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Inhibition of *Metarhizium anisopliae* infection of *Protaetia brevitarsis seluensis* larvae using several effective microorganisms

Kyu-Won Kwak[1, 2], Soon Woo Kwon[1], Sung-Hee Nam[1], Kwan-Ho Park[1], Eun-Sun Kim[1], Hee-Sam Lee[1], Ji-Young Choi[1*], and Myung-Sae Han[2*]

[1]*National Institute of Agricultural Science, Department of Agricultural Biology, 55365, Wanju, Republic of Korea*
[2]*Kyungpook National University, Department of Bio-fibers and Materials Science, 41566, Daegu, Republic of Korea*

Abstract

The purpose of this study was to determine the best method for minimizing the occurrence of *Metarhizium anisopliae* infection of *Protaetia brevitarsis seluensis* during mass breeding on agricultural farms. There is a high demand for the use of *P. b. seluensis* larvae in animal feed and as food for humans. However, mass breeding results in the entomopathogenic fungal (usually *M. anisopliae*) infection of *P. b. seluensis*. A mixture of microorganisms (*Bacillus subtilis*, *Lactobacillus plantarum*, and *Saccharomyces cerevisiae*) delayed fungal infection by *M. anisopliae*, which infected fewer *P. b. seluensis* when the microorganism mixture was added to sawdust as feed for *P. b. seluensis*. When sawdust with the effective microorganisms (EM) was given to *P. b. seluensis* for 30 d, their mortality rate was approximately 35 % less than that of the control group, which was fed sawdust without the EM. In addition, the growth of *M. anisopliae* on agar media spread with each bacterium as inhibited by up to 80 % more than those spread with 4 % sodium hypochlorite, which is a harmless fungal inhibitor generally used in agricultural farms for disinfection.

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Introduction

According to a report by the Food and Agriculture Organization of the United Nations, because of environmental variation related to climate change, there is an increasing demand for insects as an alternative protein source for human consumption, or feed for poultry, fish, and reptiles (Van Huis *et al*., 2013). The nutritional value of insects and the use of *Protaetia brevitarsis seluensis* (family Cetoniinae) as a functional food in traditional medicine have been proven (Kang *et al*., 2011). Knowledge of insect pathology is required to prevent diseases in the beetle *P. b. seluensis*, whose larvae are reported to have anti-inflammatory and anti-cancer effects (Kang *et al*., 2011; Yoo *et al*., 2007). In South Korea, an increasing demand for insects as feed, food, or functional substances has resulted in an increase in insect-growing farms. However, because of

***Corresponding author.**
Ji-Young Choi
Department of Agricultural Science, National Institute of Agricultual Science, Wanju 55365, Republic of Korea
Tel: +82-63-238-2992 / FAX: +82-53-950-6744
E-mail: choijy7@korea.kr
***Co-Corresponding author.**
Myung-Sae Han
Department of Bio-fiber and Materials Science, Kyungpook National Universtiy, Daegu 41566, Republic of Korea
Tel: +82-53-950-5738 / FAX: +82-53-950-6744
E-mail: mshan@knu.ac.kr

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increased breeding of *P. b. seluensis* larvae, the damage caused by insect pathogens has become problematic.

The fungal pathogen *Metarhizium anisopliae* infects over 200 species of insects (Pendland and Boucias, 1998). It infects insect larvae orally and transdermally, proliferating throughout the body, causing it to harden before extending its spores through the epidermis and covering the larvae with dark green spores (Chernaki-Leffer *et al*., 2007). According to an investigation on the diseases of *P. b. seluensis* larvae in insect farms, conducted from 2013 to 2015, *M. anisopliae* causes over 80 % of the fungal infections and damage to *P. b. seluensis* larvae (Kwak *et al*., 2015).

