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Management of pine wilt disease vectoring *Monochamus alternatus* adults using spray and soil application of *Metarhizium anisopliae* JEF isolates

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ABSTRACT

Chemical control is widely used to control the Japanese pine sawyer beetle, *Monochamus alternatus*, but strong chemical regulations require an environmentally sound management strategy. In this work, we investigated the use of entomopathogenic fungi and their application as a means of practical pest management. Thirty-two diverse species of fungal isolates were assayed against adult pine sawyer beetles using a contact method under laboratory conditions, and four isolates showed over 70% virulence consequently. These isolates, two each of *Beauveria bassiana* and *Metarhizium anisopliae* were sprayed on the adult beetles at 1×10^7 conidia/ml in plastic containers, respectively. The *M. anisopliae*-treated adult beetles showed 67% mortality. *M. anisopliae* isolates JEF-197 and JEF-279 demonstrated dosage-dependent insecticidal activity. Following the laboratory experiments, semi-field trials were conducted in young pine trees under high (RH 94%) and low (RH 35%) humidity conditions. In the high humidity conditions, most of the adult beetles stayed on the top of the branches. When the two *M. anisopliae* isolates were sprayed on the beetles, they showed ca. 50–70% insecticidal activity 11 days after application. In contrast, in low humidity conditions, the adult beetles tried to move off the branches and onto the soil. When the beetles reached the JEF-197 and JEF-279-treated soil, we measured > 90% insecticidal activity. This work suggests that *M. anisopliae* was the most virulent entomopathogenic fungus against adult Japanese pine sawyer beetles, and this forest insect could be ecologically controlled by the spray and soil application of the *M. anisopliae* isolates.

Introduction

The pine wilt nematode, *Bursaphelenchus xylophilus* Nickle (Aphelenchida: Aphelenchoididae) is a serious pine tree pathogen, causing pine wilt disease and damage to pine trees. Although some North American pine trees are resistant to the nematode, most pine tree species are not (Steiner and Buhrer, 1934; Mamiya and Enda, 1972; Kobayashi et al., 1984; Wingfield et al., 1984; Bergdahl, 1988; Dwinell and Nickle, 1989; Dwinell, 1993; Dwinell, 1997). The nematode is particularly damaging in Europe, mainly Portugal, and in East Asian countries such as Korea, China, Japan and Vietnam (Rutherford and Webster, 1987). In Japan, damage from the pine wilt nematode was reported as early as the 1900s. Since 1979, about 1 million m^3 of pine trees are damaged annually (Kishi, 1995). In China and Taiwan, nematode-caused pine tree damage was reported in 1982 and 1985 (Enda, 1997). In Korea, the first pine wilt nematode damage was reported in a small city in Busan in 1988, and has since spread throughout the southern part of Korea (Yi et al., 1989; Chung, 2002).

The pine tree nematode cannot move to another host tree by themselves; they need to be vectored by insects, such as *Monochamus* spp. (Evans et al., 1996; Sousa et al., 2001; Akbulut and Stamps, 2012). In East Asia, including Korea, the Japanese pine sawyer beetle, *Monochmus alternatus* HOPE (Coleoptera: Cerambycidae), is the main vector for *B. xylophilus*. Adult female of *M. alternatus* feed on pine bark, damage the endothelium and then spawn. The hatched larvae feed on the pine tree's sap wood and phloem tissue. The larvae make a space in the tree and overwinter. The following spring, the adults emerge from the pupal stage. *M. alternatus* larvae have four instars, all of which can overwinter. Some of the larvae reach the third or fourth stage over the winter and emerge without dormancy. Some of the larvae only reach the first or second stage over the winter, and take two years to become adults (Togashi, 1989; Go et al., 2019). The freshly-emerged adults

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transmit nematodes to healthy trees by feeding on twigs, which causes wounds, and through oviposition wounds (Togashi et al., 2019).

Although a variety of methods could be used to control the pine wilt nematode, it is not easy to manage the nematode in large-scale forests (Shin, 2008). Controlling the nematode's insect vector could be a more practical way to prevent the spread of pine wilt disease. *M. alternatus* is controlled mostly by synthetic chemical insecticides such as acetamiprid, clothianidin, fenitrothion, metham sodium, thiacloprid and thiamethoxam (Lee et al., 2003, Shin, 2008). For instance, metham sodium is used as a fumigation agent to control *M. alternatus* larvae in overwintering sites in damaged pine trees (Lee et al., 2003). Thiacloprid 10% SC is sprayed on pine trees in Korea to control *M. alternatus* adults (Shin, 2008). However, due to the issues with insect resistance and environmental residues. The use of chemical insecticides has been strongly regulated in many countries (Hemingway and Ranson, 2000; Whalon et al., 2008). Additionally, the law of PLS (positive list system) has been widely applied to the use of chemical pesticides, and particularly chemicals for forest insect pests should not be sprayed using airplanes, due to the unexpected chemical exposure of non-registered crops or fruit trees near to the forest (Bae and Lee, 2009). Therefore, it is important to investigate ways to control insect pests using alternatives to chemical insecticides.

Entomopathogenic fungi have been under development as a chemical agent replacement or sometimes in combination with synthetic chemicals to reduce the overall chemical burden (Glare et al., 2012; Lacey et al., 2015). Approximately 80% of entomopathogenic fungi that are used as commercially available insecticides belong to the genus of *Metarhizium* or *Beauveria* (de Faria and Wraight, 2007). In Japan there was an effort to control *M. alternatus* using the entomopathogenic fungus *Beauveria bassiana* by covering the tree with fungus-treated woven fabric or inserting fungal chips in the hole of pine tree (Shimazu and Sato, 2003). So far, much research around the world was focused on the research and development of entomopathogenic fungi for the control of this Japanese pine sawyer beetle, but consideration of the practical use and effective control in field conditions is limited.

In this work, we studied entomopathogenic fungi to develop a biopesticide to control adult stage of Japanese pine sawyer beetle, *M. alternatus*. Entomopathogenic fungi were isolated from Korean forest soil and screened out highly virulent isolates against *Tenebrio molitor* (Coleoptera: Tenebrionidae) as a preliminary bioassay insect. The adults of *M. alternatus* appear once every one or two years, and do not easily reproduce under laboratory conditions. In addition, they are very expensive to purchase. For these reasons, mealworm *T. molitor* were used in place of *M. alternatus* for the initial screening experiment.

The virulence of fungal isolates against adult *M. alternatus* was assessed using a spray method. Selected fungal isolates of *Metarhizium anisopliae* JEF-197 and JEF-279 were subjected to a dosage-dependent bioassay using the spray method. This was followed by conducting spray application using pot conditions in semi-field tests. During this experiment, interestingly, we observed the location of adults either on the potted tree or on its soil depending on environmental humidity conditions, such as low and high. Additionally, the *M. anisopliae* isolates were applied onto soil surface, which could be a novel approach here in this work. Based on this work, we suggested a new fungal application model to control the forest insect pest.

