Symbiosis, 1 (1985) 19-28
Balaban Publishers, Philadelphia/Rehovot

The Cyanobacterium *Zamia* Symbiosis: C_2H_2-Reduction and Heterocyst Frequency

P. LINDBLAD, L. HÄLLBOM and B. BERGMAN

Institute of Physiological Botany, University of Uppsala, Box 540, S-751 21 Uppsala, Sweden.
Tel. Int. + 46 18 18 28 00; Telex 76024 UniUp S (Fys. bot.).

Received October 20, 1984; Accepted February 17, 1985

Abstract

Filamentous cyanobacteria occur in a zone between the outer and inner cortex in the coralloid roots of the cycad *Zamia skinneri*. The content of cyanobiont chlorophyll *a* was essentially constant along the coralloid root even though there were considerable changes in the cyanobiont's form and function. Acetylene reduction activity, expressed either on a basis of root fresh weight or cyanobiont chlorophyll *a* content, was greatest at the growing tip of the coralloid root and declined towards basal, older parts. In contrast, the frequency of heterocysts was lowest in the tip (16.7%) and gradually increased to approximately 45%. Mixtures of single, double, triple and even quadruple heterocysts were observed. At the growing tip the majority (86%) were single; the frequency of multiple heterocysts increasing with root age. Nitrogenase activity appears to be confined primarily to sections of the coralloid root where single heterocysts predominate.

Key words: Cycad, *Zamia*, cyanobacteria, symbiosis, C_2H_2-reduction

1. Introduction

Nitrogen-fixing cyanobacteria form associations with almost all groups of plants: algae, fungi, liverworts, ferns, gymnosperms and angiosperm (Stewart et al., 1983). Cycads are the only gymnosperms which form such associations. There are 10 cycad genera with approximately 150 species (Giddy, 1974) and all of those investigated have been found to contain heterocystous cyanobacteria in, often dichotomously branched, coralloid roots. Changes in cyanobacterial forms along the coralloid roots, including an increase in heterocyst frequency away from the tip, have been described in different cycad species (Grilli Caiola, 1980; Lindblad, 1984). Increased heterocyst frequency towards more basal or older parts occurs in other plant – cyanobacterial symbioses too (Becking, 1978; Englund, 1977; Peters et al., 1982; Silvester, 1976). However, several different patterns in C_2H_2-reduction have been described. In the waterfern *Azolla* both a parallel increase in C_2H_2-reduction and heterocyst frequency (Hill, 1975, 1977; Peters et al.,

0334-5114/85/$03.00

1982) and two "peaks" in C_2H_2-reduction have been reported (Becking, 1978). The angiosperm *Gunnera* and the lichen *Peltigera aphthosa* both showed an initial increase in C_2H_2-reduction followed by a decrease in older parts (Silvester, 1976; Englund, 1977). A preliminary study of the cycad *Zamia floridana* showed a lack of correlation between C_2H_2-reduction and heterocyst frequency with C_2H_2-reduction being at a maximum just behind the growing tip of the coralloid root (Lindblad, 1984).

Here we report on the relationship of the C_2H_2-reduction to the heterocyst profile along the coralloid roots of the cycad *Zamia skinneri*.

2. Materials and Methods

Coralloid roots from the cycad *Zamia skinneri* Warsc., growing under the soil surface, were collected in the mornings of February to April 1984 in a Costa Rican forest between CATIE (Centro Agronomico Tropical de Investigacion y Ensananza), Turrialba and the river "Rio Reventazon"; 9°53″N and 83°38″E, about 950 meters above sea level. In this forest *Zamia skinneri* occurs as understorey vegetation. In the laboratory the coralloid roots were washed in distilled water and cut into sections. These sections were numbered sequentially starting with number one at the growing tip of the root. The experiments were started within one hour after collection. As coralloid roots usually were branched, some basal sections were examined more than once. The total number and numbers of different sections examined are presented for each experiment. All results are presented as means ±S.E. and the data have been tested with t-test on a 5% significance level.

Acetylene reduction activity was determined according to Stewart et al. (1967). Sections from coralloid roots were placed in 7.0 ml serum vials with a moist filter-paper (about 0.1 ml distilled water) at the bottom. Acetylene was injected to a partial pressure of 10^4 Pa and the vials were incubated in the dark, at 22°C, for six hours. One ml gas samples were withdrawn and the ethylene content analyzed. The production of ethylene was linear throughout the incubation time. The gas chromatograph used was based on the model described by Mallard et al. (1977), modified by L.-E. Henriksson (Institute of Physiological Botany, University of Uppsala, Sweden). Compressed air served as carrier gas. A standard curve was obtained with known concentrations of ethylene. Controls included samples with no acetylene added and non-coralloid cycad roots with acetylene added. None of the controls showed any ethylene production. Fresh weight of the coralloid roots was determined using a Mettler PC 220 electronic balance.

