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N_2-Fixation by Freshly Isolated *Nostoc* from Coralloid Roots of the Cycad *Macrozamia riedlei* (Fisch. ex Gaud.) Gardn.[1]

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ABSTRACT

Nitrogenase (EC 1.7.99.2) activity (acetylene reduction) and nitrogen fixation ($^{15}N_2$ fixation) were measured in cyanobacteria freshly isolated from the coralloid roots of *Macrozamia riedlei* (Fisch. ex Gaud.) Gardn. Light and gas phase oxygen concentration had marked interactive effects on activity, with higher (up to 100-fold) rates of acetylene reduction and $^{15}N_2$ fixation in light. The relationship between ethylene formation and N_2-fixation varied in the freshly isolated cyanobacteria from 4 to 7 nanomoles of C_2H_4 per nanomole $^{15}N_2$. Intact coralloid roots, incubated in darkness and ambient air, showed a value of 4.3. Maximum rates of nitrogenase activity occurred at about 0.6% O_2 in light, while in darkness there was a broad optimum around 5 to 8% O_2. Inhibition of nitrogenase, in light, by pO_2 above 0.6% was irreversible. Measurements of light-dependent O_2 evolution and $^{14}CO_2$ fixation indicated negligible photosynthetic electron transport involving photosystem II and, on the basis of inhibitor studies, the stimulatory effect of light was attributed to cyclic photophosphorylation. Nitrogenase activity of free-living culture of an isolate from *Macrozamia* (*Nostoc* PCC 73102) was only slightly inhibited by O_2 levels above 6% O_2 and the inhibition was reversible. These cells showed rates of light-dependent O_2 evolution and $^{14}CO_2$ fixation which were 100- to 200-fold higher than those by the freshly isolated symbiont. Furthermore, nitrogenase activity was dependent on both photosynthetic electron transport and photophosphorylation. These data indicate that cyanobacteria within cycad coralloid roots are differentiated specifically for symbiotic functioning in a microaerobic environment. Specializations include a high heterocyst frequency, enhanced permeability to O_2, and a direct dependence on the cycad for substrates to support nitrogenase activity.

Heterocystous, nonbranching cyanobacteria of the genus *Nostoc* and *Anabaena* form symbioses in which atmospheric N_2 fixed by the prokaryotic partner is transferred to the host (22). These symbioses include those with fungi or with fungi and green algae in lichens (17, 27), thalloid liverworts (26, 33), the aquatic fern *Azolla* (21, 22), the apogeotropic coralloid roots of cycads (15, 16, 19), and glands in the bases of the angiosperm *Gunnera* (5). With the exception of *Gunnera*, the microsymbiont is intercellular with respect to host tissues, and symbiosis takes place in normally differentiated host structures (4, 16, 21, 22, 26). However, a number of structural and/or functional alterations to the cyanobacterial components may occur, all apparently favoring symbiotic functioning (4, 13, 16, 21, 22, 26). These include changes in the gross structure of filaments and ultrastructure of vegetative cells and heterocysts, and in metabolic features associated with photosynthesis, nitrogen fixation, and nitrogen assimilation. While the microenvironment formed by the plant tissues in close contact with the cyanobacteria may well be an important component fostering symbiosis, factors in this environment specifically altering gene expression of one or the other partner remain to be identified.

In some cyanobacterial symbioses, the N_2-fixing cells are exposed to light and lie adjacent to photosynthetically active host cells. Fixation of N_2 is, therefore, likely to operate in an essentially aerobic environment, *e.g.* a gas phase O_2 pressure in the symbiotic cavities of *Azolla* fronds of 0.157 to 0.185 atm (7). Furthermore, light may potentially stimulate nitrogenase activity in these symbioses through the provision of reductant and ATP generated from photosynthetic reactions (21). In many cycad symbioses, on the other hand, the cyanobacteria are essentially excluded from light and must accordingly be dependent on heterotrophic mechanisms for reductant and ATP (15, 16, 19). While studies of intact cycad-cyanobacterial symbioses have indicated changes in filament structure and levels of nitrogenase activity in the prokaryote likely to enhance N_2 fixation (15, 16), there is little information concerning adaptations to the microenvironment of the coralloid root.

