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Characteristics of Microbial Biosurfactant as an Antifungal Agent Against Plant Pathogenic Fungus

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Abstract Characteristics of sophorolipid and rhamnolipid were evaluated as antifungal agents against plant pathogenic fungi. Eight percent of mycelial growth of plant pathogen (*Phytophthora* sp. and *Pythium* sp.) was inhibited by 200 mg/l of rhamnolipid or 500 mg/l of sophorolipid, and zoospore motility of *Phytophthora* sp. decreased by 90% at 50 mg/l of rhamnolipid and 80% at 100 mg/l of sophorolipid. The effective concentrations for zoospore lysis were two times higher than those of zoospore motility inhibition. The highest zoospore lysis was observed with *Phytophthora capsici*; 80% lysis at 100 mg/l of di-rhamnolipid or lactonic sophorolipid, showing the dependency of structure on the lysis. In the pot test, the damping-off disease incidence ratio decreased to 42% and 33% of control value at 2,000 mg/l sophorolipid and rhamnolipid, respectively. These results showed the potential of microbial glycolipid biosurfactants as an effective antifungal agent against damping-off plant pathogens.

Key words: Sophorolipid, rhamnolipid, antifungal, plant pathogen

Damping off, a soilborne root disease caused by phytopathogens such as *Pythium* sp. and *Phytophthora* sp., results in serious loss in a number of agricultural products including tomatoes and strawberry. These diseases spread rapidly through soil by migrating with water in the form of zoospore, and infect seeds, leaves, and stem of water-culturing plants [1–4]. *Oomycetes*, including *Pythium* sp. or *Phytophthora* sp., have elongated non-septum hyphae and produce dormant spores, including zoospore and zoosporangium. The zoospore moves in water with two flagellates and is the main cause of disease spreading [5, 6].

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Chemical pesticides or change of the plant-culture conditions are currently used to prevent damping-off disease. However, the latter is not effective once the disease has occurred. As for chemical pesticides, copper compound, chlorothalonil, metalaxyl, captan, and famxadone were widely used in the field [7–9]. However, owing to residual toxicity and calcitrance of these chemical pesticides, environmentally compatible control methods, such as biological control, based on the antagonism or microbial antifungal agent, have emerged as a potential candidate for the prevention of plant pathogens [10, 11].

Microbial biosurfactant is a surface-active compound produced by various microorganisms. It lowers surface tension and interfacial tension, and forms spherical micelles at critical micelle concentration (CMC). The microbial biosurfactant has an amphiphilic structure, possessing a hydrophilic moiety, such as amino acid, peptide, or polysaccharide, and a hydrophobic moiety including saturated or unsaturated lipid or fatty acids [12, 13].

Because of its unique properties such as detergency, emulsification, and dispersion, a biosurfactant has various applications, including in oil recovery, paint, cement, cosmetics and food industries [14–16]. Among various microbial biosurfactants, the glycolipid-type surfactant has gained interests, owing to its high productivity [14]. *Pseudomonas* sp. or *Candida* sp. produces more than 100 g/l of rhamnolipid or sophorolipid, using glucose as a sugar source and oil as a lipid source. Typically, glycolipid is produced during the stationary phase, when the nitrogen source is limited [17]. In addition to surface-active property, rhamnolipid and sophorolipid have been found to have antibacterial, antifungal, mycoplasmicidal, and antiviral characteristics [10, 18, 19]. The structure of glycolipid is similar to that of cell membrane, thus resulting in the perturbation of the cell membrane. As a consequence, rhamnolipid or sophorolipid has exhibited strong inhibition of Gram-positive bacteria such as *Bacillus*, *Staphylococcus*, or *Streptococcus* sp.

[18, 19]. Low cost of microbial production, low toxicity, and biodegradability make these microbial glycolipids potential ingredients in the cosmetics, health care, food, environmental clean-up, and agriculture industries.

In this study, rhamnolipid and sophorolipid were investigated as alternative antifungal agents against typical plant pathogenic fungi, including *Phytophthora* and *Pythium* sp. Results showed that these microbial glycolipids are very effective in decreasing the incidence of water-borne damping-off disease.

