Journal of Plant Biology, September 2008, 51(5) : 373-378

Induced Metabolite Changes in *Myriophyllum spicatum* during Co-existence Experiment with the Cyanobacterium *Microcystis aeruginosa*

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We conducted a 7-d co-existence experiment to verify the inhibitory activity of an aquatic vascular plant, *Myriophyllum spicatum*, on the cyanobacterium *Microcystis aeruginosa*, and to screen for allelopathic compounds. Plants were placed on a modified solid M4 medium while *M. aeruginosa* was cultured in an L16 liquid medium. Our treatments included rearing *Myriophyllum spicatum* alone (Plant-Only; PO) or with *Microcystis aeruginosa* (Plant–Cyanobacteria; PCB), as well as an untreated control, or plates with the cyanobacterium alone. In the PCB treatment, *M. aeruginosa* became transparent in three replicates on the 6th day and in four replicates on Day 7. To make the distinction in *Myriophyllum spicatum* metabolites between the PO and PCB treatments, we obtained 13 m/z profiles using a 15 T Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS). Principle component analyses were performed on those profiles, from which we obtained accurate mass values for selected peaks that had most negative loadings on the first principal component and most positive loadings on the second principal component: 282.06983 and 283.06836. We identified those peaks as $C_7H_{17}N_5O_3S_2$ and $C_{10}H_{12}N_4O_6$, respectively. The Dictionary of Natural Products database suggests that the latter is either oxanosine or xanthosine.

Keywords: allelochemicals, co-existence, *Microcystis aeruginosa*, *Myriophyllum spicatum*, oxanosine, xanthosine

Many Korean freshwater ecosystems are becoming eutrophic due to rapid industrialization and development, which has led to serious ecological and economics problems. Considerable research interests are taking physical, chemical, and biological approaches to focus on regulating phytoplankton water blooms, especially those that are cyanobacterial. Scientists are now using aquatic, especially submerged, vascular plants to control those blooms. Interactions have been described among macrophytes, phytoplankton, and periphyton, and various mechanisms have been summarized for macrophyte inhibition on phytoplankton, including shading, lowering of temperatures, nutrient competition, and allelopathy (van Donk and de Bund, 2002). Among the submerged macrophytes that have been studied, *Myriophyllum* (Planas et al., 1981; Gross and Sütfeld, 1994; Gross, 1999; Nakai et al., 2000, 2005) and *Chara* (Crawford, 1979; Jasser, 1995) have been extensively investigated for their allelopathic interactions with algae or cyanobacteria. In particular, *Myriophyllum spicatum* produces several polyphenol compounds and fatty acids to reduce *Microcystis aeruginosa* (Nakai et al., 2000, 2005). However, there is no conclusive evidence for the allelopathy of submerged macrophytes (van Donk and de Bund, 2002; Gross et al., 2007).

Co-existence experiments have been used to test reciprocal interactions between macrophytes and algae (Gross et al., 2007). Although few of those studies have screened for induced chemicals in submerged plants, Bi et al. (2007) have shown that exogenously applied methyl jasmonate and methyl salicylate can induce allelopathy.

We previously surveyed 21 reservoirs with submerged macrophytes and identified *M. spicatum* and *Hydrilla verticillata* as possible candidates for releasing allelochemical substances to diminish phytoplankton growth (Joo et al., 2007). Subsequently, we found that extracts from *M. spicatum* reduced the growth of *Microcystis aeruginosa*, with a 95% level of significance (Nam and Park, 2007). In the present study, we took a more direct approach, conducting co-existence experiments with *Myriophyllum spicatum* (aquatic plant) and *Microcystis aeruginosa* (cyanobacterium). We hypothesized that the latter would induce the former to produce allelochemical substances. We also investigated the inhibitory activity of plant on bacterium and screened for the induction of allelochemical substances.

