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Article

Screening of Entomopathogenic Fungal Culture Metabolites with Honey Bee Nosemosis Inhibitory Activity

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Simple Summary: Nosemosis of honey bees by *Nosema* spp. infection is not only related to colony collapse but also increases the susceptibility of honey bees to various pathogens, so it is necessary to develop effective control methods for nosemosis. In our study, 342 entomopathogenic fungal isolates were used to select the most effective culture metabolite for inhibiting nosemosis. The spore germination inhibitory activity of the culture metabolite against *Nosema ceranae* was evaluated, and 89 fungal isolates with an inhibitory activity of 80% or more were first selected. From these 89 fungal isolates, 44 isolates that had an inhibitory activity of 80% or more at a concentration of 1% of the culture extract were identified and selected. From these 44 isolates, 6 fungal isolates with high fungicidal activity against *Nosema* were selected. The nosemosis inhibitory activity was evaluated using 5 fungal isolates, except for 1 isolate that was negative for the lifespan of honey bees. The activity of inhibiting honey bee nosemosis by the fungal culture extract was shown only in 2 fungal isolates, and they also had the effect of prolonging the lifespan of honey bees. Culture extracts of these fungal isolates are expected to be used as good materials for controlling honey bee nosemosis.

Abstract: This study aimed to select the most effective metabolites for controlling honey bee nosemosis using culture extracts from 342 entomopathogenic fungi of 24 species from 18 genera. The germination inhibitory activity of the fungal culture extract against *Nosema ceranae* spores was evaluated using an *in vitro* germination assay method. Among 89 fungal culture extracts showing germination inhibitory activity of approximately 80% or more, 44 culture extracts that maintained their inhibitory activity even at a concentration of 1% were selected. Finally, the honey bee nosemosis inhibitory activity was evaluated using the cultured extracts of 5 fungal isolates having a *Nosema* inhibitory activity of approximately 60% or more even when the extract was removed after treatment. As a result, the proliferation of *Nosema* spores was reduced by all fungal culture extract treatments. However, only the treatment of the culture extracts from *Paecilomyces marquandii* 364 and *Pochonia bulbillosa* 60 showed a reduction in honey bee mortality due to nosemosis. In particular, the extracts of these two fungal isolates also increased the survival of honey bees.

Keywords: honey bee; nosemosis; *Nosema ceranae*; *Apis mellifera*; entomopathogenic fungi; metabolite

1. Introduction

The honey bee (*Apis mellifera* L. (Hymenoptera: Apidae)) plays an important role in the pollination of flowering plants, which are essential for the production of fruits, nuts and seeds upon which animals, including humans, rely for food [1–4]. In addition to their role in agriculture, honey bees are also important for maintaining biodiversity in natural ecosystems [5]. They help pollinate wildflowers, which provide habitat and food for a variety of other species. Honey bees are also vital to the production of honey, beeswax, and other bee products that humans have used for thousands of years [6,7]. However, unfortunately, honey bee colony collapse has become frequent, with global bee populations rapidly declining in recent years due to habitat loss, pesticide use, climate change and disease [8,9]. This is a major concern for the health of both food systems and natural ecosystems.

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Efforts are therefore underway worldwide to protect and conserve honey bee populations, including promoting habitat restoration, reducing pesticide use, and supporting research on bee health and behavior [9].

Bee colony collapse is a complex phenomenon that can be caused by multiple environmental and human-related factors [8,9]. Habitat loss and fragmentation, climate change, pesticides, mites and other parasites, and diseases and pathogens have been highlighted as major causes of honey bee colony collapse [10–13]. It is worth noting that bee colony collapse is often the result of a combination of these factors rather than a single cause. To address honey bee colony collapse, it is important to take a multipronged approach that addresses both environmental and human-related factors. Of these factors, honey bee disease, known as nosemosis, is known to have the greatest impact on honey bees [10,13–15]. *Nosema* infection can have severe negative effects on honey bee colonies, including reduced foraging activity, decreased colony growth and productivity, increased mortality, increased susceptibility to other stressors, reduced winter survival, decreased queen bee productivity, and reduced immune function [15–20]. Nosemosis is caused by two species of microsporidia, *Nosema apis* and *N. ceranae*. *Nosema ceranae* is more common and more lethal than *N. apis* [15,21–23]. Between the prevention and treatment for nosemosis, preventive methods are mainly relied on. Representative preventive methods include good beekeeping practices such as keeping the hive clean and providing adequate nutrition and moisture to the bees, hygienic behaviors such as removing diseased and dead brood from the hive, and avoiding stressors such as exposure to pesticides and poor nutrition [9,24–26]. In contrast, for direct treatment, an antibiotic called fumagillin is used, but its use is controversial due to its potential negative effects on the environment and other organisms [27–30]. Research efforts to replace it have reported that probiotic treatment, plant essential oils, propolis, plant extracts, and royal jelly, etc., can reduce the number of *Nosema* spores in bees and improve the survival rate of infected bees [31–34].