Studies with freshly isolated symbiotic cyanobacteria from coralloid roots of *Cycas circinalis* (14, 20) have shown high rates of photosynthetic O_2 evolution as well as light-stimulated nitrogenase activity in excess of those found in cultured free-living cyanobacteria originally isolated from the same source (20). In the above cycad, coralloid roots develop at or near the soil surface where cyanobacteria may be exposed to light.

The aim of the present study was to examine cyanobacteria freshly isolated from the Australian cycad *Macrozamia riedlei* in which coralloid roots develop and fix N_2 at a considerable depth (up to 0.5 m) below the soil surface (9, 19). Specifically, we have demonstrated the nature of interactions between light/darkness and oxygen levels on nitrogen and carbon fixation. Moreover, because earlier studies have shown that

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fixed nitrogen is translocated in the form of glutamine and citrulline (19), the capacity for citrulline synthesis by the isolated cyanobacterial symbiont has been investigated.

MATERIAL AND METHODS

Experimental Material

Actively growing coralloid roots were collected *in situ* from a series of shallow excavations made around mature members of a natural population of *Macrozamia riedlei* (Fisch. ex Gaud.) Gardn. in bushland in the vicinity of Perth, W.A., Australia. The harvested material was transported from the field to the laboratory within 30 min of excavation. The first 8 to 10 mm of cyanobacteria-containing lobes (16, 19) from freshly harvested samples of roots were cut into thin sections (about 1 mm each) under N_2 or Ar in degassed 10 mM Hepes buffer (pH 8.0) and suspended in this buffer, under N_2 or Ar, in the dark for 40 to 60 min. The suspension was not bubbled with gas during isolation. The cyanobacterial zone within the cortex contains a large amount of gelatinous material which apparently imbibes water and swells, forcing the cyanobacteria out of the root tissue (19) into the buffer solution. Filaments released in this way were collected by centrifugation (5000*g*, 5 min) and gently washed twice in degassed buffer under conditions of darkness and low O_2 (1–2% O_2) before they were used in experiments. The total time between cutting the coralloid roots into thin sections and commencement of experiments on the freshly isolated cyanobacteria varied from 70 to 90 min. To minimize damage to the natural population of the cycad, only small amounts of material were collected from an individual host plant at any one time. As a consequence, both the source and activity of the freshly isolated samples varied from day to day. Data are accordingly related specifically to individual experiments rather than averaged over a whole study period.

Free-living *Nostoc* PCC 73102 (an axenic, free-living strain originally isolated from *Macrozamia* [25]) were cultured photautotrophically under an atmosphere of ambient air in $BG11_0$-medium (30). Cells were harvested by centrifugation (5000*g*, 5 min) and washed once in Hepes buffer (10 mM [pH 8.0]) before they were used in experiments.

Nitrogenase Assay

Nitrogenase (EC 1.7.99.2) activity was assayed by acetylene reduction (32). Suspensions of cyanobacterial cells were incubated with shaking at 28°C in 9- or 34-mL serum vials containing 10% (v/v) acetylene in N_2 or Ar with various levels of O_2 (0.4–20%, v/v; see below). The samples were incubated in darkness or in white light (90 $\mu E \cdot m^{-2} \cdot s^{-1}$, from a mixture of cool-white fluorescent and incandescent lamps), and subsamples (0.5 or 1.0 mL) of the gas space were withdrawn at varying intervals for measurement of ethylene content by gas chromatography (1). Nitrogenase activity of intact lobes of coralloid root (5–12 mm long) was assayed in 10% (v/v) acetylene in air in darkness at 28°C. The time course of acetylene reduction was determined both for coralloid roots and suspensions of freshly isolated cyanobacteria by the "flow-through" gas exchange system described previously (3). Significant inhibition due to acetylene was not detected, and rates of nitrogenase and effects of O_2 on activity were the same as those found with assays carried out under a closed acetylene-containing atmosphere. Accordingly, only data obtained with the latter technique are reported here.

Assay of $^{15}N_2$ Fixation

Samples of the freshly isolated cyanobacterial cell suspension (10 mL) or intact lobes (5–12 mm long) of coralloid roots were exposed to $^{15}N_2$ (99 atom % excess) for 3 h. Exposure to $^{15}N_2$ was terminated by opening the vials to air and extracting cells or tissue slices in 80% (v/v) ethanol. After evaporation to dryness in a stream of N_2 at room temperature, the extracts were digested by a standard Kjeldahl procedure. Ammonia was collected by steam distillation, oxidized to N_2 for $^{14}N/^{15}N$ assay by mass spectrometry (19), and analyzed for enrichment (atom percent excess) against an ambient atmospheric standard.