Chlorophyll *a* content was estimated according to Harborne (1973). Deep frozen, 1-2 mm thick, sections from coralloid roots were placed in 7.0 ml serum vials, 1.0 ml of 80% acetone was added and the sections were cut into smaller pieces. The solution was left in the dark at 22°C for 24 hours and then analyzed in a spectrophotometer, Hitachi model 100-80. Chlorophyll recovered during a

second extraction was negligible.

Heterocyst frequency was determined according to Hitch and Millbank (1975) using CrO_3. The same technique was used to determine filament length. To calculate the heterocyst frequency only heterocysts connected to vegetative cells within or at the end of filaments were counted. The microscope used was a Carl Zeiss, binocular, $12.5 \times 40/0.65$ (and $160/0.17$).

3. Results

The cyanobacteria are found intercellularly in a zone between the outer and inner cortex in the coralloid roots of the cycad *Zamia skinneri*. The zone is transversed by cycad cells. When such roots were divided into two sections a considerably higher acetylene reduction activity was observed in the apical section as compared with the basal section, expressed either on the basis of cyanobiont chlorophyll *a* or fresh weight. A similar pattern was evident when coralloid roots were divided into three sections. The apical sections had a lower fresh weight but the chlorophyll *a* content expressed per mg (fresh weight) was similar in the different sections examined.

When coralloid roots were divided into 1-2 mm sections, each having a fresh weight of about 6 mg, the acetylene reduction activity was again most pronounced in the apical sections and then declined towards basal and older parts (Fig. 1a). However, maximal activity, expressed per fresh weight, occurred in the second section. The average activity for the first three sections was 0.7 ± 0.09 nmole $C_2H_4 \cdot$ mg (fresh weight)$^{-1} \cdot h^{-1}$ compared with 0.4 ± 0.09 nmole $C_2H_4 \cdot$ mg (fresh weight)$^{-1} \cdot h^{-1}$ being the mean activity of the whole coralloid root. In the 1-2 mm sections, the chlorophyll *a* content throughout a root was quite constant, except for the very first section which showed a lower content (Fig. 1b). Thus, the acetylene reduction activity was most pronounced in the first sections whether quantitated on the basis of coralloid root tissue or cyanobacterial chlorophyll *a* (Fig. 1c).

Figure 2 shows heterocyst frequency of CrO_3 treated roots, determined for the various sections of the treated roots. Only heterocysts within or at the end of filaments were counted. The profile of heterocyst frequency was not paralleled by that of the acetylene reduction activity. In the first section 16.7% of the cells were heterocysts and in the two following sections the heterocyst frequency more than doubled, with an average of 28.4%. As seen in Fig. 1a these sections also showed the highest acetylene reduction activity. From section four and inwards the increase in the heterocyst frequency levelled off. As free heterocysts, not connected to filaments, were often observed these were estimated separately and it was found that their number increased with root age. In spite of a continuous increase in the total heterocyst frequency, the number of single heterocysts, expressed as a percentage of the total number, continuously declined along a coralloid root,