Entomopathogenic fungi, which are fungi that infect and kill insects, have been widely studied and used as materials for microbial insecticides for pest control [35–38]. Metabolites of these entomopathogenic fungi have various physiological activities, such as insecticidal activity, antibacterial activity, antioxidant activity, immunomodulatory activity, and cytotoxic activity, and potential applications are being sought in various fields, such as agriculture, medicine, and biotechnology [39–42].

In our study, entomopathogenic fungal metabolites with proliferation inhibitory activity against *N. ceranae* were screened from various entomopathogenic fungi for the prevention and treatment of nosemosis, and the effect of improving the survival rate of bees upon *Nosema* infection was evaluated. The purpose of this study was to provide basic data on the possibility of using metabolites of entomopathogenic fungi for the prevention or treatment of nosemosis.

2. Materials and Methods

2.1. Honey bees and N. ceranae

A colony of the honey bee *A. mellifera* was reared with 50% sucrose and bee bread as the main food, and adults that emerged within 24 h were used in the experiment. *Nosema ceranae* spores were provided by the Sericulture and Apiculture Division of the Rural Development Administration, Republic of Korea.

2.2. Entomopathogenic fungal culture extract

In this study, 342 isolates of entomopathogenic fungi from 18 different genera were used (Table S1) [43]. Fungal isolates were initially suspended in 1 mL of Sabouraud dextrose broth containing yeast extract medium (SDYB: 10 g of Bacto peptone, 40 g of dextrose, 10 g of yeast extract in 1000 mL of distilled water, and pH 6.0). Cultures were inoculated with agar blocks (6 mm) of fungi collected from 2-week-old potato dextrose agar (PDA) and grown in the dark at 25 °C with shaking at 150 rpm. After 10 days, the samples were centrifuged at 10,000 x g for 10 minutes. After removing the pellet, the supernatant was filtered using a LaboPass™ Mini Plasmid DNA Purification Kit column (Cosmo

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Genetech Co. Ltd., Korea) to remove spores and mycelia. Ethyl acetate fractionation was used to separate hydrophobic substances from fungal culture filtrates. After adding the same amount of ethyl acetate as the culture medium, vortexing for 20 min and centrifugation at 4,000 rpm for 5 min, the supernatant was collected. After that, ethyl acetate was evaporated using gaseous nitrogen, and the remaining extract was dissolved in 2% acetone in the same volume as the culture filtrate and used in the next experiment. The prepared culture filtrate extract was stored at -76 °C until use. To prepare a culture filtrate extract after quantitative inoculation of entomopathogenic fungi, fungal conidia that were harvested after being cultured in PDA medium for 2 weeks were used to prepare a conidial suspension using a 0.02% Tween-80 solution. The conidia were then counted using a hemocytometer. The conidial suspension was inoculated in 30 mL PDB medium in 50 µL at a concentration of 9×10^5 conidia/mL and cultured for 10 days. After culturing, spores and mycelia were removed from the culture medium, and culture filtrate extracts were prepared using the ethyl acetate fraction in the same methods as described above.

2.3. Purification of Nosema spores

To produce infective spores, honey bees were placed in plastic cages and inoculated with 1×10^6 spores of *N. ceranae* in sucrose solution (50% v/v in water). To obtain purified *Nosema* spores, after 10 days the midgut tissues from heavily infected honey bees were individually separated using forceps and washed with phosphate buffered saline [44]. The isolated midgut was ground in 200 µL of sterile distilled water in a Bullet Blender® Homogenizer (Scientific Instrument Services Inc., Ringoes, USA) set to speed 8 with 2 mm diameter tungsten carbide beads (Sigma–Aldrich, St. Louis, USA) for 2 min. The homogenate increased the volume up to 1 mL, and the mixture was filtered through Qualitative No. 2 filter paper (Advantec MFS Inc., Dublin, USA) with an 8-11 µm pore size to remove tissue debris [45]. The filtered suspension was overlaid very gently on discontinuous 25%, 50%, 75% and 90% Percoll® (Sigma–Aldrich, St. Louis, USA) gradient and centrifuged twice at 15,000 × g for 30 min at 20 °C [46]. A small but dense band just above the bottom of the tube was collected and resuspended in sterile distilled water. After a final centrifugation at 15,000 × g for 10 min at 20 °C, the spore pellet was resuspended in sterile distilled water and stored at room temperature until use. The spore concentration was measured by counting with a hemocytometer [47], and the spore suspension was freshly prepared before use.

2.4. In vitro germination assay

Aliquots of purified *Nosema* spores (10 µL; 1×10^3 spores) were spotted onto glass slide reaction cells (12 wells; Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and air-dried for 2 h at room temperature. Germination was triggered by adding 1.5 µL of 0.1 M sucrose in distilled water to the air-dried spores, a procedure that mimics natural conditions for the germination of environmental spores [48]. After maintaining the covered glass slide at room temperature for 6 h, the germinated spores were observed under a light microscope (magnification, 400 x) (Nikon Instech Co., Ltd., Tokyo, Japan). The germination rate was calculated as the percentage of total observed spores that had germinated.