Lactobacillus plantarum (Lactobacillales: Lactobacillaceae) is a lactic acid bacterium that has antifungal properties, inhibits the growth of fungal molds, reduces mycotoxin production, and aids in food preservation. Previous studies have found that antifungal metabolites, such as organic acids, reuterin, hydrogen peroxide, and hydroxylated fatty acids, secreted by lactic acid bacteria, reduce toxin levels by binding fungal toxins, such as aflatoxin and mycotoxin (Blagojev *et al*., 2012). *L. plantarum* produces antifungal substances, such as lactic acid, phenyllactic acid, and two cyclic dipeptides cyclo (L-Leu-L-Pro) and cyclo (L-Phe-L-Pro), and inhibits growth in the genus *Fusarium* (Dal Bello *et al*., 2007). *Bacillus subtilis* (Bacillales: Bacillaceae) is a soil bacterium that produces various types of antibiotics, including a range of antifungal and antibacterial peptides, such as bacteriocins and lipopeptides (Teixeira *et al*., 2013). Of these lipopeptides, surfactin (Kluge *et al*., 1988) and mycosubtilin (Peypoux *et al.*, 1986) demonstrate antifungal activity (Majumdar and Bose, 1958). *Saccharomyces cerevisiae* (Saccharomycetales: Saccharomycetaceae) is also known to adsorb mycotoxins. The cell walls of this yeast are composed of mannan-oligosaccharides from fermentation, which bind more zearalenone and fumonisin B1 than deoxynivalenol, and can bind mycotoxins within 5 min (Shetty and Jespersen, 2006). Moreover, their antifungal activities come from potassium ion transport (Tebbets *et al*., 2013).

In this study, to prevent *M. anisopliae* infection in *P. b. seluensis* larvae, the antifungal effects of an effective microorganisms (EM) liquid mixture with three beneficial microorganisms, *B. subtilis*, *L. plantarum,* and *S. cerevisiae*, was tested. We confirmed the presence of carboxylic acids in the EM composition, using HPLC (High Performance Liquid Chromatography) and found 3 mg/mL acetic acid, 0.9 mg/mL lactic acid, and less than 0.001 mg/mL glycolic acid. In this study, to examine the antifungal activity of lactic acid produced from *L. plantarum*, the most effective inhibitor of *M. anisopliae* in the EM, an *in vivo* assay was performed to determine the concentration of lactic acid in *P. b. seluensis* and they well maintained and fungal infection was prevented.

Material and Methods

Fermented sawdust for *P. b. seluensis* larvae

To produce the fermented sawdust, 60 kg of oak sawdust (0.5–0.7 × 0.5–0.7 cm) was mixed with 1 mL of starter yeast, 375 g of sugar, 187 mL of calcium hydroxide, and 1.5 kg each of rice bran and soybeans. After spraying with water and mixing thoroughly, the sawdust was fermented for 30 d until gas was no longer produced. The fermented sawdust was purchased from the Smurf Bugs farm in Namyang-ju, Gyeonggi-do, Korea.

Effective microorganisms

B. subtilis, *L. plantarum*, and *S. cerevisiae* were cultured in R2A (Reasoner's 2A), MRS (de Man, Rogosa and Sharpe), and YEPD (Yeast Extract Peptone Dextrose) broth media, respectively. After sterilizing 4 L of each liquid medium, each microbe was inoculated in their respective liquid medium. Afterwards, 0.5 t of distilled water, 10 L of molasses extract, and 4 L of each microbial culture were placed in a 0.5-t incubator, and mixed culturing was performed at 31–32 °C for 72 h. The ratio of *B. subtilis*: *L. plantarum* : *S. cerevisiae* was 1:2:1. The EM incubator and microbes were provided by the Wanju-gun Agricultural Technology Center.

Breeding of *P. b. seluensis*

P. b. seluensis larvae were obtained from Jeju Island and grown at 22.5 ± 2.5 °C, 60 ± 5 % humidity, with a photoperiod of 16: 8 (light: dark), and raised in 400 × 270 × 230-mm transparent plastic rearing boxes. For the injection experiments, larvae were grown at 28.5 °C ± 1.5, 60 ± 5 % humidity, with a photoperiod of 16:8 (light: dark), and raised in 10-cm diameter petri dishes.

