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MORPHOLOGICAL CHARACTERISATION OF CULTURED AND FRESHLY SEPARATED CYANOBIONTS (NOSTOCALES, CYANOPHYTA) FROM *AZOLLA* SPECIES

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A comparative examination of the morphological attributes of freshly separated and cultured cyanobionts of six species of *Azolla*, namely *A. microphylla*, *A. filiculoides*, *A. rubra*, *A. caroliniana*, *A. mexicana* and *A. pinnata*, was carried out. Distinct variation with respect to colour and nature of filaments, size and frequency of heterocysts was observed when counterparts of cultured and freshly separated cyanobionts from each species of *Azolla* were compared. The cyanobionts from *A. pinnata* showed distinctly larger cells compared with the cyanobionts from other *Azolla* species, whereas the cyanobiont of *A. mexicana* resembled *Nostoc* in exhibiting coiled filaments. The other cyanobionts exhibited mostly straight filaments similar to *Anabaena*. This investigation brings out clearly the morphological variations existing among the cyanobionts belonging to the different species of *Azolla* and the transitional changes under *in vitro* conditions, need to characterise the cyanobionts at inter- and intraspecific level using molecular tools in the future for a more reliable classification system of the cyanobionts associated with *Azolla*.

Key words: *Anabaena*, *Azolla*, cyanobionts, heterocysts, *Nostoc*

INTRODUCTION

The *Azolla–Anabaena* symbiotic association is an agronomically important N_2-fixing system, exploited worldwide as a biofertilizer (Singh 1979, Pabby *et al.* 2003*a*). The N_2-fixing ability of the fern is due to the presence of the endosymbiont *Anabaena azollae* Strasburger belonging to the family Nostocaceae, and order Nostocales, which is harboured in special cavities of the dorsal leaf lobes. This endosymbiont remains associated with the fern throughout its vegetative and reproductive life cycles – thereby excluding the need for re-inoculation as required in other symbiotic associations such as the legume–*Rhizobium* symbiosis (Peters and Meeks 1989). But the major constraint for its widespread utilisation is its inability to tolerate adverse environmental conditions such as high temperature and low phosphorus availability (Tung and Watanabe 1983).

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These limitations have evoked the interest of scientific communities towards the development of sexual hybrids between different species of *Azolla* and genetic manipulation of macro- and microsymbionts (Watanabe *et al.* 1993). One of the basic and most important steps in this direction would involve the reliable and accurate taxonomic identification, isolation and cultivation of the nitrogen-fixing endophytic partner in the free-living state for manipulating its biochemical/genetic attributes.

Earlier studies have indicated that the endosymbiont of *Azolla* shows restricted growth and multiplication (Tang *et al.* 1990). But there are contrasting reports regarding successful isolation and culturing of the endosymbiont (Newton and Herman 1979, Subramanian and Malliga 1988, Kim *et al.* 1997). Investigations have shown that the free-living isolates of *Anabaena azollae* resemble *Anabaena* or *Nostoc* spp. in general morphology (Newton and Herman 1979, Zimmerman *et al.* 1989). Also, the *in vitro* cultivated filaments are morphologically distinct from filaments freshly removed from the cavities and the concept of major/minor or primary/secondary symbionts has been put forth by many workers (Plazinski *et al.* 1990, Gebhardt and Nierzwicki-Bauer 1991, Kim *et al.* 1997).

Molecular techniques have been used to look at genetic variation in the symbionts associated with the different species of *Azolla.* These experiments have been conducted by extracting symbionts/endophytes from the *Azolla* directly (without being cultured). DNA and fatty acid composition analyses have given remarkably concordant results, indicating that there are characteristic differences in the symbionts associated with each species. These findings have led to the suggestion that there may have been coevolution of host and symbiont (Caudales *et al.* 1995, Zheng *et al.* 1999, Rasmussen and Svenning 2000). However, these studies have dealt with generally one or two species of *Azolla*.

