

Influence of Nitrate, Phosphate and Herbicide Stresses on Nitrogenase Activity and Growth of Cyanobacteria Isolated from Paddy Fields*

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* This study is a unit of Ph.D.Thesis

Received : 20 June 2006

Accepted : 11 August 2006

Abstract

Samples were collected from paddy fields in Corum-TURKIYE. Nitrogen-free BG-11 medium was used for isolation of nitrogen fixing cyanobacteria. Acetylene reduction technique was used to determine the effects of different chemical agents on the nitrogenase activities of the cyanobacteria, which were identified at the genus level. *Nodularia* showed the highest nitrogenase activity (0.006 ethylene $\mu\text{l}/\text{mg}\cdot\text{h}$) at 10mM nitrate concentration. At 25mM phosphate concentration, *Nodularia* showed the highest nitrogenase activity (0.006 ethylene $\mu\text{l}/\text{mg}\cdot\text{h}$). The highest tolerances for the herbicides were present in *Nodularia* (0.06 ethylene $\mu\text{l}/\text{mg}\cdot\text{h}$) for bensulphuron (50 $\mu\text{g}/\text{ml}$) and *Nostoc* 6 ethylene $\mu\text{l}/\text{mg}\cdot\text{h}$ (for molinate 100 $\mu\text{g}/\text{ml}$).

Key words: Cyanobacteria, nitrogenase activity, isolation, environmental factors

INTRODUCTION

The utilization of nitrogen gas (N_2) as a source of nitrogen is called nitrogen fixation and it is a property of only certain prokaryotes [1, 2]. Soil algae, particularly nitrogen fixing cyanobacteria, are important photosynthetic microorganisms because they contribute to soil fertility by fixing the atmospheric nitrogen.

In the fixation process, N_2 is reduced to ammonium and the ammonium is converted to the organic form. The reduction process is catalyzed by the nitrogenase which consists of two separate proteins called dinitrogenase and dinitrogenase reductase [2, 3]. Nitrogenase activity is controlled by a complex regulon called the *nif* regulon [4, 5].

Biological processes contribute 65% of the nitrogen used in agriculture [6]. Biological nitrogen fixation contributions to rice culture up to 75kgN ha⁻¹ per culture cycle [7]. Free living microorganisms on temperate soil and waters are thought to fix as much as 45-100kg N ha⁻¹ yr⁻¹ only cyanobacteria fix as much as 28 kg N ha⁻¹ yr⁻¹ [8]. Moreover, biofertilizers have been more important because algalization may be effect plant size, nitrogen content and the number of tillers, ears, spikelets and filled grains per panicle.

Certain photosynthetic bacteria fix N_2 , but only under anaerobic conditions. The nitrogen fixation has been affected by environmental factors. Nitrate, phosphate and herbicide stresses are an important environmental factors affecting algal growth and nitrogenase activity.

Singh [9] suggested that cultures of *Nostoc* sp. rapidly and significantly lost their ability to reduce acetylene when incubated with 2mM NH_4Cl and 5mM glutamine in light. Prospero et al. [10] determined that the input of nitrogen fertilizers to field reduces nitrogen fixation since the presence of combined nitrogen inhibits nitrogenase activity, also they researched the repressive effect of ammonium on nitrogenase activity at neutral pH but not at alkaline pH, and it is so-called "fast switch-off". Singh et al. [11] found that nitrogenase and heterocyst were repressed by NH_3 at *Anabaena cycadeae*. Juan et al. [12] reported that transfer of N_2 -fixer filamentous cyanobacteria from media containing a source of combined nitrogen to a medium lacking combined nitrogen provokes the differentiation of heterocyst, specialized cells able to perform dinitrogen fixation [13, 14]. Meeks et al. [15] informed that both of species of *Anabaena* sp were maximum inhibited of