Isolation and identification of *M. anisopliae*

P. b. seluensis were collected from farms with diseased

Fig. 1. *Metarhizium anisopliae* infection of *Protaetia brevitarsis sleuensis at a farm in Korea.*
P. b. seluensis that died from *M. anisopliae* infection at *P. b. seulensis* farms in Wanju and Cheongju, Korea, show dark green fungi covering their whole bodies.

insects in Cheongju, Wanju, and Jeju. Single spores on the insects were isolated and cultured on a Sabouraud dextrose agar plate at 24°C and 60 % humidity for 15 d. The symptoms showed large black spots on the epidermis of *P. b.* eight days after *M. anisopliae* infection, the dead larvae are almost hardened, and dense green conidia are formed in the epidermis (Fig. 1).

To identify the cultured fungi, genomic DNA was purified using a power soil DNA preparation kit (MO BIO, USA) and a polymerase chain reaction was performed on the prepared template DNA using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC), or ITS1(TCCGTAGG TGAACCTGCGG) and LR3 (GTCTTGAAACACGGACC) as universal primers and an AccuPower PCR premix kit (Bioneer, Korea). The amplified polymerase chain reaction product was electrophoresed in 1 % agarose gel and purified using a QIAquick gel extraction kit (Qiagen, USA). The purified DNA was sequenced at Macrogen Co., Korea and a BLAST search confirmed that it was 99.9 % identical to that of *M. anisopliae* (JQ861946, JX110371, Gu06673, and Gu066730).

Experimental design using second instar *P. b. seluensis* infected with *M. anisopliae*

In the control group, *M. anisopliae*-inoculated sawdust was obtained after the mass death of *P. b.seluensis* caused by *M. anisopliae* infection. The sawdust was mixed with a water treatment at a ratio of 3:1 (300 g of sawdust: 100 mL of water). The plastic growth containers were either air-ventilated or sealed, and for the sealed containers, the opening was covered using a plastic wrap with several holes to minimize air ventilation, and simulate appropriate conditions for fungal growth. In the EM treatment group, *M. anisopliae*-inoculated sawdust was mixed with EM (1 × 10^7 cfu mL^{-1}; 300 g sawdust + 100 mL EM). The sawdust used in the control and EM treatment groups was fed to the insects for 30 d.

Antifungal activity of Effective microorganisms

M. anisopliae (KACC 40969), *B. subtilis* (KACC 17047), *L. plantarum* (KACC 10552), and *S. cerevisiae* (KACC 30068) were obtained from the Korean Agricultural Culture Collection (KACC), Rural Development Administration (RDA), Wanju, Korea. *B. subtilis, L. plantarum,* and *S. cerevisiae* were incubated in R2A (Reasoner's 2A), MRS (de Man, Rogosa and Sharpe), and YEPD (Yeast Extract Peptone Dextrose) broths, respectively, at 30 °C. Antagonistic activity was measured using the agar ×well diffusion assay method developed by Grover and Moore (1962). First, 20 mL of Sabouraud dextrose agar was poured into a sterile petri dish. For each EM (i.e., *B. subtilis*, *L. plantarum*, and *S. cerevisiae*), 200 μL of a concentration of 1 × 10^7 cfu mL^{-1} (0.4–0.6 at OD 600 nm, 1 mL) broth was spread evenly on the agar medium. Using a sterile steel borer, 5 mm wells of *M. anisopliae* were punched in the center of each EM smeared plate, and the antifungal activities of each EM were examined. Usually, insect farms use 4 % diluted sodium hypochlorite to prevent fungal contamination, and so it was used as the positive control in this study. The inhibitory effects of the EM against *M. anisopliae* were compared with that of the positive control and each experiment was replicated three times. The plates were checked for inhibition zones against *M. anisopliae* by measuring the surface areas of the fungi after 4 wk of growth. Furthermore, the growth inhibition rate (%) was calculated using the following equation:

$$\text{Growth inhibition rate (\%)} = (DC - DT)/DC \times 100,$$

where DC is the diameter of the control and DT is the diameter of the fungal colony in the treatment (Pandey *et al*., 1982).

