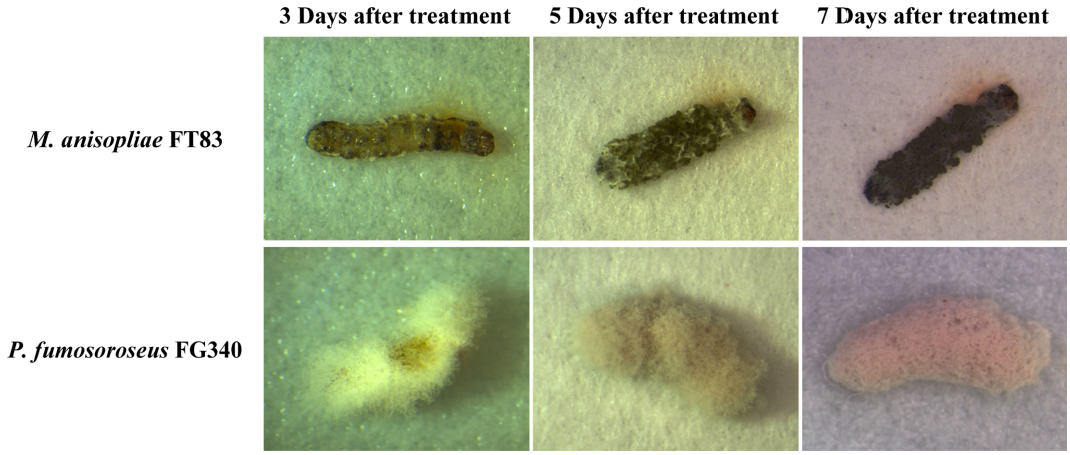

Fig. 1. Symptoms of cadavers of *Spodoptera exigua* infected by *Metarhizium anisopliae* FT83 and *Paecilomyces fumosoroseus* FG340. Cadaver infected by *M. anisopliae* FT83 were covered with mycelia 3 days after treatment and changed to dark green color by conidia from creamy white mycelia 5 days after treatment. Infection with *P. fumosoroseus* FG340 caused noticeable hyphal growth on the surface of cuticle 3 days after treatment, and the cadaver covered with conidia from white to brown color 5 days after treatment.

Table 1. Mortality of second instar larvae of *Spodoptera exigua* 6 days after treatments of different concentrations of *Metarhizium anisopliae* FT83 and *Paecilomyces fumosoroseus* and their median lethal time (LT_{50})

Concentration (conidia/mL)	*M. anisopliae* FT83		*P. fumosoroseus* FG340	
	Mortality (%)	LT_{50} (day)	Mortality (%)	LT_{50} (day)
Control	17.0 ± 4.3 d	-	0.0 ± 0.0 b	-
1×10^4	49.8 ± 8.7 c	5.7 ± 0.4 a	100.0 ± 0.0 a	4.2 ± 0.2 a
1×10^5	65.3 ± 3.0 b	4.7 ± 0.0 b	100.0 ± 0.0 a	3.8 ± 0.1 b
1×10^6	85.8 ± 5.6 a	3.6 ± 0.1 c	100.0 ± 0.0 a	3.2 ± 0.2 c
1×10^7	100.0 ± 0.0 a	2.2 ± 0.1 d	100.0 ± 0.0 a	2.9 ± 0.1 c
1×10^8	100.0 ± 0.0 a	2.1 ± 0.1 d	100.0 ± 0.0 a	2.1 ± 0.1 d
1×10^9	100.0 ± 0.0 a	1.3 ± 0.0 e	100.0 ± 0.0 a	2.0 ± 0.1 d

Values are prersented as mean ± SE. Means within the same column followed by the same letter are not significantly different using Duncan's multiple range test within the same column.

time (LT_{50}) of *S. exigua* larvae at low concentration from 10^4 to 10^6 was shorter by *P. fumosoroseus* FG340 as 4.2, 3.8, and 3.2 days compared to 5.7, 4.7, and 3.6 days by *M. anisopliae* FT83, but above 10^7 LT_{50} value was reverse. The median lethal times decreased with increase in conidial concentration.

Efficacy of entomopathogenic fungi at different temperatures and humidities. Mycelial growth of *M. anisopliae* FT83 was fastest at 25°C (32.4 ± 2.0 mm) (F = 282.92, df = 4, 8, $p < 0.0001$) and *P. fumosoroseus* FG340 was 30°C (29.04 ± 0.3 mm) (F = 60.53, df = 3, 32, $p < 0.0001$) among the tested five different temperatures (Table 2). Mortality caused by *M. anisopliae* FT83 and *P. fumosoroseus* FG340 was temperature-dependent and increased from 20 to 30°C but decreased at 35°C (Fig. 2). The median lethal time (LT_{50}) of *M. anisopliae* FT83 was 2.6, 2.2, 2.0, and 27.7 days at 20°C, 25°C, 30°C, and 35°C, respectively, and the LT_{50} of *P. fumosoroseus* FG340 was 3.0, 2.5, 2.0, and 79.7 days at 20°C, 25°C, 30°C, and 35°C, respectively. Both fungi showed the highest mortality at 30°C.