2.5. Safety test of fungal culture extract

Twenty honey bees that emerged within 24 hours were transferred to one cage and allowed to adapt for 3 days before being used in the experiment. To evaluate the safety of the fungal culture extract, 50% sucrose mixed with culture extract was fed once to the honey bees, and for comparison, 50% sucrose containing the same concentration of acetone as the treated solvent was fed once. The survival rate of honey bees was observed and recorded every day for 14 days after treatment, and the experiment was repeated three times for each treatment group.

2.6. Nosema inoculation and culture extract feeding assay

To evaluate the effect of fungal culture extract on *Nosema* infection in honey bees, 25 honey bees that emerged within 24 h were used for each experimental group. Honey bees were orally infected with purified *Nosema* spores by making a 50% sucrose suspension at 1 × 10^6 spores/mL and administering 1 mL to each experimental group. Treatment of fungal culture filtrate extract was carried out before and after *Nosema* inoculation. For the control group, honey bees were infected with *Nosema* and no treatment of culture extract was used. To evaluate the inhibitory effect of *Nosema* infection, the survival rate of honey bees was observed and recorded every day for 14 days. The proliferation of *Nosema* spores was examined by isolating and counting spores from the midgut of honey bees on the 14th day after infection to evaluate the effect of inhibition of *Nosema* growth. The experiment was repeated three times for each treatment group.

2.7. Statistical analysis

Spore germination and honey bee survival rate results were analyzed with SPSS statistical software v12.0 (SPSS, Inc., Chicago, IL, USA). Data were subjected to one-way analysis of variance (ANOVA), and comparisons among groups were performed with the SNK test. Data are expressed as the means ± standard errors (SEs), and statistical significance was set at the conventional $\alpha < 0.05$ level.

3. Results

3.1. Inhibitory activity of entomopathogenic fungal culture extracts on Nosema germination

To evaluate the inhibitory activity of the entomopathogenic fungal culture extract on *Nosema* spore germination, the effect of acetone, a solvent used in preparing the culture extract, on *Nosema* spore germination was first evaluated. After treating *Nosema* spores with various concentrations of acetone from 0.25% to 20%, no significant effect of acetone on the germination of *Nosema* spores was observed at all concentrations (Figure S1). Therefore, it was confirmed that the 0.2% concentration of acetone used in this experiment had no effect on the germination of *Nosema* spores. All fungal isolates used in the experiment were mostly only expressed with numbers for convenience, and the identification information for the fungal isolates is shown in Table S1. As a result of evaluating the germination inhibitory activity of the culture extracts of 342 fungal isolates on *Nosema* spores, the germination of spores was inhibited in a variety of ways from 0% to a maximum of 96.5% (Table S2). Most of the spores did not germinate when the germination inhibitory activity was greater than approximately 80%, some spores germinated when the germination inhibitory activity was greater than 60%, and many spores germinated when the activity was less than 60% (Figure 1). Among the 342 fungal isolates, culture extracts from 89 isolates showed spore germination inhibitory activity of more than 80%, and 20 isolates showed activity of more than 90% (Table S2). By classification of entomopathogenic fungi, approximately 80% or more of the germination inhibitory activity against *Nosema* was observed in 15 species of 10 genera among 24 species from 18 genera (Table 1). Fifty of 126 isolates of *Beauveria* spp., 18 of 81 isolates of *Metarhizium* spp., 11 of 40 isolates of *Cordyceps* spp., 3 of 17 isolates of *Pochonia* spp. and 2 of 12 isolates of *Paecilomyces* spp. showed spore germination activity against *Nosema*. As various activities were confirmed in various fungi, culture extracts of 89 isolates showing spore germination inhibitory activity of 80% or more were prepared again by quantitative inoculation of fungi, and the germination inhibitory activity of *Nosema* spores was re-evaluated.

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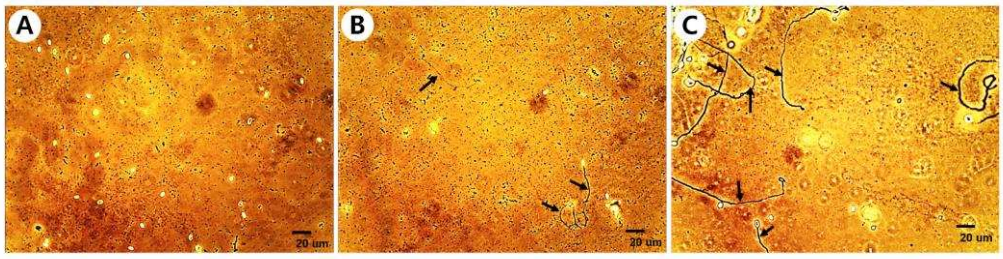

Figure 1. Spore germination inhibitory activity of fungal culture extract against *Nosema ceranae*. A, more than 80% inhibition; B, more than 60% inhibition; C, less than 60% inhibition. The scale bar is 20 μm. Arrows indicate mycelia that have grown after germination.

Table 1. Entomopathogenic fungi showing more than 80% *Nosema ceranae* germination inhibition activity.

