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N_2-Fixing Cyanobacteria as Nitrogen Biofertilizer — A Study With the Isolate *Anabaena azollae*

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Abstract

Anabaena azollae possesses several characteristic features advantageous for application as a nitrogen biofertilizer: fast growth rate (μ–0.0675 h^{-1}; doubling time of 10.2 h); tolerance to a wide range of temperatures (20–40°C); ability to grow and to fix nitrogen at optimal values (nitrogenase activity–32 μmol C_2H_4 mg^{-1}chl h^{-1}) over a broad range of pH (6 to 9); growth rate and nitrogenase activity not effected by the presence of 1% NaCl in the growth medium. Its production outdoors in 2.5 m^2 ponds was also tested over several months. Maximum yield of 17.9 g $m^{-2}d^{-1}$ was obtained during the month of August in which the morning and noon temperatures fluctuated between 21–24°C and 31–34°C, respectively the ponds being partially (30%) shaded to decrease light intensity.

Keywords: *Anabaena azollae*, nitrogen fixation, biofertilizer, biomass production, rice paddies

1. Introduction

The use of nitrogen-fixing cyanobacteria as nitrogen biofertilizer in rice fields is of great significance in many countries in the far east, where rice is the major staple diet. Indeed since the first report by De (1939) testing the potential application of these algae as a biofertilizer, many studies have been devoted to introducing this biofertilization technique, in various countries (Venkataraman, 1977, 1986; Roger and Kulasooriya, 1980; Martinez, 1984;

Ley and Qianlin, 1985; Grant et al., 1986; Roger and Watanabe, 1986). To date, however, the use of these algae as N-fertilizer still suffers from some major problems: The inability to produce good quality inocula at an economical price (Watanabe, 1984); the lack of understanding the environmental conditions prevailing in the rice ecosystem which in some cases affect the blooming of the algae, either endogenous species or inoculated ones (Roger and Kulasooriya, 1980); the low efficiency of the utilization of the fixed nitrogen by the rice plants (Watanabe et al., 1987).

This study describes the performance of *Anabaena azollae*, isolated from *Azolla filiculoides*, in relation to its application as N-fertilizer. Data concerning the effect of pH, temperature and salinity (environmental conditions which regulate the abundance of cyanobacteria in rice fields) on the growth rate and nitrogenase activity are presented. The possibility of cultivating this strain outdoors under N_2 fixing conditions during a relatively long period of time was also tested.

2. Materials and Methods

Organism

Anabaena azollae isolated from *Azolla filiculoides*, was donated by E. Tel-Or, Faculty of Agriculture of the Hebrew University at Rehovot, Israel.

Growth conditions

1. *Laboratory cultures*: The algae were cultivated in 500 ml sterilized glass columns inside a transparent plexiglass circulating water bath. Water temperature was controlled at 30°C. A constant photon flux of 175 μE $m^{-2}s^{-1}$at the surface of the growth vessel was supplied laterally by a battery of 8 cool-white fluorescent lamps. Continuous aeration was provided by bubbling filtered air containing 1.5% CO_2. Under these conditions, the pH was maintained at 6.8–7.0. The standard growth medium was BG-110 (Stanier et al., 1971). Unless otherwise stated, cultures were sampled during the logarithmic growth phase for use in the different experiments.
2. *Outdoor cultures*: 2.5 m^2 oval-shaped ponds with two channels forming a single loop were used. The culture, 250 liters in volume (medium was BG-110) and 10 cm in depth, was stirred by a paddle wheel. CO_2 was supplied to maintain the pH at a range of 6.5–7.5. *Pond maintenance:* Temperature, dissolved oxygen, and pH in the outdoor cultures were monitored daily. Light intensity ranged from 900 to 1250 μE $m^{-2}s^{-1}$between March to August, respectively.

To maintain steady state growth, the culture was bled as required. In all outdoor experiments the biomass concentration was kept between 6–8 mg·chl liter^{-1}.

