

## Full Length Research Paper

# Influence of bispyribac sodium on nitrogenase activity and growth of cyanobacteria isolated from paddy fields

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Accepted 14 March, 2019

The goal of this study was to determine the effect of bispyribac-sodium on the nitrogenase activities and growth of cyanobacteria isolated from paddy fields. Ten cyanobacterial species were used in this study. Cyanobacterial species were isolated from soil and water samples obtained from rice fields in Corum, Turkey. Among all *Anabaena* strains, the maximum activity was determined in *Anabaena* sp. O-22 (2.26  $\mu$ l ethylene/mg.h) whereas; the lowest activity was shown in *Gloeotheca* sp. O-Y (0.04  $\mu$ l ethylene/mg.h). The maximum inhibition was seen in *Anabaena* sp. O-22, *Synechocystis* sp. O-X and *Anabaena* sp. O-16 in 100  $\mu$ g/ml bispyribac-sodium concentration. Although low bispyribac-sodium concentrations somewhat stimulated growths of *Anabaena* sp. O-X2, O-Ç, O-4, O-16, O-8 and *Synechocystis* sp. O-X, the biomass of all cultures were severely inhibited at higher concentrations. The growths of *Anabaena* sp. O-22 and *Synechocystis* sp. O-X completely repressed at 100  $\mu$ g/mL and at higher bispyribac-sodium concentrations, whereas, *Anabaena* O-X2, O-6, O-4 and O-16 completely suppressed at 500  $\mu$ g/mL bispyribac-sodium concentration. The end of the study *Anabaena* sp. O-22 has been proposed as biofertilizer. The results obtained may be useful for the production of rice.

**Key words:** Cyanobacteria, nitrogenase activity, herbicide.

## 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 et al., 1997). Nitrogen fixing cyanobacteria are important photosynthetic microorganisms because they contribute to soil fertility by fixing the atmospheric nitrogen.

Nitrogen fixing cyanobacteria are found in many different ecosystems. Certain photosynthetic bacteria fix  $N_2$ , but only under anaerobic conditions. Nitrate, phosphate, light intensity, metal, osmotic and herbicide stresses are important environmental conditions affecting algal growth and nitrogenase activity (Meeks et al., 1983; Castenholz, 1988; Lehtimaki et al., 1997; Liengen, 1999; Banerjee et al., 2004; Okmen and Donmez, 2007a; Okmen et al., 2007b; Okmen et al., 2007c). These effects depend on the type and nature of environmental conditions, the organisms present as well as the experimental conditions (Tozum and Sivaci, 1993).

The herbicide Nominee commonly known as

bispyribac-sodium. Sangakkara et al. (2004) is reported to increase the rice yield by selectively eliminating weeds from paddy fields. Although, the use of the herbicide is aimed at eliminating weeds, a major portion is deposited on the surface of the soil and might adversely affect the nontarget soil microflora.

Bispyribac-sodium, sodium 2,6-bis[(4,6-dimethoxy-2-pyrimidinyl)oxy]benzoate, which was first developed by Japan Kumiai Chemical, belongs to the pyrimidinyl oxybenzoic acid group (Wu and Mei, 2011). Bispyribac-sodium has been applied post emergence to control many weeds. Rice-field herbicides while protecting rice-seedlings selectively, destroy the weeds and indirectly bring an increase in grain yield (Still and Kuziriam, 1967; Park and Park, 1971; Fischer et al., 2000). This class of herbicides (penoxsulam, imazamox and bispyribac-sodium) act through inhibition of acetolactate synthase (specific to plants and microorganisms) and thereby block the biosynthesis of the branched-chain aminoacids which lead to decreased protein synthesis and cessation of growth (Osuna et al., 2002). However, bispyribac-sodium

inhibits the synthesis of key aminoacids causing susceptible plants to stop growing and die within about two to three weeks (Slade et al., 2006).

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Many reports available indicate interaction between cyanobacteria and herbicides, including effects of herbicides on algal growth, photosynthesis, nitrogen fixation, biochemical composition and metabolic activities as well as degradation and removal of herbicides by algae and cyanobacteria (Lundqvist, 1970; Ibrahim, 1972; Singh, 1974; Dasilva et al., 1975; Tiwari et al., 1981; Maule and Wright, 1983; Stratton, 1984; Mattoo et al., 1984; El-Sawy et al., 1984; Singh et al., 1986; Goyal, 1986; Tandon and Lal, 1988; Singh and Tiwari, 1988; Mishra and Pandey, 1989a; Bhunia et al., 1991; Leganés and Fernández-Valiente, 1992; El Sheekh et al., 1994; Caux et al., 1996; Jeong-Dong and Choul-Gyun, 2006; Okmen, 2007c;).