Table 2. Mycelial growth of *Metarhizium anisopliae* FT83 and *Paecilomyces fumosoroseus* FG340 at different temperatures after 7-day cultivation on potato dextrose agar media

Temperature (°C)	Mycelial growth after 7 days (mm)	
	M. anisopliae FT83	*P. fumosoroseus* FG340
15	11.5 ± 0.5 d	12.6 ± 0.5 d
20	22.2 ± 0.8 c	18.4 ± 0.6 c
25	32.4 ± 2.0 a	24.9 ± 0.5 b
30	28.1 ± 0.7 b	29.4 ± 0.3 a
35	7.0 ± 0.0 e	7.0 ± 0.0 e

Values are prersented as mean ± SE. Means within the same column followed by the same letter are not significantly different using Duncan's multiple range test.

RH significantly affected infection by *M. anisopliae* FT83. The mortality of larvae treated with *M. anisopliae* FT83 (1×10^8 conidia/mL) at various RH was higher as RH increases: the mortality was 13.4%, 55.6%, 86.7%, and

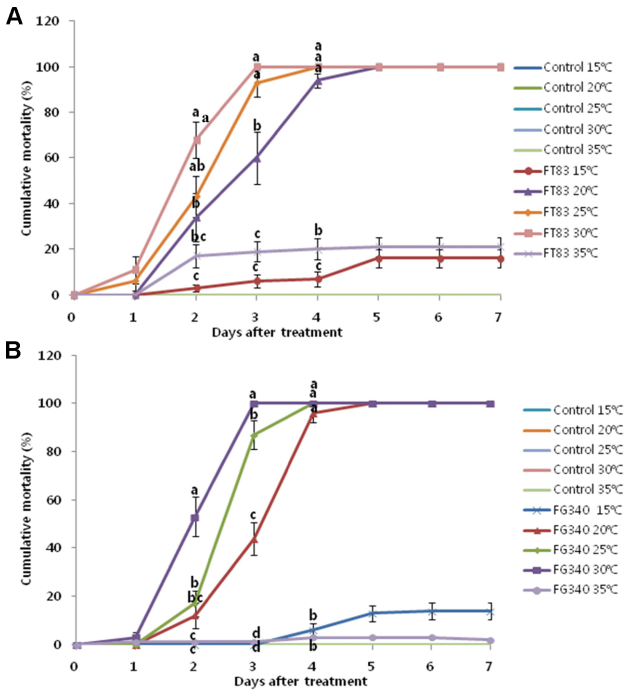

Fig. 2. Cumulative mortality of *Spodoptera exigua* larvae treated with *Metarhizium anisopliae* FT83 (A) and *Paecilomyces fumosoroseus* FG340 (B) at different temperatures: 15°C, 20°C, 25°C, 30°C, and 35°C. The conidial concentration used for each treatment was 1×10^8 conidia/mL. Control was treated with 0.01% Tween 80. Means above the line followed by the same letter are not significantly different using Duncan's multiple range test ($p > 0.05$).

97.2% at 45%, 75%, 85%, and 95% RH, respectively. *P. fumosoroseus* FG340 showed high control effects in whole ranges of RHs we conducted tests from 45% to 95% RHs; mortality of second instar larva of *S. exigua* treated with *P. fumosoroseus* FG340 (1×10^8 conidia/mL) at 45%, 75%, 85%, and 95% RH was 90.0%, 100%, 100%, and 100%, respectively ($F = 137.7$, $df = 11, 275$, $p < 0.0001$) (Table 3).

Table 3. Mortality and LT_{50} of *Spodoptera exigua* second instar larvae treated with 1×10^8 conidia/mL of *Metarhizium anisopliae* FT83 and *Paecilomyces fumosoroseus* FG340 at different relative humidities

Humidity (%)	Treatment	Mortality (%)	LT_{50} (day)
45	Control	0.6 ± 0.6 e	-
	FT83	13.4 ± 2.9 d	11.2 ± 0.8 a
	FG340	90.0 ± 5.3 ab	4.7 ± 0.5 b
75	Control	1.7 ± 0.9 e	-
	FT83	55.6 ± 6.9 c	9.0 ± 2.2 a
	FG340	100.0 ± 0.0 a	3.4 ± 0.2 b
85	Control	0.0 ± 0.0 e	-
	FT83	86.7 ± 3.7 b	4.4 ± 0.5 b
	FG340	100.0 ± 0.0 a	3.2 ± 0.1 b
95	Control	0.0 ± 0.0 e	-
	FT83	97.2 ± 1.4 ab	3.3 ± 0.3 b
	FG340	100.0 ± 0.0 a	2.9 ± 0.1 b

Values are prersented as mean ± SE. Means within the same column followed by the same letter are not significantly different using Duncan's multiple range test.

