Bull. Environ. Contam. Toxicol. (2003) 70:1052–1058

DOI: 10.1007/s00128-003-0089-y

Bulletin of Environmental Contamination and Toxicology

Survival and Growth of Diazotrophic Cyanobacterial Isolates Exposed to Rice-Field Herbicides

S. Singh, P. Datta, R. Patel

Algal Biotechnology Laboratory, Department of Biological Science, Rani Durgavati University, Jabalpur, 482 001 (M.P.), India

Received: 26 December 2001/Accepted: 29 January 2003

Cyanobacteria (blue-green algae) are a group of prokaryotic, photosynthetic micro-organisms. The dual capacity of fixing atmospheric nitrogen and carbon makes them attractive as a source of nitrogenous biofertilizer in rice agriculture (Stewart et al. 1987). Extensive use of fertilizers and herbicides for obtaining high crop yields are reported to adversely affect the diversity and survival of cyanobacterial strains (Bunt 1961; Khan and Vaishya 1995). The extreme sensitivity of diazotrophic cyanobacteria to the toxicity of rice field herbicides is a major cause of concern for successful exploitation of cyanobacteria as biofertilizers. An ideal biofertilizer strain of cyanobacteria should have ability to tolerate or resist toxic actions of herbicides. One strategy could be to isolate and screen native cyanobacterial strains against common rice-field herbicides to evaluate relative natural tolerance and to select the most tolerant or multiple herbicide-resistant strain. Such strain(s) could be further improved and exploited in rice agriculture as an efficient source of nitrogenous biofertilizer.

Efforts have been made to screen five diazotrophic cyanobacteria isolated from the rice-field and one laboratory strain *Nostoc muscorum* against four common rice-field herbicides: Arozin, Alachlor, Butachlor and 2,4-D. The isolates exhibited differential tolerance towards these herbicides. Among them, the heterocystous diazotrophic cyanobacterium *Anabaena variabilis* was found most tolerant towards all four herbicides tested. This potential strain can be further improved for biotechnological exploitation in agriculture or in environmental remediation.

MATERIALS AND METHODS

The filamentous N_2-fixing cyanobacteria *Nostoc punctiforme*, *Nostoc calcicola*, *Anabaena variabilis* and the unicellular N_2-fixing *Gloeocapsa* sp. and *Aphanocapsa* sp. were used in the present investigation. They were isolated from a local rice field during rice growing season (Singh et al. 2000), whereas the standard laboratory strain *Nostoc muscorum* ISU (ATCC 27893) was obtained from Prof. A.K. Kashyap, B.H.U., Varanasi, India. Standard microbiological methods were followed for isolation and purification of cyanobacterial strains (Rippka et al. 1979). Cultures were grown in BG_{11} medium (Rippka et al. 1979)

Correspondence to: S. Singh

devoid of any combined nitrogen source (conveniently called N_2-medium). Cultures were incubated in an air-conditioned culture room maintained at 25± 1° C fitted with cool day fluorescent light. Photon flux density of light on the surface of the vessel was 45 $\mu Em^{-2} s^{-1}$ for 18 hr/day.

All the herbicides used were of commercial grade, Arozin (30EC): Trade name Arozin; Alachlor (45.1 EC): Trade name - Lasso; Butachlor (93.34 EC): Trade name – Machete; 2,4-D Ethyl ester (38 EC): Trade name-Slash. Arozin was obtained from Agr. Evo. Ltd. (Ankleshwar, India), Alachlor and Butachlor from Evid and Co Pesticides Pvt. Ltd. (Ankleshwar, India) and 2,4-D Ethyl ester from Monsanto Chemicals of India Ltd. (Mumbai, India). Different concentrations of the respective herbicides were prepared by appropriate dilution (according to EC) in precooled double-distilled water and were filter sterilized through a millipore membrane filter.