Injection of effective microorganism into the hemolymph of the third instar *P. b. seluensis*

To simulate farm-like conditions, three replications of 10, third instar *P. b.seluensis* were grown in 400 × 270 × 230-mm transparent plastic rearing boxes. After starving the third instar *P. b.seluensis* for 72 h, the following substances were injected into the larva using a BD ultra-fine insulin syringe (31 gauge, 6 mm): 50 μL of triple distilled water, 50 μL of either R2A, MRS, or MRS broth, and 1 × 10^7 cfu per 50 μL of *B. subtilis*, *L. plantarum*, or *S cerevisiae,* respectively. After injection, individual larvae were placed in a circular, 100 mm-diameter plastic Petri dish with fermented sawdust, and their mortality rate was measured after 7 d.

Statistical data analysis

A student's *t*-test was used to assess the statistical differences among each group. Differences were statistically significant at P-values of 0.05 or less.

Lactic acid screening using M. anisopliae growth inhibition assay

The concentration of lactic acid (1.209 g/mL at 25 °C, Fluka, USA) was initially determined to be 10, 5, 2.5, and 0 % using the MIC (Minimal Inhibition Concentration). Subsequently, distilled water (DW) was added to 20 mL of the SDA medium, 5 mm wells of *M. anisopliae* were punched in the center of the plate with a sterile steel borer using 2.5, 1.25, 0.625, 0.3125, 0.15625, and 0.078125 % lactic acid. Following *M.anisopliae* inoculation, its fungicidal effect was measured at 5 d intervals for 20 d.

Electron microscopy

We used electron microscopy. Sections for electron microscopy were mounted on 200-mesh Ni grids and viewed in an H-7000 Hitachi microscope at 10 and 7.5 kV. We observed the morphology of JNK1 using scanning electron microscopy (SEM) after coating the grids with white gold.

In vivo assay using lactic acid

After measuring the effects of *M. anisopliae* on *P. b. seluensis,* lactic acid was dissolved in distilled water at a ratio of 2.5, 1.25, and 0 %, and *M. anisopliae* were added to the fungicide at a ratio of 3: 1. Their mortality rate was measured after 20 d to the early stage of the third instar larvae of the *P. b. seluensis* in a 400 × 270 × 230-mm sized sieve.

Results and Discussion

In vitro testing of the antifungal activity of effective microorganisms

The measurement of the growth inhibition zone and rate (%) against *M. anisopliae* by each EM (Fig. 2) showed that all three EM exhibited a significantly larger inhibition than the negative or positive controls (** P < 0.01). *B. subtilis* and *S. cerevisiae* inhibited 50 % of *M. anisopliae* growth in comparison to the control (no treatment), whereas *L. plantarum* inhibited approximately 40 % of fungal growth (Fig. 3). *B. subtilis* secreted numerous proteins that have antifungal activity (Moyne *et al*., 2004) and inhibited the growth of fungi in the *Fusarium*, *Pythium*, and *Rhizoctonia* genera (Li and Yang, 2005). Similarly, *S.cerevisiae* secretes numerous proteins, including the oleate-induced peroxisome *S. cerevisiae* 3-ketoacyl-CoA thiolase (ScFox3), that have antifungal activity (Li *et al*., 2008). Thus, we believed that the anti-fungal proteins secreted by the three EM synergistically and efficiently inhibited *M. anisopliae*.

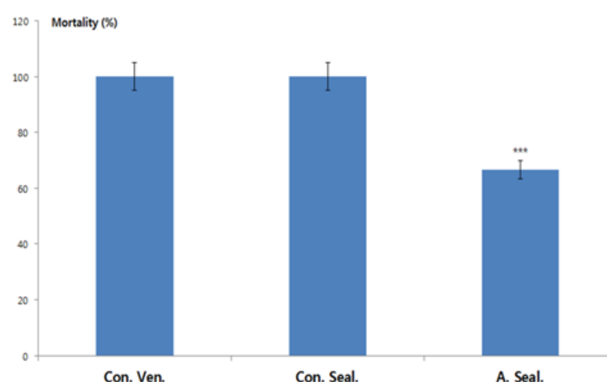

Fig. 2. Comparison of *Protaetia brevitarsis seluensis* mortality between EM treatment and control groups in *Metarhizium anisopliae* sawdust. The mortality rate of the third instar *P. b. seluensis* larvae under both the sealed and ventiliated conditions was 100% after 30 days, but the mortality rate of the EM group was 23.3%. (Con. Ven.: Control ventilation condition, Con. Seal.: Control sealed condition, A. seal.: Effective microorganism composite, sealed condition) (*t*-test: *** P < 0.001)