Fungus	No. of tested isolates	No. of isolates showing the inhibition of spore germination
All fungal isolates	342	89 (26%)*
Acremonium strictum	1	0 (0%)
Aspergillus lentulus.	5	0 (0%)
Aspergillus versicolor	3	1 (33.3%)
Beauveria bassiana	110	48 (43.6%)
Beauveria brongniartii	8	1 (12.5%)
Beauveria pseudobassiana	8	1 (12.5%)
Bionectria ochroleuca	7	0 (0%)
Clonostachys rosea	1	0 (0%)
Cordyceps farinosa	12	5 (41.7%)
Cordyceps fumosorosea	6	1 (16.7%)
Cordyceps javanica	22	5 (22.7%)
Fusarium oxysporum	2	0 (0%)
Lecanicillium spp.	8	1 (12.5%)
Metarhizium anisopliae	64	15 (23.4%)
Metarhizium lepidiotae	1	0 (0%)
Metarhizium pemphigus	16	3 (18.8%)
Mucoromycotina spp.	1	0 (0%)
Myrothecium spp.	5	0 (0%)
Paecilomyces lilacinus	7	1 (14.3%)
Paecilomyces marquandii	5	1 (20%)
Paraconiothyrium sporulosum	2	1 (50%)
Phialocephala spp.	1	0 (0%)
Pochonia bulbillosa	16	3 (18.8%)
Pochonia rubescens	1	0 (0%)
Simplicillium aogashimaense	1	1 (100%)
Simplicillium sp.	2	0 (0%)
Tolypocladium album	23	1 (4.3%)
Tolypocladium cylindrosporum	3	0 (0%)

Verticillium insectorum	1	0 (0%)

* Relative % to number of isolates (species or genus).

3.2. Nosema spore germination inhibitory activity according to the concentration of culture extract

For 89 selected entomopathogenic fungi, culture extracts were prepared by inoculation with the same conidia concentration, and then the extracts were diluted in distilled water at concentrations of 100, 10, and 1% to evaluate the germination inhibitory activity on *Nosema* spores (Figure 2). As a result, 44 of the 89 isolates showed a high spore germination inhibitory activity of 80% or more even at a diluted concentration of 1% as well as the extract stock solution. In particular, the decrease in activity according to the concentration of the extract of these isolates did not exceed 20% of the activity difference between the original solution and the diluted concentration of 1%. The activity of the remaining 45 isolates decreased significantly depending on the concentration of the extract, showing activity from approximately 50% to 5% at 1% extract concentration. Twenty-five isolates of *Beauveria* spp., 6 isolates of *Met. anisopliae*, 6 isolates of *Cordyceps* spp., 2 isolates of *Paecilomyces* spp., 2 isolates of *Pochonia* spp., and 3 other fungal isolates showed high activity similar to the stock solution even in the 100-fold diluted extract (Table 2). The following experiment was conducted using these 44 fungal isolates showing high spore germination inhibitory activity even at 100-fold dilution concentrations.

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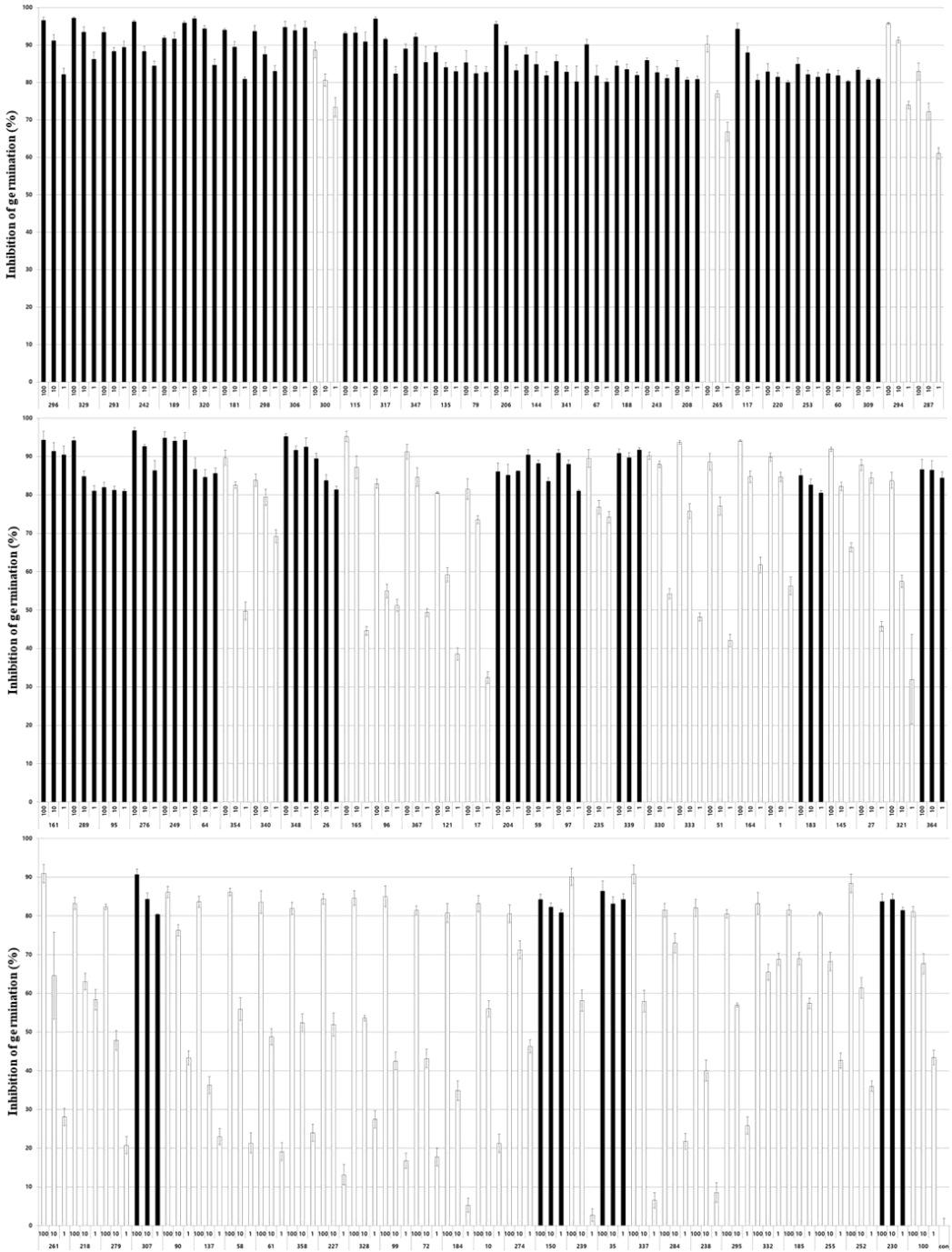