The effect of increasing concentrations (0-100 ppm) of herbicides on the growth and survival of cyanobacterial strains were determined by monitoring the changes in chlorophyll a at regular intervals of 24 hr for up to 16 days. N_2-grown exponential cells (5 d old) were harvested by centrifugation (3000 x g, 5 min), washed thrice with sterilized double-distilled water and dispensed equally in a series of 250 ml conical flasks containing 100 ml sterile N_2-medium. Graded concentrations of filter sterilized herbicides were added. Cultures without herbicide treatment were treated as the control. At indicated intervals, aliquots were withdrawn in triplicate for extraction and estimation of chlorophyll a pigment in methanol spectrophotometrically, following the method of Mackinney (1941). The experiment was repeated thrice and data were analyzed statistically by calculating standard deviation (±SD). The 50% inhibitory growth concentrations for the isolates were determined by monitoring survivability of cells on plates containing graded concentrations of herbicides. The percentage survival of the isolates were calculated by the method:

$$\frac{\text{Number of colonies on the herbicide treated plates}}{\text{Number of colonies on the untreated control plates}} \times 100$$

The concentration of the herbicide at which 50% of colonies survived as compared to untreated control culture was termed the IGC_{50} and the concentration at which no colonies survived (complete lysis) was considered the lethal concentration. The experiment was repeated thrice and every time five sets of plates were taken for each herbicide concentration. The data was analyzed by computer program and the means and standard errors were determined.

RESULTS AND DISCUSSION

The survival potential of cyanobacterial isolates under herbicide(s) stress was monitored by exposing N_2 - grown cultures to graded concentrations of herbicides

(Arozin, Alachlor, Butachlor and 2,4-D). The results are shown in Tables 1-2. Cyanobacterial isolates showed gradual but substantial inhibition in growth with increasing concentrations of herbicides. IGC_{50} concentrations of herbicides for each strain are given in Table 1. Complete lysis (lethal concentration) occurred in the cultures treated with 10 mg L^{-1} (*Aphanocapsa* sp.), 15 mg L^{-1} (*Nostoc muscorum; Gloeocapsa* sp.), 20 mg L^{-1} (*N. punctiforme, N. calcicola*), 25 mg L^{-1} (*A. variabilis*) of Arozin; 20 mg L^{-1} (*Aphanocapsa* sp; *Gloeocapsa* sp.; *N. muscorum*), 25 mg L^{-1} (*A. variabilis; N. punctiforme; N. calcicola*) of Alachlor; 15 mg L^{-1} (*N. muscorum*), 20 mg L^{-1} (*Aphanocapsa* sp.; *N. calcicola*), 25 mg L^{-1} (*A. variabilis*; *N. punctiforme*; *Gloeocapsa* sp.) of Butachlor; 10 mg L^{-1} (*Aphanocapsa sp.*), 15 mg L^{-1} (*Gloeocapsa* sp.; *N. muscorum),* 25 mg L^{-1} (*A. variabilis; N. punctiforme; N. calcicola*) of 2,4-D.

Significant variations in the relative sensitivity of the diazotrophic cyanobacterial isolates to the growth toxic effects of the herbicides tested under laboratory conditions, seems to result from interactions between mode of herbicidal actions with morphological, physiological, biochemical and genetic properties of cyanobacteria. Herbicides are reported to affect cyanobacteria in various ways (Anand and Veerappan 1980; Irisarri et al 2001). Herbicides have differential effects on various metabolic processes and the sensitivity of the strain varies depending upon the species, kind of herbicides and chemical formulations (Powell et al. 1991; Anand and Subramanian 1997; Fairchild et al. 1998).

Table 1 IGC_{50} values (mgL^{-1}) of herbicides for cyanobacterial isolates.

Cyanobacterial Isolates	Arozin	Alachlor	Butachlor	2,4-D
1. *N.muscorum*	4.9 ± 0.15	15.0 ± 0.07	10.4 ± 0.39	10.5 ± 0.69
2. *N. punctiforme*	4.7 ± 0.31	15.0 ± 0.08	14.8 ± 0.27	9.9 ± 0.09
3. *N. calcicola*	5.0 ± 0.06	14.8 ± 0.49	9.7 ± 0.45	9.7 ± 0.48
4. *A.variabilis*	9.9 ± 0.24	15.4 ± 0.36	15.0 ± 0.17	14.8 ± 0.48
5. *Gloeocapsa* sp.	5.0 ± 0.17	15.2 ± 0.07	10.0 ± 0.08	5.0 ± 0.09
6. *Aphanocapsa* sp.	5.1 ± 0.10	14.8 ± 0.35	10.3 ± 0.32	5.0 ± 0.18

The data is an average (± SEM) of three independent experiments.

