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LOCALIZATION OF NITROGENASE AND NITRATE REDUCTASE IN THE *CYCAS-ANABAENA CYCADEAE* ASSOCIATION

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Induction of nitrate reductase (NR) activity in coralloid roots of *Cycas revoluta* was observed after 8 h incubation in 0.02 M KNO_3. Other plants growing near *Cycas* showed a higher level of NR immediately when incubated in KNO_3. In contrast to NR, intact coralloid roots showed very high nitrogenase activity (~1.2 to 1.6 μmol C_2H_4 g fresh wt^{-1} h^{-1}) under both light and dark conditions as compared to transverse sections of roots. Localization of NR and nitrogenase was tested in coralloid roots using different sets of roots and also in the endophyte. Our results showed that NR activity was mainly due to the endophyte (*Anabaena cycadeae*); coralloid roots lacked it, as no NR activity was observed in chloramphenicol-treated intact root samples.

Key Words: *Anabaena cycadeae*, nitrate reductase, coralloid root, nitrogenase activity.

INTRODUCTION

Among the gymnosperms, cycads are the only group that forms a symbiotic association with nitrogen-fixing organism(s). *Anabaena*, nitrogen-fixing heterocystous cyanobacteria of the genus *Nostoc*, inhabit a cylindrical zone intercellularly in the cortex of coralloid roots. The endophytic phycobiont identified as *Anabaena cycadeae* fixes nitrogen for the plants and gets fixed carbon from the host. Intact coralloid roots showed a very high level of nitrogenase activity under light and dark conditions. Nitrogenase synthesis and/or activity is inhibited by a combined nitrogen source in a number of free-living and symbiotic cyanobacteria (Stewart et al., 1980; Sharma et al., 1988). However, there is not much information on the effect of a combined nitrogen source on nitrogenase in a cyanobiont associated with a host plant. This aspect is important because all plants, including the hosts of nitrogen-fixing microbes, grow in the presence of available combined nitrogen sources in natural systems. The correlations between NR and nitrogenase in *Rhizobium* symbiosis have been studied in greater detail by Sik and Barabas (1977). It is reported that the presence of active NR is not required for active nitrogenase activity of root nodules (Stewart et al., 1983).

Nitrate assimilation in cyanobacteria is an energy-demanding process. Conversion of nitrate into nitrogenous compounds proceeds via nitrate and ammonia, and the main enzyme is nitrate reductase. Cyanobacterial photosynthesis provides ATP and related ferredoxin for the reduction of nitrate to ammonia (Meeks et. al., 1985; Lindblad and Bergman, 1986).

The present study examines the establishment of a symbiotic association between a eukaryote and a prokaryotic partner on the basis of the NR and

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nitrogenase activity of a facultative heterotrophic endophytic alga, *Anabaena cycadeae.*

MATERIALS AND METHODS

Coralloid roots of *Cycas revoluta* collected from the botanical garden of Banaras Hindu University, India, were thoroughly rinsed with running water to remove all soil debris and then surface-sterilized by immersion in 0.5% $HgCl_2$ for five min followed by repeated rinsing with sterilized distilled water. The endophyte (a green zone in the root cortex) was teased out using a sterilized needle and scalpel and collected in sterilized water after centrifugation at 5000 rpm. The isolated endophyte was purified by streaking on nitrogen-free Allen and Arnon (1955) medium and maintained in axenic form.

Nitrogenase activity was measured by acetylene reduction technique in the standard method of Stewart et al. (1967), and NR activity was estimated according to Camm and Stein (1974). Freshly collected coralloid roots and cell suspension were separately incubated in Allen-Arnon medium supplemented with 0.02 M KNO_3 in light. The roots were crushed before enzyme estimation and total nitrite formed was measured by the diazocoupling method of Lowry and Evans (1964). Total chlorophyll *a* extracted in acetone was calculated using the specific absorption coefficients quoted by Mackinney (1941).

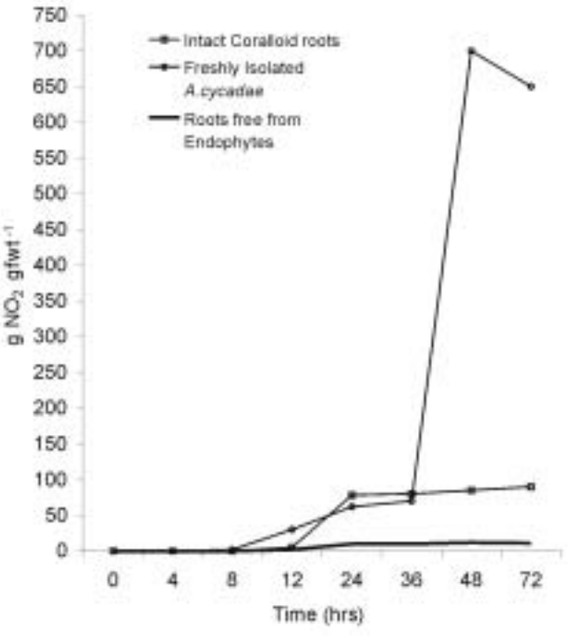

Fig. 1. Induction and localization of nitrate reductase in coralloid roots (n = 5).

