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The Anti-Termite Activity of *Bacillus licheniformis* PR2 against the Subterranean Termite, *Reticulitermes speratus kyushuensis* Morimoto (Isoptera: Rhinotermitidae)

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Abstract: Subterranean termites of the species *Reticulitermes speratus kyushuensis* Morimoto (Isoptera: Rhinotermitidae) are notoriously destructive soil-dwelling pests that feed on the cellulosic wood biomass. This leads to tremendous losses of forest trees such as *Pinus densiflora* Siebold and Zucc. (Pinales: Pinaceae) and precious wooden structures of cultural heritage. This study investigated the efficacy of chitinase and protease produced by *Bacillus licheniformis* PR2 as cuticle-degrading enzymes for the biocontrol of worker termites. *Bacillus licheniformis* PR2 produced a strong chitinase and protease activity up to a maximum of 82.3 unit/mL and 35.9 unit/mL, respectively, and caused a lethal effect on termites under laboratory conditions. Treatment of termites with the bacterial broth culture and the crude enzyme fraction of *B. licheniformis* PR2 resulted in a maximum mortality rate (with a median lethal time (ET50)) of 83.3% (3 h, 36 min) and 88.9% (2 h, 59 min), respectively. The termites treated with *B. licheniformis* PR2 exhibited loss of setae, disintegration of epicuticle, rupturing of procuticle, and swelling at sockets. The degradation of cuticular chitin and glycoprotein polymers in the termite cuticle by chitinase and protease enzymes produced by *B. licheniformis* PR2 represents an effective eco-friendly strategy for controlling termite damage in Korean cultural heritage sites and forests.

Keywords: wood structures; termite damage; entomopathogenic bacteria; cuticle-degrading enzymes; termite mortality

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1. Introduction

Termites (Isoptera) are a group of approximately 3000 species of soil-dwelling detritophagous insects with the ability to feed and decompose cellulosic biomass such as wood due to the presence of symbiotic protozoa in their hindguts [1–3]. The subterranean termites are highly eusocial insects that collectively forage by communicating the location of their food source to members of the same colony using sternal gland pheromones, which makes them highly effective foragers for specific wood substrates [2,4]. The life cycle of termites is still a subject of research, since the post-embryonic stage (early nymphs/im-matures) can undergo varying development pathways into different specialized castes in a colony [5–7]. For instance, in a young colony, the eggs hatch into nymphs (avoiding the hemimetabolous pathway to winged imago) and then molt several times into adult workers or sterile soldiers [7]. However, in old colonies, the eggs hatch into immatures that can in addition

produce winged alates (reproductive males or females) which later disperse to form new colonies at synchronized times of the year [5–7]. The alates make short dispersal/mating flights, shed off their wings (dealation), and with the help of sex-attractant pheromones, the dealates pair up on the ground (primary reproductive) to form new nests, and then mate (queen and king) to establish new colonies [5,7].

Termites have a wide range of ecological distributions, ranging from the tropics, arid and savanna vegetation, oceanic islands, and mangroves, to temperate regions, with higher diversity and abundance in the rainforests of Africa, South America, and Southeast Asia [8–10]. Despite their role in nutrient cycling through organic matter decomposition, termites are widely categorized as pests of live wood in tree trunks and wooden structures such as houses and cultural heritage sites [2,11,12]. Moreover, with the increasing changes in the global climate and substrate distribution, some termite species have invaded new ecological regions where they cause tremendous damage to wooden structures, forest trees, and crops [2,9,13]. Termites cause an estimated economic loss of approximately USD 40 billion globally per year, with a continuous increase in the scale of damage due to global warming [14–17]. They mainly move through underground tunnels to enter wooden buildings through the lower part of columns, attacking the inside of the wood in a concentric circle, which damages the original shape and destabilizes the structure of wooden buildings [2,14,18]. In the Republic of Korea, a number of subterranean termite species are known to cause substantial economic damage in agricultural/ornamental crops, live forest trees, especially *Pinus densiflora* Siebold and Zucc. (Pinales: Pinaceae), and wooden structures of cultural heritage [19,20]. The worker caste of *Reticulitermes speratus kyushuensis* Morimoto (Isoptera: Rhinotermitidae) is responsible for causing the most severe damage in the trunks of live trees and wooden structures, as observed in the live *P. densiflora* log (Figure 1).

Figure 1. (**A**) A brood of *R. speratus* worker termites on the trunk of *P. densiflora* and (**B**) Dorsal view of *R. speratus* worker termite.

There are various control strategies that have been used to reduce damage caused by termites, with synthetic pesticides or traditional wood preservatives being the most commonly preferred methods [2,16,21]. The application of synthetic pesticides (termiticides) including organophosphates (such as chlorpyrifos and endosulfan), carbamates, pyrethroids, and insect growth inhibitors have been developed, used, and phased out in succession as more effective and less harmful termiticides were developed in the mid-19th century [22,23]. More recently, termiticides such as chitin synthesis inhibitors (CSIs), neonicotinoids (imidacloprid), phenylpyrazoles (fipronil), bifenthrin, and chlorantraniliprole are among the most widely used chemical insecticides to control termites through fumigation, soil treatment, and colony removal [17,22–24]. The wooden structures are often protected from termite damage by clearing the palatable wood trees around the structure and conducting pre-treatment of the construction foundation and wood materials with termiticides [14]. Even though the use of chemical insecticides can rapidly cause termite mortality, their

continuous application, broad spectrum, and persistence in the soil cause devastating effects on human life, animal health, and to non-target organisms, including beneficial insects such as natural enemies and pollinators, and soil micro- and macrofauna [25–29]. Moreover, the indiscriminate use of chemical pesticides is also involved in the emergence of insecticide resistance in other insect pests, leading to outbreaks of secondary insect pests that would otherwise be maintained below threshold levels by natural enemies [30,31]. In addition, the continuous use of these toxicants contaminates the soil and groundwater, resulting in long-term retention and bioaccumulation of toxic residuals into the food chain, which causes health risks to humans and animals [23,32]. This creates an urgent need for developing environmentally friendly insecticides such as microbial-based biopesticides to protect non-target organisms and human health by eliminating toxic residues that are associated with conventional pesticides [8,30,33,34].