Material and methods

Insect

Mealworm *T. molitor* was used as an alternative insect to the Japanese pine sawyer beetle, *M. alternatus,* for primary screening of entomopathogenic fungi. We reared a colony of mealworms in wheat meal at 25 ± 1 °C under 50–60% relative humidity and a 16:8h light:dark photoperiod. Chinese cabbage, *Brassica rapa* subsp. *pekinensis* (Brassicaceae), leaves were placed on the wheat meal as a water source. Fifth-instar larvae were used in the experiment. *M. alternatus* adults were commercially supplied by an insect rearing company, Osang-kinsect (Guri city, Republic of Korea, http://www.k-insect.com). Final stage larvae were stored at 10 ± 1 °C for dormancy. To break dormancy, the larvae were treated at 20 ± 1 °C for 7 days and then moved to 25 ± 1 °C for emergence. The beetles were reared in 50–60% relative humidity and a 8:16 h light:dark photoperiod. All adult beetles in this study were used within 10 days of emergence.

Isolation of entomopathogenic fungi

Forest soil samples were used to isolate forest native entomopathogenic fungi to establish an entomopathogenic fungal collection against *M. alternatus*. These fungi were isolated using an insect-baiting method with *T. molitor* larvae (Kim et al., 2018). The isolated fungi were identified by morphological examination and ITS region sequencing using primers (ITS1-forward: 5′-TCC GTA GGT GAA CCT GCG G-3′, ITS4-reverse: 5′- TCC TCC GCT TAT TGA TAT GC-3′). The fungi were then stored at −70 °C in 20% glycerol stock. The isolated fungi's primary virulence screening was conducted by exposure method, where *T. molitor* larvae were released on the fungal mass in a Petri dish (60-mm diameter). *T. molitor* larvae were used to reduce the experimental cost with efficient screening of the appropriate entomopathogenic fungi. The entomopathogenic fungal isolates were cultured on a quarter sabouraud dextrose agar medium (1/4SDA, 60-mm diameter) (Difco™, Becton, USA) at 25 ± 1 °C for 14 days in order to fully induce conidiogenesis. Five of the 5th instar *T. molitor* larvae were transferred to the fungal cultured plate and kept at 25 ± 1 °C for 6 days in the dark. The number of dead larvae were counted daily. A total of 135 entomopathogenic fungal isolates were isolated from the soil samples and tested in the initial screening experiment (Table S1).

Virulence assay of M. alternatus adults

To select entomopathogenic fungal isolates that are highly virulent against *M. alternatus* adults, 2- to 3-day old *M. alternatus* adults were purchased from the insect rearing company (Osang-kinsect) and used in the bioassay. Thirty-two fungi isolates were selected from the initial screening experiment. These fungi were cultured on 1/4SDA Petri plate (90 mm dia.) at 25 ± 1 °C for 14 days. One *M. alternatus* adult was transferred to the 14-day-old fungal culture plate to induce fungal infection. After 1 h, the *M. alternatus* adult was transferred to a plastic cup (lid 100 mm dia. × bottom 60 mm dia. × height 150 mm), with a piece of filter paper (55 mm dia.; Advantec No.2, Japan) on the bottom. A fresh young pine branch (ca. 10 cm long) (*Pinus densiflora* Siebold & Zucc.) was supplied as a food source. The beetle remained in the cup at 25 ± 1 °C for 6 days with a 16:8h light:dark photoperiod. One milliliter of sterile distilled water was supplied every 2 days to maintain high humidity (> 90% relative humidity). Three of *M. alternatus* adults were used for each fungal isolate, and one fungal culture plate was used for one adult. The numbers of dead adults were observed daily. This secondary screening experiment was minimized due to the limited availability of *M. alternatus* adults.

Quantitative virulence assay of M. alternatus adults

Four selected isolates (two *B. bassiana* MJR026 and MJR065 and two *M. anisopliae* JEF-197 and JEF-279) from the secondary screening experiment were subjected to a spray assay to investigate their quantitative virulence. One adult beetle was placed into a plastic cup and a fresh young pine branch was provided as food as in the above assay method. Conidia suspensions of the isolates were prepared from 10-day-old 1/4SDA plates with a 0.03% siloxane solution (Silwet, Farmhanong Inc. Korea). The conidial concentration was adjusted to 1×10^7 conidia/ml and 1 ml of the conidia suspension was sprayed onto an *M. alternatus* adult in the plastic cup. One milliliter of sterile distilled water

was supplied every 2 days to maintain high humidity (> 90% relative humidity), and the cup was incubated at 25 ± 1 °C for 11 days with a 16:8h light:dark photoperiod. A 0.03% siloxane solution was served as a control. The bioassay was repeated three times, and 10 adults were used for each replicate. The numbers of dead adults were counted daily and this experiment was conducted with a range of conidial concentrations, 1×10^5, 1×10^6 and 1×10^7 conidia/ml. Isolates demonstrating more than 90% germination in the viability test were used in the experiments.

Semi-field test of M. alternatus adults under different humidity conditions

Selected isolates' insecticidal activities (*M. anisopliae* JEF-197 and JEF-279) were investigated in a semi-field condition with two different humidity environments. Briefly, young pine trees were planted in pots and the fungal isolates were sprayed on the trees, which were then maintained under low and high humidity conditions, respectively. More specifically, four 60–70 cm long pine trees (*P. densiflora*) were planted in plastic pots (width 30 cm × length 40 cm × height 30 cm). The pots were covered with a 20-mesh net (width 140 cm × length 110 cm × height 110 cm) for low humidity conditions (RH 34.6 ± 11.4%) and polyvinyl film for high humidity conditions (RH 93.5 ± 4.5%). The covers also prevented the adult beetles from escaping the experiment. The temperature and relative humidity for each pot were recorded every 30 min using HOBO UX100-023 External Temperature/Relative Humidity data loggers (Onset Computer Co., Bourne, MA). For each humidity condition, ten adult beetles were released into one pot and left for 2 h for settlement. Conidia were harvested from 14-days-old mycotized millet (fungal granule, GR) with a 0.03% siloxane solution and showed > 90% conidia viability using methods previously reported. Briefly, *M. anisopliae* JEF-197 and JEF-279 were cultured using the grain-based culture system on millet (*Panicum miliaceum* L.) grain purchased from a local market, Republic of Korea (Kim et al. 2011, Song et al. 2019). The two isolates were cultured at 25 ± 1 °C for 14 days and dried for 5 days to a 5% moisture content. A 50 ml conidial suspension (1×10^7 conidia/ml) was sprayed on the pine trees and adult beetles in the pots. The control group received a 0.03% siloxane solution. Each treatment was replicated three times (3 pots/replicate). After the application, all of the pots were kept in a test room at 25 ± 1 °C for 11 days. Adult beetle mortality and mycosis was checked daily.

During the pot experiments, the adult beetles' locations were monitored daily to understand their behavioral patterns and responses to the fungal application. Five locations were defined by the pot condition: 1) upper part of tree (young branch), 2) middle part of tree (between the young branch and the oldest branch), 3) lower part of tree (between the oldest branch to the soil surface), 4) soil surface, and 5) on the net or polyvinyl film at the top of the pot. The frequency at the five locations was calculated using the number of live adults and the percentage of population per location calculated as follows: (No. of live adult in specific part/No. of total live adult).