Until now, very little work has been done on the effects of rice field herbicides on nitrogen fixation and the studies carried out provide a preliminary idea about the inhibitory or stimulatory effect of the herbicides on diazotrophic growth in cyanobacteria.

Previous studies have investigated the influence of selective pesticides on the growth of cyanobacteria (Pandey, 1985; Singh and Tiwari, 1988; Mishra et al., 1989b; Prospero et al., 1993; Jin et al., 1996; Nystrom et al., 1999; Jianyi et al., 2002; Okmen et al., 2007c). Most reports demonstrated that the sensitivity of cyanobacteria toward herbicides and their growth and nitrogen fixation behavior changed in the presence of herbicides. There are no reports on the effects of bispyribac-sodium on nitrogenase activity of cyanobacteria. In this paper, we report the experimental findings obtained on the effect of a rice herbicide bispyribac-sodium, on the nitrogenase activity, growth of ten diazotrophic cyanobacterial strains, namely *Anabaena*, *Synechocystis* and *Gloeotheca* sp.

## MATERIALS AND METHODS

### Test organisms

Samples were collected from paddy fields in Osmancik, Corum-Turkey. The unicellular and filamentous, heterocystous cyanobacteria used in this study (*Anabaena*, *Synechocystis* and *Gloeotheca* sp.) were isolated from soil and water samples obtained from rice fields in Osmancik, Corum, Turkey. Nitrogen-free BG-11 medium was used for isolation of nitrogen fixing cyanobacteria. 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 (Castenholz, 1988). Temperature was maintained at 20°C and cultures were grown under a cool white light. Cells in the logarithmic phase of growth were collected from cultures and used as inocula for experiments. Experiments were conducted in batch cultures by using 10 ml of inoculated medium flasks in 25 ml. Culture media were adjusted accordingly pH 8 with 1 N NaOH and 1 N HCl. Illumination was supplied with 11  $\mu\text{mol}/\text{m}^2$  cool white light (Fogg et al., 1973; Rippka, 1988).

### Determination of nitrogenase activity

Nitrogenase activity was measured by acetylene reduction technique (Burlage et al., 1998). Cultures (10 ml) were grown under the different concentrations of bispyribac-sodium and were

enclosed by parafilm plastic. Then 1 ml of acetylene gas was injected into the serum bottles and cultures were incubated for 12 h under the experiment 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 Agilent 6890 GC-FID.

### Determination of dry weight

The pellets of centrifuged cultures were washed with distilled water three times, then dried to a constant weight at 70°C for 12 h and dry weights were measured (Castenholz, 1988; Cappuccino and Sherman, 2001).

### Influence of bispyribac – sodium on nitrogenase activity and growth

The influence of different concentrations of bispyribac– sodium (5-500  $\mu\text{g}/\text{mL}$ ) on the nitrogenase activity were also tested on *Anabaena*, *Synechocystis* and *Gloeotheca* sp. 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 cultures were grown in a liquid sterilized medium at  $20 \pm 2^\circ\text{C}$  under cool white light for 35 days. At the end of 35 days, nitrogenase activity of the cultures were determined using the acetylene reduction technique.

Appropriate control systems containing no solvent and pesticide were included in each experiment. Control and treated cultures were grown under the same temperature and light intensity as mentioned above. All experiments were performed in triplicate and the average values were presented.

## RESULTS

Cyanobacterial species were isolated from soil and water samples obtained from rice fields in Corum, Turkey. In this study, 10 cyanobacteria had studied and determined the effects of different concentrations of bispyribac-sodium on nitrogenase activities of cyanobacteria. These included 8 *Anabaena* sp., 1 *Synechocystis* sp. and 1 *Gloeotheca* sp. strains.

When *Anabaena*, *Synechocystis* and *Gloeotheca* sp. were cultured in the presence of various bispyribac-sodium concentrations, distinct effects were seen on nitrogenase activities and growths. The growths and nitrogenase activities of cyanobacteria treated with different concentrations of bispyribac-sodium under 11  $\mu\text{mol}/\text{m}^2$  light intensity are listed in Tables 1 and 2.

Among all *Anabaena* strains, the maximum activity was determined in *Anabaena* sp. O-22 (2.26  $\mu\text{l}$  ethylene/mg.h) whereas, the lowest activity was shown in *Gloeotheca* sp. O-Y (0.04  $\mu\text{l}$  ethylene/ mg.h). The nitrogenase activities of *Anabaena* sp. O-X2, O-4 and O-8 were stimulated in initial period but, increasing concentrations repressed the nitrogenase activity. Bispyribac-sodium experiments have shown that the initial nitrogenase activity of *Gloeotheca* sp. O-Y at low concentrations of bispyribac-sodium (5 to 10  $\mu\text{g}/\text{mL}$ ) did not change but, the activity repressed with increasing bispyribac-sodium concentrations in *Gloeotheca* sp. O-Y (Table 1). The maximum inhibition was

