Mechanisms of Insecticidal Action of *Metarhizium anisopliae* on Adult Japanese Pine Sawyer Beetles (*Monochamus alternatus*)

Ho Myeong Kim, Seul-Gi Jeong, In Seong Choi, Jung Eun Yang, Kwang Ho Lee, Junheon Kim, Jong Cheol Kim, Jae Su Kim, and Hae Woong Park*

ABSTRACT: Pine wilt disease, caused by *Bursaphelenchus xylophilus* (pine wood nematode), leads to severe environmental and economic damage. Here, we report the results of experiments on the biological control of pine wilt disease through termination of the insect vector of the nematode and the mechanism of the insecticidal action of *Metarhizium anisopliae* JEF-279 against *Monochamus alternatus* (Japanese pine sawyer). A combined treatment with a fungal conidia suspension and a fungal protease-containing culture filtrate caused 75.8% mortality of the insect vector. Additionally, the presence of destruxins was confirmed in the dead Japanese pine sawyer adults, and half of the 10 protein spots in proteomic analysis were identified as an actin related to muscle contraction. Based on proteomic and microscopic analyses, the infection cycle of the Japanese pine sawyer by *M. anisopliae* JEF-279 was inferred to proceed in the following sequence: (1) host adhesion and germination, (2) epicuticle degradation, (3) growth as blastospore, (4) killing by various fungal toxins (insecticidal metabolites), (5) immune response as defense mechanism, and (6) hyphal extrusion and conidiation. Consequently, the combined fungal conidia suspension and protease-containing culture filtrate treatment may be applied as an insecticidal agent, and flaccid paralysis is likely a major mechanism underlying the insecticidal action of *M. anisopliae* JEF-279 on host insects.

1. INTRODUCTION

Pine wilt disease (PWD) is a deadly forest disease that kills pine trees belonging to the genus *Pinus* around the world. PWD is caused by the pine wood nematode (PWN) *Bursaphelenchus xylophilus* Nickle (Aphelenchida: Aphelenchoiidae) and results in severe environmental and economic damages in Asia (Korea, China, Japan, and Taiwan), Europe (Spain and Portugal), and North America (the United States of America, Canada, and Mexico). Needles initially decolorize, then turn brown, and eventually die in several months. Generally, browning of plants is strongly influenced by polyphenol oxidase, which induces the oxidation of phenolic compounds during the cellular disorganization. Once pine trees are infected with PWN, increased volatile terpenes are observed in the xylem tissue, resulting in tracheid cavitation, which leads to the disruption of water influx in pine trees. The transmission of PWN to healthy pine trees is mainly performed by the insect vector *Monochamus alternatus* HOPE (Coleoptera: Cerambycidae) (Japanese pine sawyer beetle, JPS) in Korea, China, and Japan. The role of this insect species is critical to the spread of PWD, so it is important to develop an effective means of controlling it.

For this purpose, the primary modality employed has been the use of chemical pesticides such as acetamiprid, buprofezin, metam sodium, and thiacloprid. Chemicals are sprayed on adult-stage JPS in pine crowns and on larvae in dead infested logs. However, this method of control is neither effective nor practical. Furthermore, chemical pesticides pose environmental hazards, such as contamination of air, soil, and water (surface water and ground water). The current global overuse of chemical pesticides consequently represents a direct threat to human health and nontarget species. To circumvent these disadvantages, biocontrol methods employing microbial pathogens have been assessed for their capacity to control JPS. In particular, entomopathogenic fungi of the genus *Metarhizium* have been extensively studied and cultured as a means of replacing chemical insecticides or at least reducing the dosage of chemicals used to control the insect vector.

As these fungi are originally isolated from soils, they have a broad range of insect hosts. The main *Metarhizium* species developed to date for the control of various insect pests is *M. anisopliae*, which has been used to control JPS. The insecticidal action of *M. anisopliae* JEF-279 on JPS was investigated in the present study.
2. RESULTS AND DISCUSSION

2.1. Insecticidal Effect of a Fungal Conidia Suspension and a Protease-Containing Culture Filtrate Treatment on JPS Adults. Serine proteases are proteolytic enzymes that play critical roles in the immune response and signal transduction pathways in a variety of insects. An entomopathogenic strain of this species M. anisopliae JEF-279, has been previously isolated from forest soil. Insect mortality using a spraying method was observed to be dose- and time-dependent. Pathogenicity was higher under high relative humidity conditions (94%). Insecticidal proteases are cuticle-degrading proteases showing elastinolytic properties. Further, the subtilisin-like serine protease PR1A as an extracellular enzyme (Supporting Figure S1). Insect mortality differed with treatment, i.e., fungal conidia suspension (FCS), protease-containing culture filtrate, or combined FCS and protease-containing culture filtrate (CFP) ($F = 75.6, df = 3.96, P < 0.001$). Five days after the treatment, insect mortality rates caused by FCS and the protease-containing culture filtrate treatment were 42 and 18%, respectively, while that caused by CFP was 74% (Figure 1a).