Granular fungal application

Given that some of the Japanese pine sawyer beetle adults localize on the soil surface, fungal insecticidal activity was investigated by applying fungal granules of *M. anisopliae* JEF-197 and JEF-279 onto the soil surface. The soil surface of a pot (width 30 cm × length 40 cm × height 30 cm) with 200 g of soil and 200 ml of distilled water was treated with 3 g of fungal granules. After three days, allowing the fungi to colonize, five adult beetles were released onto the soil surface with young pine tree branches for food. The pots were covered with polyvinyl covers for high humidity conditions. Pots with no fungal treated granule served as a control. Each treatment was replicated three times (3 pots/replicate). The pots were kept at 25 ± 1 °C, > 90% relative humidity inside, and 25 ± 1 °C, 30% relative humidity outside for 11 days. The temperature and relative humidity were recorded every 30 min using HOBO Temperature/Relative Humidity data loggers.

Statistical analysis

The percentage of live beetle data (No. of live beetle/No. of infested beetle), both from the laboratory and semi-field conditions, were arcsine transformed and analyzed using a generalized linear model (GLM) followed by Tukey's honestly significant difference (HSD) for multiple comparisons. In the location experiments, treatment, time and location of insect were analyzed to investigate their interactions. All the analyses were conducted using SPSS ver. 19.0 at the 0.05 (α) level of significance.

Results

Isolation of entomopathogenic fungal isolates against T. molitor at 1st screening

A total of 135 fungi were isolated using the insect-baiting entomopathogenic fungi isolation method in Korean forest soil (Table S1). Of the 135 isolates, 32 isolates showed virulence against *T. molitor* larvae, killing the larvae within 5 days after fungal exposure in the laboratory conditions (Data not shown). Pathogenic species were as follows: *B. bassiana*, *B. brongniartii*, *Cordyceps confragosa, Gibberella intermedia, Isaria fumosorosea, Lecanicillium attenuatum, Metacordyceps brittlebankisoides, Metacordyceps taii, Me. anisopliae, Me. figidum, Me. lepidiotae, Paecilomyces javanicus, Pochonia bulbillosa, Pochonia suchlasporia, Purpureocillium lilacinum.* The virulence of the 32 isolates were assessed against *M. alternatus* adults (Fig. 1).

Selection of virulent fungal isolates against M. alternatus adults

Two *B. bassiana* isolates (MJR026 and MJR065) and two *M. anisopliae* isolates (JEF-197 and JEF-279) showed higher virulence against the *M. alternatus* adults than the other fungal isolates (Fig. 1). In contrast, *Gibberella intermedia* MJR061, *Isaria fumosorosea* RFC002, *Lecanicillium attenuatum* IUL014 and *Pochonia bulbillosa* AHL016, AHL034, PWS064 didn't show any virulence, although these isolates were virulent against *T. molitor* larvae. Based on this result, the two *B. bassiana* isolates (MJR026 and MJR065) and two *M. anisopliae* isolates (JEF-197 and JEF-279) were selected for further application studies.

Insecticidal activity of B. bassiana and M. anisopliae against M. alternatus adults

The fungal conidial suspension spray method was used to compare virulence against *M. alternatus* adults. Results showed that the two *M. anisopliae* isolates were more virulent than the two *B. bassiana* isolates ($F_{4,120} = 17.5$, $p < 0.001$) (Fig. 2). The *M. anisopliae*-treated adults' mortality began to increase 7 days after spraying, while the *B. bassiana* MJR065-treated adults began to die only after 9 days. Eleven days after spraying, both of the *M. anisopliae* JEF-197 and JEF-279 treatments showed similar insect mortality (67%), while the two *B. bassiana* MJR026 and MJR065 treatments showed only 0% and 7% insect mortality, receptivity. Based on these results, both of the *M. anisopliae* isolates, JEF-197 and JEF-279, were selected for further study. The beetles infected with these fungi isolates were mycotized, and fungal outgrowth was observed in the head, thorax, abdomen, antenna and leg fragments, particularly in the soft cuticular area of inter-segmentation (Fig. S1).

In a bioassay with three different conidial concentrations, the *M. anisopliae* isolates JEF-197 and JEF-279 showed high virulence against the adults in a dosage-dependent manner (Fig. 3). Eleven days after spraying, *M. anisopliae* JEF-197 showed 27% (1×10^5 conidia/ml),

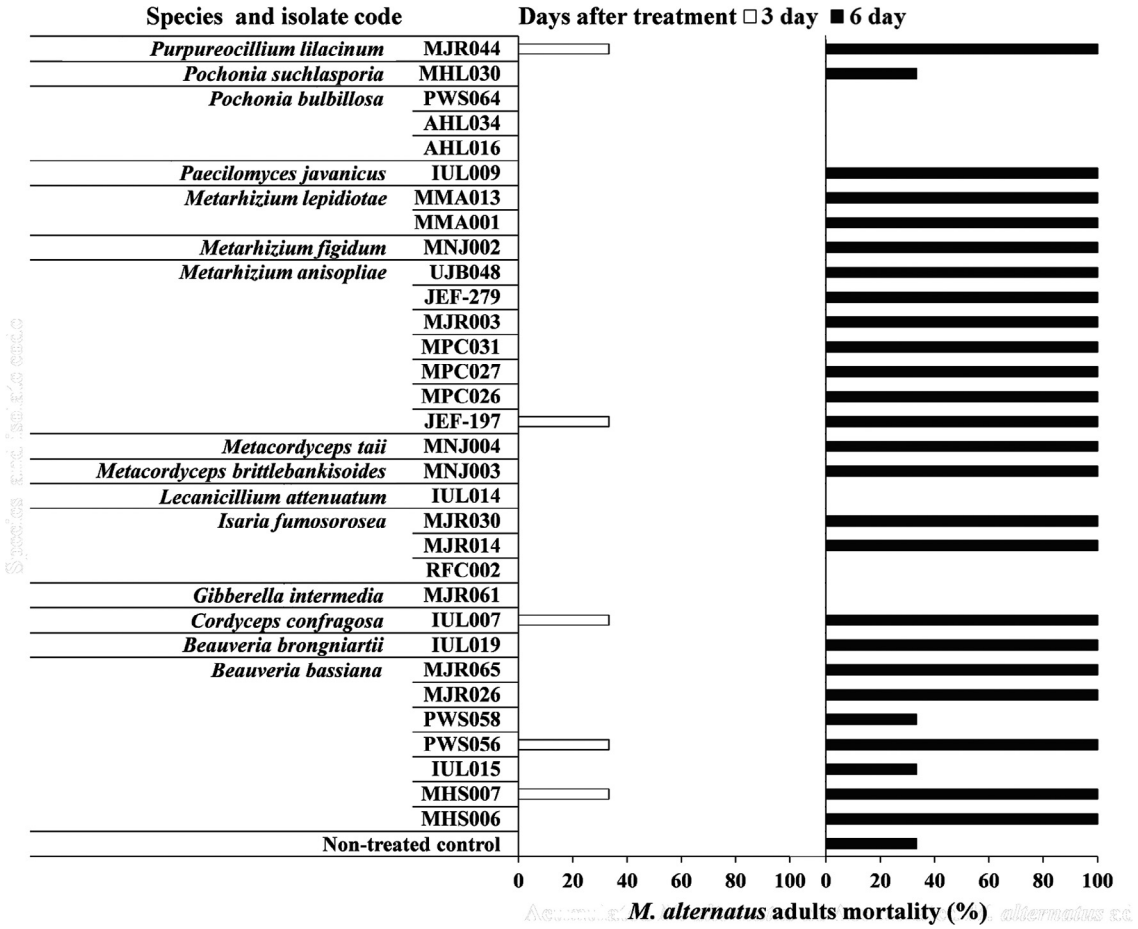

Fig. 1. Screening of entomopathogenic fungi against *M. alternatus* adults. One adult was exposed to the 14 day-cultured fungal plate. After 1 h, insect was transferred to moisturized dish conditions with a fresh young pine branch as a food source and incubated at 25 ± 1° C for 6 days. Three insects were used for each fungal isolate and no repeated experiments were performed. The number of dead adults were counted daily and the cumulative mortality (%) is calculated by adding up the daily mortality rates. The control group was treated for 1 h in a culture medium without fungi.