Several microorganisms, including bacteria, viruses, fungi, protozoa, and nematodes, are among the potential entomopathogens for controlling insect pests [8,33,35–39]. The use of bacterial entomopathogens such as *Bacillus* spp. is one of the most efficient strategies in the biological control of insect pests through various insecticidal mechanisms [35–37]. Recently, some entomopathogenic bacteria have been described to exhibit chitinolytic and proteolytic activities which cause the degradation of vital protective layers of the exoskeleton (cuticles) in insect pests [33,35,36,40]. In eusocial insects such as termites, enzymatic degradation of insect cuticles is more viable, since the cuticles of these insects are biochemically soft and poorly sclerotized, which in turn makes them more susceptible to insecticides [41–44]. In a previous study, *Bacillus licheniformis* PR2 was reported to produce the cuticle-degrading enzymes chitinase and protease. These cuticle-degrading enzymes effectively degrade cuticular chitin and glycoprotein polymers that constitute the exoskeleton of larvae of the lepidopteran insect pest *Hyphantria cunea* Drury (Lepidoptera: Erebidae) [36]. However, there is limited knowledge about the insecticidal effectiveness of this bacterial entomopathogen against isopteran insects such as termites. Therefore, the purpose of this study was to investigate the insecticidal activity of *B. licheniformis* PR2 entomopathogen as an eco-friendly alternative to synthetic insecticides in the control of *R. speratus* worker termites.

2. Materials and Methods

2.1. Bacterial Broth Culture Preparation and Collection of R. speratus Worker Termites

The entomopathogenic strain *B. licheniformis* PR2 was isolated from soil grown with pepper as previously described [45]. For the purpose of this study, the *B. licheniformis* PR2 strain was acquired from Purne Inc. (Jangseong-gun, Jeonnam-do, Republic of Korea). The bacterial strain was spread on tryptone soy agar (TSA) medium and the resulting pure single colony was re-inoculated into tryptone soy broth (TSB) medium for 48 h in an H1012 shaking incubator (Benchmark Scientific Inc., Edison, NJ, USA) set at 30 °C and 120 rpm. The culture was used for further experiments.

In April 2022, logs inhabited by *R. speratus* were collected from *P. densiflora* habitat at Chonnam National University arboretum in Gwangju-si, Korea. In order to prevent moisture loss and death of *R. speratus* worker termites used in this study, the *P. densiflora* branches were wrapped in plastic film during transportation to the laboratory (within 30 min). Then, *R. speratus* samples were immediately washed with tap water to remove adhering dust and identified based on morphological characteristics.

2.2. Cell Growth Curve of B. licheniformis PR2 and Production of Cuticle-Degrading Enzymes

For the examination of growth conditions of *B. licheniformis* PR2 and its production of chitinase and protease enzymes, 500 µL of the pre-inoculum (10^7 colony-forming unit (CFU)/mL) was pipetted into 500 mL of pink fertilizer broth medium (PB). The PB medium was composed of 1.5% NPK (20-20-20), 0.2% KH_2PO_4, 0.2% $MgSO_4$, 0.05% NaCl, 1.5% sucrose (carbon source), 0.25% chitin powder, and 0.3% yeast extract in distilled water. The bacterial strain was cultured in a shaking incubator at 120 rpm at 30 °C for 10 days,

and 1 mL samples were collected daily for CFU. Then, the samples were serially diluted for enumerating the viable cells of *B. licheniformis* PR2 with the plate spread technique on the TSA medium at 30 °C after 1 day of incubation, and the experiment was conducted in triplicate.

To determine the activity of the chitinase and protease enzymes produced by *B. licheniformis* PR2, 2 mL bacterial broth cultures collected on a daily basis for 10 days were centrifuged for 10 min at 13,000 rpm using a combi R515 centrifuge (Hanil Scientific Inc., Seoul, Republic of Korea), and the supernatants were analyzed for enzymatic activities. The activity of chitinase produced by *B. licheniformis* PR2 was determined using the method described by Hong et al. (2022) [46]. Then, 50 µL of the supernatant was added to 450 µL of 0.2 M sodium acetate ($C_2H_3NaO_2$) buffer (adjusted to pH 5.0) and then 500 µL of colloidal chitin (0.5%) was added and the mixture was incubated at 37 °C for 1 h in a temperature-controlled water bath 1660504EDU (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Then, 200 µL of 1 N NaOH was added to stop the reaction, and the solution was centrifuged at 13,000 rpm, and at 4 °C for 5 min, and 750 µL of the mixture was added to 1 mL of Schales' reagent, followed by 250 µL of distilled water. The contents were incubated in boiling water (at 100 °C) for 15 min, and the amount of reducing sugar was measured using a UV spectrophotometer (UV-1650PC, Shimadzu, Kyoto, Japan) at a wavelength of 420 nm. The experiment was repeated three times. One unit of chitinase activity was defined as the reducing activity that released 1 µmol of N-acetyl-glucosamine per hour at 37 °C.

The activity of the protease enzyme secreted by *B. licheniformis* PR2 was determined as previously described by Ghorbel-Frikha et al. (2005) [47]. The Tris buffer (100 mM) was prepared with 2 mM $CaCl_2$ and 1% casein, and the pH was adjusted to 8.0. Then, 50 µL of the bacterial supernatant was mixed with 950 µL of the buffer and the mixture was incubated for 15 min at 60 °C in a water bath. To terminate the reaction, 500 µL of 20% trichloroacetic acid (TCA) was added and the reaction mixture was centrifuged for 15 min at 13,000 rpm. Then, the absorbance of the supernatant was measured using a UV spectrophotometer at a wavelength of 280 nm, and the experiment was conducted in triplicate. One unit of protease activity was defined as the amount of enzyme required to produce 1 µg of tyrosine per min at 60 °C.

2.3. Preparation of Crude Enzyme Fraction from B. licheniformis PR2 Bacterial Broth Culture

The crude enzyme fraction of *B. licheniformis* PR2 was prepared from the bacterial culture grown in PB medium for 7 days at 30 °C and 130 rpm as previously described [48]. The bacterial broth culture was centrifuged for 30 min in a combi R515 centrifuge set at 6000 rpm and 4 °C, and the supernatant was filtered through four layers of Whatman No.6 filter paper (Whatman International Ltd., Maidstone, UK). The crude enzyme fraction was gradually precipitated from the filtrate using ammonium sulfate with gentle stirring at 4 °C, up to 80% saturation as described by Choub et al. (2021) [48]. Then, the precipitated proteins were separated by centrifugation for 30 min at 6000 rpm, after overnight stabilization of the proteins at 4 °C. Then, the pellet was dissolved in a minimum volume of 20 mM Tris-HCl buffer (pH 8.2) and the crude enzyme fraction was dialyzed at 4 °C for 24 h against the same buffer. Chitinase and protease activity in the crude enzyme fraction was determined as described above, based on the degradation of chitin and casein substrates, respectively. The total protein concentration in the crude enzyme fraction was determined based on the Bradford method, using bovine serum albumin as the standard [49]. The crude enzyme fraction was then kept at −70 °C for use in termiticidal experiments.

