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Microorganisms Against *Plasmodiophora brassicae*

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Abstract In order to find microorganisms showing antifungal activities against *Plasmodiophora brassicae*, which causes club root, Korean salt-fermented fishery products were tested. Several fermented broths of microorgansims isolated from *Ammodytes personatus* fishery products showed high antifungal activities. The identification of microorganisms and their *in vivo* antifungal activities are reported herein.

Keywords: *In vivo* antifungal activities, *Plasmodiophora brassicae*, club root, biopesticide

Club root is a disease found in many crucifers including cabbage and radish. When they are infected with club root, their roots are swollen and cannot absorb water and nutrients, resulting in withering. This disease, which is caused by *Plasmodiophora brassicae*, favors moist, cool, and acidic soil. It is a myxomycete. The spores invade root hairs and develop into a plasmodium, which is released through a pore in the host tissue. Plasmodia can penetrate host cells of the vascular system and enlarge them. Finally, plasmodia develop into resting spores within host tissue. Resting spores can survive in soil indefinitely, and they germinate and produce zoospores, whenever resting spores meet favorable environments. *P. brassicae* passes the winter as resting spores. To control the disease, two synthetic chemicals, fluazinam (3-chloro-N-[3-chloro-2,6-dinitro-4-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2-pyridinamine) and flusulfamide (4-chloro-N-(2-chloro-4-nitrophenyl)-3-(trifluoromethyl)benzenesulfonamide), have been used. However, because of consumer rejection to synthetic chemicals and their small effects, the development of a new pesticide is required. The fermented broth of microorganisms may be an alternative pesticide, which belongs to biopesticides.

In order to find microorganisms showing antifungal activities against *P. brassicae*, many microorganisms were isolated from Korean salt-fermented fishery products, *jeotkal* produced from *Ammodytes personatus*. *Jeotkal* stored at 4°C was diluted 1,000 times with distilled water. One hundred μl of the solution was spread on the nutrient agar in a Petri dish and incubated at 30°C for three days. Colonies showing typical characteristics of bacteria were selected and then transferred to new nutrient agar medium at 30°C for three days. Each colony was inoculated into LB medium and cultured in a shaking incubator at 30°C for three days. The detailed procedure for the isolation was carried out according the method reported previously [8]. Thirty-nine strains were isolated from *A. personatus*. Their fermentation broths were centrifuged, and the supernatants were collected and kept at 4°C for the activity test against *P. brassicae*.

The crop used for testing was *Brassica campestris* subsp. napus var. pekinensis, cv. Hukjinju. When the crop grew into the second leaf stage, *P. brassicae* was added. The detailed procedures to infect plants with a club root were carried out according to the method reported previously [2, 7]. The fungal control was analyzed based on the following index: 0=no clubs, 1=small galls formed on lateral roots, 2=galls formed on lateral roots or small galls formed on the main roots, and 3=many big galls formed on lateral and main roots. Since the control does not include any fermentation broth, it should show index 3 [9].

Among 39 samples screened, 7 fermented broths showed 100% control value, as listed in Table 1. Data are reported

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Table 1. The activities of fermentation broths of strains tested against *Plasmodiophora brassicae* (the treated concentration of fermentation broth was approximately 50 μg/ml).

Strain	Control value (%)
AP01	78
AP02	67
AP03	67
AP04	89
AP05	56
AP06	67
AP07	89
AP08	56
AP09	89
AP10	33
AP11	67
AP12	100
AP13	100
AP14	78
AP15	56
AP16	33
AP17	89
AP18	89
AP19	89
AP20	100
AP21	100
AP22	100
AP23	100
AP24	89
AP25	89
AP26	100
AP27	89
AP28	56
AP29	78
AP30	44
AP31	33
AP32	56
AP33	67
AP34	33
AP35	89
AP36	89
AP37	44
AP38	56
AP39	78
Fluazinam	89
Control	0

as means±standard deviations of three independent experiments, and were evaluated by Student's *t*-test. Values of $P<0.05$ were considered to be statistically significant [14]. Fluazinam (167 ppm) was used as a reference [4]. It showed 89%. Only medium without any strains showed %. Fig. 1 shows the root of the crop used for testing as a control (right), which was completely infected by *P. brassicae*, and that treated with the fermented broth of AP23 (left), one of the strains showing 100% activity.