Enzyme assays

Nitrogenase activity was estimated by the acetylene reduction method (Stewart, 1967). Samples of 4.6 ml of algal culture, washed in fresh BG-110 medium, were placed in a 25 ml Wheaton bottle sealed with a flanged rubber septum. The Wheaton bottles were subjected to rotary shaking and illuminated with a quantum flux of 75 μE m^{-2}s^{-1}, during the assay. Cell suspensions were allowed 10 min of acclimation before injection of C_2H_2. Ethylene was analyzed on an HP 5890 gas chromatograph using a stainless steel column packed with Poropack-N (0.2 cm i.d., 265 cm length). Nitrogenase activity was expressed as μ mol C_2H_4 produced per mg chlorophyll per hour.

Other methods

Ash free dry weight (AFDW) and chlorophyll-a were determined as previously described (Boussiba et al., 1987). Protein was determined according to Lowry et al. (1951). Frequency of heterocysts was calculated by microscopic countings and is expressed as % of the total number of cells in the culture.

The effect of temperature on growth was studied in a temperature block maintaining a temperature gradient from 20 to 45°C with 1.5 degree increments between adjacent test tubes. The light intensity at the bottom surface of the tubes was 110 μE m^{-2}s^{-1}.

3. Results

Effects of environmental factors

The effect of different growth conditions on the specific growth rate and nitrogenase activity of *A. azollae* were tested in the laboratory. This isolate grew relatively fast and fixed nitrogen over wide ranges of pH's (Fig. 1). The specific growth rate and the maximum nitrogenase activity being 0.065 h^{-1}, and 32 μmol C_2H_4 mg^{-1} chl h^{-1}, respectively, at PH 7.0. The same effects, high growth rate and nitrogenase activity, could be achieved in cultures growing in air, but in which the pH is controlled to 6.8–7.0. *A. azollae* can tolerate a wide range of temperatures from 20 to 40°C, without its growth rate being adversely affected (Fig. 2). This strain exhibits tolerance to NaCl up to 1% without its growth rate or its nitrogenase activity being affected (Fig. 3).

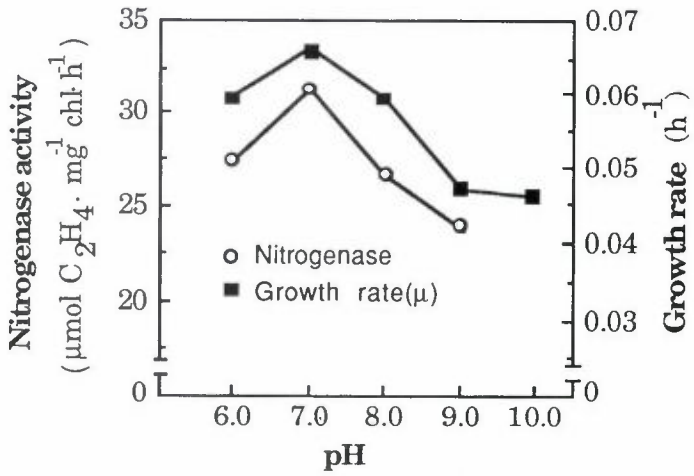

Figure 1. Effect of pH on the growth rate and nitrogenase activity of *Anabaena azollae*.

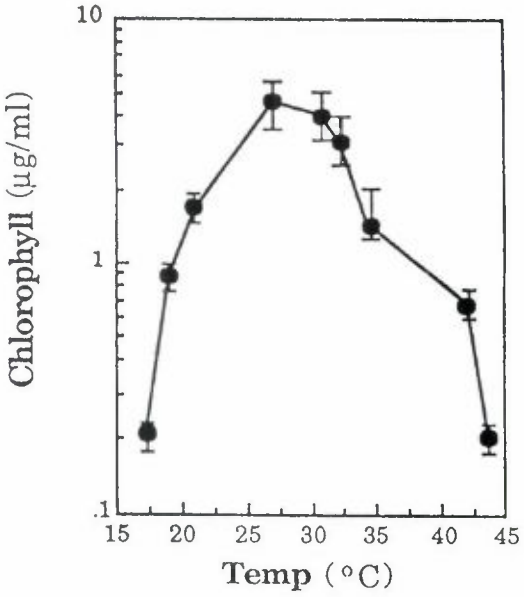

Figure 2. Effect of temperature on the growth of *Anabaena azollae*.