47% (1×10^6 conidia/ml) and 80% (1×10^7 conidia/ml) insect mortality. JEF-279 showed 47% (1×10^5 conidia/ml), 53% (1×10^6 conidia/ml) and 87% (1×10^7 conidia/ml) insect mortality. The two *M. anisopliae* isolate showed significant insect mortality compared to the control group and there was no significant difference between fungal isolates ($F_{2,168} = 5.8, p < 0.001$). The higher the concentration of conidia, the greater the virulence against the adults ($F_{2,144} = 127.805, p < 0.001$).

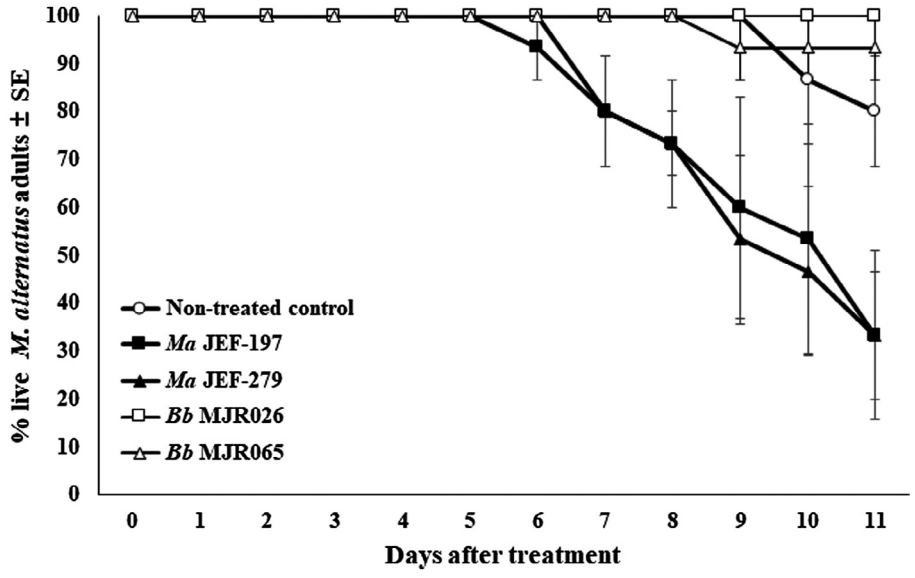

Fig. 2. Virulence of two *B. bassiana* isolates and two *M. anisopliae* isolates against *M. alternatus* adults in plastic container conditions. A conidial suspension (1×10^7 conidia/ ml) was sprayed on the adults at 1 ml/adult. This bioassay was conducted with three replicates (10 adults/replicate), and each adult was placed in a plastic container. All the containers were kept at room temperature, > 90% relative humidity for 11 days and daily the number of dead adults were counted. The values are presented as the means with standard error.

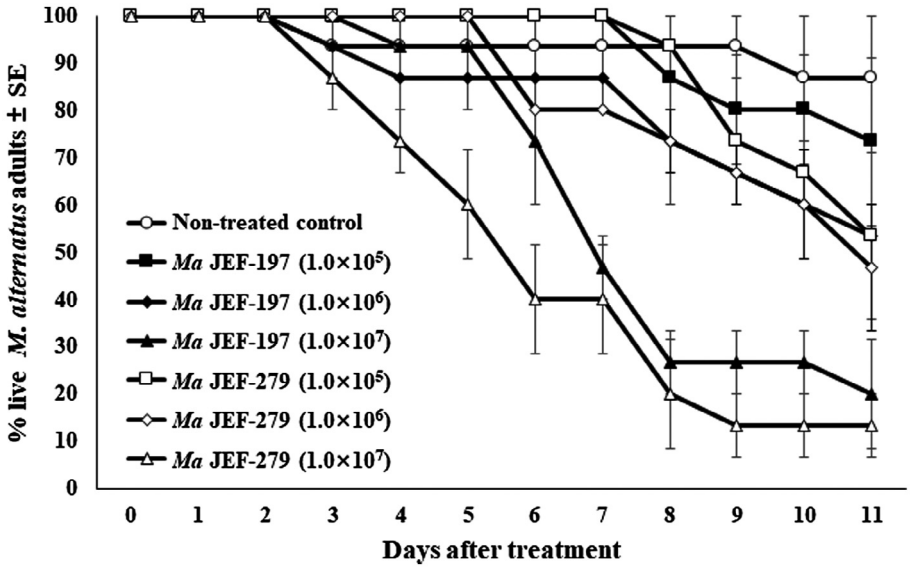

Fig. 3. Dosage-dependent virulence of two *M. anisopliae* isolates against *M. alternatus* adults in plastic container conditions. A series dilution of conidial suspension, $1 \times 10^{5-7}$ conidia/ml were sprayed on the adults at 1 ml/adult. This bioassay was conducted with three replicates (10 adults/replicate), and each adult was placed in one plastic container. All the containers were kept at room temperature, > 90% relative humidity for 11 days and daily the number of dead adults were counted. The values are presented as the means with standard error.

Insecticidal activity against M. alternatus adults in semi-field conditions

Insecticidal activity in different semi-field humidity conditions was investigated for two *M. anisopliae* isolates. In the low humidity condition (RH 34.6 ± 11.4%), the negative control group showed high insect mortality, reaching 60% in 11 days. Only *M. anisopliae* JEF-197 showed significant insect mortality ($F_{2,72} = 8.4$, $p < 0.001$) (Fig. 4A). Given the importance of relative humidity on control survival, the second round of semi-field tests was conducted in a high humidity environment (Fig. 4B). The adult beetles in the non-treated control group showed 20% insect mortality within 11 days, while on the same period, *M. ansiopliae* JEF-197 and JEF-279 showed 73% and 50% insect mortality, respectively ($F_{2,72} = 6.9$, $p < 0.005$). Most of the dead adults dropped to the soil surface. Mycosis was observed in all of the isolate-treated beetles.

