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ORIGINAL ARTICLE

Use of powdered immunized insects for inhibiting *Pectobacterium carotovorum* infestation and promoting growth in lettuce

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Abstract. We induced immune activation in mass rearing experiments in three insect species, i.e., *Gryllus bimaculatus* De Geer, 1773 (Orthoptera: Gryllidae), *Hermetia illucens* (Linnaeus, 1758) (Diptera: Stratiomyidae), and *Protaetia brevitarsis seulensis* (Kolbe, 1886) (Coleoptera: Scarabaeidae). Lysosomal staining of immune cells was a little over 6% in the control group insects, whereas it ranged from 17–35% in insects immunized with *Escherichia coli* K12 or *Bacillus thuringiensis* (Bt). The expression of attacin-like protein and defensin-like protein was also found to be upregulated at least 10-fold, and even up to 30-fold, from the third day of rearing in insects immunized with *E. coli* K12 or Bt. Non-immunized or immunized *G. bimaculatus*, *H. illucens*, and *P. brevitarsis seulensis* were freeze-dried and powdered, and these powders were then tested for protection against *Pectobacterium carotovorum* infestation. No inhibitory effects on *P. carotovorum* were observed when using all non-immunized insect powders or PBS. However, in the clear zone test treated with *H. illucens* powder at 10,000 ppm, an average size of 21.67 mm was observed. In a test using potato slices infected with *P. carotovorum*, we observed severe disease occurrence and potato weight loss in all non-immunized insect powders or PBS-treated groups. However, the group treated with *H. illucens* powder had the least potato weight loss. When tested on lettuce, the *H. illucens* powder-treated group revealed an approximately 10% increase in the fresh weight of lettuce, with both the dry weight and leaf area of lettuce increasing in comparison with those in the control group. Thus, our study proposes a novel method for the use of freeze-dried and powdered forms of insects immunized in mass rearing as effective functional fertilizers on a large scale that can also be effective in inhibiting microbial infections, overcoming the limitation of high production costs of such insect fertilizers using conventional methods.

INTRODUCTION

Insects immediately recognize pathogens such as bacteria, viruses, fungi, and parasites entering their bodies and mount an immune response (Hillyer, 2016). Although they do not have the sophisticated antigen/antibody response of vertebrates, insects rely on both cellular and humoral immune responses to fight off a variety of infectious agents (Sheehan et al., 2020).

The cellular immune response invokes immune blood cells, which recognize invading pathogens and eliminate them based on three main strategies, i.e., phagocytosis, encapsulation, and nodulation (Mahanta et al., 2023). Phagocytosis is a reaction wherein pathogens smaller than immune hemocytes, such as bacteria and fungi, are phagocytized and eliminated, whereas encapsulation is a reaction wherein immune cells surround and eliminate pathogens larger than themselves, such as parasites (Dubovskiy et al., 2016; Mahanta et al., 2023). Nodulation is a response to a rapid increase in the number of bacteria in the body, in which immune cells surround and eliminate the bacteria (Satyavathi et al., 2014; Dubovskiy et al., 2016; Mahanta et al., 2023). In contrast, the humoral immune response is the immediate response to pathogen invasion, where antimicrobial peptides (AMPs) directly eliminate pathogens (Diamond et al., 2009). These antibiotic and antimicrobial proteins are typically cationic (positively charged) and amphiphilic (containing both hydrophobic and hydrophilic regions) and interact with the microbial cell membrane to form holes and disrupt it (Lei et al., 2019; Zhang et al., 2021). A well known example of insect AMPs are

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lysozymes, which are found in a variety of insects, including flies and beetles (Wu et al., 2018). Lysozymes degrade the bacterial cell wall, leading to cell lysis (Ragland & Criss, 2017). It has been reported that various antimicrobial proteins such as attacin, defensin, holotricin, mucin, and tenascin are present in *Protaetia brevitarsis seulensis* (Coleoptera: Scarabaeidae) and are overexpressed upon pathogen invasion to eliminate pathogens (Kwon et al., 2014; Bang et al., 2015). In *Hermetia illucens* (Diptera: Stratiomyidae), a variety of antimicrobial peptides, including defensins, cecropins, attacin, and sarcotoxins, are present and actively participate in the humoral immune response (Xia et al., 2021).

Insect-based fertilizers are reported to improve soil fertility and plant growth by harnessing the rich nutrients in insects (Anyega et al., 2021; Beesigamukama et al., 2022). These are also considered raw materials that can be used as organic, renewable, and nutritious alternatives and reduce dependence on traditional chemical fertilizers, which often harm the environment. For example, the excrement produced by insects can be used as an organic fertilizer, as it contains high levels of nutrients, such as nitrogen, phosphorus, potassium, and micronutrients (Anyega et al., 2021). Moreover, insect proteins, such as defatted insect meal or hydrolyzate, can be used as a nutrient source in fertilizers and are known to complement the nitrogen needs of crops and contribute to their overall growth and development (Beesigamukama et al., 2022).

In this study, we aimed to determine the feasibility of using industrialized and mass-reared insects worldwide as functional fertilizers. To achieve this, we induced artificial immunity in mass-reared insects and identified the overexpression of various antimicrobial proteins in parallel with the activation of immune cells. Non-immunized or immunized *Gryllus bimaculatus* (Orthoptera: Gryllidae), *H. illucens*, and *P. brevitarsis seulensis* were freeze-dried and powdered, and these powders were then tested for protection against *Pectobacterium carotovorum* infestation.

MATERIAL AND METHODS

Rearing of insects and induction of immunity

Adults of *Gryllus bimaculatus* De Geer, 1773 (Orthoptera: Gryllidae), fifth-instar larvae of *Protaetia brevitarsis seulensis* (Kolbe, 1886) (Coleoptera: Scarabaeidae), and fourth-instar larvae of *Hermetia illucens* (Linnaeus, 1758) (Diptera: Stratiomyidae) were reared in sterilized cages (31.5 cm × 44 cm × 21 cm) at a temperature of 28 ± 1°C, humidity of 30–40%, and a photoperiod of 16L : 8D. The *G. bimaculatus* adults were provided with a variety of diets, including forage, vegetables, and grains, whereas *P. brevitarsis seulensis* was reared under aseptic conditions using sterilized oak sawdust. *H. illucens* individuals were provided with larval food sources with an appropriate mix of mealworms and agricultural byproducts. All insects were reared in an incubator (MIR-553; Sanyo Electric Biomedical, Japan) in a constant environment, and were then used in immunization experiments.

