

New data on the *Azolla-Anabaena* symbiosis

I. Morphological and histochemical aspects

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Abstract

Light and electron microscopy studies were made on dorsal leaves of *Azolla filiculoides* at different stages of development that corresponded to three different levels of nitrogen fixation in the plant. The morphological and histochemical studies referred to in this work showed a complex relationship between the three partners (*Azolla*, *Anabaena*, bacteria), with special emphasis on the biological recognition of these organisms in the symbiosis. The bacteria, present in all stages of leaf development, are in close association with the *Azolla* cavity cells and follow a location pattern identical to that of the cyanobacteria.

Introduction

The symbiotic association *Azolla-Anabaena* was described for the first time by Strasburger in 1873. Since then and particularly over the last few years, several studies have been made to elucidate the morphological and functional relationship between these two partners in the symbiosis (Grilli, 1964; Hill, 1975; Lumpkin and Plucnett, 1980; Neumuller and Bergman, 1981; Peters and Mayne, 1974; Peters and Calvert, 1983 and Sevillano *et al.*, 1984). Until recently it was generally accepted that this symbiosis was formed by two partners. After the isolation and identification of one type of bacteria in the leaf cavity of this fern (Wallace and Gates, 1986) the idea of a third symbiotic partner was implicitly suggested, but was not shown to be a permanent member of the prokaryotic colony at different stages of leaf development.

In this work, light and electron microscopy studies were made on dorsal lobe leaves of *Azolla filiculoides* at three different stages of development, with special attention to the relationship between the fern and the prokaryotic colony. Thus, the purpose of this work was to contribute to a better understanding of the symbiotic relationship

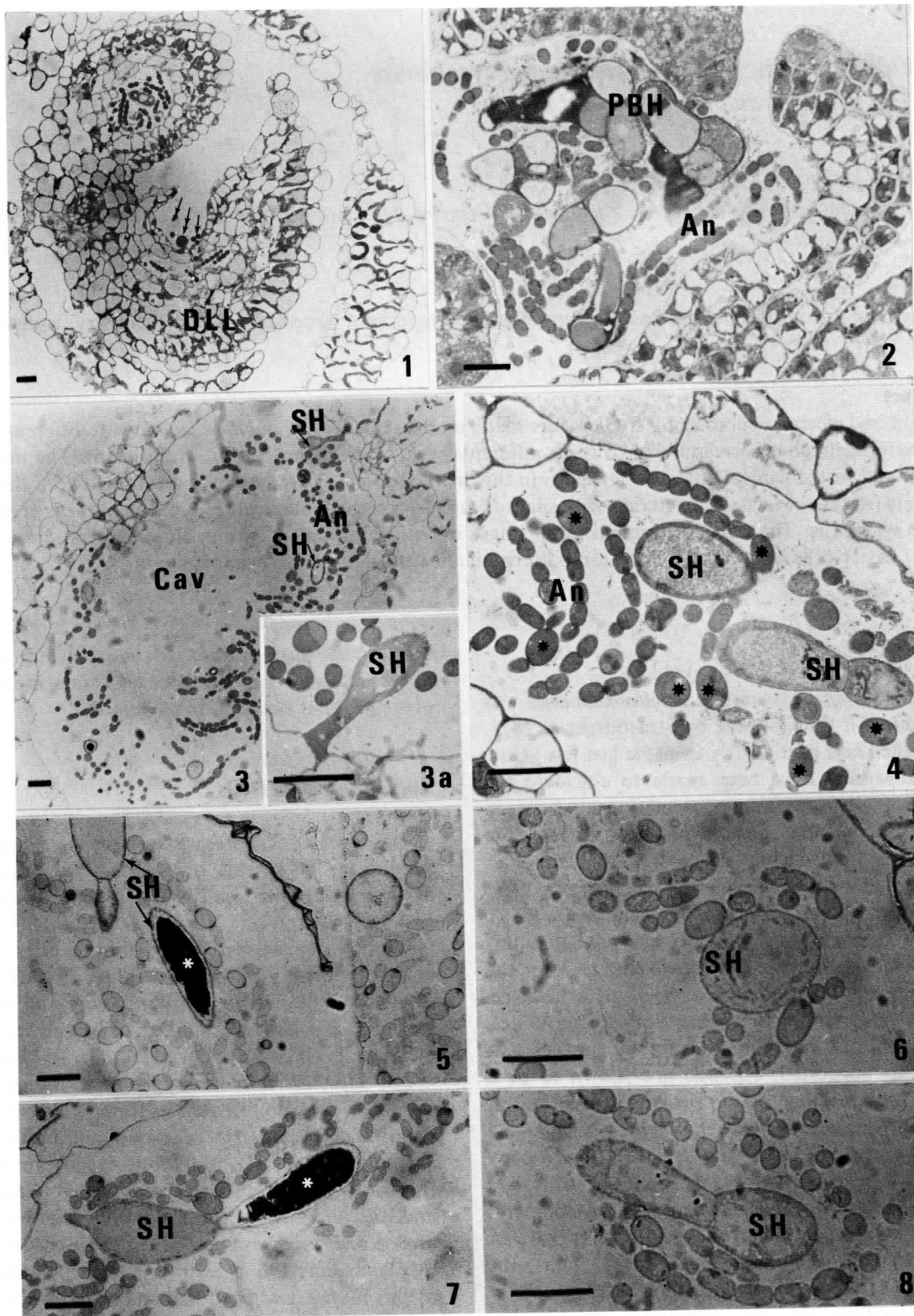
between the three partners (*Azolla*, *Anabaena*, bacteria) and to increase knowledge of some metabolic processes that occur in the symbiosis.