2.4. Anti-Termite Activity of B. licheniformis PR2 Broth Culture and Crude Enzymes on R. speratus Worker Termites

All experiments on the insecticidal activity of *B. licheniformis* PR2 against *R. speratus* worker termites were conducted in insect growth chambers (VS-91G09M-1300, VISION Co., Daejeon, Korea). Four treatment groups were used in this experiment; (1) control (only sterile distilled water), (2) PB medium without *B. licheniformis* PR2 inoculation, (3) Bacterial broth culture of *B. licheniformis* PR2 (67.4×10^7 CFU/mL), and (4) Bacterial crude enzymes of *B. licheniformis* PR2 (47.6 unit/mg of protein and 17.9 unit/mg of protein). Treatments were prepared by placing sterile, wet filter paper disks in pre-sterilized polystyrene insect-breeding dishes of 10 cm diameter and 4 cm height, with a ventilated lid, pore size = 0.053 mm (SPL Life Science Co., Ltd., Pocheon-si, Republic of Korea). Then, ten (10) worker termites were gently placed on paper in each insect-rearing dish and sprayed with 2 mL of each treatment solution. The experiment was repeated three times for each treatment. The experiment was conducted at 25 ± 2 °C, with a relative humidity of 70 ± 5%, and the light duration of 14:10 (L:D). A completely randomized design (CRD) was used and the mortality rate for *R. speratus* worker termites was evaluated hourly for 12 h after treatment.

The termite mortality was evaluated as a percentage of the number of dead termites to the initial number of termites in each treatment group. Termites were considered dead if they did not respond after being gently touched with a soft brush while being observed at a magnification of 10 × using an optical microscope (BX41TF, Olympus, Tokyo, Japan). The median lethal time (ET50) and the time required to attain 90% of the maximum response/termite mortality (ET90) were calculated from the regression curve in a Microsoft Excel spreadsheet (Microsoft Office Professional Plus 2019, Microsoft Corporation, Redmond, Washington, USA). In each treatment, dead termites were collected every day during the experimental period and kept in a fixative (4% p-formaldehyde). The surviving termites were also fixed after the study period (12 h) and all samples were stored at 4 °C in a separate sterile vial for morphological analysis.

2.5. Effect of Treatments on the Morphological Changes in Cuticle of R. speratus Worker Termites

To examine the morphological deformations in the cuticle of *R. speratus* worker termites due to treatment application, the fixed samples were washed in phosphate buffer (pH 7.4) three times to remove the paraformaldehyde. The samples were then processed through a concentration series of two dehydrating agents: ethanol (40, 60, 80, 95, and 100% at an interval of 30 min/step) and isoamyl acetate/ethanol at a concentration ratio of 1:2, 1:1, and pure isoamyl acetate (v/v), for 20, 30, and 60 min, respectively. Each sample was air-dried overnight under a fume hood. The dried sample was mounted on an aluminum pin stub with carbon conductive tape and gold coated at 60 °C. The cuticles of *R. speratus* worker termites were then observed on a GeminiSEM 500 scanning electron microscope (SEM) (Carl Zeiss AG, Oberkochen, Germany). The acceleration voltage was set at 15 kV and the magnification at 500×.

2.6. Statistical Analysis

Statistical analysis was performed using SPSS 25.0 statistical software (SPSS Inc., Chicago, IL, USA). Cell growth and the activity of cuticle-degrading enzyme produced by *B. licheniformis* PR2 were subjected to analysis of variance (ANOVA) using the Waller-Duncan test at $p = 0.01$. The data for termite mortality during the treatment period were subjected to two-way ANOVA and the results were presented as mean ± standard deviation.

3. Results

3.1. Cell Growth, Chitinase and Protease Production by B. licheniformis PR2

The cell growth of *B. licheniformis* PR2 gradually increased from day 1 to day 4 after inoculation, followed by a sharp increase from day 4 to day 5. The cell growth remained relatively constant from day 5 to day 6 before the second sharp increase to a maximum of

67.4 × 10^7 CFU/mL after 7 days post-inoculation (Figure 2A). Following the maximum cell growth, the number of viable cells sharply declined until the final sampling day (day 10).

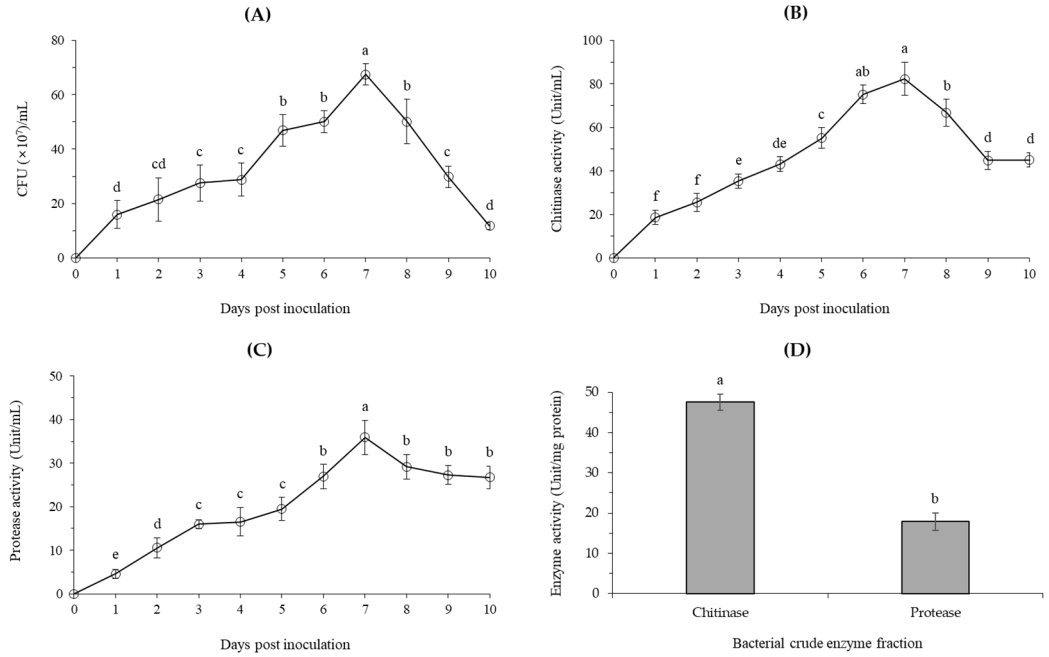

Figure 2. The viable cell count (**A**), chitinase enzyme activity (**B**), protease enzyme activity (**C**), and crude enzyme activity of *B. licheniformis* PR2 (**D**). The bacterial culture was grown in PB medium at 30 °C for 10 days. The bacterial culture for crude enzyme preparation was grown for 7 days. All points represent mean ± standard deviation (n = 3). Different superscript letters in a figure indicate that values are significantly different (p = 0.01).