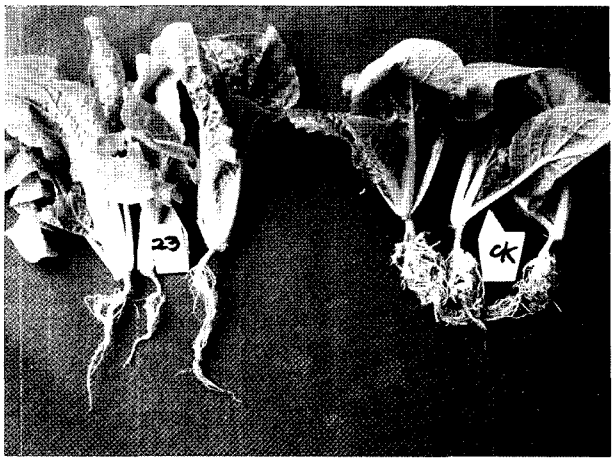

Fig. 1. The root of the crop used for testing as a control (right), which was completely infected by *P. brassicae*, and that treated with fermented broth of AP23 (left), one of the strains showing 100% activity.

On the basis of the partial 16S rDNA [3], 7 strains showing 100% of the control value were identified. The detailed procedures for 16S rDNA analysis were carried out according to the method previously published by Kim *et al.* [8]. The 16S rDNA sequences (912-bp) of strain AP12 identified from GenBank by the BLAST program showed the highest homology (99% identity) with *Kocuria rhizophila* [10]. The evolution tree constructed by using the PHYDIT program revealed that the strain AP12 is related to *K. rosea*, *K. kristinae*, and *K. varians* with a high bootstrap value (Fig. 2A) [6]. *Kocuria* is a species associated with infection [12]. *Kocuria rhizophila* was known previously as *Micrococcus luteus* [13] and isolated from the rhizoplane of the narrow-leaved cattail (*Typha angustifolia*) [10]. It is a Gram-positive, aerobic, spherical actinobacteria. The 16S rDNA sequences of strains AP13 (916 bp), AP20 (919 bp), AP21 (930 bp), AP22 (918 bp), AP23 (921 bp), and AP26 (893 bp) were searched using BLAST like AP12 and were identified to be *Cellulosimicrobium funkei* (99%), *Paenibacillus ginsengagri* (92%), *Paenibacillus lautus* (98%), *Sporosarcina ginsengisoli* (97%), *Paenibacillus* sp. 6495m-C2 (99%), and *Paenibacillus polymyxa* (95%), respectively. *Cellulosimicrobium funkei* showing 99% identity with AP13 has not yet been reported to have antifungal activity against *P. brassicae*. The evolution tree constructed by using the PHYDIT program for AP13 is shown in Fig. 2B, and shows that *Cellulosimicrobium* genus includes two species, *C. cellulans* and *C. funkei*. AP20, 21, 23, and 26 show high indentities with *Paenibacillus genus*. *Paenibacillus* is associated with infections such as septicemia, meningitis, and pneumonia [5]. It includes more than 70 species. Of these, *P. polymyxa* has a capability to suppress plant diseases and produces antimicrobial compounds [1]. The evolution trees constructed by using the