Location of M. alternatus adults in semi-field conditions

The pot location of *M. alternatus* adults was investigated under the low and high humidity conditions (Fig. 5). In the low humidity condition, before the fungal treatment, the adults were on the net (28%), top of the pine tree (43%), middle of the tree (7%), bottom of the tree (4%) and on the soil surface (18%) (Fig. 5A). After the fungal suspension treatment, the number of insects found on the soil surface increased significantly for all treatment groups ($F_{11,360} = 8.1$, $p < 0.001$). At 11 days post treatment, 62%, 88% and 59% of *M. alternatus* adults were on the soil surface in the control, *M. anisopliae* JEF-197 and JEF-279 treated group, respectively. *M. anisopliae*-treated adults showed more of a tendency to be on the soil surface compared to the control group ($F_{2,360} = 4$, $p < 0.019$). There was no significant interaction among fungal isolate, time and location of insect ($F_{88,360} = 1.1$, $p = 0.194$). Over the course of the experiment, 29%, 40%, and 38% of *M. alternatus* adults were found on the soil surface for the control group, *M. anisopliae* JEF-197 treatment and *M. anisopliae* JEF-279 treatment, respectively. A similar pattern was observed for the high humidity conditions (Fig. 5B). The adults were on the net (43%), top of the pine tree (26%), middle of the tree (10%), bottom of the tree (2%) and on the soil surface (19%) before the fungal treatment. As time passed, the number of *M. alternatus* adults on the soil surface increased ($F_{11,360} = 5.058$, $p < 0.001$); more of the *M. anisopliae*-treated adults were found on the soil surface compared to the control group ($F_{2,360} = 3.019$, $p < 0.001$). Over the course of the entire experiment, 20%, 25, and 25% of the *M. alternatus* adults were found on the soil surface in the control group, *M. anisopliae* JEF-197 treatment and *M. anisopliae* JEF-279 treatment, respectively. No significant interaction among fungal isolate, time and location of insect was observed ($F_{88,360} = 1.2$, $p = 0.132$).

Insecticidal activity of soil-treated M. anisopliae GR in semi-field conditions

Given that some of the *M. alternatus* adults localized on the soil surface, fungal granules were applied to the soil surface. *M. anisopliae* JEF-197 and JEF-279 showed 60% and 40% insect mortality 7 days after the application, and 100% and 90% of the insects died after 11 days ($F_{3,36} = 18.1$, $p < 0.001$), respectively (Fig. 6). *M. alternatus* adults' mycosis with *M. anisopliae* were also observed (Fig. S2).

Discussion

In this study, we investigated the possibility of using entomopathogenic fungi to control the adult stage of the Japanese pine sawyer beetle, *M. alternatus*. These fungi were collected from forest soil using an insect-bait method. The virulence of the isolated fungi was assayed against *T. molitor* larvae as a preliminary check before testing on the *M. alternatus* adult. *M. anisopliae* JEF-197 and JEF-279 isolates showed high virulence against *M. alternatus* adults and were used in a semi-field study of low and high humidity conditions. Results suggest a combination of fungal spray and soil application is a practical strategy for pest management.

Entomopathogenic fungi can be efficiently isolated using the insect-baiting method (Meyling, 2007; Kim et al., 2018). To isolate entomopathogenic fungi from forest soil, we used this method with the coleopteran insect, *T. molitor* larvae. The use of mealworm for fungal isolation has many advantages, such as breeding many larvae within a short time of period, survival for a long time without food and continuous soil contact. In addition, mealworm belongs to the same order, coleoptera, as *M. alternatus*, so similar virulence could be expected. Of the isolates, 32 had high virulence against *T. molitor* larvae. Among them, 81% (26 isolates) also showed virulence to *M. alternatus* adults. Thus, the mealworms were suitable insects to use for primary screening.

Entomopathogenic fungi *Beauveria* spp. and *Metarhizium* spp. have been reported as pathogenic fungi to *M. alternatus* (Shimazu et al., 1995; Shimazu and Sato, 2003; Shimazu 2004; He et al., 2008). To date, entomopathogenic fungi for biological control of *M. alternatus* have been limited to the two genera of fungi discussed above. In this study, however, *Cordyceps confragosa, Isaria fumosorosea, Metacordyceps* sp.,

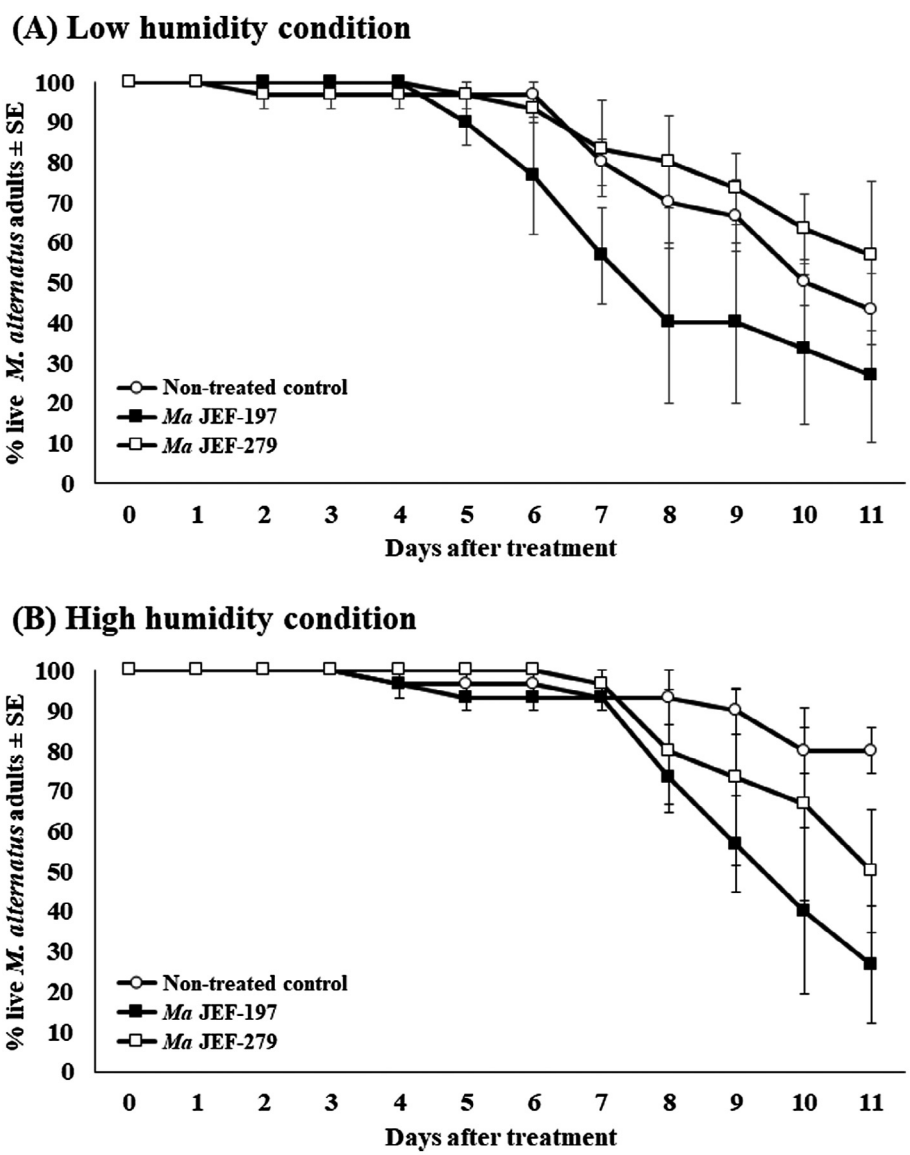

Fig. 4. Insecticidal activity of *M. anisopliae* JEF-197 and JEF-279 against *M. alternatus* adults under low (A, 34.6 ± 11.4% relative humidity) and high humidity (B, 93.5 ± 4.5% relative humidity) semi-field conditions. Four 60–70 cm long pine trees (*P. densiflora*) were planted in a plastic pot (W 30 cm × L 40 cm × H 30 cm) and ten of *M. alternatus* adults were released. A 50 ml of conidial suspension (1×10^7 conidia/ml) was sprayed on the pine trees and adults in a pot. This test was conducted with three replicates (10 adults/replicate). All the pots were kept at room temperature for 11 days and the number of dead adults were counted daily. The values are presented as the means with standard error.