MATERIALS AND METHODS

Strains and Cultivation

For the antifungal activity test, the following fungi were used: *Phytophthora capsici* was a gift from Greenbiotech. Co. Ltd.: *Phytophthora nicotianae* (KACC 40906), *Phytophthora infestans* (KACC 40718), *Pythium aphanidermatum* (KACC 40156), and *Pythium ultimum* (KACC 40705) were obtained from Korea Agriculture Culture Collection. *Candida bombicola* ATCC 22214 and *Pseudomonas aeruginosa* IGB 83 were used for sophorolipid and rhamnolipid production, respectively.

PDA medium was used for cultivation of *Phytophthora* sp. and *Pythium* sp. [2]. For zoospore or zoosporangium medium, V8 agar medium, consisting of 10% V8 juice (Campbell Soup Co., U.S.A.), 1.0 g of $CaCO_3$, and 17 g of agar was used. *Phytophthora* sp. and *Pythium* sp. were cultured at 20°C for 5–7 days and 25°C for 2 days, respectively.

Production and Extraction of Microbial Biosurfactant

Rhamnolipid or sophorolipid was produced in a 2.5-l or 5-l fermenter (Kobiotech., Seoul, Korea), using the method described by Sim *et al.* [20] or Lee *et al.* [21], respectively. For rhamnolipid recovery, following centrifugation of the cells (6,800 rpm, 15 min), residual oil in the supernatant was removed by hexane extraction. To the supernatant, 1 N HCl was added to adjust pH to 2, followed by storage at 4°C for 1 day. From this solution, rhamnolipid was extracted by the mixture of chloroform:methanol (2:1/ v:v), followed by rotary evaporation (Eyela, Tokyo Rikakikai Co., Ltd.). For sophorolipid recovery, culture broth was centrifuged (6,800 rpm, 15 min) and hexane was added to remove the residual oil. Following three times of ethyl acetate extraction, sophorolipid was recovered by rotary evaporation.

Fractionation and TLC Analysis

Extracted rhamnolipid or sophorolipid was dissolved in chloroform and eluted through a glass column (25×50 mm) packed with silica gel 60 (0.040–0.063 mm Merck Co., Damstadt, Germany), using an eluant solution (chloroform: methanol:water=65:15:20/1.75 ml/min). Fractions from this glass column were analyzed with TLC plate (silica gel 60 F254/Merck Co., aluminum sheets 20×20 cm) with the above eluant. Spots were identified by molish agent [Naphtol (15% in ethanol):sulfuric acid:ethanol:water=10.5:6.5:40.4:4] [23].

Zoospore Formation, Hyphae Growth, Mobility, and Lysis of Zoospore and Release of Zoospore

For zoospore or zoosporangium formation, fungi were cultivated in 10% V8 agar plate at 25°C for 1–3 days in the dark [2]. Distilled water (20 ml) was added to each plate and cultured for an additional 1–2 days in the light. Zoospore or zoosporangium was counted by a hemacytometer (0.0025 mm^2, Mariefeld, Lauda-Konigshofen, Germany) under a microscope (U-CMAD-2, Olympus, Tokyo, Japan). Inhibition of fungal hyphae growth by biosurfactant was determined by measuring the length of hyphae grown in the plate, which contained various concentrations of biosurfactant. For zoospore mobility and lysis test, zoospores at 100 zoospore/ml were suspended in distilled water in the well of a TC-plate (24 well) containing various concentrations of biosurfactant. After 5 min of contact, total numbers of zoospore and non-moving cells were counted and compared with the control to determine the zoospore lysis and motility inhibition by biosurfactant. For comparison with biosurfactants, chemical