MATERIALS AND METHODS

Preparation of *Myriophyllum spicatum* and Culture of *Microcystis aeruginosa*

We collected *Myriophyllum spicatum* L. from Gokneung Stream under the Bongilcheon Bridge, Gyeonggi-do, South

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Abbreviations: FT-ICR MS, Fourier transform-ion cyclotron resonance mass spectrometry; I, Initial plants before the co-existence experiment; PO, Plant-Only; PCB, Plant–Cyanobacteria; CBO, Cyanobacteria-Only; C, Control; ESI, electrospray ionization; PCA, principal component analysis; T-RFLP, terminal restriction fragment length polymorphism; NMR, nuclear magnetic resonance

Korea, in October 2007. Those plants were then maintained in an 80 L aquarium with an M4 medium (Elendt, 1990; Knauer et al., 2006). To remove other organisms, we washed the plants with 70% ethanol for 30 s, then rinsed them twice with deionized water prior to the experiments. In all, 13 plants of similar height were selected and planted on agar (M4 medium + 1.5% Bacto agar).

Microcystis aeruginosa Kützing (UTEX 2385) was obtained from the Culture Collection of Algae at the University of Texas at Austin, TX, USA. This strain was maintained in a modified L16 medium (Lindström, 1983) with vitamins and earth extract in a temperature-controlled chamber at 24°C under a 16-h photoperiod. The L16 medium was further modified by enriching it with nitrogen to optimize *M. aeruginosa* growth (Nam and Park, 2007).

Co-existence Experiment with *Myriophyllum spicatum* and *Microcystis aeruginosa*

The experiment comprised four treatments: 1) *Myriophyllum spicatum* in an L16 medium without *Microcystis aeruginosa* (Plant-Only, $n=5$; PO#1, PO#2, PO#3, PO#4, and PO#5); 2) *Myriophyllum spicatum* in an L16 medium with *Microcystis aeruginosa* (Plant–Cyanobacteria, $n=5$; PCB#1, PCB#2, PCB#3, PCB#4, and PCB#5); 3) an L16 medium with *M. aeruginosa* alone (Cyanobacteria-Only, n=3; CBO#1, CBO#2, and CBO#3); and 4) Control, treatment with an L16 medium lacking both *Myriophyllum spicatum* and *Microcystis aeruginosa* ($n=3$; C#1, C#2, C#3) (Figure 1). All tests were conducted in 500 ml glass bottles. Each day, we provided fresh L16 media to all treatments and fresh *M. aeruginosa* to the PCB test to ensure an adequate supply of nutrients and a carbon source. Plants were grown under six 20 Watt cool white fluorescent lamps, with a light intensity maintained at 74.8 ± 1.0 μE m^{-2} sec^{-1}, as measured with a quantum sensor (Licor LI-192). However, for the PO treatment, that intensity was reduced to compensate for light attenuation due to the presence of *M. aeruginosa*. Moreover, we added fresh *M. aeruginosa* at the same concentration (5.38 mg Carbon L^{-1}), which was adjusted by measuring the absorbance of the cyanobacterium at 800 nm to keep a constant light attenuation on each test date. That high concentration was chosen so that we could provide for better

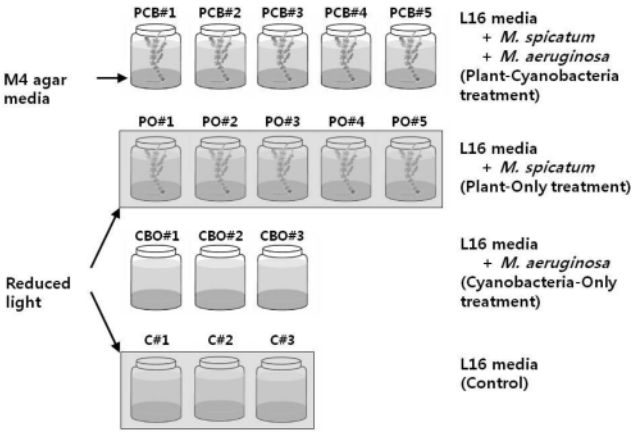

Figure 1. Design of our co-existence experiment.

induction of plant metabolites. The co-existence experiment was conducted for 7 d at 22°C under a 13-h photoperiod. Before and after the trials, selected plants were harvested, then washed with distilled water and transferred immediately to test tubes (pre-washed with acetone) at -20°C prior to the metabolite analysis. Storage periods were no longer than 3 months.