**Table 1.** Effects of bispyribac- sodium on nitrogenase activity of cyanobacteria.

Microorganisms	Ethylene amount ( $\mu\text{L}/\text{mg.h}$ )						
	Concentrations ( $\mu\text{g}/\text{mL}$ )						
	Control	5	10	25	50	100	500
<i>Anabaena</i> sp. O-X2	0.58±0.002	0.65±0.01	0.53±0	0.44±0	0.29±0	0.23±0.01	-
<i>Anabaena</i> sp. O-Ç	1.50±0.001	0.22±0.01	0.33±0.014	0.31±0.05	0.18±0	0.15±0.02	-
<i>Anabaena</i> sp. O-6	1.2±0.003	0.35±0.001	0.26±0.014	0.23±0.001	0.28±0	0.05±0	-
<i>Anabaena</i> sp. O-K	1.28±0.001	0.9±0.009	0.31±0.035	0.01±0.05	0.04±0.02	0.05±0.02	0.05±0.01
<i>Anabaena</i> sp. O-4	0.3±0.001	0.77±0.002	0.6±0.002	0.59±0.09	0.6±0.001	0.6±0.05	-
<i>Anabaena</i> sp. O-22	4.45±0.005	2.26±0.2	0.03±0.2	0.009±0	0.008±0.5	-	-
<i>Anabaena</i> sp. O-16	4.8±0.005	0.95±0.006	0.96±0.05	0.34±0.005	0.14±0.4	-	-
<i>Anabaena</i> sp. O-8	0.86±0.001	1.04±0.001	2.1±0.08	1.38±0.16	0.84±0.001	0.82±0.08	-
<i>Synechocystis</i> sp. O-X	2.62±0.002	1.18±0.008	1.40±0.001	1.45±0.003	0.9±0.04	-	-
<i>Gloeothece</i> sp. O-Y	0.30±0.004	0.25±0.001	0.27±0	0.20±0.007	0.08±0.02	0.04±0.07	0.04±0.01

(-): No effect Values are mean  $\pm$  Standard deviation.

**Table 2.** Effects of bispyribac- sodium on growth of cyanobacteria.

Microorganisms	Dry weight ( $\text{mg}/\text{mL}$ )						
	Concentrations ( $\mu\text{g}/\text{mL}$ )						
	Control	5	10	25	50	100	500
<i>Anabaena</i> sp. O-X2	1.2±5	1.8±15	1.6±10	1.5±10	1.5±0	0.7±10	-
<i>Anabaena</i> sp. O-Ç	0.4±8	0.5±20	0.5±0	0.7±10	0.4±14.5	0.4±20	0.1±12
<i>Anabaena</i> sp. O-6	5±10	0.9±10	1.1±14	1.1±4	1.4±18	0.4±20	-
<i>Anabaena</i> sp. O-K	1.4±9	0.8±5	0.6±16	0.4±6.3	0.2±17	0.01±25	0.01±20
<i>Anabaena</i> sp. O-4	0.3±10	1.2±7	1.5±0	0.8±5	0.9±15	1±1.8	-
<i>Anabaena</i> sp. O-22	0.1±15	0.1±7	0.004±10	0.004±20	0.004±20	-	-
<i>Anabaena</i> sp. O-16	0.3±2	0.8±10	0.3±10.5	0.3±24	0.1±16	0.1±20	-
<i>Anabaena</i> sp. O-8	0.7±2.5	1.1±0	1.1±0	0.9±10	0.8±20	0.7±15	0.5±20
<i>Synechocystis</i> sp. O-X	0.2±0	0.3±10	0.4±7	0.5±14	0.2±20	-	-
<i>Gloeothece</i> sp. O-Y	0.7±0	0.6±7.8	0.7±5	0.4±18	0.3±0	0.4±10	0.4±18

(-): No effect Values are mean  $\pm$  Standard deviation.

seen in *Anabaena* sp. O-22, *Synechocystis* sp. O-X and *Anabaena* sp. O-16 in 100  $\mu\text{g}/\text{ml}$  bispyribac-sodium concentration. With the exception of *Anabaena* sp. O-K and *Anabaena* sp. O-Y, the nitrogenase activities of all other strains were inhibited at 500  $\mu\text{g}/\text{mL}$  bispyribac-sodium concentration.