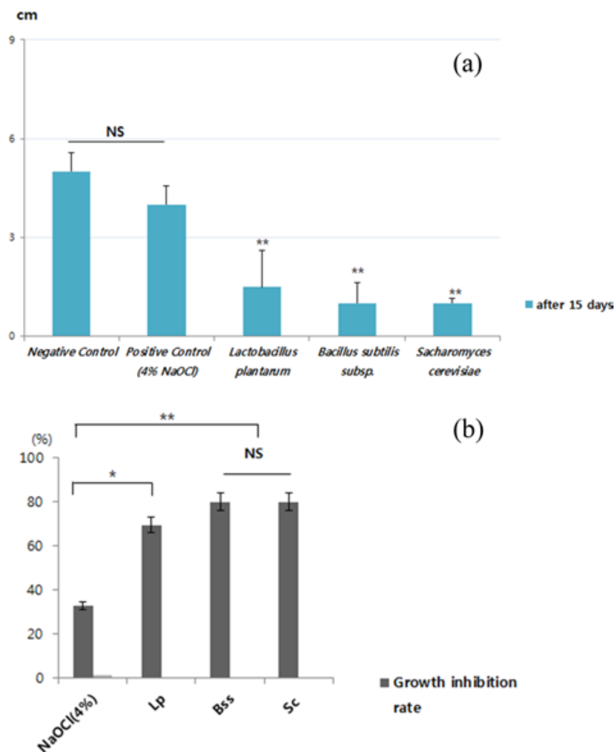

Fig. 3. Measurement of the growth inhibition zone and rate (%) against *Metarhizium anisopliae* for each EM. (A) In Sabouraud dextrose agar medium, 200 µL of triple distilled water as a negative control and 4 % sodium hypochlorite as a positive control were evenly smeared using a sterilized glass rod before seeding *M. anisopliae*. A sample of 200 µL of *Lactobacillus plantarum*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* were spread onto individual plates at a concentration of 1×10^7 cfu mL^{-1}, and *M. anisopliae* was seeded. After 15 and 30 d, the diameter of *M. anisopliae* growth was measured (*t*-test: ** P < 0.01) (B) Fungal growth inhibition (%) was calculated using the formula (DC-DT)/DC × 100. The growth inhibition (%) of EM-treated group was higher than in the sodium hypochlorite-treated group (*t*-test: * P < 0.05, ** P < 0.01).

Injection of EM into the hemolymph of the third instar *P. b.seluensis*

We examined whether EM were effective at inhibiting *M. anisopliae* by measuring *P. b. seluensis* mortality rate when the larvae were infected with EM via the hemolymph. Following the hemolymphatic injection of each EM (1×10^7 per 50 µL injection) into *P. b.seluensis*, *L plantarum* resulted in the lowest mortality rate. Our results (Fig. 4) showed that when each culture medium or microorganism was injected at a volume of 50 µL, the mortality rate increased by 10, 3, 20, and 3.3 % for the control, nutrient, MRS, and R2A broths, respectively, and 6.7, 23.3, and 10 % for *L. plantarum* (1×10^7 cfu mL^{-1}), *B.subtilis* (1×10^7 cfu mL^{-1}), and *S. cerevisiae* (1×10^7 cfu mL^{-1}), respectively. There was no significant difference between any of the groups (P > 0.05). The injection of *B. subtilis* into the hemolymph resulted in an approximately 13.3 % increase in mortality rate of *M.anisopliae* compared to that of the control group. This suggested that lowering the relative ratio of *B. subtilis* to *L. plantarum* or *S. cerevisiae* in the EM might be more effective. We confirmed that there was no effect on larvae mortality when the liquid media were injected with the effective microorganisms. Similarly, the microorganism injected into the hemolymph of the larvae had no effect on their mortality rate