Determination of Products of $^{14}CO_2$ Fixation

The incorporation of $^{14}CO_2$ into organic solutes was measured by incubating 4 to 10 mL aliquots of a suspension of cyanobacterial cells in the presence of $NaH^{14}HCO_3$ (148 kBq · mL^{-1}; specific activity 2.0 GBq · $mmol^{-1}$) in closed 34-mL serum vials at 28°C under continuous shaking in light (90 $\mu E \cdot m^{-2} \cdot s^{-1}$) or darkness. Reactions were terminated by addition of 80% (v/v) ethanol acidified with formic acid. The extract was evaporated to dryness and partitioned between distilled water and petroleum ether, and the aqueous phase was collected for analysis of labeled products. Nonamino and amino compounds were separated, and ^{14}C content of individual compounds was determined by the ion-exchange HPLC techniques described earlier (2, 3).

Assay of Oxygen Concentration

The initial O_2 contents of atmospheres of vials used subsequently for acetylene reduction assay were measured as described previously (2), with 1.0 mL samples and a gas chromatograph equipped with a thermal conductivity detector. Changes in dissolved O_2 were measured with an oxygen electrode (6), operating at 28°C both in darkness and in light (80–100 $\mu E \cdot m^{-2} \cdot s^{-1}$).

Chl *a* Content and Heterocyst Frequency

Chl *a* content was determined by extracting cyanobacterial cells or samples of coralloid roots in 80% (v/v) acetone and measuring absorbance at 663 and 645 nm (8). Heterocyst frequency was measured as described previously (11).

RESULTS

Light and oxygen partial pressure (pO_2) had marked interactive effects on the level of nitrogenase activity shown by cyanobacteria freshly isolated from coralloid roots of *Macrozamia riedlei* (Fig. 1). Rates of acetylene reduction were much higher in light than in darkness, and stimulation by light increased with decreasing pO_2 to a maximum at the lowest concentrations used (1–2% O_2). When the cyanobacteria were

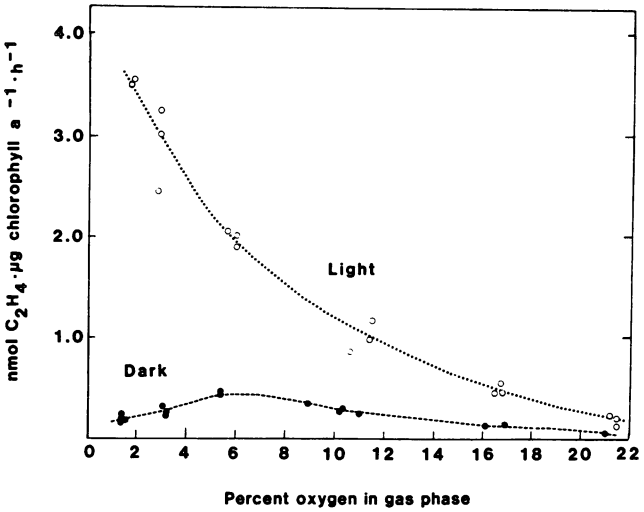

Figure 1. Acetylene reduction in cyanobacteria freshly isolated from *M. riedlei*. The washed cell suspensions were incubated for 30 min under continuous shaking in atmospheres of different oxygen concentrations in darkness (●) or in light (○) (90 $\mu E \cdot m^{-2} \cdot s^{-1}$).

incubated in darkness, a broad optimum around 5 to 8% O_2 was observed.

In an attempt to reduce O_2 concentrations in the gas-phase below 1 to 2% (as in Fig. 1), the lighter N_2 was replaced with the heavier Ar during isolation and subsequent assays. In this way, levels as low as 0.45% O_2 were achieved. Combined data from a number of separate experiments showed a peak in nitrogenase activity at around 0.6% O_2 (Fig. 2). Lower pO_2 could be achieved by vigorously degassing cyanobacterial suspensions with Ar. However, this treatment physically disrupted the filaments and resulted in an irreversible decline of more than 50% in acetylene reduction.

