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Anabaena azollae Akinetes in the Sporocarps of *Azolla filiculoides* Lam

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Abstract

At the present time the taxonomic position of the cyanobacterium symbiont of the aquatic fern *Azolla* is still controversial. Usually it is reported as *Anabaena azollae* Strasb, but recently Komàreck and Anagnostidis have assigned it to *Trichormus azollae* (Strasb.) Kom. and Anagn. Various attempts to clarify the identity of this microorganism is difficult due to the lack of information regarding the akinetes, whose developmental pattern represents a diacritic character for the *Nostocacean* taxonomy. The object of this study was to examine the akinetes in the sporocarps of two strains of *Azolla filiculoides*. Akinetes were examined using a light and a fluorescent microscope equipped with different filters and their ultrastructure was determined by TEM and SEM. Akinetes and mainly rod shaped bacteria were found under the indusium of the sporocarps. The akinetes appeared in chains without interspersed vegetative cells and they were embedded in a film of mucilagenous material. A multilayer envelope was evident and the cells contained cyanophycin, glycogen, chlorophyll and phycobiliproteins. Differences in pigment content and ultrastructure have been detected between the akinetes present in the megasporocarps and microsporocarps. In the latter the *Anabaena* cells had lower contents of chlorophyll. Based on the developmental patterns and morphological characteristics the assignment of the *Azolla* endophyte to *Trichormus azollae* Kom. and Anagn. appears to be acceptable.

Keywords: *Azolla* sporocarps, *Anabaena*, akinetes, ultrastructure

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1. Introduction

In the last decade the taxonomic position of the *Azolla* cyanobiont was discussed in many papers (Anagnostidis, 1988; Becking, 1987; Fjerdingstad, 1976; Komàreck and Anagnostidis, 1989; Plazinski et al., 1988; Plazinski et al., 1990; Tomaselli et al., 1988; Shen, 1960), and, although the symbiont was classified more often as *Anabaena*, recently it has been assigned to the genus *Nostoc* (Tomaselli et al., 1988; Plazinski et al., 1990) or to the genus *Trichormus* (Komàreck and Anagnostidis, 1989). Most of the determinations were based on the morphological characters of the vegetative cells and heterocysts, since the akinetes were seldomly found in the leaf cavities.

Komàreck and Anagnostidis (1989) in their recent classification revision of Nostacales considered the development of the akinetes as the main diacritical feature which distinguishes *Trichormus* from *Anabaena*. In *Trichormus*, akinete differentiation is apoheterocytic and the akinetes develop to form chains, while the akinete differentiation of the genus *Anabaena* is paraheterocytic and the akinetes are mainly solitary. On the other hand the *Nostoc* genus is characterized by mucilaginous sheath production and a different developmental cycle.

Although the presence of akinetes in the leaf cavities and sporocarps of *Azolla* has been reported by many authors (Strasburger, 1983; Shen, 1960; Konar and Kapoor, 1974; Ashton and Walmsley, 1976; Calvert et al., 1983; Herd et al., 1985; Cutter and Herd, 1987; Braun-Howland and Nierzwicki-Bauer, 1990; Forni et al., 1990a; Carrapiço, 1991), controversy still exists about the nomenclature used for these cells, named from time to time vegetative cells, resting cells (Dunham and Fowler, 1987) resting spores or akinetes (Herd and Cuter, 1986). Very little is known about their role in the symbiosis, due to the little attention paid to akinete ultrastructure, differentiation and germination.

In order to obtain information useful for the clarification of the taxonomy and physiology of these cells, we have undertaken this research on the akinetes of the *Azolla filiculoides* Lam. endophyte. The starting point of the study was to observe the akinetes present in the sporocarps of *A. filiculoides* in order to determine their developmental pattern, structure and ultrastructure compared to the heterocyst and vegetative cells of the phycobiont of the same *Azolla* species.

2. Materials and Methods

Sporified specimens of *Azolla filiculoides* Lam. were collected in the Botanical Garden of Naples (Italy) and in the Tiber River at Nazzano Romano

near Rome (Italy). Most of the plants differentiated micro and megasporocarps on the same individual.

Light microscopy (L.M.)

Sporocarps and leaves were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 6.9 and stored at 4°C. In order to observe the akinetes the indusium of the sporocarps was cut under the stereomicroscope and the upper parts of the indusium were squeezed to free the akinetes.

The akinetes were observed under light microscope without staining and then stained with Sudan III (Jensen, 1962), Sudan black (Jensen, 1962), Lugol's and Poinceau 2R (Flint and Moss, 1970). DAPI (4'6- diamidino-2-phenylindole) staining was made according to the method of Nagashima et al. (1983).

The observations were made using a Leitz Aristoplan microscope equipped with Ploemopack 2 System, a mercury lamp Osram HPO 100 W and the following Leitz filter combinations: green filter M^2 (excitation filter 546 nm, barrier filter 580 nm) to detect phycobiliprotein fluorescence, blue filter I_3 (excitation filter 450–490 nm, barrier filter 515 nm) to detect chlorophyll fluorescence, and UV filter A (excitation filter 340–380 nm, barrier filter 460 nm) to detect DNA, after DAPI staining.

Quantitative measurements of DNA and pigments

Measurements of autofluorescence and DAPI fluorescence intensities were made on photographic prints of the specimens, with the automatic image analysis system Quantimet 970 (Cambridge Instruments) equipped with QUIPS Version V07.00 software. The measurements were made according to the method of Canini et al. (1992).

Scanning (SEM) and transmission (TEM) electron microscopy

For SEM observations the sporocarps were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 6.9. The specimens were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in acetone and critical point dried. The indusium was cut using needles before coating. The samples were coated with gold and examined with a Scannosan Electron Microscope.

For TEM observations sporocarps were fixed as described above. Dehydration was carried out with ethanol and the samples were embedded in EPON-Araldite mixture. Thin sections were stained with toluidine blue (Feder and O'Brien, 1968) and observed by L.M. Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and observed using a Philips 300 electronmicroscope at 80 KV.