The method of immune activation using immunogenic agents involved direct injection into the insects and rearing them in cages. The needle was sterilized by finely tapering the glass tube (Haematokrit-Kapillaren), and the insects were disinfected with 70% alcohol paper before being injected dorsally to activate immunity. In the rearing cage method, immune activation was induced by deactivating all immunity activators by heating them at 100°C for at least 20 min. Subsequently, the cages were shaken at 100 rpm for 10 min once a day to artificially induce wounds on the cuticle of *G. bimaculatus, P. brevitarsis seulensis*, and *H. illucens*, exposing insect hosts through bacterial entry into mouths, spiracles, or anuses. *Bacillus thuringiensis* and *Escherichia coli*, used as immunity activators, were incubated for 15 h with shaking at 200 rpm in round bottom tubes (15 mL Falcon tubes) containing 5 mL of nutrient agar (NA) medium (3.0 and 5.0 g/L of beef extract and peptone, respectively) and LB medium (10.0, 5.0, and 5.0 g/L of casein peptone, yeast extract, and NaCl, respectively), respectively. All cultures were deactivated at 100°C for 20 min before being used as an inducer of immune activation. For the control, phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM $KH_2PO_{4;}$ pH = 7.4) was used.

Flow cytometry and reverse transcription polymerase chain reaction (RT-PCR)

Activated immune hemocytes were identified by the presence of activated lysosomes. A BD™ FACS Canto flow cytometer (BD Biosciences; San Jose, CA, USA) was used for this purpose and data were extracted using FACS Diva software by BD Biosciences. Red fluorescence was investigated by counting 10,000 hemocytes in a blood sample using the third-fluorescence channel (FL3) (610/20 bandpass).

Hemolymph was collected directly by puncturing the insect dorsally using a sterile glass Pasteur pipette (Haematokrit-Kapillaren) and capillary action. In the presence of anticoagulant solution (98, 186, 17, and 41 mM [v/v] NaOH, NaCl, EDTA, and citric acid, respectively; pH = 4.5), approximately 500 µL hemolymph samples were placed in sterile 1.5 mL tubes and mixed well. The mixed samples were centrifuged at 1,000 × *g* for 10 min, plasma was removed, and the pellet was washed with sterile water. To stain activated lysosomes, hemocytes were stained with the acidic dye LysoTracker Red (7.5 nM; Molecular Probes) for 30 min, washed thrice with PBS, fixed with 4% paraformaldehyde for 15 min, and again washed three times with PBS. The red lysosome fluorescence of 10,000 hemocytes per sample from each group was measured by flow cytometry using the FL3 channel.

Total RNA was isolated from fat bodies (SV Total RNA Isolation system kit; Promega Corp., Madison, WI, USA). Based on the isolated RNA, cDNA (GoScript TM Reverse Transcriptase; Invitrogen, Karlsruhe, Germany) was synthesized, and quantitative real-time RT-PCR (qRT-PCR) was performed using Rotor-Gene-Q (Qiagen, USA). qRT-PCR was performed using 5 µL SYBR Green master mix (Elpis-biotech, Korea), 0.2 µL of cDNA, and 0.4 µM of each antimicrobial protein gene-specific primer for a single 470 nm green fluorescence measurement (35 cycles at 94°C for 30 s, 55°C for 20 s, and 72°C for 10 s). The qRT-PCR results were normalized to the levels of β-actin gene expression and statistically analyzed using the Prism 5.0 program (GraphPad Software, San Diego, CA, USA). All data were tested using Student's two-tailed *t*-test or one-way analysis of variance with confidence intervals of 95% or better.

Freeze-drying of insects, clear zone test, potato test and growth test

The three insect species, immunized as described above, were freeze-dried for formulation. Insects, wherein immunity was induced, were washed thrice with distilled water and twice with 70% ethyl alcohol on the surface. Finally, they were washed once with 95% ethyl alcohol and packaged separately (using an autoclave to remove harmful bacteria, which have been shown to

cause a loss of function and activity of various immune-induced antibiotic and antimicrobial proteins). Before freeze-drying, they were frozen in a −80°C deep freezer and then completely freeze-dried using a freeze dryer (FDT-8650; Operon, Korea), for 12 days in the case of *G. bimaculatus* and *P. brevitarsis seulensis*, and 13 days for *H. illucens*. After freeze-drying, each insect was crushed and powdered using a pulverizer. We prepared insect powder for three species of non-immunized insects using the same method as described above. This powder served as a control in all experiments, along with PBS.

P. carotovorum was cultured on a 90 mm Petri dish with NA agar (3.0, 5.0, and 18.0 g/L of beef extract, peptone, and agar, respectively) using the streaking method for 2 days at 28°C, and then single colonies were isolated. It was prepared to a concentration of 1×10^3 cfu/mL via overnight incubation of 5 mL of NA water broth in a polystyrene round bottom tube (14 mL/ Falcon tube) in a shaking incubator at 28°C at 180 rpm. The pathogen was surface streaked onto NA agar in a 90 mm Petri dish in 30 µL increments, inoculated onto sterilized 8 mm paper discs in 20 µL increments, and incubated at 28°C for 2 days to measure the size of the clear zone.

The method employed for measuring the inhibitory effect on potatoes followed the following formula (Hadizadeh et al., 2019). Percentage of disease reduction (PDR) = (disease severity or loss (by weight) in control – disease severity or loss (by weight) in treatment)/disease severity in control × 100. Pieces of potato were placed in a 90 mm petri dish, inoculated with 30 µL of *P. carotovorum*, air-dried for 30 min, and subsequently weighed. After diluting each freeze-dried powder in sterile water at a 1 : 1 ratio, 3 mL was injected. The mixture was cultured for 3 days in a microbial incubator at 28°C under identical conditions, followed by weight measurement. All experiments were performed three times each.

Lettuce (varieties: Dongojeogchima, Dongoseed) was sown in 75 seedling trays using bed soil (Seoul Super Barokeo), and seedlings were distributed in pots (7 cm diameter × 6.5 cm height) after 7 days. Powders from each group were diluted 1 : 1 in distilled water, 100 ml of which was used for irrigation twice weekly for growth studies. The fresh weight of the lettuce was measured by weighing the aboveground and belowground parts after removing bed soil from the root, and the leaf area was measured using a Plant Canopy Analyzer (LI-3100C). The dry weight was measured after 48 h of drying at 60°C using a desiccator.

RESULTS

Immune activation through mass rearing

This study induced immune activation via mass rearing of *Gryllus bimaculatus* (Orthoptera: Gryllidae), *Hermetia illucens* (Diptera: Stratiomyidae), and *Protaetia brevitarsis seulensis* (Coleoptera: Scarabaeidae) on Luria-Bertani medium. Immune activation was induced by artificially inducing wounds in the cuticle by shaking the cage at 100 rpm for 10 min once daily with inactivated immunity inducers (dead *E. coli* and Bt) (see Material and Methods) or by introducing them into the insects via their mouths, spiracles, or anuses. In insects bred using the above methods, immune activation was checked by lysosomal staining and FASCs (Fig. 1). All experiments were performed in triplicates.

