International Journal of GEOMATE, Feb., 2017, Vol. 12, Issue 30, pp. 90 - 95
Special Issue on Science, Engineering & Environment, ISSN:2186-2990, Japan
DOI: http://dx.doi.org/10.21660/2017.30.2580

GROWTH OF THE EDIBLE MICROALGA *ARTHROSPIRA PLATENSIS* IN RELATION TO BORON SUPPLY

Shotaro Tadama[1] and *Hideaki Shiraishi[1]

[1]Graduate School of Biostudies, Kyoto University, Japan

*Corresponding Author, Received: 31 May 2016, Revised: 29 Nov. 2016, Accepted: 19 Dec, 2016

ABSTRACT: *Arthrospira* (*Spirulina*) *platensis* is an edible cyanobacterium that has been consumed worldwide as a nutrient source under the name spirulina. When preparing synthetic media for this microalga, boron is usually added to them. However, whereas boron is necessary for the N_2-fixation-dependent growth of heterocystous cyanobacteria, boron requirement by *A. platensis*, which is non-heterocystous, has not yet been carefully examined. To examine the effect of boron on *A. platensis*, we prepared a boron-depleted medium in which borate concentration was below a detection limit (0.2 μM), as determined by the spectrophotometric quantitation with H-resorcinol. Using this boron-depleted medium, *A. platensis* NIES-39 was analyzed for changes in growth, dry biomass weight and the protein, chlorophyll *a* and C-phycocyanin contents. Experimental results showed that removal of boron from the growth medium had no detectable effect on them. A control experiment with a heterocystous cyanobacterium *Anabaena* sp. UTEX 2576 showed that the growth of this cyanobacterium was suppressed in a boron-depleted medium. These experiments demonstrated that boron was not required for the growth of *A. platensis*. Our results indicate that boron supply is not necessary for the propagation of *A. platensis* even in the regions where boron deficiency in the soil deteriorates the growth of common crops. Use of boron-free media would also be helpful to improve the reproducibility of sensitive physiological experiments such as metabolome analysis, because precipitation of the insoluble salt manganese borate, which may reduce the concentration of Mn^{2+} in the medium, does not occur in boron-free media.

Keywords: Cyanobacteria, Spirulina, Arthrospira platensis, Boron, H-resorcinol

1. INTRODUCTION

Arthrospira platensis is an edible alkalophilic cyanobacterium that has been consumed worldwide as a nutrient source and food additives [1][2]. Phycobiliproteins from this cyanobacterium are also widely used as natural colorants for foods and cosmetics [2]. Products of *A. platensis* and a closely related species *A. maxima* are usually marketed under the name spirulina, because they had formerly been classified in the genus *Spirulina*.

In the artificial propagation of *A. platensis*, boric acid is usually added to its growth medium [3], as it is well known that boron is required for the growth of many photosynthetic organisms. For example, higher plants require boron for the formation and maintenance of cell wall structures [4]. Therefore, deficiency of boron in the soil causes the development of many symptoms in higher plants [4][5]. Boron is also required for the maintenance of the integrity of heterocyst cells that perform N_2 fixation in heterocystous filamentous cyanobacteria [6][7]. Boron is involved in stabilizing the heterocyst envelope in these cyanobacteria [8]. However, in contrast to higher plants and heterocystous cyanobacteria, many non-heterocystous cyanobacteria are tolerant to boron deficiency [7][9].

Since *A. platensis* is also non-heterocystous, it is possible that boron is not necessary for the growth of this cyanobacterium. However, boron requirement by *A. platensis* has not yet been carefully examined. Knowledge about the boron requirement would be helpful for the agriculture of *A. platensis*, especially in the region where boron deficiency in the soil deteriorate healthy growth of many common crops [5].

Knowledge on the boron requirement by *A. platensis* is also helpful for basic studies of this cyanobacterium. In laboratory-scale experiments, inclusion of boric acid in the growth medium results in the gradual precipitation of the insoluble salt manganese borate [10], possibly causing the change in the Mn^{2+} concentration and gradual depletion of Mn^{2+} in the growth medium. This may reduce the reproducibility of sensitive physiological experiments, *e.g.*, metabolome analysis, because manganese is an essential component of the oxygen-evolving complex in photosystem II [11], as well as many cellular enzymes (*e.g.*, xylose isomerase, glutamine synthetase and phosphoglycerate mutase [12]), and therefore alterations in the manganese concentration may affect cellular physiology. If boron is determined to be dispensable for the growth of *A. platensis*, boric acid can be omitted

from the growth medium to improve the reproducibility of sensitive physiological experiments.

