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Cycas circinalis-Anabaena cycadeae Symbiosis: Photosynthesis and the Enzymes of Nitrogen and Hydrogen Metabolism in symbiotic and cultured *Anabaena cycadeae*

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Abstract

A comparative study of photosynthesis, nitrogen and hydrogen metabolism was carried out on the cyanobiont of *Cycas circinalis* coralloid roots and the cultured isolate, *Anabaena cycadeae*. The cyanobiont showed lower rates of photosynthesis, had a higher heterocyst frequency and nitrogenase activity, and lacked uptake hydrogenase. Increase in light intensity caused stimulation of photosynthesis and nitrogenase activity of the cultured *Anabaena cycadeae* but the response in the cyanobiont was far less pronounced.

Activities of glutamine synthetase (both biosynthetic and transferase), aspartate dehydrogenase, alanine dehydrogenase and glutamate pyruvate transaminase, in the cyanobiont, were 30–60% lower than those in its free-living form *Anabaena cycadeae*. Glutamate oxaloacetate transaminase, on the other hand, was significantly higher in the cyanobiont. Glutamate dehydrogenase and nitrate reductase were undetectable.

The cyanobiont evolved hydrogen during nitrogen fixation, while the free-living isolate, *Anabaena cycadeae*, showed no hydrogen evolution, due to the presence of an uptake hydrogenase.

Keywords: *Anabaena cycadeae*, Cyanobacteria, *Cycas circinalis*, Hydrogen-metabolism, Nitrogen-metabolism, Photosynthesis, Symbiosis

Abbreviations: ADH, alanine dehydrogenase; AsDH, aspartate dehydrogenase; Chl, chlorophyll; DCMU,[3(3,4-dichlorophenyl)1,1-dimethylurea]; GDH, glutamate dehydrogenase; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; GS, glutamine synthetase; H_2ase, hydrogenase; N_2ase, nitrogenase; NR, nitrate reductase.

1. Introduction

N_2-fixing heterocystous cyanobacteria develop into association with algae, fungi, bryophytes, the water fern *Azolla*, gymnosperms and the angiosperm *Gunnera* (Stewart et al., 1983). In symbiosis the cyanobacterium becomes modified. Such modifications include cell size, ultrastructure, heterocyst frequency and enzymes of nitrogen metabolism (Stewart et al., 1980, 1983).

Cycads are the only gymnosperms involved in symbiosis with cyanobacteria. Most cycads examined have root nodules where a heterocystous cyanobacterium occurs as an endosymbiont in mucilage filled spaces of the cortex (Allen and Allen, 1965). Using $^{15}N_2$ and C_2H_2, nitrogenase activity has been demonstrated in such root nodules (Bergersen et al., 1965; Bond, 1967; Grobbelaar et al., 1971; Renaut et al., 1975; Halliday and Pate, 1976; Lindblad et al., 1985). Nitrogen, fixed by the cyanobiont, has been shown to be rapidly transferred to the remainder of the plant (Bergersen et al., 1965; Renaut et al., 1975; Halliday and Pate, 1976).

Presently, there is no information regarding the levels of nitrogen metabolizing enzymes, except nitrogenase, in the cycad cyanobionts, although, in a number of other cyanobacterial associations it has been demonstrated that some of these enzymes are modified in the cyanobiont. Similarly, no work has been done on the hydrogen metabolism in this symbiosis. In this paper we have studied photosynthetic characteristics and activities of nitrogen and hydrogen metabolism enzymes in the cyanobiont of *Cycas circinalis*; for comparison, data have also been obtained on the free-living isolate, *Anabaena cycadeae*.

2. Materials and Methods

Organisms

The cyanobacterium from *Cycas circinalis* coralloid roots (root nodules) was isolated, purified and raised in pure culture as described earlier (Singh and Singh, 1964; Singh et al., 1983). This free-living strain is referred to as *Anabaena cycadeae*. Symbiotic cyanobacterium (referred to as cyanobiont) was freshly isolated from root nodules of *C. circinalis* grown in a nursery.