The growths of *Anabaena* sp. O-X2, O-Ç, O-4, O-16, O-8 and *Synechocystis* sp. O-X were stimulated in low bispyribac-sodium concentrations, but the biomass of all cultures were severely inhibited in higher concentrations. Bispyribac-sodium experiments have shown that the initial biomass of *Anabaena* sp. O-22 at low concentrations of bispyribac-sodium (5  $\mu\text{g}/\text{ml}$ ) did not change but, the growth repressed with increasing bispyribac-sodium concentrations. The negative impacts of high bispyribac-sodium on the biomass of *Anabaena* sp. O-6 and O-K cultures were also demonstrated. The growths

of *Anabaena* sp. O-22 and *Synechocystis* sp. O-X completely repressed at 100  $\mu\text{g}/\text{mL}$  and at higher bispyribac-sodium concentrations, whereas, *Anabaena* O-X2, O-6, O-4 and O-16 completely suppressed at 500  $\mu\text{g}/\text{mL}$  bispyribac-sodium concentration (Table 2).

## DISCUSSION

Variation in growth conditions influenced the growths and nitrogenase activities of all genera. Although, the use of the herbicide is aimed at eliminating weeds, a major portion is deposited on the surface of the soil and might adversely affect the nontarget soil microflora. The nitrogen-fixing cyanobacteria are known to dominate the water-logged paddy fields and help in the nitrogen economy of rice agriculture (Singh, 1961; Stewart, 1967;

Henriksson et al., 1975). Information on resistance to herbicides, and for bispyribac in particular, is lacking. In Turkey, bispyribac-sodium is mostly used for eliminating weeds in paddy fields in the Corum- Osmancik region (THOA, 2002). For this reason, the herbicide was chosen for this study.

In this study, bispyribac-sodium stimulated nitrogenase activity of *Anabaena* sp. O-X2, O-4 and O-8 at 5 µg/ml but not in higher concentrations. It was demonstrated that *Anabaena* sp. was capable of growing both photoautotrophically and photoheterotrophically like bacteria to a great extent (Jin et al., 1996; Yan et al., 1997).

In the other cyanobacteria tested the nitrogenase activities and growths were inhibited during the initial concentration (5 µg/ml) (Table 1). Gonzalez-Barreiro et al. (2006) showed that the serious effects on growth for microalgae by herbicide added to culture medium. The main characteristic of cell death or decrease of cell viability, whether from senescence, acute stress, or aging, seems to be the loss of the ability of cells to maintain homeostasis (Gahan, 1984). Most reports have demonstrated that the inhibitory effect of herbicide became greater with an increase in herbicide concentration and suggested that the reduction in the dry matter of algae may be due to a decrease in algal photosynthesis caused by the inhibition of synthesis of chlorophyll, which is the most important pigment in algal cells for collecting solar energy for photosynthesis (Caux et al., 1996; Prospero et al., 1993).

The nitrogenase activities of *Anabaena* sp. O-K, O-6 and O-16 were more inhibited than growths. The nitrogenase activity of *Anabaena* sp. O-22 was repressed about 50% by 5 µg/ml bispyribac-sodium but growth was unaffected. In the case of *Synechocystis* sp. O-X, the herbicide also inhibited nitrogenase activity by about 50% but the dry matter increased. Powell et al. (1991) reported that nitrogenase activity was more sensitive to the isopropylamine salt of glyphosate than was photosynthetic O<sub>2</sub> evolution.

In *Anabaena* sp. strains O-X2, O-4 and O-8 it was found that very low concentrations of bispyribac-sodium (5 µg/ml) stimulated both nitrogenase activity and growth but increased concentrations repressed both nitrogenase activity and growth. The observed effects of bispyribac-sodium on cyanobacterial growth in this study are similar to those reported by Shen et al. (2005) for butachlor and acetochlor on several *Anabaena* species. Butachlor and acetochlor stimulated growth of these cyanobacteria under low concentrations (1 to 8 mgL<sup>-1</sup>), but showed high toxicity at concentrations above 16 mgL<sup>-1</sup>. Hammouda (1999), on the other hand, demonstrated the utilization of carbofuran at low concentrations by a nitrogen-fixing cyanobacterium, *Anabaena doliolum*. The 2.4D, a synthetic growth hormone analog, is reported to stimulate growth and heterocyst formation in the cyanobacterium at lower concentrations (Mishra and Tiwari, 1986). Growth studies showed that the strains used in the present study were

capable of growing both photoautotrophically and photoheterotrophically (Yan et al., 1997; Guoan et al., 1997).

The data obtained in this study provide information about the inhibitory effect of the bispyribac-sodium on growths and nitrogenase activities of cyanobacteria, which exhibits different sensitivity to the herbicide. These findings suggest a limit or avoidance of the use of bispyribac-sodium in paddy fields, due to its inhibitory effect on biological nitrogen fixation and hence a possible reduction in rice crop yields.

In this study, we have shown a clear physiologic distinction between *Anabaena* sp O-22 sp. and the other strains. Generally *Anabaena* sp O-22 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 mechanism of action of the herbicide on biological nitrogen fixation requires further study of the biochemical targets of the herbicide in cyanobacteria.

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