3. Results

Light microscope observations

In the sporocarps the akinetes appeared oval shaped (9–11 μm length and 7–8 μm width), with a light green-yellow cytoplasm showing few large cyanophycin granules (from 1 to 3), localized mainly at the cell poles. The cells were surrounded by an evident smooth colourless envelope (Fig. 1).

Although the akinetes appeared isolated after squeezing, they were embedded in a mucilagenous matrix when observed *in situ*. This material made it difficult to isolate the cells from the sporocarp indusium.

After Lugol's staining the cytoplasm appeared brown coloured (Fig. 2) due to the presence of glycogen. No staining of the cytoplasm was obtained with Sudan III and Sudan black, while the light pink colour obtained after Poinceau 2R staining indicated the presence of proteinaceus materials.

Plate I.

Anabaena azollae akinetes isolated from the sporocarps of *A. filiculoides* observed at 1 M. (bars = 10 μm).

Figure 1. Akinetes from megasporocarps. A thick envelope and few large cyanophycin granules are evident in the unstained sample

Figure 2. Akinetes after Lugol's staining. The cytoplasm appears homogenously brown coloured

Figure 3. Akinetes, observed by green filter, show an intense red autofluorescence due to the phycobiliproteins

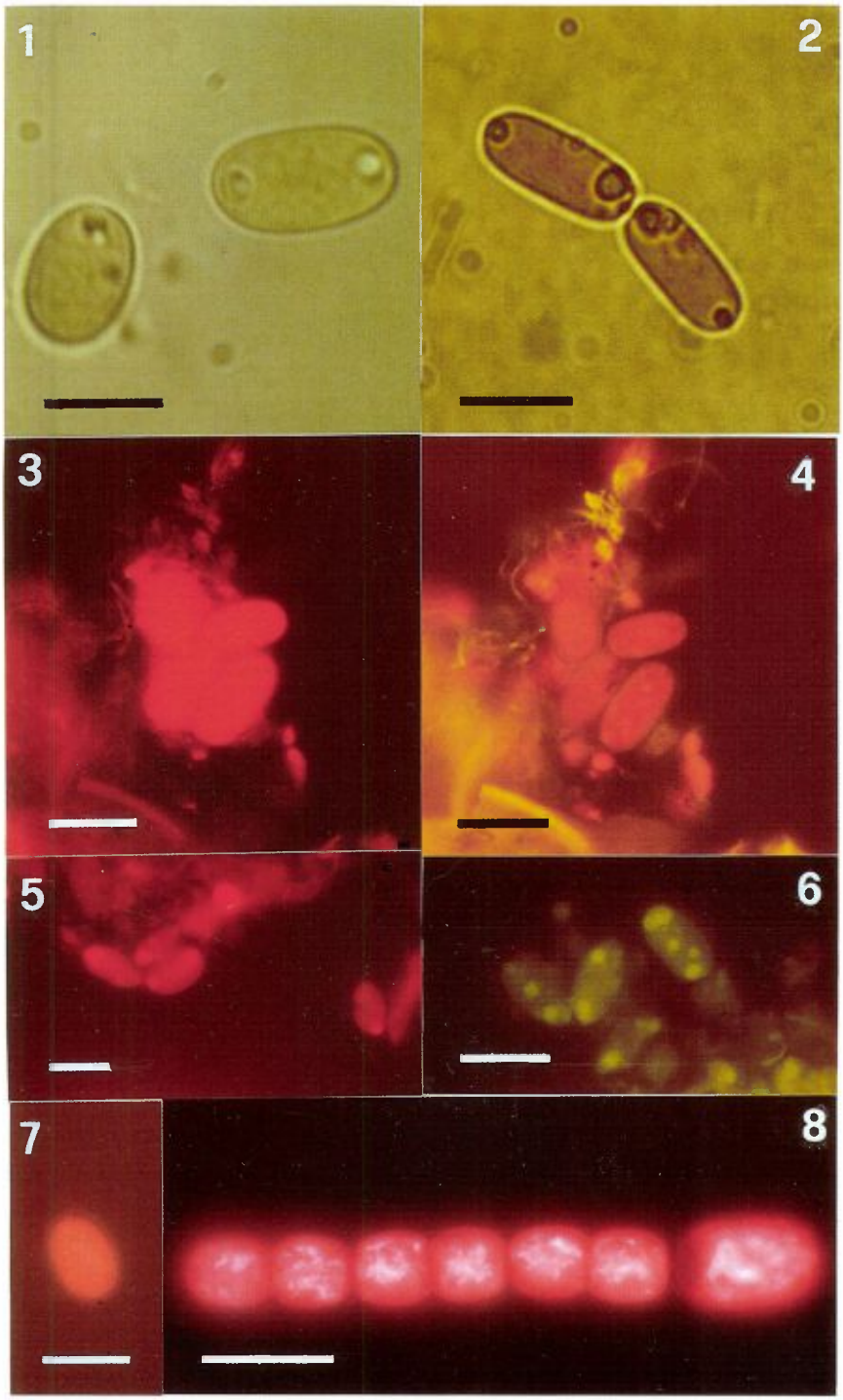
Figure 4. Red autofluorescence of chlorophyll in akinetes observed with blue filter

Figure 5. Red autofluorescence due to phycobiliproteins in the akinetes of the microsporocarps observed with green filter

Figure 6. Akinetes in the microsporocarps observed with blue filter. No red autofluorescence due to chlorophyll is detectable.

Figure 7. A weak fluorescence of DNA is evident in an akinete after DAPI staining.