Figure 2. Inhibition rate of *Nosema ceranae* spore germination in response to 89 entomopathogenic fungal culture extracts. Each extract was used to treat *Nosema ceranae* spores at concentrations of 100%, 10% and 1%, and then the germination rate was examined. Fungal isolates exhibiting an inhibition rate of 80% or more are indicated by black bars. Data show the mean ± SE.

Table 2. Entomopathogenic fungi showing the inhibition of *Nosema ceranae* spore germination by more than 80% in 1% concentration culture extract.

Fungus	No. of isolate (%)
Aspergillus versicolor	1 (2.3) *
Beauveria bassiana	24 (54.5)
Beauveria brongniartii	1 (2.3)
Cordyceps farinosa	4 (9.1)
Cordyceps fumosorosea	1 (2.3)
Cordyceps javanica	1 (2.3)

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Lecanicillium spp.	1 (2.3)
Metarhizium anisopliae	6 (13.6)
Paecilomyces lilacinus	1 (2.3)
Paecilomyces marquandii	1 (2.3)
Pochonia bulbillosa	2 (4.5)
Tolypocladium album	1 (2.3)

* Relative % to number of isolates (species or genus).

3.3. Mechanism of spore germination inhibitory activity of culture extracts

To investigate the active mechanism of inhibition of *Nosema* spore germination by 44 fungal culture extracts, the extracts of each fungal isolate were treated on *Nosema* spores for 2 h, and after removal of the extracts, spore germination was observed. As a result, the isolate showing a germination inhibition rate of approximately 60% or more even after removal of the culture extract were *M. anisopliae* 296, *Pae. marquandii* 364, *Poc. bulbillosa* 60, *Bea. brongniartii* 183, and *Bea. bassiana* 35, 161, and 59 (Figure 3). Among the remaining isolates, 5 isolates showed a germination inhibition rate of approximately 30% or more even after removing the extract, and the extracts of the other isolates showed no anti-germination activity or very low inhibition activity of approximately 5% or 20%. If the fungal culture extract showed high spore germination inhibitory activity even after removal, it was assumed that *Nosema* spores were inactivated by the culture extract, and the *Nosema* spore germination inhibitory activity of the culture extract was determined to be fungicidal activity. The inhibitory effect of *Nosema* infection on honey bees was continuously evaluated using the culture extracts of 6 fungal isolates, except for *Bea. bassiana* 161, which showed the lowest fungicidal activity.

