Colonization of Roots of Rice and Associative N_2-fixation by a Symbiotic Strain of *Nostoc*

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ABSTRACT

Nostoc sp., isolated from *Anthoceros punctatus*, colonized the roots and shoots of rice (*Oryza sativa* variety DR-92) seedlings grown in nitrogen-free medium as well as in nitrate, ammonia or urea supplemented media. However, highest level of colonization occurred in nitrate-supplemented media both under light and dark conditions. In general, colonization was higher in light than in dark condition. The kinetics of *Nostoc* sp. adsorption to the roots of rice plants was biphasic in nature during a 6 hour study period. The first rapid phase lasted for about 30 minutes followed by a slower second phase. N_2-fixation by *Nostoc* was significantly higher in association with rice plants than that in free-living conditions.

***Key words:** Nostoc, cyanobacterium, nitrogenase, colonization, Anthoceros punctatus.*

Introduction

Among the cereals, rice represents the major diet for more than two-fifths (2.4 billion) of the world's population (Ladha *et al.*, 1997, Whitton and Potts, 2000). Although N_2 is most abundant gas (80%) in the atmosphere, it is one of the most growth limiting nutrients for higher plants as they can not utilize N_2 as a source of nitrogen. Modern agricultural practices involve heavy use of chemical nitrogen fertilizers that is likely to increase further to enhance rice production in order to keep pace with increasing population. An alternative to the increased use of chemical fertilizers is to improve the ability of rice plants to obtain N from biological N_2 fixation.

Many microorganisms, both cyanobacteria and proteobacteria, have the ability to fix atmospheric N_2 either in the free-living state or in association with higher plants. Cyanobacteria are gram negative, photoautotrophic prokaryotes, and require simple inorganic medium to grow. Certain nitrogen-fixing species of cyanobacteria form symbioses with a wide variety of plants ranging from bryophytes (*Anthoceros*), water fern (*Azolla*), gymnosperms (cycads) to angiosperm (*Gunnera*) (Rai, 1990; Rai *et al.*, 2000, 2002a; Stewart *et al.*, 1983). At present, there is much interest in establishing effective N_2-fixing association between nitrogen-fixing cyanobacteria and crop plants, which would reduce the demand for nitrogenous fertilizers. With the exception of *Azolla*, none of the symbiotic plant species is currently of agronomic importance. However, the use of symbiotically competent cyanobacteria might be more advantageous since they are already adapted to a life in association with a plant (Nilson *et al.*, 2002). Creation of artificial N_2-fixing associations involving free-living cyanobacteria and crop plants has been found to be feasible (Gantar *et al.*, 1991, 2000; Svircev *et al.*, 1997; Toledo *et al.*, 1995; Ghosh and Saha, 1993; Spiller and Gunasekaran, 1990; Rai *et al.*, 2002b).

The establishment of association between rice plants and cyanobacterial strains compatible for the effective associative nitrogen-fixation could be beneficial for the growth of rice plants (Watanabe *et al.*, 1951; Singh, 1961; Ghosh and Saha, 1997). In such associations, plants may benefit by availing N from N_2-fixation by associated cyanobacteria. Here we report the ability of a *Nostoc* sp. isolated from *Anthoceros punctatus* to colonize rice plants and carry out associative N_2-fixation.

Methodology

Organisms: Rice (*Oryza sativa L.*) variety DR-92 was obtained from ICAR complex, Umiam, Shillong. The cyanobacterial strain used was isolated from *Anthoceros punctatus* collected from NEHU campus, Shillong, India.