In the control group of *G. bimaculatus*, an average of 5.64% lysosomal staining was observed, with values of 5.41, 5.74, and 5.76% for individual rearings (Fig. 1A). However, *G. bimaculatus* treated with inactivated *E. coli* K12 had an average of 17.23% lysosomal staining, with values of 17.06, 16.94, and 17.68% for individual experiments. Similarly, insects treated with inactivated *B. thuringiensis* had an average of 17.99% lysosomal staining, with values of 17.69, 17.93, and 18.34% for individual experiments (Fig. 1A). For *P. brevitarsis seulensis*, an average of 21.57% of lysosomes were stained in the *E. coli* K12-treated group, with values of 22.12, 21.22, and 21.36% for individual experiments, and an average of 30.31% in the group treated with Bt, with values of 30.35, 30.03, and 30.56% for individual experiments (Fig. 1B). For *H. illucens*, an average of 30.39% of lysosomes were stained in the *E. coli* K12-treated group, with values of 30.45, 30.33, and 30.38% for individual experiments, and an average of 35.17% in the group treated with Bt, with values of 35.31, 34.93, and 35.26% for individual experiments (Fig. 1C). The statistical analysis of the results suggested that all three insects had immune activation upon treatment with *E. coli* or Bt compared with those in the control group, with no significant difference in the degree of immune activation between *E. coli* K12 and Bt treatments (Fig. 1A–C).

Next, the expression levels of two antimicrobial proteins, i.e., attacin-like protein and defensin-like protein, were compared between the control and bacterial treatment groups of *G. bimaculatus*, *P. brevitarsis seulensis*, and *H. illucens* insect species (Fig. 2). In bacterial treatment groups of *G. bimaculatus*, the attacin-like protein was overexpressed at least 10-fold and even up to 30-fold by the third day of rearing (Fig. 2A). The attacin-like protein, known to be expressed during the invasion of gram-negative bacteria, was found to be expressed at a lower level in the group treated with *E. coli* (gram-negative bacteria) compared with that in the group treated with Bt (gram-positive bacteria) (Fig. 2A). The defensin-like protein, known to be expressed upon invasion by both gram-negative and gram-positive bacteria, was found to be overexpressed in both groups treated with *E. coli* K12 or Bt, with relatively increased expression in the group treated with Bt having insect pathogenicity (Fig. 2A). In bacterial treatment groups of *P. brevitarsis seulensis*, the attacin-like protein was overexpressed at least 5-fold and even up to 30-fold by the third day of rearing, with approximately 1.5 times higher expression in the group treated with *E. coli* K12 than in the group treated with Bt (Fig. 2B). The defensin-like protein was found to be overexpressed in both *E. coli* K12- and Bt-treated groups (Fig. 2B). In *H. illucens*-treated groups, the attacin-like protein was overexpressed at least 10-fold and even up to 35-fold by the third day of rearing, and the defensin-like protein was overexpressed 25-fold upon *E. coli* K12 invasion and 30-fold upon Bt invasion (Fig. 2C).

Clear zone test with freeze-dried and powdered immunized insects

Freeze drying involved rapid freezing of the insects and water removal through sublimation to ensure that the nutritional value of the insects was preserved and the function of the antimicrobial proteins maintained. In this study, up to 2,000 immunized *G. bimaculatus*, 1,500 immunized