During growth, *B. licheniformis* PR2 produced high concentrations of cuticle-degrading enzymes, chitinase, and protease in the broth culture. Chitinase activity showed a steady increase from day 1 up to day 7 post-inoculation, attaining a maximum activity of 82.3 unit/mL, which is consistent with the increase in number of viable cells in the same period (Figure 2B). Then, a slight decline in chitinase enzyme activity occurred from day 8 to day 9 of inoculation. The activity of chitinase then remained stable until the end of the experimental period. A similar phenomenon was observed for protease enzyme activity, which gradually increased from day 1 to day 3 and remained relatively stable up to day 5 of inoculation (Figure 2C). From day 5 onwards, the enzyme activity of protease showed a faster rate of increase and reached the peak of 35.9 unit/mL on day 7 after inoculation. Subsequently, the activity of protease slightly decreased from day 7 to day 8 and then showed a relatively stable pattern until the end of the experiment. The activity of both enzymes was positively correlated to the pattern of *B. licheniformis* PR2 cell growth in the first 7 days after inoculation. The crude enzyme fraction showed chitinase and protease enzyme activity of 47.6 units/mg of protein and 17.9 units/mg of protein, respectively. The activity of chitinase and protease enzymes did not vary before and after storage at −70 °C.

3.2. Anti-Termite Activity of B. licheniformis PR2 against R. speratus Worker Termites

Both the crude enzymes and the bacterial broth culture of *B. licheniformis* PR2 showed a high anti-termite effect on *R. speratus* worker termites under laboratory conditions (Figure 3A). The anti-termite effect of both the crude enzymes and bacterial broth culture of *B. licheniformis* PR2 increased with time from 1 h to 11 h after treatment application. Treatment with crude enzymes resulted in a faster and higher lethal effect than the bacterial broth culture treatment. After 1 h of treatment application, the mortality rate of termites in the crude enzymes and bacterial broth culture treatments were only 8.9% and 6.7%, respectively. By the end of the experiment (after 12 h), the termite mortality rate had

increased by 10.0 and 12.4 times to 88.9% and 83.3% in the crude enzymes and bacterial broth culture treatments, respectively. Based on the regression analysis for the bacterial broth culture against subterranean worker termites, the median lethal time (ET50) and the time require to attain 90% of the termite mortality (ET90) were 3 h, 36 min, and 9 h, 6 min, respectively (Figure 3B). The ET50 and ET90 for the termiticidal effect of crude enzyme fraction were 2 h, 59 min and 7 h, 41 min, respectively (Figure 3C). However, PB medium treatment and control group did not have a significant lethal effect on *R. speratus* workers during the experimental period.

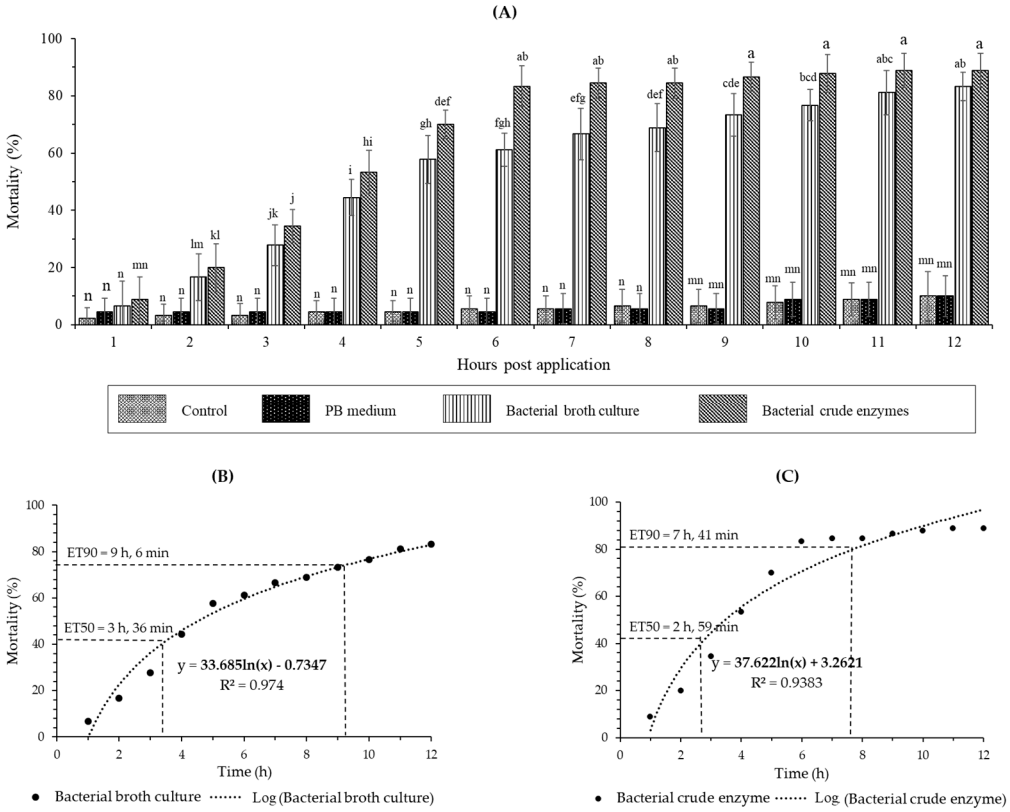

Figure 3. The mortality rate of *R. speratus* worker termites in the control group, PB medium, bacterial broth culture, and bacterial crude enzymes from *B. licheniformis* PR2 under laboratory conditions (**A**). Regression curves illustrating the median lethal time (ET50) and the time required to attain 90% of the maximum response (ET90), for the bacterial broth culture (**B**), and crude enzyme fraction (**C**). The bars represent mean ± standard deviation (n = 3). Different superscript letters a–n in the figure indicate that values are significantly different (p = 0.01).