acetylene reduction activity and heterocyst formation between 25 and 100 μM (69% and 36%), and they did not increase at higher nitrate concentrations. Moisan and Pearl [16] explained that dissolved inorganic N is a factor because it inhibits nitrogenase. Sroga [17] also indicated that nitrogenase activity of *Microcoleus* sp. was inhibited by NO_3 , NH_3 , urea under the light and dark phase. Anneliese et al. [18] reported that the effect of inhibitory of NH_3 haven't been under the anaerobic conditions. Jose et al. [19] determined that nitrogenase structural genes and some other genes related to dinitrogen fixation on repression by ammonium and different degrees of inhibition have been reported for different strains at nitrate. Bottomley et al. [20] revealed that both of NH_4NO_3 and KNO_3 completely repressed heterocyst development and nitrogenase activity at *Anabaena* sp. Valiente et al. [21] found that there was a negative correlation between ammonium and nitrogenase activity and the activation of nitrogenase was sharply inhibited. Turpin et al. [22] reported that 1mM ammonium at all pH was repressed the nitrogenase activity on *Anabaena flos-aquae* and at higher pH, the proportion of unprotonated ammonia increases and diffusion across the cell membrane can occur.

Adhya et al. [23] said that phosphorus is one of macronutrient essentials for plant growth, and addition of P to rice fields promotes root growth and rhizosphere activity and heterotrophic nitrogen fixation. Wilson and Alexander [24] established that phosphate equivalent to 30kgP/ha stimulated nitrogen fixation by about 60%, and the growth of nitrogen fixing algae was also limited in flooded paddy fields. According to Turid [25], phosphorus fertilization stimulated the nitrogenase activity, but to some other researchers; it was repressed [26, 27]. Lehtimaki et al. [28] observed that growth of *Nodularia* sp. incubation in different phosphorus concentrations was barely detectable during first 21 days. Huber [29] found that the rate of more or less than 0.9 μM the phosphate concentration is the best condition for kinetic growth at *Nodularia* sp. Leganes et al. [30] established that grain yielding on paddy field stimulated at 100kgP/ha treatments.

Jianyi et al. [31] searched on the effect of 40 herbicides on *Chlorella vulgaris* and they were determined that the photosynthetic period of *Chlorella vulgaris* was affected by molinate and the acetolactate sintase of *Chlorella vulgaris* was

effected by bensulfuron – methyl. Yan et al. [32] researched that the effects of molinate at *Anabaena sphaerica* on 300-3000 lux (5, 25, 50µg/ml) and some specific proteins were prevented functionally by toxic effect. Mansour et al. [33] and Caux et al. [34] demonstrated that toxic effect of molinate is more effective at low light intensity (300lux) than high light intensity (3000 lux) and it was related to organic carbon which was more assimilated in this condition.

Rice cultivation in India started in assured irrigation areas during the rainy summer season before 25 to 30 years ago [35]. Herbicides used in rice are categorized into preplant, pre emergence and post emergence [36 - 38]. The role of environmental factors on nitrogenase activity is not known yet. Because of this it needs to work on it. This paper summarizes effects of this nitrate, phosphate and herbicide stresses on growths and nitrogenase activities of nitrogen fixing *Anabaena*, *Nostoc* and *Nodularia* sp.

MATERIALS AND METHODS

Materials

The filamentous, heterocystous cyanobacteria were used in this study in which *Anabaena*, *Nodularia* and *Nostoc* sp. which were isolated from soil with water samples obtained from rice fields in Corum, Türkiye. *Nostoc* and *Nodularia* strains were obtained from previous studies of Prof. Dr. Gonul Donmez.

Isolation and purification were performed by dilution and plating of soil and water samples. Stock cultures were grown in the N-free BG-11 medium as previously described [10]. Temperature was maintained at 20°C and cultures were grown under a cool white light (600lux). Cells in the logarithmic phase of growth were collected from stock cultures and used as inocula for experiments.

Experiments were conducted in batch cultures by using 10 ml of inoculated medium in 25ml. Erlenmeyer flasks enclosed with cotton plugs. Culture media were adjusted accordingly pH (7, 8, 9) with 1N Na OH and 1N HCl. Illumination was supplied with 600lux cool white light [39 - 41].

Methods

Determination of nitrogenase activity

Nitrogenase activity was measured by acetylene reduction technique using in 10 ml aliquots of cell suspensions placed in stoppered 35 ml serum bottles [42]. Cultures were grown under the different environmental conditions were enclosed by plastic plugs and parafin, then 1ml of acetylene gas was injected into the serum bottles.

Cultures were incubated for 12h under the experimental conditions. After the incubation periods, samples (1ml) were

taken from serum bottles with gas-tight syringes, injected into the gas chromatograph, and ethylene concentrations were determined using a Shimadzu GC-14B.

