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Full Length Research Paper

Influence of osmotic and metal stresses on nitrogenase activity of cyanobacteria ısolated from paddy fields

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Samples were collected from paddy fields in Corum-Turkıye. 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. *Nostoc* showed the highest nitrogenase activity (0.09 ethylene µl/mg.h) at 50 mM salt concentration. At 60 mM sucrose concentration, *Nostoc* showed the highest nitrogenase activity (0.08 ethylene µl/mg.h). The highest tolerances for the metals were present in *Anabaena* (0.006 ethylene µl/mg.h) for iron (20 ppm), *Nodularia* 0.1 ethylene µl/mg.h (for manganese 20 ppm) and *Nostoc* 0.96 ethylene µl/mg.h (for zinc 5 ppm) .

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 (Manahan, 1997; Madigan, 1997). Soil algae, particularly nitrogen fixing cyanobacteria, are important photosynthetic microorganisms because they contribute to soil fertility by fixing the atmospheric nitrogen. In the fixation processs, N_2 is reduced to ammonium and the ammonium is converted to the organic form.

Biological processes contribute 65% of the nitrogen used in agriculture (Albrecht, 1998). Biological nitrogen fixation contributions to rice culture is up to 75 kgN ha^{-1} per culture cycle (Irisarri et al., 2001). Free living microorganisms on temperate soil and waters are thought to fix as much as 45 – 100 kg N $ha^{-1}yr^{-1}$, while cyanobacteria fix as much as 28 kg N ha^{-1} yr^{-1} (Metting, 1990). Moreover, biofertilizers have been more important because algalization may affect 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 is often affected by environmental factors. Osmotic and metal stresses are an important environmental factors affecting algal growth and nitrogenase activity.

Grobbelaar et al. (1987) reported that nitrogenase activity was repressed by photosynthetically produced oxygen at all concentrations. Fernandes et al. (2000) reported that when sucrose-grown (3 day old) cultures were washed off the external sucrose, resuspended in fresh medium and grown with photosynthetic inhibitors, they rapidly lost nitrogenase activity in 3 h. Madigan et al. (1997), Fernandes et al. (1993) and Vignais et al. (1985) reported that ionic and anionic compounds accumulate under the stress conditions (quaterner amines, polyols) caused switch-off effect. Murry et al. (1983) reported that oxygen inactivated the FeMo component of nitrogenase enzyme considerably faster than Fe protein component. Rippka et al. (1978) reported that molecular oxygen irreversıbly inactivate the nitrogenase synthesis.

Cavet et al. (2003) established that cyanobacteria have metal requirements often absent in other bacteria; copper in thylakoidal plastocyanin, zinc in carboxysomal carbonic anhydrase, cobalt in cobalamin, magnesium in chlorophyll, molybdenum in heterocystous nitrogenase, and manganese in thylakoidal water-splitting oxygen evolving complex. Bender et al. (1994) showed that cyanobacteria have been studied for its potential in removing zinc and manganese from contaminated water. According to Singh et al. (1992) and Jensen et al. (1982) cyanobacteria show

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Table 1. The effect of salt concentrations on nitrogenase activity in nitrogen-fixing *Anabaena*, *Nostoc* and *Nodularia* spp.

Concentration (mM)	*Anabaena* sp.		*Nostoc* sp.		*Nodularia* 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	613±40	0.27±0.04	87±3.5	2.2±0.2	280±28	0.65±0.2
10	1000±0	0.28±0.03	103±5.7	2.6±0.4	237±15	0.5±0.0
25	910±79	0.19±0.03	95±5.0	2.5±0.4	205±7.0	0.3±0.0
50	537±25	0.003±0.0	37±2.3	0.09±0.0	80±14	0.003±0.001
100	473±63	0.001±0.001	0	0	60±14	0.0005±0.0
200	0	0	0	0	0	0
400	0	0	0	0	0	0

inherent capacity to accumulate large amounts of orthophosphate in polyphosphate bodies, which in turn contribute a storage site for essential metals and also serve as a detoxification mechanism. Noriko et al. (1989) demonstrated that high concentrations of Cu, Cd and Zn caused 50% inhibition of photosynthesis in 118 isolates. Rai et al. (1989) reported that six heavy metals have antagonistic effect in *Nostoc* sp. In the same study, nitrogenase activity was inhibited with Ni (1 µg/ml) 11%, Cr (20 µg/ml) 56%, and Pb (20 µg/ml) 61% after 48 h. Van Baalen et al. (1978) reported that lower concen-trations of Ni stimulated nitrogenase activity. Takamura et al. (1989, 1990) established that Cyanophyceae were sensitive to Cu, Cd and Zn.