The effects of light and O_2 on nitrogenase activity were much the same for cyanobacteria freshly isolated from close

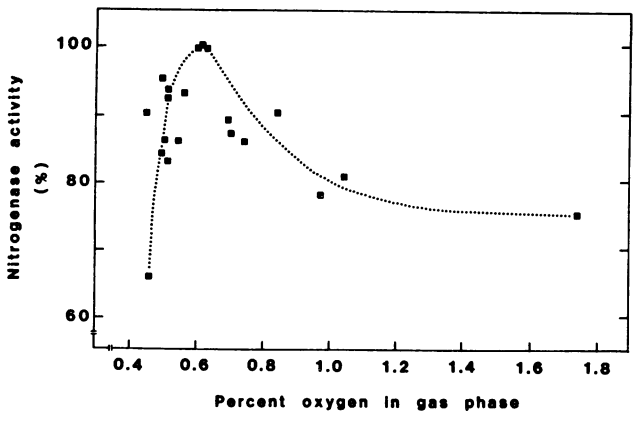

Figure 2. Acetylene reduction in cyanobacteria freshly isolated from *M. riedlei*. The cell suspensions were incubated for 10 min under continuous shaking in atmospheres of different oxygen concentrations in light (90 $\mu E \cdot m^{-2} \cdot s^{-1}$). Data are taken from two separate experiments and rates expressed relative to the maximum rate (100%).

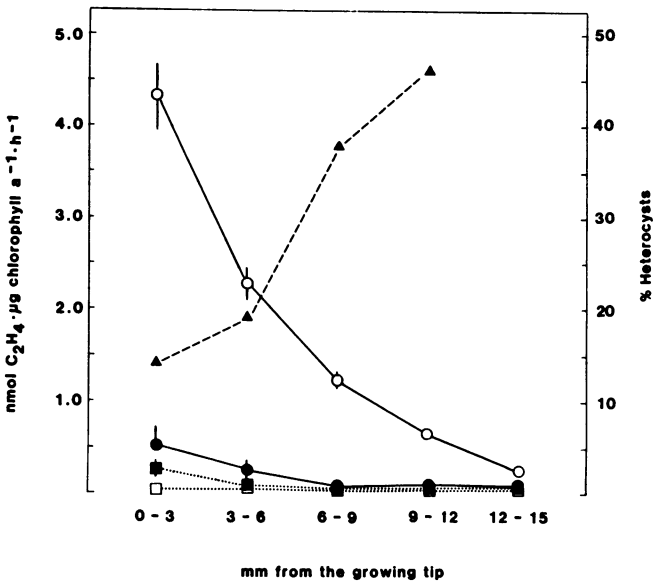

Figure 3. Acetylene reduction and heterocyst frequency in cyanobacteria freshly isolated from different sections of coralloid roots of *M. riedlei*. The coralloid roots were cut into about 3-mm-thick sections, numbered 1 to 5 beginning at the growing tip of the root and incubated in 1% O_2 or air, both containing 10% acetylene. The freshly isolated cyanobacterial cells were first incubated in darkness for 30 min (●——● = 1% O_2; ■···■ = air) and then exposed to light (90 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 30 min (○——○ = 1% O_2; □···□ = air). Values are means ± SE (n = 3). Heterocyst frequency (▲– – –▲) was measured in counts of approximately 1000 cyanobacterial cells in each tissue section.

to the apex of a coralloid root as from more basal regions (Fig. 3). Although the rate of acetylene reduction was decreased in sections progressively away from the growing tip, light stimulated activity, as did lower pO_2. The frequency of heterocysts in root segments showed an inverse relationship with nitrogenase activity, increasing from approximately 14% of total cyanobacterial cells at the growing tip (first 2 mm) to more than 45% as heterocysts 12 to 15 cm from the tip (Fig. 3).

Measurements of nitrogenase activity in freshly isolated symbiotic cyanobacteria using $^{15}N_2$ confirmed the stimulation of activity by light (Table I). Samples of the same suspensions used for $^{15}N_2$ fixation were also assayed for nitrogenase activity by acetylene reduction. The relationship between ethylene formation and N_2-fixation in these samples varied from 7 nmol C_2H_4·nmol $^{15}N_2^{-1}$ in light at 1% O_2 to 4 nmol C_2H_4·nmol $^{15}N_2^{-1}$ in darkness at 1% O_2 (Table IA). However, the rates of fixation in darkness were very low so that the calculated ratios in this case were unreliable. Intact coralloid roots, incubated in darkness and ambient O_2 level, showed a corresponding value of 4.3 (Table IB).

