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A Technique for the Prevention of Greenhouse Whitefly (*Trialeurodes vaporariorum*) Using the Entomopathogenic Fungus *Beauveria bassiana* M130

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The possibility of using hyphomycete fungi as suitable biocontrol agents against greenhouse whitefly has led to the isolation of various insect pathogenic fungi. Among them is *Beauveria bassiana*, one of the most studied entomopathogenic fungi. The objective of this study was to use *B. bassiana* M130 as an insecticidal agent against the greenhouse whitefly. M130 isolated from infected insects is known to be a biocontrol agent against greenhouse whitefly. Phylogenetic classification of M130 was determined according to its morphological features and 18S rRNA sequence analysis. M130 was identified as *B. bassiana* M130 and showed chitinase (342.28 units/ml) and protease (461.70 units/ml) activities, which were involved in the invasion of the host through the outer cuticle layer, thus killing them. The insecticidal activity was 55.2% in petri-dish test, 84.6% in pot test, and 45.3% in field test. The results of this study indicate that *B. bassiana* has potential as a biological agent for the control of greenhouse whitefly to replace chemical pesticides.

Keywords: *Beauveria bassiana*, biocontrol, chitinase, greenhouse whitefly, protease, sporulation

Introduction

The interdependence of global markets for agricultural products is necessary for the development of agricultural practices while, simultaneously, it can mitigate negative side effects on the environment. As a result, agricultural products are safer for human consumption; however, loss of agricultural products has increased because of insects, plant diseases, and weeds. This potential loss of production is up to 40% [25] despite a marked increase in pesticide use; thus, crop losses have remained relatively constant [18].

On this account, insect pest control has relied mostly on chemical insecticides since the late 1940s. Chemical controls have not only improved agricultural productivity, but can also lead to efficient cultivation. Thanks to such controls, the productivity of crops worldwide has been improved. There are also, however, serious side effects such as groundwater and soil pollution, and ecocide. To solve these problems, many advanced countries have instituted the use of biological control agents. Biological control is a method of controlling pests using other living organisms and the extraction of natural products. It relies on predation, parasitism, or other mechanisms, and there are many types. Recently, some commercial bioinsecticides have been developed and registered to control diamondback moth, beet armyworm, oriental tobacco budworm, greenhouse whitefly, and root knot nematodes [15].

Entomopathogenic fungi have been recognized as important natural enemies of insect pests and can be used as effective biological control agents against agricultural pests. Entomopathogenic fungi are usually identified based on fungal growth observed on insect cadavers. Most research

on entomopathogenic fungi, however, has been aimed at their development as biological control agents of insects, mites, and ticks, despite their great potential for use in conservation and classical biocontrol strategies. This is normally achieved through a strategy in which pest control relies on the action of the released agent but not on successive generations of the fungus. Under this paradigm, over 170 products have been developed based on at least 12 species of fungi [8]. Despite the fact that there are an estimated 700 species of entomopathogenic fungi in approximately 90 genera [21], most commercially produced fungi are species of *Beauveria*, *Metarhizium*, *Lecanicillium*, and *Isaria* that are relatively easy to mass produce [28]. Among these, *Beauveria bassiana* is a fungus with a broad natural distribution. Its potential to control more than 70 insect pests has been responsible for a substantial increase in the large-scale production of the fungus for applications in the field [26]. *Beauveria bassiana* infects the host insect by penetrating the cuticle. The fungal conidium attaches to the cuticle and then germinates. Germinating conidia produce extracellular enzymes such as lipases, chitinases, and proteases to initiate cuticle invasion. Once inside the host, the fungus develops as a yeast-like form, producing metabolites that inhibit the insect's immune system, modify the insect's behavior, or act as post-mortem antibiotics against competing microorganisms [11, 29]. After death, the fungus reverts to a filamentous form and typically digests the remaining internal organs, leaving only the chitin and protein exoskeleton [6, 13]. Moreover, this fungus appears to be harmless to most non-target organisms.