Lethal time values at 50% (LT$_{50}$) of JPS adults were 135.3 h for FCS, 160.5 h for protease-containing culture filtrate, and 100.1 h for CFP (Table 1).

The protease-containing culture filtrate treatment promoted faster development of pathogenicity, starting 4 days after treatment. Bacterial, plant, fungal, or insect proteases are candidate insecticidal agents that can destroy essential proteins in insects. Thus, for example, subtilisin-like serine proteases produced by entomopathogenic fungi are closely associated with penetration and colonization of host insects. As the cuticle of insects consists mostly of protein (70%), proteases can attack the insect cuticle. Such is the case for serine protease in M. anisopliae, which reportedly hydrolyzes cuticle proteins and penetrates the host cuticle. Therefore, the protease-containing culture filtrate treatment on JPS resulted in an increase in insect mortality, damaging the insect cuticle and facilitating penetration into the hemocoel.

Conidia of M. anisopliae JEF-279 (Figure 1e–j) were observed in JPS corpses 13 days after the conidia treatment, while no conidia were observed in nontreated JPS (Figure 1b–d). The CFP treatment resulted in a considerably higher number of conidia on the neck, thorax, and back of JPS than the FCS treatment did (Figure 1h–j). These results suggested...
that the subtilisin-like serine protease PR1A accelerated the development of pathogenicity against JPS adults more than the FCS or protease-containing culture filtrate treatment alone. Protease-containing culture filtrate could be used as a biological synergist added to FCS to kill the insects effectively. For practical use, however, the mass production process of the protease must be optimized for cost-effectiveness.

2.2. Analysis of the Mechanism of Insecticidal Action of M. anisopliae JEF-279

2.2.1. Proteomic Analysis by Two-Dimensional Gel Electrophoresis (2DE) and Liquid Chromatography-Tandem Mass Spectrometry (LC–MS/MS) Analysis

The mechanism of the insecticidal action of M. anisopliae JEF-279 against JPS adults was analyzed at the proteomic level; this can show the dynamic state of a tissue, cell, or whole organism.25 In particular, 2DE has proven to be a reliable and efficient method for the separation of proteins based on the isoelectric point and protein mass for over 4 decades.26 Here, 2DE image analysis revealed approximately 676, 657, and 611 protein spots in the RAW-JPS (control), FCS-JPS, and CFP-JPS samples, respectively (Figure 2).

Table 2. Proteins Identified by LC–MS/MS Spectrometry

| spot no. | function                        | ions score | protein ID result | protein expression (B or C/A) | references |
|---------|---------------------------------|------------|-------------------|-------------------------------|------------|
| 1       | muscle contraction              | 1534       | gi1080046002 tropomyosin-2 isoform X12 | 4.6-fold | 1, 31 |
| 2       | catalytic enzyme                | 237        | gi1080040111 fructose-bisphosphate aldolase | 4.8-fold | 32 |
| 3       | muscle contraction              | 353        | gi550248652 actin, muscle | 4.5-fold | 1, 30 |
| 4       | membrane-binding protein        | 1056       | gi550250044 annexin-B9 | 6.4-fold | 29 |
| 5       | structural molecule activity    | 73         | gi1080067306 endocuticle structural glycoprotein SgAbd-2-like | 4.9-fold | 33 |
| 6       | catalytic activity              | 571        | gi1080040022 mitochondrial-processing peptidase subunit β | 5.3-fold | 39 |
| 7       | muscle contraction              | 812        | gi1080052403 actin, musclelike | 6.2-fold | 1, 30 |
| 8       | muscle contraction              | 650        | gi550248652 actin, muscle | 8.8-fold | 1, 30 |
| 9       | muscle contraction              | 588        | gi550248652 actin, muscle | 7.3-fold | 1, 30 |
| 10      | muscle contraction              | 528        | gi550248652 actin, muscle | 5.2-fold | 1, 30 |
| 11      | muscle contraction              | 866        | gi1080052403 actin, musclelike | 6.7-fold | 1, 30 |
| 12      | ATP synthase                    | 1246       | gi550249358 ATP synthase subunit β | 2.2-fold | 38 |
| 13      | oxidoreductase                  | 401        | gi1080039074 probable isocitrate dehydrogenase | 2.1-fold | 37 |
| 14      | protein folding/cell protection | 52         | gi550251459 heat shock protein 70 | 2.3-fold | 36 |
| 15      | protein folding/cell protection | 52         | gi550251459 heat shock protein 70 | 2.1-fold | 36 |