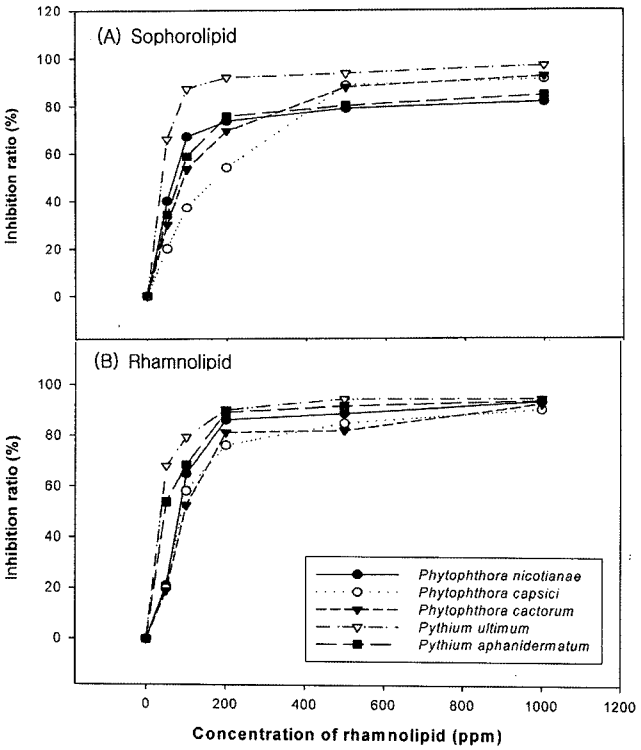

Fig. 1. Inhibitory effect of (A) sophorolipid and (B) rhamnolipid on mycelial growth of (●) *Phytophthora nicotianae*, (○) *Phytophthora capsici*, (▼) *Phytophthora cactorum*, (▽) *Pythium ultimum*, and (■) *Pythium aphanidermatum*.

surfactants obtained from Moogungwha Co. Ltd were also employed. Release of zoospore from zoosporangium was measured by counting the intact zoosporangium after 30 min at 25°C, following storage at 4°C for 1 h.

Pot Test of Damping-Off Inhibition

A pot (5×15×10 cm) was filled with sand-oatmeal (380 g sand and 20 g oatmeal), 40 g top soil, and 30 ml of various concentrations of biosurfactants. After 1 day, 10 ml of zoospore suspension (100 spore/ml) was inoculated. After 2 h, seeding (a cucumber seed per pot) was made and the pot was cultured for 7 days. All pot-tests were performed at Greenbiotech., Co. Ltd.

Results and Discussion

As shown in Fig. 1, biosurfactants showed the highest inhibition of mycelial growth of *Pythium ultimum* and the lowest inhibition of *Phytophthora cactorum*. In the case of sophorolipid, 80% of mycelial growth of *Phytophthora* sp. and *Pythium aphanidermatum* was inhibited at 500 mg/l and 90% inhibition were observed with *Pythium ultimum* at 100 mg/l of sophorolipid (Fig. 1). When rhamnolipid was tested, 80% of *Phytophthora* sp. and *Pythium aphanidermatum* was inhibited at 200 mg/l, whereas 90% of *Pythium ultimum* was inhibited at 100 mg/l (Fig. 2). This result showed that rhamnolipid inhibited mycelial growth two times more strongly than sophorolipid. It was reported that sophorolipid (0.1–2 mg/l) was more effective than rhamnolipid (35–350 mg/l) in the inhibition of Gram-positive bacteria [8]. Release of bacterial cytoplasmic enzyme upon treatment with biosurfactant implies that biosurfactant might penetrate the membrane and perturb the membrane integrity [21]. The reason for the difference in the inhibition of fungi or bacteria by rhamnolipid or sophorolipid needs to be clarified in further studies.

Motility Inhibition, Zoospore Lysis, and Inhibition of Zoospore Release

Figure 2 shows that sophorolipid inhibited the motility of *Phytophthora nicotianae* and *Phytophthora infestans* by 80% at 100 mg/l. In the case of *Pythium aphanidermatum*, more than 80% of the motility was inhibited at 200 mg/l of sophorolipid or 100 mg/l of rhamnolipid, respectively. Motility inhibition of microalgae was observed at 10 mg/l of sophorolipid [22]. Release of microalgae DNA by sophorolipid treatment indicated that disruption of membrane integrity played an important role in the motility inhibition, and release of intracellular enzyme with treatment of sophorolipid indicated disruption of the membrane integrity [21].

