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Use of Cyanobacterial Diazotrophic Technology in Rice Agriculture

Scientific Note

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Index Entries: Diazotrophic cyanobacteria; herbicide resistance; ammonia secretion; rice culture.

INTRODUCTION

Diazotrophic cyanobacteria are photoautotrophic organisms that require sunlight as a sole energy source for the fixation of carbon and nitrogen (*1*). Therefore, they have great potential as biofertilizers, and their use will decrease fuel demand for fertilizer production (*2*). The agronomic potential of heterocystous cyanobacteria, either free-living or in symbiotic association with water fern *Azolla*, has long been recognized (*3-6*). This has led to the development of small scale biotechnology involving the use of paddy soils with appropriate cyanobacterial strains as biofertilizers in rice culture, as has been reported from China (*7*), Egypt (*8*), Philippines (*9*), and India (*10,11*). Besides increasing soil fertility and sustaining rice yield, these forms are also reported to benefit rice seedlings by producing growth-promoting substances, the nature of which is said to resemble gibberellins (*12*). Whereas the incorporation of *nif* genes into the rice plants by using tissue culture and modern genetic tools remain one of the ambitious research goals, the use of cyanobacterial diazotrophic technology in rice agriculture offers an immediate or even long-term alternative to synthetic nitrogen fertilizers, particularly in developing countries and the world as a whole. However, one of the weaknesses in this technology is the heavy application of several toxic agrochemicals, especially herbicides, which are reported in most cases as inhibitors of cyano-

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bacterial diazotrophic growth (*13,14*), and in some cases as mutagenic (*15*). Naturally, a successful biotechnology requires the selection of suitable diazotrophic strains, as biofertilizers, that could tolerate the field-dose concentrations of herbicides and secrete ammonia.

Ammonia is the primary product of biological nitrogen fixation, and up to 90% of the nitrogen fixed can be released extracellularly if glutamine synthetase (GS), the primary enzyme of ammonia assimilation, is inhibited by glutamate analogues, such as L-methionine-D,L-sulphoximine (MSX) (*16*). An alternative to the use of such expensive chemical, MSX, for the induction of ammonia liberation is the use of mutant strains deficient in GS activities. Such mutant strains of heterocystous cyanobacteria defective in ammonia assimilation have been isolated, and their immobilized cells have been found suitable for the continuous photoproduction of ammonia (*17*), and thereby supporting the growth of rice plants in the absence of combined nitrogen. However, such ammonia secreting strains may not serve the practical purpose, because of their extreme sensitivity to most of the herbicides (*18,19*). Therefore, such strains, when combined with resistance to some of the commonly used herbicides, should be environmentally suitable and doubly advantageous for field application. In this paper, we describe some of our preliminary results on the isolation and development of nonheterocystous cyanobacteria that are resistant to some rice herbicides, and are able to secret ammonia produced by the fixation of dinitrogen.

MATERIALS AND METHODS

Organisms and Culture Conditions

The clonal and axenic isolate of *Gloeocapsa* (*20*) (also called *Gloeothece*), the isolates of the local rice fields of Varanasi, was grown in pure cultures in combined nitrogen-free, modified Chu-10 medium (*21*) in 100 mL batch cultures at 26°C and under illumination (14:10 h light–dark cycle) with fluorescent light intensity of nearly 45 μE m^{-2} s^{-1}. For ammonia grown cultures, the growth medium used was supplemented with 1 m*M* ammonium chloride, and pH was maintained at 7.6 with HEPES buffer (2.5 m*M*).

Herbicides

Two preemergence herbicides, butachlor (2-chloro-2′,6′-diethyl-*N* (butoxymethyl) acetanilide; active ingredient 0.5 g mL^{-1}) and fluchloralin (*N*-propyl-*N*-(2′-chloroethyl) 2,6-dinitro-*N*-trifluromethyl analine; active ingredient 0.4 g mL^{-1}), and one postemergence herbicide, propanil (3′,4-dichloropropionanilide; active ingredient 0.36 g mL^{-1}) were used as common rice-field herbicides in the present work. These herbicides were obtained from Monsanto Chemicals Pvt, Ltd. (MO), BASF Aktiengesell-

schraft (FRG), and Indofil Chemicals Ltd. (India), respectively. Differing concentrations of the respective herbicides were prepared by appropriate dilution in double-distilled water, and filter-sterilized through membrane filters (size 0.22 μM; Millipore Filter Corp., Bedford, MA), and finally added to the precooled medium at desired concentrations. The herbicide DCMU (3-(3,4-dichlorophenyl)-1,1 dimethylurea), obtained from Sigma Chemical Co., St. Louis, MO, and propanil were dissolved in absolute ethanol. The final concentration of ethanol used in the growth medium was 0.5% v/v. Control sets were also added with similar concentration of ethanol to neutralize any side effects.

