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An extra sheath around the heterocysts of *Anabaena azollae* from the aquatic macrophyte *Azolla filiculoides* Lamarck

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ABSTRACT

The fern *Azolla filiculoides* Lamarck (family Azollaceae) harbours a heterocystous nitrogen-fixing cyanobacterium *Anabaena azollae* Strasburger (Nostocales: Cyanobacteria) inside cavities on the dorsal lobes of the leaves. A new extra sheath was detected under scanning electron microscopy surrounding only the heterocysts of the cyanobiont and their chemical characterization by histochemical tests has pointed to the presence of proteins. The function of this extra sheath is unknown.

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Introduction

Azolla spp. belonging to the family Azollaceae are small heterosporic free-floating aquatic ferns with a world-wide distribution. This aquatic macrophyte is the only known fern with a permanent symbiotic association with the heterocystous nitrogen-fixing cyanobacterium *Anabaena azollae* Strasburger that inhabits cavities of the dorsal lobes of the leaf. The two partners of the symbiosis (the fern and the cyanobiont) have a synchronous development from apical meristem to mature cavities. The colony of *A. azollae* in the apical meristem has only vegetative cells but, during the development of the foliar cavities, the differentiation of heterocysts and sometimes akinetes also occurs (Carrapiço, Teixeira, and Diniz 2000; Lechno-Yossef and Nierzwicki-Bauer 2002; Carrapiço 2010). The filaments of *A. azollae* are formed by vegetative cells and heterocysts, which are among the vegetative cells. The vegetative cells have a Gram-negative cell wall type, inclusions in the cytoplasm such as carboxysomes and cyanophycin granules, and a rudimentary thylakoidal system. The heterocysts are specialized cells for nitrogen fixation, with two polar granules of cyanophycin, a thick cell wall and honeycomb-like thylakoids (Lang 1965; Neumüller and Bergman 1981; Gebhardt and Nierzwicki-Bauer 1991). Above the cell wall of free-living cyanobacteria, and also in the cyanobiont *A. azollae*, there are two more layers, a homogeneous layer formed by polysaccharides and a laminated or fibrous layer with glycolipids (Lang 1965; Adams and Duggan 1999; Brüll et al. 2000).

An extra sheath surrounding the cyanobionts inside the foliar cavities of the dorsal lobes of *Azolla filiculoides* was found during observations using scanning electron microscopy (Veys, Lejeune, and Van Hove 2000; Pereira and Carrapiço, unpublished), but nothing is known about the chemical composition of this extra sheath. So, the aim of the present research was the histochemical characterization of an extra sheath surrounding the heterocysts of *A. azollae* inhabiting the foliar cavities of *A. filiculoides*.

Material and methods

Plant material

Sporophytes of *A. filiculoides* Lam. were harvested from a water pond at the Botanical Garden of the University of Lisbon. A voucher specimen was deposited in the Herbarium of the Faculty of Sciences of University of Lisbon (LISU 191335). The sporophytes were washed in distilled water to remove epiphytes, water excess was then removed in absorbent filter paper for 5–10 min and the leaves were used immediately for analyses.

Scanning electron microscopy

The sporophytes of *A. filiculoides* (between 0.5 and 1 cm) were fixed in glutaraldehyde, dehydrated in ethanol, and dried with the CO_2 critical-point method. These dried sporophytes were cut longitudinally through the foliar cavities with a razor blade, sputter-coated with gold and observed at 15–25 kV in JEOL JSM 5200Lv and JEOL JSM T220 scanning electron microscopes as described in Pereira et al. (2001).

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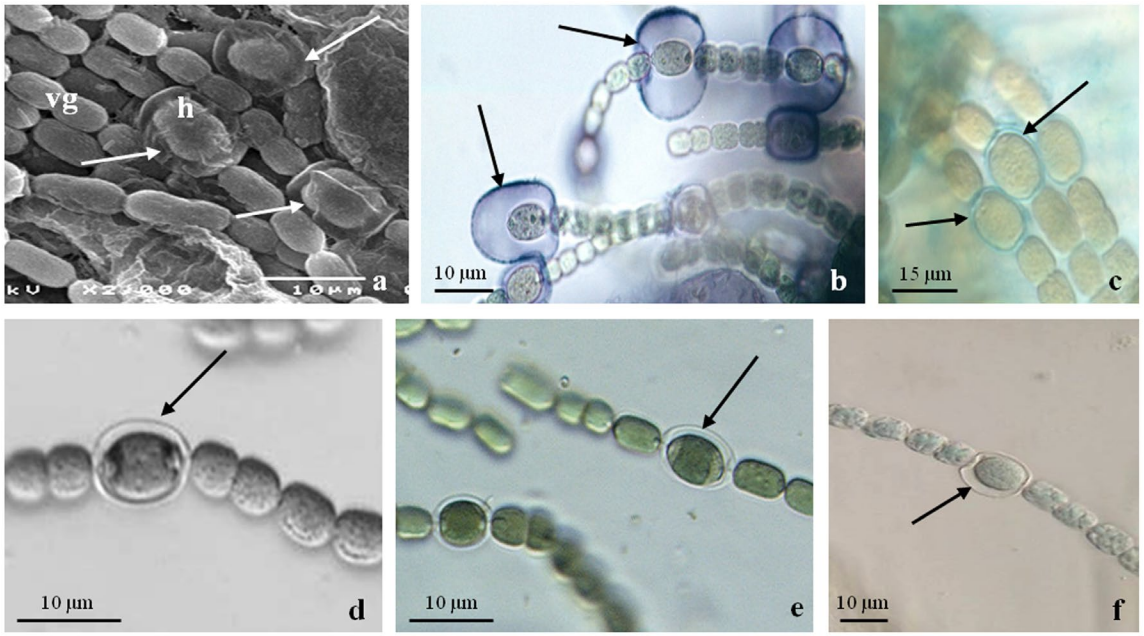