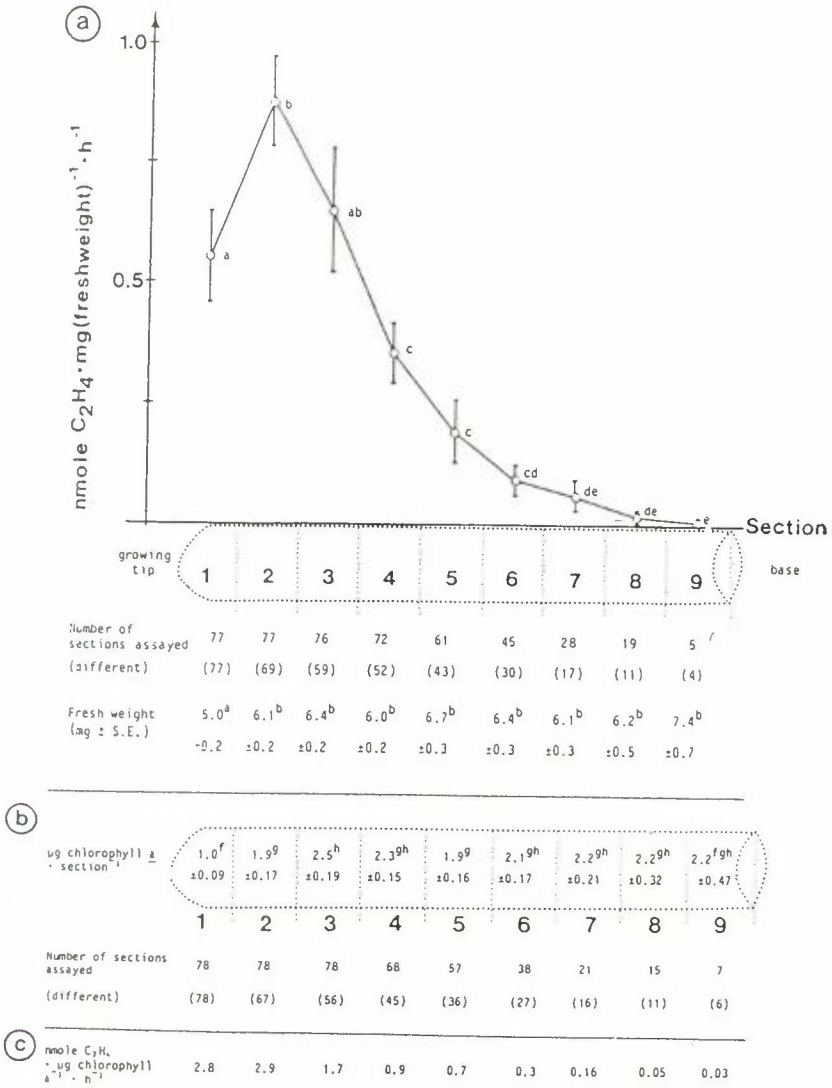
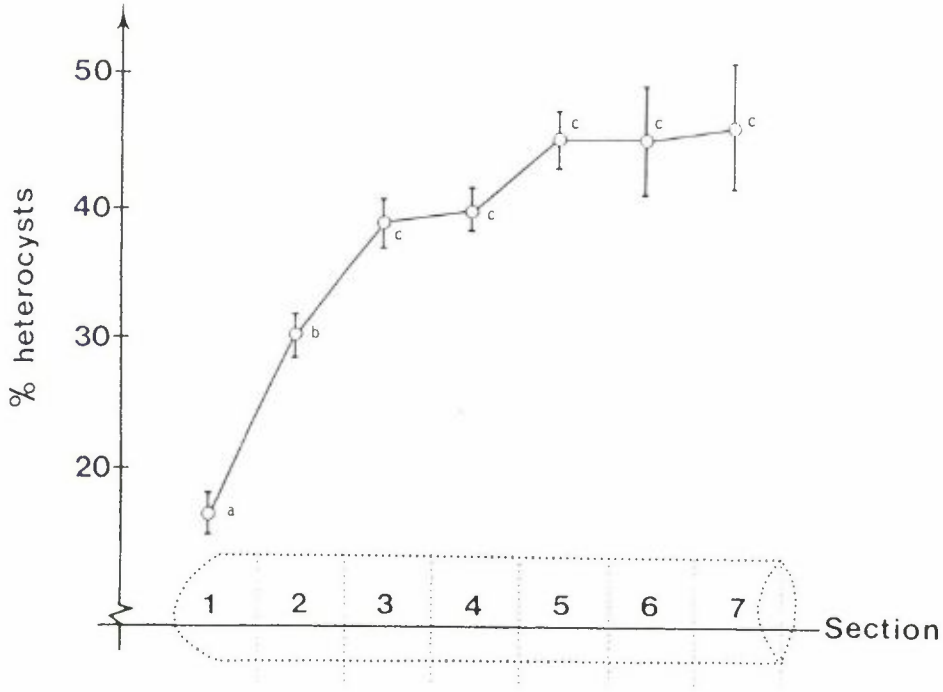


Figure 1. Acetylene reduction activity expressed per fresh weight (a), chlorophyll *a* content (b) and acetylene reduction activity expressed per chlorophyll *a* (c) in different sections of a coralloid root of the cycad *Zamia skinneri*. The level of (c) is calculated from the values in (a) and (b). The roots were cut into 1-2 mm thick sections and numbered 1-9 starting at the growing tip of the coralloid root.