Determination of dry weight

The pellets of centrifuged cultures were washed with distilled water three times, then dried to constant weight at 70°C for 12h [10, 43]. Dry weight were measured.

Influence of nitrate, phosphate and herbicides on nitrogenase activity and growth

The influence of different concentrations of KNO₃ (0.5mM-50mM), K₂HPO₄ (10µM-1M), bensulphuron-methyl (50-500 µg/ml) and molinate (5-50µg/ml) on the nitrogenase activity were also tested on *Anabaena*, *Nostoc* and *Nodularia*.

The experimental cultures were grown in 25ml flasks containing 10ml N-free BG-11 medium under the same conditions as described below. According to Rippka [41], the axenic cultures were grown in a liquid sterilized medium at 20 ± 2°C under fluorescent light (600lux) for 35days. At the end of 35 days, nitrogenase activity of cultures was determined using the acetylene reduction technique. For dry weight was made as determination described by Cappucino et al. [43]. All experiments were performed in triplicate and parallel conditions.

RESULTS

When *Anabaena*, *Nostoc* and *Nodularia* sp was cultured in the presence of various nitrate, phosphate and herbicide concentrations, distinct effects were seen on nitrogenase activities and growths.

Effects of nitrate on nitrogenase activity and growth

The growths and nitrogenase activities of *Anabaena*, *Nostoc* and *Nodularia* sp treated with different concentrations of nitrate under 600 lux light intensity are listed in Table 1. It can be seen that the nitrate markedly inhibited the growths and nitrogenase activities of all cultures. The inhibitory effect increased with the increase in nitrate concentration. Under 100mM nitrate concentration, the nitrogenase activities of all cultures were completely reduced. The highest nitrogenase activity of *Nostoc* sp at different concentration were registered with 1 mM nitrate (0.12µl ethylene / mg.h). The lowest nitrogenase activity of *Nodularia* sp at different concentration were found with 10mM nitrate (0.006µl ethylene / mg.h). The growths of *Anabaena* and *Nostoc* sp. completely repressed at 10mM, but the growth of *Nodularia* sp suppressed at 100mM nitrate concentration.

Table 1. Effects of nitrate on nitrogenase activity and growth of cyanobacteria **

Treatment	<i>Anabaena</i> sp.		<i>Nostoc</i> sp.		<i>Nodularia</i> sp.	
	Dry weight (mg/l)	Ethylene amount (µl / mg.h)	Dry weight (mg/l)	Ethylene amount (µl / mg.h)	Dry weight (mg/l)	Ethylene amount (µl / mg.h)
Control	520±52	0,37±0,05	30±1,5	11±2,13	280±28	0,74±0,24
10 µM	470±15	0,003±0	22±1,4	0,17±0	290±10	0,36±0,011
100 µM	396±5,7	0,003±0	17±1,4	0,16±0,007	115±7,07	0,016±0,0007
1 mM	225±7,07	0,003±0	15±1,4	0,12±0,021	90±5,7	0,008±0,0006
10 mM	0	0	0	0	50±0	0,006±0,0007
100 mM	0	0	0	0 0 0		
1M	0	0	0	0 0 0		

** Nitrate effects on the growth (p < 0.01).

Effects of phosphate on nitrogenase activity and growth

The effect of phosphate on nitrogenase activities and growths of all cultures shown (Table 2). *Anabaena* and

Nostoc sp. were shown to tolerance to 10mM phosphate concentration and *Nodularia* sp. was shown to tolerance to 25mM. Nitrogenase activity of *Anabaena* sp. was stimulated at 500µM phosphate concentration but increasing

concentrations repressed the nitrogenase activity. In *Nostoc* and *Nodularia* sp., the activities repressed with increasing phosphate concentrations during the initial period. The growths of *Anabaena* and *Nostoc* sp. completely repressed

at 25mM and higher phosphate concentrations, and *Nodularia* sp. completely suppressed at 50mM phosphate concentration (Table2).