Isolation of the cyanobiont

Root nodules were washed in distilled water, cut into small pieces and then gently crushed, using a mortar and pestle, in Chu-10 medium (Gerloff et al., 1950) containing 1% PVP, to release the cyanobiont. Bulk of the host tissue was removed by passing the suspension through 2, 4, and 8 layers

of muslin cloth. The filtrate was then repeatedly centrifuged at 500×g to remove remaining host tissue debris.

Heterocyst frequency

Heterocyst frequency was calculated as percentage of total cells, by light microscope observations of the filaments of *A. cycadeae* and the cyanobiont.

N_2ase activity

N_2ase activity of intact coralloid roots, freshly isolated cyanobiont, and cultured *A. cycadeae* was measured using acetylene reduction assay. One ml cyanobacterial culture, or 1 g coralloid roots were placed in 7 ml serum vials. Coralloid roots were kept moist by placing a distilled water soaked filter paper in the vial. Acetylene was injected to a final concentration of 10% (v/v) of the air phase in the vial and the production of ethylene estimated, after incubating the vial for 30 min at 25°C and desired light intensity (see text), as described before (Stewart et al., 1967) except that the column packing material was Porapak T.

Chl estimations

Cells were filtered on a Whatman GF/C filter paper and chlorophyll extracted in methanol at 4°C for 12 h in darkness. Chl content was calculated from absorption readings at 663 nm according to Mackinney (1941).

Protein estimations

The method of Lowry et al., (1951) was followed using bovine serum albumin as standard.

Oxygen exchange

O_2-evolution, by the cultured isolate and by the cyanobiont in intact root nodules, was measured polarographically at 25°C and desired light intensity (see text) using a Clark-type oxygen electrode as before (Rao et al., 1984).

Enzyme assays

NR was assayed in whole cells according to Manzano et al., (1976). Other enzymes were assayed in cell-free preparations. Cells were washed in 50 mM Tris-HCl buffer, pH 7.5, centrifuged and then ruptured by passage through a French Pressure Cell at 110 MPa. The extracts were then centrifuged at 30,000×g for 20 min and the supernatant liquids dialyzed overnight at 4°C

against the same buffer. Enzyme activities in such extracts were then assayed by coupling the reactions to NADH oxidation (NADPH in the case of GDH) followed at 340 nm, except in the case of GS transferase assays which were done by colourimetric measurements of γ-glutamylhydroxamate formation. GS (biosynthetic and transferase) activities were measured according to Sampaio et al., (1979), AsDH according to Haystead et al., (1973), and GPT according to Jäger and Weigel (1978). GDH (NADPH-dependent), ADH and GOT were measured according to Stewart and Rowell, (1977).

Hydrogen exchange measurements

This was done according to Tel-Or et al., (1977). Samples were placed in 15 ml capacity sample tubes sealed with rubber stoppers. H_2 gas was injected into these tubes to a final concentration of 2% (v/v) followed by incubation in a BOD incubator, at 3 klux light intensity and 27±1°C temperature. At 30 min time intervals, 0.5 ml gas samples were withdrawn and analysed on a Perkin-Elmer Sigma 3B Gas Chromatograph fitted with a MS 5A column and a thermal conductivity detector. Argon served as a carrier gas and tubes containing N_2-medium and H_2 served as control. The rates of decrease and/or increase in the H_2 gas phase, relative to the control, were calculated and are expressed as H_2 uptake and/or H_2 evolution rates, respectively.

Analysis of photosynthetic pigments

The absorption spectrum of methanol-soluble and water-soluble photosynthetic pigments was obtained using a Gilford spectrophotometer scanning from 400 nm to 700 nm. Methanol-soluble pigments were extracted as above and water-soluble pigments were extracted by freezing-thawing the cyanobacterial cells suspended 0.05 M potassium phosphate buffer (pH 6.7). The *in situ* pigment composition of *A. cycadeae* and the cyanobiont, in thin slices (0.1 mm thick) of root nodules, was analysed using a photoacoustic spectrometer (E.G. and G. Princeton Applied Research Corporation, USA; model 6001), scanning from 500 nm to 700 nm wavelengths, at room temperature, with 40 Hz modulation frequency as detailed by Balasubramanian and Rao, (1982).

