Engineering of mutants of Anabaena *PCC7120 (cyanobacterium) constructed by DNA-plasmid transfer and UV-radiation resistant to the herbicide Diuron capable of ammonia production*

✉ Mahasneh IAK [1, 3], Tiwari DN[2], AL-Thani RF[3], Qandeel M[1, 3]

[1]Biotechnology laboratory, Department of Biological Sciences, Al al-Bayt University, PO Box 130040, Mafraq City, Jordan
[2]Center of Advance Study in Botany, Banaras Hindu University, Varansi 221005, India
[3]Department of Biological Sciences, Faculty of Science, University of Qatar, PO Box 2713, Doha City, Qatar
Phone: +974-4852132 (ext. 1148), 4794655, mobile: 5381560;
Fax: +974-4835111; E-mail: Mahasneh2002@yahoo.co

ABSTRACT

Ten mutants of *Anabaena* PCC7120 were successfully constructed to express two genetic traits, ammonia production and the resistance to the Diuron herbicide, using transposon induced mutagenesis and UV-radiation. The ranges for heterocyst frequency and ammonia production of mutants were 4.2%-6.8% and 2.3-6.6 μmol mg dry wt^{-1} h^{-1} respectively. The herbicide-resistance threshold for all mutants was 2.0 mg L^{-1}, above which the mutants started to decline in health as observed microscopically. The dry weight of the mutants reached 300-400 mg L^{-1} with growth patterns similar to those of the wild-type after 20 days of growth in a batch culture in the presence of Diuron. In contrast, the addition of herbicide to the wild-type has led to a drastic reduction in O_2-evolution followed by cellular vaculation, breakdown of filaments and cell death within 24 h (data not shown; microscopic inspection).

Keywords: DNA-plasmid transfer, ammonia production, herbicide-resistant, *Anabaena*

Biotecnología Aplicada 2004;01:13-15

RESUMEN

Ingeniería de mutantes de Anabaena PCC7120 (cianobacteria) resistentes al herbicida Diuron y productores de amoníaco construidos por transferencia de ADN plasmídico y radiación UV. Diez mutantes de Anabaena PCC7120 se construyeron con éxito para expresar dos rasgos genéticos, producción de amoníaco y la resistencia al herbicida Diuron, usando mutagénesis inducida por transposon y radiación UV. Los rangos de frecuencia para la heterocistesis y la producción de amoníaco de los mutantes fueron 4.2%-6.8% y 2.3-6.6 μmol mg de peso $seco^{-1}$ h^{-1} respectivamente. El umbral de resistencia a herbicida para todos los mutantes fue de 2.0 mg L^{-1}, por encima de éste los mutantes empezaron a decaer en viabilidad, como se observó microscópicamente. El peso seco de los mutantes alcanzó de 300-400 mg L^{-1} con patrones de crecimiento similares a aquellos del tipo salvaje después de 20 días de crecimiento en un medio de cultivo en presencia de Diuron. En contraste, la adición de herbicida al tipo salvaje llevó a una reducción drástica en la evolución del O_2, seguido por la vaculación celular, rompimiento de filamentos y muerte celular dentro de las 24 h (no se muestran datos; inspección microscópica).

Palabras claves: Transferencia de ADN plasmídico, producción de amoníaco, resistente a herbicidas, *Anabaena*

*I*ntroduction

Cyanobacteria as N_2-fixers are widely spread around all environments particularly in the rice fields where they can be used as biofertilizer [1, 2]. The biotechnological applications of cyanobacteria have always faced major obstacles since they are unable to externally excrete the fixed-N in the field [3, 4, 5]. To overcome this, several attempts have been made to create cyanobacterial mutants which can excrete ammonia externally. These have been carried out using UV-radiation [6], plasmid transfer [7] and the manipulation of the key genes (HetR and nif) in the nitrogen-fixation process [8, 9]. The suitability of the strains as biofertilizers is also restricted by their inability to excrete ammonia as well as to resist herbicides, which are used against weeds in the rice fields [2]. In our previous work [5] we showed that mutants AK101 and AK102 of *Anabaena* PCC7120 were able to excrete ammonia but were not herbicide–resistant, which limited their application as biofertilizers. The aim of the present study is to engineer cyanobacterial mutants capable of ammonia production and at the same time herbicide-resistant.

*M*aterials and methods

Cyanobacterial culture and growth conditions

The wild-type strain of *Anabaena* PCC7120 (ATCC27893) was obtained from Wolk CP, Michigan State University, USA. Cultures of the wild-type and mutants strains were grown photoautotrophically at 30 °C in a Chu-(10)-N medium as described previously [5]. The morphological changes of the cyanobacterial cultures under several treatments were inspected microscopically to examine possible cell damage. Growth of both wild-type and mutants were determined and expressed as dry weight (dry wt mg L^{-1}).