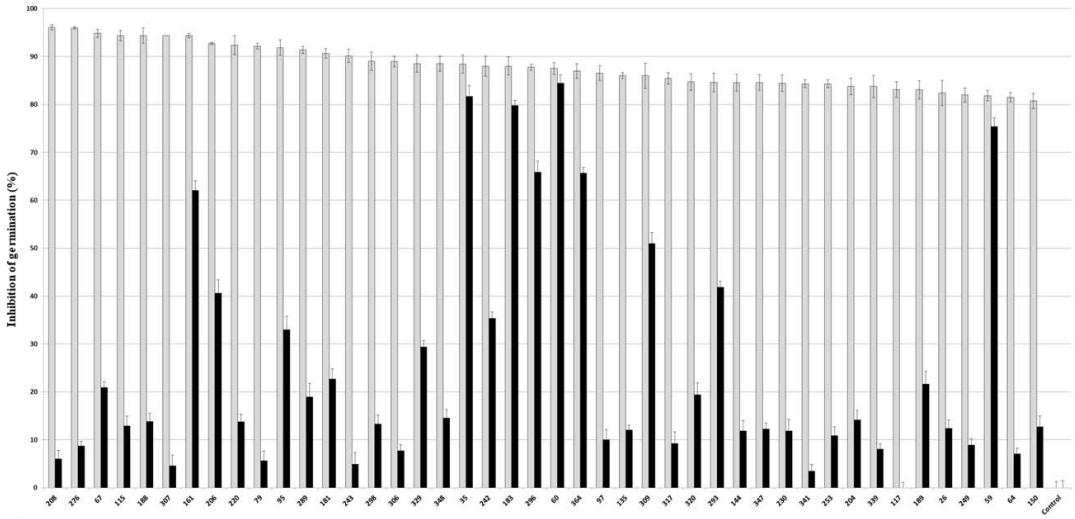

Figure 3. Evaluation of the fungistatic and fungicidal activities of entomopathogenic fungal culture extracts against *Nosema ceranae* spores. After treatment with the fungal culture extract on *Nosema ceranae* spores, germination was observed without removing the extract (white bar) or after removing the extract (black bar). Data show the mean ± SE.

3.4. Effects of culture extracts on honey bees

Prior to the evaluation of the inhibitory activity of the culture extract against *Nosema* infection in honey bees, the effect of the extracts on the survival of honey bees was evaluated. Acetone, used as a solvent for the culture extract, showed a similar survival rate for the untreated group up to 2% concentration, but at a concentration of 4%, the survival rate decreased from 4 days after treatment, and the final survival rate showed a difference of approximately 5% from the untreated group (Figure S2). In the *Nosema* infection experiment of the culture extract for honey bees, the extract that was diluted more than 10 times was used, so the actual concentration of acetone did not exceed 0.2%, confirming that the possibility of acetone affecting the lifespan of honey bees was very low. The

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effects of culture extracts on 6 fung6al isolates in honey bees were evaluated by dividing the concentrations of the extracts into 1% and 10%. As a result of treatment with 1% diluted extract, 5 fungal isolates except *Bea. brongniartii* 183 had no significant effect on the lifespan of honey bees (Figure 4A). Even at 10% dilution, only *Bea. brongniartii* 183 partially reduced the survival rate of honey bees, so it was excluded from further experiments (Figure 4B).

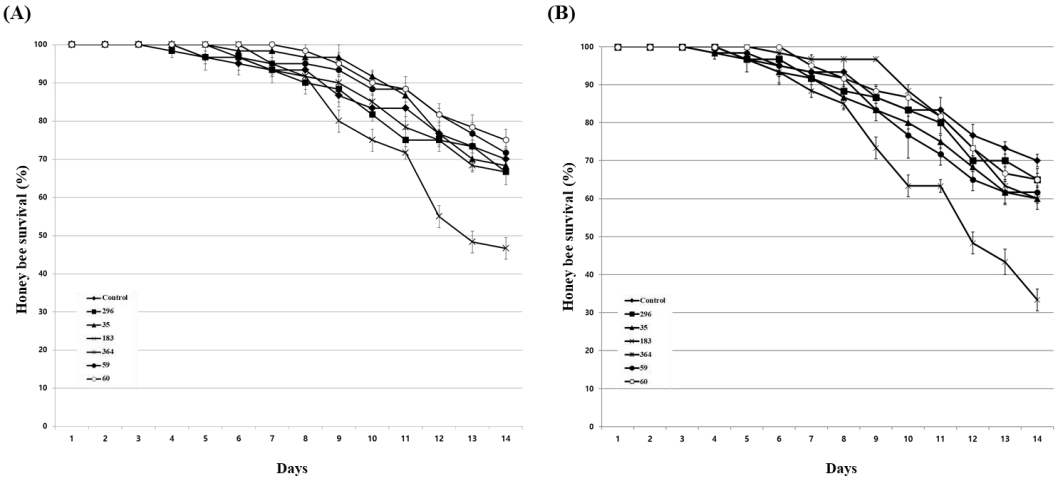

Figure 4. Honey bee survival under 1% (A) and 10% (B) fungal culture extract treatments. A mixture of fungal culture extract and 50% sucrose solution was fed to honey bees. After that, the survival rate of honey bees was determined for 14 days. The control group was fed only a 50% sucrose solution. Data show the mean ± SE.

3.5. Inhibitory effect of culture extract on honey bee nosemosis

To evaluate the effect of inhibiting honey bee nosemosis by fungal culture extracts, honey bees were treated with each extract at a concentration of 5%, and the survival rate of honey bees and *Nosema* spore production were evaluated. The effect of the culture extract was evaluated in two ways: by treating the extract before *Nosema* infection of honey bees and by treating the extract after *Nosema* infection. After treating each culture extract before infection with *Nosema*, *Pae. marquandii* 364 culture extract only improved the survival rate of honey bees by approximately 13% compared to *Nosema*-infected honey bees (Figure 5A). Other fungal culture extracts showed similar or lower survival rates of honey bees compared to *Nosema*-infected honey bees. Treatment of culture extracts of *M. anisopliae* 296, *Pae. marquandii* 364, *Bea. bassiana* 59 and *Poc. bulbillosa* 60 reduced spore production by approximately 65%, 32%, 80% and 33%, respectively, compared to that of honey bees infected with Nosema alone (Figure 6A).