TABLE 1. Nitrogenase activity in intact coralloid roots, freshly isolated and laboratory grown *Anabaena cycadeae* (n = 5)

Experimental Conditions	Nitrogenase Activity*
Intact coralloid roots	1.5
Transverse section of root	0.83
Laboratory grown *A. cycadeae*	0.43
Endosymbiont (freshly isolated)	0.12

* µmol C_2H_4 µg^{-1}Chl *a* h^{-1}

RESULTS AND DISCUSSION

Under aerobic conditions, freshly collected coralloid roots of *Cycas revoluta* had high nitrogenase activity in both light and dark. Activity varied from 1.0 µm C_2H_4 g wt^{-1}h^{-1} to 2.5 µm C_2H_4 g wt^{-1}h^{-1} (Sharma et al., 1988). Transverse sections of coralloid roots and the isolated endophyte did not show significant C_2H_2 reduction. Laboratory-grown algae showed a C_2H_2 reduction rate of 5–7 nmol C_2H_4 µg^{-1} chl *a* h^{-1} in light under aerobic conditions. Lindblad (2002) also reported nitrogenase activity in coralloid roots of *Cycas* spp., and specific activity of 40 nmol C_2H_4 µg^{-1} chl *a* h^{-1}. Unlike in free-living cyanobacteria, nitrogenase activity was the same in light or dark in coralloid roots. This may be due to the presence of the bulk of reductant in the root and/or endophyte, which is utilized whenever the supply of reductant becomes limiting. Costa et al. (1999) detected equal nitrogen fixation in dark and up to 2.5 K lux light intensity in coralloid roots of *C. circinalis*. All symbiotic cyanobacterial species exhibit high nitrogenase activity due to the high frequency of heterocysts, a suitable anaerobic environment within the host tissues, and derepression of nitrogenase activity/synthesis due to continuous excretion of ammonia by the endophyte (Rai et al., 1986; Peter and Meeks, 1989).

Intact coralloid roots revealed maximum nitrogenase activity of 1.5 µmol C_2H_4 µg^{-1} chl *a* h^{-1}, as compared to 0.83 µmol C_2H_4 µg^{-1} chl *a* h^{-1} in transverse sections of roots, 0.43 µmol C_2H_4 µg^{-1} chl *a* h^{-1} in laboratory-grown *Anabaena cycadeae,* and 0.12 µmol C_2H_4 µg^{-1} chl *a* h^{-1} in the endosymbiont (freshly isolated algae) (Tab. 1).

TABLE 2. Nitrate reductase activity in intact coralloid roots, freshly isolated and laboratory grown *Anabaena cycadeae* (n = 5)

Type of tissue/cells material	NR Activity*		
	Time (h)		
	0	12	24
Intact coralloid roots	0	43.0	80.0
Isolated *A. cycadeae*	0	0.24	0.52
Intact coralloid roots + chloramphenicol (200 µg/ml)	0	0.00	0.00
Freshly isolated *A. cycadeae* + chloramphenicol (25 µg/ml)	0	0.00	0.00
Laboratory grown *A. cycadeae*	0	0.36	0.65
Laboratory grown *A. cycadeae*+chloramphenicol (25 µg/ml)	0	0.00	0.05

* Nitrate Reductase (NR) activity expressed in µg NO_2^- formed g fresh wt^{-1}
One-gram healthy roots showed nearly 15–20 µg chlorophyll *a*

Induction of NR activity was tested individually in intact coralloid roots, endophyte-free coralloid roots, and freshly isolated endophyte. Figure 1 reveals that induction of NR occurred only in coralloid roots with the endophyte after 8–12 h incubation. Freshly isolated endophyte also showed very high NR activity after 8 h incubation, gradually increasing to 680 µg NO_2 g f wt^{-1} at 48 h. Endophyte-free coralloid roots showed very low NR activity, perhaps due to contamination by some endophyte left after teasing. This result suggests that NR induction took place due only to the endophyte of the coralloid roots.

Addition of chloramphenicol to freshly isolated endophyte and intact coralloid roots inhibited NR activity completely (Tab. 2). Chloramphenicol, a potent inhibitor of protein synthesis in prokaryotes, was added to coralloid roots and isolated endophyte (200 and 25 µg ml^{-1}, respectively) along with NO_3^- during NR induction. Laboratory-grown *A. cycadeae* also showed inhibition of nitrate reductase following chloramphenicol treatment. This observation revealed differential synthesis of nitrate reductase in the *Cycas-Anabaena* association, occurring solely in the endophyte. If nitrate reductase synthesis really had occurred in the host tissue, one might expect activity even after chloramphenicol treatment. The implication of this finding is that the coralloid root is the site of N_2 fixation and that probably the main root performs the function of NO_3^- assimilation, if required. Our data on the enzyme distribution in *Cycas Anabaena* symbiosis resembles that on lichen symbiosis, where GS glutamine synthetase (ammonia-assimilating enzyme) activity was not detected in the symbiotic alga (Rai et al., 1983). Furthermore, the GS-linked methyl ammonium transport system has also been reported to be absent in this symbiotic system. These findings indicate that, similarly to the lichen *Peltigera* (Rai et al., 1983), morphological, physiological and biochemical modifications in cyanobacteria are involved in the establishment of symbiosis and sustained N_2 fixation in *Cycas revoluta*. The absence of nitrate reductase in coralloid roots seems to have a significant role in the regulation of N_2 fixation in this xerophytic plant. Our results are comparable to earlier findings that *Azolla* fronds did not show much inhibition of nitrogenase activity by combined nitrogen sources (Srivastava et al., 1988). This character probably enables the cyanobiont to fix N_2 even in the presence of NO_3^- in natural conditions.

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