Isolation and purification of Nostoc sp. *from Anthoceros punctatus*: Gametophytes of *Anthoceros punctatus* were washed with sterile water and then treated with 0.5% sodium hypochlorite solution for surface sterilization for 5 min. Cyanobacterial colonies visible with naked eyes were excised with sterile needle and washed with sterile water. The colonies were then plated on BG-11_0 nutrient medium with 1% agar (Rippka *et al.*, 1979). Plates were then incubated in the culture room under fluorescent light (photon fluence rate of 50 µmol m^{-2} s^{-1}) at 25°C. After two weeks of incubation a single colony was picked up and made axenic by repeated plating on BG-11_0 nutrient medium with 1% agar containing antibiotic polymixin-B-sulphate (0.01mg ml^{-1}) and cycloheximide (0.1 mg ml^{-1}). Finally, cultures were raised in liquid BG-11_0 medium.

Germination of rice: Rice seeds were surface-sterilized by washing in sterilized distilled water followed by 1% (W/V) sodium hypochlorite for 5 min, and finally thorough rinsing in sterilized distilled water. Germination was carried out on autoclaved perlite in glass beakers. The perlite was irrigated with a 10-fold dilution of autoclaved BG-11_0 medium containing 2mM $NaNO_3$. The experiments were carried out in a growth chamber at 25°C, saturating relative humidity, and a 12 h light/dark cycle at a light intensity of 50 µmol photons m^{-2} s^{-1}.

Co-cultivation of Nostoc with rice plants: 10 days old seedlings of rice were uprooted and washed with sterilized distilled water to remove perlite. Seedlings were transferred to 50 ml capacity tubes each containing 30 ml of different N-media, keeping the roots submerged. The media used were 10-fold dilutions of BG-11_0 (N_2-medium), BG-11_0 + 2mM $NaNO_3$ (nitrate-medium), BG-11_0 + 2mM NH_4Cl (ammonium-medium), and BG-11_0+1mM Urea (Urea-medium) buffered with equimolar concentration of HEPES (pH 7.5). All glasswares and media used were sterilized prior to use. 4 day old N_2-growing cyanobacterial culture was added to each tube (final concentration 1µg chl *a* ml^{-1}) containing seedlings and co-cultivation carried for 7 days under light or darkness.

Chlorophyll a estimation: Chlorophyll *a* content of cyanobacterial cells associated with the roots of rice was measured using the method described by Mackinney (1941).

Nitrogenase activity measurement: Nitrogenase activity was measured using the acetylene reduction technique (Stewart *et al.*, 1967). After 7 days of co-culture with *Nostoc*, rice roots were excised and washed for 1 min in ultrasonic bath (Power sonics 405) to remove loosely associated cyanobacterial cells and then nitrogenase activity was measured.

Phycocyanin estimation: Phycocyanin content of cyanobacterial cells associated with the shoots of rice plants was measured using the method described by Bennett and Bogorad (1973).

Measurements of shoot and root dry weight: The shoots and roots of rice plants from which the phycocyanin and chl *a* of associated cyanobacteria had been extracted, were oven-dried at 80°C for 72 h and their dry weight determined.

Results

Nostoc sp. isolated from the gametophyte of *Anthoceros punctatus* could colonize the roots and shoots of rice seedlings grown in nitrogen-free medium as well as in the presence of nitrate, ammonia and urea as nitrogen sources. The extent of cyanobacterial colonization was determined by measuring the amount of chl *a* (µg Chl *a* g^{-1} root dry wt.) in root samples and phycocyanin content (µg phycocyanin g^{-1} root dry wt.) in the shoot samples of rice seedlings. Roots and shoots of rice seedlings lacked chlorophyll *a* and phycocyanin, respectively, in the absence of cyanobacteria. Chlorophyll *a* content of *Nostoc* sp. associated with rice roots was highest in nitrate-containing media followed by nitrogen-free media, ammonia-media, and urea containing media (Fig. 1). A similar pattern was found in case of phycocyanin content of *Nostoc* sp. associated with the shoots (Fig. 2). In general, colonization was higher in light than in darkness. Since *Nostoc* sp. used in this study was isolated from a symbiotic host plant (*Anthoceros punctatus*), in order to check efficiency in terms of quick association, the kinetics of cyanobacterial adsorption to the roots of rice plants was studied over a period of 6 hours. The adsorption of *Nostoc* sp. to the roots of rice plants exhibited biphasic kinetics (Fig. 3). The first rapid phase lasted for about 30 minutes followed by a slower second phase. *Nostoc* sp. also showed higher nitrogenase activity when associated with rice roots than when in free-living condition. Nitrogen sources like nitrate, urea and ammonia suppressed nitrogenase activity of free-living *Nostoc* sp. but significant nitrogenase activity was found when associated with rice roots in light as well as in dark (Fig. 4).

