Mycobiology 36(4) : 242-248 (2008)

Inhibitory Effect of Algal Extracts on Mycelial Growth of the Tomato-Wilt Pathogen, *Fusarium oxysporum* f. sp. *lycopersici*

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(Received June 4, 2008. Accepted October 20, 2008)

The present study was undertaken to explore the inhibitory effect of cyanobacterial extracts of *Nostoc commune* FA-103 against the tomato-wilt pathogen, *Fusarium oxysporum* f. sp. *lycopersici*. In an optimal medium, cell growth, antifungal activity, and antifungal compound production could be increased 2.7-fold, 4.1-fold, and 13.4-fold, respectively. A crude algal extract had a similar effect as mancozeb at the recommended dose, both in laboratory and pot tests. *In vitro* and *in vivo* fungal growth, spore sporulation and fungal infection of wilt pathogen in tomato seeds were significantly inhibited by cyanobacterial extracts. *Nostoc commune* FA-103 extracts have potential for the suppression of *Fusarium oxysporum* f. sp. *lycopersici*.

KEYWORDS : Algae, Algal extract, Antifungal activity, Cyanobacteria, *Fusarium oxysporum* f. sp. *lycopersici*, *Nostoc commune*, Tomato

Fusarium spp. are an abundant saprophyte in soil and organic matter and are found worldwide. Some strains cause a vascular wilt disease in crops, including vegetables, bananas, and date palms. However, some strains can protect plants from wilt. The pathogenic stains of this fungus have numerous specialized forms, known as *formae specials* (f. sp.), that cause diseases such as vascular wilt, corm rot, root rot, or damping-off (Olivain and Alabouvette, 1999). *Fusarium oxysporum* f. sp. *lycopersici* is known to affect tomato plants which is a crop plant of great economic importance (Suárez-Estrella *et al*., 2007). Tomato production is significantly reduced by *Fusarium oxysporum* f. sp. *lycopersici* because it can destroy roots of tomatoes at growth stages. Many strategies to control this fungal pathogen have been investigated in the field (Biondi *et al.*, 2004; Khan *et al.*, 2007). Currently, the most effective method in preventing tomato from *Fusarium* wilt is to mix the seed with chemical fungicides. The application of chemical fungicide induces other problems, such harm to other living organisms and the reduction of useful soil microorganisms (Khalifa *et al*., 1995; Lewis *et al.*, 1996). Therefore, public concern is focused on alternative methods of pest control, which can play a role in integrated pest management systems to reduce our dependence on chemical pesticides (Sutton, 1996). As with other vascular plant-diseases and sanitation measures are difficult to apply (Brayford, 1992). A promising strategy for the replacement of chemical pesticides has been the implementation of biological control. The recent development in the commercialization of biological control products has accelerated this approach (Fravel *et al*., 2003).

Algae are one of the chief biological agents that have been studied for the control of plant pathogenic fungi, particularly soil borne disease (Abdel-Kadar, 1997; Hewedy *et al.*, 2000). For example, cyanobacteria (blue-green algae) and eukaryotic algae produce biologically active compounds that have antifungal activity (Kulik, 1995; Schlegel *et al.*, 1998), and antibiotic and toxic actvity (Bonjouklian *et al*., 1991; Kiviranta *et al*., 2006) against plant pathogens.

Anabaena spp. (Moore *et al*., 1986; Farnkmolle *et al*., 1992), *Scytonema* spp. (Chetsumon *et al*., 1993), and *Nostoc* spp. (Bloor and England, 1989) were shown to be efficient in the control of damping-off as well as the growth of soil fungus *Cunninghamella blakesleana*. Especially, culture filtrates or cell extracts from cyanobacteria and algae applied to seeds protect them from damping-off fungi such as *Fusarium* sp., *Pythium* sp. and *Rhizoctonia solani* (Kulik, 1995). In a previous study (Kim, 2006), antifungal activities were found in 29 strains of the 298 microalgal strains tested. *Nostoc communm* FA-103 was selected as the subject of this study because of its broad spectrum of antifungal activity on plant pathogenic fungi, especially *F. oxysporum* f. sp. *lycopersici*.

In this study, we investigated the optimal conditions for producing the antifungal compounds and the application of algal extracts for control of *Fusarium* wilt in tomato seeds.