3.3. Morphological Cuticle Deformation of R. speratus Worker Termites after Treatment

The SEM analysis of the morphological changes in the cuticle of *R. speratus* worker termites treated with the bacterial broth culture and the crude enzyme treatments (Figure 4C,D) revealed deleterious degradation of the termite cuticle compared to PB medium treatment and the control group (Figure 4A,B). The morphological characteristics of dead *R. speratus* worker termites from the bacterial broth culture treatment revealed a ruptured epicuticle and some broken setae. In the crude enzyme treatment, the dead termites exhibited complete rupturing of the epicuticle and the procuticle, some broken setae, and abnormal swelling at sockets. However, the cuticle of dead *R. speratus* worker termites in the PB medium treatment and control group showed normal cuticle morphologies with healthy setae (Figure 4).

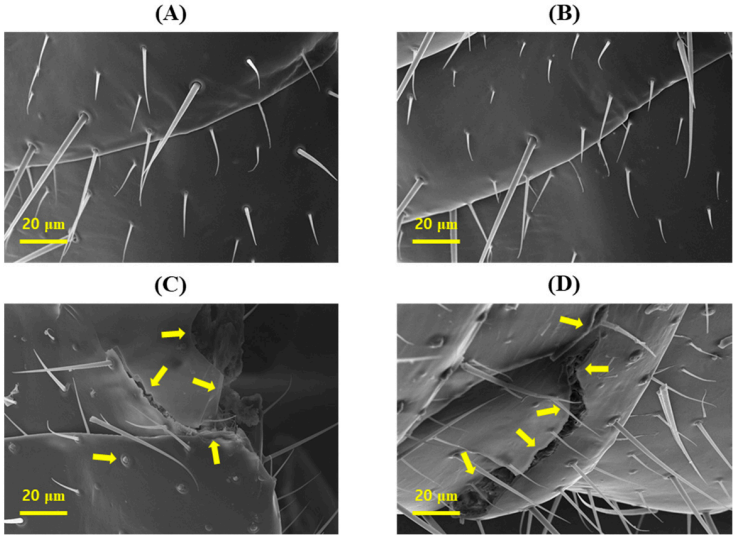

Figure 4. The scanning electron micrographs indicating the morphological characteristics in the cuticles of *R. speratus* worker termites in the different treatment groups; control (**A**), PB medium (**B**), bacterial broth culture (**C**), and bacterial crude enzymes (**D**) from *B. licheniformis* PR2 under laboratory conditions. The yellow arrows indicate cuticle deformations such as rupturing, swollen socket and broken setae due to treatment application.

4. Discussion

The arthropod cuticle (especially in insects) is a biological composite material that contains fibrous chains of alpha-chitin which combine to form crystalline structures with H-bonded sugar residues [50]. These cuticular chitin nanofibrils are interlinked to silk-like collagen and globular proteins to provide for vital structural, protective, and sensory functions of the insect cuticle [50,51]. For instance, the insect cuticle serves multiple life functions, including body shape stabilization by holding appendages and internal organs in their right positions, aids in locomotion, prevents desiccation, and serves as a physical barrier against toxins and pathogenic infections [50–52]. In most terrestrial insects, such as the Coleoptera order, the epicuticle is covered by a single waxy layer that prevents water loss [53,54]. The wax is mainly composed of surface lipids and hydrocarbon mixtures which play a waterproofing role on the surface of the epicuticle of insects [53,54]. Thus, the disruption or degradation of insect cuticles is one of the most promising strategies in the control of insect pests [35,36,40,55]. The role of cuticle-degrading enzymes such as chitinase and protease has been elucidated as a mechanism by which some entomopathogenic microbes control insect pests in agriculture and forestry [35,36,40,56]. Among the bacterial entomopathogens, *Bacillus* spp. are the most prospective biological control agents against insect pests, which in part is attributed to the secretion of cuticle-degrading enzymes such as chitinase and protease [35,36]. In this study, *B. licheniformis* PR2 secreted cuticle-degrading enzymes such as chitinase and protease into bacterial broth culture during its growth phase. These cuticle-degrading enzymes have the potential to hydrolyze the cuticular chitin polymers and structural proteins that are vital for the survival of insects [40,57–59]. The SEM analysis of the cuticles in *R. speratus* worker termites treated with the crude enzyme fraction (containing chitinase and protease) and bacterial broth culture of *B. licheniformis* PR2 revealed morphological deformation such as the loss of setae, rupturing of the cuticle surface, and swelling at sockets. The degradation of the cuticle by the crude enzyme fraction of chitinase and protease indicates the surface of the termite epicuticle could be largely composed of chitin and glycoprotein fibers. Both the crude enzymes and the bacterial broth culture of *B. licheniformis* PR2 showed a high anti-termite effect on *R. speratus* worker termites. Thus, the production of cuticle-degrading chitinase and protease enzymes by *B. licheniformis* PR2 could play a significant role in the biological control of insect pests such as *R. speratus* worker termites.