Figure 8. DAPI staining of trichome of vegetative cells and heterocyst of *A. azollae* from the leaf cavities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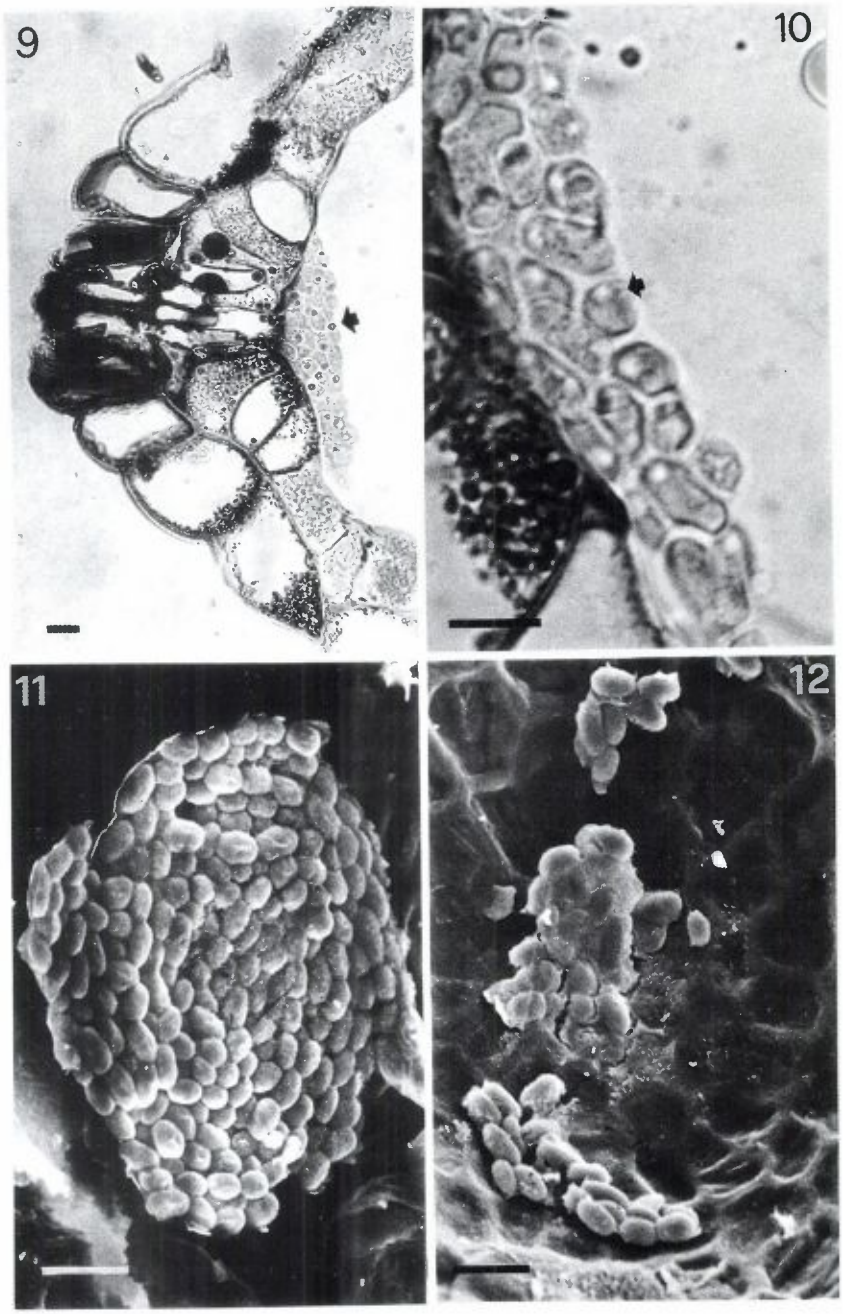
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Plate II.

Figure 9 . Thin section of a microsporocarp observed at 1 M. Akinetes (arrow) are visible of the indusium. (Bar = 10 μm)

Figure 10. Akinetes (arrow) at a higher magnification. The akinetes show a fine granular material and few cyanophycin granules. (Bar = 10 μm)

Figure 11. SEM micrograph of chains of akinetes with no heterocysts and vegetative cells from microsporocarps. (Bar = 5 μm)

Figure 12. Akinetes under the indusium of microsporocarps. (Bar = 5 μm)

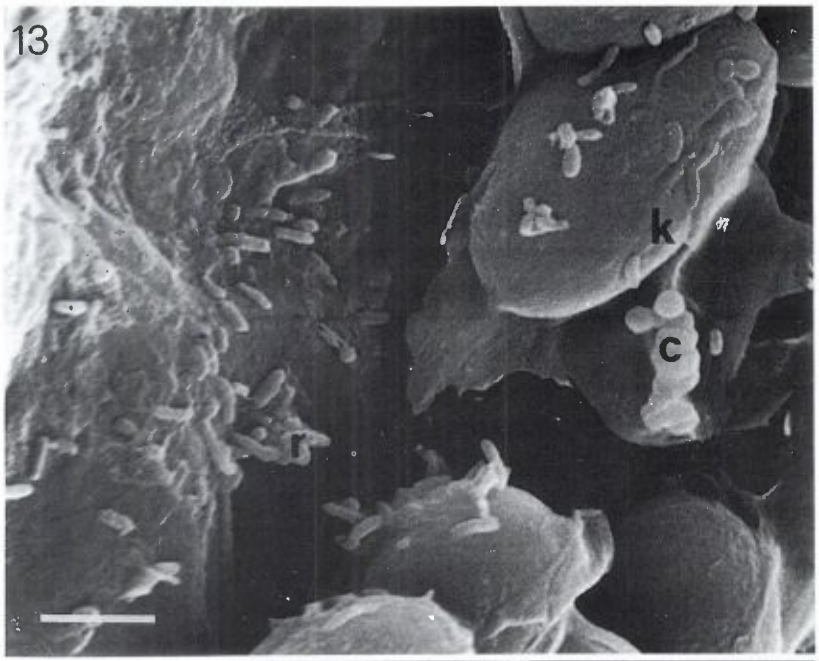
The intense red autofluorescence observed with the green filter revealed the presence of phycobiliproteins (Fig. 3). The presence of chlorophyll was determined evaluating its autofluorescence due to excitation obtained using the blue filter (Fig. 4).

After DAPI staining the cyanobiont akinetes (excited with UV light) showed the autofluorescent cytoplasm surrounding light pink areas indicating the presence of DNA nucleoplasm (Fig. 7). A light red autofluorescence was also detected in the samples treated by DAPI and observed with the green filter. Generally an evident yellow envelope and yellow cyanophycin granules in the red fluorescent cytoplasm were noted in DAPI treated specimens excited with the blue filter, but in some samples a yellow fluorescent cytoplasm, probably due to polysaccharides, was observed.