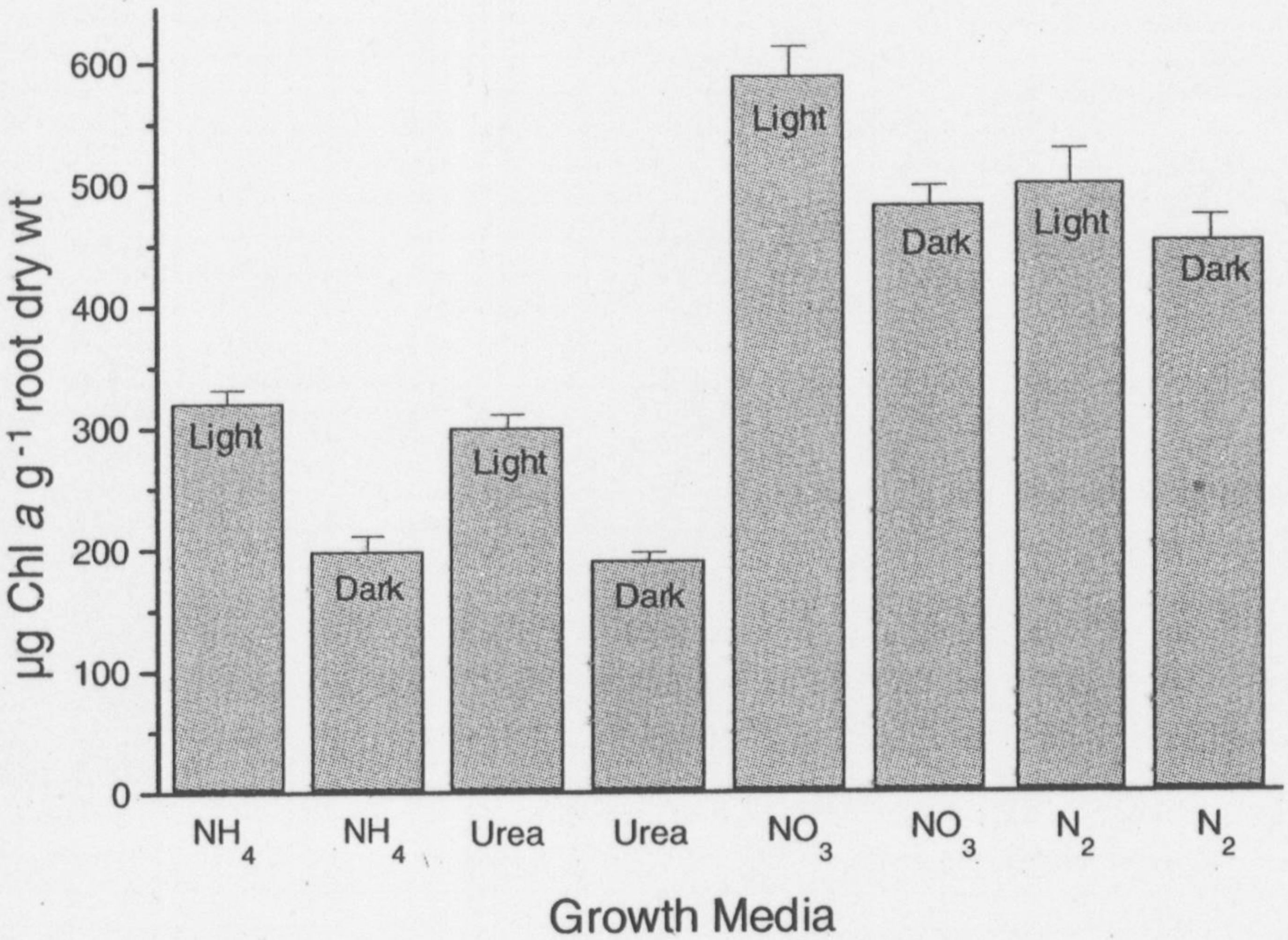

Fig. 1. Chlorophyll *a* content of *Nostoc* sp. associated with rice roots after co-cultivation in various media for 7 days at 25°C. The values are the means ± SE of three experiments.

