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Laboratory and Field Evaluations of Entomopathogenic *Lecanicillium attenuatum* CNU-23 for Control of Green Peach Aphid (*Myzus persicae*)

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An entomopathogenic fungus was isolated from an infected aphid. The isolate conformed most closely to *Lecanicillium attenuatum* CBS 402.78 (AJ292434) based on the internal transcribed spacer (ITS) region of its 18S rDNA, and thus was designated *L. attenuatum* CNU-23. Laboratory and field evaluations of CNU-23 blastospores were carried out for the control of green peach aphids. The laboratory evaluations of CNU-23 revealed an aphid mortality of about 80% with an estimated LT_{50} of 3.72 days after the application of CNU-23 at 1×10^6 blastospores/ml. Meanwhile, the field evaluations of CNU-23 performed on greenhouse pepper plants during the rainy season showed an aphid mortality ranging from 72% to 97%. Significant sporulation was observed in the aphids treated with CNU-23. Therefore, the results suggest that *L. attenuatum* CNU-23 can be used as a biocontrol agent for green peach aphids on greenhouse pepper plants.

Keywords: Aphids, biocontrol, entomopathogens, fungi, *Lecanicillium*

In South Korea, the green peach aphid (*Myzus persicae*) is an important pest for greenhouse crops, such as peppers, tomatoes, strawberries, and watermelons. The aphid feeds on plant nutrients, resulting in curled and deformed leaves. Moreover, the aphid secretes sugar, which supports the growth of plant pathogens, and transmits many viral diseases, including the cucumber mosaic virus and potyvirus, in several crops. Although a range of broad-spectrum synthetic insecticides are widely used to control aphids, their intensive use is of concern owing to the potential side effects on human health. The development of pesticide-resistant insects is also a worry for pest management strategists. Thus, recent research has focused on the development of alternative control methods in an effort to reduce the use of synthetic insecticides.

An environmentally friendly alternative to synthetic pesticides for the control of crop pests is the use of entomopathogenic fungi [13, 18, 20]. For example, *Lecanicillium* spp., which comprise the principal mitosporic microorganism commonly found in aphids, exhibit high pathogenic activity against aphids [4, 6]. Vertalec is an example of a commercial *Lecanicillium* product developed for aphid control. Yet, relatively few data are available on the use of entomopathogenic fungus-based products in South Korea. Accordingly, the present study evaluated an entomopathogenic isolate, *Lecanicillium attenuatum* CNU-23, for the control of *M. persicae* in pepper plants under laboratory and greenhouse conditions.

An entomopathogenic fungus was isolated from an infected green peach aphid on pepper plants in 2005, transferred onto potato dextrose agar (PDA), and incubated at 25°C for 7 days. After several transfers onto a cellophane disc (4 cm in diameter) on new PDA plates, the total genomic DNA was extracted from the mycelia using an AccuPrep DNA extraction kit (Bioneer Corp., Daejeon, South Korea). The internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) of the nuclear rDNA was then amplified from the genomic DNA with primers ITS5 and ITS4 [19] using a Quick PCR Premix (GENENMED, Seoul, South Korea). The PCR reaction was initiated at 90°C for 10 min, followed by a thermal cycle of denaturation for 1 min at 94°C, annealing for 1 min at 52°C, and extension at 72°C for 1 min. The amplified PCR products were then electrophoresed on a 0.75% agarose gel, and the checked amplicons were purified using an Accuprep PCR purification kit (Bioneer Corp., Daejeon, South Korea). Next, the purified PCR products were sequenced using an AB13700 model DNA sequencer (Applied Biosystems Inc., CA, U.S.A.). For sequencing the ITS and 28S regions, the primer pairs ITS4 (TCCTCCGCTTATTGATATGC) and LR5 (TCCTGAGGGAAACTTCG) were used, as described

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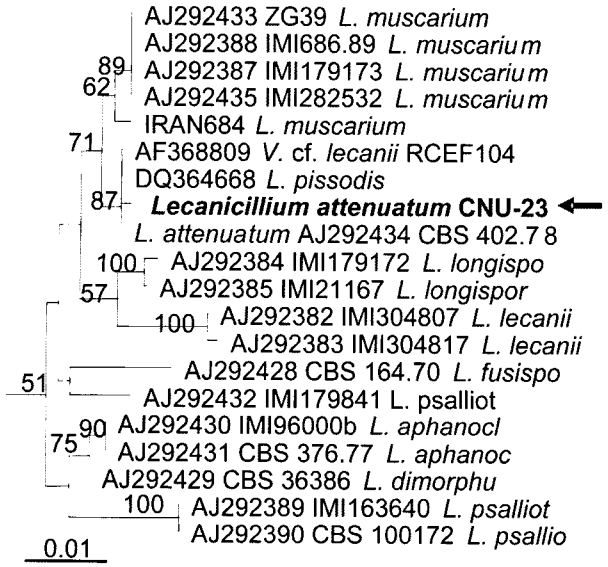

Fig. 1. Phylogenetic tree of *Lecanicillium attenuatum* CNU-23 based on ITS sequence analysis.