To determine chlorophyll *a* concentrations, we filtered 20 to 30 mL of sample water through 47 mm GF/C glass microfiber filters (Whatman International Ltd., England). Afterward, the filters were refrigerated at –20° in film canisters prewashed with 90% acetone. Chlorophyll *a* was measured using a fluorometer (Turner Designs, Trilogy™) according to the EPA Method 445.0, but without the acidification step (Turner Designs, 2006).

Plant Extraction and Metabolite Analysis Using High-resolution Fourier Transform-ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS)

The protocol of Lisec et al. (2006) was followed to extract metabolites from 3 intact plants of *Myriophyllum spicatum* collected before the co-existence experiment began and from 10 plants harvested afterward.

Extracts were diluted 100-fold with a water:methanol solution (1:1, v:v). Prepared samples were then analyzed by negative ion mode electrospray ionization (ESI), with an automated chip-based nano electrospray system (Triversa Nanomate; Advion BioSciences). This system comprised a 96-well plate, a rack of 96 conducting pipette tips, and chip spray needles. Crossover contamination was avoided by using a new tip and spray needle for each sample. The electrospray process utilized a –1.4 ~ –1.6 kV spray voltage and about 0.5 psi of nitrogen pressure. ESI is known as 'soft' ionization; fragmentation of analyte molecules is considered nonexistent (Sleighter and Hatcher, 2007). The ionized samples were analyzed by state-of-the-art KBSI 15T FT-ICR MS, which has the largest magnet in the world, thereby enabling one to determine the m/z of analyzed ions with sub-ppm accuracy. Individual spectra for these extracts were studied to yield 2M word time-domain data. Those data sets were co-added (100 acquisitions) and then Hanning-apodized, followed by a single zero-fill before rapid Fourier transformation and magnitude calculation. Each frequency was converted to a mass-to-charge ratio via quadrupolar electric trapping potential approximation (Ledford et al., 1984).

Identification of Changed Metabolites Using Principal Component Analysis (PCA) and a Natural Product Database

Thirteen m/z peak profiles from FT-ICR MS were compiled and manually aligned to produce large data matrices (13 observations x 479 peak variables). To avoid the variability of primary metabolites that would blur their induced changes, we systematically removed peaks responsible for primary metabolites that occur in most profiles. Finally, we decided to remove those peaks that occurred in more than 10 profiles from the original data matrix. Peak intensity data were centered but not normalized. All statistical analyses, including PCA, were performed with S-Plus 6 for Windows

(Insightful Corp., USA). From our PCA analysis, we chose mass peaks with high loading values on their principle component axis, thereby separating the scores for Plant-Only and Plant–Cyanobacteria treatments. Because FT-ICR MS provides chemical formulae directly, we used them to search for matches of candidate substances from the Dictionary of Natural Products database (http://www.chemnetbase.com/).

RESULTS

Concentrations of *Microcystis aeruginosa* did not change much until Day 5 of the co-existence experiment. On Day 6, *M. aeruginosa* in PCB#1, PCB#2, and PCB#5 became transparent while *M. aeruginosa* in PCB#3 and PCB#4 maintained color. On Day 7, *M. aeruginosa* in PCB#1, PCB#2, PCB#4, and PCB#5 became transparent while color was maintained in PCB#3.

Chlorophyll *a* analysis confirmed our observations (Figure 2). Initial concentrations ranged from 150 to 250 μg L^{-1} until the fourth day, when they began to decline in all experimental units within the PCB treatment. Chlorophyll *a* concentrations dropped below 30 μg L^{-1} in PCB#1, PCB#2, and PCB#5 on Day 6 and in PCB#4 on Day 7. The concentration in PCB#3 also decreased to less than 100 μg L^{-1} on Day 7.