Pochonia sp. and *Purpureocillium lilacinum* also showed virulence to *M. alternatus* adults. Among them, several fungi have been evaluated for their potential as biological control agents against invertebrate pests (Zimmermann, 2008; Luangsa-ard et al., 2011; Manzanilla-López et al., 2013; Vongsangnak et al., 2017). This result suggests that *C. confragosa, I. fumosorosea, Metacordyceps* sp., *Pochonia* sp. and *P. lilacinum* are candidates for potential control of *M. alternatus,* even though these fungal species were not selected as candidates for further experiments in this study.

The *M. anisopliae* JEF-197 and JEF-279 isolates showed a high virulence against *M. alternatus* adults using a 1×10^7 conidia/ml spray method. *B. bassiana* MJR026 *and* MJR065 isolates showed a low virulence by the same method even though the isolates showed high virulence during the secondary screening in the Petri dishes. This result can be explained, the adults *M. alternatus* were exposed to much more *Beauveria* conidia (not quantified) than *Metarhizium* conidia in the secondary screening experiment. It supported that conidia yields were higher in *B. bassiana* than *M. anisopliae* in same cultured conditions with various substrates (Song et al., 2019). Therefore, *M. anisopliae* JEF-197 and JEF-279 isolates are much stronger than that of the *B. bassiana* isolates in this study. In other words, the *M. anisopliae* isolates can kill *M. alternatus* adults with smaller numbers of conidia compared to *B. bassiana* conidia. However, the *B. bassiana,* isolated from *M. alternatus* larvae remains, has a high virulence and has been utilized as a means for controlling *M. alternatus* in Japan (Shimazu et al., 1995; Shimazu and Sato, 2003). Although our two selected *B. bassiana* isolates, MJR026 and MJR065, showed low virulence against *M. alternatus* adults by the spray method, confirmation of virulence against *M. alternatus* larvae is still required in further experiments.

The virulence of *M. anisopliae* JEF-197 and JEF-279 isolates was evaluated against *M. alternatus* adults with different conidia concentrations. We achieved high insect mortality (> 80%) only when 1×10^7 conidia/ml was used. This concentration of conidia has been used for virulence assays in many studies and is being evaluated at available concentrations that can be used for practical entomopathogenic fungal industrialization (Barson et al., 1994; Kassa et al., 2002; Park et al., 2018). Production of conidia suspensions at concentrations higher than 1×10^7 conidia/ml are not only difficult to make for some entomopathogenic fungi isolates but can also provide disadvantages in terms of unit cost. In addition, when smaller doses ($1 \times 10^{4-6}$ conidia/ml) are applied in the forest, it's possible the conidia could spread and colonize, and would not be effective as a pest control. Although the concentration of conidia suspension for *M. alternatus* adult control is 1×10^7 conidia/ml, various other aspects should be considered before its application in the forest.

In the semi-field conditions, we compared the insecticidal activity of

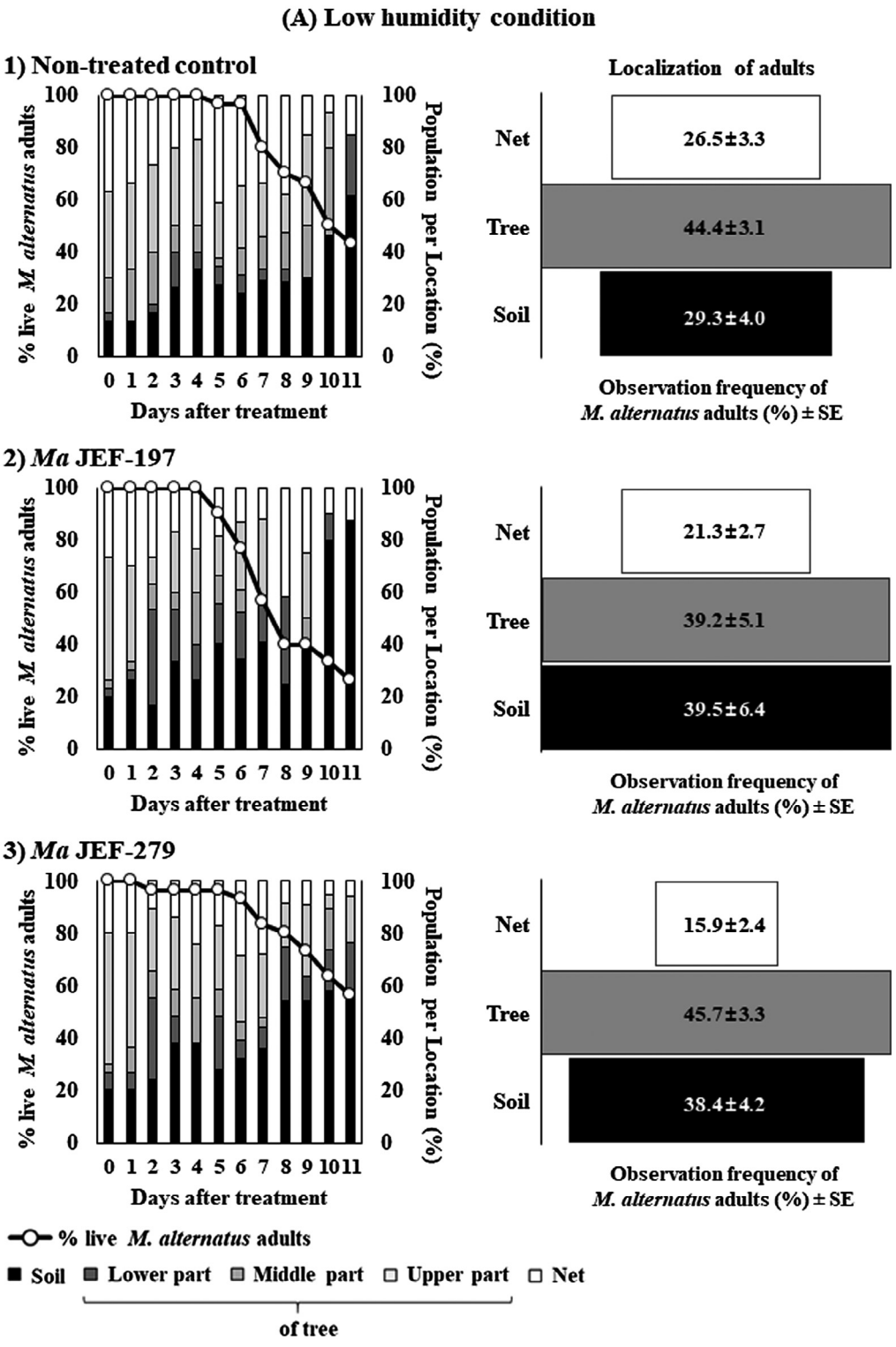

Fig. 5. Localization of alive *M. alternatus* adults in the low and high humid semi-field conditions. In the time of semi-field assay, localization of adults was monitored daily by checking the 1) upper part of tress (newly generated branch), 2) middle part of tree (from the under newly generated branch to the oldest branch) and 3) lower part of tree (from the under oldest branch to the soil surface), 4) soil surface, and 5) on the net or polyvinyl film. The percentage of population per location was calculated by ratio of live adults in specific parts to total live adults.