Therefore, for the first time, an investigation was undertaken to compare freshly separated and *in vitro* cultured cyanobionts from six species of *Azolla* in terms of their morphological attributes, as a prelude to their future characterisation at the physiological and molecular level. Physiological characterisation of these cyanobionts has revealed interesting differences, which have been attributed to several factors-including signals from the host to its endosymbiont (Pabby *et al.* 2003*b*).

MATERIAL AND METHODS

Growth and maintenance of Azolla

Six species of *Azolla* – *A. microphylla* Kaulfuss, *A. filiculoides* Lamarck, *A. rubra* R. Brown, *A. caroliniana* Willdenow and *A. mexicana* Presl belonging to section *Euazolla* and *A. pinnata* R. Brown placed in section *Rhizosperma* were obtained from the *Azolla* germplasm of the National Centre for Conservation and Utilisation of Blue-green Algae, Indian Agricultural Research Institute, New Delhi, India. *A. caroliniana* 3001 was originally procured from the International Rice Research Institute, Philippines and the other species originally belong to the germplasm of the Central Rice Research Institute, Cuttack, India. They have been maintained on chemically defined nitrogen free Espinase and Watanabe medium at 25±2 °C and a light intensity of 52 µmol photon m^{-2} s^{-1} and 16:8 h light:dark cycles (Watanabe and Espinas 1976).

Isolation and maintenance of cyanobionts

For the isolation of endosymbiont(s), fronds of various species of *Azolla* grown under sterile laboratory conditions were blot dried using sterile filter paper and their roots dissected in a sterile inoculation chamber. These fronds were surface sterilised with 0.01% mercuric chloride for half an hour, and then washed thoroughly with sterile distilled water. After drying on sterilised filter paper, the fronds (up to the 4th branch) were homogenised using a mortar and pestle with sterile distilled water and filtered twice through four layers of cheesecloth. The entire procedure was carried out in a UV sterilised chamber in a laminar flow. The freshly separated cyanobiont(s) were then spread on nitrogen supplemented BG–11 agar plates. The plates were incubated under controlled laboratory conditions (as mentioned above). Colonies of the cyanobiont(s) appeared after three weeks. The isolated cyanobionts were maintained in nitrogen supplemented medium. Successive subculturing was required and these colonies were re-inoculated in fresh nitrogen deficient medium (once in every 7 days) for healthy growth of the cultures.

A modified method (Nierzwicki-Bauer and Haselkorn 1986) based on the gentle rolling procedure of Peters and Mayne (1974) was used to separate freshly separated cyanobionts after sterilisation of different species of *Azolla* (15 days old) as described above.

Microscopic examination of the cyanobiont

Both freshly separated and *in vitro* cultured cyanobionts were viewed under a Nikon (Microphot-FX) light microscope for examining of the nature of

filaments, shape and size of vegetative cells, heterocysts and akinetes, which were described using keys of Desikachary (1959). Cell dimensions given in Tables 1 and 2 are the range in size observed in a total of one hundred cells.

RESULTS

Morphological studies were carried out with one cultured cyanobiont each from *A. microphylla*, *A. filiculoides*, *A. rubra*, *A. caroliniana*, *A. mexicana* and two from *A. pinnata* (AP1 and AP2). The growth pattern and cell characteristics of cultured cyanobionts isolated from different species of *Azolla* showed considerable variation. Growth of the cultured cyanobionts on solid medium (agar based BG–11 medium) was generally of spreading type, except of AP1, which formed pinpoint-like colonies. This dissimilar behaviour was reflected in growth of this cultured endosymbiont in liquid medium – as it formed irregular aggregates uniformly suspended throughout the medium. On the other hand, the other cultured cyanobionts formed free-floating aggregates, mostly observed on the surface of the medium.

