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Screening of Cyanobacteria (Blue-Green algae) from Rice Paddy Soil for Antifungal Activity against Plant Pathogenic Fungi

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Soil cyanobacteria isolated from the rice paddy fields of 10 different locations across Korea were evaluated by agar plate diffusion test for antifungal activity. Aqueous, petroleum ether, and methanol extracts from one hundred and forty two cyanobacterial strains belonging to the 14 genera were examined for antifungal properties against seven phytopathogenic fungi causing diseases in hot pepper (*Capsicum annuum* L). Of total cyanobacteria, nine cyanobacteria (6.34%) exhibited antifungal effects. The nine cyanobacteria selected with positive antifungal activities were two species of *Oscillatoria*, two of *Anabaena*, three of *Nostoc*, one of *Nodularia*, and one of *Calothrix*. *Alternaria alternata* and *Botrytis cinerea* were inhibited by nine and eight species of cyanobacteria, respectively. *Rhizopus stolonifer* was suppressed by only methanol extract of *Nostoc commune* FK-103. In particular, *Nostoc commune* FK-103 and *Oscillatoria tenuis* FK-109 showed strong antifungal activities against *Phytophthora capsici*. Their antifungal activity at the late exponential growth phase is related to the growth temperature and not associated with the growth parameters such as cell biomass and chlorophyll-*a* concentration. The high inhibition levels of antibiotics were 22.5 and 31.8 mm for *N. commune* FK-103 and *O. tenuis* FK-109, respectively. The optimal temperature for antibiotic productivity was 35°C.

KEYWORDS: Antibiotics, Antifungal activity, Cyanobacteria, Rice paddy filed, Soil

The screening, isolation, and physiology of antibiotic producing microorganisms have been focused on heterotrophic. However, cyanobacteria have very little attention because they are able to grow under diverse nutritional conditions photoautotrophically or chemoheterotrophically (Smith, 1982) and are among the oldest photoautotrophic organisms. Cyanobacteria are a main component of microbial community in rice paddy fields, contribute to the fertility of agricultural ecosystems (Roger and Kulasoorija, 1980) and produce a potential source of biological active secondary metabolites, which are compounds that are not essential for cell metabolism (Dodds *et al.*, 1995). Many of these compounds have been reported to possess antibiotic and pharmacological effects such as toxicity for eukaryotic organisms and antibacterial (Bloor and England, 1989), antifungal (Kulik, 1995), antiviral (Patterson *et al.*, 1993), and enzyme-inhibiting activities (Cannell *et al.*, 1987; Sveshnikov *et al.*, 1997). Most of cyanobacterial metabolites are accumulated in the cyanobacterial biomass. Moreover, cyanobacteria excrete various organic compounds, the hepatotoxic microcystins and nodularins or the neurotoxic anatoxins and saxitoxins, into their environment (Carmichael, 1992; Rinehart *et al.*, 1994). Cyanobacteria are one of the major components in rice paddy fields in Korea (Kim and Lee, 2006) and their beneficial effects on growth, yield and nitrogen fixation of such ecosystems have been reported (Pedurand and Reynaud, 1987). Some potential applications to consider for cyanobacteria are the production of antimicrobial compounds for the pharmaceutical industry and the agricultural sector as both bio-fertilizers and biocontrol agents. It is necessary to screen many cyanobacteria before suitable strain can be selected to application. The aim of the present work was to screen many cyanobacterial strains against plant pathogenic fungi associated with hot pepper to identify strains containing potentially useful secondary metabolites.

