Isolation and Identification of Entomopathogenic Fungus from the Pine Wilt Disease Vector, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) in Korea

Tae-Young Shin, Jae-Bang Choi, Sung-Min Bae, Ye-Rim Cha, Jeong-Mi Oh, Hyun-Na Koo and Soo-Dong Woo*

Department of Agricultural Biology, College of Agriculture Life & Environment Sciences, Chungbuk National University, Cheongju 361-763, Korea

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Entomopathogenic fungi were isolated directly from a cadaver of adult *Monochamus alternatus* supporting fungal sporulation, using a semi-selective medium and then screened several fungal colonies. The pathogenicity of each fungus was tested using oak longicorn beetle, *Moechotypa diphysis*, as substitutive insect. As the result, only one of them showed high pathogenicity against *M. diphysis*, with up to 100% mortality within 21 days of inoculation. Selected fungus was named as MaW1 and identified by *Beauveria bassiana* using microscopic examination and DNA analysis. Pathogenicity was also evaluated to *M. alternatus*.

Key words: *Monochamus alternatus*, *Beauveria bassiana*, Entomopathogenic fungi, *Moechotypa diphysis*

**Introduction**

Pine wilt is the most important disease of pine trees in Korea, Japan, China and recently has been introduced into Portugal (Mota et al., 1999). The pathogen causing this disease, *Bursaphelenchus xylophilus*, the pinewood nematode is vectored by adults of some cerambycid beetle species and the Japanese pine sawyer, *Monochamus alternatus*, which is the major vector species in Korea (Korea Forest Research Institute, 2003). The Korea government has established a pine wilt center for integrated research on pine wilt disease and development of several control methods (fumigation, aerial application of pesticides, injection of nematocides into tree trunks and use of the vector’s natural enemies). However, the cost is too high and difficult to apply in large areas (Shin, 2008). The common method to prevent this disease is to control the vector insects. In the most of countries with pine wilt disease, chemical insecticides have been used to kill vector insect and thus prevent transmission of the pathogen (Maehara et al., 2007). However, in Japan, to reduce the use of chemicals, an entomopathogenic fungus *Beauveria bassiana* was studied and developed as an insecticide (Shimazu and Kushida, 1983; Shimazu et al., 1992 and 1995; Shimazu, 1994 and 1997; Shimazu and Sato, 2003). Entomopathogenic fungi were natural enemies of insects and contribute to the role of their host population. Several fungal species in genus *Beauveria* commonly occurs in environment and has been used as a pest control agent in a number of countries (Xavier and Khachatourians, 1996). Recently, Portugal researchers also studied entomopathogenic fungus *B. bassiana* against the pine wilt disease vector, *M. galloprovicialis* (Francardi et al., 2003). This is thought to be the convenient and effective method to control *M. alternatus*. Biological control for *M. alternatus* is necessary for integrated pest management. Therefore, the objective of this study was to isolate and identify entomopathogenic fungus from a cadaver of adult *M. alternatus* and select high pathogenic fungus in Korea.

**Material and Methods**

**Insect and fungi**

Only adults of *M. alternatus* and *M. diphysis* were field-
collected or obtained from the culture of insect eco-toxicology laboratory, Chungbuk National University, Korea and used to bioassay. Four strains of entomopathogenic fungi and three strains of non-entomopathogenic fungi were used to this study. All strains are listed in Table 1.

**Isolation of entomopathogenic fungi and morphological examination**

The fungi were isolated directly from a cadaver of adult *M. alternatus* supporting fungal sporulation, using a semi-selective medium consisting of potato dextrose agar (PDA; Difco™, USA) containing penicillin-streptomycin solution (Sigma, USA). The fungi were grown on PDA for 2 weeks at 25°C in the dark and then stored at 4°C until use. To isolate entomopathogenic fungi against *M. alternatus*, *M. diphysis* adults were used as substitutive insect for bioassay. Each fungal suspension was prepared by scraping conidia from the surface of 2 weeks old culture dish and used to bioassay. Four strains of entomopathogenic fungi and three strains of non-entomopathogenic fungi were used to this study. All strains are listed in Table 1.