Suspensions of conidia are spread by spray applications for the control of insect pests and require improved dispersion. To improve dispersion, hydrophobic conidia are often formulated in oil or added to spray mixes containing wetting agents as adjuvants. Since spore persistence of fungi on the foliar surface is affected by solar radiation, considerable effort has focused on the protection of these entomopathogens by incorporating solar blockers and sunscreens [14].

Meanwhile, the greenhouse whitefly (*Trialeurodes vaporariorum*) has been focused on as a major harmful insect in many countries [2, 4]. Adults and immature flies are phloem feeders and reduce productivity of agricultural products. Furthermore, they produce large amounts of honeydew on the leaf and this honeydew reduces leaf photosynthesis [3]. Crops in the families Cruciferae, Leguminosae, Malvaceae, and Solanaceae are mainly targeted [2]. Tomato, cucumber, and tobacco, which are cultivated widely in Korea, are also seriously affected by the greenhouse whitefly. These insects have been controlled primarily using various chemical insecticides [12, 20]. However, the greenhouse whitefly continues to constitute a major problem on crops because insecticide-resistant populations have developed [5, 20].

Thus, the objective of this study was to effectively control the greenhouse whitefly using entomopathogenic fungi. To achieve this goal, a strain with a high pathogenicity was isolated from infected insects. The phylogenetic relationships of selected entomopathogenic fungi were analyzed. Additionally, proteolytic enzyme and chitinase enzyme activities, which are involved in insect pathogenicity, were measured. Finally, a bioassay was conducted in order to investigate the potential of the control agent.

Materials and Methods

Microorganism

Beauveria bassiana M130 was isolated from an infected insect. It was stocked at −80ºC in 30% glycerol containing sterile 0.1% Tween 80 and mineral oil. The fungal strain was cultured on potato dextrose agar (PDA; Difco, USA) at 28ºC for 8 days and stored at 4ºC until use.

Identification of *Beauveria bassiana* M130

For identifying its phylogenetic classification, the morphological features of M130 were determined through the use of a light microscope (Olympus CX21, Japan), scanning electron microscope (Hitachi S-2500, Japan), and 18S rRNA sequencing.

Chitinase Activity

Chitinase activity was determined by a modified dinitrosalicylic acid (DNS) method [17]. This method is based on the concentration of *N*-acetyl glucosamine (NAG) that is released as a result of enzymatic action [16, 27]. The 2.5 ml reaction mixture contained 0.5 ml of 1% colloidal chitin in 1 ml of phosphate buffer (0.1 M, pH 7.0), and 1 ml of crude enzyme. The mixture was vortexed well and incubated in a water bath at 40ºC for 30 min. About 1 ml of the reaction was arrested by the addition of 3 ml of DNS reagent, followed by heating at 100ºC for 5 min. The colored solution was centrifuged at 10,000 rpm for 5 min and the absorption of the appropriately diluted test sample was measured at 545 nm using a UV Spectronic Genesys 5 (Milton Roy, USA) along with substrate and enzyme blanks. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μM of *N*-acetyl-D-glucosamine per hour [9, 24].

Protease Activity

A 0.6% (w/v) casein solution (prepared by mixing 6 mg/ml casein in 1/15 potassium phosphate buffer and adjusting to pH 7.0 with 1 N NaOH; 3 ml) was incubated with 1 ml of enzyme at 30ºC for 10 min. It was then added to 0.4 M trichloroacetic acid

(TCA) solution, which was incubated at 30°C for 30 min. Afterwards, the product was centrifuged for 5 min at 10,000 rpm. To 0.5 ml of supernatant was added 1.25 ml of 0.4 M Na_2CO_3 and 0.25 ml of 2 N Folin. The final product was measured at 660 nm [1, 9]. One unit of protease was defined as the amount of enzyme that released 1 μM of tyrosine per hour.

Optimization of Culture Temperature

Spore suspension (1.0×10^6 conidium/ml) was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of potato dextrose broth (PDB; Difco, USA), and the flasks were incubated at 23°C, 25°C, 28°C, and 33°C at 130 rpm. The dry cell weight (DCW) of mycelia was measured at 48 h intervals on a regular basis for 8 days .

