Assessment of the Effects of Temperature and Relative Humidity on the Pathogenicity of Metarhizium anisopliae Strain FRM515 to Plautia crossota stali

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Abstract

We evaluated the effects of temperature and relative humidity (RH) on the pathogenicity of Metarhizium anisopliae strain FRM515 to Plautia crossota stali. Low temperature decreased the pathogenicity of the fungal strain: its pathogenicity at 15°C was lower than that at higher temperatures. Mortality rates differed little among stink bugs kept at 80, 60, and 40% RH after treatment with a suspension containing 1 × 10⁸ conidia/ml; however, when 1 × 10⁷ conidia/ml was used, the LT₅₀ value at 40% RH was 1.7 times that at 80% RH. Among three species of stink bugs, the susceptibility of Glaucias subpunctatus to M. anisopliae strain FRM515 was similar to that of P. c. stali, whereas Halyomorpha halys was relatively resistant to infection with this strain. [³⁵S]Cysteine-labeled exuviae were degraded by a conidial suspension of M. anisopliae strain FRM515. The radioactivity in the medium increased from 24 to 30 h after treatment to the time when conidia germinated on the surface of the exuviae.

Key words: Stink bug; Plautia crossota stali; entomopathogenic fungi; microbial control; Metarhizium anisopliae

INTRODUCTION

Three species of stink bugs—Plautia crossota stali (Scott), Halyomorpha halys (Stal), and Glaucias subpunctatus (Walker)—are well known as serious fruit tree pests (Hasegawa and Umeya, 1974). P. c. stali is widely distributed throughout Japan, and G. subpunctatus develops in warm regions, usually along with P. c. stali (Ide, 1997). H. halys develops mainly in cool regions and damages apple fruits (Funayama, 2003). Stink bugs develop in forests and invade orchards because of their high mobility (Moriya, 1996); therefore, frequent spraying of chemical pesticides in orchards is necessary to prevent damage to fruit. However, repeated application of chemical pesticides can cause the resurgence or development of secondary pest problems (Metcalf, 1986). With increasing demand for IPM (integrated pest management) strategies and ecologically friendly measures, alternative methods for controlling stink bugs have been undertaken.

Entomopathogenic fungi are promising microbial control agents. Because stink bugs are plant-sucking insects, entomopathogenic fungi likely would be more suitable agents for their control than other microbial agents, such as bacteria and viruses; bacteria and viruses must be ingested to cause disease, whereas entomopathogenic fungi infect insects through their skin. A previous investigation evaluated the fungus Beauveria bassiana as a microbial pesticide for the control of P. c. stali (Tsuda et al., 1996, 1997). Further, the high pathogenicity of Metarhizium anisopliae strain FRM515 to P. c. stali (Ihara et al., 2001) suggests that this organism is a candidate microbial control agent for stink bugs. However, other characters (i.e., factors affecting pathogenicity) of the fungus are currently unknown and must be addressed to clarify its suitability for this application. Here we investigated the ability of M. anisopliae strain FRM515 to infect stink bugs in the laboratory.

MATERIALS AND METHODS

Fungal strain and medium. We used M. anisopliae strain FRM515, previously identified as a strain with high pathogenicity to P. c. stali (Ihara et al., 2001). Sabouraud maltose medium (SMY) con-
taining 4% maltose, 1% peptone, and 1% yeast extract was used for liquid cultivation of the strain, and SMY solidified with 1.5% agar was used for solid cultivation. The fungus was grown in pure culture on SMY slants in test tubes for 2 wk at 25°C. After the cultures had developed conidia, the test tubes were kept at −30°C as a stock culture.

Insects. Laboratory-reared P. c. stali and H. halys were used. They were originally collected in Tsukuba, Ibaraki, Japan, were maintained at 23°C with a 16L : 8D photoperiod, and were provided with peanuts, soybeans, and distilled water (Moriya et al., 1985).

New generations of G. subpunctatus adults were collected from a citrus field in Minami-shimabara, Nagasaki, Japan, and kept in the laboratory for 1 wk under conditions of 25°C with a 16L : 8D photoperiod, and peanuts, soybeans, and distilled water before they were used in bioassays.

