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Research Article

Entomopathogenic Fungi as Dual Control Agents against Both the Pest *Myzus persicae* and Phytopathogen *Botrytis cinerea*

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Abstract The green peach aphid (*Myzus persicae*), a plant pest, and gray mold disease, caused by *Botrytis cinerea*, affect vegetables and fruit crops all over the world. To control this aphid and mold, farmers typically rely on the use of chemical insecticides or fungicides. However, intensive use of these chemicals over many years has led to the development of resistance. To overcome this problem, there is a need to develop alternative control methods to suppress populations of this plant pest and pathogen. Recently, potential roles have been demonstrated for entomopathogenic fungi in endophytism, phytopathogen antagonism, plant growth promotion, and rhizosphere colonization. Here, the antifungal activities of selected fungi with high virulence against green peach aphids were tested to explore their potential for the dual control of *B. cinerea* and *M. persicae*. Antifungal activities against *B. cinerea* were evaluated by dual culture assays using both aerial conidia and cultural filtrates of entomopathogenic fungi. Two fungal isolates, *Beauveria bassiana* SD15 and *Metarhizium anisopliae* SD3, were identified as having both virulence against aphids and antifungal activity. The virulence of these isolates against aphids was further tested using cultural filtrates, blastospores, and aerial conidia. The most virulence was observed in the simultaneous treatment with blastospores and cultural filtrate. These results suggest that the two fungal isolates selected in this study could be used effectively for the dual control of green peach aphids and gray mold for crop protection.

Keywords Antifungal activity, *Botrytis cinerea*, Dual control, Entomopathogenic fungi, *Myzus persicae*

The green peach aphid, *Myzus persicae*, destroys agricultural crops directly through feeding and indirectly by transmitting viral vectors [1]. The most commonly used field strategy for the population reduction of *M. persicae* is based on chemical insecticides. However, this aphid has rapidly developed multiple resistances to many chemical insecticides including carbamates, pyrethroids and neonicotinoids [2]. Additionally, the overuse of pesticides may cause harmful pollution and harmful effects on humans and other organisms, raising serious environmental and health concerns [3]. Therefore, the potential use of entomopathogenic fungi as a biological aphid control method offers a favorable alternative to chemically derived pest management strategies [4, 5].

Gray mold, caused by the fungus *Botrytis cinerea*, seriously damages over 200 crop species in commercial greenhouses and fields worldwide. Gray mold affects several plant organs, not only flowers, fruits and leaves but also shoots and soil storage organs [6, 7]. This mold is difficult to control because of its various modes of attack and wide host range of inoculum sources [7]. The method used to control *B. cinerea* generally depends on the application of fungicides. Because of consumer needs worldwide for a reduction in the use of chemical fungicides and the emergence of pathogens resistant to these chemicals, there is a need to develop a substitute method for the control of *B. cinerea* [8].

Entomopathogenic fungi play an important role in the control of pest populations [9]. Importantly, these fungi are generally harmless to humans and are not known to

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negatively impact the environment. These fungi can also effectively control sucking pests because they interfere with penetration into the insect cuticle [10, 11]. More than 170 pest control products have been developed based on at least twelve species of fungi. For example, various entomopathogenic fungi, such as *Beauveria* spp., *Lecanicillium* spp., *Metarhizium* spp., and *Paecilomyces* spp., have been used to control the green peach aphid and other pests [12]. Moreover, it has been recently reported that many entomopathogenic fungi can play unexpected roles in nature, including as fungal endophytes, phytopathogens antagonists, beneficial rhizosphere-associates and plant growth promoters [13]. For example, *B. bassiana* exists as an endophyte in various plants and has the potential for plant disease and insect regulation [14]. These diverse effects of entomopathogenic fungi result from the production of varied metabolites, such as antibiotics, bioactive volatile compounds and enzymes [13]. Secondary metabolites produced by entomopathogenic fungi exhibit various insecticidal, antimicrobial, anticancer, and antioxidant properties. They have also been recommended as potential alternatives to the development of new bioactive agents [15-17]. The potential for dual control against insect pests and plant pathogens has also been reported for entomopathogenic fungi [17-19].

Recently, we reported several fungi having high virulence to the green peach aphid from 342 isolates of entomopathogenic fungi isolated in Korea [5, 20]. In this study, we evaluated the antifungal activity of these selected fungi to explore the potential for the dual control of the phytopathogen *B. cinerea* as well as the pest *M. persicae*. This study proposes the possibility of entomopathogenic fungi as plant protection agents not only against the green peach aphid but also against gray mold.