1. Mahasneh IA, Tiwari DN (1992). The use of biofertilizer of *Calothrix* sp. M103, enhanced by addition of iron and siderophore production. J. Appl. Bacteriol. 73:286-9.

2. Whitton BA (2000) Soils and rice-fields. In: Whitton BA, Potts M, eds. The Ecology of Cyanobacteria. The Netherlands: Kluwer Academic Publishers:233-55.

3. Thomas SP, Zaritsky A, Boussiba S, (1990). Ammonium excretion by an L-methionine-DL-sulfoximine-resistant mutant of the rice field cyanobacterium *Anabaena siamensis*. Appl. Environ. Microbiol. 56: 3499-504.

4. Singh S, (1992). Photobiological production of ammonia by an L-methionine-DL-sulfoximine-resistant mutant of cyanobiont *Nostoc* ANTH. Indian J. Exp. Biol. 30:284-7.

✉ Corresponding author

Construction of mutants

Herbicide-resistant mutants were constructed using a UV-induction program and the diuron herbicide [3-(3,4-dichloropheny)-l,1-dimethylurea); a.i. 98.6%] (DCMU). The screening and selection of herbicide-resistant mutants were carried out by plating serial dilutions of the wild-type on the Chu-(10)-N medium petridishes which were then exposed to the UV radiation program including time and distance. After the incubation period of the inoculated plates, some of the few colonies (which appeared yellow in color) of the herbicide-resistant mutants were selected and grown in liquid medium of Chu-(10)-N medium containing the corresponding concentration of herbicide Diuron to that of the plates. Thereafter the mutant cultures which showed healthy growth (compared to the wild-type and after the microscopic inspection) were maintained viable as herbicide-resistant mutants of Anabaena PCC7120.

Mutants for ammonia production were constructed by DNA-plasmid transfer (plasmid RP4 and helper: Cargo plasmids pRL 528: pRL 1063) into the herbicide–resistant mutants of *Anabaena* PCC7120 using the triparental conjugation technique as described before [7]. The exconjugant colonies of mutants containing plasmid and defective in glutamine synthatase (GS) were selected, grown and maintained in Chu-(10)-N medium in the presence of glutamine (50 mg L^{-1}) and streptomycin (10 mg L^{-1}). Consequently, 22 mutants were selected and tested for the presence of both traits (ammonia production and herbicide-resistance).

Results and discussion

Ten mutants of *Anabaena* PCC7120 were successfully constructed for the presence of two genetic traits; ammonia production and herbicide-resistance (Table 1). The ranges for heterocyst frequency and ammonia production of all mutants were 4.2%-6.8% and 2.3-6.6 µmol mg dry wt^{-1} h^{-1} respectively (Table 1). Ammonia production could occur if the key enzyme in nitrogen assimilation (glutamate synthatase; GS coded by the gln-A gene) has become partially-defective. Apparently this is the case with our mutants where the disruption by transposon occurs possibly in the same locus of the mutant genome leading to the GS-defective phenotype.

Table 1. Ammonia production and heterocyst frequency of the mutants of *Anabaena* PCC7120 during growth in batch culture in the presence of the herbicide Diuron (2.0 mg L^{-1}) compared with the wild-type in the absence of the latter. (n=4 for each treatment).

Strains	Ammonia production (µmol mg dry wt^{-1} h^{-1})	heterocyst frequency(%)
Wild-type	0.0	6.5
M1	2.6	4.2
M3	3.2	4.8
M12	2.3	4.5
M14	6.6	4.8
M16	2.6	4.2
M17	4.8	5.6
M18	2.9	5.9
M19	4.2	4.8
M20	3.6	6.5
M22	3.2	4.4

After 20 days of growth in batch culture at 2.0 mg L^{-1} of herbicide Diuron (Figure 1) the dry weight of the mutants had reached 300-400 mg L^{-1} which was similar to the growth pattern of the wild-type in the absence of Diuron. The M14 showed a normal and health growth (Figure 2) under different concentrations of herbicide (0.5, 1.25, 2.0 mg L^{-1}) (Figure 2), which was similar to that of the remaining mutants (data not shown). The herbicide-resistance threshold was 2.0 mg L^{-1} above which level mutants started to become unhealthy. Microscopic inspection revealed however that no damages occurred to the cells growing in media at concentrations below the herbicide-resistance threshold. Above the threshold, mutants were able to tolerate up to 4.0 mg L^{-1} but this was accompanied by a reduction in O_2-evolution (data not shown) and some morphological symptoms were also revealed. Eventually the higher level of herbicide stopped growth. This may well be compared with the herbicide-resistant red microalga *Porphyridium aerugineum* [10].