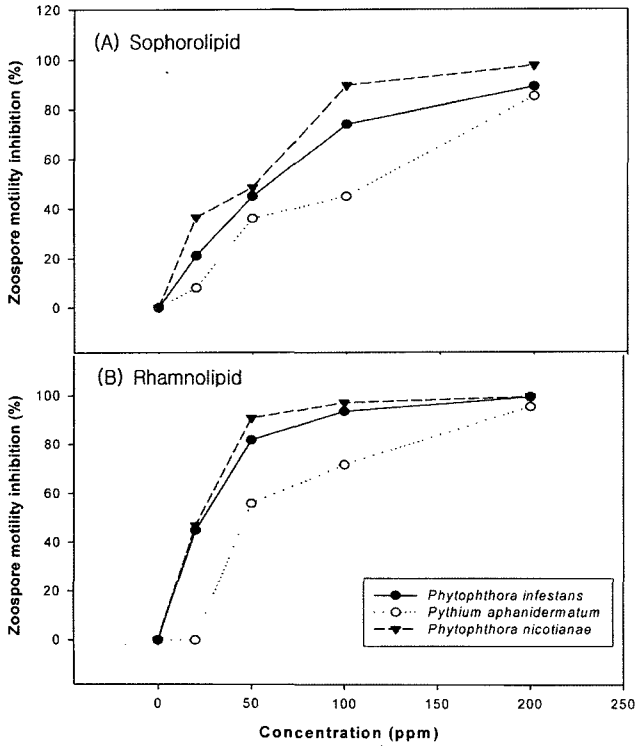

Fig. 2. Inhibitory effect of (A) sophorolipid and (B) rhamnolipid on (●) *Phytophthora infestans*, (○) *Pythium aphanidermatum*, and (▼) *Phytophthora nicotianae*.

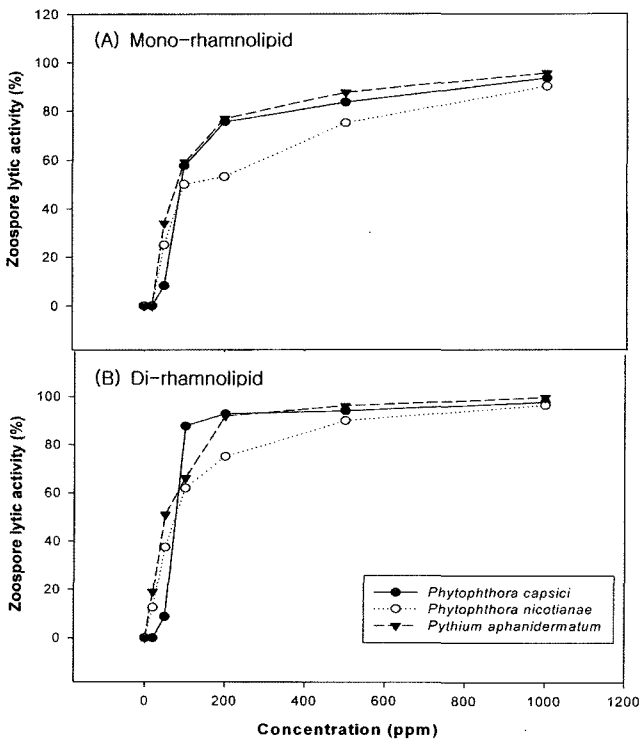

Fig. 3. Zoospore lysis effect of (A) mono-rhamnolipid and (B) di-rhamnolipid on (●) *Phytophthora capasici*, (○) *Phytophthora nicotianae*, and (▼) *Pythium aphanidermatum*.