MATERIALS AND METHODS

Aphid source and growth conditions. Green peach aphids were collected from a colony in the insect ecotoxicology laboratory at Chungbuk National University, Korea. The green peach aphid population was raised and maintained on Chinese cabbage at 25 ± 1°C with 50%–60% relative humidity and a 16 : 8 (L : D) photoperiod. Adult stages were tested against fungi in the laboratory.

Fungal growth and generation of culture filtrates. Fungal isolates with high virulence to green peach aphids were cultured on potato dextrose agar (PDA) plates for 14 days at 25°C. Then, 14-day-old cultures were used for each experiment and stock cultures of each isolate were stored at −70°C until further use. Two-week-old fungal conidia were collected by scraping fungi from PDA plates and resuspending the material in a 0.02% Tween-80 (Difco, Detroit, MI, USA) solution. The conidial suspension was vigorously stirred and filtered through cotton to remove the mycelial debris. The conidial concentration was adjusted using a hemocytometer, and viability was determined on PDA with 0.05% benomyl (95% active ingredient, Sigma, St. Louis, MO, USA). Then, 50 µL of the conidial suspension (9×10^5 conidia/mL) with more than 90% viability was inoculated in 30 mL of potato dextrose broth (PDB; pH 5.6) in a 100 mL flask. The samples were cultured at 25°C in the dark with shaking at 150 rpm for 14 days. After 2 wk, the samples were centrifuged at 10,000 ×g for 20 min at 4°C, and the supernatants were filtered via a membrane filter paper (Advantec No. 2; Advantec, Tokyo, Japan) to separate the mycelial and spore masses. The culture filtrate for the antifungal assay condition was adjusted to pH 5.6 with HCl and NaOH and re-filtered through a 0.45 µm membrane filter (28 mm syringe filter; Corning, New York, NY, USA). All fungal culture filtrates were stored at −70°C until further use.

Antifungal activity assay. The fungal isolate *B. cinerea* T3-4 was obtained from the plant fungal disease laboratory of Chungbuk National University, Korea, and used for the antifungal assay. The isolate was cultured on PDA medium at 22°C in the dark. The dual culture method was used to detect antifungal activity against *B. cinerea* on PDA. A 6 mm agar plug of entomopathogenic fungi and *B. cinerea* were placed on PDA 30 mm away from the edge of a 90-mm Petri dish. The plate was incubated at 22°C in the dark for 4 days, and the inhibition zone was measured. To determine the antifungal activities of fungal culture filtrates, the conidial suspensions of *B. cinerea* were diluted with PDB (pH 5.6) to 2×10^4 conidia/mL. Then, 100 µL of the conidial suspension was placed in each well of 96-well plates with 100 µL of fungal culture filtrates at different concentrations (1%, 10%, and 100% diluted with sterilized distilled water) and incubated at 22°C for 48 hr. The absorbance of each well was measured at a wavelength of 595 nm using a microplate reader. For controls, 100 µL of sterilized distilled water instead of fungal culture filtrates were treated with 100 µL of the *B. cinerea* conidial suspensions in PDB. The assay was repeated three times. To determine whether antifungal activities were fungicidal or fungistatic, 50 µL of the conidial suspension (2×10^5 conidia/mL) of *B. cinerea* was inoculated in 1 mL of fungal culture filtrate in 1.5 mL microtubes and cultured at 22°C in the dark. They were collected at different hours (1, 2, 4, 8, 16, 24, 32, and 48 hr), and 100 µL of the solution diluted to 10% was smeared onto PDA plates. The appearance of *B. cinerea* growth was confirmed after 4 days. For controls, 50 µL of the *B. cinerea* conidial suspensions were treated in 1 mL of sterilized distilled water instead of fungal culture filtrates.

Bioassay. Bioassays were used to compare aphid control efficacy among fungal culture filtrates, blastospores and conidia. The conidia suspensions in 0.02% Tween-80 were adjusted to 1×10^8 conidia/mL using a hemocytometer. The blastospore suspensions were also adjusted by the same method. Their mixtures were made by mixing fungal culture

filtrates with pellet which the conidia or blastospores were precipitated, and were also adjusted by the same method. Bioassays were conducted in 60-mm petri dishes containing a thin layer of 1.5% agar, and a 60 mm detached leaf disk of Chinese cabbage was placed on the plate. Twenty green peach aphid adults were placed on the leaf disks using a hair brush pen, and then 1 mL of suspensions containing each fungal culture filtrates, blastospores, conidia and their mixtures were sprayed into each dish by an SD tower sprayer [5, 21]. Aphids were examined daily for mortality, and treatments were maintained at over 90% relative humidity in a chamber at 25 ± 1°C for 7 days. The bioassay was repeated three times.