Preparation of *Beauveria bassiana* M130 Conidia

An inoculum of conidia for liquid culture was obtained from a 14-day sporulated culture on a PDA plate at 28 ± 1°C. After 14 days of incubation at 28°C, sterile 0.1% (v/v) Tween 80 was poured onto the culture on the surface of the PDA and the conidia were collected by scraping the culture with a glass hockey stick and suspension-filtering using glass wool. The number of conidia in the spore suspension was counted using a hemocytometer (Superior, Germany). The filtrate was thoroughly vortexed and diluted to achieve a suspension of 1.0×10^6 conidia/ml.

Incubation of Greenhouse Whitefly

Greenhouse whiteflies were bred on potted pepper at 28 ± 1°C in a 16L/8D (L: light; D: darkness) photoperiod. All insects were grown on pepper leaves until use. All potted plants were covered with an insect rearing cage to prevent escape.

Greenhouse Whitefly Pot Test

Tomato pots were prepared in an environment with a relative humidity of 85% and temperature of 28°C or 30°C, and whiteflies were released in each pot. After 3 days, the spore concentration was adjusted to 1.0×10^8 conidia/ml to conduct the bioassay on the greenhouse whitefly. Greenhouse whiteflies were sprayed with spore suspension, and after 8 days, mortality rates were identified.

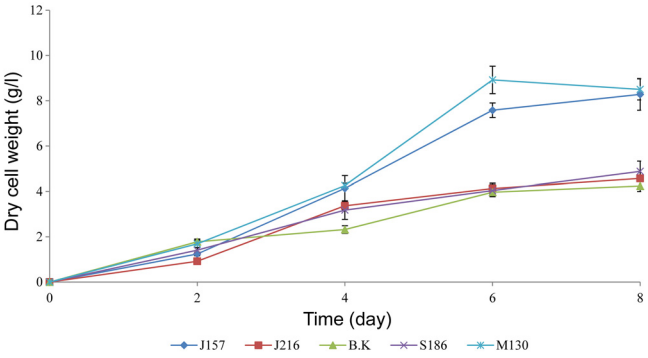

Fig. 1. Mycelial growth of five strains at 28°C for 8 days.
Vertical lines indicate standard deviations.

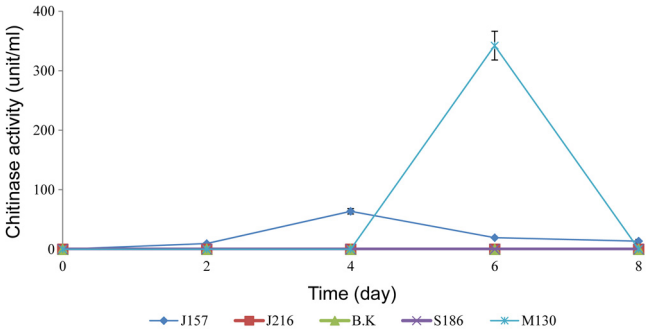

Fig. 2. Chitinase activity produced by isolated strains.
Vertical lines indicate standard deviations.

Greenhouse Whitefly Field Test

The experiment was conducted in greenhouses to identify the greenhouse whitefly control effect of M130. To this end, a spore suspension was sprayed, and after 8 days, the greenhouse whitefly mortality rate was identified.

Scanning Electron Microscopy

The samples were fixed for 1 h in a solution of glutaraldehyde (2.5% in 0.1 M phosphate buffer, pH 6.8), and then dehydrated in an ethanol gradient (50%, 60%, 70%, 80%, 90%, 95%, and 99.9%). In the next step, ethanol was replaced by CO_2, followed by "critical-point drying". In a sputter coating process, infected eggs, nymphs, and greenhouse whitefly were covered with a gold layer to increase their electrical conductivity.

