Chiang Mai J. Sci. 2014; 41(5.1) : 1150-1156
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Inhibition of Seedling Growth in Giant Mimosa and Reduction of Mitotic Activity in Onion Root Tips Caused by Cyanobacterial Extract

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Received: 20 March 2012
Accepted: 27 November 2012

ABSTRACT

Phytotoxicities of crude extracts from four cyanobacteria, *Nostoc* sp., *Scytonema* sp., *Lyngbya* sp.1, and *Lyngbya* sp.2, were investigated with seedling growth bioassays. The results show all extracts did not affect seed germination, but caused root and shoot growth suppression. The extract from *Nostoc* sp. was found to inhibit root and shoot growth with the lowest half-maximum inhibitory concentration. Only *Nostoc* sp. extract caused obvious root crack formation when exposed to high concentrations of the extract. The extract also inhibited root cell division by the reduction of the mitotic index and the number of cells in all mitotic phases. Crude extract from *Nostoc* sp. is the most effective in root growth inhibition and root cell damage. Decrease of root length is probably caused by the inhibition of cell division which blocks the cell mitosis.

Keywords: cyanobacterial extract, *Nostoc*, *Scytonema*, *Lyngbya*, root growth inhibition, mitotic index

1. INTRODUCTION

Cyanobacteria (prokaryotic blue-green algae) are known to produce various kinds of secondary metabolites that can affect many biochemical processes in cells. These secondary metabolites are sometimes called allelochemicals because they can influence the growth of surrounding organisms [1-2]. There are some reports about a wide range of allelochemicals and toxins from cyanobacteria that have an inhibitory effect on the growth of many organisms such as bacteria, microalgae, fungi, invertebrates, and some plants [2]. For example, *Nostoc spongiaeforme* TISTR 8169 has an inhibitory activity on the root elongation of barnyard grass (*Echinochloa crus-galli* (L.) P. Beauv.) [3]. The metabolite from *Scytonema hofmanni* can cause root damage in *Lemna* species (Lemnaceae) [4]. Entzeroth *et al.* [5] found that bioactive compounds from *Lyngbya aestuarii* are able to inhibit the growth of *Lemna minor* L. The cyanobacterium *Microcystis aeruginosa* can produce microcystin that causes the accumulation of reactive oxygen species (ROS), well-recognized triggers of cell death [6-7], in tobacco cell suspension [8] and reduces growth and chlorophyll content in the aquatic plant *Lemna gibba* L. [9]. These findings indicate the potency of cyanobacterial extracts which suppress vital physiological processes in some plants. Due to its efficiency, cyanobacterial

extracts can be used as bio-control agents to decrease the use of chemical herbicides which are toxic to users, consumers, and environment for decades. In this experiment, a noxious giant mimosa (*Mimosa pigra* L.) was used as a test plant because the seeds are homogeneously in germination and it is an invasive weed in Thailand. For the effect on root cell division, we used onion (*Allium cepa* L.) which is widely used for the study on mitosis because it is readily available and the chromosomes are large and few in number.

There is a need to identify natural pesticides for application in agriculture. Extracts from cyanobacteria are an option for development as a bioherbicide. Studies of bioactive compounds from cyanobacteria and modes of action of cyanobacterial extracts are few. The objectives of this study are to compare the phytotoxic effects of the extracts from cyanobacteria, viz., *Nostoc* sp., *Scytonema* sp., *Lyngbya* sp.1, and *Lyngbya* sp.2 on giant mimosa seedling growth and to determine effects on root cell division by cyanobacterial species which had the highest inhibitory effect on seedling growth from the first experiment.