Among the four herbicides tested Arozin showed the most deleterious effect on the growth of the cyanobacterial isolates. The maximum reduction in chlorophyll a content was recorded in *N. muscorum* ISU (Arozin-66.6%, Alachlor-63%, Butachlor-67.5%, 2,4-D-61.7%) and the lowest in *Anabaena variabilis* (Arozin-29.7%, Alachlor-21.3%, Butachlor-48%, 2,4-D-25.7%) at IGC_{50} concentrations by the end of 8^{th} day of growth. A more or less similar pattern of growth inhibition could be observed (Table 2) for other herbicides, i.e., 2,4-D, Butachlor or Alachlor. In the present study, chlorophyll synthesis was found to be severely affected in graded concentrations and complete lysis of cultures occurred between 10 - 25 mg L^{-1} of Arozin in different isolates. On the contrary, stimulation in chlorophyll a synthesis up to 10 mg L^{-1} and survival up to 100 mg L^{-1} of Arozin in *A. variabilis* ARM 310 but not in *Tolypothrix tenuis* ARM 76 was reported by Goyal et al. (1991). It is also clear from the results that among the isolates, the

unicellular diazotrophs *Gloeocapsa* sp. and *Aphanocapsa* sp. are more sensitive to Arozin than multicellular heterocystous forms. In graded concentrations of 2,4-D, cultures survived between 10 - 25 mg L^{-1} as compared to 175 mg L^{-1} for

Table 2 Effects of graded concentrations of herbicides on percent inhibition of chlorophyll a content of cyanobacterial isolates at the end of 8th day of diazotrophic growth.

Cyanobacterial Isolates	Herbicides (mgL^{-1})				
	Arozin				
	5.0	10.0	15.0	20.0	25.0
1. *N. muscorum* ISU	67 ± 1.0	75 ± 2.0	99 ± 1.5	ND	ND
2. *N. punctiforme*	32 ± 0.6	49 ± 1.0	78 ± 1.0	89 ± 2.6	ND
3. *N. calcicola*	45 ± 2.6	51± 2.0	64 ± 1.7	87 ± 1.7	ND
4. *A. variabilis*	30 ± 0.6	30 ± 1.0	51 ± 1.0	59 ± 1.0	ND
5. *Gloeocapsa* sp.	65 ± 0.6	81 ± 1.0	92 ± 2.6	ND	ND
6. *Aphanocapsa* sp.	62 ± 2.5	89 ±1.0	95 ± 2.0	97 ± 1.0	ND
	2,4-D				
	5.0	10.0	15.0	20.0	25.0
1. *N. muscorum* ISU	58 ± 2.6	61 ± 0.6	87 ± 0.6	ND	ND
2. *N. punctiforme*	30 ± 1.5	27 ± 2.5	35 ± 1.0	46 ± 2.0	83 ± 1.0
3. *N. calcicola*	32 ± 0.6	34 ± 2.6	44 ± 2.6	66 ± 0.6	85 ± 0.6
4. *A. variabilis*	21 ± 0.6	31 ± 2.0	26 ± 2.0	61 ± 3.2	86 ± 1.0
5. *Gloeocapsa* sp.	53 ± 2.0	73 ± 1.7	78 ± 2.0	ND	ND
6. *Aphanocapsa* sp.	59 ± 2.6	85 ± 2.6	94 ± 2.3	94 2.0	ND
	Butachlor				
	5.0	10.0	15.0	20.0	25.0
1. *N. muscorum* ISU	45 ± 2.6	67 ± 2.0	84 ±1.7	96 ± 1.5	ND
2. *N. punctiforme*	20 ± 1.5	20 ± 2.0	42 ± 2.0	84 ± 2.0	ND
3. *N. calcicola*	41 ± 1.0	57 ± 1.7	64 ± 2.6	85 ± 0.6	ND
4. *A. variabilis*	13 ± 0.6	43 ± 3.0	48 ± 2.0	72 ± 2.5	95 ± 1.0
5. *Gloeocapsa* sp.	15 ± 2.6	21 ± 1.1	54 ± 1.7	62 ± 1.0	83 ± 2.0
6. *Aphanocapsa* sp.	46 ± 2.3	49 ± 0.6	73 ± 1.5	83 ± 2.6	ND
	Alachlor				
	5.0	10.0	15.0	20.0	25.0
1. *N. muscorum* ISU	42 ± 1.0	71 ± 1.7	63 ± 1.0	85 ± 0.6	ND
2. *N. punctiforme*	14 ± 1.0	20 ± 1.0	23 ± 0.6	69 ± 1.6	69 ±1.0
3. *N. calcicola*	20 ± 0.6	29 ± 1.7	32 ± 2.5	39 ± 1.0	ND
4. *A. variabilis*	23 ± 1.7	46 ± 1.7	21 ± 2.0	63 ± 0.6	ND
5. *Gloeocapsa* sp.	15 ± 1.0	36 ± 2.0	36 ± 1.7	67 ± 1.0	87 ±1.0
6. *Aphanocapsa* sp.	49 ± 2.0	48 ± 1.5	47 ± 1.7	86 ± 2.0	ND

