A novel technique to inoculate conidia of entomopathogenic fungi and its application for investigation of susceptibility of the Japanese pine sawyer, *Monochamus alternatus*, to *Beauveria bassiana*

Mitsuaki SHIMAZU*

Forestry and Forest Products Research Institute; Tsukuba, Ibaraki 305–8687, Japan

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Abstract

A novel technique to measure the virulence of an entomopathogenic fungus, *Beauveria bassiana* by exposing tarsi of adults to dry conidia was developed to evaluate effectiveness of nonwoven fabric strip formulation of this fungus for controlling adults of the Japanese pine sawyer, *Monochamus alternatus*. To regulate inoculum density without suspending conidia in water, conidia were killed by heating at 100°C for 1 h and a step dilution series of conidia was prepared by mixing dead conidia with live conidia at different ratios. The conidial mixtures were attached to tarsi of CO₂-anesthetized adults with a fine hairbrush. The 50% lethal doses determined by this method on 14 d were 5.5×10^6 conidia/individual for aged adults and 1.9×10^6 conidia/individual for young adults, and on 30 d were 2.8×10^5 conidia/individual for aged adults and 2.4×10^4 conidia/individual for young adults. The number of conidia produced on a nonwoven fabric strip was 3.5×10^8 conidia/cm², and 8.5×10^5 conidia/individual were attached to adult beetles which walked on the strip. Based on these results, the validity of a biological control method for *M. alternatus* to prevent vectoring of the pine wilt disease is discussed.

Key words: Bioassay; inoculation; conidia; nonwoven fabric; *Beauveria bassiana*

INTRODUCTION

The pine wilt disease is a most important disease of pine forests in Japan. This disease is caused by the pinewood nematode, *Bursaphelenchus xylophilus*, which is thought to have originated in North America (Nickle et al., 1981). This nematode is vectored by adults of some cerambycid beetle species and the Japanese pine sawyer, *Monochamus alternatus*, is the major vector species in Japan. Chemical insecticides have mainly been used to kill vectors and prevent prevalence of diseases. As one microbial control agent for *M. alternatus*, an entomopathogenic fungus, *Beauveria bassiana*, has been considered promising (Shimazu and Kushida, 1983). Shimazu et al. (1995) developed a method to utilize this fungus for the control of *M. alternatus* larvae by applying the fungus cultured on nonwoven fabric strips. This method is the most effective microbial control technique for *M. alternatus* larvae available to date. If the adults can

also be targeted by *B. bassiana*, the use of chemical pesticides can be greatly reduced.

Actually, adults of *M. alternatus* are relatively resistant to *B. bassiana* (Shimazu and Kushida, 1983). Therefore it is difficult to control the adults by simple spraying of conidial suspension. However, use of this fungus to control *M. alternatus* adults by compelling direct contact with conidia instead of spraying a conidial suspension is being investigated (Okitsu et al., 2000; Okabe et al., 2001, 2002). These studies using nonwoven fabric strips with *B. bassiana* conidia on the surface. For this method, quality control of the strips for insecticidal activity is important. However, the “virulence” of such non-liquid materials is difficult to measure and no convenient bioassay method has been established.

In the present study, inoculation experiments by contaminating the tarsi of *M. alternatus* adults with conidia were carried out. For these experiments, inoculum doses were controlled by altering the den-

*E-mail: shimazu@ffpri.affrc.go.jp
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sity of live conidia by mixing with dead conidia, allowing a 50% lethal dose to be calculated on the basis of mortalities at different densities of live conidia. This unique method for controlling conidial densities was established, and the possibility of controlling *M. alternatus* adults with conidia-forming nonwoven fabric strips is discussed.

MATERIALS AND METHODS

Newly emerged adults of *M. alternatus* were used for the experiments. To obtain the beetles, dead *Pinus thunbergii* inhabited by *M. alternatus* were collected at Shirako, Chiba Prefecture on April 14, 2003. Logs were cut and placed in a screened cage at Tsukuba, Ibaraki Prefecture. The adults emerging in the cage were collected and reared individually in a plastic cup at 25°C with twigs of *Pinus densiflora* as food.