Materials and Methods

Microorganisms. The N_2-fixing blue-green alga (cyanobacterium), *Nostoc commune* Vaucher ex Bornet et Fla-

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hault strain FA-103, was isolated from rice paddy fields in Korea. The strain FA-103 was cultured at 25°C in the N-free BG-11 (basal medium) liquid medium (Kim, 2006; Kim and Lee, 2006) under the illumination of cool-white fluorescent lamps at a photon flux density of 40 μE/m^2/s. Algal growth was determined by measuring the cell concentration and chlorophyll *a* concentration according to the method of Parsons and Strickland (1963). The culture of the strain FA-103 was filtered by GF/C (Whatman, Uppsala, Sweden) and extracted with 90% methanol at 60°C for 10 min. After extraction, the solid suspension was removed by centrifugation. Then, absorbance of extracts was measured at 665, 645, and 635 nm using a spectrophotometer (model HP8453, Hewlett Packard, MI, USA). The concentration of chlorophyll *a* (Chl-*a*) was calculated using the following equation.

$$\text{Chl-}a\ (\text{mg/l}) = 11.6\ A_{665} - 1.31\ A_{645} - 0.14\ A_{515}$$

The plant pathogenic fungus, *Fusarium oxysporium* f. sp. *lycospersici* KACC40032 was kindly supplied by the National Institute of Agricultural Biotechnology in Suwon, Korea. Tomato seeds (*Lycopersicon esculentum* cv. Pink charm) were purchased by Nongwoobio Co. Ltd., Suwon, Korea.

Optimization of antifungal compound production. To optimize culture conditions for production of antifungal compound, approximately 1.0×10^4 cells/*ml* of algae in the exponential growth phase was inoculated into 500 *ml*-Erlenmeyer flasks containing 250 *ml* of medium. The flasks were incubated at 28°C with agitation (150 rpm in a rotary shake incubator) under the continuous illumination of cool-white fluorescent lamps with light intensity of 40 μE/m^2/s. In this study, the culture media such as N-free BG-11 (Kim and Lee, 2006), BG-11 (Rippka *et al*., 1979), BG-11+N, and Allen's medium (Allen, 1952) were applied to algal cell culture for algal cell growth, antifungal activity and production of antifungal compounds and then we focused on the concentrations of $NaNO_3$, K_2HPO_4 and NaCl including initial pH. BG-11 included $NaNO_3$ 1.5 g, K_2HPO_4 0.04 g, $MgSO_4 \cdot 7H_2O$ 0.075 g, $CaCl_2 \cdot 2H_2O$ 0.036 g, EDTA-disodium 0.001 g, Na_2CO_3 0.02 g, citric acid 0.006 g, ferric ammonium citrate 0.006 g, and micronutrient 1 *ml* per liter (Rippka *et al*., 1979). The composition of micronutrient was H_3BO_3 2.86 g, $MnCl_2 \cdot 4H_2O$ 1.81 g, $ZnSO_4 \cdot 7H_2O$ 0.222 g, $Na_2MoO_4 \cdot 2H_2O$ 0.39 g, $CuSO_4 \cdot 5H_2O$ 0.079 g, $Co(NO_3)_2 \cdot 6H_2O$ 0.0494 g per liter (Rippka *et al*., 1979).

The optimal culture condition found in each step was applied to the next step. After selection of the optimal medium for production antifungal compounds in Erlenmeyer flasks, The combined effects obtained from the optimization were consequently applied to antifungal compound production in 10 *l* bubble column photobioreactors (BCPs) (Lee *et al*., 2006). A pre-culture of 250 *ml* was prepared in a 500-*ml* Erlenmeyer flask. The cells were transferred to 10 *l* BCPs containing the optimal medium with 0.2 vvm flow-rate-aeration and 5% CO_2 gas and 95% air under a constant continuous light intensity of 40 μE/m^2/s. The temperature and pH were kept constant at 25°C and 7.0 ± 0.5, respectively, during the period of cultivation.

Preparation of algal extracts. The freeze-dried algal cells (40 g) were extracted twice with 80 *ml* of methanol for 24 h at room temperature. The extract was separated from the cell residue by filtration (Whatman filter paper no. 3). The filtrate was concentrated at 40°C under reduced pressure in a rotary evaporator (EYELA N-1000S, Tokyo RikakiKai Co. Ltd., Japan). The 285 mg of pale brown extract was collected, suspended in 80% ethanol, and kept at 4°C.