In contrast, addition of 2.0 mg L^{-1} (the threshold of herbicide) to the wild-type led to a drastic reduction

5. Mahasneh IA, Mishra AK, Tiwari DN, (1994). Transposon-induced mutants of the cyanobacterium *Anabaena* sp. PCC7120 capable of ammonia liberation. Biotechnol. Lett. 16:765-70.

6. Wolk CP, Ernst A, Elhai J, (1994). Heterocyst metabolism and development. In: Bryant DA, eds. The Molecular Biology of Cyanobacteria. The Netherlands: Kluwer Academic Publishers:769-823.

7. Elhai J, Vepritskiy A, Muro-Pastor AM, Flores E, Wolk CP, (1997). Reduction of conjugal transfer efficiency by three restriction activities of *Anabaena* sp. strain PCC7120. J. Bacteriol. 179:1998-2005.

8. Buikema WJ, Haselkorn R, (1991). Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena* 7120. Genes. Dev. 5: 321-30.

9. Dong Y, Huang X, Wu XY, Zhao J, (2000). Identification of the active site of HetR protease and its requirement for heterocyst differentiation in the cyanobacterium *Anabaena* sp. Strain PCC7120. J. Bacteriol. 182:1575-9.

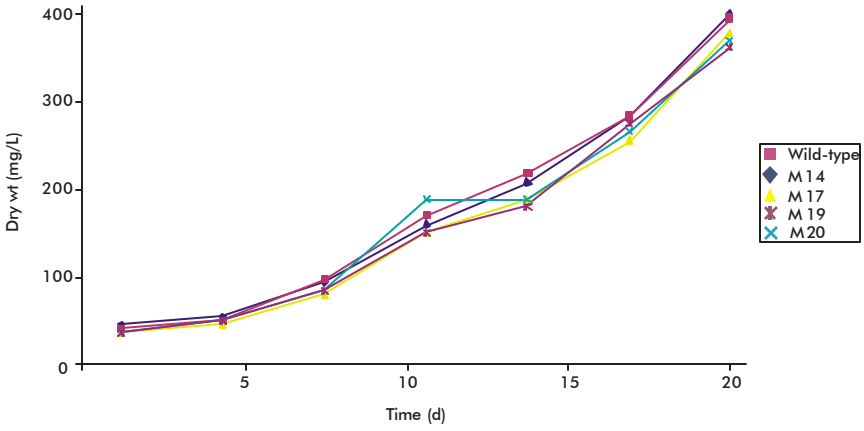

Figure 1. Growth patterns of the herbicide-resistant mutants of *Anabaena* PCC 7120 during growth in batch culture in presence of the herbicide Diuron (2.0 mg l^{-1}) compared with the wild-type in absence of the latter.

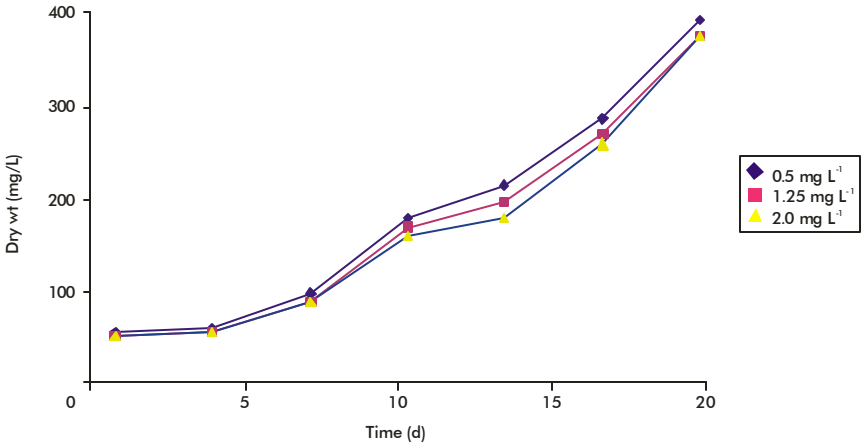

Figure 2. Growth patterns of the herbicide-resistant mutant M14 of *Anabaena* PCC7120 at various concentrations of herbicide Diuron.

in O_2-evolution followed by cellular vaculation, breakdown of filaments and death of cells within 24 h. These data show that both traits (herbicide-resistance and ammonia production) have become integral part of the mutants genome representing a marked contrast to the normal circumstances in which cyanobacterial strains are neither capable of ammonia production nor herbicide-resistance. Therefore, these mutants can potentially serve as biofertilizers in the rice fields or other crops.

Acknowledgements

The authors would like to thank Professor C. Peter Wolk for providing the strain PCC7120 and Al al-Bayt University, Banaras Hindu University and University of Qatar for providing facilities and support.

10. Boura-Halfon S, Rise M, Arad SM, Sivan A, (1997). Characterization of mutants of the red microalga *Porphyridium* sp. (Rhodophyceae) resistant to DCMU and atrazine. Phycol. 36:479-87.

Accepted for publication in december, 2003.