B. bassiana F-263, which was originally isolated from a cadaver of *M. alternatus* larva in Kumamoto Prefecture, Japan and exhibits strong virulence to this insect, was used in the experiments.

The fungus was cultured on 45×5 cm nonwoven fabric strips (Nitto Denko Co.). The strip was cut into a 2.5 cm square, soaked with 100 ml of 300 ppm aqueous solution of Tween 80 and agitated vigorously with a vortex mixer for 1 min to prepare a conidial suspension. Conidial density in the suspension was measured using a Thoma's hemocytometer and the value was converted to conidial density per unit area of nonwoven fabric strip.

To estimate the number of conidia on adult beetles after walking on the strips, the beetles were allowed to walk on the strip for more than 5 cm and were then anesthetized with CO₂. The 6 tarsi were cut from the insect and placed in a test tube containing 0.5 ml of 70% ethanol to remove the hydrophobic property of the conidia. Then, 4.5 ml of 300 ppm aqueous solution of Tween 80 was added and the mixture agitated vigorously with a vortex mixer for 1 min to produce a suspension of conidia from the tarsi. Conidial density in the suspension was measured using a Thoma's hemocytometer, from which the amount of inoculated conidia was estimated. Five adults were used for the measurements.

The fungus was cultured in Sabouraud's saccharose broth with 1% yeast extract in a shaker-incubator at 25°C for 5 d. One part of the cultured

material was mixed with 5 parts of Sabouraud's saccharose agar (1%) containing 1% yeast extract, the mixture was poured into plastic boxes of 23 cm×16 cm×4.5 cm and the boxes were incubated at 25°C for 21 d under continuous lighting with fluorescent lamps to facilitate sporulation. The culture was then air-dried for 5 d and dried conidia were harvested with a brush and stored at 5°C until use.

Dry conidia preparations containing different concentrations of live conidia were inoculated on tarsi of adult insects. Amount of live conidia inoculated onto tarsi of adults was controlled by mixing dead conidia with live ones. The conidia were killed by heating in a drying oven at 100°C for 1 h in a beaker covered with aluminum foil. The mass of heat-killed conidia was reduced to 99%. Based on this, the non-diluted (1/1×), 1/10×, 1/100×, and 1/1,000× plots were created by mixing live conidia with dead ones in weight ratios of 1,000:0, 100:891, 10:980.1 and 1:989, respectively. To evaluate actual ratios of active conidia in each plot, small portions of each conidial mixture were spread onto agar plates of Sabouraud's dextrose medium containing 1% yeast extract with a glass rod, incubated for 20 h at 25°C and germination rates measured by checking germ tube development with a microscope.

Dry conidial mixtures were attached to all tarsi of CO₂-anesthetized adult insects with a fine brush (Fig. 1). Five extra adult insects were inoculated with dead conidia on all tarsi with a fine brush and different amounts of inoculated conidia were estimated in the manner described above.

Two groups of adult insects, young insects within 4 d after emergence, and aged insects over 10 d after emergence were subjected to the inoculations. Thirty young and 30 aged insects were employed for each inoculum plot.

The inoculated insects were placed on tissue paper to remove excess conidia, replaced into plastic cups and reared individually at 25°C. Mortality was inspected every day. When an insect died, pine twigs were removed from the cup, a moistened piece of tissue paper (Kimwipe[®]) placed in the cup and the cadaver was kept at 25°C for more than 14 d. Whether the insect was killed by *B. bassiana* was determined by growth of aerial mycelia and sporulation.

Fig. 1. Inoculation of conidial mixtures with a fine hairbrush.

Fig. 2. Cumulative mortalities of young and aged adults of *Monochamus alternatus* inoculated with conidia of *Beauveria bassiana* at different densities.