The role of environmental factors on nitrogenase activity is not known yet. This paper summarizes effects of osmotic and metal stresses on growth and nitrogenase activity of nitrogen-fixing *Anabaena*, *Nostoc* and *Nodularia* sp.

MATERIALS AND METHODS

Materials

The filamentous, heterocystous cyanobacteria used in this study were *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 (Prosperi et al., 1993). Temperature was maintained at 20°C and cultures were grown under a cool white light (600 lux). 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 25 ml. Erlenmyer flasks were enclosed with cotton plugs. Culture media were adjusted accordingly pH (7, 8, 9) with 1 N NaOH and 1 N HCl. Illumination was supplied with 600 lux cool white light (Castenholz, 1988; Fogg et al., 1973; Rippka, 1988).

Determination of nitrogenase activity

Nitrogenase activity was measured by acetylene reduction technique using in 10 ml aliquots of cell suspensions placed in stoppered 25 ml serum bottles (Burlage et al., 1998). Cultures were grown under the different environmental conditions were enclosed by plastic plugs and parafin, then 1 ml of acetylene gase was injected into the serum bottles. Cultures were incubated for 12 h under the experimental conditions. After the incubation periods, samples (1 ml) 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 12 h (Prosperi et al., 1993; Cappuccino et al., 2001). Dry weight were measured.

Influence of osmotic and metal stresses on nitrogenase activity

The influence of different concentrations of NaCl (10 – 400 mM), sucrose (10 – 60 mM), iron (0.625 – 80 ppm), manganese (0.625 – 40 ppm) and zinc (0.625 – 40 ppm) on the nitrogenase activity were also tested on *Anabaena*, *Nostoc* and *Nodularia*.

Cultures in log phase were used in this study. The experimental cultures were grown in 25 ml flasks containing 10 ml N-free BG-11 medium under the same conditions as described below. According to Rippka (1988), the axenic cultures were grown in a liquid sterilized medium at 20 ± 2°C under fluorescent light (600 lux) for 35 days. At the end of 35 days, nitrogenase activities of cultures were determined using the acetylene reduction technique. Measur-ement of dry weight was made as determination described by Cappucino et al. (2001). All experiments were performed in tripli-cate and parallel conditions.

RESULTS AND DISCUSSION

When *Anabaena*, *Nostoc* and *Nodularia* sp. were cultured in the presence of various salt, sucrose and metal concentrations, distinct effects were seen on nitrogenase activities and growths. The growths and nitrogenase activities of *Anabaena*, *Nostoc* and *Nodularia* sp. treated with different concentrations of NaCl under 600 lux light intensity are listed in Table 1. It can be seen that the salt markedly inhibited the growths and nitrogenase activities of all cultures. The nitrogenase activities of *Anabaena*

Table 2. The effects of sucrose concentrations on nitrogenase activity in nitrogen-fixing *Anabaena*, *Nostoc* and *Nodularia* spp.

Concentration (mM)	*Anabaena* sp.		*Nostoc* sp.		*Nodularia* 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)
Kontrol	473±25	0.23±0.028	86±3.5	2.2±0.2	280±28	0.7±0.2
10	1115±35	0.01±0.0007	95±7.0	0.10±0.007	275±7.0	0.04±0.001
20	1645±77	0.012±0.0007	95±7.0	0.11±0.014	330±10	0.03±0.002
40	1326±66	0.008±0.0005	100±7.0	0.10±0	675±21	0.01±0.001
60	1275±35	0.008±0.002	125±7.0	0.08±0	766±15	0.004±0.0005

Table 3. The effects of iron concentrations on nitrogenase activity in nitrogen-fixing *Anabaena*, *Nostoc* and *Nodularia* spp.