2. MATERIALS AND METHODS

2.1 Cyanobacteria Culture and Extraction

The cyanobacteria, *Nostoc* sp., *Scytonema* sp., *Lyngbya* sp.1, and *Lyngbya* sp.2, were collected in December 2009 and isolated with the application of the streak plate method. Their genera were identified according to Desikachary [10]. Each isolated cyanobacterium was cultivated in BG-11 liquid media [11], pH 6.5, 8.5, 6.5, and 9.5, respectively (the optimal pH for each species) under daylight fluorescent lamps (300 μmol $m^{-2}s^{-1}$) at ambient 9.5, respectively (the optimal pH for each species) under daylight fluorescent lamps (300 μmol $m^{-2}s^{-1}$) at ambient temperature (32 ± 3 °c). The cells in the exponential phase of growth (15, 12, 19, and 25 days after cultivation, respectively) were harvested by means of filtration and subsequently dried at 50 °C for 72 hours. The dried cells were ground to a powder and extracted with 80% methanol for 24 hours at ambient temperature (32 ± 3 °c). The solution was centrifuged at 3000 rpm for 15 minutes and the supernatant was evaporated in a rotary evaporator to obtain a crude brown gum. The residue was extracted twice and the supernatant was combined with the first one.

2.2 Seedling Growth Bioassay

Each gummy extract was dissolved in aqueous of 0.1% dimethyl sulfoxide (DMSO) in concentrations of 0, 0.1, 0.2, 0.3, 0.4, and 0.5%. The solutions of these concentrations (1.5 ml) were added to Petri dishes (5 cm diameter) containing filter paper. Six seeds of *Mimosa pigra* L. were placed on the filter paper and kept in the dark at ambient temperature (32 ± 3 °c). The solution of 0.1% DMSO was served as an untreated control. Seed germination, root, and shoot (hypocotyl) lengths were measured after three days of exposure to the extracts. Consequently, the half maximal inhibitory concentration (IC_{50}) of each cyanobacterial crude extract was calculated.

2.3 Analysis of Mitotic Index

Mitotic index (MI) was investigated by the modified Armbruster *et al.* [12] method. Equal-sized onion bulbs were allowed to produce roots in distilled water for 24 hours prior to their placement in glass bottles which contained the extracts of *Nostoc* sp. at concentrations of 0, 0.1, 0.3, and 0.5% for 72 hours. The roots were cut, fixed in an absolute ethanol: glacial acetic acid solution (3: 1 v/v) for 24 hours, and transferred to 70% ethanol for storage until the mitotic index determination was performed. For microscopic analysis, the root tips were placed in distilled water for 20 minutes to remove the ethanol and subsequently hydrolyzed in 5 N HCl for 5 minutes.

A 1-mm long tip was squashed into separated cells in a drop of aceto-orcein for 10 minutes. A cover slip was immediately placed over the preparation and the slides were heated for a few minutes. The slides were assessed for their mitotic index (percentage of mitotic cells per total cells (2500 cells/slide). The means were obtained from cell counts from three slides.

2.4 Statistical Analyses

The data was presented as the means ± standard deviation (SD) of three replicates. The relationships between the extract concentration and the root or shoot length were demonstrated by regression analysis. The percentages of dividing cells in each concentration were compared by ANOVA test at the 0.05 level and comparisons of means with Duncan's multiple range test.

3. RESULTS AND DISCUSSION

3.1 Effects of Cyanobacterial Extracts on Seedling Growth

The methanolic extracts from all four cyanobacterial species had no activity on seed germination (data not shown). Distinct phytotoxic effects on seedling growth, a reduction of both the shoot and root lengths, were detected. Both the shoot and root lengths were found to be approximately five and two cm, respectively in the controls. *Nostoc* sp. extract caused rapid inhibition of seedling growth in the concentration range 0.1-0.3% and was relatively constant in root and shoot length at 0.3-0.5%. At the highest concentration, 0.5%, both shoot and root lengths were dramatically reduced to 1.39 and 0.19 cm, respectively (Figures 1 and 2a). The extract

