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# Antifungal Property of Microorganisms against Korea Oak Wilt Pathogen, *Raffaelea quercus-mongolicae*

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Five strains out of 200 candidate strains (SG 1-9, 1-12, SG 2-8, 2-10, and 2-17) were selected to determine their antifungal activity against *Raffaelea quercus-mongolicae*. The 16S rDNA sequences of the five strains were determined by sequencing analysis and analyzed by the homology of the blast program at NCBI. The homology search showed that SG 1-9 and 1-12 had a 98% homology with *Streptomyces cinnamoneus* and 98% homology with *Burkholderia cepacia*, while SG 2-8, 2-10, and 2-17 had a 99% homology with *Streptomyces fradiae*, a 97% homology with *Staphylococcus epidermidis*, and a 99% homology with *Staphylococcus epidermidis*. Out of the five selected strains, organic extract and protein extracts of SG2-17 strain broth were employed to determine antifungal activity against *Raffaelea quercus-mongolicae*. The organic extract exhibited antifungal activity, but the protein extracts did not demonstrate such an activity. Three organic solvents, butanol, benzene, and ethyl acetate, were also used for determination of antifungal activities. The activity measurements revealed that benzene extract possessed the greatest inhibitory effect on the growth of *Raffaelea quercus-mongolicae*, with the next highest being butanol extract, and ethyl acetate extract being the lowest.

**Keywords:** An antifungal activity, organic extract, *Raffaelea quercus-mongolicae*, 16S rDNA sequences

Oak trees are one of important trees in Korea for planting. Oak wilt caused by *Raffaelea quercus-mongolicae* has became a major disease of oak in Korea since reported firstly in 2004, and increased in Koreawide. In case of Japan, oak wilt disease was reported firstly in1934, and appeared in 1980s, became a major disease of oak [1, 6]. *Raffaelea* sp. is an ambrosia fungus that invades water conducting tissue of diverse tree species and has a symbiotic relationship with ambrosia beetle [2, 3]. The insects developed in galleries use fungi as nutrition source and disperse new habitats, and a specialized insect organ was adapted to transport symbiotic fungi [4]. A good prevention against *Raffaelea* sp. is to eliminate all of diseased trees in appearance areas and treat with fumigants. This method prevents the dissemination of the disease tree to uninfected trees. Few fungicides also have been effective in diseased trees, and however there are fungicides limited in use due to high cost.

Microorganisms have been considered as a good antifungal resource, especially in human and agriculture. *Streptomyces* species have been produced medicinally important antibiotics including amphotericin B, streptomycin, and so on [7].

To isolate microbe strains from Seosan, Korea, serially diluted soil were spread on nutrient agar media for two weeks at 25°C, and isolated strains were inoculated on the same fresh media.

*Raffaelea quercus-mongolicae* was grown on PDA media (Difco, USA) with 1.5% agar for two weeks at 25°C. For measurement of antifungal activity, fungal pathogen was inoculated on the side of the same fresh media, and also isolated strains were inoculated at other side of the same plates. Strains showing the high antifungal activity

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were collected and cultured in broth media. The cultured broths were extracted with organic solvents for metabolites compounds and ammonium phosphate (pH 7.0) for proteins.

To identify the isolated strains, 16S rDNA sequences were amplified and were analyzed by DNA sequencing analysis. Genomic DNAs of isolated strains were isolated using the genomic DNA extraction kit (QIAGEN, Hilden, Germany), and amplified by PCR (1 cycle of denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, and additional extension at 72°C for 10 min) with 16S rDNA primers [5]. The 16S rDNA sequences were analyzed by the homology search of the blast program at NCBI after the sequence analysis of amplified 16S rDNAs.

Morphological characteristics were observed by using FE-SEM (Hitachi S-4300), which followed the procedure described by Williams *et al*. [9]. The strains for SEM observation were cultured on a small 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 hours and followed washing three times at 4°C for 10 minute in 0.05 M sodium cacodylate buffer (pH 7.2). Samples were post-fixed in 1% aqueous osmium tetraoxide in 0.05 M sodium cacodylate buffer (pH 7.2) for 2 hours at 4°C, following two times washing with distilled water. Samples were dehydrated in a graded ethanol series (30, 50, 70, 80, 90, 100, 100, and 100%), and critical point dried in liquid $CO_2$ using the Balzers CPD 010 (Balzers Instruments, Liechtenstein) after the treatment of isoamyl acetate for 15 minutes two times, and mounted on aluminum stubs, and coated with gold using the Polaron SEM Coating Unit E5100 (Thermo VG Scientific, Beverly, MA).

Samples serially diluted from soil were spread on Bennet's media for two weeks 25°C, and two hundreds strains were isolated and incubated on the same fresh media. The strains were cocultured with *Raffaelea quercus-mongolicae* on PDA media for two weeks. Five strains, SG 1-9, 1-12, SG 2-8, 2-10, and 2-17, were collected by determination of antifungal activity (Fig. 1A). The 16S rDNA sequences of the above five strains were determined by the sequencing analysis**.** The 16S rDNA sequences were analyzed by the homology of BLAST program at NCBI. The homology search showed that SG 1-9 and 1-12 had 98% homology with *Streptomyces cinnamoneus* and 98% homology with *Burkholderia cepacia*, SG 2-8, 2-10, and 2-17 had 99% homology with *Streptomyces fradiae*, 97% homology with *Staphylococcus epidermidis*, and 99% homology with *Staphylococcus epidermidis*.

Morphological characteristics were observed by the procedure previously described by Williams *et al.* [8]. Morphological images of samples were obtained and shown in Fig. 1B.