The akinetes were present in the sporocarps of the strain of *Azolla* from Naples Botanical Garden as well as in the strain from the Tiber River. In both strains the akinetes were similar, but megasporocarp akinetes differed from those of the microsporocarps. In fact in the latter, the symbiont cells were generally colourless, fluorescence with the green filter (Fig. 5), indicating the presence of phycobiliproteins, but they appeared less red fluorescent with the blue filter (Fig. 6).

For a comparison we observed also the symbiont cells in the leaf cavities of the same plant, where only trichomes of vegetative cells and heterocysts were found. The vegetative cells appeared barrel-shaped (4–10 μm×4–5.5 μm) and intensively fluorescent with the green filter, but less fluorescent with the blue filter.

Vegetative cells showed an intense white-pink fluorescence, after DAPI staining and after being excited with UV light (Fig. 8). No envelope was evidenced and the number and size of cyanophycin granules were variable. The heterocysts measured 10–12 μm×7–8 μm and after DAPI staining the white fluorescent nucleoplasm was clearly distinguishable from the rest of the cytoplasm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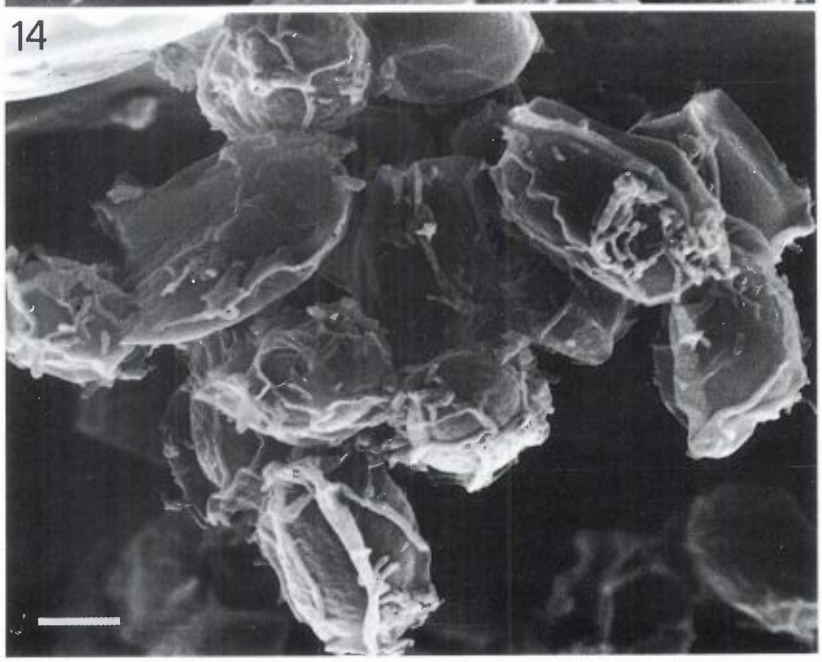
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Plate III.
SEM micrographs. (Bars = 10 μm)
Figure 13. Cyanobiont akinetes (k), bacterial rods (r) and cocci (c) from a microsporocarp
Figure 14. Shrunken akinetes in the megasporocarp

(Fig. 8). The comparative data obtained with the different cytochemistry tests used and with the brightness measurements by Quantimet, are reported respectively in Table 1 and Table 2.

In thin sections of micro and megasporocarps, akinetes form a layer (Fig. 9) under the pore or cap of the indusium and these cells show few big granules of cyanophycin and granular material (Fig. 10).

SEM and TEM observations

At SEM the cyanobiont cells appeared as a layer under the pore of the microsporocarps and the indusium cap of the megasporocarps (Figs. 11, 12). They were embedded in a mucilagenous material containing also bacteria. In the megaspocarps the akinetes always presented a wrinkled or broken envelope (Fig. 14). Up to now we do not know the cause of this aspect. Moreover, in the ultrathin sections, the akinetes from megasporocarps appeared deeply embedded in the mucilage (Fig. 15) together with numerous bacterial rods and rare cocci. No heterocysts were observed. Each akinete was surrounded by a multilayered envelope, which was often detached from the cell wall as already reported with SEM. The cell wall structure did not differ from that of the vegetative cells of the endophyte living in the leaf cavities. The cytoplasm showed a network system of thylakoids among which very numerous glycogen granules were visible; whereas at the periphery, few large cyanophycin granules and some carboxysomes were present (Fig. 16). Moreover, the cytoplasm showed very numerous areas with electrontransparent material of unknown nature and origin (Fig. 17). We did not detect phycobilisomes on the thylakoids and we never observed a distinct nucleoplasmic area with carboxysomes and polyphosphate granules. This suggests that the DNA and carboxysomes were scattered within the cytoplasm confirming the reaction given by DAPI staining, which showed a diffused white-blue fluorescence of the cytoplasm.

Akinetes from microsporocarps were sometimes accompanied by vegetative cells (Fig. 18) and they showed a thick electrontransparent envelope and electrondense material between the envelope and the cell wall (Fig. 19). The cytoplasm appeared less structured and contained only few thylakoids, cyanophycin