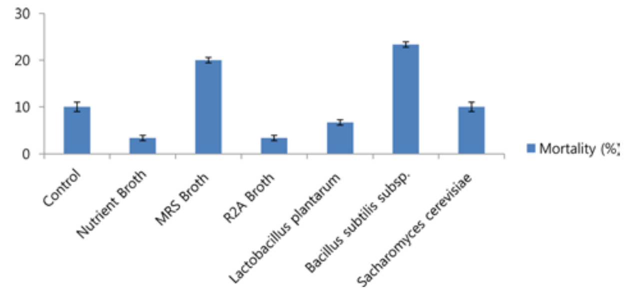

Fig. 4. Measurement of mortality rate when EM was injected into *Protaetia brevitarsis seluensis by hemolymphatic* injection.
Mortality rate was examined when each EM was injected into third instar *P. b. seluensis* at a concentration of 1×10^7 cfu mL-1, and a volume of 50 µL per insect. There was no significant difference between injection of EM and the respective EM culture media. However, the highest mortality rate was caused by *Bacillus subtilis* at 23.3 %. In addition, the mortality rate caused by the *Lactobacillus plantarum* injection was the lowest, at 6.7 %, but it did not significantly differ from the control (* P < 0.05).

Using a petri dish per larvae was an experimental method employed to reduce the influence of the insects on each other. Insects have an acquired and innate immune system, and the innate immune system (cellular and humoral system) is more developed. In the cellular immune system, when the activated hemocytes interact with pathogens, phagocytosis occurs (Kwon *et al*., 2014). Therefore, the cellular immune response is processed faster than the humoral immune response. Thus, the hemocytes in the hemolymph of *P. b.seluensis* were increased by these immune reactions. Exposure to EM might enhance the immune system of larvae, thereby reducing fungal infection. The hemocyte encounters bacteria and consequently fungal infections may be reduced when the larvae are exposed to the fungus.

Treatment of second instar *P. b.seluensis* with *M. anisopliae*

In this treatment, sawdust obtained from the mass death of *P. b.seluensis* by *M. anisopliae* infection was divided into EM-treated and untreated (control) groups in 400 × 270 × 230-mm rearing boxes. The control group was subdivided into an air-ventilated and a sealed group. In this study, when *P. b. seleusnsis* was grown at 20–25 °C and 60 % humidity in a temporary building, the mortality rate of second instar *P. b. seluensis* larva was 100 % after 30 d in both the air-ventilated and the sealed rearing boxes. In contrast, the EM-treated group showed only a 67 % mortality rate after 30 d even under the sealed condition. Likewise, the mortality rate of the third instar *P. b. seluensis* larvae under both the sealed and ventilated conditions was 100 % after 30 d, but the mortality rate of the EM group was 23.3 % (Fig. 2). These sexual spore- producing fungi show reduced pathogenic activities after subculturing, which affects spore production and results in the loss of pathogenic activities (Kim *et al.,* 2013, Silman *et al.,* 1991). Thus, it was very difficult to induce infection using the *M.anisopliae* inoculation of *P. brevitarsis* larva or sawdust. Therefore, we used sawdust in which a mass of *P. b. seluensis* larvae were killed. Aldehydes, short chain fatty acids, and wax esters that are secreted from larval epithelial tissue protect the skin of the insects from penetration by pathogenic fungi. It has previously been reported that the fungal infection of *P. b. seluensis* larvae generally occurs 20 h after contact with *M. anisopliae* (Zimmerman, 2007). In this study, when *P. b.seluensis* was grown at 20–25 °C and 60 % humidity in a temporary building, the mortality rate of second instar *P. b.seluensis* larva was 100 % after 30 d in both the air-ventilated and the sealed rearing boxes. In contrast, the EM-treated group showed only a 67 % mortality rate after 30 d even under the sealed condition. Likewise, the mortality rate of the third instar *P. b. seluensis* larvae under both the sealed and ventilated conditions was 100 % after 30 d, but the mortality rate of the EM group was 23.3 % (Fig. 2). *Thus, EM inhibited the growth of M. anisopliae* and lowered the rate of infection when single larva was grown in 10-cm diameter petri dishes at a constant temperature of 29–30 °C and 60 % humidity. Temperature seemed to be an important environmental factor affecting infection rate, and the insects' physiological properties, such as biological growth and molecular factors that sensed and changed with the temperature (Zars, 2003). Weight gain of *P. b.seluensi*s larvae at the breeding temperatures of 27.5 and 30 °C was greater than at 25 °C (Kim *et al*., 2015). The mortality rate differed with changing temperatures even though all other factors were controlled in the EM treatments. Moreover, the mortality rate of *P.b.seluensis* was 2-times lower in the EM-treatment group than in the control group (Fig. 2). The individually reared insects showed less fungal infection than mass reared insects. Thus, *B. subtilis*, *L. plantarum*, and *S.cerevisiae* may be able to inhibit fungal growth because various types of volatile compounds are produced when these bacteria are cultured and fermented in sawdust. This was especially true for lactic acid bacteria, such as *L. plantarum*, by which, antifungal substances, such as cyclo (L-Phe-L-Pro), cyclo (L-Phe-trans-4-OH-L-Pro), and phenyllactic acid, are produced (Ström *et al*., 2002).