In this study, we prepared a boron-depleted medium, and effects of boron on the growth and some biochemical properties of *A. platensis* were examined to determine whether boron depletion had any effect on this cyanobacterium.

2. MATERIALS AND METHODS

2.1 Reagents

H-resorcinol, or 1-(2,4-Dihydroxy-1-phenyl-azo)-8-hydroxynaphthalene-3,6-disulphonic acid disodium salt, was purchased from Tokyo Chemical Industry Co. (Tokyo). Other chemicals were purchased from Nakalai Tesque (Kyoto) and Wako Pure Chemical Industries (Osaka). All chemicals were reagent grade or better. Water was purified with the Milli-Q Advantage A10 Ultrapure Water Purification System (Merck Millipore, Darmstadt) before use. Polypropylene plasticware was used to prepare reagent solutions, to avoid boron contamination from glassware [13]

2.2 Cyanobacterial strains

A. platensis NIES-39 was obtained from the Microbial Culture Collection at the National Institute for Environmental Studies, Tsukuba (MCC-NIES). *Anabaena* sp. UTEX 2576 was obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX).

2.3 Growth media and growth conditions

SOT medium and boron-depleted SOT medium for *A. platensis* were prepared as described [14][15], except that the macroelement solution used to prepare the latter medium did not contain boric acid. *Anabaena* sp. UTEX 2576 was cultured either in $BG11_0$ medium [16] or in the boron-depleted $BG11_0$ medium that contained no added boric acid. Cyanobacterial cells were cultured in 250-mL polycarbonate Erlenmeyer flasks (Thermo Scientific Nalgene, Waltham) containing 200 mL each of growth medium. Conditions for light and temperature were as described [15][17]. *A. platensis* NIES-39 was pre-cultured in the boron-containing medium, harvested by filtration on nylon mesh as described [17], and washed on it with boron-depleted medium before inoculation into each growth medium. *Anabaena* sp. UTEX 2576 was cultured in the same way except that pre-cultured cells were collected and washed by centrifugation rather than by filtration.

2.4 Quantitation of boron in the medium

To draw standard curves, 900 µL of 0.11 mM H-resorcinol in 0.65 M ammonium acetate-acetic acid buffer (pH 5.5) was mixed with 100 µL of SOT medium containing various concentrations of boric acid. The mixtures were incubated at 25°C with constant shaking at 140 rpm. After 60 h, optical density at the wavelength of 510 nm (OD_{510}) was determined with a spectrophotometer (Novaspec II, Pharmacia-LKB Biotechnology, Uppsala). Samples for quantitation were reacted with H-resorcinol in the same way, and OD_{510} was determined. Means and the standard errors of means were determined from 4 samples.

2.5 Determination of growth curves

Cyanobacterial cells were cultured in four polycarbonate flasks for each set of culture conditions. The means and the standard deviations of the OD_{730} values and the trichome concentrations were determined over time. The numbers of trichomes in cultures were determined as described [18].

2.6 Determination of the amounts of biomass, protein, chlorophyll *a*, and C-phycocyanin

Samples were taken from cultures during the late logarithmic phase when the OD_{730} values of the cultures were approximately 1.0–1.2. Data were obtained from four independent cultures for each set of culture conditions, and the means and the standard deviations were determined.

To determine the amount of biomass, 10 mL of cultures were centrifuged at 4°C for 20 min at 3,000 *g*, and the collected biomass was washed with 1 mL each of distilled water for 5 times. The washed biomass was dried at 85°C for 72 h, cooled down, and weighed.