M. anisopliae JEF-197 and JEF-279 depending on the humidity on *M. alternatus* adults. At low humidity, the mortality of *M. alternatus* adults treated the fungal isolates was lower than expected, and by 11 days post treatment, the mortality of adults that were not treated with the fungal isolates was as high as 60%. This is because the fungal isolates' activity decreases at low humidity, and *M. alternatus* adults have also decreased activity at low humidity. Therefore, we confirmed the insecticidal activity at high humidity condition. The mortality of *M. alternatus* adults treated with the fungal isolates was like that of insects at low humidity conditions, but relatively low mortality was observed in the control group than at low humidity conditions. Korea normally has two periods of heavy rain in the summer – the monsoon season in June/July and

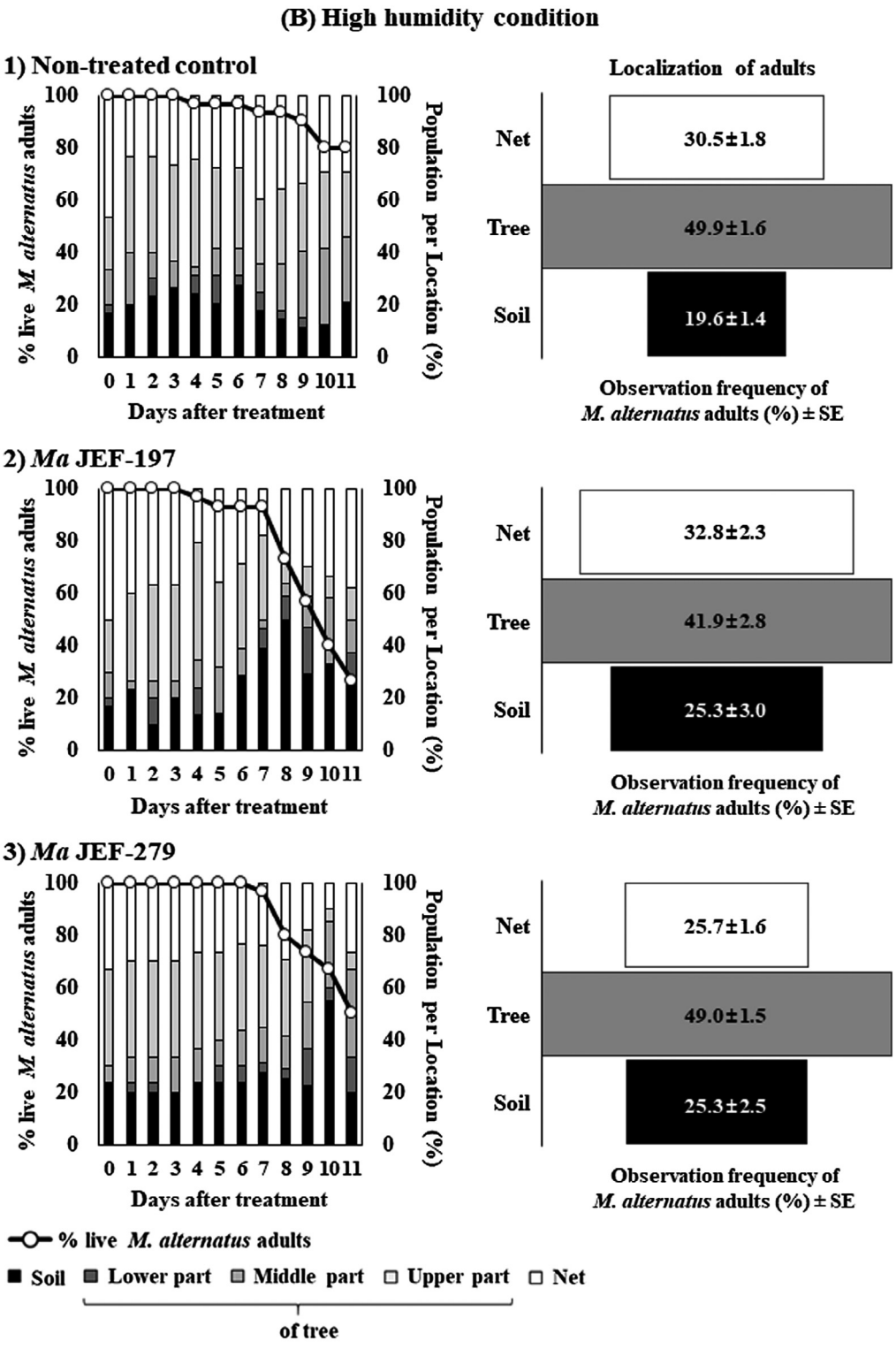

Fig. 5. (*continued*)

typhoon season in August/September. These tend to make summers very humid, and *M. alternatus* adults are active during these seasons (Kwon et al., 2006; Shin, 2008). The National Institute of Forest Science, Republic of Korea also reported that Korean forests maintain higher than 70% relative humidity throughout the year (http://mtweather.nifos.go.kr/current/mount_current_main.html#). This suggests that the control efficacy of *M. alternatus* adults by entomopathogenic fungi will be high for the high humidity conditions of Korean forests.

The *M. alternatus* adults' distribution seen in the semi-field conditions showed that most adults were found on the pine trees, but about 19–40% were on the soil surface. In addition, *M. anisopliae*-infected live adults tended to be on the soil surface in this study. The host-specific entomopathogenic fungus *Ophiocordyceps unilateralis* has been reported to control insect brains and manipulate their behavior to reach death locations that are optimal for spore dispersal (Shang et al., 2015). Information on changes in insect behavior patterns due to entomopathogenic fungus *M. anisopliae* infections are limited. Further