Table 2. Effects of phosphate on nitrogenase activity and growth of cyanobacteria **

Treatment	<i>Anabaena</i> sp.		<i>Nostoc</i> sp.		<i>Nodularia</i> sp.	
	Dry weight (mg/l)	Ethylene amount (µl / mg.h)	Dry weight (mg/l)	Ethylene amount (µl / mg.h)	Dry weight (mg/l)	Ethylene amount (µl / mg.h)
Control	540±80	0,26±0,06	29±3,6	146±1,15	280±28	0,72±0,22
500 µM	500±20	0,45±0,08	25±4,5	0,03±0,02	160±0	0,002±0
5 mM	355±7,07	0,40±0	26±4,6	0,02±0	153±5,7	0,002±0
10 mM	296±23	0,30±0	22±3,6	0,02±0	125±7,07	0,002±0
25 mM	0	0	0	0	113±20,8	0,002±0
50 mM	0	0	0	0	0	0

** Phosphate effects on the growth (p < 0.01).

Effects of bensulfuron-methyl on nitrogenase activity and growth

Table 3 summarise the effects of bensulfuron- methyl concentrations on all of the cultures. The maximum tolerance (0.06µl ethylene / mg.h) was seen at *Nodularia* sp. in 50µg/ml bensulfuron- methyl concentration. In *Anabaena* and *Nostoc* sp., the tolerances were found in 40 µg/ml bensulfuron- methyl concentration.

Although a low bensulfuron- n-methyl concentration (5µg/ml) so mewhat stimulated nitrogenase activity, at higher concentrations nitrogenase activity was severely inhibited at *Anabaena* sp. In *Nodularia* sp., the nitrogenase activity inhibition with bensulfuron-methyl at 5µg/ml was severely. For *Nostoc* sp., the highest nitrogenase activity was seen at 30 µg /ml bensulfuron-methyl. The negative impact of high bensulfuron-methyl on the biomass of all cultures was also shown.

Table 3. Effects of bensulfuron-methyl on nitrogenase activity and growth of cyanobacteria **

Treatment (µg/ml)	<i>Anabaena</i> sp.		<i>Nostoc</i> sp.		<i>Nodularia</i> sp.	
	Dry weight (mg/l)	Ethylene amount (µl / mg.h)	Dry weight (mg/l)	Ethylene amount (µl / mg.h)	Dry weight (mg/l)	Ethylene amount (µl / mg.h)
Control	420±20	0,24±0,04	45±5,6	6,25±0,9	210±10	1±0,2
5	480±30	0,8±0,03	50±1,5	5,8±1,0	145±7,07	0,09±0
10	450±10	0,25±0,03	41±3,5	4,6±0,2	143±5,7	0,08±0,011
20	415±35	0,26±0,014	30±0,7	4,5±0	130±14	0,07±0,02
30	250±30	0,25±0,02	19±2,6	4,5±0	115±7,07	0,07±0,007
40	0	0	0	0	105±7,07	0,07±0
50	0	0	0	0	90±10	0,06±0,006

**Bensulfuron-methyl effects on the growth (p < 0.01).

Effects of molinate on nitrogenase activity and growth

The results in Table 4 show that the nitrogenase activities and growths decreased at all molinate levels. The minimum activity was determined at *Anabaena* sp. (0.12µl ethylene / mg.h) whereas, the highest activity was shown at *Nostoc* sp. (6µl ethylene / mg.h). The maximum tolerance

were seen at all of cultures in 100µg/ml molinate concentration.

Molinate experiments have shown that the initial nitrogenase activity of *Nodularia* sp. at low concentration of molinate (50µl / ml) does not change. The nitrogenase activities of all cultures completely repressed at 200 µg/ml molinate concentration.

Table 4. Effects of molinate on nitrogenase activity and growth of cyanobacteria **

Treatment (µg/ml)	<i>Anabaena</i> sp.		<i>Nostoc</i> sp.		<i>Nodularia</i> sp.	
	Dry weight (mg/l)	Ethylene amount (µl / mg.h)	Dry weight (mg/l)	Ethylene amount (µl / mg.h)	Dry weight (mg/l)	Ethylene amount (µl / mg.h)
Control	420±20,0	0,24±0,04	43±5,6	8,15±1,7	365±7,07	0,27±0,014
50	406±20,8	0,14±0,04	31±1,7	7±0,45	180±14,00	0,26±0,014
100	320±30,0	0,12±0,035	27±2,0	6±0,25	150±0,00	0,20±0,035
200	0	0	0	0	0	0
300	0	0	0	0	0	0
500	0	0	0	0	0	0

** Molinate effects on the growth ($p < 0.01$).

DISCUSSION

As stated in the introduction, soil algae are grown in different environmental factors. Variation in growth conditions influenced the growths and nitrogenase activities of all genera. Nitrate is an important one that affects the algal growth. Generally, the addition of nitrate inhibited both the algal growth and nitrogenase activity. All nitrogenase activities were sharply repressed and the algal growth was partly inhibited by nitrate (Table 1).