“Ion score is calculated as follows: −10 × log (P), where P is the probability that the observed match is a random event. Individual ion scores >30 indicate identity or extensive homology (P < 0.05).”

In a comparison between FCS-treated JPS and RAW-JPS samples, 483 paired and 367 unpaired protein spots were found (Figure 2a,b). In the FCS-treated JPS sample, 52 protein spots indicated an over 2-fold increase in the protein expression level, whereas 61 protein spots indicated a 2-fold decrease in the protein expression level, compared to the RAW-JPS adults. The expression of proteins in FCS-treated JPS adults increased by approximately 4.5-fold, respectively, compared to RAW-JPS adults, and they were determined as tropomyosin-2 isoform X12, fructose-bisphosphate aldolase, actin, annexin-B9, and endocuticle structural glycoprotein SgAbd-2-like (Table 2). Annexins have been related to DNA replication, exocytosis and endocytosis, inhibition of phospholipase activity, resistance to reactive oxygen species, and signal transduction.25–28 In particular, insect annexin-B9 is a calcium-dependent membrane-binding protein affecting protein transport processes.29 Actin and tropomyosin were expressed for muscle contraction as a defense mechanism.30,31 In addition, fructose-bisphosphate aldolase and endocuticle structural...
glycoprotein SgAbd-2-like function as catalytic enzymes in muscles and formation of endocuticle, respectively.\textsuperscript{32,33}

In another comparison between CFP-treated JPS and RAW-JPS, 482 paired and 323 unpaired protein spots were found (Figure 2a,c). In CFP-JPS adults, the expression level of 37 proteins was increased more than 2-fold, whereas the expression level of 52 proteins decreased more than 2-fold compared to that in the RAW-JPS adults. Protein expression in the FP-JPS adults increased by approximately 2.1-fold compared to the RAW-JPS, and they were identified as mitochondrial-processing peptidase subunit $\beta$, actin, ATP synthase subunit $\beta$, isocitrate dehydrogenase, and heat shock protein 70 (Table 2). In particular, half of the 10 protein spots were identified as actin because flaccid paralysis may be expected as the major insecticidal mechanism on JPS. Additionally, the presence of destruxins in the dead JPS adults after the treatment with FCS or CFP was confirmed (Supporting Figure S2), whose mode of action causes flaccid paralysis and inhibits the synthesis of DNA, RNA, and proteins in insect cells.\textsuperscript{34,35} Treatment of JPS adults with insecticidal metabolites (destruxins and protease) produced by M. anisopliae JEF-279 caused tetanic paralysis, followed by flaccid paralysis. JPS adults overexpressed actin and tropomyosin for muscle contraction as a defense mechanism.\textsuperscript{3} The mechanism underlying the insecticidal effect of spraying conidia of M. anisopliae JEF-279 was similar to that observed for the treatment with destruxins or protease. Heat shock protein 70 (HSP70) contributes to insect survival under stress conditions by promoting protein integrity and cell homeostasis.\textsuperscript{36} Most flight-related insect muscles contain high activities of NAD$^+$-linked isocitrate dehydrogenase to generate energy via the citric acid cycle.\textsuperscript{37} In addition, the mitochondrial-processing peptidase subunit $\beta$ and ATP synthase subunit $\beta$ function in catalytic activity and mitochondrial ATP synthase, respectively.\textsuperscript{38–40} Thus, JPS utilizes its immune system to overcome flaccid paralysis and environmental stress and to obtain energy in response to the treatment with FSC or CFP. Flaccid paralysis is speculated to be a major element of the mechanism underlying the insecticidal action of M. anisopliae JEF-279 in host insects.