In general, the cultured cyanobionts appeared dull green in colour except AP1 (Fig. 1h) which was bluish green in appearance. The freshly separated cyanobionts uniformly exhibited a bluish green colour (Tables 1 and 2). Interestingly, the freshly separated cyanobionts (except that from *A. mexicana*) appeared as straight filaments, lying loosely scattered in the host cell tissue (Fig. 2a–d, f). But, the filaments of cyanobionts from *A. mexicana* showed a coiled appearance (Fig. 2e). The cultured cyanobionts, contrastingly, consisted of a mixture of mainly straight and a few coiled filaments (Fig. 1a–g). However, the cultured cyanobiont AP1 were observed to grow as only straight filaments (Fig. 1h).

Freshly separated cyanobionts consisted of filaments with a high percentage of heterocysts but no akinetes were observed (Fig. 2a–f). But in cultured cyanobionts, except AP1, akinete formation and irregularities in filament structure were observed after seven days of growth in nitrogen deficient medium (Fig. 1a–g). The shape of vegetative cells in freshly separated cyanobionts was, in general, cylindrical with slight variations such as cells with rounded edges in *A. filiculoides* and *A. pinnata* or short cylindrical cells were observed in *A. rubra* and *A. caroliniana* (Table 1). Variation in the size of vegetative cells was comparatively less and maximum size of vegetative cells was observed trichomes of cyanobionts from *A. pinnata* (8.85–12.25 × 5.7–7.6 μm) (Table 1).

Vegetative cell shape showed distinct variation within the cultured cyanobionts isolated from a particular species as well as between species. The shape of vegetative cells ranged from short cylindrical in *A. caroliniana* and *A.*