In addition, treatment of *R. speratus* worker termites with crude enzymes and bacterial broth culture from *B. licheniformis* PR2 caused a maximum mortality rate of 88.9% and 83.3%, respectively. Specifically, treatment with crude enzymes had a higher rate of termite mortality and showed a rapid anti-termite activity compared to bacterial broth culture. From 5 h to 10 h after termite treatment, the mortality rate in the group treated with crude enzymes was significantly ($p < 0.01$) higher than that in the group treated with the bacterial broth culture. Treatment with crude enzymes caused a significant change in termite mortality in each successive hour from 1 h to 6 h after treatment application. Treatment of subterranean worker termites with the crude enzyme fraction reduced the ET50 by 36 min, and ET90 by 1 h 25 min compared to bacterial broth culture treatment. This indicates a higher efficacy of the crude enzyme fraction due to the increased concentration of the chitinase and proteinase enzymes. The termites in the PB medium and the control group showed a negligible mortality rate of only 10.0% in each group by the end of the experiment. This was also consistent with SEM analysis, which revealed that dead termites in the PB medium and the control group exhibited normal cuticle shapes with healthy sockets and setae. Thus, the observed termite mortality in both the crude enzymes and bacterial broth culture treatments could be a direct result of the activity of cuticle-degrading enzymes such as chitinase and protease. Unlike in other insects such as the Coleoptera, the nature of termite cuticles could have unique weaknesses that may contribute to the susceptibility to cuticle degradation [60,61]. For instance, wet wood termites are extremely prone to desiccation and require high relative humidity to survive, which indicates a potential deficiency in the development of a waterproof waxy layer on the surface of the epicuticle [60,61]. This could also reduce the barrier and allows the contact of treatments with the epicuticle, which consists of a cuticulin bilayer of chitin, lipoproteins, and protein-polyphenol sandwiching fatty acid polymers [34,40,62]. The activity of chitinase and protease on the epicuticle causes the degradation of cuticular chitin and protein polymers [34,62]. Moreover, the termite procuticle is also unsclerotized, and thus, the procuticle (potentially dominated by weaker arthropodin protein) could be more susceptible to the hydrolyzing activity of proteases compared to other insect cuticles that contain sclerotin proteins [44,60,61]. The degradation of termite cuticles causes rupturing and subsequent loss of moisture which in turn leads to rapid termite mortality due to their low tolerance to desiccation [35,36,40,60,61]. Moreover, the rupturing of the termite cuticles also exposes internal tissues to environmental aggressions, such as microbial infections and pressure, while the damage to the sensory organs/setae reduces the insect's mobility, which could all cumulatively accelerate termite mortality [35,36,40,51]. These findings are consistent with previous studies that demonstrated the potential of cuticle-degrading enzymes such as chitinase and protease from entomopathogenic bacteria. For instance, in the biological control of various insect pests such as the larvae of the fall webworm *H. cunea* (Lepidoptera: Erebidae) [36], the jujube gall midge *Dasyneura jujubifolia* Jiao and Bu (Diptera: Cecidomyiidae) [35], and subterranean termites, (Isoptera: Rhinotermitidae) [33,62]. Moon et al. (2022) demonstrated the insecticidal and repellent effect of *B. licheniformis* PR2 against fall webworm larvae by inducing cuticle degradation, which was linked to the activities of chitinase and protease enzymes [36]. Therefore, the results of this study confirm that *B. licheniformis* PR2 could be effectively used to control several insect pests, including *R. speratus,* through the production of cuticle-degrading chitinase and protease enzymes.

5. Conclusions

Bacillus licheniformis PR2 produced cuticle-degrading chitinase and protease enzymes during its growth. Treatment of termites with the bacterial broth culture and crude enzyme fraction effectively degraded the termite cuticle and lead to a rapid rate of termite mortality. The degradation of termite cuticles could in part be attributed to the degradation of chitin and protein fibers in the soft unsclerotized nature termite cuticles. The treatment with crude enzyme fraction substantially lowered the ET50 and ET90 values compared to the bacterial culture broth, which indicates a higher termiticidal efficacy of the purified chitinase and

protease enzymes. The rapid and high termiticidal efficacy of *B. licheniformis* PR2 highlights its potential for field application to control termite damage and reduce the use of harmful chemical pesticides. This is the first report to demonstrate the potential of cuticle-degrading chitinase and protease enzymes secreted by *B. licheniformis* PR2 as an effective and eco-friendly biological control strategy against *R. speratus* worker termites. Further studies such as the chemical analysis of the cuticle before and after treatment could help to elucidate the precise mode of cuticle degradation.

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References

1. Bignell, D.E.; Eggleton, P. Termites in ecosystems. In *Termites: Evolution, Sociality, Symbioses, Ecology*; Abe, T., Bignell, D.E., Higashi, M., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000; pp. 363–387. [CrossRef]
2. Kim, S.; Lee, S.; Lim, I. Study of minimum passage size of subterranean termites (*Reticulitermes speratus kyushuensis*). *J. Cult. Herit. Stud. (Munhwaje)* **2020**, *53*, 188–197. [CrossRef]
3. Moore, B.P. Biochemical studies in termites. In *Biology of Termites*; Krishna, K., Weesner, F.M., Eds.; Academic Press: New York, NY, USA, 1969; pp. 407–432.
4. Traniello, J.F.A.; Leuthold, R.H. Behavior and ecology of foraging in termites. In *Termites: Evolution, Sociality, Symbioses, Ecology*; Abe, T., Bignell, D.E., Higashi, M., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000; pp. 141–168. [CrossRef]
5. Lainé, L.V.; Wright, D.J. The life cycle of *Reticulitermes* spp. (Isoptera: Rhinotermitidae): What do we know? *Bull. Entomol. Res.* **2003**, *93*, 267–278. [CrossRef]
6. Engel, M.S.; Barden, P.; Riccio, M.L.; Grimaldi, D.A. Morphologically specialized termite castes and advanced sociality in the early cretaceous. *Curr. Biol.* **2016**, *26*, 522–530. [CrossRef] [PubMed]
7. Roisin, Y. Diversity and evolution of caste patterns. In *Termites: Evolution, Sociality, Symbioses, Ecology*; Abe, T., Bignell, D.E., Higashi, M., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000; pp. 95–119. [CrossRef]
8. Sindhu, S.S.; Rakshiya, Y.S.; Verma, M.K. Biological control of termites by antagonistic soil microorganisms. In *Bioaugmentation, Biostimulation and Biocontrol*; Singh, A., Parmar, N., Kuhad, R.C., Eds.; Springer: Berlin, Germany, 2011; pp. 261–309. [CrossRef]
9. Eggleton, P.; Tayasu, I. Feeding groups, lifetypes and the global ecology of termites. *Ecol. Res.* **2001**, *16*, 941–960. [CrossRef]
10. Eggleton, P. Global patterns of termite diversity. In *Termites: Evolution, Sociality, Symbioses, Ecology*; Abe, T., Bignell, D.E., Higashi, M., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000; pp. 25–54. [CrossRef]
11. Holt, J.A.; Lepage, M. Termites and soil properties. In *Termites: Evolution, Sociality, Symbioses, Ecology*; Abe, T., Bignell, D.E., Higashi, M., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000; pp. 389–407. [CrossRef]
12. Su, N.-Y.; Scheffrahn, R.H. Termites as pests of buildings. In *Termites: Evolution, Sociality, Symbioses, Ecology*; Abe, T., Bignell, D.E., Higashi, M., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000; pp. 437–453. [CrossRef]
13. Buczkowski, G.; Bertelsmeier, C. Invasive termites in a changing climate: A global perspective. *Ecol. Evol.* **2017**, *7*, 974–985. [CrossRef]
14. Ghaly, A.; Edwards, S. Termite damage to buildings: Nature of attacks and preventive construction methods. *Am. J. Eng. Appl. Sci.* **2011**, *4*, 187–200. [CrossRef]
15. Indrayani, Y.; Setyawati, D.; Maryani, Y.; Simangunsong, B. A termite attack on rubber plantation on peat soil: Level of damage and identification of pest species. *IOP Conf. Ser. Earth Environ. Sci.* **2022**, *976*, 012006. [CrossRef]
16. Park, I.K.; Shin, S.C. Fumigant activity of plant essential oils and components from garlic (*Allium sativum*) and clove bud (*Eugenia caryophyllata)* oils against the Japanese termite (*Reticulitermes speratus Kolbe*). *J. Agric. Food Chem.* **2005**, *53*, 4388–4392. [CrossRef]