Fig. 2. Morphology of *Lecanicillium attenuatum* CNU-23. Conidia, violet-colored arrow; Conidiophores, red-colored arrows.

previously [19]. The sequence of the isolate was aligned against sequences from the GenBank database, and phylogenetic trees were inferred by neighbor joining, as described previously [3, 15]. The phylogenetic analyses were performed as described previously [8–10], and the degrees of sequence similarities were analyzed using the similarity comparison feature of BioEdit ver. 5.0.9.

Based on the phylogenetic analysis, the fungal isolate was most closely clustered with *L. attenuatum* CBS402.78 (AJ292434) retrieved from GenBank (Fig. 1), and the sequence identity values obtained from NCBI BLASTN searching were phylogenetically identical to the sequence of *L. attenuatum* CBS402.78 (AJ292434). Although the isolate was also related to other groups, including *L. muscarium* IMI282532 (AJ292435), *L. lecanii* IMI304807 (AJ292382), and *L. longisporum* IMI179172 (AJ292384), it was still distinct based on an ITS sequence analysis and morphological observations. Thus, the isolate was named *L. attenuatum* CNU-23, and has been deposited in the GeneBank database under Accession No. EF192939. When CNU-23 was cultured on PDA, the colonies reached a diameter of 12 mm within 10 days and were white with a black-brown color in the center with a yellowish white reverse. Microscopic observations of CNU-23 showed three to five phialides per node on prostate hyphae or secondary branches, measuring 9–16 μm in length and 1–2 μm wide. The conidia of CNU-23 were cylindrical with an attenuated base and slightly curved in the middle. Two-cell stages in the size range 4–6 μm×1.5–2.0 μm were also observed. Conidiophores arose from aerial hyphae, usually prostrate and little differentiated from the subtending hyphae (Fig. 2). The isolate was matched to *L. attenuatum* based on a comparison of its morphological and molecular phylogenetic characteristics [21]. CNU-23 did not grow at 35°C, and was found to utilize Tween 80 and soybean oil as sole carbon sources, which has not been previously reported for other *Lecanicillium* species.

Laboratory bioassays of CNU-23 against aphids were performed using 2-week-old Chinese cabbages [7]. Chinese cabbage seeds were disinfected with 70% ethanol for 1 min, followed by rinsing with sterile distilled water. The disinfected seeds were then planted in commercial compost in square plastic insect-breeding dishes (80×80×100 mm) and grown in a greenhouse at 25±2°C and 60±5% relative humidity for 2 weeks. Twenty green peach aphids (*M. persicae*) were reared on the Chinese cabbage leaves and allowed to produce progeny. Following a 24-h of incubation, the adult aphids were removed from the leaves; each dish had 20–25 2nd instar nymphs. To prepare the fungal agent, isolate CNU-23 was grown in a mineral salt medium (MSM) with 0.2% (v/v) soybean oil and 1% (v/v) Tween 80 in a shaking incubator at 150 rpm at 25°C for 7 days. The MSM also included the following constituents (g/l, pH 7.2): K_2HPO_4 2.4, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 0.2, and $CaCl_2 \cdot 2H_2O$ 0.025. The cultures were filtered through sterilized cheesecloth and the blastospores were collected by vacuum-filtering through two sterilized Whatman No. 2 filter papers. The blastospores, for use as the fungal agent, were counted on a hemacytometer under a microscope. To confirm their viability, the blastospores were also grown on PDA plates for 7 days. The blastospores were then diluted to give a concentration of 10^4 to 10^8 blastospores/ml and sprayed onto the nymphs on the Chinese cabbage leaves using a small handheld sprayer. Thereafter, the plants were maintained in a growth chamber at 25±2°C and 85±5% relative humidity with a 16 h:8 h light:dark

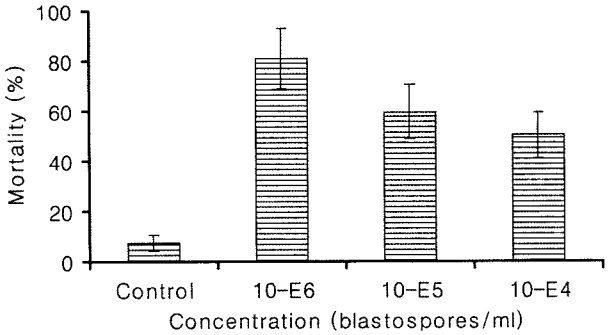

Fig. 3. Laboratory mortalities of *Myzus persicae* treated with *Lecanicillium attenuatum* CNU-23 at different blastospore concentrations.
The data shown are means±SD of triplicate experiments.