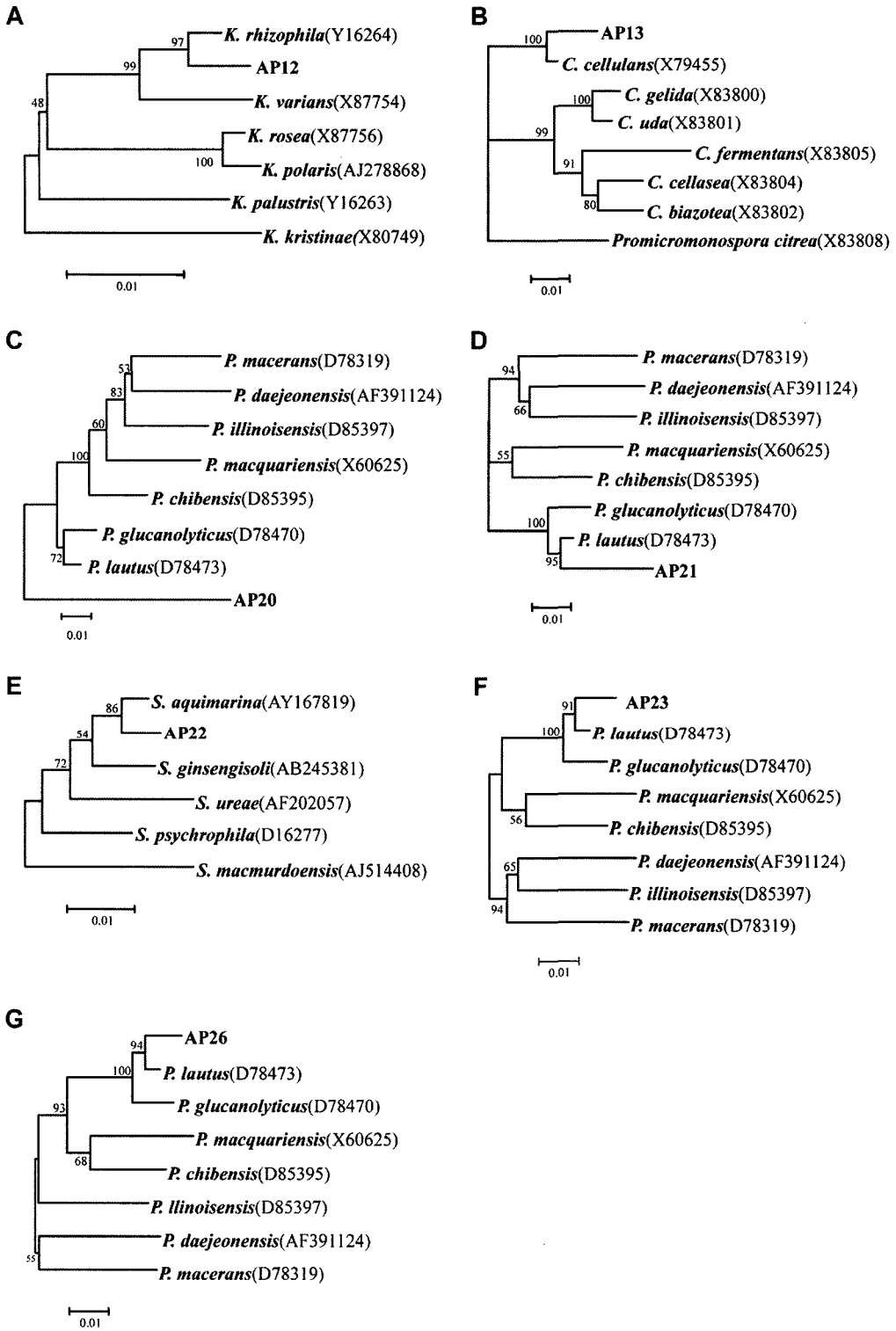

Fig. 2. Phylogenetic trees of 16S rDNA of AP12 (**A**), AP13 (**B**), AP20 (**C**), AP21 (**D**), AP22 (**E**), AP23 (**F**), and AP26 (**G**), based on the neighbor-joining method.