3. Results and Discussion

Photosynthetic characteristics

We measured the absorption spectra of methanol-soluble and water-soluble pigments of the cyanobiont and the cultured isolate and found the phycoerytrin peak to be absent in the cyanobiont (data not shown). To ensure

that the absence of the phycoerythrin peak was not due to the limitation of the extraction procedure we also measured *in situ* composition of the photosynthetic pigments using photoacoustic spectroscopy. Again, phycoerythrin was found to be absent in the cyanobiont, otherwise, *A. cycadeae* and the cyanobiont were similar in respect of Chl *a* and phycocyanin spectral characteristics (Fig. 1). The significance of the absence of phycoerythrin, in the cyanobiont, is not clear at present, however, it may indicate the nitrogen-limiting status of the cyanobiont. Similar results have been found in the case of *Peltigera aphthosa* cyanobiont (Rai, 1980; Stewart et al., 1981).

The free-living *A. cycadeae* showed a progressive increase in the O_2-evolution rate with increase in light intensity from 0.5 klux to 15 klux (Fig. 2). No further increase was observed beyond 15 klux light intensity (data not shown). In contrast, the intact root nodules, containing cyanobiont, did not show any O_2-evolution upto 2 klux light intensity but showed a relatively low level of O_2-evolution thereafter. O_2-evolution in response to increasing light intensity was also far less pronounced as compared to that in the free-living *A. cycadeae*. DCMU was found to inhibit O_2-evolution in both cases, suggesting the occurrence and operation of PS II in the cyanobiont when provided with light. That is, the cyanobiont retains the photosynthetic capacity although it occurs in root nodules under the soil where the availability of light would be negligible. The fact that the rate of O_2-evolution in intact root nodules was low and showed a slow response to increase in light intensity may have two explanations. First, previous studies on cyanobacteria indicate that under low light conditions of growth there are more PS I reaction centres than PS II, and that in high light both PS I and PS II reaction centres are similar in number (Kawamura et al., 1979; Vierling and Alberte, 1980). Since the cyanobiont functions under heterotrophic growth conditions in root nodules, with negligible light availability, the number of PS II reaction centres may be limiting. Second, there may be a limitation of light energy reaching the cyanobiont within the root nodules because of the intervening cycad root tissues. However, when the cyanobiont was separated from the root nodules and O_2-evolution measured under saturating light conditions (15 klux) in such freshly isolated cyanobiont cells, a rate of 45μmol oxygen evolved mg^{-1} Chl *a* h^{-1} was found. This was less than half of the O_2 evolution rate in the free-living *A. cycadeae* under similar conditions (111μmol O_2 evolved mg^{-1} chl *a* h^{-1}). This clearly indicated that even with full light availability the rate of photosynthetic evolution in the cyanobiont was much lower. Thus the low number of O_2-evolving PS II reaction centers may be the main reason for low rates of photosynthetic O_2 evolution in the cyanobiont, as discussed above.

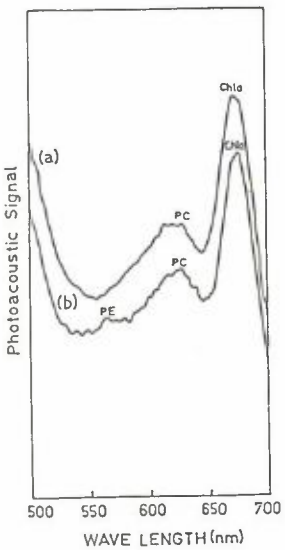

Figure 1. Photoacoustic spectrum of cyanobiont in intact root nodules (a) and the free-living *Anabaena cycadeae* (b). PE, phycoerythrin; PC, phycocyanin; Chl, chlorophyll *a*.