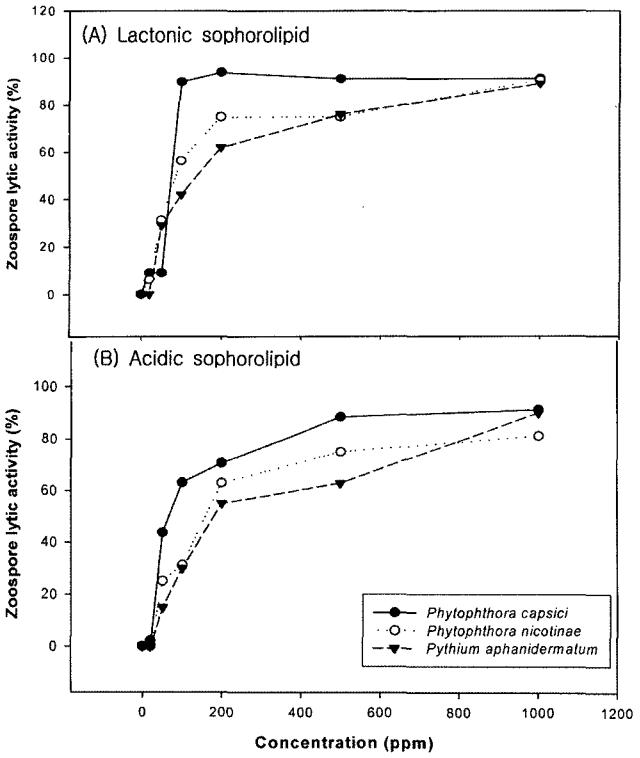

Fig. 4. Zoospore lysis effect of (A) lactonic sophorolipid and (B) acidic sophorolipid on (●) *Phytophthora capsici*, (○) *Phytophthora nicotinae*, and (▼) *Pythium aphanidermatum*.

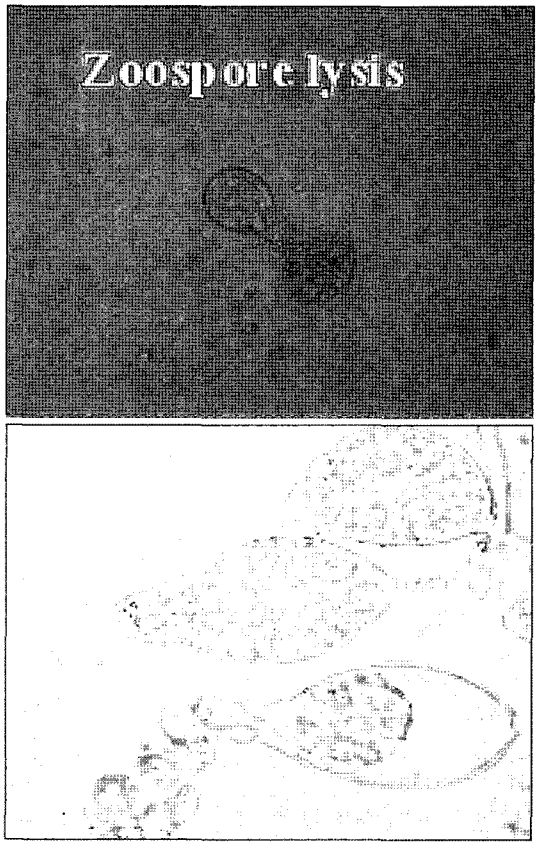

Fig. 5. Photograph showing the release and lysis of zoospore.

Zoospore lysis was observed following thinning and disturbing of the membrane by biosurfactant treatment (Fig. 5). It was reported that zoospore of oomycetes had no cell wall before forming a cyst, a hard spore. Therefore, the membrane of zoospore is exposed and fragile to external stresses such as treatment with surfactants, including sophorolipid or rhamnolipid.

Zoospore lysis was investigated using different types of sophorolipid (lactonic or acidic) or rhamnolipid (di- or mono-rhamnolipid). The highest zoospore lysis ratio was observed with *Phytophthora capsici*; 80% lysis at 100 mg/l of di-rhamnolipid or lactonic sophorolipid and 40% lysis at 100 mg/l of acidic sophorolipid or mono-rhamnolipid. Ninety percent of *Pythium aphanidermatum* were lysed at 100 mg/l of di-rhamnolipid and 60% at 500 mg/l of acidic sophorolipid. Antibacterial activity has been reported to depend on the structure of microbial biosurfactant [21]. Against species of *Bacillus*, *Staphylococcus*, and *Streptococcus*, lactonic sophorolipid and di-rhamnolipid showed higher activity than acidic sophorolipid and di-rhamnolipid. In the present study, a similar structural dependency of zoospore lysis was observed, indicating that a similar mechanism might be involved in the zoospore lysis. These characteristics of biosurfactant can be applied for the development of a bactericidal agent [23].