FT-ICR MS profiles showed clear differences among the initial plants, plants in the PO treatment, and those in the PCB treatment (Figure 3). Generally, PO extracts had fewer peaks than from the other treatments. The first and second principal components explained 27.8% and 21.6% (total 49.4%), respectively, of all variability in the MS profiles. Scores from our principal component analysis of those profiles produced a congruent pattern, with the concentration of chlorophyll *a* changing because of the co-existence experiment. Although the #4 units in the PO treatment appeared to be outliers (Figure 4), all other scores from the initial, PO, and PCB plants were well-aggregated according to treatment groups. Among those from the PCB trial, scores for PCB#3 were closest to values from the PO treatment. On the first principal component axis, PCB treatment showed lower scores than either PO treatment or initial plants while, on the second principal component axis, PCB treatment had higher scores than the other two. In all, 282.06981, 283.06837, 317.05484, and 281.06199 [M-H^+] were the most negative loadings on the first axis but the most positive on the second (Figure 5).

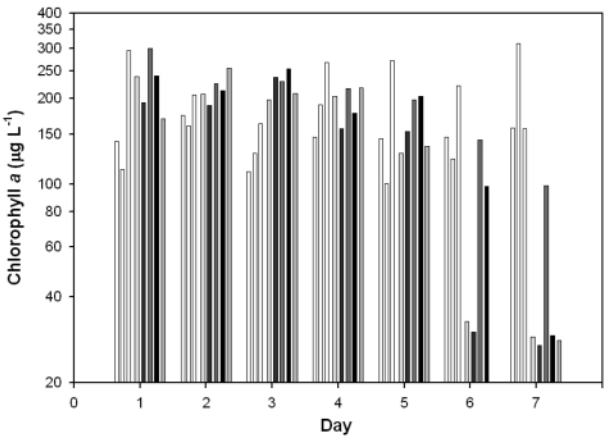

Figure 2. *Microcystis aeruginosa* biomass changes in terms of chlorophyll *a* concentration by Cyanobacteria-Only (CBO) treatment (open bars) or Plant–Cyanobacteria (PCB) treatment (black bars). From left to right, open bars represent CBO#1 to CBO#3; black bars, PCB#1 to PCB#5.

Using the formulae provided from FT-ICR MS, we searched the candidate substances responsible for reducing *M. aeruginosa* in this co-existence experiment. Unfortunately, we identified only one formula that matched with those included in the Dictionary of Natural Products: $C_{10}H_{12}N_4O_6$ (283.06837 [M-H^+]) for either oxanosine or xanthosine (Table 1). We checked the area of that peak and found that its pattern matched well with the *M. aeruginosa* clearance pattern in our co-existence experiment (Figure 6). Among other selected m/z peaks, only 282.06981 [M-H^+] showed a similar matched pattern with the *M. aeruginosa* clearance pattern (data not shown).

DISCUSSION

Our results showed that the biomass of *Microcystis aeruginosa* was reduced when co-existing with *Myriophyllum spicatum*, as manifested by changes in metabolite profiles. Subsequent analysis suggested that several substances, including oxanosine or xanthosine, are candidates for the allelochemical substances that are induced during such co-existence.

We attempted to alleviate confounding factors, e.g., the presence of other organisms or competition for light and nutrients (Gross et al., 2007). For example, the *M. spicatum* tissues were washed with ethanol to remove potential impacts of other epiphytic algae or bacteria before the experiments began. To control the lighting environment, intensities were reduced during the Plant-Only treatment (Figure 1). Furthermore, fresh L16 media and *Microcystis aeruginosa* were supplied each day to avoid any limitations on nutrients for the cyanobacterial cells. Likewise, the M4 medium in our agar plates provided nutrients for maintaining *Myriophyllum spicatum*. In fact, those plants grew as well in the Plant–Cyanobacteria treatment as did those in the Plant-Only treatment over the test period (data not shown). Therefore, we might conclude that the clearance of *Microcystis aeruginosa* was mainly due to the presence of *Myriophyllum spicatum*.