Statistical analysis. The virulence and antifungal activities were analyzed by SPSS statistics ver. 24 (IBM Corp., Armonk, NY, USA). Data were subjected to one-way analysis of variances (ANOVA) and comparisons between groups were performed with analysis of Tukey's studentized range (honestly significant difference). Data were expressed as means ± standard error (SE) and statistical significance was set at the conventional $\alpha < 0.05$ level.

RESULTS

Antifungal activities of entomopathogenic fungi. Twelve fungal isolates with high virulence to the green peach aphid [5] were investigated for antifungal activity against *B. cinerea* on PDA plates. Differing antifungal activities were observed in each treatment (Fig. 1). Anti-*B. cinerea* activity was verified for most isolates with the exception of *L. attenuatum* SDMp1 and *Purpureocillium lilacinum* SD18 (Table 1). Three levels of anti-*B. cinerea* activity were observed: high activity with greater than 2.0 mm of inhibition zone (4 isolates), middle activity with 1.0 to 1.9 mm of inhibition zone (2 isolates) and low activity with an inhibition zone smaller than 1.0 mm (6 isolates). The antifungal activity to *B. cinerea* was further evaluated using the fungal culture filtrates of 12 isolates in 96-well plates. Seven fungal culture filtrates, *B. bassiana* SD1, SD7, SD8, SD12, SD14, and SD15, and *M. anisopliae* SD3, showed significantly high antifungal activities at 100% concentration (Fig. 2). Four of these fungal culture filtrates, *B. bassiana* SD8, SD12, SD14, and SD15, and *M. anisopliae* SD3, exhibited significant difference for the suppression growth of *B. cinerea* at lower concentrations, indicating that suppression was attributable to the presence of antifungal substances in the fungal culture filtrate (Fig. 2). These results are consistent with those of the dual culture assay, except on *B. bassiana* SD8.

The two fungal isolates, *B. bassiana* SD15 and *M. anisopliae* SD3, which showed the 81.6 and 100% mortality against the green peach aphid respectively [5] and the highest antifungal activities against *B. cinerea* (Table 1, Fig. 2), were selected for further studies. The antifungal activities of the two selected fungal culture filtrates were confirmed as

Table 1. Comparative evaluation of antifungal activities of the indicated isolates

Species/Isolates	Anti-*Botrytis cinerea* (clear zone, mm)[a]	GenBank accession Nos.[b]
Beauveria bassiana		
SD1	2.3 ± 0.4 d	KC551965
SD7	2.5 ± 0.1 d	KC551959
SD8	0.5 ± 0 abc	KC551958
SD9	0.2 ± 0.2 ab	KC551957
SD12	2.4 ± 0.2 d	KC551954
SD14	1.0 ± 0.1 c	KC551952
SD15	5.2 ± 0.3 e	KC551951
Lecanicillium attenuatum		
SDMp1	0 ± 0 a	KC551947
SDMp2	0.6 ± 0.2 abc	KC551946
Metarhizium anisopliae		
SD3	1 ± 0 bc	KC551963
Purpureocillium lilacinum		
SD17	0.2 ± 0.2 ab	KC551949
SD18	0 ± 0 a	KC551948
Control	0 ± 0 a	

Values are presented as mean ± SE.

[a]Values followed by different letters are significantly different (Tukey test, $p < 0.05$).

[b]GenBank database accession numbers are assigned by the internal transcribed spacer sequence of isolates to the National Center for Biotechnology Information (NCBI) GenBank database.

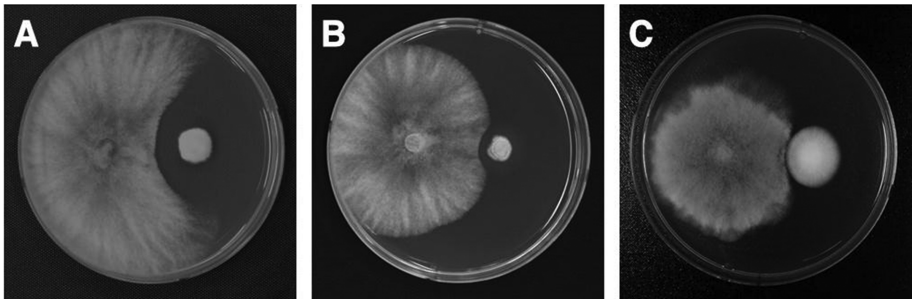

Fig. 1. Representative antifungal activities of entomopathogenic fungi against *Botrytis cinerea* on potato dextrose agar plates. A, High activity of *Beauveria bassiana* SD15; B, Middle activity of *Metarhizium anisopliae* SD3; C, Low activity of *Purpureocillium lilacinum* SD18.