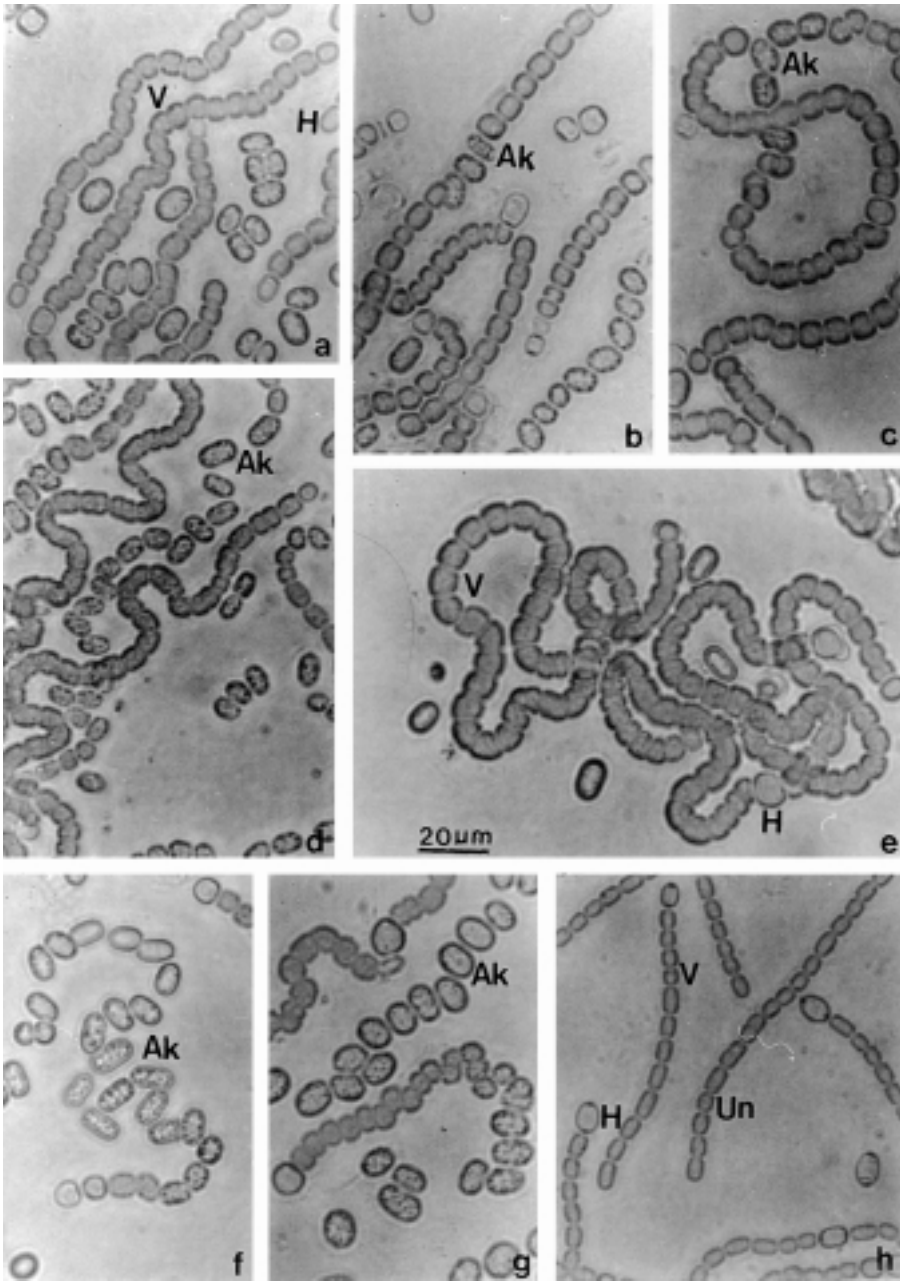

Fig. 1. Photomicrographs of cultured cyanobionts (a) *Azolla microphylla*; (b) *A. rubra*; (c) *A. filiculoides*; (d) *A. caroliniana*; (e–f) *A. mexicana*; (g) *A. pinnata* 2; (h) *A. pinnata* 1 grown in nitrogen deficient medium showing vegetative cell (V), heterocyst (H), akinete (Ak) and undifferentiated filaments (Un)

pinnata 1 (AP1), spherical to barrel in *A. microphylla*, quadratic barrel to cylindrical in *A. filiculoides*, quadratic barrel in *A. mexicana* and spherical to quadratic in *A. pinnata* 2 (AP2) (Table 2). It was clearly visible that the vegetative cells of cultured cyanobionts of *Azolla* spp. were smaller in size compared to their freshly separated counterparts.

The most characteristic types of cells observed in the trichomes were heterocysts. Both terminal as well as intercalary heterocysts were observed in cultured and freshly separated cyanobionts in all species of *Azolla* (Tables 1 and 2). The shape of heterocysts varied from spherical to globose in freshly