Multivariate analyses, such as PCA, have been widely used for analyzing chromatograms. These include terminal restriction fragment length polymorphism (T-RFLP) (Park et al., 2006), fatty acid biomarker research (Ju et al., 2004), metabolic fingerprinting with NMR (Kim et al., 2006), and MS m/z spectrum analysis in metabolomics (Xie et al., 2008). In a usual metabolomics approach, the high dimensionality of chromatograms is reduced to a few important principal components in score plots, and researchers look for peaks with high loading values for those components (Roessner et al., 2001; Allwood et al., 2006; Xie et al.,

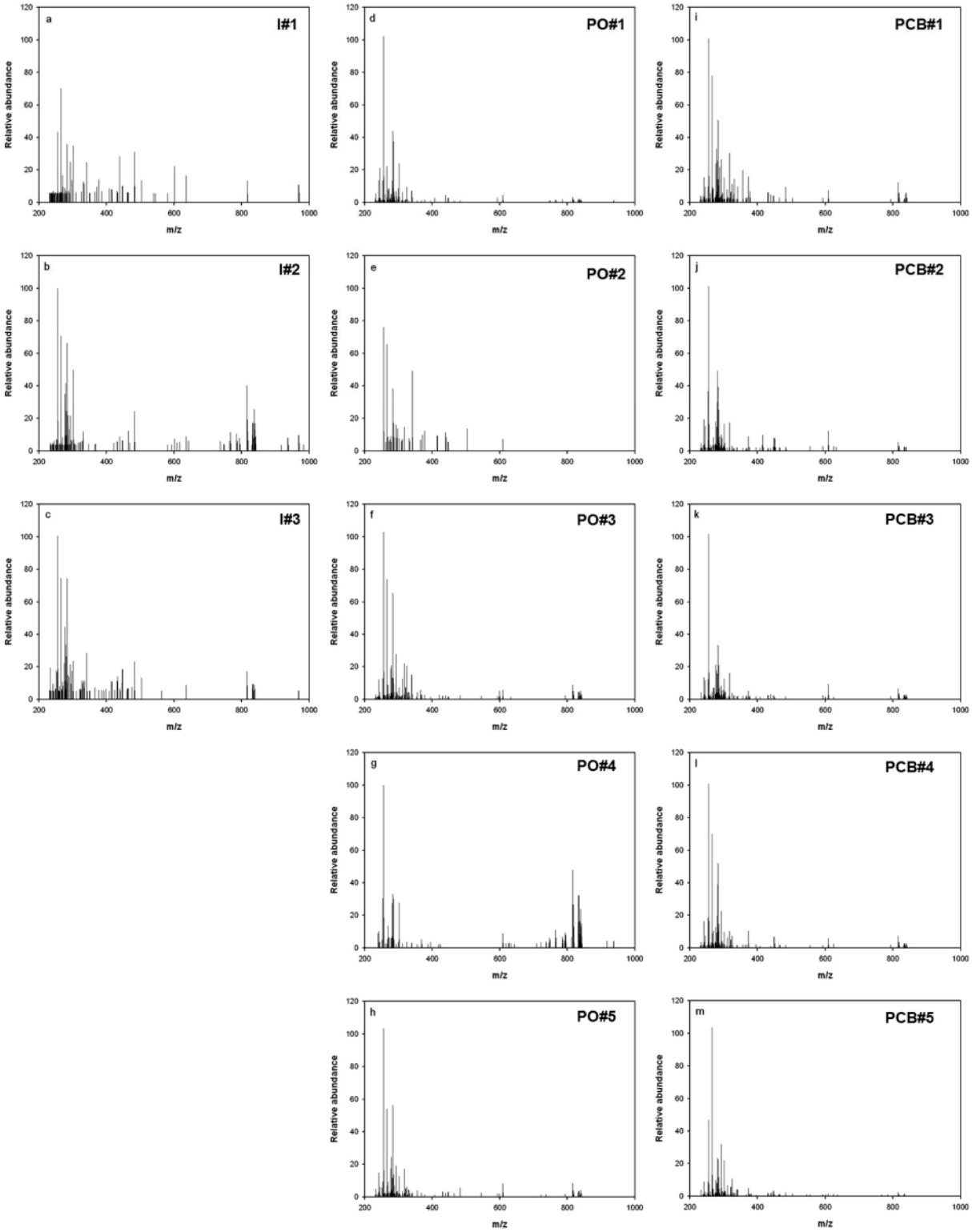

Figure 3. FT-ICR MS m/z profiles: a – c, initial plants (I); d – h, Plant-Only (PO) treatment; and i –m, Plant–Cyanobacteria (PCB) treatment. Replication numbers are indicated by # and numerals.