Concentration (ppm)	*Anabaena* sp.		*Nostoc* sp.		*Nodularia* 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	613±40	0.27±0.04	48±1.7	2.4±0.07	280±28	0.65±0.2
0.625	456±11	0.007±0.0	35±1.1	0.055±0.007	280±20	0.83±0.06
1.25	483±10	0.006±0.0	24±3.0	0.02±0.0	226±20.8	0.9±0.07
2.5	440±0	0.006±0.0	22±1.1	0.02±0.0	196±23	0.7±0.07
5	430±0	0.006±0.0	18±1.1	0.02±0.0	135±35	0.7±0.014
10	390±10	0.006±0.0	16±1.1	0.01±0.0	0	0
20	180±10	0.006±0.0005	0	0	0	0
40	0	0	0	0	0	0
80	0	0	0	0	0	0

and *Nostoc* sp. were partly stimulated at lower salt concentration (10 mM). The inhibitory effect increased with the increase in salt concentration. Under 200 mM salt concentration, the nitrogenase activities of all cultures were completely reduced. The highest nitrogenase activity of *Nostoc* sp. at different concentration was registered with 50 mM salt (0.09 µl ethylene/mg.h). The lowest nitrogenase activity of *Nodularia* sp. at different concentration was found with 100 mM salt (0.0005 µl ethylene/mg.h). The growths of *Anabaena* and *Nodularia* sp. were completely repressed at 200 mM, but the growth of *Nostoc* sp. was suppressed at 100 mM salt concentration (Table 1).

The effects of sucrose on nitrogenase activities and growths of all cultures are shown in Table 2. The nitrogenase activities of all cultures were inhibited at different sucrose concentrations. However, the growths of all cultures were not repressed at higher sucrose concentrations. The minimum activity was obtained in *Nodularia* sp. (0.004 µl ethylene/mg.h) while the highest activity was seen in *Nostoc* sp. (0.08 µl ethylene/mg.h) (Table 2).

Table 3 summarise the effects of iron concentrations on the cultures. Nitrogenase activity of *Nodularia* sp. was stimulated at low iron concentrations (0.625 and 1.25 ppm), but the activity was inhibited at higher iron concentrations. In *Anabaena* and *Nostoc* sp., the activities were repressed with increasing iron concentrations during the initial period. For *Nodularia* sp., the highest nitrogenase activity was seen at 5 ppm iron concentration. The maximum tolerance was seen in *Anabaena* sp. (20 ppm).

The effect of manganese on nitrogenase activities and growths the cultures are shown in Table 4. In this study, the nitrogenase activities of *Anabaena* and *Nostoc* sp. were stimulated at low manganese concentration (0.625 ppm) but the activities were inhibited at higher manganese concentrations. In *Nodularia* sp., the activity was repressed with increasing manganese concentrations during the initial period. The growths of all cultures were completely repressed at 40 ppm manganese concentration. Therefore, the growths of *Nostoc* and *Nodularia* sp. were stimulated at low manganese concentration (0.625 ppm) but increasing concentrations repressed the growths. In *Anabaena* sp., the growth was inhibited at all of concentrations (Table 4).

The growths and nitrogenase activities of all cultures treated with different concentrations of zinc under 600 lux light intensity are depicted in Table 5. It can be seen that zinc markedly inhibited the growth and nitrogenase activities of *Anabaena* and *Nodularia* sp. Under 10 ppm zinc concentration, the growths and nitrogenase activities of all cultures were completely reduced. The nitrogenase activity of *Nostoc* sp. was stimulated at 0.625 ppm zinc concentration but increasing concentrations repressed the activity. The highest activity of *Nostoc* sp. is at 5 ppm zinc concentration (0.96 µl ethylene/mg.h).

As stated in the introduction, soil algae are grown in

Table 4. The effects of manganese concentrations on nitrogenase activity in nitrogen-fixing *Anabaena*, *Nostoc* and *Nodularia* spp.

Concentration (ppm)	*Anabaena* sp.		*Nostoc* sp.		*Nodularia* 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	613±40	0.27±0.04	48±1.73	2.3±0.3	280±28	0.65±0.2
0.625	600±42	0.31±0.07	82±1.4	2.8±0.014	340±14	0.55±0.07
1.25	546±11	0.29±0.02	81±0.7	2.7±0.01	215±7.0	0.3±0
2.5	560±0	0.29±0.007	70±0	2.4±0.14	196±15	0.26±0.06
5	0	0	50±0	0.025±0.007	130±0	0.2±0
10	0	0	50±0	0.03±0.03	120±5.7	0.2±0
20	0	0	30±0	0.006±0.005	67±15	0.1±0
40	0	0	0	0	0	0

Table 5. The effects of zinc concentrations on nitrogenase activity in nitrogen-fixing *Anabaena*, *Nostoc* and *Nodularia* spp.