Results and Discussion

Selection of *Beauveria bassiana*

The fungal growth of five strains was evaluated and the results are shown in Fig. 1. Results for measurements of enzyme activity are shown in Figs. 2 and 3. Among the five selected strains, J157 and M130 showed similar fungal

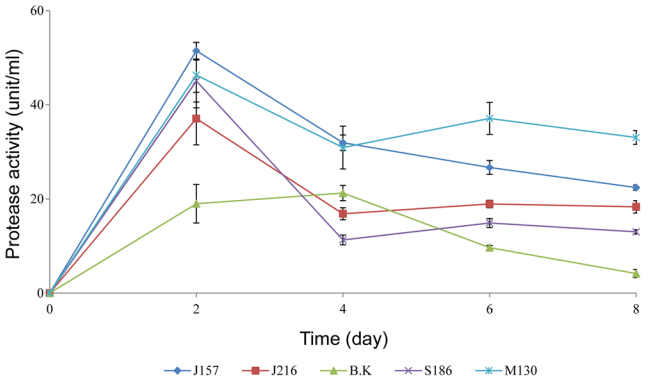

Fig. 3. Protease activity produced by isolated strains.
Vertical lines indicate standard deviations.

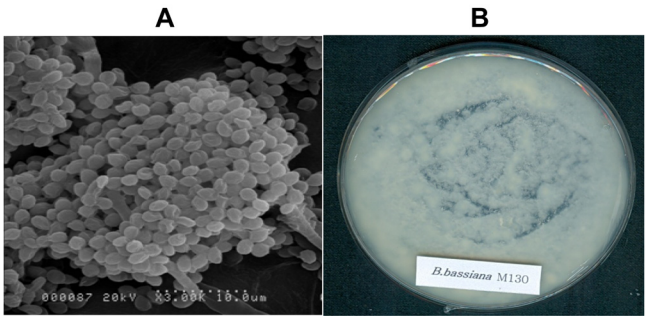

Fig. 4. Scanning electronic microscopy of strain M130 (× 200, 72 h).
(**A**) The spores showed ellipsoidal morphology. (**B**) Isolated *Beauveira bassiana* M130 in petri dish state.

growth patterns over 4 days, but afterwards, M130 fungal growth was greater than that of J157.

Until day 2, the five strains did not show chitinase activity, but as time elapsed, J157 and M130 showed activity. However, strain J157 showed the maximal enzyme activity (63.74 unit/ml) at 2 days, which gradually decreased. M130 showed the maximal enzyme activity (342.29 unit/ml) at 6 days.

In the case of protease, all strains showed maximum enzyme activity at 2 days, and the activity gradually decreased. However, strain M130 maintained high enzyme activity after 4 days. Based on the above results, *B. bassiana* M130 was selected for use in this study.

Identification of *Beauveria bassiana* M130

The selected strain, M130, was cultured in PDB at 25°C to determine its phylogenetic classification and investigate its biochemical features. The mycelium color of M130 is white, the conidial shape is cylindrical, the spore size is 2.0–5.0 μm, and the appressorium is almost cylindrical (Fig. 4). These results are similar to those of Stephen and Rehner [23]. The sequences could be identified in NCBI database. A comparative 18S rRNA gene sequence analysis revealed that strain M130 is the most closely related to members of the genus *Beauveria*. Moreover, in a neighbor-joining phylogenetic tree constructed on the basis of the 18S rRNA gene sequences, strain M130 also fell within the radiation of the cluster comprising the *Beauveria* species. Strain M130 exhibited 18S rRNA gene sequence similarity values of 100% with *B. bassiana* IMI 393155. Therefore, strain M130 was identified as *B. bassiana* M130 (Fig. 5).