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Materials and Methods

Cyanobacteria and Culture conditions. One hundred and forty two cyanobacterial strains from 14 genera (Table 1) were screened from 100 soil samples taken from rice paddy fields in 10 different locations across Korea such as Ahandong, Buyeo, Gimjae, Hongcheon, Icheon, Jincheon, Jinju, Kimhae, Naju, and Seosan (Kim and Lee, 2006). For identification of cyanobacterial isolates, cyanobacterial chromosomal DNA was isolated as previously described (Hong *et al.*, 2002b). The 16S rDNA was PCR amplified with primers 27F, 5'-AGAGTTTGATCATGGCTCAG-3' and 1492R, 5'-GGTTACCTTGTTACGACTT-3' in 50 μl reactions containing 20 ng template DNA, 1× PCR buffer, 5 mM $MgCl_2$, 10 mM dNTP, 10 pM of each primer and 2.5 units of *Taq* DNA polymerase. The PCR was run with 35 thermal cycles of denaturation for 1 min for 94°C, annealing for 2.5 min at 55°C, extension for 2.5 min for 72°C, and with a final elongation step of 7 min at 72°C in

Table 1. Cyanobacterial strains tested in the screening for antifungal activity

Genus	Cellular shape	Family (Order)	Other characteristic	No. of strains
Aphanocapsa	Spherical	(Chroococcales)	Cell division by binary fission	1
Dactylococcopsis	Elongate cell with pointed ends	(Chroococcales)	Unicellular or colonial	1
Merismopedia	Oval, spherical	(Chroococcales)	Form flat sheet of cells	1
Microcystis	Oval, spherical	(Chroococcales)	Unicellular or colonial	2
Synechocystis	Coccoid	Chroococcaceae	Unicellular or colonial	1
Anabaena	Cylindrical, ovoid	Nostocaceae	Filamentous, heterocystous	40
Anabaenopsis	Helical	Nostocaceae	Filamentous, heterocystous	2
Calothrix	Cylindrical	Rivulariaceae	Unbranched untapered	6
Cylindrospermum	Cylindrical	Nostocaceae	Filamentous, heterocystous	2
Nostoc	Cylindrical	Nostocaceae	Formation of thallus	55
Nodularia	Discoid	Nostocaceae	Chemoheterotroph	3
Tolypotrix	Spherical	Scytonematoceae	Rarely geminate false branches single or absent	3
Oscillatoria	Ovoid	Ocillatoriaceae	Transverse septa visible	24
Anthrospira	Helical	Ocillatoriaceae	Helical coil	1
Genera-14				Strains-142

a DNA thermal cycler, (Genetic analyser 377; Perkin-Elmer, Boston, MA, USA), employing the thermal profile. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by automated DNA sequencer (Bionex, Seoul, Korea) using the SequiTherm EXCEL II Labelled Primer Sequencing Kit (LI-COR Inc., Lincoln, NE, USA) with the T3F and M13R primers. Identification of cyanobacteria isolated was determined as compared the full-length sequencing of this bacterium with a collection of 16S rDNA obtained from the DDMJ/EMBL/Genbank database.

The cyanobacteria were grown in batch cultures at 25~28°C under a constant light intensity of 35~50 $\mu E/m^2/s$ in a 500-*ml* Erlenmeyer flask containing 250 *ml* nitrogen-free BG-11 liquid medium (Allen, 1968). The growth was determined by measuring the culture density at 680 nm using a spectrophotometer (model HP8453, Hewlett Packard, MI, U.S.A.). BG-11 contains 1.5 g of $NaNO_3$, 0.04 g of $K_2HPO_4 \cdot 3H_2O$, 0.075 g of $MgSO_4 \cdot 7H_2O$, 0.036 g of $CaCl_2 \cdot 2H_2O$, 0.006 g of citric acid, 0.006 g of ferric ammonium citrate, 0.001 g of EDTA (disodium salt), 0.02 g of $NaCO_3$, and 1 *ml* of trace metal mix A5, 1,000 *ml* of distilled water litter without nitrogen source (Allen, 1968). Trace metal mix A5 is composed of H_3BO_3, 2.86 g; $MnCl_2 \cdot 4H_2O$, 1.81 g; $ZnSO_4 \cdot 7H_2O$, 0.222 g; $NaMoO_4 \cdot 2H_2O$, 0.39 g; $CuSO_4 \cdot 5H_2O$, 0.079 g; $Co(NO_3)_2 \cdot 6H_2O$, 49.4 mg; and distilled water 1,000 *ml*. The pH of the media should be 7.0 ± 0.2 after autoclaving and cooling. Only axenic strains were used. A diluted algal suspension was sprayed onto the surface of solidified enriched medium with 0.5% glucose, 0.5% peptone and 1% yeast extract. The absence of bacterial and fungal colony for 5~7 days incubations indicated that the culture was axenic.