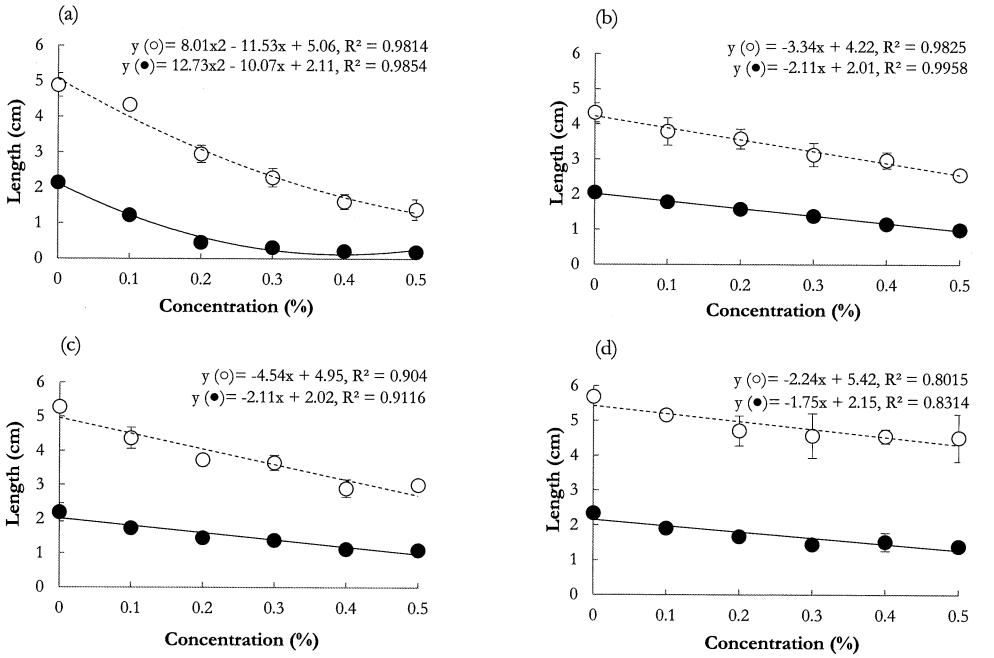

Figure 1. Effects of crude extracts of four cyanobacteria on root (●) and shoot (○) lengths of giant mimosa seedlings. Each bar represents standard deviation (SD) of the mean. IC_{50} values of each cyanobacteria were established as follows: (a) *Nostoc* sp.: 0.12% (root), 0.27% (shoot); (b) *Scytonema* sp.: 0.48% (root), 0.63% (shoot); (c) *Lyngbya* sp.1: 0.48% (root), 0.55% (shoot); (d) *Lyngbya* sp.2: 0.61% (root), 1.21% (shoot).

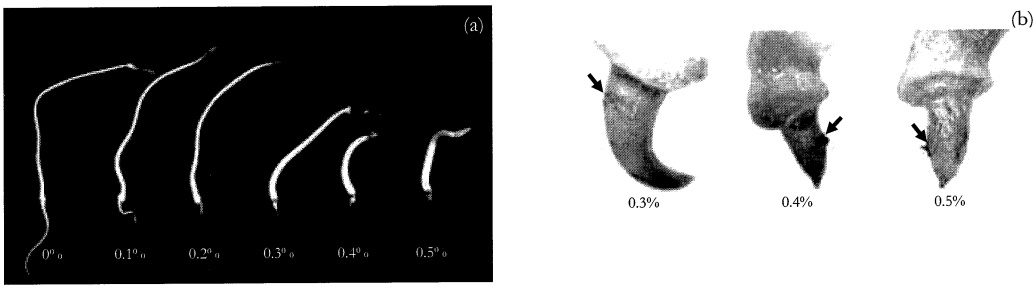

Figure 2. Effects of crude extracts of *Nostoc* sp. on giant mimosa seedling growth (a) and root cell damage (b); arrows indicate root cracking subsequent to exposure to high concentrations of the extracts for three days.