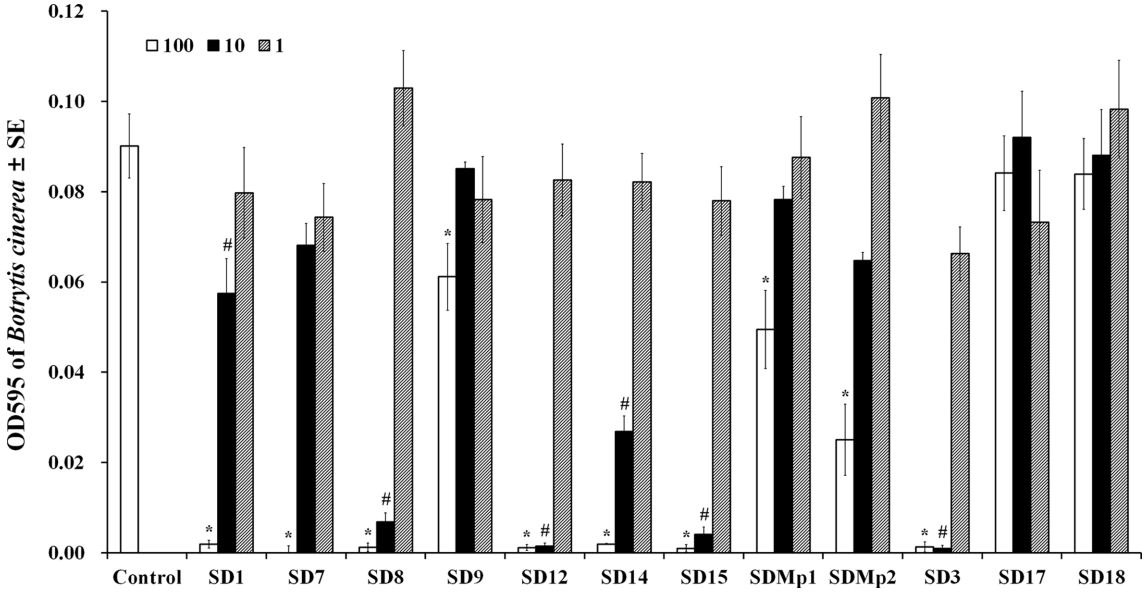

Fig. 2. Antifungal activities of fungal culture filtrates against *Botrytis cinerea* for 48 hr. The absorbance of each sample was measured at 595 nm using a microplate reader. The control is the conidial suspension treated with sterilized distilled water, and treatments are the conidial suspension treated with each *Beauveria bassiana* (SD1, SD7, SD8, SD9, SD12, SD14, and SD15), *Lecanicillium attenuatum* (SDMp1 and SDMp2), *Metarhizium anisopliae* SD3, and *Purpureocillium lilacinum* (SD17 and SD18). The fungal culture filtrate was diluted to 10% and 1% concentration with sterilized distilled water. The asterisk (*) and pound (#) represent significant data at 100% and 10% concentration, respectively (Tukey test, $p < 0.05$). Vertical bars correspond to standard error (SE).