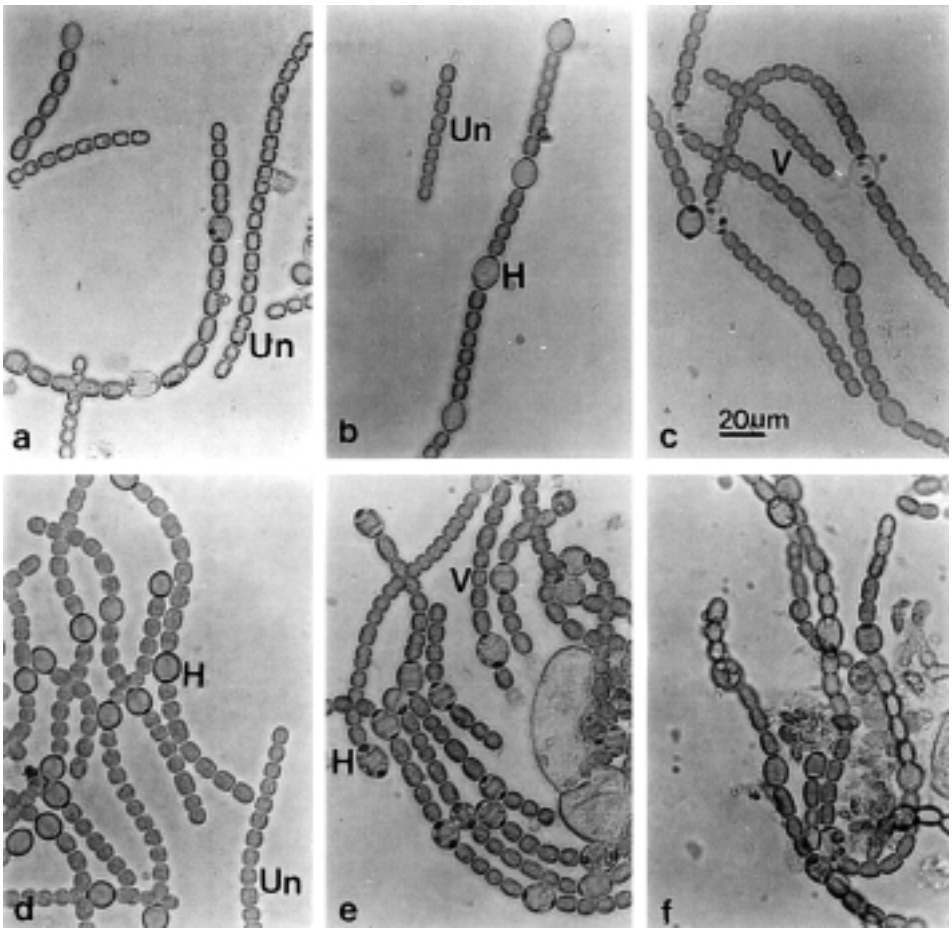

Fig. 2. Photomicrographs of freshly separated cyanobionts (a) *Azolla microphylla*; (b) *A. rubra*; (c) *A. filiculoides*; (d) *A. caroliniana*; (e) *A. mexicana*; (f) *A. pinnata* showing vegetative cell (V), heterocyst (H) and undifferentiated filaments (Un)

Table 1
Morphological characters of freshly separated cyanobionts of *Azolla* species

Parameters	*A. microphylla*	*A. rubra*	*A. filiculoides*	*A. caroliniana*	*A. mexicana*	*A. pinnata*
Filaments						
Colour	Bluish-green	Bluish-green	Bluish-green	Bluish-green	Bluish-green	Bluish-green
Nature	Straight	Straight	Straight	Straight	Slightly coiled	Straight
Cell type						
Vegetative cell						
Shape	Oblong–cylindrical	Short cylindrical	Cylindrical with rounded edges	Short cylindrical	Cylindrical	Cylindrical with rounded edges
Size (µm)	7.6–11.4 × 5.7–7.6	5.7 –11.4 × 5.7	7.6–9.5 × 5.7–7.6	6.65 –11.4 × 4.75–7.6	9.5–11.4 × 5.7–7.6	8.85–12.25 × 5.7–7.6
Heterocyst						
Shape	Spherical	Spherical-globose	Spherical	Globose	Spherical–globose	Variable, mainly globose
Size (µm)	8.55–11.4 × 7.6–9.5	9.5–13.3 × 7.6–10.45	10.5–13.3 × 7.6–10.45	10.45–11.4 × 7.6–8.55	11.4–15.2 × 9.5–11.4	11.4–15.2 × 10.45–11.4
Frequency (%)	22.30	21.35	24.28	13.38	27.53	22.53

Table 2
Morphological characters of cultured cyanobionts isolated from *Azolla* species

Parameters	*A. microphylla*	*A. rubra*	*A. filiculoides*	*A. caroliniana*	*A. mexicana*	*A. pinnata 1*	*A. pinnata 2*
Filaments							
Colour	Dull green	Dull green	Dull green	Dull green	Dull green	Bluish-green	Dull green
Nature	Coiled/ straight	Straight/ coiled	Slightly coiled	Coiled/ straight	Coiled	Straight	Coiled/ straight
Cell type							
Vegetative cell							
Shape	Spherical–barrel	Quadratic–cylindrical	Quadratic barrel–cylindrical	Short–long cylindrical	Quadratic barrel	Short–long cylindrical	Spherical–quadratic
Size (µm)	3.8–5.7 × 3.8–4.75	7.6 × 4.75–6.65	3.8–5.7 × 3.8	4.75–5.7 × 3.8–4.75	3.8–7.6 × 3.8–7.6	2.85–5.7 × 2.85–3.8	5.7–6.65 × 3.8–5.7
Heterocyst							
Shape	Cylindrical	Spherical to globose	Spherical or cylindrical	Spherical or cylindrical	Variable (oval or barrel or cylindrical)	Spherical–oblong	Spherical–short barrel
Size (µm)	7.6 × 7.6–4.75	7.6–11.4 × 5.7–7.6	7.6–8.55 × 4.75–6.65	5.7–7.6 × 4.75–7.6	7.6–15.2 × 5.7–11.4	3.8–11.4 × 3.8–7.6	7.6 × 3.8–6.65
Frequency (%)	13.56	7.92	8.77	7.73	10.55	7.20	9.92