Selection of Herbicide Resistant Strains

Cyanobacterial samples collected from the local rice plots were homogenized, washed, and diluted in sterilized growth medium, and aliquots (0.2 mL) were spread on agar nutrient plates, supplemented or not with 25 $\mu g\ mL^{-1}$ of one of the herbicides, butachlor, fluchloralin, and propanil, used singly or collectively. After 15 d of incubation, these plates were examined for cyanobacterial colonies under the binocular microscope.

Selection of Ammonia Secreting Mutants

Log cultures of *Gloeocapsa,* resistant to butachlor and fluchloralin (*20*); were treated with 100 $\mu g\ mL^{-1}$ of nitrosoguanidine (NTG) at pH 8.5 (buffered with 20 m*M* Tris-HCl) and 30°C. After thorough washing, the cultures were allowed to multiply and segregate for 15 d, and finally screened on the agar nutrient plates, supplemented with 15 $\mu g\ mL^{-1}$ MSX. Several clones, presumed to be MSX resistant, were picked up and individually grown in the medium containing 100 $\mu g\ mL^{-1}$ glutamine. Clone number GS-9, GS-13, GS-21, and GS-22 were finally selected as stable clones under culture conditions, and their ability to liberate ammonia in the N_2-fixing medium was evaluated.

Acetylene Reduction

Nitrogenase activity of cyanobacterial cultures (in vivo) was measured by the method of Stewart and Lex (22), using acetylene reduction. The ethylene produced from acetylene reduction by the cultures was analyzed on a HP 5840 Gas Chromatograph with Stainless Poropack N Column, where nitrogen gas served as carrier. The analysis was performed at 100°C, and the nitrogenase activity was expressed as n mol ethylene produced/μg Chl $a^{-1}h^{-1}$.

Ammonia Estimation

Ammonia liberated in culture medium was measured colorimetrically by the color development with Nessler's reagent. The results were expressed in terms of $\mu mol\ mg^{-1}$ cell protein.

In Vivo Glutamine Synthetase Activity

Glutamine synthetase activity was measured in terms of Mn^{2+}-dependent *r*-glutamyl hydroxamate produced during the reaction, following the methods of Sampio et al. (*23*).

Chlorophyll *a* and Protein Estimation

The chlorophyll contents of cultures were estimated by the method of Mackinney (*24*). The Lowry method was used to measure cell protein, with bovine serum albumin as standard.

Growth

Growth of homogeneous suspension cultures was measured either turbidometrically with a Photochem Colorimeter (MK 111) using red filter (650 nm), or by counting the number of cells using a hemocytometer.

RESULTS AND DISCUSSION

Diazotrophic cyanobacteria, being the O_2-evolving procaryotes and the major components of wetland rice ecosystems, serve as the easily available and cheapest sources of natural biofertilizers. Butachlor and other related α-chloroacetamides are reported to exert their inhibitory effects primarily on protein synthesis in weed plants (*25*) and cyanobacteria (*26*), whereas the mode of action of fluchloralin is not known. Trifluralin, closely related to fluchloralin, has been reported as a growth inhibitor of higher plants, by interfering with the synthesis and assembly of microtubules and RNA synthesis (*27*). In contrast, propanil is reported as an inhibitor of photosynthesis (*28*), like that of DCMU, which is known to interfere with PS II system (*29*).

A strain of *Gloeocapsa* from rice-fields, which is extremely resistant to butachlor and fluchloralin and efficient to diazotrophy under aerobic conditions, has been reported (*20*). The rich population of *Gloeocapsa* found in rice-fields treated with field doses of butachlor (20 μg mL^{-1}) and fluchloralin (15 μg mL^{-1}) prompted us to examine its resistance to these herbicides under laboratory conditions. This unicellular cyanobacterium with firm sheath (Fig. 1) was selected by growth on agar medium containing the desired concentration of butachlor (25 μg mL^{-1}) or fluchloralin (25 μg mL^{-1}), or both. None of the heterocystous forms could be selected for isolation under similar conditions. In contrast, none of cyanobacterial species produced colonies in the presence of propanil. The strain of *Gloeocapsa* exhibited resistance to the field doses of butachlor and fluchloralin, which far exceeded that of the field application rates, with the exception of fluchloralin. Propanil inhibited the diazotrophic growth even at concentration (10 μg mL^{-1}) lower than the field dose rate (35 μg mL^{-1}).