PHYDIT program for AP20 (919 bp), AP21 (930 bp), AP22 (918 bp), AP23 (921 bp), and AP26 (893 bp) are shown in Figs. 2C, 2D, 2E, 2F, and 2G, respectively. Of 7 strains showing antifungal activity against *P. brassicae*, AP20 showed only 92% identity with *Paenibacillus ginsengagri* [11]. Therefore, it appears to be a novel strain,

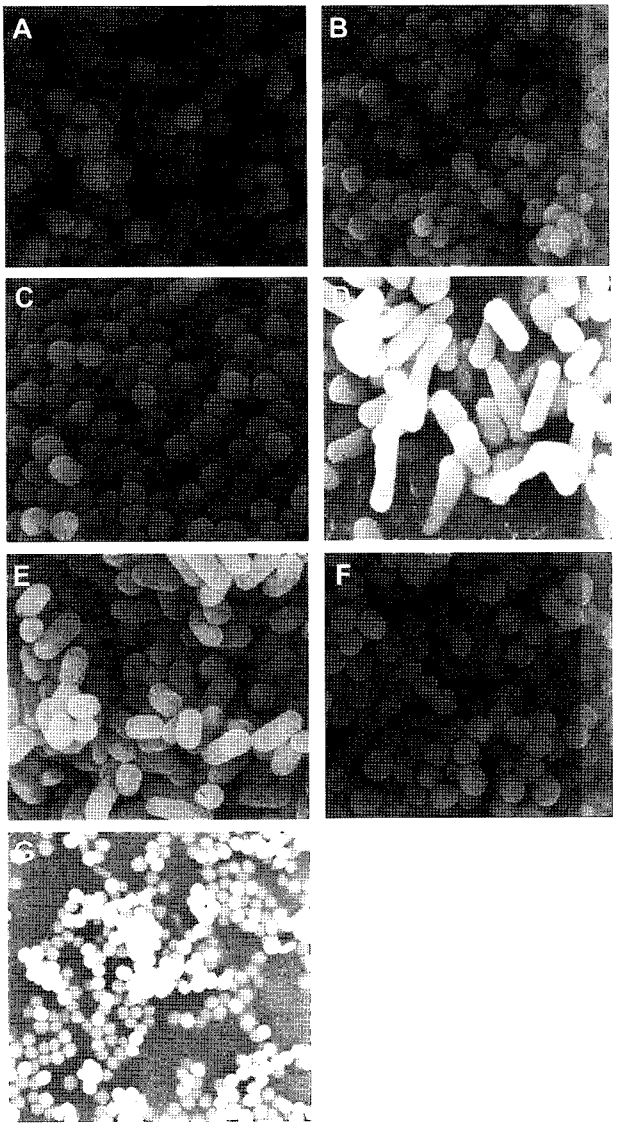

Fig. 3. Morphologies of AP12 (**A**), AP13 (**B**), AP20 (**C**), AP21 (**D**), AP22 (**E**), AP23 (**F**), and AP26 (**G**), observed by scanning electron microscopy.

and its identification remains to be determined in a future study.

In order to elucidate the morphology of the strain Ap12, scanning electron microscopy was used. The strain Ap12 was cultured on LB medium at 30°C for a day. The sample was then fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) and post-fixed in 1% aqueous osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2). The prepared sample was dehydrated in a graded ethanol series (30, 50, 70, 80, 90, 100, 100, and 100%), and mounted on aluminum stubs coated with gold (Polaron SEM Coating Unit E5100, Thermo VG Scientific, MA, U.S.A.). The image obtained is shown in Fig. 3A. In addition to the AP12, the images of the other six strains obtained from SEM are also shown in Figs. 3B, 3C, 3D, 3E, 3F, and 3G, respectively.

A typical naturally occurring soil bacterium is *Bacillus thuringiensis*. Present authors have earlier reported biological effects of a few fermented broths of *Streptomyces* isolated from soil against plant pathogenic fungi. Similar to synthetic pesticides, their toxicological test cannot be waived for registration, because they were found from soil. However, the strains used in these experiments were isolated from food, a Korean salt-fermented fishery product, *jeotkal* produced from *Ammodytes personatus*; therefore, it should be safer than synthetic pesticides. In addition, it is not known that fermented products from *Ammodytes personatus* show an activity against fungi, especially *P. brassicae*. Since the fermented broths (50 mg/ml) have a higher activity than fluaziman (167 ppm=167 μg/ml) used as a control, the improvement of their activities is undoubtedly required. Nevertheless, the observation that fermented broths obtained from food showed strong antifungal activities against club root is highly valuable.

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