As coralloid roots usually were branched, basal sections have been assayed more than once. The figures in brackets give the number of different sections assayed. Values given are means ± S.E. and those with the same letters are not significantly different at $P < 0.05$.



Number of sections counted	25	24	23	19	13	4	3
(different)	(25)	(22)	(20)	(17)	(13)	(4)	(3)
Number of cells counted	10261	6450	5441	4099	2526	801	560
(different)	(10261)	(5991)	(4750)	(3643)	(2526)	(801)	(560)
Fresh weight (mg ± S.E.)	4.7^{a} ±0.3	5.8^{b} ±0.3	6.5^{b} ±0.3	6.2^{b} ±0.3	6.5^{b} ±0.7	7.0^{ab} ±2.0	6.3^{ab} ±1.5

Figure 2. Heterocyst frequency in different sections of a coralloid root of the cycad *Zamia skinneri*. The roots were cut into 1-2 mm thick sections and numbered 1-7 starting at the growing tip of the coralloid root.

As coralloid roots usually were branched, basal sections have been counted more than once. The figures in brackets give the number of different sections counted. Values given are the means ± S.E. and those with the same letters are not significantly different at $P < 0.05$.

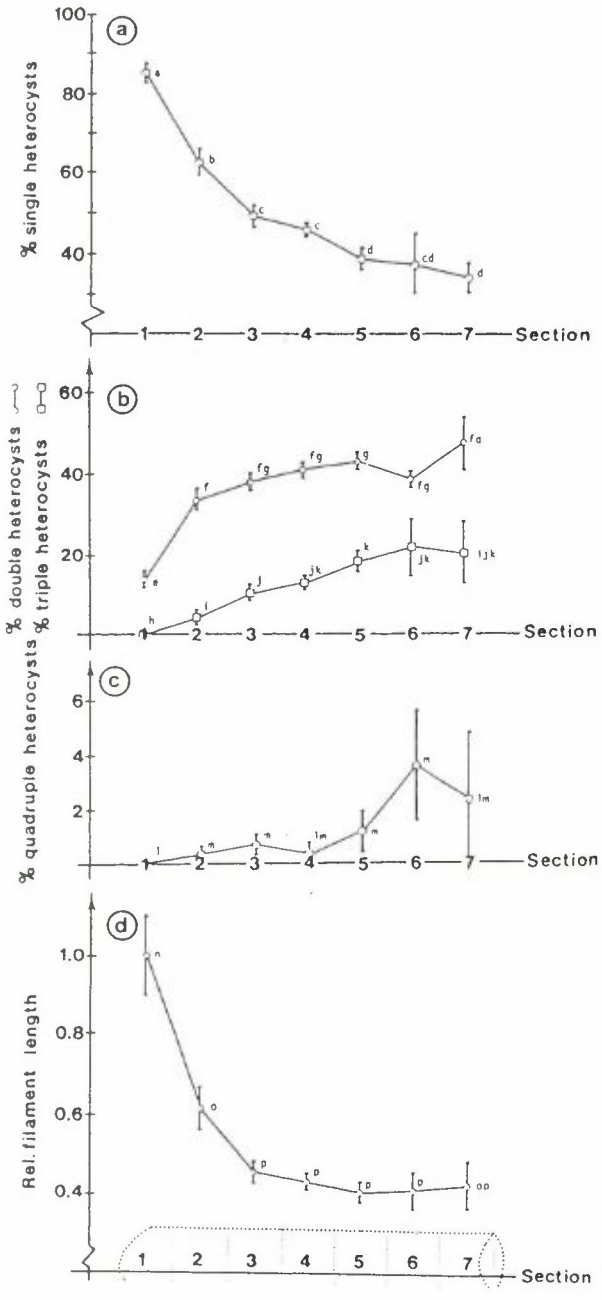


Figure 3. Percentages of single (a), multiple (b,c) heterocysts of the total number of heterocysts and relative filament length (d) in different sections of a coralloid root of the cycad *Zamia skinneri*. Otherwise as in Fig. 2.

decreasing from 85.6% at the root tip to 31% at the base (Fig. 3a). In contrast the frequency of double and triple heterocysts increased from 14.3 to 47% and 0 to 19.7% in the tip and at the base respectively (Fig. 3b). Even some quadruple heterocysts were found (Fig. 3c). Simultaneously, the relative filament length decreased from 1.0 (corresponding to 14.5 cells) in the first section to approximately 0.4 (Fig. 3d). A relative value is used as filaments easily break during treatment with CrO_3 and handling for the light microscope. Normally the filaments consist of more than 100 cyanobacterial cells.