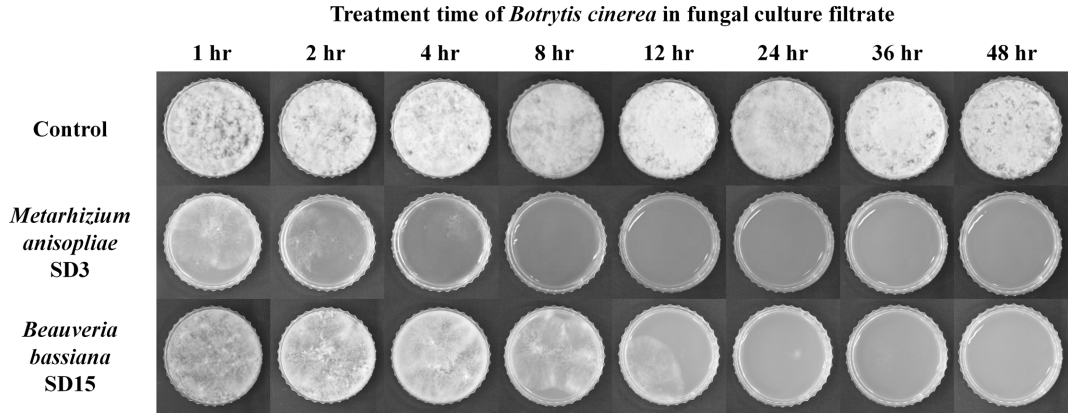

Fig. 3. Fungicidal activities of fungal culture filtrate against *Botrytis cinerea* by treatment time. After treatment of the conidial suspension of *Botrytis cinerea* with fungal culture filtrate, the conidial suspension was collected at different hours and incubated on potato dextrose agar for 4 days. The control is the conidial suspension treated with sterilized distilled water.

fungicidal activity. *M. anisopliae* SD3 had stronger activity than *B. bassiana* SD15 (Fig. 3).

Virulence of culture filtrates, blastospores and aerial conidia. The virulence of *B. bassiana* SD15 and *M. anisopliae* SD3 against green peach aphids was further evaluated for activity specific to their fungal culture filtrates, blastospores and aerial conidia. Treatment with the fungal culture filtrate alone showed comparably high virulence to aerial conidia alone in both fungi (Fig. 4). Blastospore treatment alone showed the lowest virulence in both fungi. The filtrate and aerial conidia combination treatment could not increase the mortality at 7 post-treatment days when compared with the fungal culture filtrate or aerial conidia alone. However, blastospores significantly enhanced the killing of aphids when used with fungal culture filtrate. The synergistic effect of aerial conidia was lower than that of blastospores. *M. anisopliae* SD3 showed higher virulence for all treatments compared to *B. bassiana* SD15. Cadavers from all treatments, except fungal culture filtrate alone, showed visible fungal growth on the body surfaces (data not shown).