RESULTS

Conidial density on the nonwoven fabric strips was $3.5 \times 10^8 \pm 4.0 \times 10^7$ (mean $\pm$ s.d.)/cm² and the number of conidia on adult beetles that walked on the strips was $8.5 \times 10^5 \pm 4.5 \times 10^5$ (mean $\pm$ s.d.)/individual.

Germination rates of the conidial mixtures in the non-diluted plot, 1/10 $\times$ plot, 1/100 $\times$ plot and 1/1,000 $\times$ plot were 92.3, 16.3, 1.60 and 0.25%, respectively. The number of conidia attached onto

tarsi of the inoculated adults was $4.7 \times 10^6 \pm 1.7 \times 10^6$ (mean $\pm$ s.d.)/individual. Active conidial doses per individual in the non-diluted plot, 1/10 $\times$ plot, 1/100 $\times$ plot and 1/1,000 $\times$ plot were estimated to be 4.3×10^6 , 7.7×10^5 , 7.5×10^4 , and 1.2×10^4 , respectively.

The adults inoculated with more live conidia showed higher mortalities in shorter periods (Fig. 2). With the same dose, young beetles suffered higher mortalities in shorter periods than aged beetles. As for the adult insects that died within 90 d

Table 1. Mortalities of *Monochamus alternatus* adults and 50% lethal dose for *Beauveria bassiana* conidia

Plots	Inoculum size (conidia/ individual)	Number inoculated	Mean days to death	14 d		30 d	
				Mortality (%)	LD ₅₀ (conidia/ individual)	Mortality (%)	LD ₅₀ (conidia/ individual)
Aged 1/1,000×	1.2×10 ⁴	30	48	(−0.12)		0.24	
Aged 1/100×	7.5×10 ⁴	30	33	13.69	5.47×10 ⁶	36.18	2.84×10 ⁵
Aged 1/10×	7.7×10 ⁵	29	27	14.29		53.85	
Aged 1/1×	4.3×10 ⁶	30	14	51.67		100.00	
Young 1/1,000×	1.2×10 ⁴	30	32	11.11		42.59	
Young 1/100×	7.5×10 ⁴	30	26	14.81	1.85×10 ⁶	61.73	2.39×10 ⁴
Young 1/10×	7.7×10 ⁵	30	15	37.04		96.30	
Young 1/1×	4.3×10 ⁶	30	11	62.96		100.00	

after inoculation, their mean life spans ranged from 14 to 48 d for aged adults and from 11 to 32 d for young adults. When the inoculum dose was greater the life span tended to become shorter (Table 1). On the basis of cumulative mortalities at 14 d for young adults and at 30 d for aged adults, 50% lethal doses (LD₅₀) were calculated (Table 1). About half of the inoculated insects produced *B. bassiana* conidia on the whole body surface, but others produced conidia only on the legs or antennae, or produced no conidia. In this study, the total dead insects were counted for calculation of mortalities regardless of conidia production.

DISCUSSION

Adults of *M. alternatus* stay in wood about 7 d after eclosion (Makihara, 1997), then emerge from the tree and feed on the bark of live pine twigs. This behavior is called maturation feeding. Pinewood nematodes are carried by the adult beetles and infect healthy pine trees during feeding. Namely, *M. alternatus* itself does not kill pine trees, but is a pest insect because it vectors the pinewood nematode. In the laboratory, adult beetles begin maturation feeding immediately after emergence, but feed most actively about 20 d after emergence (Okuda, 1974). Exit of the nematode also shows a peak 2 to 3 weeks after emergence of *M. alternatus* adults (Enda, 1972; Hosoda and Kobayashi, 1978; Mineo 1983). In the case of *Monochamus carolinensis*, which vectors the pinewood nematode in North America, the nematode exit was also maximum 3 weeks after emer-

gence of the beetle (Linit, 1989). The infection rate was the highest 3 weeks after emergence of the beetle when healthy pine trees were experimentally fed to *M. alternatus* adults (Mineo and Kontani, 1975). Therefore, it is necessary to kill the vector adults before the peak period of maturation feeding to suppress the pinewood nematode. Takeshita (1978) investigated the surface area of scars on pine twigs made by maturation feeding of *M. alternatus* and proposed that to suppress the pine wilt disease chemical pesticides should be used to kill the adult beetles within 15 d after emergence. It has also been reported that the pre-oviposition period of *M. alternatus* is around 20 d (Ochi, 1969; Enda and Nobuchi, 1970; Iwasaki and Morimoto, 1973; Ido and Takeda, 1975). All these data consistently indicate that *M. alternatus* can be effectively controlled if the adult beetles are killed within 14 d.