4. Discussion

It is apparent that both the biomass of the coralloid roots of *Zamia skinneri* (Figs. 1a and 2) and, based on chlorophyll *a*, the cyanobacterium content (Fig. 1b) are approximately constant from just behind the growing tip to the basal regions. However, the cyanobacterium undergoes considerable changes both in form and function. Such changes have earlier been observed in some other symbiotic cyanobacteria (see e.g. Stewart et al., 1983).

Maximum acetylene reduction activity close to the growing tip and a gradual decline along the root corresponds to results found in a closely related cycad, *Zamia floridana* (Lindblad, 1984). The decline in acetylene reduction activity along the cycad roots is probably due to aging of the cyanobacteria, as shown by the decrease in filament length (Fig. 3d) and an increase in the number of free, i.e. not filament connected, heterocysts which are probably not active, and by lysis of vegetative cells (data not shown). At the growing tip the filaments may be several hundred cells long.

The several fold increase in total heterocyst frequency from younger towards older parts of the coralloid roots of *Z. skinneri* is a pattern found in several other cyanobacterial symbioses (Becking, 1978; Grilli Caiola, 1975, 1980; Hill, 1975, 1977; Lindblad, 1984; Silvester, 1976). Exceptions are lichens with one autotroph only, e.g. *Peltigera canina*, where a gradient in heterocyst frequency from younger to older parts occurs, but the frequency never exceeds 5% (Bergman and Hällbom, 1982). Spratt (1911) reported on a high frequency of heterocysts in *Cycas*, particularly in old material, and this was confirmed by Grilli Caiola (1975, 1980) and Zhu (1982). As we also observed double, triple and even quadruple heterocysts in *Z. skinneri*, it is worth noting that in 1894 Schneider showed drawings depicting what may be double and triple heterocysts in *Cycas*. Spratt (1911) and Grilli (1963a; 1963b) reported the occurrence of double heterocysts in the same genera. Multiple heterocysts have also been reported in *Gunnera* (Silvester, 1976). Cyanobacterial filaments without heterocysts, similar to those observed in *Peltigera canina* (Bergman and Hällbom, 1982), were never observed in the present study of *Z. skinneri*.

The fact that the acetylene reduction activity decreased towards basal and older parts of the coralloid roots contrasts with the more than threefold increase

in the total heterocyst frequency observed (Fig. 2). Thus, the number of potential nitrogen-fixing (acetylene reduction) cells correlates inversely with the activity of the enzyme nitrogenase. Our results, however, show that there is a good general correlation between acetylene reduction activity and the percentage of single heterocysts (Fig. 3a); while an inverse relation is apparent between acetylene reduction activity and the frequency of multiple heterocysts (Figs. 3b and c). The latter observation indicates a high frequency of inactive heterocysts in the multiple complexes.

The acetylene reduction activity by the *Z. skinneri* symbiont observed in the present study is lower than observed in free-living cyanobacteria and in other symbiotic systems (Ray et al., 1978; Becking and Donze, 1981) but similar to that found in *Peltigera canina* (Bergman and Hällbom, 1982). The slightly reduced acetylene reduction activity and chlorophyll *a* content (Figs. 1a and b) at the very growing tip apparently reflects a smaller cyanobacterial zone.

Acknowledgements

This study was sponsored by the Swedish Agency for Research Cooperation with Developing Countries (SAREC) and the Lennanders Fund, University of Uppsala, Sweden.

We want to express our sincere gratitude to Dr. G. Budowski, Dr. R. Borel, Dr. J. Fargas and R. Russo, all working at CATIE, Costa Rica for providing the possibilities to perform this study, and to Dr. J.E. Nylund, Swedish University of Agricultural Sciences, Uppsala, for initiating, encouraging, and supporting this expedition.

References

Becking, J.H. 1978. Ecology and physiological adaptions of *Anabaena* in the *Azolla-Anabaena azollae* symbiosis. *In*: Environmental Role of Nitrogen-Fixing Bluegreen Algae and Asymbiotic Bacteria, Symposium, Sweden, Sept. 1976. *Ecol. Bull.* (Stockholm) **26**: 266-281.

Becking, J.H. and Donze, M. 1981. Pigment distribution and nitrogen fixation in *Anabaena azollae*. *Plant and Soil* **61**: 203-226.

Bergman, B. and Hällbom, L. 1982. *Nostoc* of *Peltigera canina* when lichenized and isolated. *Can. J. Bot.* **60**: 2092-2098.