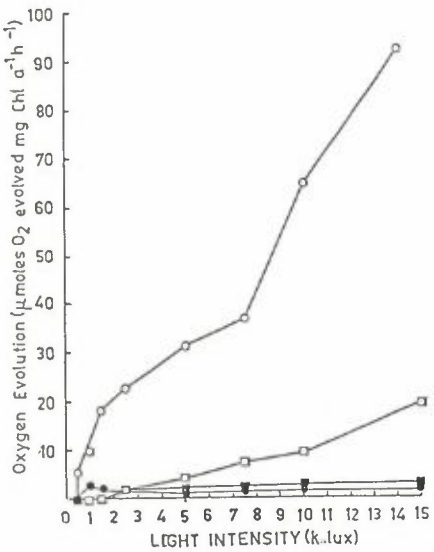

Figure 2. Effect of light intensity on photosynthetic O_2 evolution. ○, free-living *Anabaena cycadeae*; □, cyanobiont in intact root nodules; ●, free-living *Anabaena cycadeae*, 5μM DCMU added; ■, cyanobiont in intact root nodules, 5μM DCMU added.

Hydrogen metabolism

H_2 evolution was undetectable in the free-living N_2-fixing cultures of *A. cycadeae* but an H_2 evolution rate of 4μmol mg^{-1} Chl *a* h^{-1} was found in intact root nodules. In contrast, H_2 uptake was undetectable in the intact root nodules while free-living *A. cycadeae* showed an H_2 uptake rate of 28μmol mg^{-1} Chl *a* h^{-1}. These findings clearly indicate that the development of symbiotic association between *A. cycadeae* and *C. circinalis* results in the loss of the cyanobacterial uptake H_2ase activity.

The reasons for the absence of uptake H_2ase activity in the cyanobiont are not yet clear. However, catabolizable organic carbon substrates are known repressors of uptake H_2ase activity in *Azotobacter* and *Rhizobium* (Partridge et al., 1980; Maier et al., 1979). Keeping in view the heterotrophic mode of cyanobiont's nutrition in the root nodules, it is tempting to suggest that the loss of uptake H_2ase activity in symbiosis may have been caused by the inhibitory effect of catabolizable organic carbon moving from the cycad to the cyanobiont as in the case of *Azotobacter* and *Rhizobium* mentioned above.

Nitrogen metabolism

The N_2ase activity of the cyanobiont, freshly removed from the cycad root nodules, was much higher than that of the free-living *A. cycadeae*. The cyanobiont showed a N_2ase activity of 40 nmol C_2H_2 reduced μg^{-1} Chl *a* h^{-1} as compared to 4 nmol C_2H_2 reduced μg^{-1} Chl *a* h^{-1} in the case of free-living *A. cycadeae*. This compared poorly with the optimum N_2ase activity of the cyanobiont in intact root nodules (see Fig. 3), prompting us to use intact root nodules in further experiments. High levels of N_2ase activity observed with the cyanobiont can be explained by the fact that the cyanobiont showed an average heterocyst frequency of 25%, as against a value of 5% detectable in the free-living *A. cycadeae*. Most free-living cyanobacteria show a heterocyst frequency of the 5–6% (Stewart, 1980). Our values for the heterocyst frequency of the cyanobiont are comparable to those reported by Grilli-Caiola (1980). However, it should be emphasized that our values are for a mixed population of the cyanobiont, isolated from whole root nodules. These values are likely to vary along the root as reported for *Zamia* (Lindblad et al., 1985). A similar argument applies to our N_2ase values.

Further studies with intact root nodules, in order to avoid disturbing the cyanobiont, showed that upto 24 h the N_2-fixation activity of the cyanobiont was rather similar both in dark and in upto 2.5 klux light intensity (40 nmol C_2H_2 reduced μg^{-1} Chl *a* h^{-1}). In contrast N_2ase activity of the free-