2008). In our present study, the loading plot in PCA (Figure 5) screened more than 100 peaks to select important peaks matching the observed *Microcystis aeruginosa* clearance pattern (Table 1, Figure 2). However, we are still cautious in using loading values because such an analysis is biased for changes in major peaks with wider areas on the chromatograms. Those major peaks are thought to represent primary metabolites. Therefore, we must develop methods to find changes in smaller peaks from secondary metabolites, especially when searching for specific allelochemical substances.

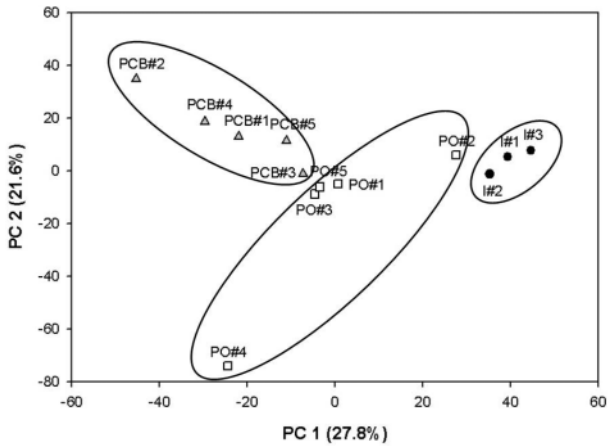

Figure 4. PCA scores for FT-ICR MS m/z profiles for initial plants (I), Plant-Only (PO) treatment, and Plant–Cyanobacteria (PCB) treatment. Replication numbers are indicated by # and numerals.

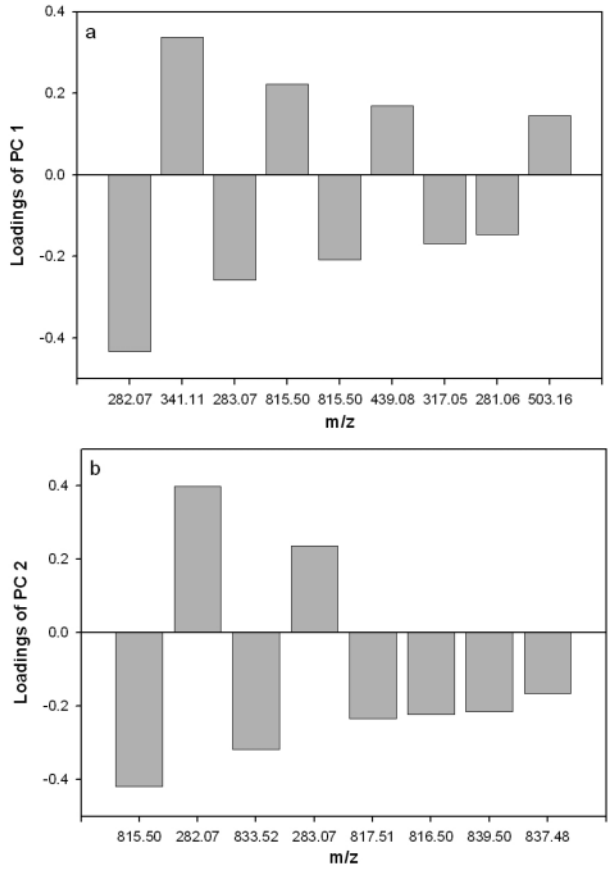

Figure 5. Loading values for first principal component (a) and second principal component (b).

Although loading values from our PCA analysis suggested several molecular formulae (Figure 5), we were able to identify only one (Table 1). Considering the high accuracy of FT-ICR MS, it is possible that those unidentified formulae represent unknown secondary metabolites induced in *Myriophyllum spicatum*. We cannot exclude the possibility that one or some of these are potential allelochemical substances, but this will require further investigation.