On the other hands, *S. cerevisiae*–innoculated sawdust delayed *Beauveria bassiana*, the insect pathological fungi infection and reduced *P. b. seluensis* mortality by white muscardine *in vivo* and *S. cerevisiae* also no harmful effect when injected into the hemolymph of *P. b. seluensis* (Kwak *et al*., 2016). In addition, in yeast, organic acids such as pyruvic acid, malic acid, succinic acid, tartaric acid, oxalic acid, fumaric acid, citric acid, and acetic acid are produced when cultured in aerobic conditions and are known to inhibit fungi.

Therefore, this verifies that the supernatant from the EM cultured liquid media was enough to inhibit fungal growth because of interactions between complex organic compounds, such as organic acids and antifungal peptides. From these results, the EM used in this study appears to be effective at preventing fungal infection during *P. b. seluensis* breeding, and feeding them with EM-treated sawdust inhibited the growth of *M. anisopliae* thereby decreasing mortality. Further analyses of various organic acids and antifungal metabolites from each bacterium are required to determine whether they can synergistically inhibit the growth of insect pathogenic fungi. Based on this research, EM, which are known to have very low toxicity in humans, can be used to effectively prevent insect pathogenic fungi as the insect production market continues to grow.

Antifungal effect of lactic acid and proper concentration

We confirmed the presence of carboxylic acids in the EM composition, using HPLC (High Performance Liquid Chromatography) and found 3 mg/mL acetic acid, 0.9 mg/mL

lactic acid, and less than 0.001 mg/mL glycolic acid. Lactic acid was added at concentrations of 0.025, 0.01, 0.001, 0.003, 0.006, 0.0007, and 0 % to determine whether it could inhibit fungal growth. Their antifungal activities were compared in media with and without lactic acid. From measuring the length of the mycelium 5, 10, 15, and 20 d after the completion of the experiment, we confirmed that the mycelium treated with 0.025 % lactic acid did not completely grow. Mycelia growth was measured every 5 d and a regression analysis revealed that the value of R^2 was close to 1. Thus, mycelia growth was significantly inhibited by lactic acid. Hence, it was possible to predict the inhibition rate on the length of the mycelium depending on the concentration of lactic acid.