Especially, the two cyanobacteria, *Nostoc commune* FK-103 and *Oscillatoria tenuis* FK-109, showing strong antifungal effect on *Phytophthora capsici* were grown at different temperatures (20, 25, 30, 35, and 40°C) to investigate influence of temperature on the production or accumulation of antibiotics. Dry weight of cellular biomass was estimated after the cells were centrifuged, washed and dried to constant weight at 105°C. Chlorophyll *a* was determined by extracting cells with 96% methanol (Lorenzen, 1967).

Antifungal bioassay. Cyanobacteria cells were harvested in their early stationary phase (or late exponential phase) of growth by centrifugation at 10,000×g for 15 min and the supernatants-free biomass was freeze-dried (Clean Vac 8, BioTron Inc., Pucheon, Korea) for 22 h at 0.035 mbar and stored at −20°C until used. Algal extracts were prepared by resuspending the freeze-dried algal supernatants in distilled water, and 500~1,000 mg of the freeze-dried algal supernatants were extracted with 30 *ml* 100% ethanol for 3 h and then centrifuged at 15,000×g at 4°C for 15 min. The extract was purified using a C_{18} column to remove any chlorophyll and then freeze-dried. The freeze-dried extracts were dissolved in 1 *ml* methanol or petroleum ether. Antifungal activity was evaluated by agar plate diffusion test (Lorain, 1996). The fungi used for the antifungal test were *Alternaria alternata*, *Botrytis cineria*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Phytophthora capsici*, *Pythium ultimum* and *Rhizopus stolonifer* that were grown on potato dextrose agar (PDA; Difco, U.S.A.). To obtain spores of *Phytophthora capsici*, mycelia were grown on potato-dextrose liquid medium at 28 ± 0.5°C for 7 days and the spores were collected by filtration and centrifugation for 10 min at 1,000×g and 4°C. Filter paper disks (6.5 mm) were saturated with 100 μl of the test solution, dried, and placed on PDA plates, which were inoculated with a standardized quantity of suspension containing 1.0×10^5 spores/*ml*. Plates were incubated at 28 ± 0.5°C for 5 days in dark. Nystatin disks (10 μg,

Sigma, St, Luis, MO, U.S.A.) were used as positive controls. The distinct and circular radii of inhibition zones were measured. The antifungal activity by the resuspending-lyophilized algal supernatants was classified as no inhibition (–; ≤ 5 mm), week inhibition (+; 6~10 mm), moderate inhibition (++; 10~20 mm), or strong inhibition (+++; ≥ 20 mm). For each resuspending-lyophilized algal supernatant and fungus, there were three replicates per assay and each assay was repeated three times.