The peak for $C_{10}H_{12}N_4O_6$ (m/z = 283.06837 [M-H$^+$]) was identified as either oxanosine or xanthosine. The former was first isolated as a nucleoside antibiotic from *Streptomyces capreolus* (Shimada et al., 1981). It also blocks the growth of *HeLa* cells *in vitro* (Majumdar et al., 2005) as well as gram-negative bacteria (Kato et al., 1984). Because cyanobacteria are also gram-negative, we can assume that oxanosine inhibits the growth of *Microcystis aeruginosa*. However, we were unable to find any literature indicating plant production of oxanosine, which means that this potential inhibitory property of oxanosine needs more examination. In plants, xanthosine is the first intermediate metabolite in the caffeine biosynthesis pathway (Ashihara and Crozier, 2001). Because caffeine is considered an allelopathic substance that prohibits seed germination (Harborne, 1993), it might also inhibit the growth of *M. aeruginosa*. Further studies will investigate the possibilities that oxanosine and xanthosine are allelopaths that suppress such development.

In conclusion, our co-existence experiment demonstrated the clearance of *M. aeruginosa* as well as the induction of metabolite changes in *Myriophyllum spicatum*. Although we

Table 1. Selected peaks based on signs and magnitude of loading values. FT-ICR MS provided a unique molecular formula for each m/z value, except that of 281.06199 m/z, which was not assigned by the machine. Candidate substances are listed according to the Dictionary of Natural Products database

Peaks [M-H$^+$]	Candidate molecular formula [M]	Candidate substances
281.06199	-	
282.06981	$C_7H_{17}N_5O_3S_2$	Not found in Dictionary
283.06837	$C_{10}H_{12}N_4O_6$	Oxanosine
		Xanthosine
317.05484	$C_9H_{18}O_{10}S_1$	Not found in Dictionary
373.34775	$C_{26}H_{46}O_1$	Not found in Dictionary
609.14671	$C_{41}H_{26}N_2S_2$	Not found in Dictionary
815.49919	$C_{43}H_{76}O_{12}S_1$	Not found in Dictionary
817.51488	$C_{44}H_{74}N_4O_8S_1$	Not found in Dictionary

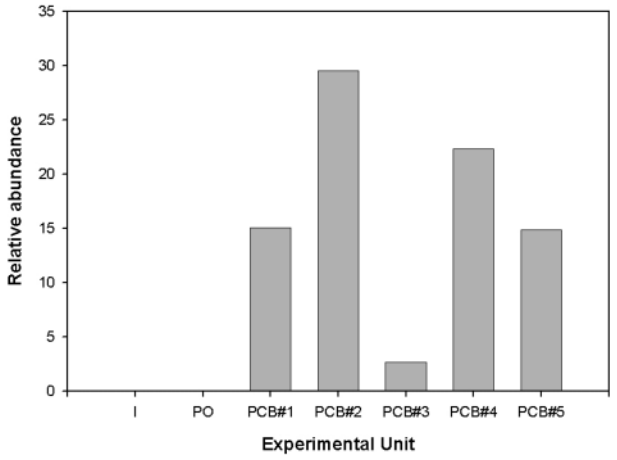

Figure 6. Relative abundance in terms of peak areas for m/z = 283.06837 [M-H$^+$] from initial plants (I), Plant-Only (PO) treatment, and Plant–Cyanobacteria (PCB) treatment. Replication numbers are indicated by # and numerals. Three replicates of (I) and (PO) showed no relative abundance for that peak.

performed PCA and a database search to match possible candidate formulae for allelochemical substances, including oxanosine and xanthosine, we need traditional fractionation and bioassay evidence plus information on accurate mass and formulae. For the mass induction of allelochemical substances, as part of such a fractionation and bioassay approach, we must conduct outdoor co-existence experiments on a much larger scale.

ACKNOWLEDGEMENT

This work was supported by a Korea Science and Engineering Foundation (KOSEF) grant funded by the Ministry of Science and Technology of the South Korea government (MOST) (No. R01-2006-000-11096-0), and by Intramural Research Funds by Ajou University given in 2005. We are grateful for two anonymous reviewers for improvements of an earlier manuscript.

Received July 1, 2008; accepted July 17, 2008.

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