This confirms the report by Huber [44] for the *Nodularia*, and it is similar to the reports Bottemley et al. [20], who found that the nitrogenase activity of *Nodularia* suppressed by the addition of nitrate.

According to the literature, the maximal inhibition of acetylene reduction and heterocyst formation in *A. cylindrica* occurred between and 25 and 100 µM and did not increase at higher nitrate concentrations [15]. Its results are similar to those of our studies. These results can be explained in this way: the nitrogenase activity inactivated by nitrate, which resembles the so-called "switch-off", observed in phototrophic bacteria.

The comparison of nitrogenase activities of algal cells under the different phosphate concentration, the nitrogenase activity of *Anabaena* sp. stimulated at 500µM phosphate concentration, whereas the nitrogenase activities of the other two species inhibited (Table 2).

In addition, the algal growths of all the cultures were partly suppressed. These results may be described like the following: phosphate is necessary for the algal growth but it is not necessary for the nitrogenase activity [23]. According to the research [25], phosphorus fertilization stimulated the nitrogenase activity and the highest activity was obtained with about 300 µM (200 µE/m².s) at *Anabaena* sp., also the nitrogenase activity of *Nostoc* sp. stimulated at 12mM phosphate concentration, however more phosphate concentrations repressed the nitrogenase activity, the result of which is similar to this study. In *Nostoc* and *Nodularia* sp., the nitrogenase activities inhibited at the beginning (Table2). These results seem to suggest that phosphorus stimulated nitrogenase activity in P- starved cells but not in P- sufficient cells [44].

It is anonymously reported that [45], bensulfuron-methyl and molinate are mostly used for eliminating weeds in paddy fields in Corum-Osmancık in Türkiye. For this reason, two herbicides were chosen for this study. In herbicide treatments, bensulfuron-methyl stimulated nitrogenase activity of *Anabaena* sp. at 5µg/ml but not in

higher concentrations. Whereas the nitrogenase activities and growths of other two species were inhibited during the initial concentration (5µg/ml) (Table3), it was demonstrated that *Anabaena* sp. was capable of growing both photoautotrophically and photoheterotrophically like bacteria to a great extent [32, 46].

In molinate treatments, all genera demonstrated tolerance to 100 µg/ml level of molinate concentration. In addition, the nitrogenase activities and growths of all genera completely repressed with an increase in molinate concentration. Yan et al. [32] reported that *A. sphaerica* kept growth rate at 100 µg/ml molinate concentration. This result is similar to our studies.

Most reports demonstrated that the inhibitory effect of herbicide became greater with an increase in herbicide concentration and suggested that the reduction in the growth rate of algae may be due to a decrease in algal photosynthesis caused by the inhibition of synthesis of chlorophyll, the most important pigment in algal cells for collecting solar energy for photosynthesis [47, 48].

The data obtained in this study provide information about the inhibitory effect of the different environmental factors on growths and nitrogenase activities of all genera, under which the cyanobacteria exhibits different sensitivity to the factors. These findings suggest a ban on the use of molinate and bensulfuron-methyl in paddy fields, owing to its inhibitory effect. Moreover, these results showed that nitrate and phosphate fertilizers could be applied under lower concentrations to rice fields.

Several differences in the growth and nitrogenase activity rates of *Nodularia*, *Nostoc* and *Anabaena* sp. were observed, which may explain the different vertical, horizontal and temporal distribution of the three genera in paddy fields. In this study, we have shown a clear physiologic distinction between *Nostoc* sp. and the other strains. Generally *Nostoc* sp. had the best optimal performance of nitrogenase activity in all environmental conditions, so it is thought that it is a suitable genus for biofertilizer. A better understanding of the mechanisms require further study about the nitrate, phosphate and herbicide stresses.

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