**Table 1. Fungal strains used in this study.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFB-13</td>
<td><em>B. bassiana</em></td>
<td>Entomopathogen</td>
</tr>
<tr>
<td>KCTC 6300</td>
<td><em>B. Brociciartii</em></td>
<td>Entomopathogen</td>
</tr>
<tr>
<td>KACC40217</td>
<td><em>Metahizium</em> sp.</td>
<td>Entomopathogen</td>
</tr>
<tr>
<td>SFP-198</td>
<td><em>Paeilomyces fumosoroseus</em></td>
<td>Entomopathogen</td>
</tr>
<tr>
<td>GKPE-1</td>
<td><em>Alternaria alternata</em></td>
<td>Non-entomopathogen</td>
</tr>
<tr>
<td>GBYP-8</td>
<td><em>Botrytis cinerea</em></td>
<td>Non-entomopathogen</td>
</tr>
<tr>
<td>JC-24</td>
<td><em>Colletotrichum acutatum</em></td>
<td>Non-entomopathogen</td>
</tr>
</tbody>
</table>

**Isolation of genomic DNA and PCR**

All fungal genomic DNA were extracted using an I-genomic BYF DNA extraction mini kit (iNtRON Co., Korea) and identification of entomopathogenic fungus was conducted by previous reported specific primers, P1-forward (5'-AAGCTTTCGACATGGTCTG-3'), P3-backward (5'-GGAGGTGGTACAGGTCTG-3'), P5-forward (5'-AAGCTTTCGACATGGTCTG-3') (Hegedus and Khatourians, 1996). The reaction mixture consisted of: AccuPOWER™ PCR PreMix (Bioneer Co., Korea), 100 ng DNA and 10 pmol of each primer in a 20 ul volume. The reaction parameters for P1-P3 primer set were as follows; initial denaturation for 3 min at 94°C, followed by 35 cycles of 94°C for 30 sec, 53°C for 30 sec, and 72°C for 45 sec, and a final 10 min extension at 72°C using a thermal cycler (Takara, Japan). After PCR, the amplified DNA fragments were separated by electrophoresis in 1.0% agarose gel and extracted from agarose gel by using Power Gel Extraction Kit (Dyne Bio Inc., Korea).

**Cloning of PCR product and sequence analysis**

The PCR products were cloned into the pCR2.1-TOPO (Invitrogen Co., USA) by following manufacturer’s protocol. Recombinant colonies were randomly selected and the plasmid DNA was isolated by using LaboPass™ Mini Plasmid DNA Purification Kit (Cosmogenetech Co., Korea). The sequence of cloned DNA was deposited in SolGent Co. (Korea). Sequences were aligned using DNAANALYST (Lymnon BioSoft, Quebec, Canada) and compared with previous reported sequences using hierarchical clustering (Corpet, 1988).

**Bioassay of *M. alternatus* and *M. diphyisc**

Conidia obtained from 14-day-old PDA plate were suspended in 0.02% Tween-80 solution. Inoculums were prepared from 1 × 10^4 to 1 × 10^6 conidia/ml by direct counting on a haemocytometer. Adults of *M. diphyisc* were inoculated by dipping for 15–20 sec into the conidia suspension and were maintained in sterilized individually insect breeding dish at 90% plus relative humidity in an incubator at 25°C. 10 insects were used for each test and the mortality was checked daily. Pathogenicity against *M. alternatus* was same to above mentioned methods except concentration of conidia with only 1 × 10^5 conidia/ml.

**Result and Discussion**

**Isolation of entomopathogenic fungi and morphological examination**

We collected a cadaver of adult *M. alternatus* infected white fungi to control it (Fig. 1A). Several fungal colonies were isolated from the cadaver and their pathogenicity was evaluated against to *M. alternatus* for the selection of entomopathogenic fungus. *M. diphyisc* is also serious pest to various trees in forest. The result showed that only one colony has high pathogenicity against *M. diphyisc*. This was named as MaW1. To examine the morphology of MaW1, the growth of colony on PDA and arrangement of conidia were observed. MaW1 showed that slow growing,