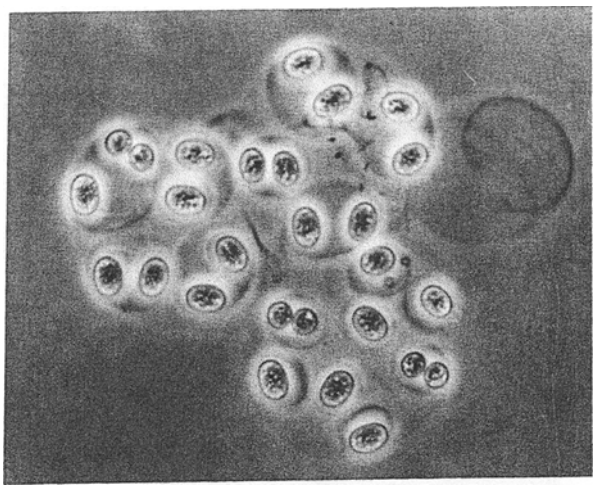

Fig. 1. Photomicrograph of *Gloeocapsa* showing firm sheath around each cell or group of cells (×500).

Table 1
Growth and Nitrogenase Activity of *Gloeocapsa* (Her^r)
Under the Herbicide Stress (24 h treatment)

Herbicides (μg mL^{-1})	Optical density (650 nm)	Chl *a* (μg mL^{-1} culture)	Nitrogenase activity (nmol C_2H_4 μg Chl a^{-1} h^{-1})
Control	0.185	3.250	16.90
Butachlor (25)	0.100	3.240	18.26
Fluchloralin (25)	0.095	3.248	26.20
Butachlor (25) + Fluchloralin (25)	0.085	3.242	20.20
Propanil (25)	0.030	0.410	0.00
DCMU (15)	0.020	0.368	0.00

Results are averages of three independent experiments.

Table 1 shows the effect of these herbicides on diazotrophic growth and acetylene reduction by *Gloeocapsa* under photosynthetic conditions. Growth in terms of Chl *a* (acetone extract) and nitrogenase activity were normal in the presence of butachlor or fluchloralin, either individually or in combination, compared to that of control cultures. Addition of these herbicides into 10 d-grown log cultures caused clumping of cells into small balls, and direct measurement of optical density of such cultures gave growth difference from control cultures which produced homogeneous suspension of cultures. Propanil and DCMU did not cause clumping of cells, but drastically reduced both the processes. It is interesting to note that unlike the former herbicides, these two herbicides are inhibitors of higher plants photosynthesis (*27*). Further experiments on nitrogenase activity with these herbicides have shown that propanil had little effect

Table 2
Nitrogenase Activity of *Gloeocapsa* Under Varied Growth Conditions
(nmol C_2H_4 μg Chl a^{-1} h^{-1})

Time (h)	Light	Dark	DCMU + light	Propanil + light	DCMU + glucose + light	Propanil + glucose + light	Glucose + dark
0	12.60	11.50	11.58	12.11	11.78	12.22	12.11
4	16.80	6.09	6.80	5.85	13.13	11.50	6.52
8	20.62	1.02	2.16	1.35	12.85	12.20	4.32
12	18.58	0.00	0.00	0.00	13.22	11.44	2.15

Results are averages of three independent experiments. 100 μg of glucose added in each case.

on nitrogenase activity during the 4 h following its addition; inhibition was observed only in the longer term treatments (Table 2). Similar results were noted in cultures either treated with DCMU or incubated in dark, where nitrogenase activity decreased with time reaching to zero after 12 h. The eventual inhibition of nitrogen fixation by propanil or DCMU does suggest that PS II supplies reductant for nitrogenase activity. If the photosystem II was directly supplying reductant from water, the effect of DCMU or propanil should be immediate. It appears that these herbicides inhibit nitrogen fixation indirectly by inhibiting photosynthetic CO_2 assimilation. As long as intracellular carbon reserves are available, dinitrogen fixation can be supported, but once they are exhausted, nitrogenase activity stops. This hypothesis is supported by the ability of glucose to prevent the gradual inhibition of nitrogen fixation by both DCMU and propanil; glucose serves as an energy source itself. The persistent nitrogenase activity in light and glucose is consistent with report of others, in which photoheterotrophic assimilation of glucose by cyanobacteria has been demonstrated to generate ATP and reductants to support growth and nitrogenase activity, albeit at reduced rate, compared to that of control cultures (*30*). In dark, glucose could not support nitrogenase activity significantly. Using batch cultures of *Gloeothece* (*Gloeocapsa*) spp., Gallon et al. (*31*) demonstrated the independence of photosynthesis and nitrogen fixation, the two processes taking place in the same cells, during the light and dark periods of growth, respectively. Under continuous light, the temporal separation of these two processes during the cell cycle is the mechanism by which these cells can grow photoautotrophically under aerobic dinitrogen fixation conditions (*32*).