Determination of algal cell growth and antifungal compound production. Cell growth of *N. commune* FA-103 was estimated using cell dry weight. Cells were harvested by centrifugation, washed twice with distilled water, and dried to constant weight at 85°C for 12 h. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone formed around the disk. The experiment was performed in triplicate, and the mean of the diameter of the inhibition zones was calculated. Quantitative bioassay of the antifungal compound was confirmed by comparing with a standard curve of commercial antifungal compound concentration, which was modified from Loo's method (Loo *et al*., 1945).

Microalgal methanol extract (500 μg) was applied to sterile paper disks (8 mm in diameter) for antifungal assay. Paper disks containing only methanol were used as control. *Fusarium oxysporium* f. sp. *lycopersici* (1.0×10^6 spores/*ml*) were added to molten potato dextrose agar at 40°C. The seeded agar was poured into 9-cm Petri dishes. The algal extract treated paper disks were placed in the center of the seeded agar plates. Plates were incubated for 3 days at 28°C, and then inhibition zones of mycelial growth around the paper disks were measured. The commercial fungicide mancozeb, a polymeric complex of zinc and manganese salts of ethylene bisthiocarbamate (EBDC), (Sungbo Chemical Co. Ltd., Seoul, Korea) was used as reference standard of antifungal activity. The inhibition zone produced by the crude extract was converted to the concentration of mancozeb with the standard curve. Antifungal activity and antifungal compound production are presented as mg equivalent to mancozeb/g of algal dry weight and mg equivalent to mancozeb/*l* of medium, respectively.

Effects of algal extracts on the inhibition of *F. oxysporum* f. sp. *lycopersici* infection in tomato seeds. Experiments to determine the effect of algal extract concentrations on

the inhibition of infection of tomato seeds were carried out in Petri dishes. The surface of tomato seeds was sterilized with 10% sodium hypochloride (v/v) then coated with algal extract. The coated seed were incubated at 25°C for 24 h, and then the fungal spores were spread on the seeds. The inoculated seeds were laid on moist seed-cultivating paper in Petri dishes, and were incubated at 30°C in dark conditions. After 7 days-incubation, seeds or seedling were observed.

The influence of algal extract concentration on *F. oxysporum* f. sp. *lycopersici* cell growth in tomato seeds was investigated at several different concentrations, ranging from 100 to 500 μg/seed. The algal extract was coated with methanol base, zeolite mixing, and dissolving in Tween 20 (10%, v/v) to enhance the antifungal activity. The same vehicles without the addition of the algal extract were used as controls. The fungal inoculum concentrations used in these experiments were 10, 100, and 500 spores/seed. Each experiment was performed in 10 replicates.

***In vivo* anti-*Fusarium* wilt activity by microalgal extract.** The anti-fungal activity of algal extract in tomato plant was evaluated in a growth room. Tomato seeds (*Lycopersicon esculentum* cv. Pink charm) were prepared as previously described. We compared two different inoculation methods: (i) seeds were directly inoculated by fungal spores with the concentration of 500 spores/seed before they were sown in plastic pots (5 × 15 × 10 cm) containing steam-sterilized soil mixture (peat moss, perlite and vermiculite, 5 : 3 : 2, v/v/v), sand, and loam soil (1 : 1 : 1, v/v/v). It is representative model of direct inoculation (seed inoculation). (ii) In indirect inoculation, soil mixture was inoculated with the fungal spores (1.0×10^3 spores/g of steam-sterilized soil mixture) before the seed were sown. The tested tomato seed were cultivated as representative model of mixing with soil mixture inoculated pathogen (soil inoculation). Tomato plants were raised in a growth room at 30°C with 80 μE/m^2/s and 60~90% relative humidity. Plants were watered regularly. After 14 days of sowing, infection was determined.

Results and Discussion

Optimization of culture medium to produce antifungal compound. BG-11+N medium (addition of 3.0 g/*l* $NaNO_3$) showed the best results among the media tested (data not shown). However, the optimal culture condition for producing antifungal substances from *N. communes* FA-103 was further investigated by using BG-11+N medium as basal medium. The optimal medium for production of antifungal compounds was prepared by adding $NaNO_3$ as N source to the concentration of 3.0 g/*l*, increasing K_2HPO_4 concentration to 0.15 g/*l* and reducing $CaCl_2$ concentration to 0.015 g/*l* with the initial pH 7.0.