Bioassay. Conidial suspensions (1×10⁷ and 1×10⁸ conidia/ml) of M. anisopliae strain FRM515 for use in bioassays were prepared as described previously (Ihara et al., 2001). Each adult stink bug was dipped into 5 ml of conidial suspension in a 20-ml plastic vessel for 10 s at room temperature. After excess drops of suspension were wiped from the surface of the stink bug with a paper tissue, the insect was transferred into a plastic cup (φ70 × 30 mm) lined with filter paper. A total of 10 P. c. stali, 5 H. halys, or 5 G. subpunctatus insects were put into each plastic cup. For the standard conditions, they were kept for 10 or 15 d at 25°C and 85–90% relative humidity (RH; model MLR-350HT Growth Cabinet, SANYO, Tokyo, Japan) with a 16L : 8D photoperiod, and were provided with peanuts and water. Dead stink bugs were counted every 24 h, and mycelial growth and conidial formation on the dead stink bugs were confirmed by further incubation at 25°C. As a negative control, a solution of 0.2% Tween 80 and 0.89% NaCl was used instead of conidial suspension. To calculate LT₅₀ values, the bioassay results were subjected to probit analysis (Finney, 1971), and the probit mortality obtained during the experiment was regressed against the number of post-treatment days.

Mycelial growth of M. anisopliae strain FRM515 at different temperatures was examined according to the method of Shimazu (2004). The conidial suspension (10 µl, 1×10⁷ conidia/ml) was placed at the center of the SMY plate. Plates were incubated for 6 days at various temperatures (15, 20, 25, 30 and 35°C). The bottom of the plate was marked with crossed lines through the center of the colony, and the diameter of the colony along the lines was measured every 24 h. Because the initial diameter varied among colonies, these values were subtracted from the measured values, and the resulting differences were divided by 2. The test was performed four times.

Degradation of radiolabeled exuviae. To obtain radiolabeled last-stadium exuviae of P. c. stali, nymphs were orally labeled with [³⁵S]cysteine (Ihara et al., 2002). Two radiolabeled exuviae were put into each 8.0-ml tube, and 5.0 ml of conidial suspension (1×10⁷ or 1×10⁸ conidia/ml) of M. anisopliae strain FRM515 was added. The reaction tubes were incubated at 25°C with reciprocal shaking at 100 spm (strokes per minute). Every 6 h, 100 µl was withdrawn and the radioactivity level was measured in a liquid scintillation counter (LSC-3500, Aloka, Tokyo, Japan). As a negative control, a solution of 0.2% Tween 80 and 0.89% NaCl was used instead of conidial suspension. Quenching was corrected according to the external standard channel ratio method. The measuring time was 1 min, and ACSII (Aqueous Counting Scintillant, Amersham Bioscience, USA) was used as the scintillation cocktail. The test was performed three times.

Electron microscopy. The experimental procedure was the same as that for evaluating the degradation of radiolabeled exuviae, except that non-labeled exuviae were used. After incubation for 24 h, the exuviae were rinsed once with 0.2% Tween 80 and 0.89% NaCl. The part of the exuvia corresponding to the thorax notum was excised and coated with gold. Samples were examined under a scanning electron microscope.

RESULTS AND DISCUSSION
Effect of temperature and RH on pathogenicity
Environmental conditions that affect the pathogenicity of M. anisopliae need to be identified before the fungus can be used as a microbial pesticide. Because temperature and RH have pronounced effects on the germination and mycelial growth of M. anisopliae (Walstad et al., 1970; Hywel-Jones and Gillespie, 1990), we tested the
effects of these factors on the pathogenicity of *M. anisopliae* strain FRM515 to *P. c. stali*.

Adults of *P. c. stali* were treated with a conidial suspension (1×10⁸ conidia/ml) of *M. anisopliae* strain FRM515 and were incubated at 15, 20, 25, or 30°C. The pathogenicity of strain FRM515 was decreased at low temperature (i.e., 15°C). The mortality rate profiles at 20, 25, and 30°C differed little and indicated the high pathogenicity of *M. anisopliae* strain FRM515 to stink bugs (Fig. 1). Whereas the mortality rate reached 100% within 10 d at 20, 25, and 30°C, that at 15°C on day 10 was 30.0%. The LT₅₀ values of *M. anisopliae* strain FRM515 at 20, 25, and 30°C were 5.2 (95% FL, 4.8 to 5.6), 4.4 (4.1 to 4.6), and 4.0 d (3.8 to 4.3 d), respectively, but the LT₅₀ value at 15°C was 12.1 d (11.2 to 13.1 d).