2.2.2. Biological Analysis by Transmission Electron Microscopy (TEM). The infection cycle of M. anisopliae JEF-279 was investigated by microscopic observation. Particularly, TEM analysis was performed to identify the conidia and hyphae of M. anisopliae JEF-279. The conidia are relatively constant in size, and the cell walls appear black, whereas hyphae show white cell walls and vary in size (Supporting Figure S3). The infection cycle of M. anisopliae JEF-279 in JPS was observed. TEM analysis of the JPS epicuticle facilitated the detection of a rigid structure prior to the FCS treatment (Figure 3a). Five days after the treatment, the JPS adult was still alive but showed the hemocoel filled with single-cell hyphal bodies called blastospores (Figure 3b).\textsuperscript{41} Eight days after the treatment, the JPS adult was dead and the hyphae were able to penetrate the cuticle and move out from the cuticle (Figure 3c). Finally, hyphal extrusion by penetrating peg formation and conidiation 13 days after the treatment were observed (Figure 3d). Entomopathogenic fungi, such as Beauveria bassiana and M. anisopliae, have been widely used for biological control.\textsuperscript{32,42} In a similar study, the infection cycle of B. bassiana in insects was reported to occur in the following sequence of events: (1) host adhesion, (2) germination, (3) cuticle degradation, (4) growth as blastospores, (5) host colonization and killing, (6) immune response interactions, and (7) hyphal extrusion and conidiation.\textsuperscript{41} On the other hand, the infection cycle of M. anisopliae in termites (Nasutitermes exitiosus) reportedly occurs in the following sequence: (1) germination of conidia, (2) appressoria and penetration of the cuticle, (3) penetration of the cuticle, (4) development in the hemolymph, and (5) invasion of the host tissue.\textsuperscript{44} The microscopic and proteomic results of the current study suggest that the infection cycle of M. anisopliae JEF-279 in JPS adults proceeds as follows: (1) host adhesion and germination, (2) epicuticle degradation, (3) growth as blastospore, (4) killing by fungal toxins (insecticidal metabolites), and (5) hyphal extrusion and conidiation.

3. CONCLUSIONS

This study aimed to investigate the mechanism of the insecticidal action of M. anisopliae JEF-279 against JPS adults. When CFP was used to treat JPS adults, insect mortality was higher than when conidia or FP treatment was used. The insecticidal protein PR1A produced by M. anisopliae JEF-279 accelerated the development of pathogenicity against JPS adults, assisting insect body penetration by conidia. During the infection process, several proteins were overexpressed in JPS adults and identified mainly as actin. In addition, destruxins were confirmed in the dead JPS adults. We propose that the infection cycle of JPS adults by M. anisopliae JEF-279 occurs as per the following sequence of events: (1) host adhesion and germination, (2) epicuticle degradation, (3) growth as blastospore, (4) killing by insecticidal metabolites, and (5) hyphal extrusion and conidiation. In addition, the CFP treatment can be applied as an insecticide in the field of biological control.
4. METHODS

4.1. Protease-Containing Culture Filtrate Production. *M. anisopliae* JEF-279 (KFCC11721P), which was isolated from the soil from Mt. Jiri, Korea, was provided by Chonbuk National University, Korea. For maintenance of the fungus, conidia of *M. anisopliae* JEF-279 were stored in 1.2 mL cryovial tubes (Sigma, Canada) at −70 °C. When necessary, the stock was cultured in a 500 mL flask containing 100 mL of 1/4 Sabouraud dextrose broth (SDB) under agitation at 200 rpm for 3 days. To produce the protease-containing culture filtrate, a 1% (v/v) seed culture was inoculated in a 5 L jar bioreactor (MARADO-05D-XS, BioCnS, Daejeon, Korea) in a total volume of 3 L containing 1% (w/v) wheat bran, 1% (v/v) soybean protein, 0.42% (NH₄)₂SO₄, 0.2% KH₂PO₄, 0.1% protease peptone, 0.02% urea, 0.03% CaCl₂, 0.03% MgSO₄·7H₂O, 0.2% Tween 80, and 0.2% trace element. *M. anisopliae* was cultivated at 28 °C for 5 days under agitation at 300 rpm and an aeration rate of 1.0 vvm (air volume added to the liquid volume per minute).

4.2. Insecticidal Effect of Conidia and Protease-Containing Culture Filtrate on JPS Adults. To evaluate the insecticidal effect of fungal metabolites, three treatments (sterile distilled water, FCS at a concentration of 1 × 10⁻⁴, Sabouraud dextrose broth (SDB) under agitation at 200 rpm for 3 days) were included per treatment. A total of 50 JPS adults (10 + 10 + 10 + 10) were tested per treatment, and sterile distilled water was used as a negative control. The sprayed JPS adults (380–1700 m/z), followed by 10 MS/MS scans. For MS₁ full scans, the orbitrap resolution was 15 000 and the AGC was 2 × 10⁶. The AGC was 1 × 10⁴ for MS/MS in the LTQ.