The cell growth of *N. commune* FA-103 in N-free BG-11 and BG11+N media in 10 *l* bubble column photobioreactors sparged with 5% CO_2 and incubated at the controlled conditions were compared. Algal cell growth, antifungal activity, and antifungal compound production of the strain FA103 in BG11+N media could be increased 2.7-fold, 4.1-fold, and 13.4-fold, respectively (Table 1).

Inhibition of *F. oxysporum* f. sp. *lycospersici* in tomato by crude algal extracts. The algal extract concentration of 150 μg/seed is the minimal concentration (Table 2) that has an effect similar to mancozeb. The antifungal activity of the crude algal extract could be maintained at 50°C for more than 2 h, and the optimal pH ranges were from 5 to 8 (data not shown). These results are similar to those reported by Cano *et al.* (1990), Caire *et al.* (1993), Fish and Cood (1994), Stratmann *et al.* (1994), and Borowitzka (1995), who reported that the extracts of *Nostoc muscorum* significantly inhibited the growth of *Candida albicans* and *Sclerotinia sclerotiorum*.

Cyanobacteria produce exopolysaccharides that can function as energy sources for fungi and produce plant growth regulators, which are abscisic acid, ethylene, jasmonic acid, auxin, and cytokinin-like substances, the cytokinin isopentenyl adenine. These substances can influence fungal growth (Ördög and Pulz, 1996; Stirk *et al.*, 1999).

Effect of formulation of algal extract on *F. oxysporum* f. sp. *lycospersici* in tomato. The crude algal extract by

Table 1. Growth, antifungal activity, and antifungal compound production by *Nostoc commune* FA-103 in different media.

Medium	N source	Medium $NaNO_3$ (g/*l*)	Composition K_2HPO_4 (g/*l*)	$CaCl_2$	pH	Dry weight of algae[a] (g/*l*)	Antifungal	
							Activity (mg/g)[b]	Compound production (mg/*l*)
N-free BG-11	–	0.0	0.04	0.036	7.5	0.87	2.54	0.29
BG-11	$NaNO_3$	1.5	0.04	0.036	7.5	1.21	2.88	0.57
BG-11+N	$NaNO_3$	3.0	0.15	0.015	7.0	2.34	10.32	3.90

[a]To determine significant differences among the treatments, one-way ANOVA tests were performed. Correlation coefficients among the experiments were analysed using SAS Systems v. 8.12 (SAS Institute Inc., Cary, NC).
[b]Antifungal activity by *Nostoc commune* FA-103 against *Fusarium oxysporum* f. sp. *lycopersici in vitro*.

Table 2. Effect of crude algal extract concentration on the inhibition of *Fusarium* wilt by *F. oxysporum* f. sp. *lycospersici* in tomato seeds.

Treatment	Average no. of infected seedling[a]
Seed + pathogen[b]	20.0
Seed + algal extract[c] (50 μg/seed) + pathogen	17.6
Seed + algal extract (100 μg/seed) + pathogen	8.5
Seed + algal extract (150 μg/seed) + pathogen	0.8[e]
Seed + algal extract (200 μg/seed) + pathogen	0.6[e]
Seed + algal extract (250 μg/seed) + pathogen	0.4[e]
Seed + algal extract (300 μg/seed) + pathogen	0.3[e]
Seed + algal extract (350 μg/seed) + pathogen	0.1[e]
Seed + algal extract (400 μg/seed) + pathogen	0.0[e]
Seed + mancozeb (200 μg/seed)[d] + pathogen	0.0[e]

[a]Means from 20 replicates, each replicate containing 20 seeds
One-way ANOVA tests were performed to determine significant differences among the treatments. Correlation coefficients among the experiments were analyzed using SAS Systems v. 8.12 (SAS Institute Inc., Cary, NC).
[b]*Fusarium oxysporum* f. sp. *lycospersici* at the concentration of 500 spores/seed
[c]Algal extract formulated using surfactant (0.1% Tween 20; v/v).
[d]Dose of mancozeb as recommended by Sungbo Chemical Company
[e]Any two means are not significantly different at the 5% level of significance by Duncans's multiple rang test (Gomez and Gomez, 1984)

methanol coated with Tween 20 (0.1%; v/v) inhibited *F. oxysporum* f. sp. *lycospersici* in tomato seeds at the same rate as mancozeb. The length of roots, the number of hairy roots and the stem length were significantly increased. These results suggest that the algal extract coated with Tween 20 (0.1%; v/v) showed the best results not only in control of the pathogen but also in stimulating tomato seedling growth.