Mycelial growth of *M. anisopliae* strain FRM515 on SMY plates at different temperatures was examined (Fig. 2). Mycelial growth rates of the fungus at 15, 20, 25, 30, and 35°C were 0.6, 1.3, 2.2, 1.4, and 0.6 mm/d, respectively. The maximum growth rate occurred at 25°C, but the highest pathogenicity occurred at 30°C. The disagreement between the growth rate of *M. anisopliae* strain FRM515 and its pathogenicity to *P. c. stali* suggests that the physiologic conditions of the stink bug at various temperatures need to be considered. The fact that the mean mortality rate of the control on day 7 was 33.3% at 30°C but 14.8% at 25°C supports the hypothesis that the physiologic state of the insect contributes to its sensitivity to *M. anisopliae* strain FRM515.

To examine the effect of RH on mortality due to *M. anisopliae* infection, stink bugs treated with a conidial suspension (1×10⁸ or 1×10⁷ conidia/ml) of *M. anisopliae* strain FRM515 were incubated at 25°C and 80, 60, or 40% RH. When the conidial suspension containing 1×10⁸ conidia/ml was used for the assay, the susceptibility of the insects to infection differed little among RHs (Fig. 3A): the LT₅₀ values at 80, 60, and 40% RH were 15.8, 10.5, and 11.1%, respectively.
FL, 5.2 to 6.0), 5.8 (5.5 to 6.0), and 6.6 d (6.2 to 7.0 d), respectively; however, when 1×10^7 conidia/ml was used, the LT_{50} value at 40% RH (9.9 d; 95% FL, 9.0 to 11.2 d) was 1.4 to 1.7 times that at the higher RHs (80% RH, 5.8 [5.3 to 6.2] d; 60% RH, 7.1 [6.6 to 7.7] d) (Fig. 3B). The fact that RH alters the pathogenicity of *M. anisopliae* strain FRM515 at decreased conidial dosage may indicate that the pathogenicity of the fungus to stink bugs depends on the number of conidia that attach to the insect’s skin rather than RH.

**Infection of two other stink bug species**

High pathogenicity toward the target insect is an important characteristic in the development of a microbial pesticide. *M. anisopliae* strain FRM515 showed the highest pathogenicity to *P. c. stali* among the 711 isolates tested (Ihara et al., 2001). Two additional species, *G. subpunctatus* and *H. halys*, are (like *P. c. stali*) fruit-piercing stink bugs (Hasegawa and Umeya, 1974) that damage apple (Funayama, 1996), citrus (Ide, 1997), pear (Sato, 1997), and persimmon (Yanase, 1997). We therefore tested the pathogenicities of *M. anisopliae* strain FRM515 to *G. subpunctatus* and *H. halys*.

The susceptibility of *G. subpunctatus* to *M. anisopliae* strain FRM515 was similar to that of *P. c. stali*, leading to LT_{50} values of 4.7 (4.4 to 5.0) and 4.4 d (4.1 to 4.6 d), respectively (Fig. 4). In comparison, *H. halys* was markedly less sensitive to infection by *M. anisopliae* strain FRM515: the LT_{50} value for this stink bug was 10.4 d (9.1 to 12.3 d).

Sosa-Gómez and Moscardi (1998) reported similar results after they tested the ability of *M. anisopliae* to infect three species of soybean stink bugs. The pathogenicities of *M. anisopliae* on *Piezodorus guildinii* and *Nezara viridula* were significantly higher than that on *Euschistus heros*. The authors noted that the differing susceptibilities of stink bugs to *M. anisopliae* represent important information for the development of entomopathogenic fungi as microbial pesticides of these pests, because species with decreased susceptibility to *M. anisopliae* (*E. heros*) and those with increased susceptibility (*P. guildinii*) usually occur as a complex in soybean fields. In the case of fruit-piercing stink bugs, *P. c. stali*, *G. subpunctatus*, and *H. halys* as a complex can severely damage various fruit crops when outbreaks occur concurrently (Hasegawa and Umeya, 1974); therefore, given the generally high pathogenicity of *M. anisopliae* to stink bugs, an effective method of applying the organism should be developed so that the microbial control of stink bugs can be practically considered.

**Degradation of radiolabeled exuvia**

In our previous study, fourth-stadium nymphs of *P. c. stali* were orally labeled with [35S]cysteine, and intact 35S-labeled exuvia was used as a substrate for enzymatic degradation (Ihara et al., 2002). We applied this same methodology to the detection of cuticle degradation resulting from the invasion of *P. c. stali* by *M. anisopliae* strain FRM515 after germination of the conidia.