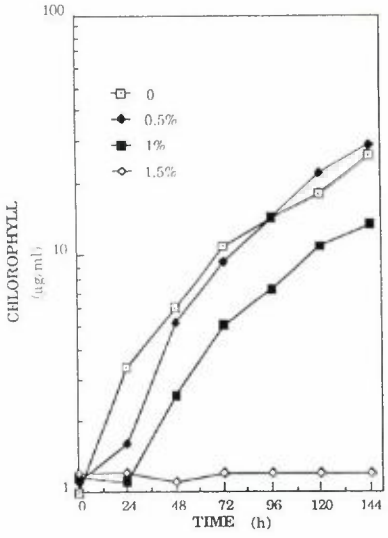

Figure 3. Effect of NaCl on the growth of *Anabaena azollae*. NaCl was added to final concentrations (w/v) as indicated.

Table 1. Outdoor production of *Anabaena azollae* in 2.5 m^2 ponds

Month (1987)	Temperature (°C)	Output rate (g $m^{-2}d^{-1}$)	Nitrogenase* activity	Heterocysts (% of total cells)
March	12–15[1] 23–26[2]	5.6	4.7	4–5
June	18–21 28-31	12.3	5.2	5–6
August	21–24 31–34	17.9	5.8	5–6

[1]morning; [2] noon

*nitrogenase activity – μmol C_2H_4 mg^{-1} chl h^{-1}

Outdoor mass production of Anabaena azollae

Data accumulated at Sede-Boker concerning the mass production of *A. azollae* during several seasons of the year are presented in Table 1. A major effect which controlled the mass production of this nitrogen fixing strain was the temperature fluctuation during the months of production. Maximum yield of 17.9 g $m^{-2}d^{-1}$ was obtained during the month of August during which the morning and noon temperatures were close to optimum. No difference between C/N ratios were observed in the material grown in the lab or outdoors (data not shown).

Some characteristic features of outdoor cultivation of this strain were observed: (1) Relatively small amounts of ammonia were present in the medium during growth (between 0.1 and 0.3 mM). This phenomenon was not observed in the laboratory; (2) significant reduction in frequency of heterocysts (5–7%) and nitrogenase activity (4–7 μmol $C_2H_4 mg^{-1}$chl h^{-1}) during growth, compared with laboratory cultures; (3) sensitivity to solar irradiance (1250 $\mu E\ m^{-2}s^{-1}$) which necessitated continuous shading of the pond in the summer (August), reducing light intensity by 30%.

The release of ammonia to the surrounding environment

In one event during the course of growth in outdoor ponds, a sudden drastic drop in temperature occurred (below 10°C). This caused rapid decomposition of the *Anabaena* cells and an increase of ammonia in the growing medium (Fig. 4).

The released ammonia was consumed and promoted the establishment of new species of algae (green) as revealed by microscopic observation and by the total loss of nitrogenase activity (Fig. 4). This situation is comparable to the decomposition of cyanobacteria in rice fields, when the nitrogen compounds are utilized by the rice plants.

4. Discussion

Rice fields continuously undergo environmental changes during maturation of the rice plants (Roger and Kulasooriya, 1980). During the growth cycle of the rice plants, light becomes limiting due to tillering development, and there is an increase of pH from 6 to 7 in the inoculation stages, to 8–9.5 towards the end of growth. Also, due to evaporation, there is a constant increase in salt concentration, while temperature may also fluctuate over a wide range (Venkataraman, 1986). Clearly, these environmental factors may directly affect the growth and development of cyanobacteria in rice fields. In particular, these factors may control nitrogenase activity and therefore affect the performance of these microorganisms as nitrogen biofertilizers. The search for suitable strains which can perform well under the different environmental conditions prevailing in rice fields, should therefore by considered, as the first stage in the development of biotechnology using cyanobacteria as biofertilizer.