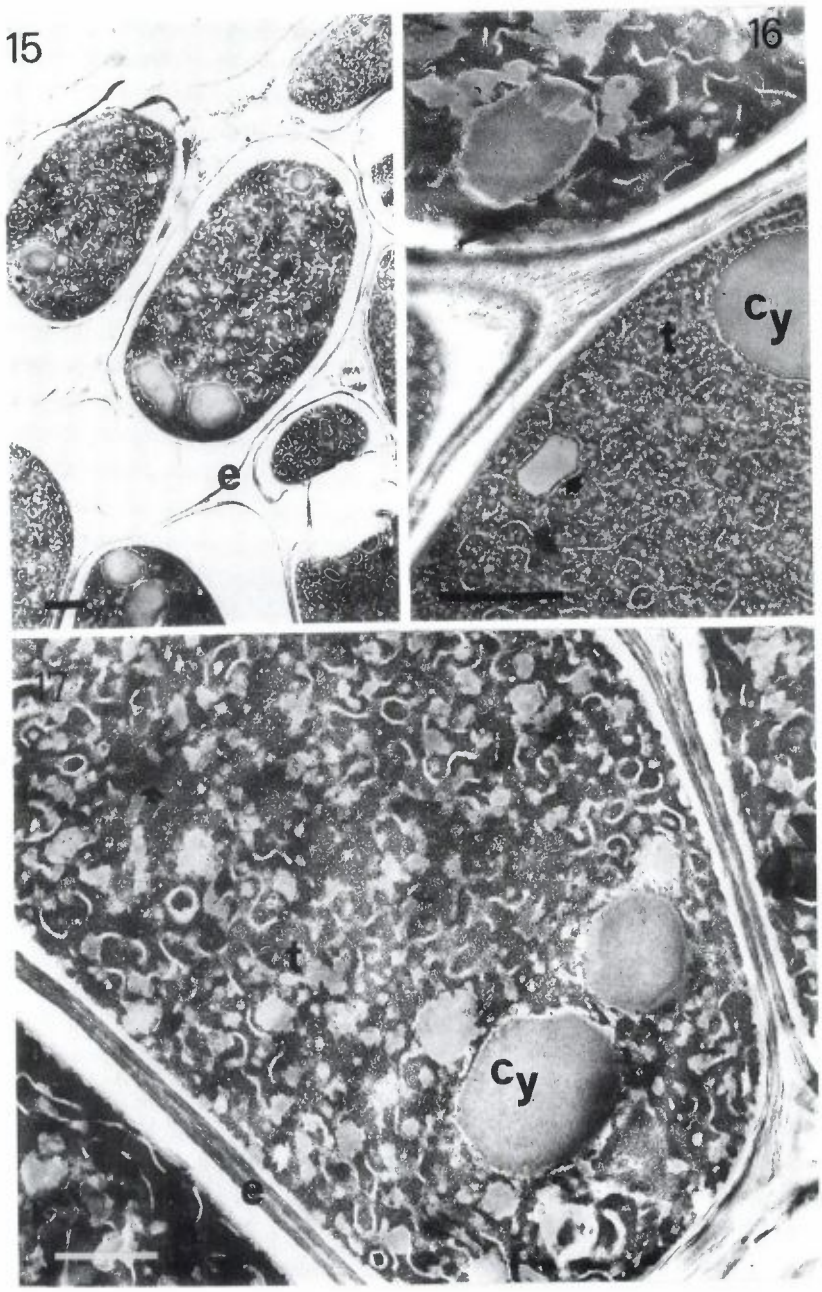
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Plate IV.
TEM micrographs
Figure 15. Akinetes with a detached envelope (e) in a megasporocarp. Bar = 20 μm)
Figure 16. Akinetes showing electrondense granular masses (arrow), large cyanophycin granules (cy) and many electrontransparent areas (a) containing unknown material. (Bar = 10 μm)
Figure 17. Akinete showing a multilayered structure of envelope (e), granular material (arrow), cyanophycin granules (cy) and thylakoidal membranes (t). (Bar = 10 μm)

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and glycogen granules. Among the akinetes, electrondense material probably due to degenerated cytoplasmic material was observed.

In the sporocarps the bacteria were localized mainly among the akinetes (Fig. 20). The bacteria were mostly rod shaped, about 2 μm long and 0.5 μm diameter, but some larger cocci were visible. They were similar to those isolated by Forni et al. (1990b) from the sporocarps of different strains of the same *Azolla* species and identified as different species of the genus *Arthrobacter* Conn and Dimmick. They had a cytoplasm with few electron dense masses and sometimes granules.

The ultrastructural aspect of the vegetative cells of cyanobiont in the leaf cavities of the same *Azolla* species is shown in Fig. 21. The lack of an envelope surrounding the cell wall and the absence of electrontransparent areas within the cell can be seen, while the carboxysomes granules are numerous and scattered in the cytoplasm together with small vacuoles and electrondense granules.

The heterocyst ultrastructural organization (Fig. 22) was different from that reported for akinetes of *A. azollae* present in the sporocarps. The cell wall was surrounded by a multilayered envelope. In the cytoplasm the thylakoids were rare, while at the poles the thylakoid membranes formed the characteristic honeycomb structure.

4. Discussion

The cyanobiont cells present in the sporocarps are akinetes and not vegetative cells because of the cell size, the presence and structure of a thick envelope and the presence of cyanophycin and glycogen granules. Their characteristics are very similar to those reported by Herd and Cutter (1986) in megasporocarps of *Azolla microphylla* Kaulfus and by Lin and Watanabe (1988) in *Anabaena azollae* isolated from *Azolla microphylla* and grown in culture.