4.3. Transmission Electron Microscopy (TEM). Samples were fixed with 1% (v/v) glutaraldehyde and 2% paraformaldehyde (v/v) in 0.05 M cacodylate buffer (pH 7.2) at 25 °C for 4 h. Samples were washed and postfixed with 1% OsO₄ in 0.05 M cacodylate buffer at 25 °C for 1 h. The fixed samples were washed using the phosphate buffer and then dehydrated in a graded ethanol series (30–100%). Then, samples were embedded in LR White resin at 50 °C for 24 h, and ultrathin sections (80–100 nm thick) were prepared with an ultramicrotome using a diamond knife. The thin sections were stained with uranyl acetate and lead citrate. A transmission electron microscope (JEM-2400F; Jeol, Tokyo, Japan) was used to visualize the samples.

4.4. Two-Dimensional Gel Electrophoresis (2DE) and Gel Image Analysis. Two-DE was performed essentially as previously described. Aliquots in sample buffer (7 M urea, 2 M thiourea, 4.5% CHAPS, 100 mM DTE, 40 mM Tris, pH 8.8) were applied to immobilized pH 3–10 nonlinear gradient strips (Amersham Biosciences, Uppsala, Sweden). Isoelectric focusing (IEF) was conducted at 80 000 Vh. The second dimension was performed on 9–16% linear gradient polyacrylamide gels (18 cm × 20 cm × 1.5 mm) at a constant 40 mA per gel for 5 h. After protein fixation in 40% methanol and 5% phosphoric acid for 1 h, the gels were stained with Coomassie Brilliant Blue (CBB) G-250 for 12 h. Subsequently, gels were destained using H₂O and scanned in a Bio-Rad (Richmond, CA) GS710 densitometer. The program Image Master Platinum 5.0 image (Amersham Biosciences) was used for image analysis.

4.5. Protein Identification by LC–MS/MS. Nano LC–MS/MS analysis was conducted with the EASY-nLC (Thermo Fisher Scientific, San Jose, CA) and the LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a nanoelectrospray source. Samples were separated on a C18 nanobore column (150 mm × 0.1 mm, 3 μm pore size; Agilent) with mobile phase A (0.1% formic acid and 3% acetonitrile in deionized water) and mobile phase B (0.1% formic acid in acetonitrile). The chromatography gradient was designed for a linear increase from 0% B to 60% B in 9 min, 60% B to 90% B in 1 min, and 3% B in 5 min. The flow rate was maintained at 1800 nL/min. Mass spectra were acquired using data-dependent acquisition with a full mass scan (380–1700 m/z), followed by 10 MS/MS scans. For MS₁ full scans, the orbitrap resolution was 15 000 and the AGC was 2 × 10⁶. The AGC was 1 × 10⁴ for MS/MS in the LTQ.

4.6. Database Searching. The mascot algorithm was used to identify peptide sequences present in a protein sequence database from NCBI. Database search criteria used were as follows: taxonomy, *Anoplophora glabripennis*; fixed modification, carboxymethylated at cysteine residues; variable modification, oxidized at methionine residues, maximum allowed missed cleavage, 2; MS tolerance, 10 ppm; MS/MS tolerance, 0.8 Da. The peptides were filtered with a significance threshold of P < 0.05.

4.7. Statistical Analysis. Data were analyzed using PASW software (version 17, SPSS Inc., CA). Analysis of variance was used to determine significant treatment effects at P < 0.05 using Tukey’s honestly significant difference test. LT₅₀ and LT₉₀ values of the insects were evaluated using Probit analysis. Data are shown as means ± standard error (SE).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c03585.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and protein identification by matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)/TOF mass spectrometry (MS) (Figure S1), content of destruxins analyzed by high-performance liquid chromatography (HPLC) (Figure S2), and transmission electron microscopy image analysis for hypha and conidia of *M. anisopliae* JEF-279 (Figure S3) (PDF)

AUTHOR INFORMATION

Corresponding Author

Hae Woong Park – R&D Division, World Institute of Kimchi, Gwangju 61755, Republic of Korea; orcid.org/0000-0002-5181-1255; Phone: +82-62-610-1728; Email: haewoong@wikim.re.kr; Fax: +82-62-610-1850

Authors

Ho Myeong Kim – R&D Division, World Institute of Kimchi, Gwangju 61755, Republic of Korea
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