17. Ahmad, F.; Fouad, H.; Liang, S.Y.; Hu, Y.; Mo, J.C. Termites, and Chinese agricultural system: Applications and advances in integrated termite management and chemical control. *Insect Sci.* **2021**, *28*, 2–20. [CrossRef]
18. Lee, J.M.; Kim, Y.H.; Hong, J.Y.; Lim, B.; Park, J.H. Exploration of preservatives that inhibit wood feeding by inhibiting termite intestinal enzyme activity. *J. Korean Wood Sci. Technol.* **2020**, *48*, 376–392. [CrossRef]
19. Im, I.G.; Cha, H.S.; Kang, W.C.; Lee, S.B.; Han, G.S. The status of damage and monitoring of subterranean termite (*Reticulitermes* spp.) (Blattodea: Rhinotermitidae) for wooden cultural heritage in Korea. *J. Conserv. Sci.* **2021**, *37*, 191–208. [CrossRef]
20. Han, S.H.; Lee, K.S.; Chung, Y.J. Characteristic of termite inhabits in South Korea and the control. *Conserv. Stud.* **1998**, *19*, 133–158.
21. Kim, S.H.; Chung, Y.J. Ingestion toxicity of fipronil on *Reticulitermes speratus kyushuensis* (Isoptera: Rhinotermitidae) and its applicability as a termite bait. *J. Korean Wood Sci. Technol.* **2017**, *45*, 159–167. [CrossRef]
22. Lee, S.; Im, I.; Kim, S. A history of termite control and improvements to prevent termites in wooden architectural heritage. *J. Cult. Herit. Stud. (Munhwaje)* **2021**, *54*, 194–215. [CrossRef]
23. Lemus, R.; Abdelghani, A. Chlorpyrifos: An unwelcome pesticide in our homes. *Rev. Environ. Health* **2000**, *15*, 421–433. [CrossRef]
24. Gordon, J.M.; Velenovsky IV, J.F.; Chouvenc, T. Subterranean termite colony elimination can be achieved even when only a small proportion of foragers feed upon a CSI bait. *J. Pest Sci.* **2022**, *95*, 1207–1216. [CrossRef]
25. He, B.; Ni, Y.; Jin, Y.; Fu, Z. Pesticides-induced energy metabolic disorders. *Sci. Total Environ.* **2020**, *729*, 139033. [CrossRef]
26. Meftaul, I.M.; Venkateswarlu, K.; Dharmarajan, R.; Annamalai, P.; Megharaj, M. Pesticides in the urban environment: A potential threat that knocks at the door. *Sci. Total Environ.* **2020**, *711*, 134612. [CrossRef]
27. Carvalho, F.P. Pesticides, environment, and food safety. *Food Energy Secur.* **2017**, *6*, 48–60. [CrossRef]
28. Mahmood, I.; Imadi, S.R.; Shazadi, K.; Gul, A.; Hakeem, K.R. Effects of pesticides on environment. In *Plant, Soil and Microbes*; Hakeem, K., Akhtar, M., Abdullah, S., Eds.; Springer: Cham, Switzerland, 2016; pp. 253–269.
29. Gargouri, B.; Yousif, N.M.; Bouchard, M.; Fetoui, H.; Fiebich, B.L. Inflammatory and cytotoxic effects of bifenthrin in primary microglia and organotypic hippocampal slice cultures. *J. Neuroinflamm.* **2018**, *15*, 159. [CrossRef]
30. Rezende-Teixeira, P.; Dusi, R.G.; Jimenez, P.C.; Espindola, L.S.; Costa-Lotufo, L.V. What can we learn from commercial insecticides? Efficacy, toxicity, environmental impacts, and future developments. *Environ. Pollut.* **2022**, *300*, 118983. [CrossRef] [PubMed]
31. Osbrink, W.L.; Lax, A.R.; Brenner, R.J. Insecticide susceptibility in *Coptotermes formosanus* and *Reticulitermes virginicus* (Isoptera: Rhinotermitidae). *J. Econ. Entomol.* **2001**, *94*, 1217–1228. [CrossRef] [PubMed]
32. Singh, N.S.; Sharma, R.; Parween, T.; Patanjali, P.K. Pesticide contamination and human health risk factor. In *Modern Age Environmental Problems and Their Remediation*; Oves, M., Zain, K.M., Ismail, I.M.I., Eds.; Springer: Cham, Switzerland, 2016; pp. 49–68. [CrossRef]
33. Rakshiya, Y.S.; Verma, M.K.; Sindhu, S.S. Efficacy of antagonistic soil bacteria in management of subterranean termites (Isoptera). *Res. Environ. Life Sci.* **2016**, *9*, 949–955.
34. Jabeen, F.; Hussain, A.; Manzoor, M.; Younis, T.; Rasul, A.; Qazi, J.I. Potential of bacterial chitinolytic, *Stenotrophomonas maltophilia*, in biological control of termites. *Egypt. J. Biol. Pest Control* **2018**, *28*, 86. [CrossRef]
35. Choi, S.-I.; Ajuna, H.B.; Won, S.-J.; Choub, V.; Kim, C.-W.; Moon, J.-H.; Ahn, Y.S. The insecticidal potential of *Bacillus velezensis* CE 100 against *Dasineura jujubifolia* Jiao & Bu (Diptera: Cecidomyiidae) larvae infestation and its role in the enhancement of yield and fruit quality of jujube (*Zizyphus jujuba* Miller var. *inermis* Rehder). *Crop Prot.* **2023**, *163*, 106098. [CrossRef]
36. Moon, J.-H.; Won, S.-J.; Choub, V.; Choi, S.-I.; Ajuna, H.B.; Ahn, Y.S. Biological control of fall webworm larva (*Hyphantria cunea* Drury) and growth promotion of poplar seedlings (*Populus*×*canadensis* Moench) with *Bacillus licheniformis* PR2. *For. Ecol. Manag.* **2022**, *525*, 120574. [CrossRef]
37. Ajuna, H.B.; Kim, I.; Han, Y.S.; Maung, C.E.H.; Kim, K.Y. Aphicidal activity of *Bacillus thuringiensis* strain AH-2 against cotton aphid (*Aphis gossypii*). *Entomol. Res.* **2021**, *51*, 151–160. [CrossRef]
38. Kalha, C.; Singh, P.; Kang, S.; Hunjan, M.; Gupta, V.; Sharma, R. Entomopathogenic viruses and bacteria for insect–pest control. In *Integrated Pest Management: Current Concepts and Ecological Perspective*; Abrol, D.P., Ed.; Elsevier: Amsterdam, The Netherlands, 2014; pp. 225–244. [CrossRef]
39. Wang, W.; Zhou, L.; Dong, G.; Chen, F. Isolation and identification of entomopathogenic fungi and an evaluation of their actions against the larvae of the fall webworm, *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae). *Biocontrol* **2020**, *65*, 101–111. [CrossRef]
40. Leger, R.S.; Cooper, R.M.; Charnley, A.K. Cuticle-degrading enzymes of entomopathogenic fungi: Cuticle degradation in vitro by enzymes from entomopathogens. *J. Invertebr. Pathol.* **1986**, *47*, 167–177. [CrossRef]
41. Bordereau, C.; Andersen, S.O. Structural cuticular proteins in termite queens. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **1978**, *60*, 251–256. [CrossRef]
42. Ye, C.; Song, Z.; Wu, T.; Zhang, W.; Saba, N.U.; Xing, L.; Su, X. Endocuticle is involved in caste differentiation of the lower termite. *Curr. Zool.* **2021**, *67*, 489–499. [CrossRef] [PubMed]
43. Mahapatro, G.K. Can insecticide resistance be developed in termites? *Curr. Sci.* **2017**, *112*, 1097–1098. [CrossRef]
44. Kakkar, G.; Chouvenc, T.; Su, N.-Y. Postecdysis sclerotization of mouthparts of the formosan subterranean termites (Isoptera: Rhinotermitidae). *J. Econ. Entomol.* **2016**, *109*, 792–799. [CrossRef]
45. Kwon, J.-H.; Won, S.-J.; Moon, J.-H.; Lee, U.; Park, Y.S.; Maung, C.E.H.; Ajuna, H.B.; Ahn, Y.S. *Bacillus licheniformis* PR2 controls fungal diseases and increases production of jujube fruit under field conditions. *Horticulturae* **2021**, *7*, 49. [CrossRef]
46. Hong, S.; Kim, T.Y.; Won, S.-J.; Moon, J.-H.; Ajuna, H.B.; Kim, K.Y.; Ahn, Y.S. Control of fungal diseases and fruit yield improvement of strawberry using *Bacillus velezensis* CE 100. *Microorganisms* **2022**, *10*, 365. [CrossRef]