DISCUSSION

Entomopathogenic fungi are important factors regulating insect populations. *M. anisopliae*, *I. fumosorosea, B. bassiana* and *Lecanicillium* sp. are important natural control agents and sources of mycopecticides for many Noctuidae pests management worldwide [19]. Several studies have examined their potential use as biological control agents. Lin *et al.* [18] compared the pathogenicity of several fungal species against *S. litura*. *B. brongniartii* and *N. rileyi* showed 100% and 95.2% mortality after treatment with 8×10^7 conidia/mL and LT_{50} was 3.0 and 4.1 days against larvae of *S. litura*, respectively. The cumulative mortality of *S. litura* exposed to *I. fumosorosea* and *B. bassiana* was 85.7% and 71.4%, respectively, and the LT_{50} values were 4.9 and 6.3 days, respectively. Asi *et al.* [19] also examined the susceptibility of *S. litura* to *M. anisopliae* and *I. fumosorosea*. These two fungi caused mortalities of 53.5% and 41.2%, respectively after 10 days of treatment at a concentration 10^7 conidia/mL. Dose-mortality assays using these two isolates revealed that the mortality of third instar larvae was 15%, 21%, 52%, and 58% when applied with *I. fumosorosea* at 10^5, 10^6, 10^7, and 10^8 conidia/mL, respectively, and 9.3%, 12.0%, 36.2%, and 43.0% with *M. anisopliae* 10 days after treatment. We observed that *M. anisopliae* FT83 and *P. fumosoroseus* FG340 which are used in this study, showed higher mortality than other results. All second instar larvae of *S. exigua* died after exposure to 10^7 conidia/mL of *M. anisopliae* FT83 and 10^4 conidia/mL of *P. fumosoroseus* FG340 within 6 days after application.

The pathogenicity of the fungi is primarily mediated by entry through the external larval integument [23]. Conidia attachand germinate on cuticle and penetrate into insect body. Upon entry into the hemocoel, the mycelia grow and spread throughout the whole body and then form hyphae and produce blastospores. Host death often occurs due to a combination of fungal toxins, physical obstruction of blood circulation, nutrient depletion and organ invasion. Efficacy of control agents against insect pests is influenced by abiotic environmental factors such as temperature, RH, water, and solar radiation which have effect on germination, vegetative growth and viability of entomopathogenic fungi. In this study, mycelial growth of the *M. anisopliae* FT83 and *P. fumosoroseus* FG340 on PDA media and control efficacy to beet armyworm were affected by temperature but influence of RHs on control efficacy was differed from two isolates.

Several studies have examined the insecticidal activity of metabolites produced by entomopathogenic fungi including *M. anisopliae* and *P. fumosoroseus*. Beauvericin, beauverolides, 2,6-pyridinecarboxylic acid and dipicolinic acid are metabolites isolated from *P. fumosoroseus* [24-26]. Beauvericin is a cyclic hexadepsipeptide that has insecticidal activity against

Aedes aegypti mosquito larvae [27]. Dipicolinic acid is toxic to the blowfly *Calliphora erythrocephala* [28] and third instar nymphs of *Bemisia tabaci* type B [29]. The toxic crude proteins produced by *I. fumosorosea* have insecticidal and antifeedant activity against *Plutella xylostella* [30]. The toxic crude proteins showed 83.3% mortality of third instar larvae 6 days after treatment. In general, entomopathogenic fungi have low control efficacy in low RH or dry condition such as 45% RH or under 75% RH. For example, *L. attenuatum* having high pathogenicity against cotton aphid showed 49% mortality at 85% RH but 97% mortality at 97% RH [31]. *M. anisopliae* FT83 also showed low mortality (13.4%) at 45% RH and high mortality (97.2%) at 95% RH. However *P. fumosoroseus* FG340 showed high mortality (90%) at 45% RH compared with other fungal isolates. We suppose *P. fumosoroseus* FG340 at low RH was influenced by direct fungal infection as well as the secondary metabolites for high mortality.

Based on our results, we suggest that *M. anisopliae* FT83 and *P. fumosoroseus* FG340 have good potential to develop mycopesticides to control beet armyworm. Furthermore, we will conduct further study to test the control efficacy of these fungal isolates in greenhouses to control *S. exigua* larva.

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