When honey bees were infected with *Nosema* and then treated with culture extracts, the extracts of *Poc. bulbillosa* 60 and *Pae. marquandii* 364 showed approximately 11% and 8% increases in honey bee survival rates, respectively, while the survival rates of the others decreased slightly (Figure 5B). *Nosema* spore production was reduced compared to *Nosema*-infected honey bees in all fungal culture extract treatments (Figure 6B). These results suggested that the culture extracts of *Pae. marquandii* 364 and *Poc. bulbillosa* 60 were effective in inhibiting honey bee nosemosis.

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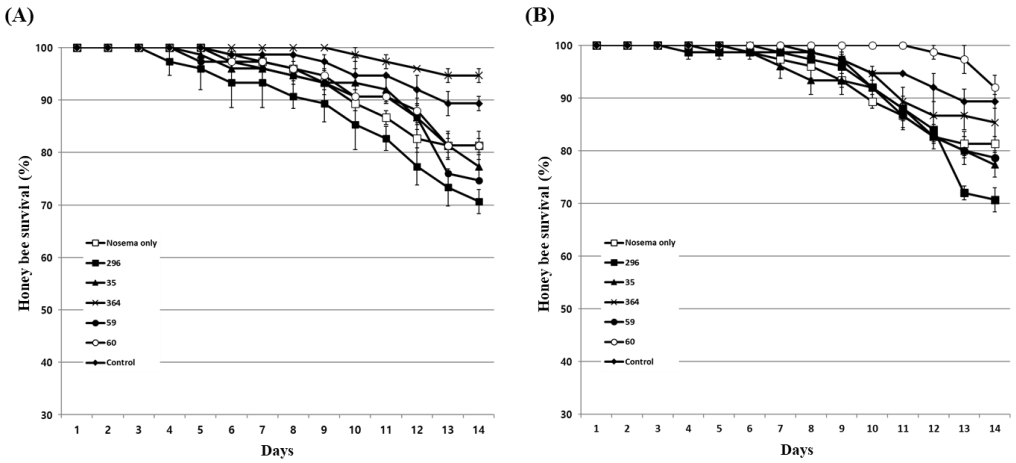

Figure 5. Honey bee survival against *Nosema ceranae* infection after treatment with 5% fungal culture extract before (A) or after (B) infection. Nosema only is a control inoculated with a mixture of 50% sucrose solution and spores to honey bees. The control group was fed only a 50% sucrose solution. Data show the mean ± SE.

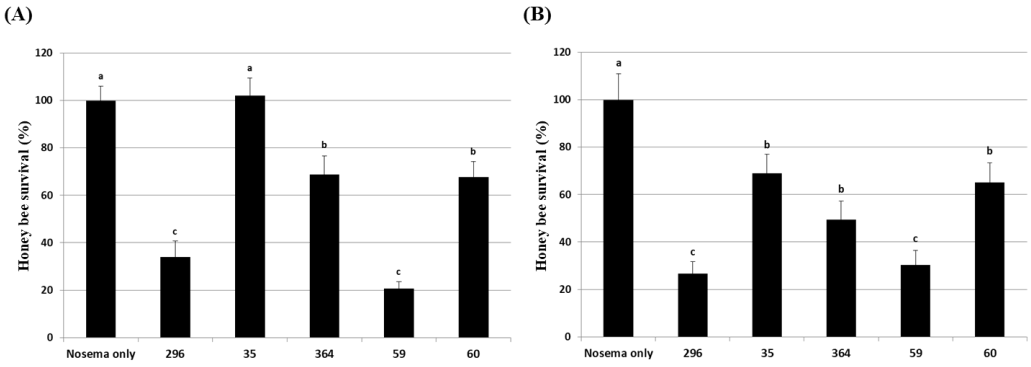

Figure 6. Spore proliferation in Nosema-infected honey bees treated with 5% fungal culture extract before (A) or after (B) *Nosema ceranae* infection. Data show the mean ± SE. Values with different letters are significantly different ($p < 0.05$, SNK test in one-way ANOVA).

4. Discussion

This study was conducted to screen and select metabolites of entomopathogenic fungal cultures that are effective for the control of honey bee nosemosis. Among the 342 entomopathogenic fungal isolates of 24 species from 18 genera tested, the germination inhibitory effect of *Nosema* was shown in the culture extracts of more than 95% of the isolates (Tables 1 and S2). These results suggested that the germination inhibitors of *Nosema* were contained in the fungal culture extract and were consistent with previous studies showing that entomopathogenic fungal metabolites had various biological activities. It has been reported that entomopathogenic fungal metabolites have antimicrobial activity against various bacteria and fungi, so such antifungal activity could be sufficiently expected for fungi such as *Nosema* in our study [39–42]. To the best of our knowledge, this is the first report that various diverse types of fungal metabolites have antifungal activity against *Nosema*.