separated cyanobionts of different *Azolla* species while the variation in heterocyst shape was much more pronounced in cultured cyanobionts (Table 2). Variability existed within filaments of cultured symbionts of *A. mexicana,* which exhibited oval or barrel or cylindrical shaped heterocysts (Fig. 1e). Heterocysts in general were much smaller in size in cultured cyanobionts of different *Azolla* species. The heterocysts in freshly separated cyanobionts ranged in size from 11.4–15.2 × 9.5–11.4 µm (length × breadth) in *A. mexicana* and 11.4–15.2 × 10.5–11.4 µm in *A. pinnata* (Table 1) compared with their cultured counterparts 7.6–15.2 × 5.7–11.4 µm in *A. mexicana* and 3.8–11.4 × 3.8–7.64 µm in *A. pinnata* 2. Both terminal and intercalary heterocysts were observed and their frequency in freshly separated cyanobionts was also distinctively higher compared with the cultured cyanobionts. Heterocyst frequency varied from 21–28% in cyanobionts freshly separated from different species of *Azolla,* except in the cyanobiont from *A. caroliniana* (Table 1). In cultured cyanobionts, it was mainly in the range of 7–11%, with only the cyanobiont of *A. microphylla* exhibiting a heterocyst frequency of 13.56% (Table 2). Paired heterocysts were present in AP1 but rarely observed in cyanobionts of other species, except in *A. rubra* and *A. pinnata*. The freshly separated cyanobionts exhibited prominent polar granules and distinct inner content in the heterocysts (Fig. 2a–f).

DISCUSSION

The relatively poor knowledge regarding the identity and taxonomic nature of cyanobionts of *Azolla* has limited genetic manipulation of this association. Controversies exist regarding the obligate or culturable nature of the cyanobionts and the existence of similar/different species in different strains of *Azolla* as well as the host housing one or more than one species of cyanobiont (Lechno-Yossef and Nierzwicki-Bauer 2002, Pabby *et al.* 2003*a*). In the present investigation a morpho-taxonomic comparison between freshly separated cyanobionts and their culturable counterparts was carried out, in order to understand the changes occurring on transition from symbiotic to free-living state.

Distinct morphological differences were observed between and among the cultured and freshly separated cyanobionts of *Azolla*. Such differences have been reported earlier (Newton and Herman 1979) based on antigenic similarities/dissimilarities and were attributed to developmental changes or selection of mutant strains capable of *in vitro* growth. Certain researchers are also of the view that in addition to the primary symbiont, there exist certain minor (secondary) symbionts, which are phenotypically similar to each other

(Meeks *et al.* 1988, Zimmerman *et al.* 1989, Gebhardt and Nierzwicki-Bauer 1991). Such cultured endosymbionts are known to be different from their freshly separated counterparts, not only in their morphological characteristics but also with respect to their organisation of *nif* HDK genes (Meeks *et al.* 1988, Coppenolle *et al.* 1995).

It has been suggested that free-living isolates may not be true isolates or symbionts from the dorsal leaf cavities but contaminating epiphytic organisms. However, in the present study, the *Azolla* accessions were maintained in sterilised medium under controlled temperature and light conditions in the laboratory. Under such conditions, no growth of contaminating algae was observed even on prolonged incubation for more than three months with successive subculturing every three weeks. Additionally, before separation of cyanobionts from *Azolla* species, a stringent sterilisation procedure was followed to rule out the possibility of epiphytic contaminants. Therefore, the freshly separated cyanobionts cannot be considered as epiphytic contaminants.