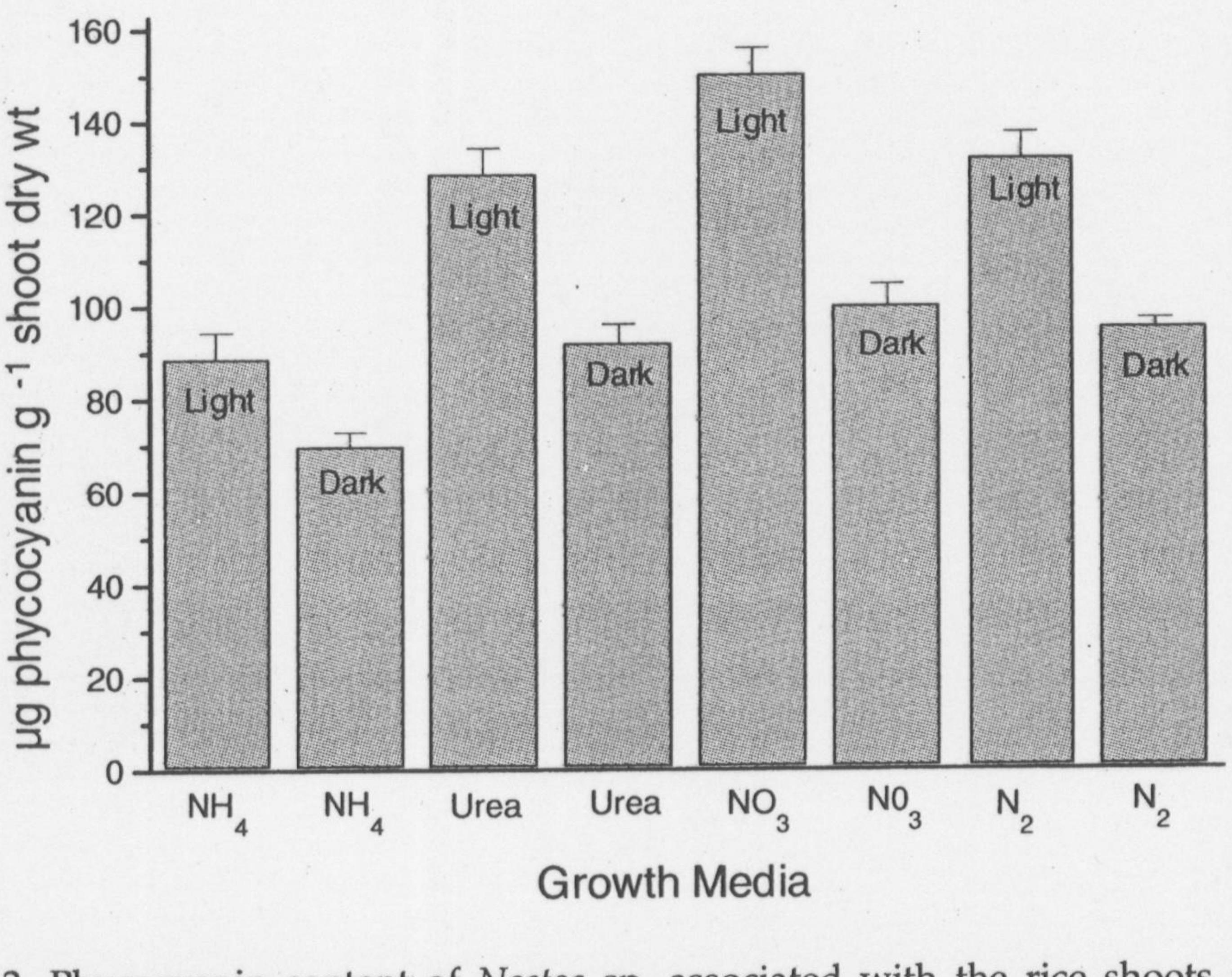

Fig. 2. Phycocyanin content of *Nostoc* sp. associated with the rice shoots after co-cultivation in various nitrogen media for 7 days at 25°C. The values are the means ± SE of three experiments.