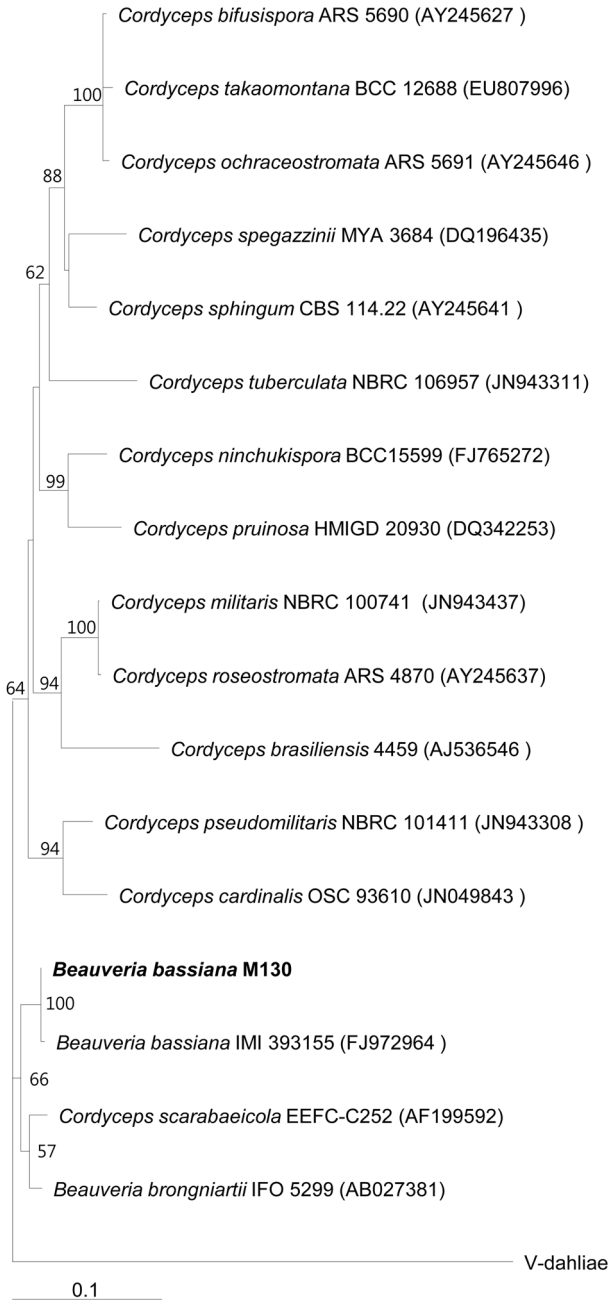

Fig. 5. Neighbor-joining tree based on 18S rDNA gene sequences, showing phylogenetic relationships between strain M130 and species from *Beauveria*.
Numbers at nodes indicate levels of bootstrap support (%p) based on 1,000 resembled data sets; only values above 70% are given. Bar, 0.1 substitutions per nucleotide position.

Optimization of *Beauveria bassiana* M130 Culture Temperature

The mycelial growth of *B. bassiana* M130 was examined at different temperatures for 8 days and the results are provided in Fig. 6. The optimal temperature for mycelial growth was 28°C and mycelial growth of *B. bassiana* M130

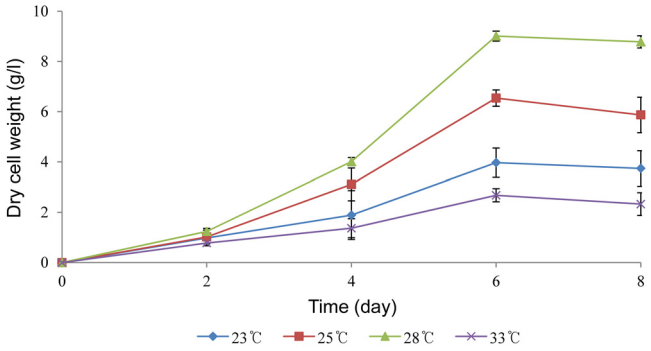

Fig. 6. Effect of temperature on mycelial growth of *B. bassiana* M130 over 8 days.
Vertical lines indicate standard deviations.