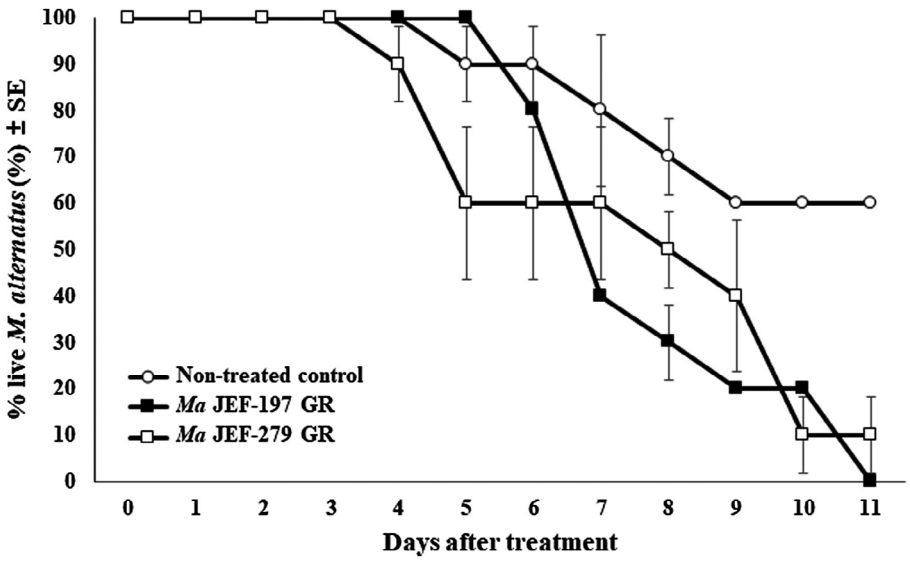

Fig. 6. Insecticidal activity of fungal granules of *M. anisopliae* JEF-197 and JEF-279 against *M. alternatus* adults on soil. Ten of *M. alternatus* adults were released onto the granules of *M. anisopliae* JEF-197 and JEF-279-treated soil. This test was conducted with three replicates (10 adults/replicate). All the pots were kept at room temperature for 11 days and the number of dead adults were counted daily. The values are presented as the means with standard error.

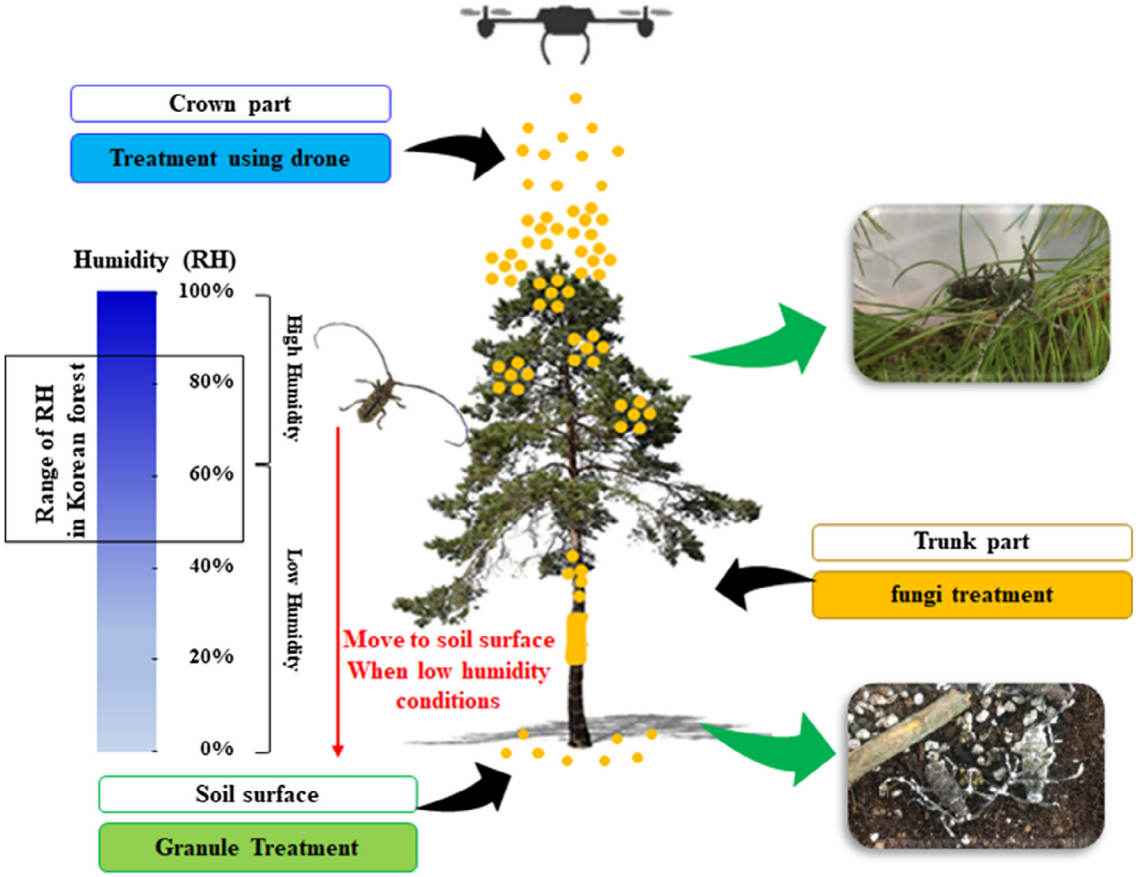

Fig. 7. A suggested entomopathogenic fungal application strategy to control Japanese pine sawyer beetle *M. alternatus* in forest. Entomopathogenic fungi can be applied three different ways to control *M. alternatus* adults. 1) Spraying of fungal conidial suspension by drone; 2) Colonization of entomopathogenic fungi on pine tree; 3) Treatment of fungal granules on pine forest. A powerful strategy to control this forest pest insect can be developed using these three methods in combination.

studies are needed to clarify the insect patterns observed in this experiment. Through these behavior patterns, it was confirmed that the adults can move between the pine trees and soil surface. This suggests the possibility of controlling *M. alternatus* adults through treatment of the fungal isolates on the soil surface. *M. alternatus* movement on the soil surface in forest fields has not been reported. This study's results are not proven because we confirmed the location of *M. alternatus* adults when they were in the pot conditions only. It may be possible to control *M. alternatus* adults by treating the soil surface with fungal granules once the moving pattern of the adults from the trees to soil surface is confirmed in the forest conditions. If confirmed, complex fungi treatment can be a more effective strategy of *M. alternatus* management (Fig. 7). First, as an aerial treatment, the entomopathogenic fungi can be applied to a wide area of forest, however, it would be necessary to have a means to protect non-target insects. A second method is to treat pine trees in a certain area with the

entomopathogenic fungi so that the *M. alternatus* adults come into contact with it while feeding on the pine tree. This method will have less impact on non-target insects than aerial application. A third method is to treat the fungal granules in the soil around the pine trees. As *M. alternatus* adults move to the soil, they will make contact with the fungi. This method should be used in combination with the other two methods rather than on its own. Further studies on the effects of *M. anisopliae* against *M. alternatus* adults in field conditions are needed along with fungal effect on non-target organisms.

In conclusion, this study investigated the possibility of controlling *M. alternatus* adults using entomopathogenic fungi, *M. anisopliae* JEF-197 and JEF-279, focusing on spray and novel soil application strategies. We constructed an entomopathogenic fungal library against Japanese pine sawyer beetle and selected highly virulent JEF-197 and JEF-279. Additionally, we figured out that in high humidity condition the adults stayed on branches of pine tree, but in low humidity condition they moved to soil surface. Experimentally, spray on tree branches and soil application could be effective pest management strategies. We suggest that Japanese pine sawyer beetle adults could be ecologically controlled by the spray and soil application of the *M. anisopliae* isolates. Although the entomopathogenic fungi took more time to show insecticidal effects against *M. alternatus* adults compared to chemical agents, they control insects in a more environmentally friendly way.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aspen.2019.12.012.

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