Inhibition of nitrogenase by pO_2 greater than 2 to 3% O_2 in the light was effectively irreversible. Freshly isolated suspensions, assayed for acetylene reduction over 10 min at low (2–4%) O_2 and then exposed to air (21% O_2) for 30 min before being reassayed at low O_2, were inhibited by as much as 94% (Table II). Inhibition did not increase following sub-

Table I. *Acetylene Reduction Activity and $^{15}N_2$-Fixation by Freshly Isolated Cyanobacteria (A) and Intact Coralloid Roots (B) of M. riedlei*

Rates of acetylene reduction were measured over 2 h and $^{15}N_2$ fixation over 3 h periods. Chl *a* was assayed at the end of the incubation period. The cyanobacterial suspension used in this experiment was prepared from the same sample of material collected from the field which provided the intact coralloid roots for assay.

Incubation Conditions	Acetylene Reduction	$^{15}N_2$ Fixation	C_2H_4/N_2
	nmol C_2H_4·(μg Chl a)$^{-1}$·h^{-1}	*nmol $^{15}N_2$·(μg Chl a)$^{-1}$·h^{-1}*	*nmol C_2H_4· nmol $^{15}N_2^{-1}$*
A. Freshly isolated cyanobacteria			
Light, 1–2% O_2	2.10 ± 0.20[a]	0.30 ± 0.07	7.0 ± 0.8
Dark, 1–2% O_2	0.02 ± 0.01	0.005 ± 0.005	4.0 ± 6.0
B. Intact coralloid roots			
Dark, Air	6.27 ± 0.36	1.47 ± 0.18	4.3 ± 0.8

[a] Values are means ± SE (n = 3).

sequent cycles in air. However, exposure to pO_2 levels intermediate between 2 and 3 and 40% O_2 resulted in intermediate levels of inhibition of nitrogenase (Table II). These features of the response to O_2 were essentially the same for cyanobacteria freshly isolated from the tip region or from more basal sections of coralloid root lobes (data not shown).

Light also stimulated acetylene reduction by free-living cyanobacteria which had been grown photoautotrophically with ambient air (Fig. 4). Like freshly isolated cell suspensions from coralloid roots, the free-living organism showed increased activity in subambient O_2. However, in this case, the stimulation was only twofold compared to the activity in air (Fig. 4). Inhibition due to air was completely reversible (data not shown). The free-living organism showed evidence of a broad optimum between 5 and 10% O_2 for activity in darkness (Fig. 4).

The effect of light in stimulating nitrogenase activity of freshly isolated cyanobacteria was inhibited by addition of 100 μM of the photophosphorylation uncoupler, carbonylcyanide *m*-chlorophenyl-hydrazone (21.3 ± 3.7 [means ± SE, n = 6]% compared with control), but not by addition of 100 μM of the PSII inhibitor, 3-(3^1, 4^1-dichlorophenyl)-1, 1-dimethylurea (89.1 ± 8.8% compared with control). Assays of O_2 exchange showed that light-dependent net O_2 evolution was negligible in freshly isolated cyanobacteria, being 5.6 (±0.3, means ± SE) nmol O_2·(μg Chl *a*)$^{-1}$·h^{-1}, compared to 1.4 μmol O_2·(μg Chl *a*)$^{-1}$·h^{-1} by the free-living cultured organism.

Table II. *Effect of pO_2 on Nitrogenase Activity in Cyanobacteria Freshly Isolated from Coralloid Roots of M. riedlei*

The cyanobacteria were isolated over a 1-h period under N_2 in the dark and then assayed (Assay 1 = 100% activity) for C_2H_2-reduction over a 10-min period in low pO_2 (2–4%) in the light (90 μE·m^{-2}·s^{-1}; 10 min). Vials were then flushed with this atmosphere minus C_2H_2 and exposed to a range of pO_2 from 2 to 3% to 40% O_2 for 30 min in the light after which they were again flushed with low O_2 and C_2H_2 reduction assayed again (Assay 2) as above. Subsequently, the vials were flushed and exposed to the same range of O_2 levels used in the first transfer. After 30 min in the light, the atmosphere was replaced with low pO_2 for a second time and nitrogenase was assayed again (Assay 3).

pO_2	Nitrogenase Activity	
	Assay 2	Assay 3
	% of control (=Assay 1)	
2–3% O_2	97.2 ± 5.6[a]	95.0 ± 3.9
3–4% O_2	87.3 ± 7.2	82.5 ± 6.0
6% O_2	47.4 ± 2.9	43.4 ± 13.1
9% O_2	18.9 ± 4.3	14.2 ± 0.9
11% O_2	19.1 ± 3.0	8.5 ± 0.3
21% O_2	6.7 ± 1.5	6.0 ± 1.0
40% O_2	3.7 ± 0.5	3.3 ± 0.3

[a] Values are means ± SE (n = 4).