47. Ghorbel-Frikha, B.; Sellami-Kamoun, A.; Fakhfakh, N.; Haddar, A.; Manni, L.; Nasri, M. Production and purification of a calcium-dependent protease from *Bacillus cereus* BG1. *J. Ind. Microbiol. Biotechnol.* **2005**, *32*, 186–194. [CrossRef]
48. Choub, V.; Ajuna, H.B.; Won, S.-J.; Moon, J.-H.; Choi, S.-I.; Maung, C.E.H.; Kim, C.-W.; Ahn, Y.S. Antifungal activity of *Bacillus velezensis* CE 100 against anthracnose disease (*Colletotrichum gloeosporioides*) and growth promotion of walnut (*Juglans regia* L.) trees. *Int. J. Mol. Sci.* **2021**, *22*, 10438. [CrossRef] [PubMed]
49. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
50. Vincent, J.F. Arthropod cuticle: A natural composite shell system. *Compos. Part A Appl. Sci. Manuf.* **2002**, *33*, 1311–1315. [CrossRef]
51. Vincent, J.F.; Wegst, U.G. Design and mechanical properties of insect cuticle. *Arthropod Struct. Dev.* **2004**, *33*, 187–199. [CrossRef]
52. Moussian, B. The arthropod cuticle. In *Arthropod Biology and Evolution: Molecules, Development, Morphology*; Minelli, A., Boxshall, G., Fusco, G., Eds.; Springer: Berlin, Germany, 2013; pp. 171–196. [CrossRef]
53. Gibbs, A. Physical properties of insect cuticular hydrocarbons: Model mixtures and lipid interactions. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **1995**, *112*, 667–672. [CrossRef]
54. Mitov, M.; Soldan, V.; Balor, S. Observation of an anisotropic texture inside the wax layer of insect cuticle. *Arthropod Struct. Dev.* **2018**, *47*, 622–626. [CrossRef] [PubMed]
55. Merzendorfer, H. Chitin synthesis inhibitors: Old molecules and new developments. *Insect Sci.* **2013**, *20*, 121–138. [CrossRef] [PubMed]
56. De Moraes, C.K.; Schrank, A.; Vainstein, M.H. Regulation of extracellular chitinases and proteases in the entomopathogen and acaricide *Metarhizium anisopliae*. *Curr. Microbiol.* **2003**, *46*, 205–210. [CrossRef]
57. Stoykov, Y.M.; Pavlov, A.I.; Krastanov, A.I. Chitinase biotechnology: Production, purification, and application. *Eng. Life Sci.* **2015**, *15*, 30–38. [CrossRef]
58. Fang, W.; Feng, J.; Fan, Y.; Zhang, Y.; Bidochka, M.J.; Leger, R.J.S.; Pei, Y. Expressing a fusion protein with protease and chitinase activities increases the virulence of the insect pathogen *Beauveria bassiana*. *J. Invertebr. Pathol.* **2009**, *102*, 155–159. [CrossRef] [PubMed]
59. Mótyán, J.A.; Tóth, F.; Tőzsér, J. Research applications of proteolytic enzymes in molecular biology. *Biomolecules* **2013**, *3*, 923–942. [CrossRef]
60. Zukowski, J.; Su, N.-Y. Survival of termites (Isoptera) exposed to various levels of relative humidity (RH) and water availability, and their RH preferences. *Fla. Entomol.* **2017**, *100*, 532–538. [CrossRef]
61. Zukowski, J.; Su, N.-Y. A comparison of morphology among four termite species with different moisture requirements. *Insects* **2020**, *11*, 262. [CrossRef]
62. Fu, R.; Luo, J.; Feng, K.; Lu, X.; Tang, F. Termite-killing components in *Serratia marcescens* (SM1). *J. For. Res.* **2020**, *32*, 1739–1744. [CrossRef]