The effects of pathogen inoculum and algal extract on the inhibition of fungal infection in tomato seed were shown in Table 4. The infection of tomato seed increased with increasing concentration of fungal pathogen inoculum, and the tomato seeds were completely infected at the inoculum concentration of 500 spores/seed. The treatment of algal extract at a concentration lower than the minimum inhibitory concentration (150 μg/seed) showed inhibitory effect on fungal growth in tomato seeds, but no statistical differences were observed when inhibition was compared at higher inoculum concentrations. These results indicate that the application of algal extract concentration lower than the minimum inhibitory concentration (150 μg/seed) could mitigate the infection of *F. oxysporum* f. sp. *lycospersici* in tomato seeds to certain level.

Control of seed-borne and soil-borne *F. oxysporum* f. sp. *lycospersici*-diseases by microalgal extract. In the direct inoculation of *F. oxysporum* f. sp. *lycospersici* with the concentration of 500 spores/seed on tomato to seeds (seed inoculation), the same degree of infection was observed when the seeds were inoculated indirectly (soil inoculation) by a mixture of fungal inoculum (1.0×10^3 spores/g of soil) and steam-sterilized soil mixture. However, the crude algal extract of 200 μg/seed had the same controlling effect as mancozeb at the recommended dose in both the seed and soil inoculation. This suggests that the crude algal extract of *N. commune* FA-103 is a potential natural fungicide that could effectively control the infection of *F. oxysporum* f. sp. *lycospersici* in tomato at a concentration similar to the commercial fungicide, mancozeb. Zulpa *et al.* (2006) studied the effect of *Nostoc muscorm* on the growth of some plant pathogens. Abo-Shady *et al.* (2007) also reported that cyanobacteria filtrates strongly inhibited the phytopathogenic fungi isolated from leaves, stems, and roots of Faba bean. Moreover, mycelial growth of several phytopathogenic fungi such as *Fusarium oxysporum*, *Penicillium expansum*, *Phytophthora cinnamomi*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Verticillium albo-atrum* were inhibited by the methanol extracts of the cyanobacterium *Nostoc* strain ATCC 53789 (Biondi *et al*., 2004).

Nostoc muscorum filtrates have potential for the sup-

Table 3. Effect of algal extract formulation on the inhibition of *F. oxysporum* f. sp. *lycospersici* in tomato seeds 14 days after treatment.

Treatment	Length of root (cm)	No. hairy roots	Length of stem (cm)	Average no. of infected seedling[a]
Seed + pathogen[b]	0.00	0.00	0.00	20.0
Seed + algal extract[c] (CH_3OH) + pathogen	10.47	2.82	10.99	0.6
Seed + algal extract (zeolite) + pathogen	15.08	2.18	11.12	1.8
Seed + algal extract (0.1% Tween 20) + pathogen	9.16	4.03	13.88	0.2
Seed + mencozeb[d] + pathogen	10.66	3.04	9.02	0.5

[a]Means from 20 replicates, each replicate containing 20 seeds.
One-way ANOVA tests were applied to determine significant differences among the treatments. Correlation coefficients among the experiments were analyzed using SAS Systems v. 8.12 (SAS Institute Inc., Cary, NC).
[b]*Fusarium oxysporum* f. sp. *lycospersici* at the concentration of 500 spores/seed
[c]Algal extract at the concentration of 150 μg/seed formulated using methanol, zeolite, or surfactant (0.1% Tween 20, v/v) as vehicles.
[d]Mancozeb at the recommended concentration of 200 μg/seed.

Table 4. Effect of fungal pathogen inoculum and algal extract concentration on the inhibition of *Fusarium oxysporum* f. sp. *lycopersici* in tomato seeds.