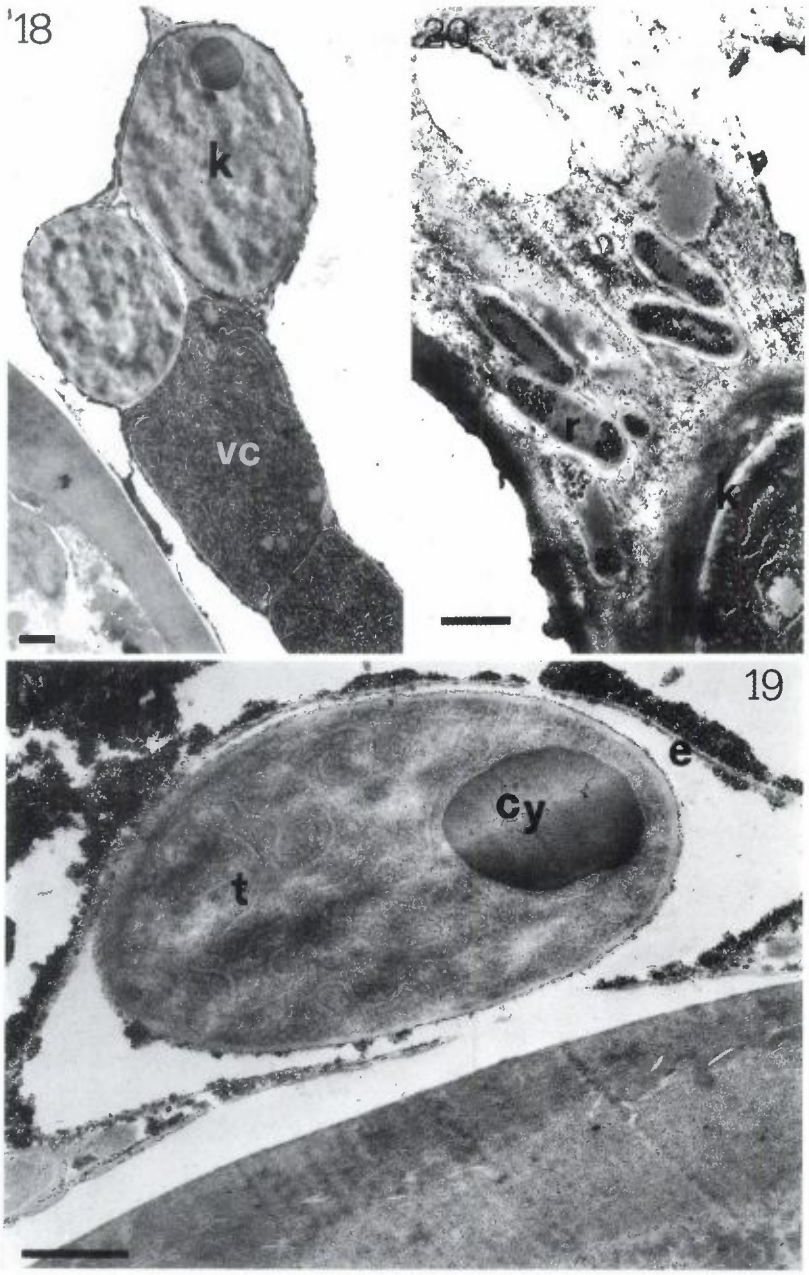
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Plate V.
TEM Micrographs
Figure 18. Two akinetes (k) and vegetative cells (vc) in microsporocarps. (Bar = 1 μm)
Figure 19. Akinetes from microsporocarps showing electrondense material outside of the cell. Envelope appears detached from the cell wall. Cyanophycin granules (cy) and thylakoids (t) are visible. Bacteria are visible among the endophyte cells. (Bar = 10 μm)
Figure 20. Bacteria rods (r) within mucilagenous matrix surrounding akinetes. The bacterial cytoplasm contains electrondense granules. (Bar = 5 μm)

Becking (1987) reported the presence of the akinetes only in the megasporocarps and in the oldest leaves of *Azolla filiculoides*, since he never found any cyanobacterium cells within the microsporocarps. On the contrary, we have found that the endophyte differentiates the akinetes in both mega and microsporocarps as already reported in the same species by Carrapiço (1991) and in *Azolla microphylla* by other authors (Herd and Cutter, 1986; Lin and Watanabe, 1988). However the fate of the microsporocarp akinetes is unknown.

In addition we confirm Becking's observations (1987) regarding the absence of heterocysts and vegetative cells among the akinetes in the sporocarps. In fact, according to our observations, in the mature megasporocarps of *A. filiculoides*, the akinetes are present in chains without any other cell types, although some vegetative cells might occur in the microsporocarps. The lack of heterocysts and vegetative cells seems to indicate that in the sporocarps an entire trichome differentiates into akinetes, as occurs in *Trichormus*. Based on this character the assignment of the *Azolla* endophyte to *Trichormus azollae* Kom. and Anagn. (1989) appears acceptable.

The presence of chlorophyll in the akinetes, as revealed by their autofluorescence, is in agreement with the detection of 32-Kda Q_B binding protein of the photosystem II found in the akinetes of *A. azollae* in the leaf cavities of *A. caroliniana* (Braun-Howland and Nierzwicki-Bauer, 1990). Generally the pigment and DNA contents of the akinetes is highly variable in cyanobacteria. Some authors (Sutherland et al., 1979) have reported a higher content of chlorophyll in akinetes in comparison to the vegetative cells, while others have reported the absence of the photosynthetic pigments in the akinetes of *Anabaena doliolum*, a species isolated from the coralloid roots of *Cycas circinalis* (Rao et al., 1984). Our data show that phycobiliproteins and chlorophyll contents of the megasporocarp akinetes, determined by autofluorescence, are