Out of the above five strains, organic extract and proteins extract of SG2-17 strain broth were determined the antifungal activity against *Raffaelea quercus-mongolicae* because SG2-17 was faster growth rate than GS2-10 at the initial stage of growth. The organic extract showed the antifungal activity, but the proteins extract didn't show it (data not shown). Based on the above data, the effects of organic solvents used for extraction were determined. Three organic solvents, butanol, benzene, and ethyl acetate, were used for the determination. The determination showed

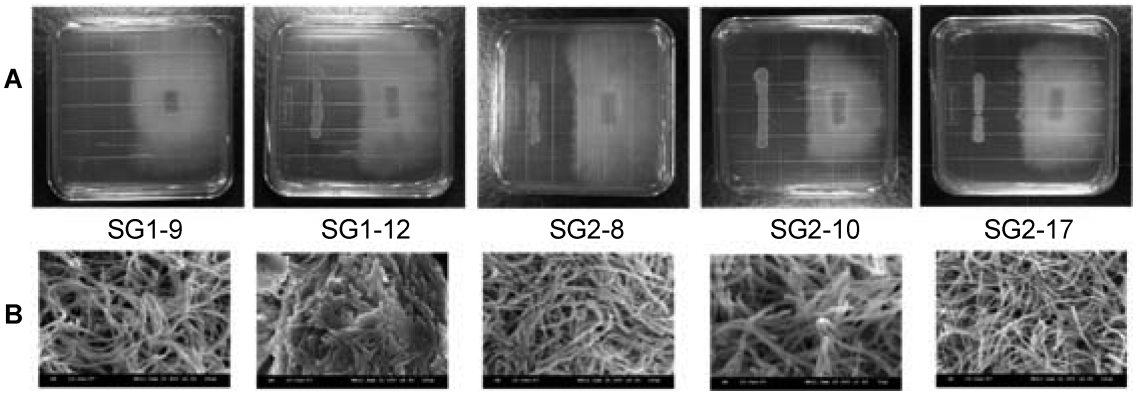


**Fig. 1. Antifungal activity and morphological characteristics of microorganisms against *Raffaelea quercus-mongolicae*.** A: assay of antifungal activity, B: morphological characteristics by scanning electron microscopy.

**Table 1. Antifungal activity of organic solvent extracts of SG2-17 strain against *Raffaelea quercus-mongolicae*.**

| Extraction solvent | Activity[a] |
|---|---|
| benzene layer | +++ |
| butanol layer | ++ |
| ethyl acetate layer | + |

[a] relative activity

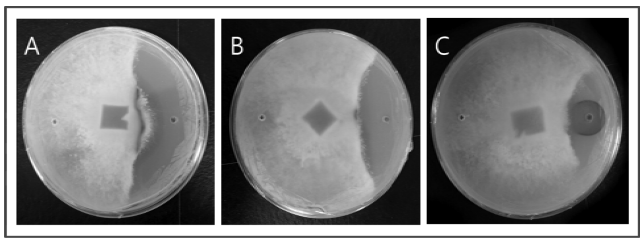


**Fig. 2. Antifungal activity against Korea oak wilt pathogen, *Raffaelea quercus-mongolicae* by SG2-17.** A: benzene layer; B: butanol layer; C: Ethyl acetate layer.

that benzene extract was highest in the inhibition of growth of *Raffaelea quercus-mongolicae*, and next was butanol extract, and ethyl acetate extract was lowest (Table 1, Fig. 2).

A variety of tree diseases was spread to cause an ecological damage in nature. Among the diseases, *Raffaelea quercus-mongolicae* have become major pathogen of tree, causing wilt disease. There have been many attempts to inhibit plant diseases in biological control.

Silver nanoparticles were attempted to control *Raffaelea quercus-mongolicae*, and showed a good inhibition [3]. In the case, the antifungal activity was depended on different forms of silver nanoparticles. The another attempt was to use the antagonistic properties of mushroom strain against Korean oak wilt pathogen, *Raffaelea quercus-mongolicae* [1]. The results showed that the species of mushrooms living on different host plants had the antagonistic properties of the pathogen. As a result of the above attempt, microbes have been used as good resources for biological control to inhibit the growth of pathogen.

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**국문초록**

## 참나무시들음 병원균 *Raffaelea quercus-mongolicae*에 대한 항균미생물 분리

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*Raffaelea quercus-mongolicae*에 대한 항균작용을 하는 미생물을 분리하기 위하여 200개의 균주를 분리하였으며 이 중 SG 1-9, 1-12와 SG 2-8, 2-10, 2-17 5개의 균주에서 항균활성을 확인하였다. 5개의 균주는 확인을 위하여 16S rDNA 염기서열 분석을 하였으며, SG 1-9는 *Streptomyces cinnamoneus*와 98%의 homology가 SG 1-12는 *Burkholderia cepacia*와 98% homology가 같은 것으로 나타났다. 또한 SG 2-8은 *Streptomyces fradiae*와 99%의 homology를 SG 2-10은 *Staphylococcus epidermidis*와 97%를 SG 2-17은 *Staphylococcus epidermidis*와 99%의 homology를 나타내었다. 위 5개의 균주 중 활성이 가장 강한 SG 2-17의 유기용매추출물과 단백질추출물을 분리하여 활성을 조사하였으며 유기용매추출물에서 강한 활성을 확인하였다. 3개의 유기용매 중 benzene 추출물이 가장 높은 *Raffaelea quercus-mongolicae*의 균사성장을 억제하였다.