The results obtained in this investigation and previously (Zimmerman and Boussiba, 1987), regarding the effect of environmental factors on the growth rate and nitrogenase activity of *A. azollae* give support to the possibility of

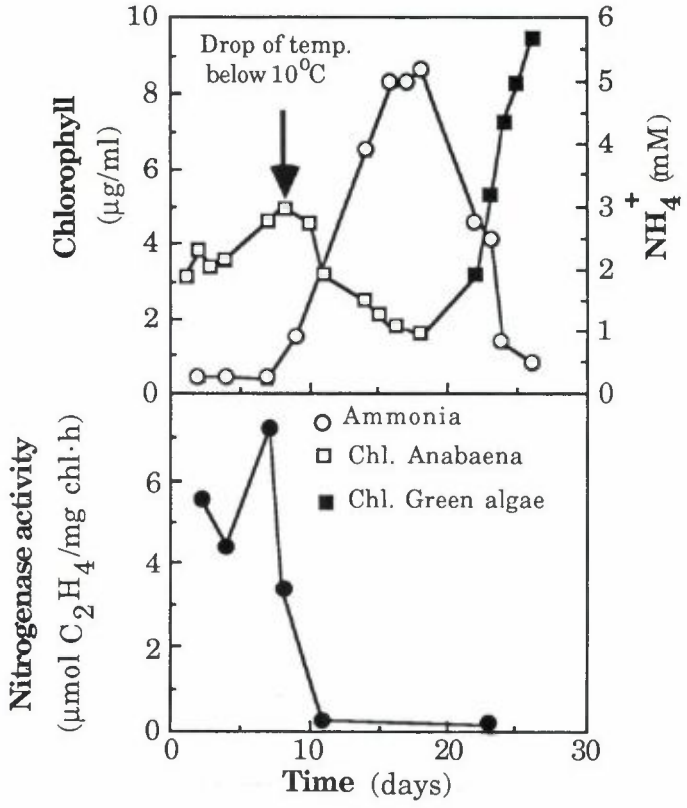

Figure 4. Growth and ammonia release of *Anabaena azollae* cultivated outdoors in 2.5 m^2 pond.

using this isolate as a nitrogen biofertilizer. This strain grows relatively fast (dt of 10.2 hr), but slower than *Anabaena siamensis* another potential biofertilizer strain which grows much faster (dt of about 4.0 hr) (Antarikanonda, 1985). *A. azollae*, however, possesses several other characteristic features which may be considered advantageous; it fixes nitrogen at almost optimal rates over a broad range of pH; tolerates a wide range of temperatures; and can withstand up to 1% NaCl in the growth medium without its growth or its nitrogenase activity being significantly affected. Indeed, these features of *A. azollae* have been described previously (Antarikanonda and Lorenzen, 1983) as the desirable ones, when considering natural isolates of N_2-fixing cyanobacteria to serve as nitrogen biofertilizer.

The next important stage when considering application of N_2-fixing cyanobacteria as nitrogen biofertilizer is mass production of high quality inoculum of the desirable strains (Watanabe, 1984). Data concerning mass production of nitrogen-fixing cyanobacteria are still limited, and the rate of reported production 6-8 g $m^{-2}day^{-1}$ is relatively low (Watanabe, 1959). Recently Fontes et al. (1987) obtained higher rates of production 8 to 13 g (dry weight) $m^{-2}day^{-1}$ using *Anabaena variabilis*. It is imperative to consider these data with great caution since they were obtained in a small scale (0.25 m^2) and over a relatively very short period of time. The highest rate of production in a bigger reactor 2.5 m^2 obtained in this work was 17.9 g (A.F.D.W.) $m^{-2}day^{-1}$. The rate of production was calculated from a culture being at steady state of at least 25 days.

The third stage, which should perhaps be considered the critical one in the selection of the desirable strain, to be used as a nitrogen biofertilizer, is its performance under field conditions. Important factors which should be taken into consideration are: competition with endogenous strains, resistance to pesticides and grazers and, finally, the effectiveness of fixed nitrogen transfer, to the benefit of the rice plants (an example of such a flow of nitrogen during the decomposition of *A. azollae* is documented in Fig. 4). The performance of *A. azollae* in rice fields is now being investigated.