Results and Discussion

Screening for antifungal activity. One hundred and forty two cyanobacterial strains were tested (ref. Table 1) and nine strains were selected for the antifungal activity of their aqueous extract against various red pepper pathogenic fungi (Table 2). Two species belonged to the genus *Oscillatoria* and six of them were included in the family Nostocaceae (two *Anabaena*, three *Nostoc*, and one *Nodularia*). Others belonged to the genus *Calothrix*. The ten-cyanobacterial strains whose water extracts had antifungal activity were identified. Among the tested fungi, *R. stolonifer* was more resistant because it was inhibited by only one species, *Nostoc commune* FK-103 (Table 2). The extracts and culture medium (resuspending in H_2O) of nine cyanobacterial strains showed antifungal activity against *Alternaria alternata*. However, only the petroleum ether and methanol extracts of *Nostoc commune* FK-103 and *Oscillatoria tenuis* FK-109 revealed a strong antifungal effect on *Phytophthora capsici*. In addition, *Nostoc commune* FK-103 indicated a widespread spectrum on antifungal activities. Cano *et al.* (1990) have been evaluated the antifungal activity of terrestrial cyanobacterium *N. commune* against the *Candida albicans* by 20.83% and Mule *et al.* (1991) also reported that extract of *N. commune* inhibited the growth of same fungus. These antifungal activities are very interesting in the perspective of cyanobacterial research and possibly are important in commercial. Nevertheless, the antifungal activities of cyanobacterial metabolites were rarely studied. Only three groups have reported antifungal activity of cyanobacteria metabolites (Cannell *et al.*, 1987; Kellam *et al.*, 1988; Welch, 1962) while several groups have reported antimicrobial activity against bacteria (Cannell *et al.*, 1988), cyanobacteria and algae (Flores and Wolk, 1986), and virus (Starr *et al.*, 1962). The antifungal activity of the antibiotics from the nine-cyanobacterial strains selected were tested on seven species of plant pathogenic fungi

Table 2. Results of antifungal activity by different extracts from cyanobacteria

Cyanobacterial strain (Origin)	Extract	Bioautography						
		Alternaria alternata	*Botrytis cinerea*	*Colletotrichum gloeosporioides*	*Fusarium oxysporium*	*Phytophthora capsici*	*Pythium ultimum*	*Rhizopus stolonifer*
Anabaena solitaria FK-023	H_2O	–	–	–	+	–	–	–
(Hongcheon)	PE	–	–	–	+	–	–	–
	MeOH	++	+	+	–	–	–	–
Anabaena sp. FK-030	H_2O	+	+	-	–	–	–	–
(Kimhae)	PE	++	-	-	–	–	–	–
	MeOH	–	–	–	–	–	–	–
Calothrix brevissima FK-056	H_2O	+	+	–	–	+	+	–
(Buyeo)	PE	++	+	+	+	–	–	–
	MeOH	+++	++	+	++	–	–	–
Nostoc commune FK-103	H_2O	+	–	–	–	–	–	–
(Jincheon)	PE	+	–	–	–	+++	++	–
	MeOH	+	+	+	++	+++	+	+
Nostoc commune FK-089	H_2O	–	–	+	–	–	–	–
(Ahandong)	PE	++	–	+	+	+	–	–
	MeOH	–	–	–	–	–	–	–
Nostoc muscorum FK-134	H_2O	–	–	–	–	+	–	–
(Seosan)	PE	–	–	–	–	+	+	
	MeOH	++	++	+++	+	–	–	–
Nodularia sp. FK-077	H_2O	–	+	+	+	–	–	–
(Jinju)	PE	–	–	–	–	–	–	–
	MeOH	+	+	+	++	–	–	–
Oscillatoria angustissima FK-113	H_2O	+	–	–	–	–	–	
(Icheon)	PE	+	+	++	++	–	–	–
	MeOH	++	+	++	+	–	–	–
Oscillatoria tenuis FK-109	H_2O	–	+	–	–	–	–	–
(Naju)	PE	++	–	–	–	+++	–	–
	MeOH	+	–	–	–	+++	+	–

related to *Capsicum annuum* L. (Table 2). However, the potential activity of cyanobacteria to inhibit certain soil-borne diseases needs further investigation before they can be accepted as biocontrol agents for agriculture. A further advantage of these cyanobacterial strains is that they are known to produce a wide range of plant growth regulators such as abscisic acid, ethylene, jasmonic acid, auxin, and cytokinin-like substances as well as the cytokinin isopentenyl adenine (Ördög and Pulz, 1996; Strik *et al.*, 1999), and these substances can also influence fungal growth (Zulpa *et al.*, 2003).