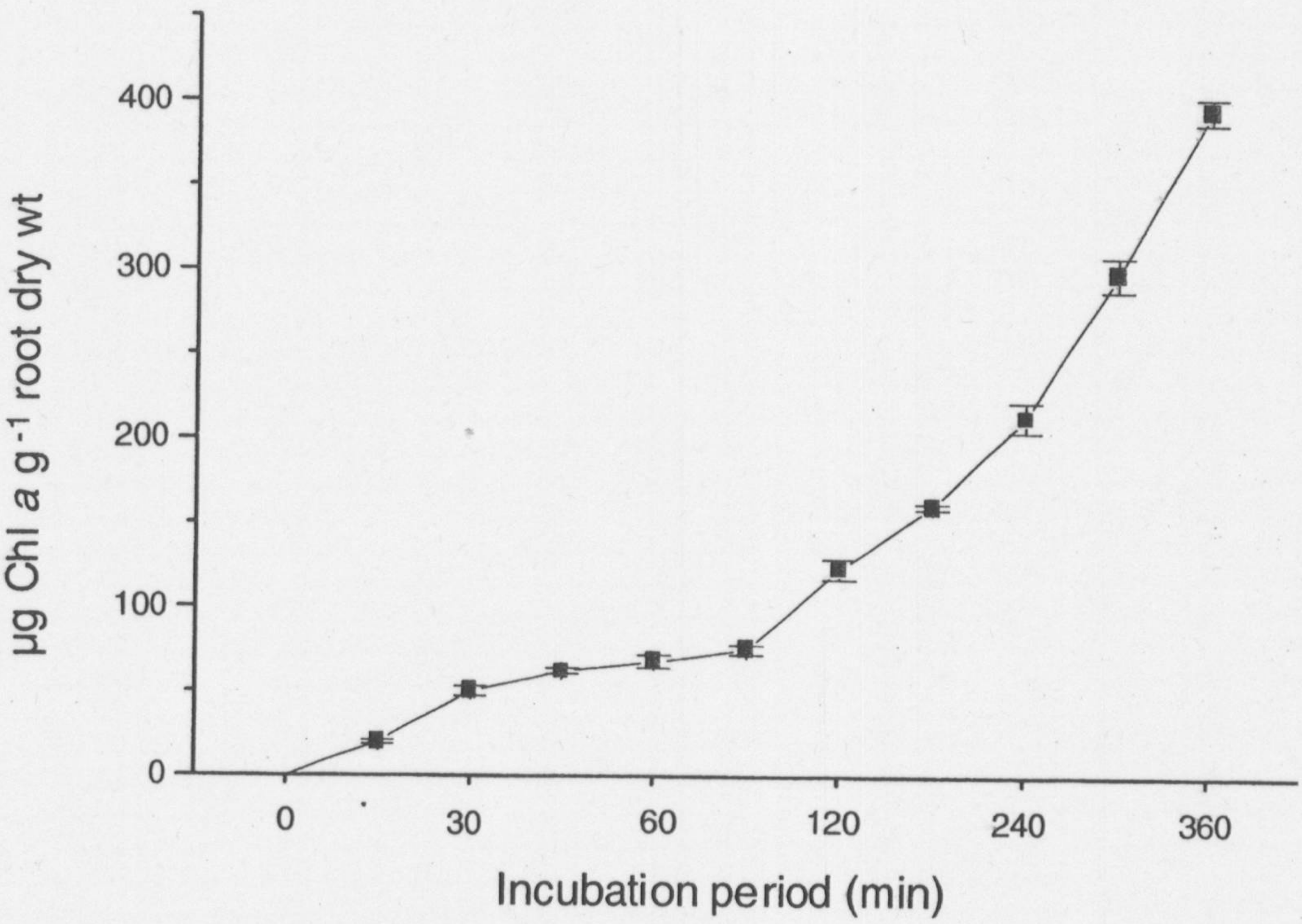

Fig. 3. Adsorption of *Nostoc* to rice roots. The values are the means ± SE of three experiments.