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REFERENCES

Antarikanonda, P. 1985. A new species of the genus *Anabaena: Anabaena siamensis* sp *Novo* (Cyanophycease) from Thailand. *Nova. Hedwigia* **41:** 343–352.

Antarikanonda, P. and Lorenzen, H. 1983. Highly efficient N_2 fixing blue green algae in Thailand: A possible remedy to nitrogen scarcity. *Bangkok MIRCEN News Letters* **3:** 1–6.

Boussiba, S., Vonshak, A., Cohen, Z., Avissar, Y., and Richmond, A. 1987. Lipid and biomass production by the halotolerant microalgae *Nannochhoropsis salina. Biomass* **12:** 37–48.

De, P.K. 1939. The role of blue green algae in nitrogen fixation in rice fields. *Proc. Roy. Soc. Lond. B.* **127:** 121–139.

Fontes, A.G., Vargas, M.A., Moreno, J., Guerrero, M.G., and Losada, M. 1987. Factors affecting the production of biomass by a nitrogen fixing blue green alga in outdoor culture. *Biomass* **13:** 33–43.

Grant, I.F., Roger, P.A., and Watanabe, I. 1986. Ecosystem manipulation for increasing biological N_2 fixation by blue-green algae (Cyanobacteria) in lowland rice fields. *J. Biol. Agricult. Horticul.* **3:** 299–315.

Ley, H.S. and Qianlin, W. 1985. Nitrogen fixing blue green algae a source of biofertilizer. In: *Proceedings of the Joint CHINA-U.S. Phycology Symposium*, pp. 479–496.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.

Martinez, M.R. 1984. Algae: biofertilizer for rice. Philippines Council for Agriculture Research and Resources Development (PCARRD). *PCARRD Monitor* **12:** 9.

Roger, P.A. and Kulasooriya, S.A. 1980. *Blue-Green Algae and Rice.* The International Rice Research Institute, Los Banos, Philippines.

Roger, P.A. and Watanabe, I. 1986. Techniques for utilizing biological nitrogen fixation in wetland rice: potentialities, current usage, and limiting factors. *Fertilizer Res.* **9:** 39–77.

Solorzano, L. 1969. Determination in natural waters by the phenolhypochlorite method. *Limnol. Oceanogr.* **14:** 799–801.

Stanier, R.Y., Kunisawa, M.M., and Cohen-Bazire, G. 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriol. Rev.* **35:** 171–205.

Stewart, W.D.P., Fitgerald, G.P., and Burris, R.H. 1967. *In situ* studies on N_2-fixation using the acetylene reduction technique. *Proc. Nat. Acad. Sci. USA* **58:** 2071–2078.

Venkataraman, L.V. 1986. Blue-green algae as biofertilizers. In: *CRC Handbook of Microalgal Mass Culture.* A. Richmond, ed. CRC Press, Inc. Boca Raton, FL, pp. 455–471.

Venkataraman, G.S. 1977. Blue green algae as a biological N-input rice cultivation. In: *Proceedings, National Symposium, Nitrogen Assimilation and Crop Productivity.* Hissar India, pp. 132–141.

Watanabe, A. 1959. On the mass culturing of a nitrogen fixing blue-green algae *Tolypothrix tenuis. J. Gen. Appl. Microbiol.* **5:** 85–91.

Watanabe, I. 1984. Use of symbiotic and free-living blue-green algae in rice culture. *Outlook of Agriculture* **13:** 166-172.

Watanabe, I., de datta, S.K., and Roger, P.A. 1987. Nitrogen cycling in wetland rice soils. In: *International Symposium on Advance in Nitrogen Cycling in Agricultural Ecosystem*, CSIRO, Australia, pp. 1–23.

Zimmerman, W.J. and Boussiba, S. 1987. Ammonia assimilation and excretion in a symbiotic strain of *Anabaena azollae* from *Azolla filiculoides*. *Lam. J. Plant Physiol.* **127:** 443–450.