To determine the amount of proteins, cells in 1 mL of samples were collected by centrifugation as above and washed five times with 1 mL each of 25 mM Tris-HCl (pH 7.4), 138 mM NaCl, 2.68 mM KCl (Tris-buffered saline). Then, they were disrupted by the Mini-Beadbeater-1 (Biospec Products, Bartlesville) using the 0.1 mm diameter glass beads. In the disruption, shaking the samples on the equipment for 30 sec and cooling them on ice for 1 min were repeated 7 times. Protein concentrations in these samples were determined by Bradford's method [19] using Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules). As a standard for protein determination, bovine γ-globulin (Bio-Rad Laboratories) was used.

Concentrations of chlorophyll *a* and C-phycocyanin were determined from the OD_{620} and OD_{678} values of the cultures as described [20].

3. RESULTS

3.1 Quantitation of boron concentration in the growth medium

In the experiments to follow, *A. platensis* was propagated in the medium with no added boric acid. However, it is known that considerable amount of boron is released from borosilicate glass into water when solutions are stored in glassware [13]. Therefore, the medium and all solutions were prepared in plasticware to avoid boron contamination from glassware. Even with this care, it was still possible that reagents used to prepare the medium were contaminated with boron. Therefore, boron concentration in the prepared growth medium was examined before performing the main experiments. To determine boron concentration, a spectrophotometric quantitation method with H-resorcinol was employed. This reagent forms a 1:1 complex with boron under weakly acidic conditions with an optimum at pH 5.5, and the complex can be determined spectrophotometrically at 510 nm [21]–[23].

Results of the boron quantitation in the boron-depleted medium and in the usual medium (SOT medium) are shown in Fig. 1. As shown in the upper left panel, experiments to draw a calibration curve showed that the standard solutions containing as low as 0.2 µM boric acid (12.4 ng mL^{-1}) exhibited significant OD_{510} values, indicating that detection limit for boric acid with this method was lower than 0.2 µM. In contrast,