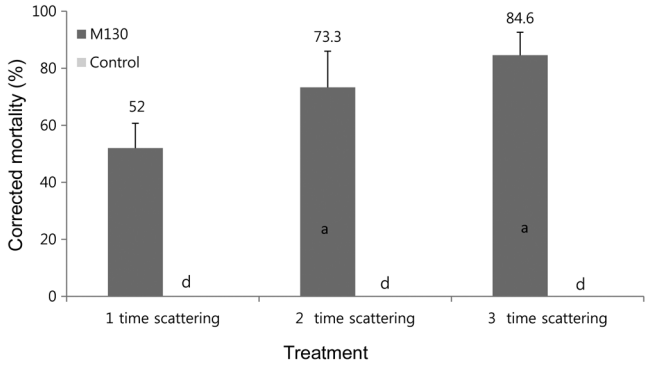

Fig. 7. Corrected mortalities of greenhouse whiteflies treated with *B. bassiana* M130 at a concentration of 1×10^8 conidia/ml in pots.
The different letters above the error bars indicate the statistically significant differences analyzed by Duncan's multiple range test ($p \leq 0.05$). Vertical lines indicate standard deviations.

was best at 28°C. Previous studies [7] have shown that the optimal temperature for mycelial growth of *B. bassiana* is generally around 25–28°C and the findings of the present study are consistent with these results.

Sato *et al*. [22] implied that the optimal growth temperature of *B. bassiana* varies depending on the geographic origin of the isolate. *B. bassiana* isolated from Okinawa, the southernmost island and warmest region of Japan, exhibited the best growth at 30°C, whereas strains isolated in warmer regions tended to have higher optimal temperatures for mycelial growth. In contrast, Fargues *et al*. [7] observed no evident relationship between relative growth and climatic origin of *B. bassiana* isolates. *B. bassiana* M130 was isolated in Yecheon, Gyeongbuk. Yecheon is not a warmer region of Korea. Therefore, the *B. bassiana* M130 growth pattern was similar to that reported by Fargues *et al*. [7].

Bioassay

Pot test. Pot test results are shown in Fig. 7. The control value increased with the number of applications of the biological control agent. With three applications, the maximal control value was up to 84.6%. Generally, *B. bassiana* spores are known to germinate best at 15–30°C and a relative humidity of 80% [10]. In this study, the temperature was adjusted to 28°C and the relative humidity to 85%. Proper temperature and relative humidity appear to be factors in the ability of *B. bassiana* M130 to control populations of greenhouse whitefly. A control value of 84.6% indicates that *B. bassiana* M130 may possibly be substituted for chemical pesticides.

Field test. The results of the field test as shown in Fig. 8 are consistent with patterns identified in the pot test. The control value increased with increasing number of applications. The maximum control value was up to 45.3%. The lower control value was due to poor conditions for germinating *B. bassiana* spores.

In this study, the highest greenhouse temperature was 28°C. The lowest temperature was 12°C, and the average relative humidity was 60%. We suspect that if the number of applications of the biological control were increased, the control value will increase in the field test.

Insecticidal Process of *Beauveria bassiana* M130

In Fig. 9, images 1, 2, and 3 show M130 conidia attached to the surface of an egg. Thereafter, they formed appressoria and invaded the egg. Images 4–7 show the internal invasion

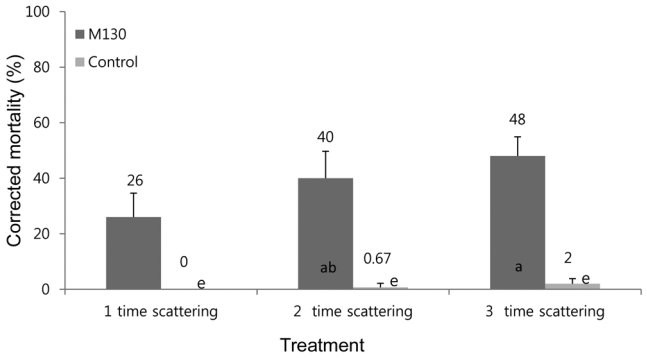

Fig. 8. Corrected mortalities of greenhouse whiteflies treated with *B. bassiana* M130 at a concentration of 1×10^8 conidia/ml in the field.
The different letters above the error bars indicate the statistically significant differences analyzed by Duncan's multiple range test ($p \leq 0.05$). Vertical lines indicate standard deviations.