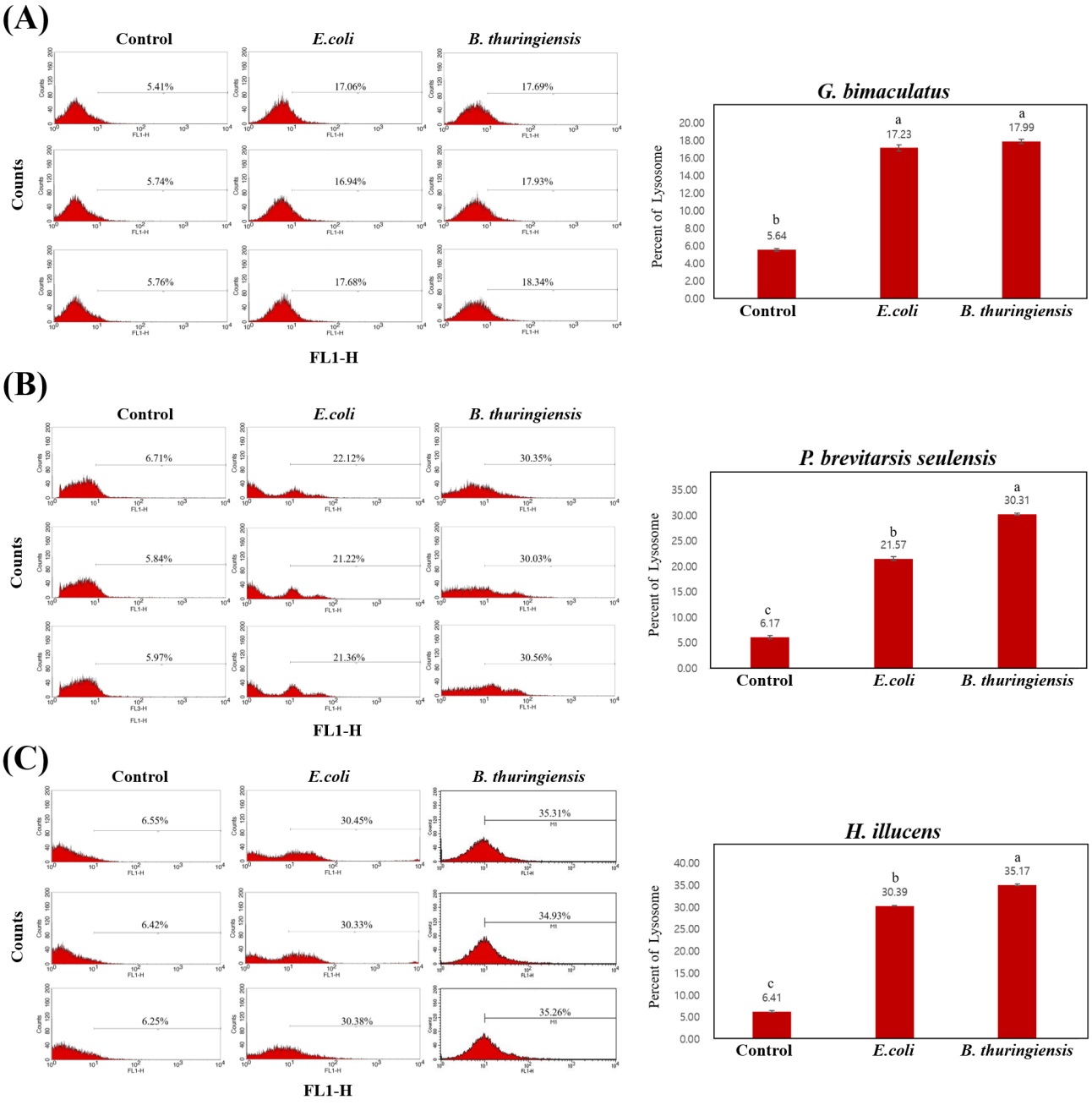


Fig. 1. The results indicate the activation status of the immune system following treatments with *E. coli* K12 and *B. thuringiensis* (Bt) for each insect, confirmed through lysosome staining and Flow Cytometry (FACS) analysis (A–C). The control group of *G. bimaculatus* showed an average of 5.64% lysosomal staining; insects reared with heat-killed *E. coli* K12 showed an average of 17.23% lysosomal staining; and insects reared with heat-killed Bt showed an average of 17.99% lysosomal staining. For *P. brevitarsis seulensis*, an average of 21.57% of lysosomes were stained in the *E. coli* K12 treatment group and an average of 30.31% in the Bt treatment group (B). The same was true for *H. illucens*, with an average of 30.39% in the *E. coli* K12 treatment group and 35.17% in the Bt treatment group (C). The results obtained from the flow cytometry cell counting method are presented as the means of three independent experiments, with each mean calculated from 10,000 hemocytes. Statistical analysis was performed using Duncan's multiple range test or one-way analysis of variance (ANOVA). A p-value of ≤ 0.05 was considered statistically significant.

P. brevitarsis seulensis, and 5,000 immunized *H. illucens* were freeze dried. Non-immunized *G. bimaculatus*, *P. brevitarsis seulensis*, and *H. illucens* were also prepared as controls with PBS.

With an average drying time of 7 days, most of the insects were not completely dry, causing odors and the growth of bacteria and mold on the crops after treatment (data not shown). Next, similar to the seven-day drying treatment of insects, odors and bacterial growth were observed even upon drying them for 9 days, which was not considered an appropriate drying time (data not shown). With a drying time of 12 days, *G. bimaculatus* and *P. brevitarsis seulensis* could be dried adequately, but moisture was still present in *H. illucens*. Therefore, for *H. illucens*, 13 days was considered an appropriate freeze-drying period.

A clear zone test was performed against *P. carotovorum* infection to determine whether the lyophilized insect powders have antimicrobial activity. No inhibitory effects on

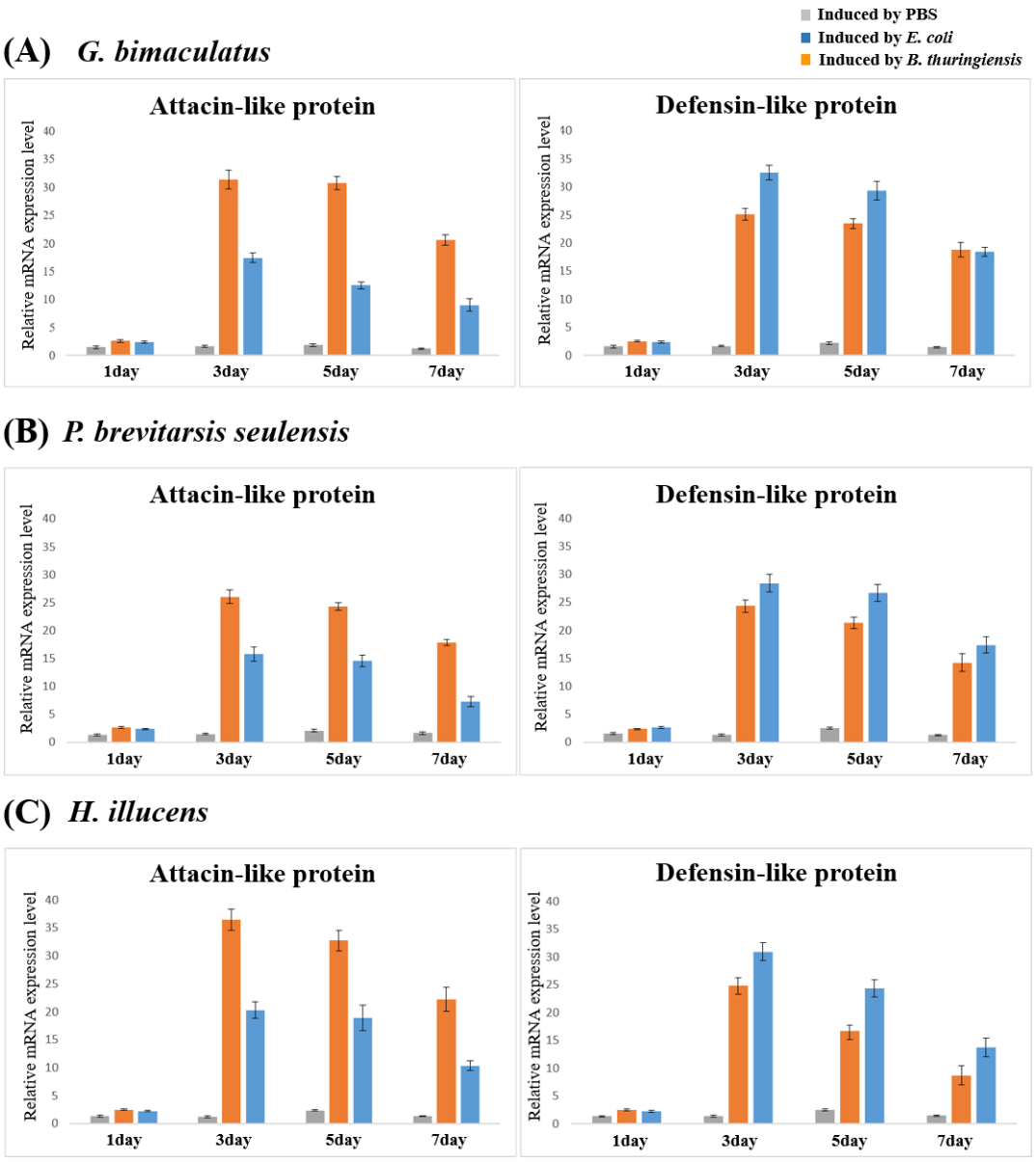
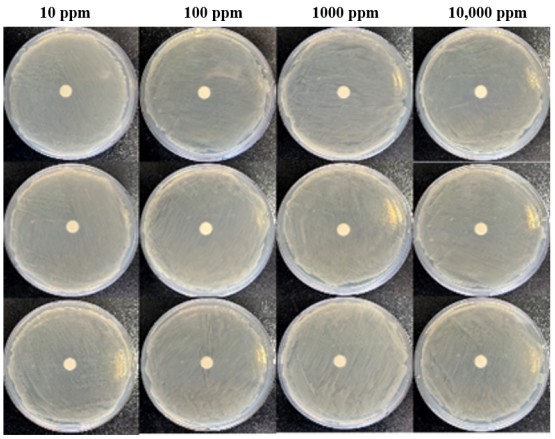