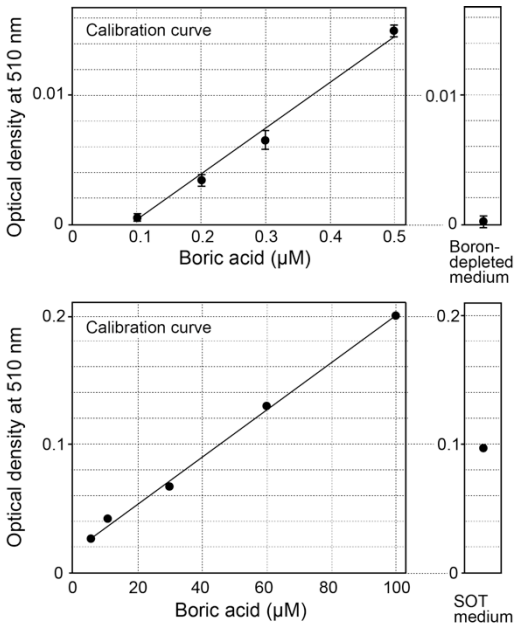

Fig.1 Quantitation of boron concentrations

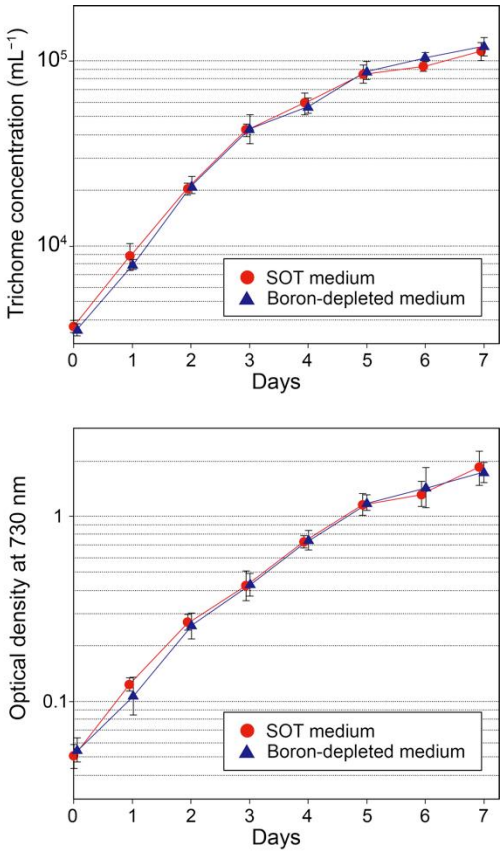

Fig. 2 Growth of *A. platensis* in relation to boron

samples prepared with the boron-depleted medium exhibited hardly detectable absorption at 510 nm (Fig. 1, upper right panel). Therefore it was clear that boric acid concentration in this medium was far lower than 0.2 µM. In this respect, it has been reported that, when water was stored in glass bottles, concentration of boron released from glass into water reached 5.2 µM [13]. Therefore, the boron level in the boron-depleted medium (<0.2 µM) was far lower than the residual level of boron in water stored in glass bottles. Concentration of boric acid in the SOT medium used in this study was also examined and determined to be approximately 45 µM (Fig. 1, lower panels).

3.2 Effect of boron depletion on the growth of *A. platensis* NIES-39 and *Anabaena* sp. UTEX 2576

To examine the effect of boron on the growth of *A. platensis*, *A. platensis* NIES-39 was cultured in the usual SOT medium and in the boron-depleted medium prepared as above. Trichome concentrations and the OD_{730} values were determined over time, and growth curves were drawn. As shown in Fig. 2, there were no significant differences between the growth curves

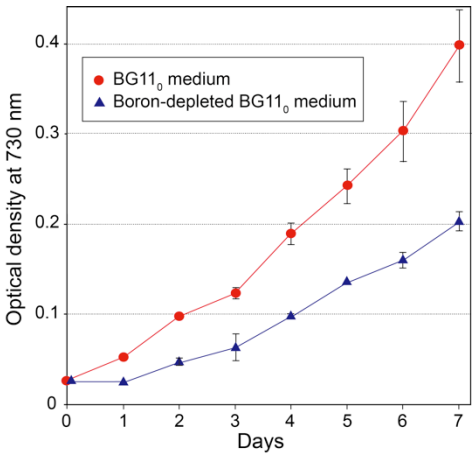

Fig. 3 Growth of *Anabaena* sp. in relation to boron

determined in the presence and absence of boron, indicating that boron depletion had no detectable effect on the growth of *A. platensis* NIES-39.

As a control experiment, a heterocystous cyanobacterium *Anabaena* sp. UTEX 2576 was cultured in the boron-containing medium ($BG11_0$ medium) and in the boron-depleted $BG11_0$ medium. As shown in Fig. 3, growth of this cyanobacterium was suppressed in the boron-depleted medium. Statistical analysis by the Student's *t*-test indicated significant differences ($P<0.05$) between the cultures in the $BG11_0$ medium and in the boron-depleted $BG11_0$ medium at and after 24 h. This control experiment confirmed that boron had been effectively depleted in the boron-depleted media prepared in this study. It is worth noting that the culture of *Anabaena* sp. UTEX 2576 grown in the boron-depleted medium exhibited a yellowish color, similar to other heterocystous cyanobacteira grown under boron-depleted conditions [6][7]. In contrast, the culture of *A. platensis* NIES-39 had the usual blue-green color even when it was propagated in the boron-depleted medium.

The experiments in Fig. 2 and Fig. 3 clearly indicated that boron depletion had no detectable effect on the growth of *A. platensis* whereas it deteriorated the growth of *Anabaena* sp. that requires boron for the N_2-fixation-dependent growth.

3.3 Effect of boron depletion on the biomass and the protein, chlorophyll *a*, and C-phycocyanin contents

A. platensis trichomes propagated in the boron-depleted medium were visually indistinguishable from those propagated in the usual SOT medium, when their morphologies were observed under a dissecting microscope. To examine whether there were any differences in their cellular physiology, some biochemical properties were determined next.

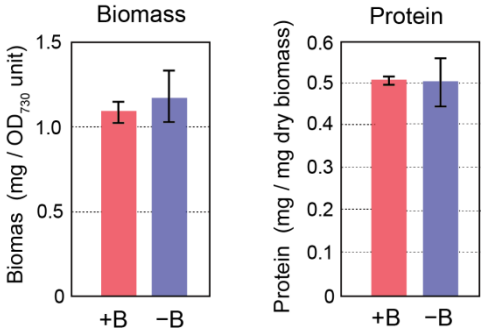

Fig.4 Biomass and the protein content

Fig. 4 shows the amount of biomass and the protein content of *A. platensis* NIES-39 propagated in the presence and absence of boron. There were no significant differences between them as analyzed by the Student's *t*-test with significance level set at 0.05. The protein content of *A. platensis* NIES-39 was approximately 50% (w/w) both in the presence and absence of boron (Fig. 4, right panel). These values were comparable to the values (50–55%) that had been determined with another *A. platensis* strain [24].