Treatment	Average no. of infected seedlings[a]
seed + pathogen[b] (10 spores/seed)	13.2
seed + pathogen (100 spores/seed)	17.2
seed + pathogen (500 spores/seed)	20.0
seed + algal extract[c] (100 μg/seed) + pathogen (10 spores/seed)	2.9
seed + algal extract (100 μg/seed) + pathogen (100 spores/seed)	6.2
seed + algal extract (100 μg/seed) + pathogen (200 spores/seed)	8.6
seed + algal extract (200 μg/seed) + pathogen (10 spores/seed)	0.3
seed + algal extract (200 μg/seed) + pathogen (100 spores/seed)	0.4
seed + algal extract (200 μg/seed) + pathogen (200 spores/seed)	0.2

[a]Means from 20 replicates, each replicate containing 20 seeds
One-way ANOVA tests were applied to determine significant differences among the treatments. Correlation coefficients among the experiments were analyzed using SAS Systems v. 8.12 (SAS Institute Inc., Cary, NC).
[b]*Fusarium oxysporum* f. sp. *lycopersici*
[c]Algal extract formulated by surfactant (0.1% Tween 20, v/v)

Table 5. Effect of algal extract on tomato wilt pathogen control in tomato seeds cultivated in soil.

Fungal pathogen inoculation	Treatment	Average no. of infected seedling[a]
Seed inoculation	seed + pathogen[b] (500 spores/seed)	15.50
	seed + algal extract (150 μg/seed) + pathogen (500 spores/seed)	2.24
	seed + algal extract (200 μg/seed) + pathogen (500 spores/seed)	0.15
Soil inoculation	seed + pathogen (1×10^3 spores/g of soil)	15.60
	seed + algal extract (150 μg/seed) + pathogen (1×10^3 spores/g of soil)	3.10
	seed + mancozeb[d] (200 μg/seed) + pathogen (1×10^3 spores/g of soil)	0.20

[a]Means from 20 replicates, each replicate containing 20 seeds
One-way ANOVA tests were applied to determine significant differences among the treatments. Correlation coefficients among the experiments were analyzed using SAS Systems v. 8.12 (SAS Institute Inc., Cary, NC).
[b]*Fusarium oxysporum* f. sp. *lycopersici*
[c]Algal extract formulated by surfactant (0.1% Tween 20, v/v)
[d]Mancozeb at the recommended concentration of 200 μg/seed

pression of phytopathogenic fungi such as the sugarbeet pathogens *Fusarium verticillioides*, *Rhizoctonia solani* and *Sclerotium rolfsii* (Rizk, 2006). *In vitro* and *in vivo* growth, sporulation, and sclerotial production were significantly inhibited with *Nostoc muscorum*. *In vivo* studies showed that *F. oxysporum* was very sensitive to cyanobacteria species (Rizk, 2006). The maximum inhibition of *Fusarium* growth in soil was 81% with *Anabaena flos-aquae*. In addition, the growth activities of *F. oxysporum* f. sp. *betae*, *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *vasinfectum* were strongly inhibited with increasing concentration of cyanobacterial extracts (Moussa and Shanab, 2001). Tomato *Fusarium* wilts due to *F. oxysporum* f. sp. *lycopersici* were reduced significantly by all formulation of algal extracts (Tables 4 and 5). Tomato seeds treated by 200 μg/seed algal extract showed the best control effect comparing with the control inoculated by the fungal pathogen only (Table 5). However, most concentrations of algal extracts have a significant effect on the growth of *Rhizoctonia solani* in soil (data not shown). This is in agreement with results obtained by Pushparaj *et al*. (1999), Tahmida Begum *et al*. (1999), and Rizk (2006). Nonetheless, Kulik (1995) reported that the growth of *R. solani* on PDA was significantly inhibited by using *N. muscorm* extract.

In conclusion, the sensitivity to cyanobacteria metabolites not only depends on the fungal genus but also on the species and on the mode of growth. Therefore, elucidation of chemical structure of the antifungal compounds produced by *N. commune* FA-103 is required and the mode of action of algal extracts to inhibit the mycelial growth of *F. oxysporum* f. sp. *lycopersici* should be investigated.

Acknowledgement

This work was supported by the 21C Frontier Microbial Genomics and Applications Center Program (MG08-0305-1-0), Ministry of Education, Science & Technology, Republic of Korea for which the authors are thankful.

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