Figure 1. Filaments of *Anabaena azollae* inhabiting the mature foliar cavities of *Azolla filiculoides*. (A) Cyanobacterial filaments formed by vegetative cells (vg) and heterocysts (h) with an extra barrel-shape sheath (arrow). (B) Dark blue colour in the extra sheath around the heterocysts (arrow) stained with Coomassie Brilliant Blue. (C) Cell wall of the heterocysts with faint blue colour (arrow) stained with Alcian Blue. Extra sheath around the heterocyst is not visible. (D) Extra sheath around the heterocysts (arrow) without black colour in Sudan black B stain. (E) Extra sheath around the heterocysts (arrow) without green colour in copper acetate/rubeanic acid stain. (F) Extra sheath surrounding the heterocysts (arrow) without orange colour in antimony trichloride stain.

Histochemical tests

The cavities of the dorsal lobes of washed *A. filicu-loides* sporophytes were squeezed to isolate the cyanobiont *A. azollae*. The histochemical tests were: (i) Coomassie Brilliant Blue for proteins (Fisher 1968, (ii) Sudan black B for total lipids (Bronner 1975, (iii) copper acetate/rubeanic acid for fatty acids (Ganter and Jollès 1969), (iv) antimony trichloride for sterols (Hardman and Sofowora 1972), and (v) Alcian Blue in acetic acid for mucopolysaccharides (Ling-Lee, Ashford, and Chilvers 1977). The histochemical tests were compared with their controls. An Olympus BX60 (Olympus, Essex, UK) light microscope coupled with a Leica DP50 camera (Leica Microsystems, Wetzlar, Germany) was used for image acquisition.

Results and Discussion

The observation of the foliar cavities of *A. filiculoides* under scanning electron microscopy allowed the detection of an extra barrel-shape sheath surrounding only the heterocysts of the *A. azollae* filaments (Figure 1A). This extra sheath was not found in the cyanobionts of *Cycas* species and in free-living cyanobacteria (data not shown).

Histochemically, this new extra barrel-shape sheath stained dark blue with Coomassie Brilliant Blue, indicating a proteinaceous composition (Figure 1B). However, there is no report of those compounds in the cell wall of the *A. azollae* cells (vegetative cells and heterocysts). As this extra sheath is distinct from the special cell wall of the *A. azollae* heterocysts, it probably does not correspond to the homogeneous and laminated or fibrous layers above the cell for *A. azollae* described by Lang (1965), and in free-living cyanobacteria, as described by Adams and Duggan (1999) and Brüll et al. (2000).

The Alcian Blue gave a faint blue staining in the cell wall of the heterocysts, indicating the presence of mucopolysaccharides (Figure 1C), which may be in accordance with the acidic mucopolysaccharides present in the fibrous layer as described by Brüll et al. (2000), and it probably does not correspond to the extra sheath as viewed under scanning electron microscopy.

In addition, the extra sheath did not show the presence of lipids, fatty acids and sterols given the absence of staining with Sudan Black B (Figure 1D), copper acetate/rubeanic acid (Figure 1E) and antimony trichloride (Figure 1F).

The origin and function of this extra sheath are unknown, but as it is specific to the heterocysts, it is probably synthesized by the cyanobiont to assist in the maintenance of an oxygen-free microenvironment.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Notes on contributors

Both authors are plant biologists with a deep interest in plant symbiosis especially the symbiosis between the fern *Azolla* and the cyanobacterium *Anabaena azollae*.

Ana. L. Pereira made the research, discussed the results and wrote the manuscript.

F. Carrapiço supervised the research and discussed the results.

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