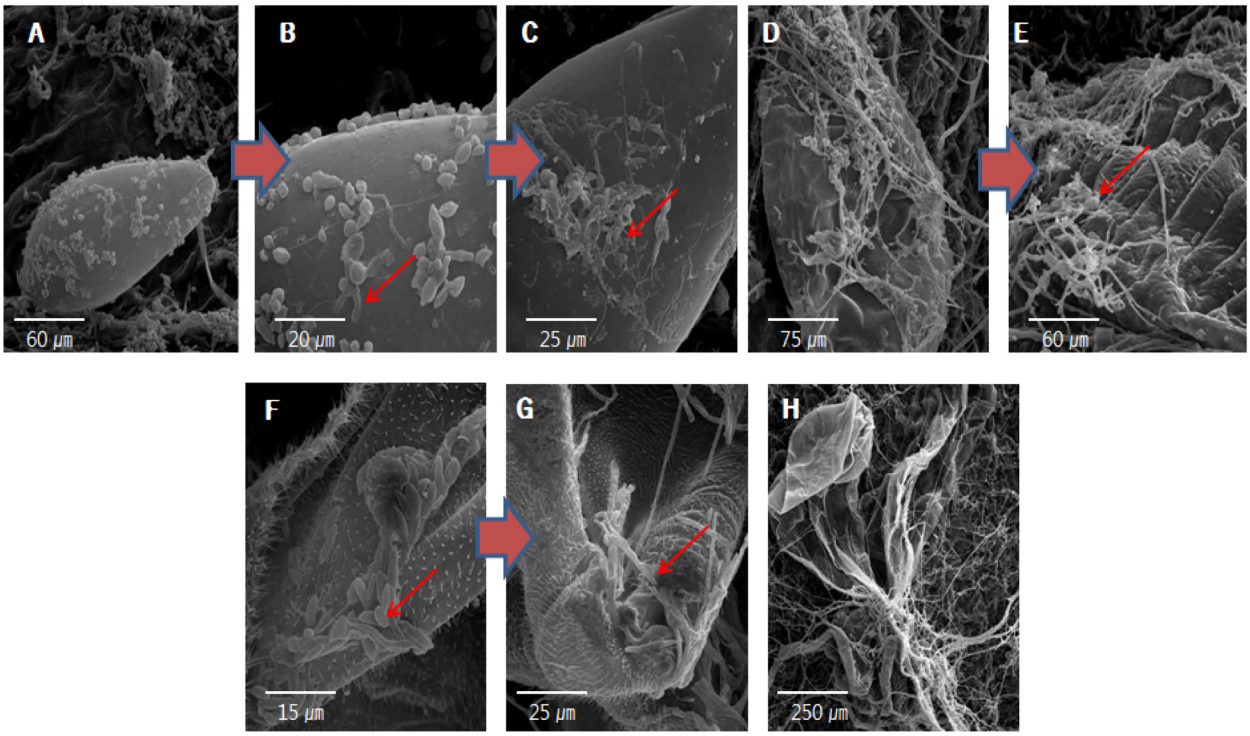

Fig. 9. Scanning electron micrographs of *B. bassiana* M130 on greenhouse whitefly egg, nymph, and imago.
(**A**) Conidia adhered to the surface of egg (×500, 0 h after inoculation); (**B**) germinating conidium, with penetration of the germ tube (×1,300, 24 h); (**C**) growth of the germ tube (×1,200, 48 h); (**D** and **E**) extrusion of the mycelium with degradation of the cuticle of the nymph (×400, 72 h and ×500, 96 h); (**F** and **G**) conidia adhered to the tegument of greenhouse whitefly and extrusion of the mycelium on segment (×2,000, 24 h and ×1,200, 96 h); and (**H**) whole body covered in mycelia of *Beauveria bassiana* M130 (×120, 144 h).

process in nymphs and imagos. These results illustrate the invasion process of general entomopathogenic fungi.

Acknowledgments

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