The Chlorophyll *a* and C-phycocyanin contents were also determined and compared (Fig. 5). Analysis by the Student's *t*-test with significance level set at 0.05 indicated that there were again no significant differences in their contents between the cells cultured in the presence and absence of boron.

4. DISCUSSION

The boron requirement by *A. platensis* has not been carefully examined. Whereas there has been a report that boron can be omitted from the growth medium to culture *A. platensis* PCC 8005, experiments in that study were performed using a glass fermenter [25]. Since water becomes contaminated with boron when stored in glass containers and that the boron release from glass is accelerated under alkaline conditions [13], it was

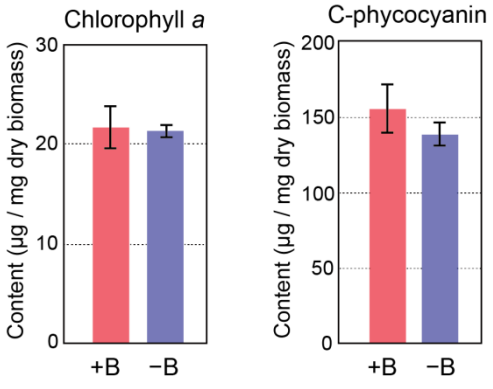

Fig. 5 Chlorophyl *a* and C-phycocyanin contents

possible that the boron released from the glass vessel into the alkaline-leaned medium for *A. platensis* was sufficient to support the normal growth of *A. platensis* in that study. In contrast, our study was carefully performed using plasticware to avoid boron contamination. Our experimental results clearly demonstrated that even a residual amount of boron is not necessary for the growth of *A. platensis* (Fig. 2). In addition, there were no detectable differences in the biochemical properties of the cells grown in the presence and absence of boron (Fig. 4 and Fig. 5).

It has been reported that the C-phycocyanin content of many *Arthrospira* strains significantly increased when they were cultured in a medium that was not supplemented with microelements (Fe, B, Mn, Zn, Cu, Mo) [26]. This effect was most likely caused by the limitation of the element(s) other than boron, since our study demonstrated that boron depletion had no significant effect on the C-phycocyanin content.

A. platensis has been used for commercial propagation. In addition to commercial usage, it has also been used as a small-scale crop providing nutritional supplements for the communities where the staple diet is poor or inadequate [27][28]. The results of our study suggest that *A. platensis* can be an excellent crop that can be grown without boron supplementation even in the region where boron deficiency in the soil deteriorates the healthy growth of many common crops.

In the studies of *Arthrospira*, synthetic growth media used in biological experiments have been supplemented with boric acid [3][14]. However, boric acid gradually forms an insoluble salt manganese borate [10], since the growth media contain manganese ion. Formation of the precipitate of manganese borate may gradually deplete manganese ions in the media during prolonged storage. This may affect the results of sensitive physiological experiments, *e.g*., metabolome analysis, because manganese is an essential cofactor of many metabolic enzymes [11][12]. Since our study showed that boron is not necessary for the growth of *A. platensis*, the use of boron-free media is recommended in sensitive physiological studies of *A. platensis*.

5. CONCLUSION

Boron depletion had no detectable effect on the growth and biochemical properties of *A. platensis*. This result indicates that boron supply is not necessary for the propagation of *A. platensis* even in the regions where boron deficiency in the soil deteriorates the growth of common crops. Since boron is not necessary for the growth of *A. platensis*, the use of boron-free media is recommended when performing sensitive physiological experiments, to avoid the precipitation of manganese borate that may reduce effective concentration of manganese ions in the media.

6. ACKNOWLEDGEMENTS

This work was supported by the Grant-in-Aid for Challenging Exploratory Research from the Japan Society for the Promotion of Science [JSPS KAKENHI Grant Number 26660063] and the Management Expenses Grants for National University Corporations from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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