Fig. 2. Graph comparing the expression of the antimicrobial proteins attacin-like protein and defensin-like protein. The attacin-like protein in *G. bimaculatus* was overexpressed 10- to 30-fold from the third day of rearing and was expressed at a lower level compared to the group treated with gram-positive bacteria (Bt) (A). The defensin-like protein was found to be overexpressed in both gram-negative (*E. coli* K12) and gram-positive (Bt) treated groups (A). In *P. brevitarsis seulensis*, the attacin-like protein was overexpressed approximately 5- to 30-fold from the third day of rearing, and defensin-like protein was overexpressed in both groups treated with gram-negative (*E. coli* K12) and gram-positive (Bt) bacteria. In *H. illucens*, attacin-like protein was overexpressed at least 10-fold and up to 35-fold by the third day of rearing, and defensin-like protein was overexpressed 25-fold upon gram-negative bacterial invasion and 30-fold upon gram-positive bacterial invasion.

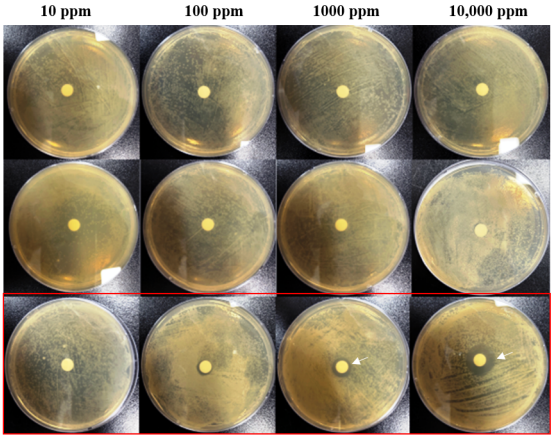
P. carotovorum were observed when employing all non-immunized insect powders, including *H. illucens* (Fig. 3A). Next, we investigated the inhibitory effects using immunized insect powders (Fig. 3B–D). No clear zones were formed at 10, 100, 1,000, and 10,000 ppm dilutions of *P. brevitarsis seulensis* powder (Fig. 3B and 3C). For *G. bimaculatus*, clear zones of 9, 8, and 9 mm, with an average size of 8.67 mm, were observed at 10,000 ppm (Fig. 3B and 3C). For *H. illucens*, no clear zones were formed at 10 ppm, but at 100 ppm, clear zones of 10, 10, and 11 mm formed, with an average size of 10.33 mm, whereas, at 1,000 ppm, clear zones of 13, 12, and 13 mm formed, with an average size of 12.67 mm (Fig. 3B and 3C). Moreover, at 10,000 ppm, clear zones measuring 22, 21, and 22 mm formed, with an average size of 21.67 mm (Fig. 3B and 3C). The error bars have been statistically processed to represent the 95% confidence interval for the binomial distribution (Fig. 3D).

Next, these insect powders were tested for the inhibition of *P. carotovorum* infection in potato slices (Fig. 4). The reduction in potato weight resulting from *P. carotovorum* infection was quantified through the utilization of three varieties of nonimmunized insect powders (Fig. 4A and B). Across all experimental treatments, the average reduction in potato weight ranged from 2.1 to 2.4 g, suggesting the absence of any inhibitory effect against *P. carotovorum*.

(A)



(B)



(C)

Immunized Insect powder	Con. (ppm)	Clear zone size (mm)		
Control	10	0	0	0
	100	0	0	0
	1,000	0	0	0
	10,000	0	0	0
P. brevitarsis seulensis	10	0	0	0
	100	0	0	0
	1,000	0	0	0
	10,000	0	0	0
G. bimaculatus	10	0	0	0
	100	0	0	0
	1,000	0	0	0
	10,000	9	8	9
H.illucens	10	0	0	0
	100	10	10	11
	1,000	13	12	13
	10,000	22	21	22

(D)

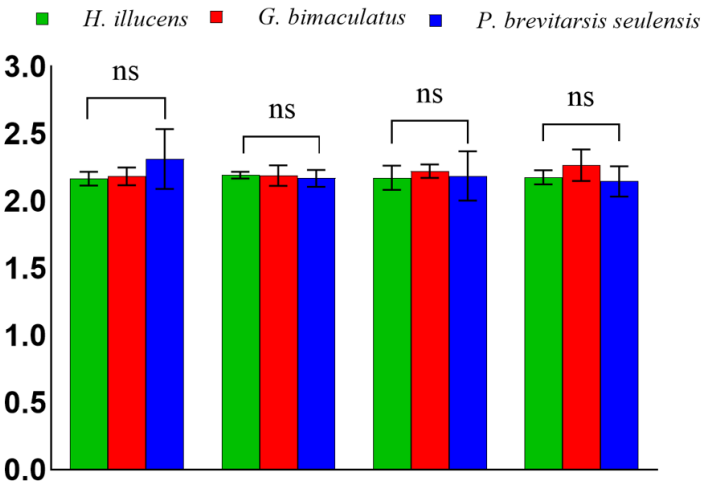
Fig. 3. The clear zone test results for *P. carotovorum* at various concentrations (ppm) of freeze-dried powder from each insect, when mixed in a light solution, are as follows. No inhibitory effects on *P. carotovorum* were observed when employing all non-immunized insect powders, including *H. illucens* (A). Dilution of the powder of *P. brevitarsis seulensis* resulted in no clear zones at 10, 100, 1,000, and 10,000 ppm (B). For *G. bimaculatus*, clear zones of 9, 8, and 9 mm, with an average size of 8.67 mm, were observed at 10,000 ppm (B). For *H. illucens*, no clear zones formed at 10 ppm, but at 100 ppm, clear zones with an average size of 10.33 mm were formed. At 1,000 ppm, clear zones with an average size of 12.67 mm were formed. At 10,000 ppm, clear zones with an average size of 21.67 mm were formed (B). The error bars represent the 95% confidence interval for the binomial distribution (C). 1 ppm means 1 g of insect powder for every 1 million parts of the solution.