Radioactivity was released into the media in an incubation time-dependent manner, and the amount of radioactivity in the media increased after 24 to 30 h of incubation (Fig. 5). To confirm the conidial stage responsible for this effect, the experiment was repeated, except that non-labeled exuviae were used. The reaction was stopped at various times, and the surface of the exuvia was observed by electron microscopy. Conidia on the surface of the exuvia germinated after incubation for 24 h (Fig. 6A), corresponding to the time at which the level of radioactivity in the media began to increase. Although the shapes of the conidia and germ tube of *M. anisopliae* were altered somewhat under the vacuum necessary for microscopy, the tips of the germ tubes seemed to become wider than the germ tube and adherent to the surface of the exuvia (arrows in Fig. 6A). The germinated conidia of *M.
anisopliae on the skin of western flower thrips showed similar changes in shape (Vestergaard et al., 1999; Leemon and Jonsson, 2007); therefore, the experimental system that involves exuviae and conidia seems to reflect the natural infection process of M. anisopliae. Further, microscopic analysis revealed the degradation and fungal penetration of the epicuticle of the wireworm after germination of M. anisopliae (Zacharuk, 1970). The increase in the amount of radioactivity released (Fig. 5) appears to coincide with the stage at which the exuvia is degraded.

Germination of M. anisopliae strain FRM515 on the surface of the exuvia began after 24 h of incubation, but germination on 1.5% agar solid began 12 h after inoculation (Fig. 6B). Leemon and Jonsson (2007) reported that conidia of M. anisopliae produced a germ tube after incubation for 11 h at 25°C on the surface of the cattle tick (Boophilus microplus). That the delay in germination on the exuvia of stink bugs might be caused by the presence of inhibitory materials is one possibility to explain the phenomenon. For example, (E)-2-decenal, a cuticle component of the southern green stink bug (Nezara viridula), is known as a material that inhibits the germination of M. anisopliae conidia (Sosa-Gómez et al., 1997); however, the possibility that other factors are involved in the germination of M. anisopliae conidia on stink bug exuviae has not been ruled out.

Assumable application of M. anisopliae strain FRM515 for control of fruit-piercing stink bugs

The seasonal occurrences of the fruit-piercing stink bugs have been investigated in a wide area in Japan, such as in Saga (Ide, 1997), Fukuoka (Sato, 1997), Gifu (Yanase, 1997), Ibaraki (Moriya et al., 1987) and Akita (Funayama, 2003). Stink bugs develop in woodlands or uncultivated areas, and continuously enter orchards (Moriya, 1996). Although the annual number of developed stink bugs and their seasonal occurrence fluctuate each year, they possibly invade various orchards from late May to mid-October. Since the flight potential of P. c. stali increases when temperature becomes higher than 20°C (Moriya, 1995), the temperature in orchards with stink bugs should be around 20°C or higher. That M. anisopliae strain FRM515 showed high

Fig. 5. Solubilization of radioactivity from radiolabeled exuviae incubated in conidial suspension (1×10⁸ conidia/ml) of M. anisopliae strain FRM515.

Fig. 6. Germination of conidia on the surface of exuviae of P. c. stali (A) and on solid agar (B). (A) Electron microscopy of conidia germination on exuviae after incubation for 24 h at 25°C. (B) Phase-contrast microscopy of germination on 1.5% solid agar. The conidial suspension (in 0.2% Tween 80 and 0.89% NaCl) was spread on 1.5% solid agar, and the culture was incubated for 12 h at 25°C.
pathogenicity to \( P. \) c. \( stali \) at higher than 20°C (Fig. 1) suggests that the strain might be suitable to use in orchards as a microbial control agent throughout their damage season. The effect of RH on pathogenicity might be small when an application method for attaching a large number of conidia to insect skin is developed. The pathogenicity of \( M. \) anisopliae strain FRM515 depended on the dose of conidia attaching on the skin rather than RH (Fig. 3). The auto-dissemination system combined with an aggregation pheromone for controlling \( P. \) c. \( stali \) (Tsutsumi et al., 2003) would be an effective method for infection.

\( H. \) halys is the dominant species of fruit-piercing stink bug in northern Japan (Funayama, 2003), and \( P. \) c. \( stali \) and \( G. \) subpunctatus are the major populations in the west Kanto area of Japan (Ohira, 2003). The result that the susceptibility of \( H. \) halys to \( M. \) anisopliae strain FRM515 was relatively lower than those of \( P. \) c. \( stali \) and \( G. \) subpunctatus (Fig. 4) suggests that the strain could be applied in the east Kanto area to control \( P. \) c. \( stali \) and \( G. \) subpunctatus. To control \( H. \) halys using this strain, an effective infection method to increase pathogenicity is necessary.

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