from *Scytonema* sp. had a moderate inhibitory effect comparable to those of the extract of *Lyngbya* sp.1. The *Lyngbya* sp.2 extract had a weak effect on seedling growth. As shown in Figure 1, the extracts of *Nostoc* sp., *Scytonema* sp., *Lyngbya* sp.1, and *Lyngbya* sp.2 inhibited root growth with IC_{50} at 0.12, 0.48, 0.48, and 0.61%; while the IC_{50} of shoot inhibition were at 0.27, 0.63, 0.55, and 1.21%, respectively. Based on this variable, the following order of potency was noted: *Nostoc* sp. > *Lyngbya* sp.1 > *Scytonema* sp. > *Lyngbya* sp.2. These results indicate that all four species share a common effect on giant mimosa seedling growth inhibition with different efficacies. These results correlate with previous findings of *Nostoc spongiaeforme* TISTR 8169 which was had an inhibitory activity on root elongation of barnyard grass. [3]. Gleason and Case [4] reported that the metabolite, cyanobacterin, from *Scytonema hofmanni* caused root damage in *Lemna* species. As for *Lyngbya*, Entzeroth *et al.* [5] found that 2, 5-dimethyldodecanoic acid of *Lyngbya aestuarii* is capable of inhibiting the growth of *Lemna minor* L. The effectiveness of the *Nostoc* sp. extract caused shortening, browning, and cracking of roots (Figures 2a-b). Cracking was also reported in *Pisum sativum* L. cv. Alaska roots subsequent to treatment with aluminum [13] and *Zea mays* L. roots treated with coumarin [14]. The cracks are caused by the inhibition of surface cell expansion while the internal cells expand normally. These cracks lead to the loss of the plasma membrane integrity in roots.

Other reports on the effects of cyanobacterial extracts on root growth inhibition suggest the inhibition of protein phosphatase 1 and 2A activity [15-16]. Protein phosphatases have been reported to be correlated with the regulation of auxin transport in *Arabidopsis thaliana* (L.) Heynh. and organization of microtubules [17-18].

3.2 Effects of *Nostoc* sp. Extract on Cell Division

The effects of the *Nostoc* sp. extract on cell division were further investigated because of having the most effective activity on root growth inhibition. This experiment was performed on onion root tips because of the large chromosomes and few in number. The results show that the mitotic index decreases subsequent to the exposure of the roots to elevated concentrations of the extract, especially at 0.3%. The mitotic index was found to be reduced to 0.1% and was significantly dissimilar to that of the control (Table 1 and Figure 3). This shows the ability of the extract to inhibit cell division in onion root tips. Sanevas *et al.* [19] showed that the crude extract of the cyanobacterium, *Hapalosiphon* sp., can reduce the mitotic index of onion and cause root growth inhibition. Smith *et al.* [24] noted that cryptophycin from *Nostoc* sp. GSV224 inhibits