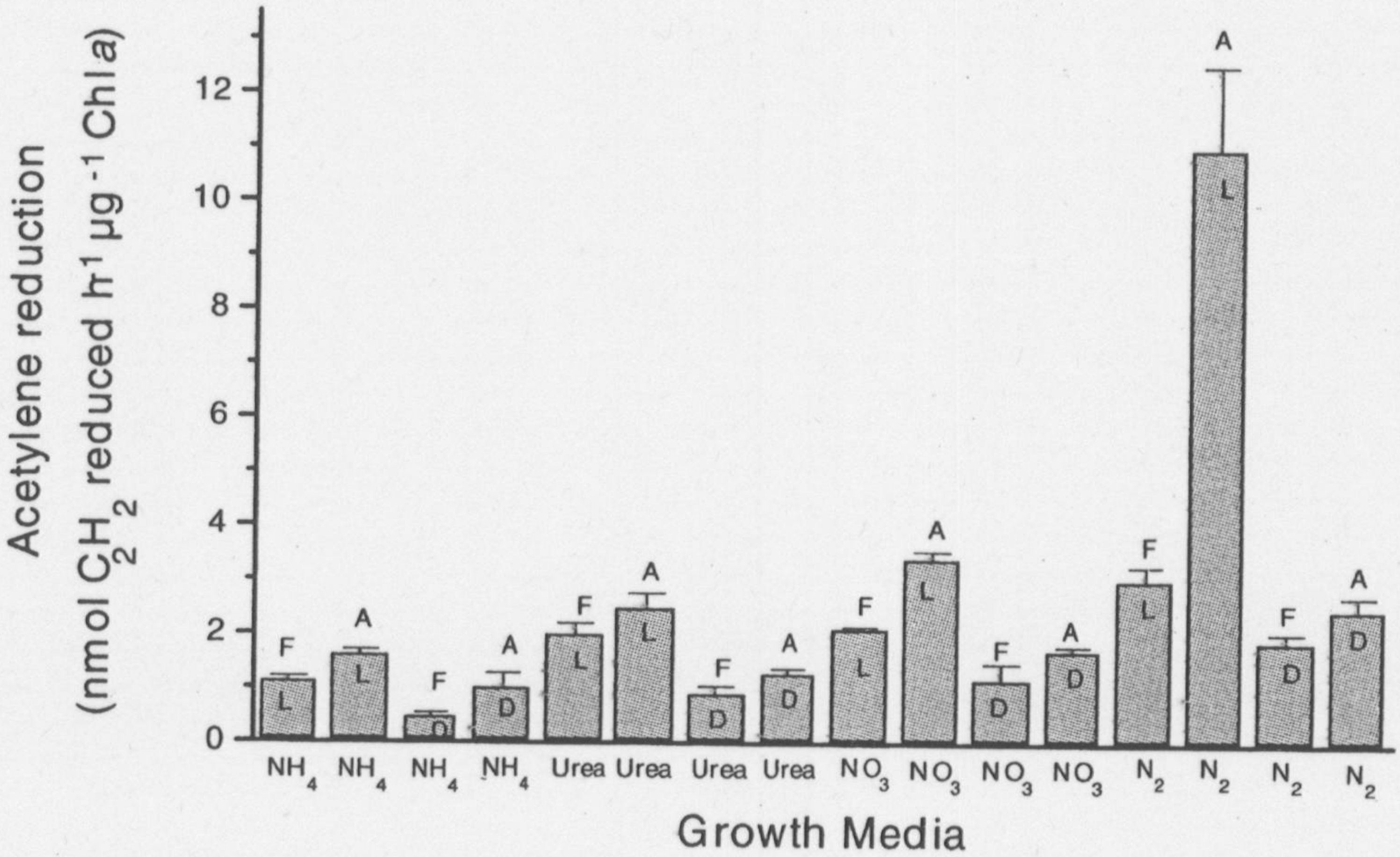

Fig. 4. Nitrogenase activity of *Nostoc* sp. associated with the rice roots in various nitrogen media after 7 days of co-cultivation. The values are the means ± SE of three experiments. F = Free-living *Nostoc* sp., A = Associated *Nostoc* sp., L = Light, D = Dark.

Discussion

Free-living cyanobacteria when used as biofertilizer fix N_2 in the light (during day) and release much of the nitrogen in soil on death and decay (Eskew *et al.*, 1981; Ito and Watanabe 1985; Tirol *et al.*, 1982; Rai *et al.*, 2002b). This then becomes available to all organisms in the field including rice plants. In addition, presence of combined nitrogen in the form of chemical nitrogen fertilizers in the field, suppresses N_2-fixation by such cyanobacteria (Kamuru *et al.*, 1997; Rai *et al.*, 2002b; Bhattacharya *et al.*, 2002). Our findings presented in this paper clearly showed that the *Nostoc* sp. is able to colonize roots and submerged shoots of the rice plants and carry out associative N_2-fixation. Furthermore, the rates of associative N_2-fixation, N_2-fixation during darkness and N_2-fixation in presence of combined nitrogen were all much higher in comparison to that in free-living condition. These attributes clearly show that the *Nostoc* sp. used in the present study is likely to be a much better biofertilizer because of the fact that it would associate with rice plants, fix nitrogen at the plant surface thereby enhancing availability of fixed nitrogen directly