Chemical surfactant was reported to inhibit the bacterial growth by disturbing the membrane protein or membrane lipid, and higher growth inhibition was observed with cationic surfactant than nonionic surfactant [24]. In the present study on zoospore lysis, similar results were obtained:

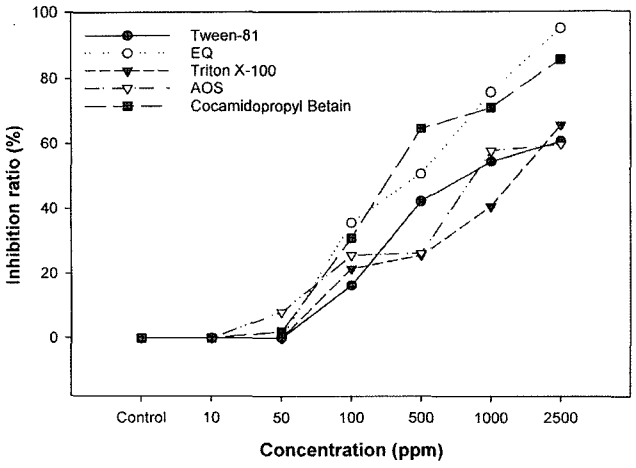

Fig. 6. Lysis of zoospore (*P. capsici*) by chemical surfactant. EQ, EsterquartMPS-814S); AOS-1416, α-Oleffinsulfornate.

Table 1. Inhibition of zoospore release.

Concentration (mg/l)	Inhibition ratio (%)		
	Sophorolipid	Rhamnolipid	Metalaxyl
200	17	ND	50
500	24	17	75
2,000	42	50	ND

ND; Not determined.

80% of zoospore lysis was observed at 1,000 mg/l of cationic surfactant, EQ, whereas 60% of lysis occurred at 2,500 mg/l of nonionic surfactant, Triton X-100 (Fig. 6). Compared with chemical surfactants (metalaxyl), sophorolipid or rhamnolipid showed a lower inhibition of zoospore release (Table 1).

Pot Test of Prevention of Damping-Off Disease

In the pot test for the prevention of damping off incidence, 72% and 68% of seed growth ratio were observed at 2,000 mg/l of sophorolipid and rhamnolipid, respectively (Table 2). As shown in Table 1, metalaxyl was very effective in the inhibition of the zoospore release, compared with biosurfactants. Since zoospore release is the most efficient disease-spreading mechanism, a lower inhibition by sophorolipid and rhamnolipid in the pot test might have been due to low inhibition of zoospore release by the biosurfactant. A better preventive method might be designed with biosurfactants if zoospore release could be inhibited by other types of biosurfactants. Screening of biosurfactants for inhibition of zoospore release is underway in the laboratory.

In this study, the potentials of microbial biosurfactants, such as sophorolipid and rhamnolipid, as an antifungal agent were evaluated. Mycelial growth of plant pathogenic fungi was significantly inhibited at low concentration of biosurfactants. These biosurfactants were also very effective in the inhibition of motility as well as in the lysis of zoospore. The degree of inhibition or lysis depended on the structure of biosurfactants. In the pot test, damping-off disease incidence ratio decreased to 42% and 33% of control value at 2,000 mg/l of sophorolipid and rhamnolipid, respectively. Along with the low toxicity and biodegradability of biosurfactants, the antifungal characteristics showed that microbial glycolipid biosurfactants possess great potential as promising and environment-compatible biocontrol agent.

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Table 2. Prevention of damping-off disease by biosurfactant treatment.

Treatment (mg/l)	Pot number (%)								
	Control*	Sophorolipid			Rhamnolipid			Metalaxyl		Sterile**
		100	500	2,000	100	500	2,000	200	500	
Growth	52	60	68	72	52	60	68	68	72	93
Infected	32	28	24	28	40	24	20	16	8	7
No growth	16	12	8	0	8	16	4	8	0	0

*Control; no treatment. **Sterile; Soil was sterilized by autoclaving at 121°C, 10 min.

phytopathogenic fungi causing damping-off disease. *J. Microbiol. Biotechnol.* **14:** 599–607.
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