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**Short Communication**

# Soil Microorganisms against *Cryphonectria parasitica*

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*Cryphonectria parasitica* is the filamentous ascomycetes that causes chestnut blight and first occurs in New York Zoological Garden in 1904. Since then, the pathogen has spread to Europe and caused serious damage to the chestnut trees. In Korea, the disease was first reported as chestnut stem blight in 1925 [Martha and Gary, 1986]. Chestnut trees are economically important trees, especially in Korea, where the chestnut fruits are forest product exported to Japan.

Early attempts at biological control in North America and Europe involved the isolation of fungal strains with severely reduced virulence and weakened mycelia growth and sporulation, because they perturb the developmental processes of fungal host [Baoshan and Dorald, 1999; Gary, 1994; Ursula and Daniel, 1994]. However, hypovirulent *C. parasitica* field isolates exhibit a wide range of variability in the virulence levels. Recently, results of genetic diversity on the hypovirus isolates that hypovirulent *C. parasitica* was useful and stable as biological control agent at a geographic scale were questoned [Tobin *et al.*, 2000]. In Korea, hypovirulent *C. parasitica* also was proposed as one of attempts to protect chestnut trees from chestnut blight fungus. But *C. parasitica* isolated in Korea showed a wide genetic variation that could reduce the effectiveness of biological control. Therefore, in the study attempts were made to isolate bacteria showing antifungal activity for biological control against *C. parasitica.*

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To isolate strains from soils of two areas, Gapyeong and Suncheon, Korea, serially diluted soil samples were plated on potato dextrose agar plates at 25°C for 3 days, and the resulting colonies were incubated with *C. parasitica* for determination of antifungal activity. Morphological characteristics were observed by FE-SEM (S-4300, Hitachi, Japan). The strains for SEM observation were cultured on a Petri dish containing Bennet's medium at 28°C for 7 days. Samples were fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) at room temperature for 4 h, followed by washing three times at 4°C for 10 min in 0.05 M sodium cacodylate buffer (pH 7.2). Samples were post-fixed with 1% aqueous osmium tetraoxide in 0.05 M sodium cacodylate buffer (pH 7.2 at 4°C) for 2 h, following two times washing with distilled water. Samples were serially dehydrated in ethanol (30, 50, 70, 80, 90, 100, 100, 100%), treated with isoamyl acetate for 15 min two times, in liquid $CO_2$ using the Balzers CPD 010 (Balzers Instruments, Liechtenstein), mounted on aluminum stubs, and sputter-coated with gold and palladium using the Polaron SEM Coating Unit E5100 (Thermo VG Scientific, Beverly, MA, USA).

To identify strains showing antifungal activity, genomic DNA was isolated using the genomic DNA extraction kit (QIAGEN, Hilden, Germany) for 16S rDNA analysis. 16S rDNA was amplified by PCR using PCR reaction kit (Qiagen, Germany). The primer sequences were: forward, 5'-AGA GTT TGA TCC TGG CTC AG-3'; and reverse, 5'-ACG GCT ACC TTG TTA CGA CTT-3'. Thermal cycle conditions were as follows: 1 cycle of denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and final extension at 72°C for 10 min. The PCR products were cloned using a pGEM-T cloning kit (Promega, Madison, WI, USA) according to the manufacture's instruction. The determined sequences were analyzed by the homology search of the BLAST program at National Center for Biotechnology Information and aligned with ClustalW software.

All manipulations for test of antifungal activity of broths were carried out under sterile condition. Fungal spores were harvested from colonies and suspended in sterile water. The broths of 4 strains showing the highest antifungal activity were precipitated with 50% ammonium sulfate and the precipitated proteins were centrifuged. The centrifuged protein pellets were diluted to 0.1 M Tris buffer (pH 8.0) followed by dialysis. In order to estimate the antifungal activity, the protein solution was filtered through 0.45 nm sterile membrane and added 100 μL of

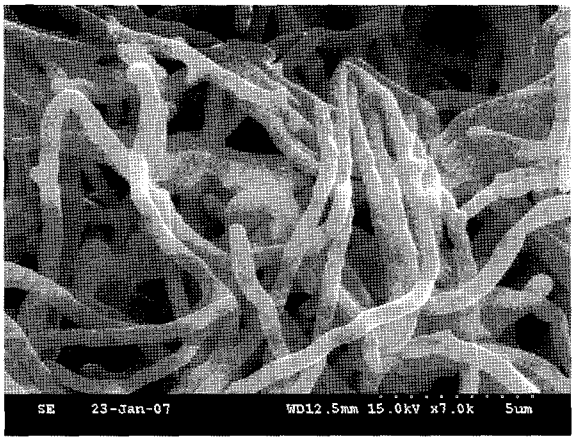


**Fig. 1. The morphological characteristics of strain S 1-9 by scanning electron microscopy.**

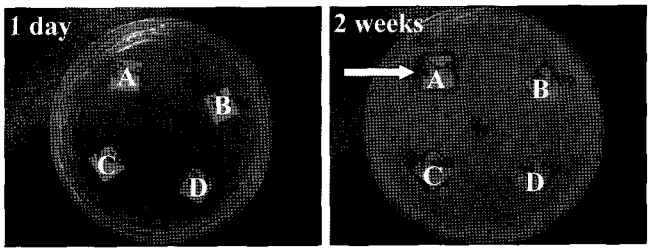


**Fig. 2. Effect of 4 strains protein solution on growth inhibition in dual culture (clear zone showing growth inhibition of mycelium is indicated by arrow; A, Gapyeong 1-9; B, Suncheon 1-10; C, Suncheon 1-20; D, Gapyeong 1-12).**

each samples to steriled paper disc on agar plates containing fungal spores. The plates were incubated at 30°C for 4 days.

The isolated strains from soil of Gapyeong and Suncheon showed antifungal activities against *C. parasitica*, chestnut tree pathogen. Among 217 isolated strains, 44 strains showed high growth inhibition against *C. parasitica*. Four strains showed higher growth inhibition against *C. parasitica*. S 1-9 indicated growth inhibition showing the clearest zones against the pathogen. For the identification, the morphological characteristic and 16S rDNA sequences of 4 strains have been determined. The morphological characterization was obtained by a method of previously described (Fig. 1). The 16S rDNA sequences of three strains showed the highest homology with the 16S rDNA sequences of *Streptomyces cinnamoneus, S. griseus,* and *S. fungicidicus* and one strain with *Burkholderia cepacia* by the BLAST search program. The 16S rDNA sequences of isolated stain S 1-9, which indicated the best growth inhibition of *C. parasitica*, were aligned with 16S rDNA sequences of *S. cinnamoneus* (accession number EU140333).

To determine the antifungal activity of broths, proteins of the above isolated 4 strains were prepared and cultured with *C. parasitica.* S 1-9, one of 4 strains, showed the best growth inhibition of *C. parasitica* (Fig. 2).

Genus *Streptomycetes* are widely used in industries due to their ability to produce chemical compound, antibiotics, and anti-tumor agent. [Janos, 1995; Yaowel *et al.*, 1998; Michael *et al.*, 1998]. In particular, *Streptomycetes* have been reported as genus of antifungal bacteria to protect the plants from the disease caused by fungal genus [Jung *et al.*, 2002]. It was that *B. cepacia* isolated from the rhizosphere could protect the plants as antagonistic bacteria against the pathogen in agriculture. Therefore, previous reported 4 strains could be used as potential biocontrol agents to protect chestnut trees from *C. parasitica*.

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