M. anisopliae controlling effect by lactic acid treatment

In the *in vitro* experiments, we confirmed that *M. anisopliae* was significantly suppressed under concentrations of 0.01 and 0.025 % lactic acid, and lactic acid was effective at controlling insect pathogenic fungi. Lactic acid was dissolved at concentrations of 1.25 and 2.5 % and treated with the contaminated sawdust to determine if it could effectively control fungal infection in *P. b. seluensis* After 20 d, the mortality of *P. b. seluensis* was 82.2 %, for 1.25 % lactic acid, 75.5 % for 2.5 % lactic acid, and 100 % in the control group. In the lactic acid-treated group, we found there was a significant difference at $P < 0.05$. As a result, we found that *P. b. seluensis* in the lactic acid-treated group had a 24.5 % lower mortality rate than those in the control group. Therefore, lactic acid was effective at inhibiting the growth of *M. anisopliae* in *P. b. seluensis*.

M. anisopliae sexual reproduction inhibited by lactic acid treatment

The morphology of *M. anisopliae* treated with lactic acid appeared not to be conidia but rather mycelia, and fungal sexual reproduction decreased. When lactic acid was introduced at different concentrations, from 0.0007 to 0.025 %, the size of the fungal spores increased and the mycelium became denser (Fig. 5). This indicated that fungal conidial production was affected by specific concentrations of lactic acid. Lactic acid was dissolved at concentrations of 0.01% and 0.025 % and treated with the contaminated sawdust to determine if it could effectively control fungal infection in *P. b. seluensis*.

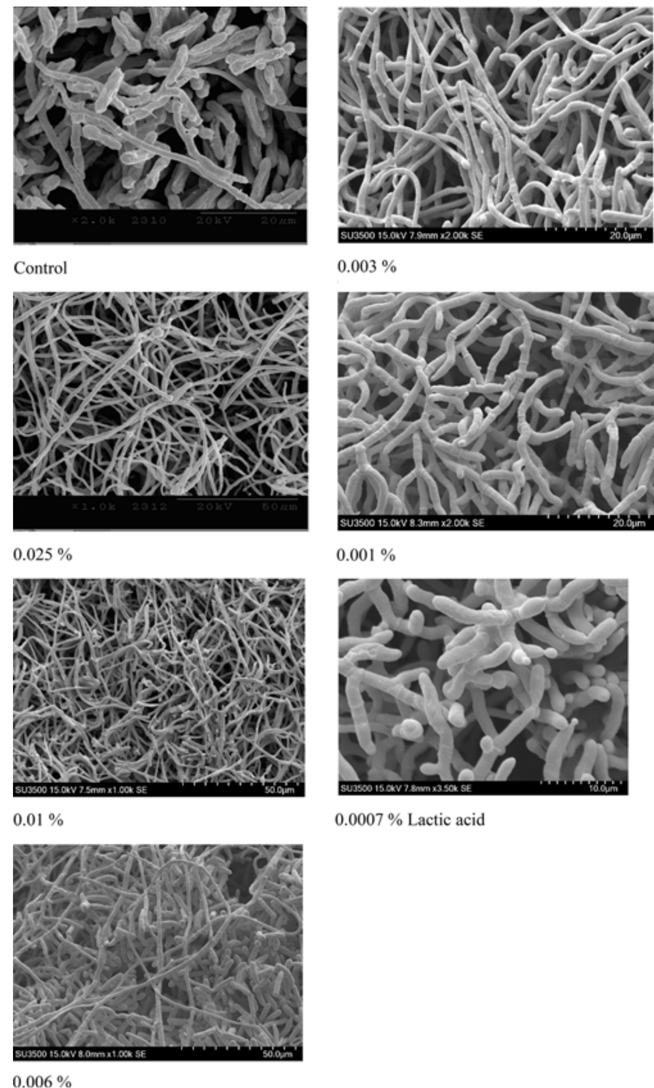

Fig. 5. SEM of *Metarhizium anisopliae* treated the specific concentrations of lactic acid The morphology of *M. anisopliae* on SEM appeared not to be conidia but rather mycelia, and fungal sexual reproduction decreased. The size of spores increased and the mycelium became denser when treated lactic acid concentration to *M. anisopliae* was lower. This indicated fungal conidial production was affected by specific concentration of lactic acid.

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Conflicts of interest

The authors have no conflict of interest to declare.

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