Influence of temperature on antifungal activity of cyanobacteria. The antifungal activity of the antibiotics at the late exponential/early stationary phase of growth (for 10 days culture) against *Phytophthora capsici* in relations to temperatures and growth measurements by *Nostoc commune* and *Oscillatoria tenuis* is shown in Fig. 1. Antifungal activities increase in the medium by cyanobacteria, although not in direct proportion to growth parameters such as biomass and chlorophyll *a*. The high inhibition levels of antibiotic, 22.5 and 31.8 mm for *Nostoc commune* and *Oscillatoria tenuis,* respectively, were observed at 25°C, while the cyanobacterial biomass and chlorophyll *a* reached the maximum at 30°C along with the increasing in proportion to the increase of temperature. Gromov *et al.* (1991) reported that the production of cyanobacterin Lu-1 by *Nostoc linckia* was to be temperature-dependent. The antibiotic production from *N. muscorum* was dependent on the limitation of one or more nutrients in the growth medium and not in direct accordance to cell concentration. The optimal temperature for antibiotic productivity and for growth of *N. muscorum* was 35°C (Bloor and England, 1991; El-Sheekh *et al.*, 2006). The conditions under which the antibiotics are produced by the cyanobacteria must still be determined in order to maximize the production of the useful antibiotics, which can be used as biological material with potential application in agriculture. Work is now in progress to intensify the chemical studies in order to determine which is the most appropriate faction regarding the inhibitory substances and also which concentration will assure the growth inhibition of the plant pathogenic fungi in hot pepper and to develop suitable cyanobacterial strains that improve crop growth and control disease in a cost effective, environmental-friendly manner.

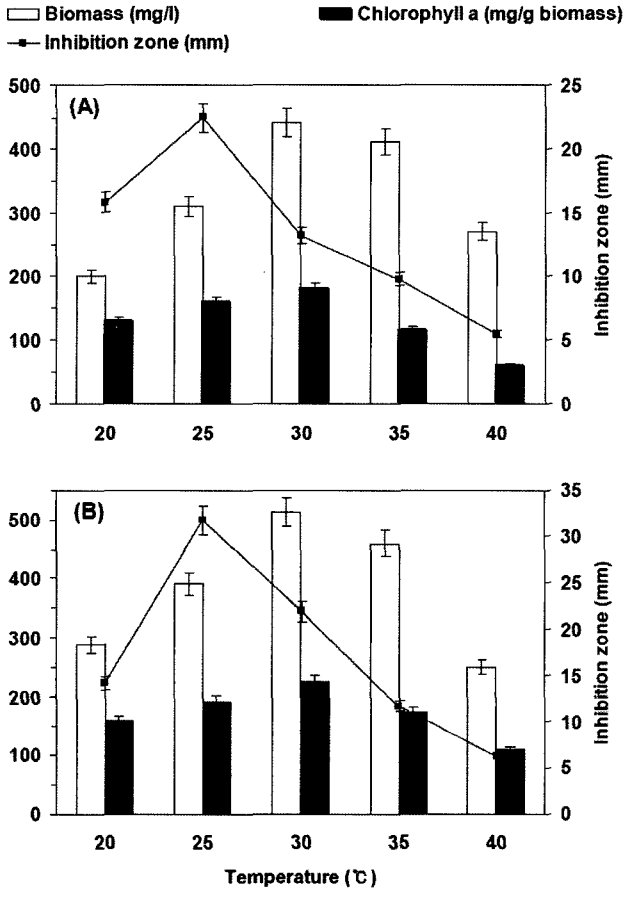

Fig. 1. Growth and production of antibiotics by (A) *Nostoc commune* FK-103 and (B) *Oscillatoria tenuis* FK-109 in batch culture at different temperatures (mean of five values for the late exponential phase of growth ± S.E.). For the inhibition test, filter paper disks (6.5 mm) were saturated with 100 μl of the test solution, dried, and placed on PDA plates, which were inoculated with a standardized quantity of suspension containing 1.0×10^5 spores/*ml*. Plates were incubated at 28 ± 0.5°C for 5 days in dark.

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