To determine the most effective culture extract among fungal isolates with antifungal activity against *Nosema*, 6 isolates showing high anti-germination effects at a 100-fold diluted concentration and even when removed after treatment with the extract were selected and used for the evaluation of honey bee nosemosis inhibitory activity (Figures 2 and 3). In our study, the inhibitory activity of the culture extract against *Nosema* spores was evaluated by dividing the fungistatic activity, which is inhibited only when the extract is present, and the fungicidal activity, which inactivates the spores by the extract. It was judged that the fungicidal active culture extract was more effective in controlling nosemosis, and such isolates were selected. In addition, the nosemosis inhibitory activity of the

culture extract showed a preventive effect by the treatment before infection with *Nosema* in one fungal isolate and a therapeutic effect after infection in two fungal isolates (Figure 5). However, in the experimental groups treated with the culture extract, lower honey bee survival rates were observed compared to the control group in which the culture extract was not treated, and these results were presumed to be due to the toxicity of the culture extract. Although the honey bee survival rate was not significantly reduced compared to that in the control under treatment with 10% of each culture extract, it is presumed that the low toxicity of the culture extract increased the susceptibility of honey bees to *Nosema*, thereby lowering the survival rate. However, it was confirmed that the production of *Nosema* spores was reduced by treatment with the culture extract, and it was confirmed that all the culture extracts could inhibit the growth of *Nosema* (Figure 6). As a particularly noteworthy result, treatment with the culture extracts of *Poc. bulbillosa* 60 and *Pae. marquandii* 364 showed a higher honey bee survival rate than uninfected honey bees, confirming that these culture extracts not only inhibit nosemosis but also increase the lifespan of honey bees. However, it has been shown that these effects may vary depending on the treatment concentration of these culture extracts. When only the culture extract was treated at a 10% concentration, the survival rate of honey bees was not higher than that of the control group, but at a 1% concentration, the culture extracts of *Poc. bulbillosa* 60 and *Bea. bassiana* 59 partially increased the survival rate of honey bees (Figure 4). To date, there has been no report on substances showing positive activity on the lifespan of bees among entomopathogenic fungal metabolites. In our results, further research is needed to determine whether the metabolite that shows *Nosema* inhibitory activity and the metabolite that increases the lifespan of honey bees are the same.

Various entomopathogenic fungal metabolites have been reported, and the most representative substance is beauvericin from *Bea. bassiana* and destruxins from *Metarhizium* spp., which are the most actively studied and utilized [40,49–52]. These substances have both antifungal activity and insecticidal activity. Other substances known to have antifungal activity include muscodorin, oosporein, patulin, enniatins, pradimicin, flavoglaucin, and terpenoids [40,51,53–55]. However, among these substances, inhibitory activity against *Nosema* has not been reported. Furthermore, since various fungal metabolites show various antifungal activities, the possibility that anti-*Nosema* active substances exist among fungal metabolites is considered sufficient. In a previous report on *Poc. bulbillosa* and *Pae. marquandii*, which showed the highest *Nosema* inhibitory activity in our study, there was no result for *Nosema* inhibitory activity. However, the antimicrobial activity, including antifungal activity and insecticidal activity, of the metabolites in *Poc. chlamydosporia*, *Pae. variotii*, and *Pae. lilacinus* have been reported [56–58]. Therefore, it is considered sufficient that the 2 fungal isolates in our study also have antifungal activity against *Nosema*. Through further research on the metabolites of *Poc. bulbillosa* 60 and *Pae. marquandii* 364, it may be possible to develop an effective control agent for honey bee nosemosis.

5. Conclusions

Metabolites of 342 entomopathogenic fungal isolates of 24 species from 18 genera were used to evaluate the germination inhibitory activity of *Nosema* to search for fungal metabolites with effective inhibitory activity against *Nosema*. As a result, inhibitory activity was observed in all cultured metabolites except for 2 genera and 1 species of fungus, and high inhibitory activity of approximately 80% or more was shown in 15 species and 10 genera. Among them, the culture extracts of *Poc. bulbillosa* 60 and *Pae. marquandii* 364 had fungicidal activity at low concentrations and effectively inhibited honey bee nosemosis.

Supplementary Materials: Table S1. The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Entomopathogenic fungal isolates used in the experiment. **Table S2.** Germination inhibition activity of entomopathogenic fungal culture extracts against *Nosema ceranae* spores. **Figure S1.** Germination of *Nosema ceranae* spores by acetone solvent treatment. After treating the spores with acetone at each concentration, the germination rate was determined by an *in vitro* germination assay. **Figure S2.** Honey bee survival by acetone treatment at different concentrations. A mixture of acetone and 50% sucrose

solution was fed to honey bees. After that, the survival rate of honey bees was determined for 14 days. The control group was fed only a 50% sucrose solution. Data show the mean ± SE.

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