Concentration (ppm)	*Anabaena* sp.		*Nostoc* sp.		*Nodularia* 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	1100±141	0.35±0.07	90±5.7	2.23±0.2	210±10	1.06±0.2
0.625	875±7.07	0.25±0.04	90±7.07	2.75±0.21	143±28	0.83±0.06
1.25	300±14.1	0.25±0.03	60±0	1.95±0.07	83±5.7	0.50±0
2.5	163±47	0.23±0.06	51±3.5	1.30±0.021	44±15	0.01±0.003
5	80±10	0.014±0.0017	23±2	0.96±0.11	0	0
10	0	0	0	0	0	0
20	0	0	0	0	0	0
40	0	0	0	0	0	0

different environmental factors. Variation in growth conditions influenced the growths and nitrogenase activities of all genera. Osmotic and metal stresses are important factors that affects the algal growth.

Generally, the addition of salt inhibited the nitrogenase activities but the growths were partly stimulated by lower salt concentration (10 mM) (Table 1). For this reason, the ammonium compounds accumulated under the stress conditions repressed the nitrogenase activity and is called “ammonia switch off effect” reversible mechanism (Madigan et al., 1997; Vignais et al., 1985; Howard et al., 1983). Diazotrophic growth need sodium but sodium do not play a part in heterocyst formation or protection of nitrogenase from oxygen (Rai et al., 1999).

At the end of this studies it was determined that *Anabaena* sp. showed a tolerance of 100 mM salt concentration. In addition, algal growth and nitrogenase activity of *Anabaena* were completely inhibited at 200 mM salt concentration which is similar to the reports of Fernandes et al. (2000) and Selwin et al. (1991). According to the literature, nitrogenase activity of *Anabaena* sp. is stimulated by 50 mM salt concentration (Fernandes et al., 2000); however, more salt concentrations repressed the nitrogenase activity. Turid (1999) reported that nitrogenase activity of *Nostoc* sp. is stimulated at 10 mM salt concentration but increasing salt concentration resulted in a significant decrease in nitrogen fixation. According to the literature (Moisander et al., 2002), *Anabaena* sp. grew at concentrations up to 15 g/L salt concentration but inhibited at 20 g/L NaCl. *Nodularia* sp. can also grow in wide range of salt (0 - 20 g/L) and nitrogenase activity is stimulated in 5 g/L salt concentration but repressed in higher salt concentrations.

The comparison of nitrogenase activities of all cultures under the different salt concentrations show some variation which may be due to the fact that the isolates were taken from difference fields and the halotolerant species were differently adapted to these fields. Species in same genus respond differently to salt (Fernandes et al., 1993; Apte et al., 1987; Moisander et al., 2000). San-gaeta et al. (1999) demonstrated that nitrogenase activity of *Nostoc* sp. is lower at 10 mM sucrose concentration than the contro, which is similar to this study.

Fang et al. (2000) and Marscher (1995) indicated that the critical iron toxicity contents of paddy are above 500 µg Fe per gram leaf dry weight. In this study, the nitrogenase activities of *Anabaena* and *Nostoc* sp. were suppressed at low iron concentration (0.625 ppm). In this study, the nitrogenase activities of all cultures were completely inhibited at 10 ppm zinc concentration. Taban

et al. (2003) reported that the maximum tolerance of paddy is 2.0 mg Zn/kg. In this study, the highest tolerance of *Nostoc* sp. was 5 ppm zinc concentration. These results will be useful in iron and zinc treatments on paddy fields.

Most reports demonstrated that the inhibitory effect of stress become greater with an increase in osmotic and metal concentrations 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 (Van Baalen et al., 1978; Takamura et al., 1990).

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. According to this study, there is a clear physiologic distinction between *Nostoc* sp. and the other strains. Generally *Nostoc* sp. had the best optimal performance of nitrogenase activity in all stress conditions, making it a suitable candidate for biofertilizer. High salt and metal concentrations can be expected in rice fields when it is contaminated, but it is diluted or consumed in a short time, particularly if it is applied before flooding. More studies will be needed in order to determine the characteristics of osmotic and metal sensitivities of all the genera.

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