The powders of immunized *G. bimaculatus*, *P. brevitarsis seulensis*, and *H. illucens* were treated at concentrations of 10, 100, 1,000, and 10,000 ppm to determine the reduction in the weights of potato slices (Fig. 4C and D). The results showed an average reduction of 2.18 g in the control group, regardless of concentration. At 1,000 ppm concentrations, reductions averaging 1.8 and 1.86 g were found with *G. bimaculatus* and *P. brevitarsis seulensis*, respectively, with

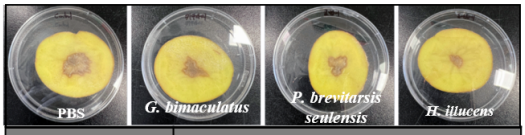
(A)

Nonimmunized Insect powder	Concentration (ppm)			
	10	100	1,000	10,000
G. bimaculatus	▽2.20g	▽2.22g	▽2.20g	▽2.21g
	▽2.19g	▽2.18g	▽2.22g	▽2.30g
	▽2.15g	▽2.16g	▽2.24g	▽2.28g
P. brevitarsis seulensis	▽2.22g	▽2.14g	▽2.21g	▽2.19g
	▽2.40g	▽2.17g	▽2.10g	▽2.14g
	▽2.31g	▽2.19g	▽2.24g	▽2.10g
H. illucens	▽2.14g	▽2.20g	▽2.21g	▽2.15g
	▽2.18g	▽2.19g	▽2.14g	▽2.18g
	▽2.17g	▽2.18g	▽2.16g	▽2.19g

(B)



(C)



Immunized Insect powder	Concentration (ppm)			
	10	100	1,000	10,000
PBS (control)	▽2.12g	▽2.23g	▽2.24g	▽2.18g
	▽2.21g	▽2.13g	▽2.15g	▽2.20g
	▽2.19g	▽2.17g	▽2.22g	▽2.11g
G. bimaculatus	▽2.02g	▽2.08g	▽1.82g	▽1.46g
	▽2.09g	▽1.98g	▽1.75g	▽1.39g
	▽2.02g	▽1.99g	▽1.83g	▽1.34g
P. brevitarsis seulensis	▽2.10g	▽2.13g	▽1.88g	▽1.53g
	▽2.13g	▽2.05g	▽1.81g	▽1.57g
	▽2.10g	▽2.05g	▽1.88g	▽1.49g
H. illucens	▽1.82g	▽1.47g	▽1.04g	▽0.58g
	▽1.91g	▽1.32g	▽0.92g	▽0.55g
	▽1.84g	▽1.34g	▽0.97g	▽0.53g

(D)

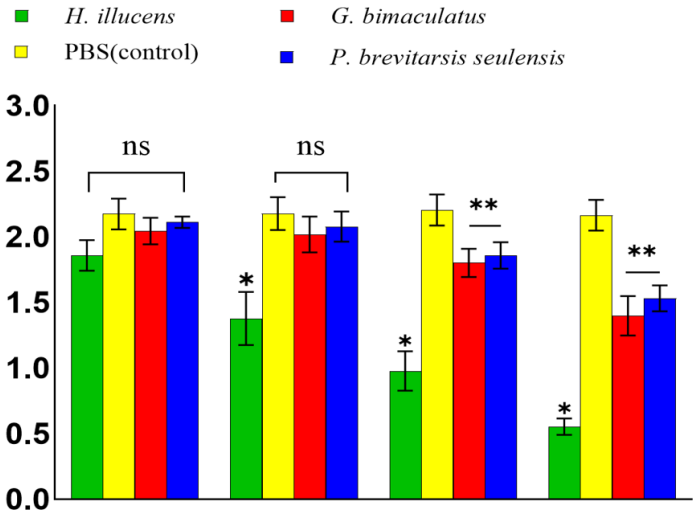


Fig. 4. Experimental results on the inhibition of *P. carotovorum* by varying concentrations (ppm) of insect powder, using potato slices. The reduction in potato weight resulting from *P. carotovorum* infection was quantified through the utilization of three varieties of nonimmunized insect powders (A and B). Across all experimental treatments, the average reduction in potato weight ranged from 2.1 to 2.4 g, suggesting the absence of any inhibitory effect against *P. carotovorum*. Reductions averaging 1.8 and 1.86 g were found with 1,000 ppm of *G. bimaculatus* and *P. brevitarsis seulensis*, respectively, with the smallest reduction averaging 0.98 g. In addition, with 10,000 ppm of *G. bimaculatus*, *P. brevitarsis seulensis*, and *H. illucens*, the weight losses averaged 1.4, 1.53, and 0.55 g, respectively, with the smallest weight losses observed with *H. illucens* (C and D). The experiment was repeated three times. Error bars indicate Mean ± SEM. *P < 0.05 (t-test). ns – not significant (D). 1 ppm means 1 g of insect powder for every 1 million parts of the solution.

the least reduction averaging 0.98 g for *H. illucens*. In addition, with 10,000 ppm of *G. bimaculatus*, *P. brevitarsis seulensis*, and *H. illucens*, weight losses averaged 1.4, 1.53, and 0.55 g, respectively, with the least weight loss observed in *H. illucens* (Fig. 4C). In comparison with the cohort subjected to immunized *H. illucens* powder treatment, a significant reduction in potato weight was observed in the group treated with control (PBS), as well as in the groups treated with non-immunized *G. bimaculatus*, non-immunized *P. brevitarsis seulensis*, and non-immunized *H. illucens* powder. Additionally, significant reductions were observed in

(B)

Treatment	Concentration (ppm)	Fresh weight(g)	Dry weight(g)	Leaf area(cm²)
Control	1,000	89.48	2.10	85.32
		91.37	2.34	89.13
		87.51	1.95	81.51
	10,000	89.37	2.11	86.42
		93.14	2.34	90.31
		86.47	1.98	83.14
G.bimaculatus	1,000	90.87	2.16	90.30
		88.73	2.02	85.46
		92.18	2.27	94.02
	10,000	95.30	2.37	95.29
		95.53	2.12	93.17
		97.86	2.39	98.82
P.brevitarsis seulensis	1,000	89.48	2.11	89.58
		85.14	2.03	87.17
		93.24	2.21	91.24
	10,000	90.73	2.13	91.63
		94.26	2.26	94.34
		86.25	1.99	88.47
H.illucens	1,000	106.86	3.78	111.97
		102.37	3.41	107.64
		109.34	3.91	114.37
	10,000	115.20	4.51	122.37
		112.83	4.41	121.49
		118.72	4.63	124.35

(C)