to the rice plants, and can fix significant amount of nitrogen during darkness. Our results showing quick adsorption of *Nostoc* to the rice root is noteworthy since this can be a way to associate the cyanobacterial strains on the uprooted rice seedlings.

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References

Benett, A. and L. Bogorad. 1973. Complementary chromatic adaptation in a filamentous blue-green alga. *Journal of Cell Biology* **58**:419–435.

Bhattacharya, J., A.K. Singh and A.N. Rai. 2002. Nitrogen nutrition in the cyanobacterium *Nostoc* ANTH, a symbiotic isolate from *Anthoceros*: Uptake and assimilation of inorganic-N and amino acids. *Indian Journal of Biochemistry and Biophysics* **39**:163–169.

Eskew, D.L., A.R. Eaglesham and A.A. App. 1981. Heterotrophic $^{15}N_2$ fixation and distribution of newly fixed nitrogen in a rice-flooded soil system. *Plant Physiology* **68**: 48–52.

Gantar, M., N.W. Kerby, P. Rowell, and Z. Obreht. 1991. Colonization of wheat (*Triticum vulgare* L.) by N_2-fixing cyanobacteria I. A survey of soil cyanobacterial isolates forming association with roots. *New Phytologist* **118**:477–483.

Gantar, M. 2000. Co-cultivation of N_2-fixing cyanobacterium *Nostoc* sp. strain 2S9B and wheat callus. *Symbiosis* **29**:1–18.

Ghosh, T.K. and K.C. Saha. 1993. Effects of inoculation with N_2-fixing cyanobacteria on the nitrogenase activity in soils and rhizosphere of wetland rice (*Oryza sativa* L.). *Biology and Fertility of Soils* **16**:16–20.