Strains of diazotrophic cyanobacteria deficient in GS activity are important for the production of extracellular ammonia. GS deficient strains can be easily obtained by the selection of MSX-resistant colonies that

Table 3
Photoproduction of Ammonia by GS-defective Strains of *Gloeocapsa* (Her^r)

Strains	Rate of ammonia produced (μmol mg Chl a^{-1} h^{-1})		
	Control	Butachlor (50 μg mL^{-1})	Fluchlorolin (50 μg mL^{-1})
Parent (Her^r)	—	1.2	—
GS-9	18.0	17.0	20.4
GS-13	16.5	18.2	18.8
GS-21	16.9	17.6	17.5
GS-22	17.5	16.8	17.5

Results are averages of three independent experiments.

Table 4
Glutamine Synthetase Activity (In Vivo) of the Various Strains of Gloeocapsa

Strains	GS-transferase activity (nmol *r*-glutamylhydroxamate mg protein^{-1} min^{-1})		
	Control	Butachlor	Fluchlorolin
Parent (Her^r)	907.2	1015.20	1090.7
GS-9	260.2	210.0	244.0
GS-13	288.5	180.0	210.4
GS-21	230.8	211.0	221.6
GS-22	294.1	233.5	247.7

liberate ammonia (*33*). All the MSX-resistant isolates of *Gloeocapsa*, GS-9, GS-13, GS-21, and GS-22, were found capable of releasing high level of ammonia in N_2-medium, even in the presence of butachlor or fluchloralin, when compared to the parent strain that is resistant only to butachlor and fluchloralin (Table 3). Stock cultures of GS-defective strains were maintained in the growth medium containing glutamine (100 μg mL^{-1}). The ammonia liberating property of MSX-resistant strains of *Gloeocapsa* confirms that they are GS-defective mutants, and they do not represent mutation to MSX-transport. MSX resistance can also result from impaired transport of MSX into the cyanobacterial cells (*17*). The GS activity of the mutants studied is much decreased when compared with the parent strain, as measured by transferase activities (Table 4). Reduction of GS-transferase and biosynthetic activities in similar type mutants of *Anabaena variabilis* (AICC 29413) has been reported (*17,34*). However, such strains of heterocystous cyanobacteria are, no doubt, more beneficial than *Gloeocapsa* in

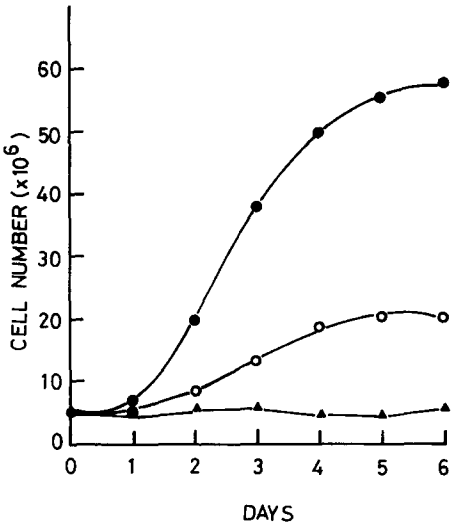

Fig. 2. Growth behavior of the nondinitrogen fixing cyanobacterium *Anacystis nidulans* in presence of NH_4^+ (●), N_2 (△), and *Gloeocapsa*-biofertilizer (○).

terms of specific rate of ammonia production, but their extreme sensitivity to the rice-field herbicides (butachlor and fluchloralin) raises doubt about their practical applicability. Therefore, the combination of GS-defective mutation with herbicide resistance in the diazotrophic cyanobacterium, *Gloeocapsa,* is of greater importance for field application as suitable biofertilizers.

Although we have not evaluated the impact of the present strains of *Gloeocapsa* in field trials, its ammonia liberating effect has been found to support photosynthetic growth of another unicellular cyanobacterium, *Anacystis nidulans,* that is incapable of dinitrogen fixation. This experiment was performed involving both ammonia liberating mutant of *Gloeocapsa* and *A. nidulans* in mixed cultures in the absence of combined nitrogen. Growth of *A. nidulans* was much better in the presence of combined ammoniacal nitrogen (1 m*M*) than the growth observed in the presence of the *Gloeocapsa*-biofertilizer (Fig. 2). The absence of both nitrogen sources prevents growth. Growth of *A. nidulans,* supported by the *Gloeocapsa*-biofertilizer, continued up to 5 d and further remained constant. It appears that such biofertilizers are able to liberate ammonia in N_2-medium at a slow rate with limited period of activities.

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