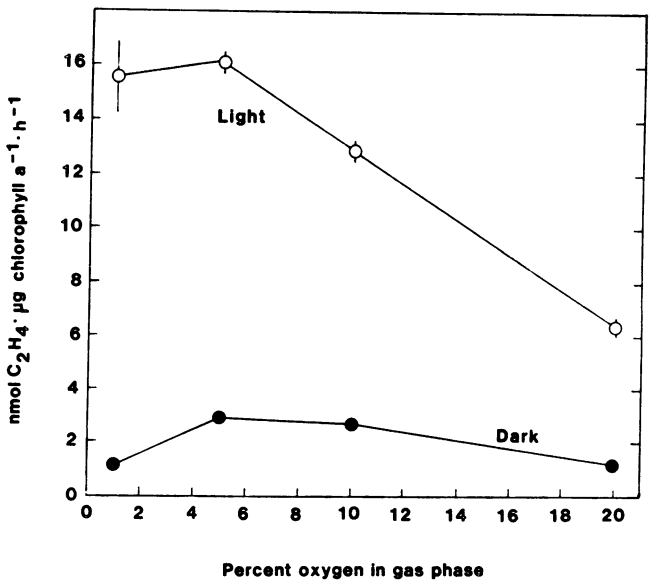

Figure 4. Acetylene reduction by free-living cyanobacteria (*Nostoc* PCC 73102) cultured under photoautotrophic conditions in a medium free from combined N. Suspensions were assayed in darkness (●) or in light (○) (90 μE·m^{-2}·s^{-1}) with a range of O_2 levels in the gas phase. Values are means ± SE (n = 3).

Light-stimulated $^{14}CO_2$ fixation by both freshly isolated and photoautotrophically cultured cell suspensions, but, whereas the rate was increased 4- to 7-fold in the freshly isolated organism, fixation by the cultured cyanobacteria was stimulated more than 50-fold (Table III). Low pO_2 (5% O_2) had no effect on $^{14}CO_2$ fixed by the cultured free-living cyanobacteria, but, in freshly isolated cyanobacteria, 5% O_2 or air resulted in doubling or more of fixation compared to that in 1% O_2, both in light and darkness. Addition of ornithine also increased fixation in the symbiotic (freshly isolated) organism but had almost no effect on rates in cultured cyanobacteria (Table III). Both cyanobacterial suspensions formed [^{14}C] citrulline and [^{14}C]arginine as products of $^{14}CO_2$ fixation. The highest rates of synthesis were found for the free-living cyanobacteria in light, especially following ornithine addition (Table IIIB). Ornithine also stimulated labeling of both citrulline and arginine by the freshly isolated organism. The highest rates in this case was in the light, but, in darkness, as much as 37% of the total ^{14}C which was fixed was recovered in these two amino compounds (5% O_2 + Ornithine; Table IIIA).

DISCUSSION

This study demonstrates that freshly isolated washed cell suspensions of symbiotic cyanobacteria from coralloid roots of *Macrozamia riedlei* retain nitrogenase activity when separated from their host as long as they are not exposed to levels of O_2 above about 1%. Rates of C_2H_2 reduction and $^{15}N_2$ fixation by these cyanobacteria, incubated in darkness at the optimal pO_2 level (5–8% O_2), are, however, low (less than 0.5 nmol $C_2H_4 \cdot \mu g$ Chl $a^{-1} \cdot h^{-1}$) compared with the activity initially recorded (in darkness) in the intact coralloid roots from which they had been isolated (6 nmol $C_2H_4 \cdot \mu g$ Chl $a^{-1} \cdot h^{-1}$). Nevertheless, the potential for fixation at rates equal to those in the intact symbiosis must be retained following isolation, because C_2H_2 reduction upon illumination became as great or greater than that recorded for the intact symbiosis. Furthermore, the highest values recorded in light and optimally low pO_2 (14 nmol $C_2H_4 \cdot \mu g$ Chl $a^{-1} \cdot h^{-1}$, see Fig. 2) are almost equal to those exhibited by the free-living isolate cultured under photoautotrophic conditions (16 nmol $C_2H_4 \cdot \mu g$ Chl $a^{-1} \cdot h^{-1}$).