The data is an average (±SD) of three independent experiments.
ND = not detectable.

Gloeocapsa sp. (Tozum and Sivaci 1993), 800 mg L^{-1} for *Anabaenopsis raciborskii* (Das and Singh 1977) and 10mM for *Anabaena* UAM 202 (Leganes and Fernandez-Valientl 1992). At the increasing concentration of 2,4-D, growth inhibition in isolates of *Gloeocapsa* sp. and *Aphanocapsa* sp. along with the standard laboratory strain *N. muscorum* was more severe than other isolates (Table 2). *Gloeocapsa* sp. on the other hand exhibited higher tolerance to Butachlor (Machete) as compared to other isolates. Similarly another isolate of *Gloeocapsa* sp. showed only partial inhibition of growth and photosynthesis upon exposure to Butachlor but at much higher concentration, i.e., 100 mg L^{-1} (Singh et al. 1986). Lethal concentration (25 mg L^{-1}) of Butachlor for *A. variabilis* is much higher than reported earlier i.e., 1.0 mg L^{-1} for *A. variabilis* (Pandher et al. 1994) isolated from different agro-climatic zones. Alachlor proved to be less toxic to cyanobacterial strains as compared to Arozin, Butachlor and 2,4-D (Table 2). The lethal concentrations of Alachlor for these strains range between 20 - 25 mg L^{-1} which is however, very low as compared to 80 mg L^{-1} for *A. doliolum, N. muscorum* and *Aphanothece stagnina* (Singh et al. 1978). The overall sensitivity of cyanobacterial isolates and the standard laboratory strain towards all four herbicides tested declined in the following order:
N. muscorum ISU > *Aphanocapsa* sp. > *Gloeocapsa* sp. > *N. calcicola* > *N. punctiforme* > *A. variabilis.*

Highest sensitivity of the laboratory strain *N. muscorum*, as compared to rice-field isolates, confirm our notion that strains which are exposed to periodic applications of herbicides are more tolerant and could be an ideal source of any herbicide resistant gene pool. Among the natural isolates, unicellular nitrogen fixing cyanobacteria, i.e., *Aphanocapsa* sp. and *Gloeocapsa* sp., are less tolerant to toxic actions of the herbicides tested as compared to filamentous heterocystous nitrogen fixers, i.e., *N. calcicola, N. punctiforme* and *A. variabilis.* Such differential tolerance between unicellular and multicellular cyanobacteria could not be assigned to any one factor but needs in-depth investigation. The variations in survival potential and lethal dosages of herbicides for different strains, either laboratory or natural, suggest the presence of differing degrees of inherent natural tolerance among strains from diverse ecosystems. Such reported variations also necessitate comprehensively conducting herbicide toxicity experiments with all the potential strains originating from various sources under similar sets of conditions to understand the basis of such variations.