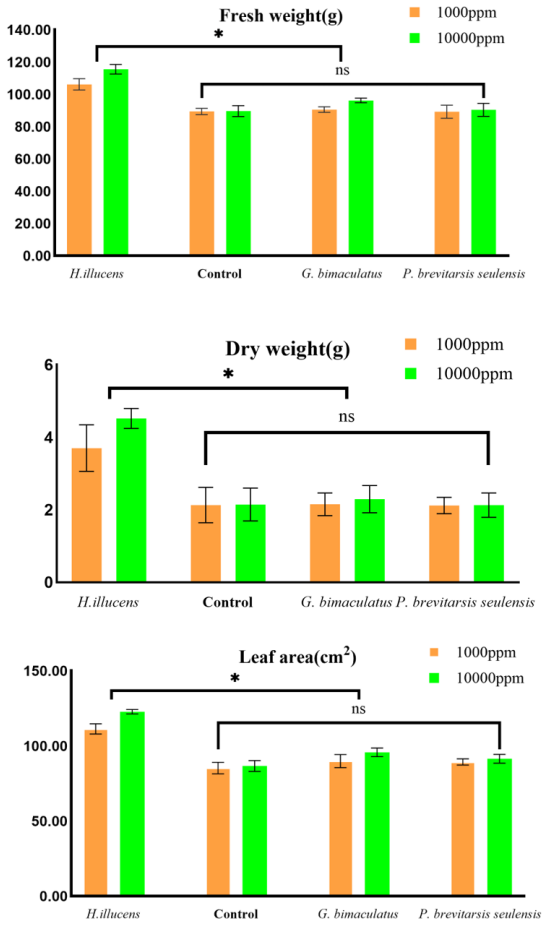


Fig. 5. The outcomes of treatments with insect powder at concentrations of 1,000 and 10,000 ppm, exhibiting allelopathic effects on lettuce. Lettuces subjected to control (PBS), *G. bimaculatus*, *P. brevitarsis seulensis*, and *H. illucens* powders exhibit symptoms of *P. carotovorum*-induced disease, characterized by wilting leaves. Lettuce treated with immunostimulated *H. illucens* powder demonstrates healthy growth (A; marked by white arrow). It was observed that the fresh weight and dry weight were highest in the *H. illucens* powder treatment group, and similarly, the leaf area was also noted to be the widest in this group (B and C). Compared to the group treated with control, *P. brevitarsis seulensis*, and *G. bimaculatus* powder, the growth status of lettuce in the group treated with *H. illucens* powder was significantly increased (C). The experiment was repeated three times. Error bars represent Mean ± SEM. *P < 0.05 (t-test). ns – not significant (C). 1 ppm means 1 g of insect powder for every 1 million parts of the solution.

the groups treated with immunized *P. brevitarsis seulensis* and immunized *G. bimaculatus* powder (Fig. 4D).

Impact of freeze-dried insect powders on lettuce growth parameters

After treating lettuce with freeze-dried insect powders at concentrations of 1,000 ppm and 10,000 ppm, which had antagonistic effects as described in Material and Methods, we compared the treated groups by measuring their fresh weight, dry weight, and leaf area (Fig. 5). Figure 5A illustrates the growth status of lettuce post-treatment with immunostimulated insect powders. Lettuces subjected to control (PBS), *G. bimaculatus*, and *P. brevitarsis seulensis* powders exhibit symptoms of *P. carotovorum*-induced disease, as evidenced by wilting leaves (Fig. 5A; marked by white arrow). Conversely, lettuce treated with immunostimulated *H. illucens* powder demonstrated healthy growth (Fig. 5A). Subsequent analyses involved the examination and comparison of dry weight, fresh weight, and leaf area among lettuce samples in each treatment group (Fig. 5B and C). In the control group, the fresh weight of lettuce averaged 89.45 g at 1,000 ppm. At comparable treatment concentrations, the fresh weights of lettuce in the groups treated with *G. bimaculatus* and *P. brevitarsis seulensis* powders were similar to those in the control group, averaging 90.59 g and 89.29 g, respectively. However, the fresh weight of lettuce in the group treated with *H. illucens* powder at 1,000 ppm averaged 106.19 g >16 g higher than that in the control group (Fig. 5B). The dry weight of lettuce at 1,000 ppm averaged 2.13 g in the control group. While the dry weights in the groups treated with *G. bimaculatus* and *P. brevitarsis seulensis* powders were similar to those in the control group, averaging 2.15 and 2.12 g, respectively, that in the group treated with *H. illucens* powder averaged 3.7 g >1.57 g higher than in the control group (Fig. 5B). The leaf area of lettuce at 1,000 ppm averaged 85.32 cm^2 in the control group. While the leaf area of lettuce in the groups treated with *G. bimaculatus* and *P. brevitarsis seulensis* powders averaged 89.93 and 89.33 cm^2, respectively, ~11 cm^2 wider than that in the control, that in the group treated with *H. illucens* powder was even wider and averaged 111.33 cm^2 (Fig. 5B). The fresh weight of lettuce at 10,000 ppm averaged 89.66, 90.41, 91.56 g in the control group, the group treated with *P. brevitarsis seulensis* and *G. bimaculatus* powder, respectively (Fig. 5B). However, it increased to an average 115.58 g in the groups treated with *H. illucens* powders (Fig. 5B). The dry weight of lettuce at 10,000 ppm averaged 2.14 g in the control group. The group treated with *P. brevitarsis seulensis* and *G. bimaculatus* powder had a similar average dry weight of lettuce, whereas that treated with *H. illucens* powder showed the largest difference (Fig. 5B). The leaf area of lettuce at 10,000 ppm measured an average of 87.91 cm^2 in the control group, and that in the group treated with *H. illucens* powder was the highest with an average of 122.73 cm^2 (Fig. 5B). Compared to the group treated with control, *P. brevitarsis seulensis*, and *G. bimaculatus* powder, the growth status of lettuce in the group treated with *H. illucens* powder was significantly increased (Fig. 5C).

DISCUSSION

In general, each insect is immunized via individual injections of immunogenic substances in appropriate doses (Fig. S1). It has been shown that direct injection methods lead to high activation of the cellular and humoral immune responses in various insects (Kwon et al., 2014; Lee et al., 2014; Bang et al., 2015; Hwang et al., 2017; Cho & Cho, 2019, 2021; Go et al., 2022). However, this method has a major drawback, as it requires a lot of labor and time to immunize insects in large numbers. In addition, in cases where the needle is injected rather strongly and deeply into *Protaetia brevitarsis seulensis*, it can damage organs and cause melanization throughout the body, resulting in reduced activity and death (Fig. S1). To overcome these shortcomings, we sought to activate immunity by rearing insects with immunity inducers in their cages, shaken at 100 rpm for 10 min once a day to artificially induce wounds in the cuticle or to introduce the immunity inducers into the insects through their mouths, spiracles, or anuses. Although it was not feasible to induce immunity in all individuals, as is the case when injecting into each insect, the activation of lysosomes (cellular immunity) and overexpression of antimicrobial proteins (humoral immunity) demonstrated activation of immunity in insects bred using the above method.