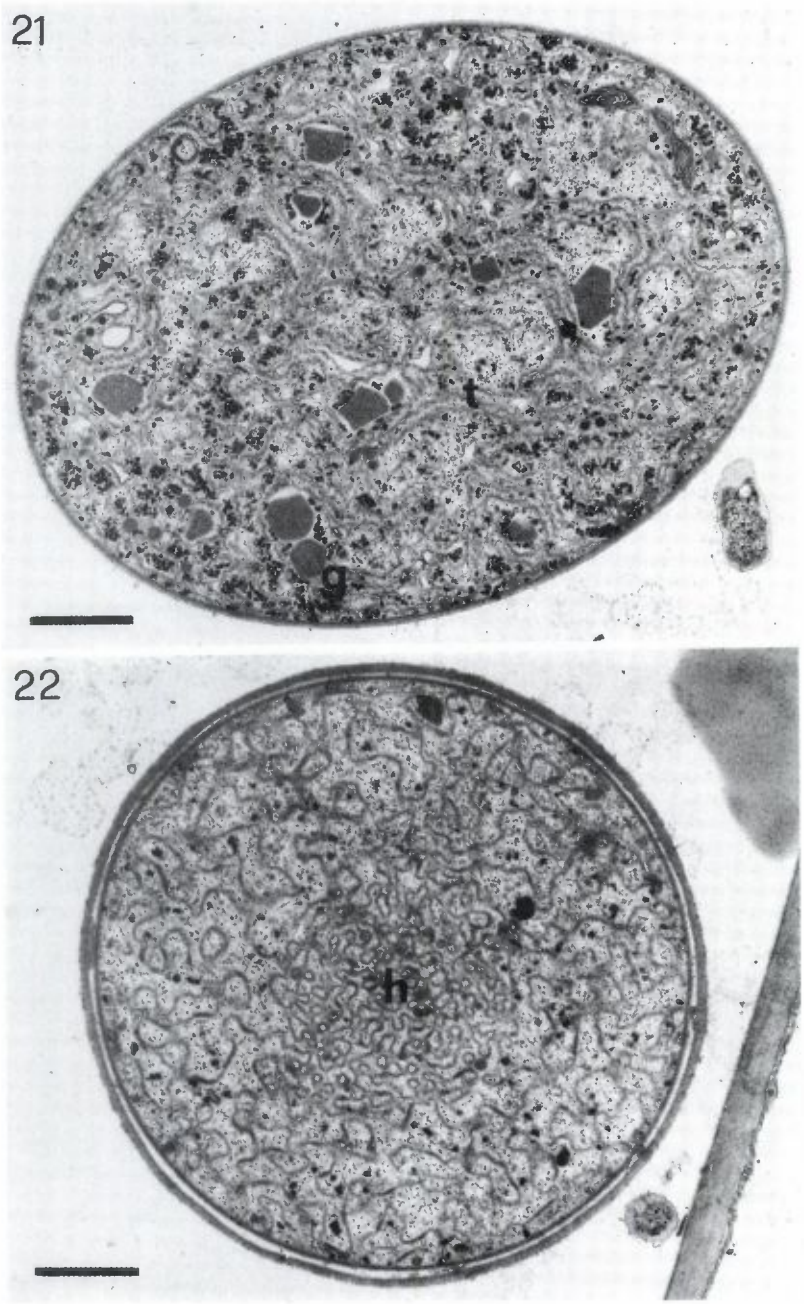
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Plate VI.
TEM micrographs (Bars = 10 μm)
Figure 21. A vegetative cell of cyanobiont in a leaf cavity of *A. filiculoides*. The cell wall is lacking of an envelope and cytoplasm contains many carboxysomes (arrow), glycogen granules (g) and thylakoidal membranes (t).
Figure 22. Transverse section of an heterocyst of *A. azollae* in the leaf cavity of *A. filiculoides*. The envelope doesn't have multilayered structure and in central area of the cytoplasm the honey-comb structure (h) is visible.

Table 1. Cytochemical analyses of the akinetes (ak) in the micro and megasporocarps and of the vegetative cells (vc) and heterocysts (h) of cyanobiont in the leaf cavities of *A. filiculoides*

Cytochemistry	*A. azollae*			
	ak		vc	h
	Microsporocarps	Megasporocarps	Leaf cavities	
Lugol's	++	+++	+++	–
Sudan III	–	–	–	–
Sudan black B	–	–	–	–
Poinceau 2R	+	+	+	+
Toluidine blue	++	++	++	+

– = no reactions, + = weak, ++ = good, +++ = very good

Table 2. Phycobiliproteins and chlorophyll autofluorescence and DAPI fluorescence brightness determined by Quantimet in the cyanobiont akinetes (ak) from micro and megasporocarps and of the vegetative cells (vc) and heterocysts (h) in the leaf cavities of *A. filiculoides*.

	Brightness			
	ak		vc	h
	Microsporocarps	Megasporocarps	Leaf cavities	
Phycobiliproteins (M_2 filter block)	39500±4500	43100±5000	39000±4000	38000±4000
Clorophyll (I_3 filter block)	20870±3500	24018±4000	21100±2000	21600±2000
DNA (A filter block)*	0	0	1664±73	2830±200

* after DAPI staining

higher than those of microsporocarp akinetes. The very low akinetes fluorescence after DAPI staining, not detectable by Quantimet, may be due to the difficulties of fluorochrome penetration and not to a low DNA content of these cells.

The ultrastructure of the akinetes shows a multilayered envelope, in which some lamellar layers are distinguishable. This envelope appears less thick compared to the one usually formed by the akinetes of free-living *Nostoc* and *Anabaena* (Grilli Caiola and De Vecchi, 1980).

The cytoplasm of megasporocarps akinetes is rich in glycogen granules with just a few large cyanophycin granules and many electron transparent vacuole-like areas, whose content is unknown. This aspect has been already reported for the akinetes of the endophyte of *A. microphylla* (Herd and Cutter, 1986). On the basis of the fluorescence and the staining with Poinceau 2R we can hypothesize the presence of polysaccharides and proteic material in the cytoplasm. The cytoplasm of microsporocarp akinetes appears less structured and does not show electrotransparent areas.

Lucas and Duckett (1980) made cytochemistry tests with the cells of the cyanobiont in the sporocarps of *A. filiculoides*, and reported the positive reaction of the cells to periodic acid Schiff, to blue bromophenol mercury and to Sudan black B. Unfortunately these authors did not indicate the cell type, i.e. if they were akinetes or vegetative cells, so it is not possible to compare our results with theirs.

Acknowledgements

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REFERENCES

Anagnostidis, K., Economou-Amilli, A., and Overbeck, J. 1988. *Anabaena azollae* Strassb. and the periphyton of *Azolla filiculoides* Lam. in Lake Trichonis and the lagoon of Aetolikon, Hellas (Greece). *Arch. Hydrobiol.* **113:** 321–343.

Ashton, P.J. and Walmsley, R.D. 1976. The aquatic fern *Azolla* and its *Anabaena* symbiont. *Endeavour* **35:** 39–43.

Becking, J.H. 1987. Endophyte transmission and activity in the *Anabaena-Azolla* association. *Plant Soil* **100:** 183–212.

Berliner, M.D. and Fisher, R.W. 1987. Surface lectin binding to *Anabaena variabilis* and to cultured and freshly isolated *Anabaena azollae*. *Curr. Microbiol.* **16:** 149–152.