The effect of light on acetylene reduction by the freshly isolated organism is unlikely to be due to photosynthetic generation of reductant or to the provision of oxidizable substrates through enhanced fixation of CO_2. Light-dependent O_2 evolution was negligible, and addition of 100 μM DCMU had no significant effect on acetylene reduction. Furthermore, rates of $^{14}CO_2$ fixation, though stimulated by light, were relatively low in the isolated organism compared to free-living cyanobacteria. These observations are consistent with the symbiotic cyanobacteria having little or no PSII activity and contrast to the free-living organism, which showed high rates of photosynthesis (O_2 evolution and CO_2 fixation). Interest-

Table III. *Fixation of [^{14}C]CO_2 and Formation of [^{14}C]Citrulline and [^{14}C]Arginine by (A) Cyanobacteria Freshly Isolated from Coralloid Roots of M. riedlei, and (B) Nostoc PCC 73102 Cultured Under Photoautotrophic conditions*

The cell suspensions were incubated with [^{14}C]CO_2 in light (90 $\mu E \cdot m^{-2} \cdot s^{-1}$) or darkness, with 1% O_2, 5% O_2, or air in the gas phase and with 0 or 10 mM ornithine (orn) added for 1 h at 30°C.

Incubation Conditions	Total $^{14}CO_2$ Fixed	[^{14}C]Citrulline	[^{14}C]Arginine
	[Bq·(μg Chl a)$^{-1}$·h^{-1}]		
A. Freshly isolated cyanobacteria			
Light 1% O_2	25.8	1.3	ND[a]
Light 5% O_2	49.2	0.7	0.2
Light Air	42.0	2.0	0.9
Light 1% O_2 + orn	52.8	7.1	1.8
Light 5% O_2 + orn	115.8	13.7	4.6
Light Air + orn	82.8	11.0	3.6
Darkness 1% O_2	3.6	ND	ND
Darkness 5% O_2	12.0	0.6	ND
Darkness Air	9.0	1.2	ND
Darkness 1% O_2 + orn	6.0	1.4	0.9
Darkness 5% O_2 + orn	23.4	5.5	3.2
Darkness Air + orn	13.8	4.9	1.4
B. Cultured free-living cyanobacteria			
Light 5% O_2	2460	ND	62.4
Light Air	2252	ND	27.6
Light 5% O_2 + orn	2880	38.4	57.6
Light Air + orn	3120	57.7	64.8
Darkness 5% O_2	48.6	0.7	0.7
Darkness Air	39.0	0.5	0.8
Darkness 5% O_2 + orn	66.0	2.5	3.3
Darkness Air + orn	39.0	0.9	1.5

[a] Not detectable.

ingly, a freshly isolated organism from *Cycas circinalis* differed from that of *M. riedlei* and showed high rates of photosynthetic O_2 evolution under saturating light conditions (20).

The stimulation of nitrogenase by light is probably due to increased ATP supply through cyclic photophosphorylation in heterocysts. High rates of photophosphorylation, despite reduced photosynthetic CO_2 fixation, have been demonstrated in a number of other symbiotic cyanobacteria (21, 22), indicating that electron transport supporting ATP synthesis in these systems relies on heterotrophic mechanisms (21). Although the present experiments with cyanobacteria freshly isolated from *M. riedlei* fail to identify the substrates used to support nitrogenase, it seems likely that they were supplied by the host prior to isolation. Such substrates may include low mol wt solutes translocated from the host and possibly used in part to synthesize carbohydrate polymers (28).