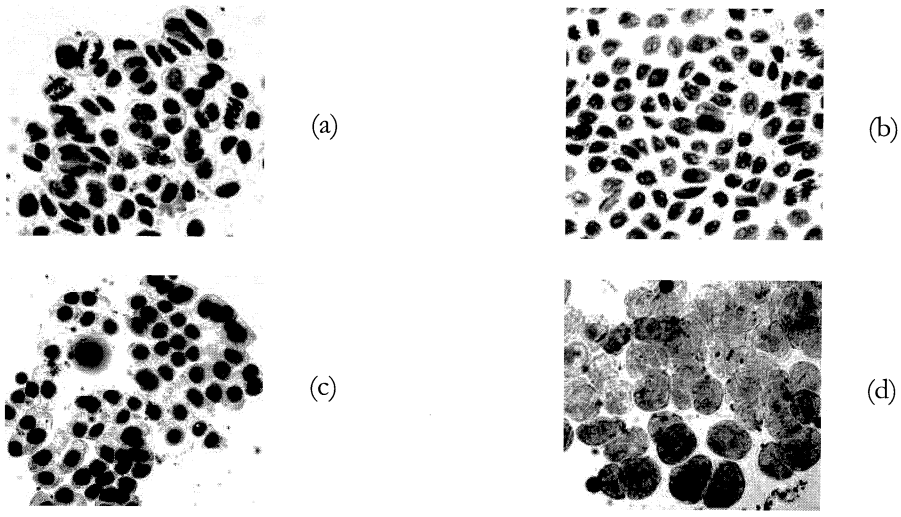

Figure 3. Photographs of cell division in onion root tips after exposure to *Nostoc* sp. extract at different concentrations. (a) control (b) 0.1% (c) 0.3% and (d) 0.5%

leukemia cell proliferation by the depletion of microtubules in cells.

The number of dividing cells in prophase, metaphase, anaphase, and telophase decreased remarkably when the roots were exposed to the extract at higher concentrations (Table 1). No chromosome aberration was detected in those samples. These results indicate that the extract causes an arrest of cells in interphase and an inhibition of cells which enter mitosis. Some researchers have reported that under stress conditions, redox sensing is potentially the key to control the cell cycle progression. Reactive oxygen species are the second messengers in the activation of resistant genes which block the cell cycle from G1 to S, retard DNA replication, and delay the start of mitosis [20-21]. Each phase of the cell mitosis is initiated when the previous phase is complete. There are 2 major checkpoints, the DNA structure and the spindle assembly, which control cell cycle progression [22]. In the study by Sánchez-Moreiras *et al.* [23] on the effects of natural compounds, benzoxazolin-2(3H)-one (BOA), on lettuce (*Lactuca sativa* L.) root meristem, flow cytometry analyses and mitotic index revealed

Table 1. Effects of crude extracts of *Nostoc* sp. on the mitotic index and number of cells in each mitotic phase of onion root tips after three days of exposure.

Extract concentration (%)	Dividing cells (%)				Mitotic index (%)
	Prophase	Metaphase	Anaphase	Telophase	
0	1.63 ± 0.38 a	0.78 ± 0.42 a	0.40 ± 0.08 a	0.53 ± 0.30 a	3.29 ± 0.95 a
0.1	1.39 ± 0.19 a	0.53 ± 0.16 a	0.24 ± 0.04 b	0.33 ± 0.14 ab	2.49 ± 0.24 a
0.3	0.10 ± 0.04 b	0.00 ± 0.00 b	0.00 ± 0.00 c	0.00 ± 0.00 b	0.10 ± 0.04 b
0.5	NDC	NDC	NDC	NDC	NDC

Note: Identical letters in each column are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test. NDC–no dividing cells.

retarding of the cell cycle in a BOA-treated meristem at the G2/M checkpoint. A similar phenomenon was reported for ambiguine D isonitrile, phytotoxic compound from *Hapalosiphon* sp., which inhibited lettuce growth caused by ROS-induced lipid peroxidation and subsequently by mitosis inhibition [25]. It is possibly that the extract causes stress conditions and inhibits the particular state in interphase prior to mitosis.

4. CONCLUSIONS

In the search of natural compounds, root growth can be used as a bioassay guide for the isolation of the phytotoxins. From the experiments described herein, the extract from *Nostoc* sp. was found to be the most effective on inhibiting seedling growth which caused by root cell damage and suppression of root cell division. Although there are some reports about the effects of cyanobacterial extract to inhibit the growth of some plants, this is the first report that *Nostoc* sp. extract can inhibit root and shoot growth of giant mimosa. The search for bioactive compounds from this species and their modes of action should be continued.

ACKNOWLEDGEMENTS

The authors are grateful to The Faculty of Science, Kasetsart University and Kasetsart University Research and Development Institute (KURDI) for their financial supports.

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