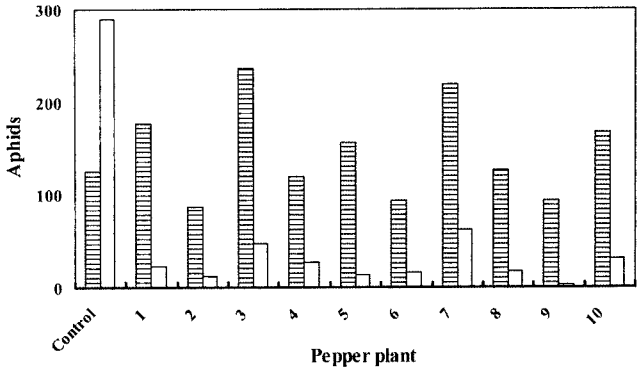

Fig. 5. Field mortalities of *Myzus persicae* in greenhouse pepper plants before (□) and after (■) application of *Lecanicillium attenuatum* CNU-23.

photoperiod. The control nymphs were treated with an MSM mixture without the fungal blastospores. The aphid mortality was monitored for 7 days after the spray application as follows; mortality (%)=(number of insects on control leaf–number of insects on treated leaf)/(number of insects on control leaf and treated leaf). The median lethal time (LT_{50}) was estimated as described previously [7]. Among the dead aphids, only those exhibiting fungal sporulation were considered to have died of fungal infection. In addition, field evaluations of the fungal isolate were performed on green peach aphids occurring naturally on greenhouse pepper plants at Bannam, Naju City, Chonnam, South Korea, during the rainy season in July 2006. The greenhouse had previously suffered from serious green peach aphid damage every year during this same season, thereby allowing the fungal isolate to be assessed for aphid control. Ten pepper plants with high aphid populations on three leaves per plant were sprayed with the fungal isolate at 10^7 to 10^8 blastospores/ml. A second application was carried out 3 days later, as described above, and aphid mortality was investigated for 12 days after the final application. Only aphids exhibiting fungal sporulation were considered as having died from fungal infection. The temperature and relative humidity ranged from 23 to 28°C and 80% to 90%, respectively, during the experiments.

The laboratory evaluations of CNU-23 showed that it was effective in controlling aphids when sprayed in a blastospore-based formulation, as about 80% aphid mortality was observed when CNU-23 was sprayed at 1×10^6 blastospores/ml (Fig. 3), whereas only 10% aphid mortality was observed in the control samples without the application of CNU-23. More than 60% aphid mortality was observed 4 days after the application of CNU-23, giving an LT_{50} value of 3.72 days (Fig. 4). Meanwhile, in the field evaluations, the aphid populations increased from 125 to 289 on the control pepper plants, yet decreased significantly following CNU-23 application, with mortalities of 72% to 97% (Fig. 5). Significant inhibition of aphid reproduction was observed with the sporulation of CNU-23 (Fig. 6).

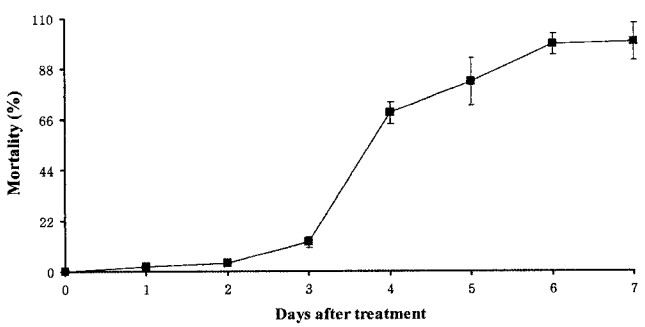

Fig. 4. Time-course mortalities of *Myzus persicae* treated with *Lecanicillium attenuatum* CNU-23 at 1×10^6 blastospores/ml.
The data shown are means±SD of triplicate experiments.

Fig. 6. Photograph showing fungal sporulations on aphids infected with *Lecanicillium attenuatum* CNU-23 in greenhouse pepper plants.
The arrows indicate examples (in white) of fungal sporulation on the pepper leaf.

Although the parasitization mechanism of *M. persicae* is unclear, CNU-23 may adhere to and penetrate the host tegument, as described in other entomopathogens [1, 5], or produce insect cuticle-degrading enzymes, such as chitinases and protease [14, 16].

Management and control of aphid infestation in organic greenhouse farming is a challenging research field. The use of biocontrol agents is a promising approach acceptable to organic farming. To date, at least 60 biocontrol products, including 38 species or varieties of fungi, have been developed and used as biocontrol agents against pests [2]. Despite attempts to screen low-cost substrates for the mass production and commercialization of fungal agents [12, 17], few investigations on selective substrates for fungal growth have been performed. When CNU-23 was grown on a mixture of soybean oil and Tween 80, its blastospore concentrations ranged from 10^{10} to 10^{12}/ml. Thus, a mixture of soybean oil and Tween 80 can be used as a substrate for the mass production of CNU-23. CNU-23 can also be selectively cultivated in agricultural water containing a mixture of soybean oil and Tween 80, in which microorganisms capable of degrading these substrates are not commonly found.

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