In view of the apparent close dependence of the freshly isolated organism on ATP supply, it is reasonable to suppose that low levels of nitrogenase activity in darkness reflect loss or damage of heterotrophic mechanisms previously functioning to provide ATP within the intact coralloid root. Separation of the cyanobacteria from the host tissue disrupts the intercellular microenvironment and any biochemical interactions which the cyanobacteria might have experienced *in situ* in the coralloid root (19). Damage to cyanobacterial filaments during their separation from the host might also eliminate previous metabolic interactions between heterocysts and vegetative cells. Addition of ornithine to the freshly isolated preparation increases $^{14}CO_2$ fixation specifically into citrulline and arginine. The very marked stimulation of citrulline synthesis by exogenous ornithine does, however, raise the possibility that this precursor might normally be provided by the host. Although citrulline synthesis is a prominent feature of the carbon and nitrogen metabolism of the isolated cyanobacteria, the cultured free-living organism also exhibited this propensity, suggesting that an explanation other than simple enhancement of this metabolic sequence is needed to account for the marked predominance of citrulline in the translocated products of this symbiosis (19).

The coralloid roots of *M. riedlei* exhibit a pattern of filament differentiation similar to that of a number of other cycads (15, 16) in that the frequency of heterocysts, and especially multiple heterocysts, increases progressively from the root apex. However, as shown for *Cycas* and *Zamia* (15, 16), nitrogenase activity decreases in progressively older segments, and this inverse correlation between acetylene reduction and heterocyst frequency also holds for *M. riedlei*. Lindblad (15) has suggested that the decline in acetylene reduction along cycad coralloid roots is probably due partly to aging of the cyanobacteria and to the fact that multiple and single heterocysts are intrinsically inactive. Similar changes have been noted for old fronds of *Azolla filiculoides* (4). However, despite the decline in nitrogenase activity in cyanobacteria freshly isolated from older tissues of *M. riedlei,* all show pronounced stimulation by light and a requirement for low O_2 to promote maximum rates of activity.

There have been few measurements which compare acetylene reduction and $^{15}N_2$ fixation by intact cycad-cyanobacterial symbioses and, as far as we are aware, none has been conducted with the freshly isolated diazotrophic partner. With coralloid roots of *M. riedlei* collected in the field, values in the range of 2.27 to 11.78 mol C_2H_2 reduced:mol N_2 fixed have been reported (9). The average, 5.8:1.0, as well as that of 4.3 for intact coralloid roots obtained in the present study, suggests that the symbiosis evolves H_2 at rates at least equivalent to those of N_2 reduction. These observations are consistent with those for the freshly isolated organism from *C. circinalis,* which appears to have lost uptake hydrogenase activity (14, 20). The ratio of C_2H_2 reduced:N_2 fixed by intact *Azolla-Anabaena* has been found to range from 3.2 in 0.3 atm N_2 to 1.7 in 0.8 N_2, while values for the freshly isolated cyanobacteria from this symbiosis vary from 4.4 to 2.5 (23). Peters *et al.* (23) also showed that H_2 evolution by the *Azolla* symbiosis varied with pN_2, but that the presence of uptake hydrogenase limited H_2 evolved under ambient conditions.

A particularly interesting feature of freshly isolated cyanobacteria of *M. riedlei* is the very marked and irreversible inhibition of light-stimulated nitrogenase activity with increasing gas phase pO_2. This property is not shared by cyanobacteria freshly isolated from other symbioses (5, 26, 33) or by free-living cultures and appears to be due to inactivation of nitrogenase. An alternative explanation for the effect of increasing pO_2 in light is that under the conditions, particularly relevant for cyanobacteria suddenly exposed to light, O_2 caused photoinhibition of pigments. However, any photooxidation would have to have been selective, affecting only those pigments involved in photophosphorylation supporting nitrogenase and not those supplying reductant and ATP to CO_2 fixation. In fact, in the light, increasing pO_2 stimulated $^{14}CO_2$ fixation.

Except for certain mutants of *Anabaena* which fail to form heterocysts (29), in which the heterocysts are deficient in envelope glycolipids (10), or in which normal protective mechanisms for nitrogenase are absent (34), light-dependent nitrogenase activity by free-living heterocystous cyanobacteria has generally been reported to be only slightly increased, or even decreased, by subambient O_2 (12, 31). O_2-sensitive nitrogenase activity has, however, been reported for some free-living cyanobacteria in which diazotrophy had been induced under anaerobic conditions of growth (18, 24, 29). Thus, the cycad coralloid root appear to provide a low O_2 environment, to which the cyanobacteria in turn respond by forming heterocysts which are more permeable to gases than those forming in ambient air. In view of the importance of the interactions between O_2 and nitrogenase activity in determining the effectiveness of symbiotic N_2-fixation, the cycad-*Nostoc* association provides a most useful model for studying the relationship in an intercellular partnership within a plant root.

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