Braun-Howland, E.B., Lindblad, P., Nierzwicki-Bauer, S.A., and Bergman, B. 1988. Dinitrogenase reductase (Fe-protein) of nitrogenase in the cyanobacterial symbionts of three *Azolla* species: localization and sequence of appearance during the heterocyst differentiation. *Planta* **176:** 319–322.

Braun-Howland, E.B. and Nierzwicki-Bauer, S.A. 1990. Occurrence of 32-K Da Q_B-binding protein of photosystem II in the vegetative cells, heterocysts and akinetes of *Azolla caroliniana* cyanobionts. *Planta* **180:** 361–371.

Canini, A., Grilli Caiola, M., and Ferrucci, L. 1992. Quantitative and qualitative DNA variations in *Anabaena azollae* Strasb. living in *Azolla filiculoides* Lam. *Cytometry* **13:** 299–306.

Carrapiço, F. 1991. Are bacteria the third partner of the *Azolla-Anabaena* symbiosis? In: *Nitrogen Fixation.* M.Polsinelli et al., eds. Kluwer, Dordrecht, pp. 453–456.

Cutter, E.G. and Herd, Y.R. 1987. Morphogenesis of sporocarps of *Azolla microphylla.* In: Azolla *utilization.* IRRI. *Proc. of the Workshop on* Azolla *use.* Fuzhou, Fujian, China, 1985, pp. 39–45.

Dunham, D.G. and Fowler, K. 1987. Megaspore germination, embryo development and maintenance of the symbiotic association in *Azolla filiculoides* Lam. *Bot. J. Linnean Soc.* **95:** 43–53.

Feder, N. and O'Brien, T.P. 1968. Plant microtechnique: some principle and new methods. *Amer. J. Bot.* **55:** 123–142.

Fjerdingstad, E. 1976. *Anabaena variabilis* status *azollae. Arch. Hydrobiol., Supp. 49, Algological Studies* **17:** 377–381.

Flint, F. and Moss, R. 1970. Selective staining of protein and starch in wheat flour and its products. *Stain Technology* **45:** 75–79.

Forni, C., Grilli Caiola, M. and Gentili, S. 1990a. Bacteria in the megasporocarps of *Azolla filiculoides* Lam. *Giornale Botanico Italiano* **124:** 678–679.

Forni, C., Gentili, S., Van Hove, C., and Grilli Caiola, M. 1990b. Isolation and characterization of the bacteria living in the sporocarps of *Azolla filiculoides* Lam. *Ann. Microbiol.* **40:** 235–243.

Grilli Caiola, M. and De Vecchi, L. 1980. Akinete ultrastructure of *Nostoc* species isolated form cycad coralloid roots. *Can. J. Bot.* **58:** 2513–2519.

Grilli Caiola, M. 1990. Structural arrangement of phycobionts in coralloid roots of Cycads. *Memoirs N.Y. Bot. Garden* **57:** 94–103.

Herd, Y.R., Cutter, E.G., and Watanabe, I. 1985. A light and electron microscopic study of microsporogenesis in *Azolla microphylla. Proc. Royal Soc. Edinburgh* **86B:** 53–58.

Herd, Y.R. and Cutter, E.G. 1986. An ultrastructural study of postmeiotic development in the megasporocarps of *Azolla microphylla. Can. J. Bot.* **64:** 822–833.

Jensen, W.A. 1962. *Botanical Hystochemistry.* Freeman, San Francisco. 408 pp.

Komàrek, J. and Anagnostidis, K. 1989. Modern approach to the classification system of cyanophytes. 4. Nostocales. *Arch. Hydrobiol. Suppl. Algological Studies* **82(3):** 247–345.

Konar, R.N. and Kapoor, R.K. 1974. Embriology of *Azolla pinnata*. *Phytomorphology* **24:** 228–261.

Lin, C. and Watanabe, I. 1988. Study on the association between *Anabaena azollae* and *Azolla microphylla* during the germination of megasporocarps. *Symbiosis* **5:** 199–208.

Lucas, R.C. and Duckett, J.G. 1980. A cytological study of the male and female sporocarps of the heterosporous fern *Azolla filiculoides* Lam. *New Phytol.* **85:** 409–418.

Nagashima, H., Kuroiwa, T., and Fukuda, I. 1983. Chloroplast nucleoids in a unicellular hot spring alga *Cyanidium caldarium* and related algae. *Experientia* **40:** 563–564.

Newton, J.W. and Herman, A.I. 1979. Isolation of cyanobacteria from the aquatic fern *Azolla*. *Arch. Microbiol.* **120:** 161–165.

Plazinski, J., Franche, C., Liu, C.C., Shaw, W., Gunning, B.E.S., and Rolfe, B.G. 1988. Taxonomic status of *Anabaena azollae*: an overview. *Plant Soil* **108:** 185–190.

Plazinski, J., Zheng, Q., Taylor, R., Croft, L., Rolfe, B.G., and Gunning, B.E.S. 1990. DNA probes show genetic variation in cyanobacterial symbionts of the *Azolla* fern and a closer relationship to free-living *Nostoc* strains than to free-living *Anabaena azollae* strains. *Appl. Environ. Microbiol.* **56:** 1263–1270.

Rao, V.V., Rai, A.N., and Singh, H.N. 1984. Metabolic activities of akinetes of the cyanobacterium *Anabaena doliolum:* oxygen exchange, photosynthetic pigments and enzymes of nitrogen metabolism. *J. Gen. Microbiol.* **130:** 1299–1302.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell. Biol.* **17:** 208–212.

Shen, Y.E. 1960. *Anabaena azollae* and its host *Azolla pinnata*. *Taiwania* **7:** 1–7.

Strasburger, E. 1873. *Über Azolla*. Jena.

Sutherland, J.M., Herdman, M., and Stewart, W.D.P. 1979. Akinetes of the cyanobacterium *Nostoc* PCC 7524: macromolecular composition, structure and control of differentiation. *J. Gen. Microbiol.* **115:** 273–287.

Tomaselli, L., Margheri, M.C., Giovannetti, L., Sili, C., and Carlozzi, P. 1988. The taxonomy of *Azolla* spp. cyanobionts. *Ann. Microbiol.* **38:** 157–161.