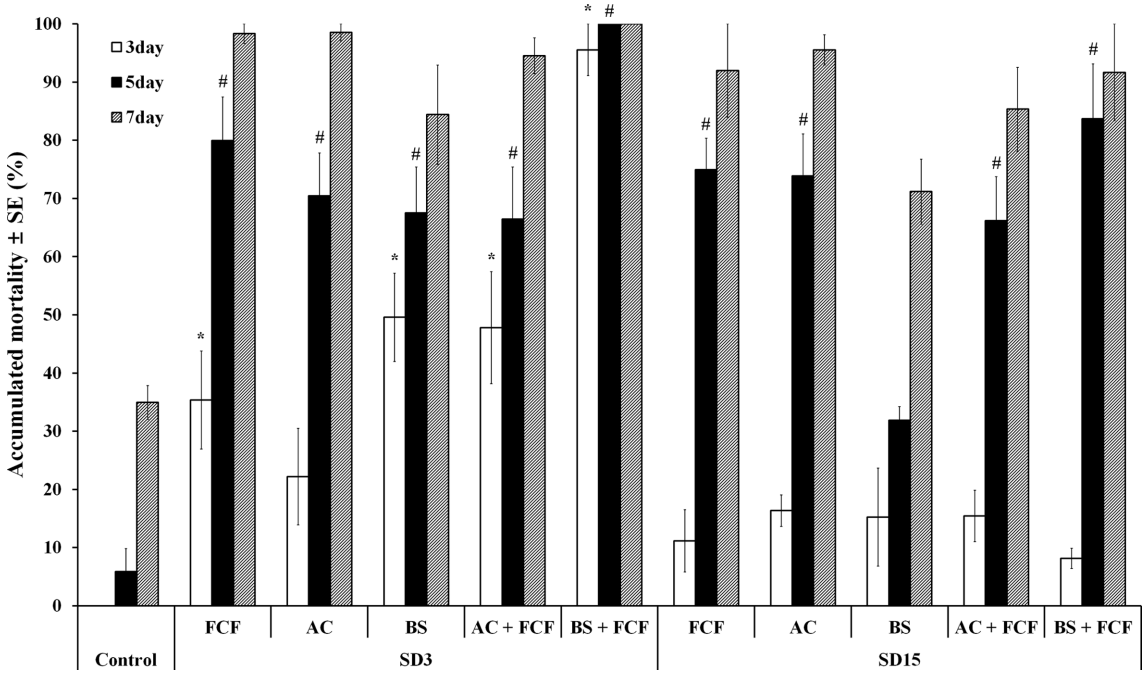

Fig. 4. Control efficacy of green peach aphids by the fungal culture filtrates, blastospores and aerial conidia of *Metarhizium anisopliae* SD3 and *Beauveria bassiana* SD15. Control, 0.02% tween-80; FCF, fungi cultural filtrate; AC, aerial conidia; BS, blastospore. The asterisk (*) and pound (#) represent significant data on 3 and 5 days respectively after treatment (Tukey test, $p < 0.05$). Vertical bars correspond to the standard error.

DISCUSSION

The main goal of this study was to identify entomopathogenic fungal isolates with high antimicrobial activity against certain fungal plant pathogens and high virulence against the green peach aphid. Generally, control of the green peach aphid, a major greenhouse pest, with entomopathogenic fungi has proven to be more difficult than the control of pests in other cropping systems because of their rapid reproduction and development. Many researchers suggest the importance of evaluating environmental factors, as well as virulence, to select an ideal biocontrol agent for these pests [5, 17, 22]. Entomopathogenic fungi for the control of aphids, therefore, should have not only a high virulence but also high stabilities against various environment factors such as temperature variability and UV radiation. The fungal isolates used in this study were selected from our previous study to satisfy these requirements [5]. However, these fungal isolates still have a critical disadvantage in practical application because they require high humidity. Most entomopathogenic fungi used as biocontrol agents generally need humidity conditions near saturation for spore germination. However, high humidity can also encourage the growth of phytopathogens such as powdery mildew and gray mold, so growers tend to avoid high moisture [23]. The recent research trend in entomopathogenic fungi has concentrated on identifying protective agents against plant pathogens and biocontrol agents. Many researchers have suggested that entomopathogenic fungi have the ability to simultaneously control pests and phytopathogens [9, 14, 17, 18, 24, 25]. If our isolates may serve as dual control agents against both pest and plant pathogens, it would overcome the high humidity problem and offer a great alternative for crop protection. Therefore, we evaluated the twelve entomopathogenic fungal isolates identified in our previous study for their antifungal activities against the major phytopathogen, *B. cinerea*. All fungal isolates showed various antifungal activities against *B. cinerea* according to our experiments. *B. bassiana* SD15 and *M. anisopliae* SD3 showed high antifungal activities (Table 1, Figs. 1 and 2), specifically due to fungicidal activity (Fig. 3). As previous studies for antagonistic effect of entomopathgenic fungi, many researchers have reported that *Beauveria* spp., *Isaria* spp., *Lecanicillium* spp. and *Metarhizium* spp. with virulence to pest were antagonistic against plant pathogens, *B. cinerea*, *Fusarium* spp., *Phytophthora megasperma*, *Podosphaera fuliginea*, *Pythium* spp., *Rhizoctonia solani*, *Sphaerotheca fuliginea*, and *Verticillium dahliae* [9, 14, 17-19, 24-26]. However, our study is the first report about the dual activity of entomopathogenic fungi against both green peach aphid and *B. cinerea* as far as we know. Moreover, fungal culture filtrates of these isolates were selected finally to solve a problem by high humidity and may one day be used as a fungicide to protect against plant disease.

Cultural filtrates of entomopathogenic fungi have various effects for pests as insecticidal or feeding deterrence [27]. These filtrates include several enzyme activities, such as chitinase, protease and lipase, and are useful as pesticidal

agents [28]. Additionally, the fungal blastospores are known for germinating more rapidly than conidia, and this could be effective to control insect pests [29, 30]. Although the high virulence of aerial conidia against aphids and the high antifungal activity of cultural filtrates were confirmed from two selected fungal isolates in this study, the virulence of blastospores and cultural filtrates, as well as aerial conidia, was further investigated and enhanced the usefulness of selected fungal isolates as the simultaneous control agents. High virulence was observed on not only cultural filtrates but also blastospores, and their synergistic effect was also shown (Fig. 4). This result suggests that the mixture of blastospores and cultural filtrates from the two fungal isolates, *B. bassiana* SD15 and *M. anisopliae* SD3, would be powerful for the simultaneous control of the green peach aphid and phytopathogen *B. cinerea* without the problem of high humidity. For the practical application of these fungal isolates, further studies of the dual activities in crops and the formulation of a mixture of blastospores and cultural filtrates are needed.

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