The present finding further suggests that *A. variabilis* is the most tolerant to all four herbicides tested and a promising strain as an ideal cyanobacterial biofertilizer inoculant to improve soil fertility and crop productivity. Natural diazotrophic cyanobacterial strain(s) will have more potential to establish and grow in the field and serves as a biofertilizer.

Acknowledgments. We thank the Head, Department of Biological Sciences, R.D.University Jabalpur and U.G.C. New Delhi for financial assistance.

REFERENCES

Anand N, Subramanian TD (1997) Effect of certain pesticides on the physiology of *Nostoc calcicola*. Phykos 36: 15-20

Anand N, Veerappan B (1980) Effect of pesticides and fungicides on blue green algae. Phykos 19: 210-212

Bunt JS (1961) Nitrogen fixing blue green algae in Australian rice soils. Nature 192: 479-480

Das B, Singh PK (1977) The effect of 2,4-D on growth and nitrogen fixation of bluegreen alga *Anabaenopsis raciborskii*. Arch Environ Contam Toxicol 5: 437-445

Fairchild JF, Ruessler DS, Carlson AR (1998) Comparative sensitivity of five species of macrophytes and six species of algae to atrazine, metribuzin, alachlor and metachlor. Environ Toxicol Chem 17: 1830-1834

Goyal D, Roy Choudhury P, Kaushik BD (1991) Effect of two new herbicides on the growth and nitrogen fixation in *Anabaena* and *Tolypothrix*. Acta Bot Indica 19: 25-28

Irrisari P, Gonnet S, Monza J (2001) Cyanobacteria in Uruguayan rice fields: diversity, nitrogen fixing ability and tolerance to herbicides and combined nitrogen. J Biotechnol 91: 95-103

Khan AH, Vaishya RD (1995) The sensitivity of blue green algae to paddy herbicides. Weed News 2: 28-30

Leganes F, Fernandez-Valientl E (1992) Effect of phenoxy acetic herbicides on growth photosynthesis and nitrogenase activities in cyanobacteria from rice field. Arch Environ Contam Toxicol 22: 130-134

Mackinney G (1941) Absorption of light by chlorophyll solutions. J Biol Chem 140: 315-325

Pandher MS, Gurmehar K, Gosal SK, Gupta RP, Kaur G (1994) Effect of herbicides on the growth and nitrogen fixation by blue green algae. Res Develop Rep 11: 9-13

Powell HA, Kerby NW, Rowell P (1991) Natural tolerance of cyanobacteria to the herbicide glyphosate. New Phytol 119: 421-426

Rippka R, Dervelles J, Waterbury JB, Herdman M, Stainer RY (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111: 1-61

Singh LJ, Tiwari DN, Singh HN (1986) Evidence for genetic control of herbicide resistance in a rice field isolate of *Gloeocapsa sp.* capable of aerobic diazotrophic under photoautotrophic conditions. J Gen Appl Microbiol 32: 81-88

Singh S, Datta P, Patel R (2000) Cyanobacterial flora and properties of rice field soils of Jabalpur and Katni districts of Madhya Pradesh. Phykos 39: 135-140

Singh VP, Singh BD, Singh RB, Dhar B, Singh RM, Shrivastava JS (1978) Effect of herbicide Alachlor on growth and nitrogen fixation in cyanobacteria and rhizobia. Indian J Exp Biol 16: 1325-1327

Stewart WDP, Rowell P, Kerby NW, Reed RH, Machray GC (1987) A century of nitrogen fixation. Phil Trans R Soc London 317: 245-258

Tozum Calgan SRD, Sivaci Guner S (1993) Effects of 2,4-D and methylparathion on growth and nitrogen fixation in cyanobacterium, *Gloeocapsa*. Internat J Environ studies 43: 307- 311