Today, chemical insecticides such as MEP are sprayed on healthy pine crowns to prevent maturation feeding and transmission of the nematodes. Use of *B. bassiana* for controlling the adult beetles would lead to reduction in the use of chemical insecticides. As an example of fungal control of Cerambycid adults, a nonwoven fabric strip formulation of *Beauveria brongniartii* has been commercially used against *Anoplophora malasiaca* on citrus and some garden trees (Kashio and Ujiie, 1988; Hashimoto et al., 1989; Kashio and Tsutsumi, 1990; Tsutsumi et al., 1990) and *Psacothia hilaris* on mulberry (Kawakami and Shimane, 1986; Ishishikawa et al., 1988). However, dipping inoculation experiments indicated that *M. alternatus* is relatively resistant to *B. brongniartii* (Shimazu and

Kushida, 1983).

B. bassiana F-263 isolated from a *M. alternatus* larva is highly virulent to larvae, but requires a longer time to kill adults. According to Shimazu and Kushida (1983), the mortality of *M. alternatus* adults exposed to 10^5 conidia/ml of this fungus by dipping inoculation was 50% with an average incubation period of 37 d and with 10^6 conidia/ml was 100% with an average incubation period of 25 d. The long periods required for the death of adults allows them to vector pinewood nematodes and lay eggs. Therefore, *B. bassiana* is not likely suitable for controlling the adults of *M. alternatus* as long as ordinary application methods such as spraying of conidial suspensions are used. However, recent studies revealed that *B. bassiana* achieves high mortality of *M. alternatus* adults in a short period when the adults are exposed to the conidia directly by using a trap in which they were forced to walk on a nonwoven fabric strip covered with *B. bassiana* conidia (Okitsu et al., 2000; Okabe et al., 2001, 2002). The infested trees were covered with a large plastic sheet and the adults that emerged from the trees were forced to walk on the strips before escaping from the cover. It is thought that walking on the strips with *B. bassiana* conidia resulted in high mortality of *M. alternatus* adults, because the adults were exposed to many more conidia on the tarsi than when dipped in a conidial suspension.

For insecticidal assay of entomopathogenic fungi, dipping the host insect into conidial suspensions has been a standard method. However, recent findings in which adults of *M. alternatus* were effectively killed by direct contact with conidia of *B. bassiana* formed on nonwoven fabric strips (Okitsu et al., 2000; Okabe et al., 2001, 2002) show the necessity for a bioassay technique applicable to dry conidia preparations. In the present study, the method of diluting live conidia with heat-killed conidia was established and enabled control of the dose of live conidia with a dry-conidia inoculating system leading to quantitative evaluation of insecticidal activity.

Young adults within 4 d after emergence were killed within 11 d on the average by inoculation with non-diluted conidia equivalent to 4.3×10^6 conidia per individual, and were killed within 15 d on the average by inoculation with 1/10 diluted conidia equivalent to 7.7×10^5 conidia per individ-

ual. These results indicate that the number of conidia attached to *M. alternatus* adults by walking on a nonwoven fabric strip, 8.5×10^5 per individual, was sufficient to kill young adults within two weeks. Even aged adults over 10 d after emergence were killed within 14 d on the average by inoculation with non-diluted conidia. These data suggest the possibility that the nonwoven fabric strip formulation of *B. bassiana* can be utilized for controlling the pine wilt disease as long as *M. alternatus* adults are somehow able to be forced to contact the formulation in the field.

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