Englund, B. 1977. The physiology of the lichen *Peltigera aphthosa*, with special reference to the bluegreen phycobiont (*Nostoc* sp.). *Physiol. Plant.* **41**: 298-304.

Giddy, C. 1974. *Cycads of South Africa.* Purnell, Cape Town. SBN 360 00274 9.

Grilli, M. 1963a. Osservazioni sulle infrastrutture di *Anabaena cycadinae* e delle cellule dei tubercoli radicali di *Cycas revoluta*. *Giorn. Bot. Ital.* **70**: 460-466.

Grilli, M. 1963b. Richerche al microscopio elettronico sulle cianoficee viventi nei tubercoli radicali di *Cycas revoluta*. *Ann. Microbiol.* **13**: 109-124.

Grilli Caiola, M. 1975. A light and electron microscopic study of the bluegreen algae growing in the coralloid roots of *Encephalartos altensteinii* and in culture. *Phycologia* **14**: 25-33.

Grilli Caiola, M. 1980. On the phycobionts of the cycad coralloid roots. *New Phytol.* **85**: 537-544.

Harborne, J.B. 1973. Recommended techniques, chlorophyll estimation. *In*: J.B. Harborne, *Phytochemical Methods*, pp. 205-207. Chapman and Hall, London. ISBN 412-10540-3.

Hill, D.J. 1975. The pattern of development of *Anabaena* in the *Azolla-Anabaena* symbiosis. *Planta* (Berlin) **122**: 179-184.

Hill, D.J. 1977. The role of *Anabaena* in the *Azolla-Anabaena* symbiosis. *New Phytol.* **78**: 611-616.

Hitch, C.J.B. and Millbank, J.W. 1975. Nitrogen metabolism in lichens. VI. The bluegreen phycobiont content, heterocyst frequency and nitrogenase activity in *Peltigera* species. *New Phytol.* **74**: 473-476.

Lindblad, P. 1984. Diversion between C_2H_2-reduction and heterocyst frequency in a cycad root. *In*: C. Veeger and W.E. Newton, eds. *Advances in Nitrogen Fixation Research*, p. 511. Wageningen.

Mallard, T.M., Mallard, C.S., Holfeld, H.S. and LaRue, T.A. 1977. Portable gas chromatograph for the acetylene reduction assay for nitrogenase. *Analyt. Chem.* **49**: 1275-1277.

Peters, G.A., Calvert, H.E., Kaplan, D., Ho, O. and Toia, R.E., Jr. 1982. The *Azolla-Anabaena* symbiosis: Morphology, Physiology and Use. *Isr. J. Bot.* **31**: 305-323.

Ray, T.B., Peters, G.A., Toia, R.E. and Mayne, B.C. 1978. *Azolla-Anabaena* relationship. VII. Distribution of ammonia-assimilating enzymes, protein, and chlorophyll between host and symbiont. *Plant. Physiol.* **62**: 463-467.

Schneider, A. 1984. Mutualistic symbiosis of algae and bacteria with *Cycas revoluta*. *Bot. Gaz.* January: 25-32.

Shi, D., Li, J.G., Zhong, Z.P., Wang, F.Z., Zhu, L.P. and Peters, G.P. 1981. Studies on nitrogen fixation and photosynthesis in *Azolla imbricata* (Roxb) and *Azolla filiculoides* Lam. *Acta Bot. Sinica* **23**: 306-315.

Silvester, W.B. 1976. Endophyte adaption in *Gunnera-Nostoc* symbiosis. *In*: P.S. Nutman, Ed. *Symbiotic Nitrogen Fixation in Plants*, Vol. 7, pp. 521-539.

Spratt, E.R. 1911. Some Observations in life-history of *Anabaena cycadeae*. *Ann.*

Bot. **25**: 369-380.

Stewart, W.D.P., Fitzgerald, G.P. and Burris, R.H. 1967. *In situ* studies on N_2-fixation using the acetylene reduction technique. *Proc. Natl. Acad. Sci.* U.S.A. **58**: 2071-2078.

Stewart, W.D.P., Rowell, P. and Rai, A.N. 1983. Cyanobacteria-Eucaryotic plant symbioses. *Ann. Microbiol.* (Inst. Pasteur) **134B**: 205-228.

Zhu, C. 1982. Fine structure of blue-green algae and the cells lined along the endophyte cavity in the coralloid root of *Cycas*. *Acta Bot. Sinica* **24**: 109-114.