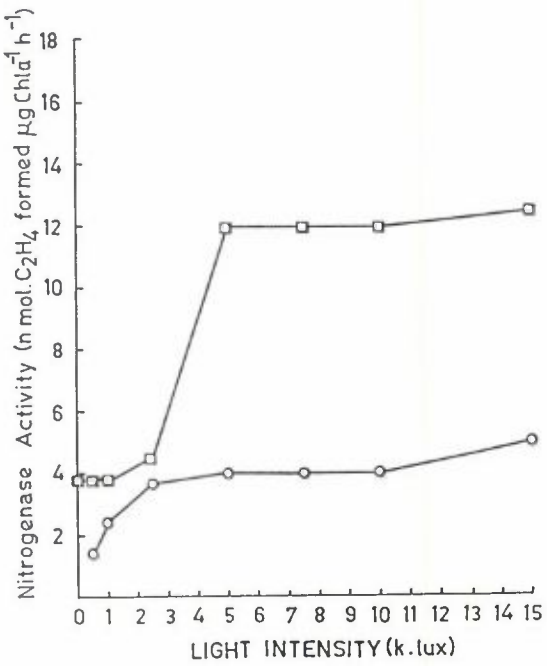

Figure 3. Effect of light intensity on nitrogenase activity in free-living *Anabaena cycadeae* and the cyanobiont in intact root nodules. o, free-living *Anabaena cycadeae*; □, cyanobiont in intact root nodules ($\times 10^{-1}$). Please note that the N_2ase values of the cyanobiont are reduced by factor of 10 before plotting.

living *A. cycadeae* declined sharply and became undetectable within 3 h of darkness (data not shown). It is evident that N_2ase activity in the cyanobiont was sustained through chemoheterotrophic metabolism, a mode of nutrition prevailing under natural conditions where the cyanobacterium occurs in root nodules below the soil surface in darkness.

When root nodules were subjected to light intensity above 2.5 klux, N_2ase activity increased rapidly reaching a maximum at 5 klux and remaining constant thereafter (Fig. 3). The lack of a significant effect of light intensity below 2.5 klux on the N_2ase activity of the cyanobiont may be due to the cyanobiont's inability to receive enough light because of the intervening cycad root tissues. Free-living *A. cycadeae*, in contrast, showed a light intensity optimum of 2.5 klux for maximum N_2ase activity.

The fact that O_2-evolution and N_2ase activity do not seem to show an identical response to increase in light intensity (see Fig. 2 and 3), may suggest that N_2ase is more directly dependent on the non-O_2-evolving PS I, rather

Table 1. Activities of various nitrogen-metabolising enzymes in *Cycas circinalis* cyanobiont and in free-living *Anabaena cycadeae*. (values are mean ±SEM; n=5).

Enzymes	Enzyme Activity (nmol product formed min^{-1} mg^{-1} protein)	
	Cyanobiont	*Anabaena cycadeae*
GS		
transferase	966±20	1474±30
biosynthetic	43±2	60±2
ADH (aminating)	10±1	18±1
GDH (NADPH-dependent)	ND	30±2
AsDH (aminating)	7±1	19±1
GPT	27±2	54±3
GOT	22±1	12±1
NR (Fd-dependent)	ND	ND (0.2)[a]

[a] Represents activity in cells grown on medium supplemented with 20 mM KNO_3.

ND: not detectable.

than the O_2-evolving PS II. PS I has been shown to be able to supply N_2ase with ATP and reductant from a pool of organic carbon intermediates (Tel-Or and Stewart, 1976).

Other nitrogen-metabolising enzymes, involved in primary ammonia assimilation and transamination reactions, also showed differences between the free-living *A. cycadeae* and the cyanobiont in the root nodules (Table 1). NR was found to be absent in the cyanobiont as well as in the free-living *A. cycadeae*. However, NR activity was detectable when the free-living *A. cycadeae* was grown on nitrate-supplemented medium, supporting the earlier contention that NR of *A. cycadeae* is nitrate-inducible (Bagchi et al., 1985). NADPH-dependent GDH activity was present in the free-living *A. cycadeae* but undetectable in the cyanobiont. There was a reduction in the activities of AsDH, ADH, GPT and GS (both biosynthetic and transferase), and an increase in the activity of GOT, in the cyanobiont as compared to those in the free-living *A. cycadeae*. It is interesting to note here that while in lichens and *Azolla* the GS levels are reduced by over 90% and 70%, respectively, here in the case of the *C. circinalis* cyanobiont the reduction level of GS seems much smaller (Table 1). This points to a possibility that the cycad cyanobiont, unlike those in lichens and *Azolla* (Rai et al., 1981, 1983; Peters et al., 1980),

transfers fixed-N_2 to its eukaryotic partner not entirely as ammonia but does so, at least partially, in the form of organic-N. This should merit further research studies.

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