All immune cells typically show increased numbers of intracellular lysosomes after phagocytosis to eliminate pathogens (Kinchen & Ravichandran, 2008; Fu & Harrison, 2021; Lancaster et al., 2021). In the past, various insects have been evaluated based on the increase in the staining of lysosomes (Cho & Cho, 2019, 2021; Go et al., 2022). Moreover, defensin and attacin, the representative antimicrobial peptides in insects, are regulated by innate immune pathways, such as the Toll and Imd pathways, and expressed in various organs such as the fat body, hemocytes (immune cells), gut, respiratory tract, and reproductive system (De Gregorio et al., 2002; Kleino et al., 2005; Ilyasov et al., 2012; Parvy et al., 2019). These antimicrobial peptides directly bind to the pathogen cell membrane, forming a hole and killing the cell. They regulate the overall insect immune system by affecting immune cell activation, nodulation, and encapsulation and controlling the production of other immune-related molecules (Lavine & Strand, 2002; Cole & Nizet, 2016; Manniello et al., 2021). This study identified the overexpression of attacin and defensin proteins, which are typical antimicrobial proteins in insects. The expression of these representative antimicrobial proteins suggests that the mass-rearing method induced sufficient immunity in each insect. An overall increase in immunity in the insects in the treatment groups could be inferred compared with those in the control group, although the degree of immune activation varied for each insect.

A variety of methods were used to powder the non-immunized or immunized insects (see Material and Methods). For example, insects were boiled or heated, which led to a loss of function of the antimicrobial proteins (data not shown). It also created several problems, including odors, environmental pollution, complicated procedures, and

large amounts of carbon emissions. However, freeze-drying generally minimized protein degradation and preserved structure, thus maintaining the functional structure of the protein, and the overexpressed antimicrobial proteins were speculated to be functional. A variety of factors, including various cryostabilizers and additives used, proper pH and buffer systems, cooling and freezing rates, drying temperatures, pressures, and drying times, must be checked (Nowak & Jakubczyk, 2020). For example, although the average drying time for crops, reagents, and bacteria is reported to be around 3 days (Nowak & Jakubczyk, 2020), insects need more drying days than crops, reagents, and bacteria, as the wax in their cuticles prevents the evaporation of water from their bodies. Therefore, an appropriate timeframe was selected, as drying for excessively long periods could result in the loss of activity of the antibiotic and antimicrobial proteins activated by inducing immunity. Finally, these methods could maintain the function of proteins in the bodies of immunized and mass-reared insects to a certain extent.

Bacterial blight is a common plant disease caused by a variety of bacteria, including *Pectobacterium* spp. and *Dickeya* spp. (Ma et al., 2007; Shaw, 2008; Diallo et al., 2009; Czajkowski et al., 2011). These bacteria infect crops on a large scale, causing the tissue to decay and soften, ultimately leading to wilting and death (Kim et al., 2011; Motyka et al., 2017; Mejía-Sánchez et al., 2019). Currently, measures to control bacterial blight often involve the use of fungicides and products containing copper compounds, such as copper hydroxide or copper sulfate (Lee et al., 2012; Husak, 2015; Osdaghi et al., 2020). Although an inhibitory effect was observed in the clear zone test in the laboratory, further research seems to be needed to determine which of the various antimicrobial proteins expressed in the insect body are responsible for the effect. The powdered insects have the potential to be used as crop fertilizer, containing a variety of nutrients (protein, nitrogen, phosphorus, potassium, and micronutrients) (Hodge et al., 2022; Carroll et al., 2023). It is speculated that these nutrients increase soil fertility, promote healthy crop growth, and improve soil structure, microbial activity, and nutrient availability. Hitherto, the biggest disadvantage of insect-based fertilizers is the high cost of production. However, functionalized insect-based fertilizers (e.g., with antibiotic and antimicrobial properties) are environmentally friendly and increase crop growth and productivity, which is expected to contribute to the continuous development and growth of the insect fertilizer industry.

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Supplementary Fig. S1 follows on next page.

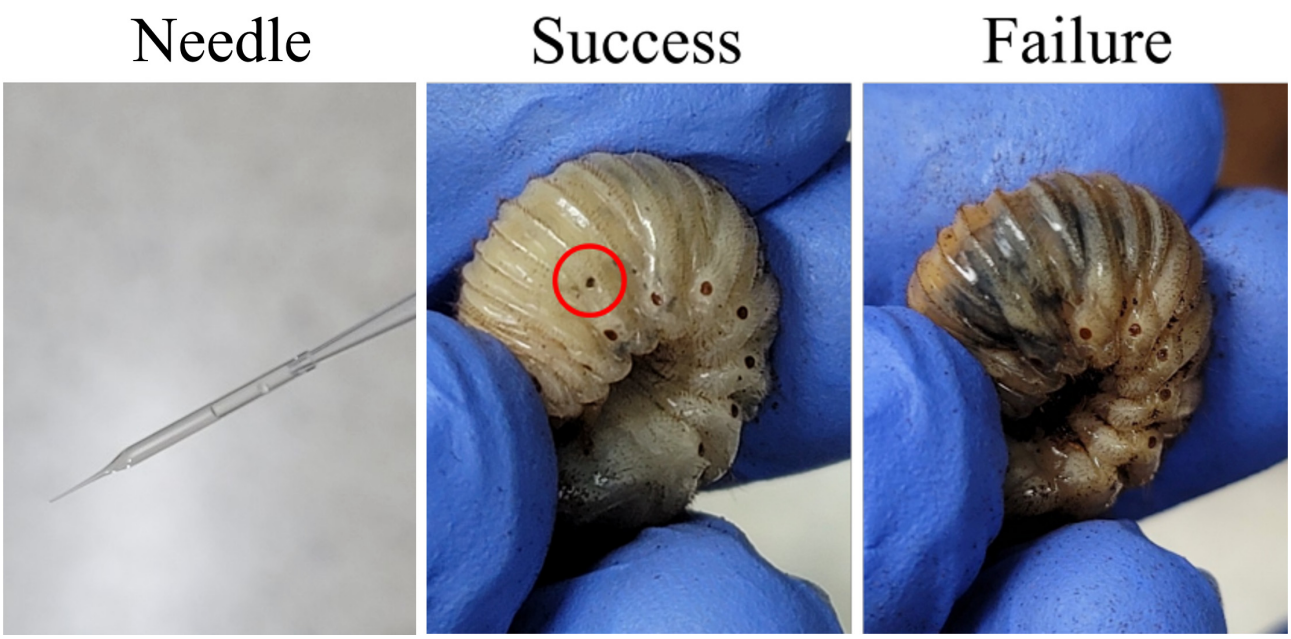

Fig. S1. The injections into the target insects were made with a glass-tipped needle. Injections were made differently depending on the state of the insects, whether they had undergone incomplete or complete metamorphosis, or how soft or hard the insect bodies were (A). If the needle goes deep into the insect's body or is forcefully injected, the insect may die from the wound, greatly affecting the results of the experiment (B and C). Successful injection can be confirmed by extremely small melanization that occurs on the insect's body after injection (B).