Ghosh, T.K. and K.C. Saha. 1997. Effects of inoculation of cyanobacteria on nitrogen status and nutrition of rice (*Oryza sativa* L.) in an Entisol amended with chemical and organic sourses of nitrogen. *Biology and Fertility of Soils* **24**:123–128.

Ito, O. and I. Watanabe. 1985. Availability to Rice Plants of Nitrogen Fixed by Azolla. *Soil Science and Plant Nutrition* **31**:91–104.

Kamuru, F., S.L. Albrecht, L.H. Allen and K.T. Shanmugan. 1997. Growth and accumulation of ^{15}N in rice inoculated with the parent and nitrogenase-depressed mutant strain of *Anabaena variabilis*. *Applied Soil Ecology* **5**:189–195.

Ladha, J.K., Bruijn, F.J. de and K.A. Malik. 1997. Assessing opportunities for nitrogen fixation in rice: A frontier project. *Plant Soil* **194**:1–10.

Mackinney, G. 1941. Absorption of light by chlorophyll solutions. *Journal of Biological Chemistry* **140**:315–322.

Nilson, M., J. Bhattacharya, A.N. Rai and B. Bergman. 2002. Colonization of roots of rice (*Oryza sativa*) by symbiotic *Nostoc* strains. *New Phytologist* **156**:517–525.

Rai, A.N. 1990. CRC *Handbook of symbiotic cyanobacteria*. CRC Press, Boca Raton.

Rai, A.N., E. Soderback and B. Bergman. 2000. Cyanobacterial-plant symbiosis, review. *New Phytologist* **147**:449–481.

Rai, A.N., B. Bergman and U. Rasmussen. 2002a. *Cyanobacteria in symbiosis*. Kluwer Academic Publishers, Neitherland.

Rai, A.N. and B. Bergman. 2002b. Creation of new nitrogen-fixing cyanobacterial associations. *Proceedings of the Royal Irish Academy* **102B**:65–68.

Rippka, R., J. Deruelles, J.B. Waterbury, M. Herdman and R.Y. Stanier. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology* **111**:1–61.

Singh, R.N. 1961. *Role of blue-green algae in nitrogen economy of Indian agriculture*. New Delhi, India: Indian Council of Agricultural Research.

Spiller, H. and M. Gunasekaran. 1990. Ammonia-excreting mutant strain of the cyanobacterium *Anabaena variabilis* supports growth of wheat. *Applied Microbiology and Biotechnology* **33**:477–480.

Stewart, W.D.P., G.P. Fitzgerald and R.H. Burris. 1967. *In situ* studies on N_2-fixation using the acetylene reduction technique. *Proceedings of the National Academy of Sciences, USA* **58**:2071–2078.

Stewart, W.D.P., P. Rowell and A.N. Rai. 1983. Cyanobacteria-eukaryotic symbiosis. *Annals of Microbiology* (Inst Pasteur) **145B**:205–228.

Svircev, Z., I. Tamas, P. Nenin and A. Drobac. 1997. Co-cultivation of N_2-fixing cyanobacteria and some agriculturally important plants in liquid and sand cultures. *Applied soil Ecology* **6**:301–308.

Tirol, A.C., P.A. Roger and I. Watanabe. 1982. Fate of nitrogen from a blue-green alga *Nostoc* sp. in a flooded rice *Oryza-sativa* soil. *Soil Science and Plant Nutrition* **28**:559–570.

Toledo, G., Y. Bashan, and A. Soeldner. 1995. *In-vitro* colonization and increase in nitrogen-fixation of seedling roots of black mangrove inoculated by filamentous cyanobacteria. *Canadian Journal of Microbiology* **41**:1012–1020.

Watanabe, A., R. Ito and C. Konishi. 1951. Effect of nitrogen-fixing blue-green algae on the growth of rice plants. *Nature* **168**:748–749.

Whitton, B.A. and M. Potts (eds.). 2000. *The ecology of cyanobacteria*. Dordrecht. Kluwer.