Growth pattern and morphological characteristics of cultured cyanobionts in the present study were in general quite similar and regular, having initially straight filaments (with no heterocysts) which gradually converted into a mixture consisting of both straight and coiled filaments with heterocysts. The presence of undifferentiated filaments in the freshly separated cyanobionts and their similarity (albeit larger in size) to the cultured cyanobionts during the early stages of culturing perhaps are indicative of only such filaments (which perhaps have not as yet come under the developmental control of the host) being able to survive and grow under *in vitro* conditions. It has been observed by earlier workers that the freshly separated cyanobionts are irregular in size and shape but *in vitro* cultures are very regular and much smaller in size (Newton and Herman 1979). The mature cavities of *Azolla* are known to contain a heterogeneous population of cyanobacterial filaments which show a low morphological variability within a filament but shape and size between filaments are extremely variable (Braun-Howland and Nierzwicki-Bauer 1990). In our investigation, variation in size of vegetative cells belonging to different filaments was relatively low and no akinetes were observed in the freshly separated cyanobionts. This can be attributed to their isolation from the relatively younger parts of *Azolla*, i.e. leaf lobes up to the 4th branch point.

It is interesting to note that the cyanobiont of *A. pinnata* was different from the other cyanobionts, and reflects the taxonomic inclusion of *Azolla pinnata* in section *Rhizosperma* as against section *Euazolla,* to which the other species belong (Peters and Meeks 1989). Another much investigated aspect related to the taxonomic status of the cyanobionts was also brought out to focus during this study. All the cultured and freshly separated cyanobionts, except

those from *Azolla mexicana,* resembled *Anabaena* in their morphology. The cyanobiont from *A. mexicana* exhibited coiling which was more suggestive of its being related more to *Nostoc* sp. than *Anabaena* sp. A similar morphological assessment in this regard has been provided earlier (Meeks *et al.* 1988, Plazinski *et al.* 1990, Gebhardt and Nierzwicki-Bauer 1991). Physiological characterisation of these cultured and freshly separated cyanobionts has showed distinctly different growth attributes and enzymatic activity of N and P metabolism. The amount of chlorophyll *a,* proteins, sugars and ammonia excreted were observed to be significantly higher in freshly separated cyanobionts, whereas their cultured counterparts exhibited higher activities of N-assimilating enzymes (Pabby *et al*. 2003*b*).

Taxonomists have also recognised only one species – *A. azollae* as the symbiont on the basis of limited available evidence. Serological surveys using polyclonal antibodies have shown that the similarities among 32 isolates of *A. azollae* far outweighed differences (Gates *et al.* 1980, Ladha and Watanabe 1982). But recent studies using monoclonal antibodies, have concluded that there are at least four subgroups of *Anabaena azollae* in *Azolla* species (Liu *et al.* 1989). Hence, a definite need exists for comparative characterisation of fresh isolates *vs.* cultured isolates, firstly at the morphological level and later at the molecular level. Our findings are clearly illustrative in this regard, as there was distinct variation not only among the cultured and freshly separated cyanobionts but also among the cyanobionts from different species of *Azolla*. This lends weight to the conclusions regarding the need for a number of subgroups of *Anabaena azollae*.

Presently, molecular profiling of these strains is being carried out in our laboratory, which will further help to elucidate the relationships/relatedness of cultured cyanobionts to the endosymbionts in *Azolla* species. The present study, involving morphological characterisation of the cyanobionts of *Azolla* at the interspecific level therefore provides a starting point for further analysis at intraspecific and molecular level. This can provide a more comprehensive and reliable classification of this association, which can be utilised in genetic manipulation of this association for increased temperature and pest tolerance and thereby wider exploitation of *Azolla* in agriculture and industry.

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