Materials and methods

The *Azolla filiculoides* plants were collected in Alcochete (70 km from Lisbon) and maintained in natural conditions in the Botanical Garden of Lisbon.

Light and electron microscopy studies were made on dorsal lobe leaves of this fern at different stages of development (apical meristem, full mature leaf and senescent leaf) that correspond to three different levels of nitrogen fixation in the plant.

For electron microscopy studies, leaves were submitted to a double step fixation process by 4% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) and in 1% OsO₄ in 0.05 M cacodylate buffer (pH 7.4) with or without ruthenium red (Colombo and Rascio, 1977). The samples were dehydrated in an acetone series and embedded in Epon-Araldite (Mollenhauer, 1964). Semi-thin sections (800 nm) of this material without ruthenium red, were used in lipid histochemistry by sudan black B staining



(Bronner, 1975) and in the detection of insoluble polysaccharides by the periodic acid Schiff (PAS) technique (Feder and O'Brien, 1968). For further electron microscopic morphological data, impregnation with KI-OsO_4 mixture was made (Carrapiço *et al.*, 1984).

Thin sections (60–80 nm) of the specimens were cut with a glass knife and Porter-Blum MT-2 ultramicrotome and mounted on copper grids. The sections were post-stained (except KI sections) and the observations were made in a Hitachi-12 electron microscope operating at an accelerating voltage of 75 Kv.

Results and discussion

In early stages of cavity formation, at the apical meristem, several filaments of undifferentiated cells of *Anabaena* and bacteria were seen in the proximity of epidermal cells or near the primary branched hair (PBH) cells (Figs. 1, 2, 9, 10, 12).

The blue-green alga (cyanobacterium) filaments were mainly characterized by the absence of heterocysts (Figs. 2, 9) and the PBH cells showed a typical transfer hair morphology (Fig. 10).

The next stages of leaf development (mature and senescent leaves) were characterized by the presence of other types of transfer hairs, namely the simple hairs (SH), which also showed a transfer cell morphology (Figs. 3, 3a, 11). These trichomes appeared at different stages of development in the

same leaf cavity and they seemed not to be related to the age of the leaf. The prokaryotic colonies (cyanobacteria and bacteria) appeared to be more numerous and the blue-green alga was characterized by the presence of a great number of heterocysts in the filaments (Fig. 4). A middle-age trichome revealed the presence of labyrinthine cell wall ingrowths, whose contours were closely followed by the plasmalemma (Fig. 11); a large central vacuole with deposits (Figs. 4, 5, 7); other different cytoplasmic organelles, in particular, a poorly developed endomembrane system revealed by KI-OsO_4 impregnation (Figs. 13, 14); some plastids with rudimentary tylakoid system; numerous mitochondria with developed cristae, and a few peroxisomes (Fig. 11). It seems that no significant morphological difference exists between these two levels of leaf development, with the main exception that the number of heterocysts in the blue-green alga present in senescent leaves decreases.

The presence of some compounds in the cell vacuoles of simple hairs are clearly evident in electron and light microscopy observations. The use of PAS and sudan black B tests in semi-thin sections revealed the polysaccharidic and lipophilic nature of these substances accumulated in the terminal cells of these trichomes (Figs. 5, 6, 7, 8). This accumulation may be the result of an autolytic or a secretion process. It is not clear whether the same hair can accumulate these two kinds of substance

Fig. 1. Early stage of leaf development. A depression, which corresponds to the cavity before closing, can be seen in the ventral side of the dorsal leaf lobe (DLL). This depression contains several filaments of *Anabaena* vegetative cells (arrows) and the primary branched hair (double arrow).

Fig. 2. The apical colony of *Anabaena* cells (An) is maintained in the proximity of the primary branched hairs (PBH). In this stage of leaf development no heterocyst are observed in the cyanobacteria.

Figs. 3–4. Several aspects of a cavity (Cav) in a mature leaf. Sections of simple hairs (SH) can be seen. The presence of a great number of heterocysts in *Anabaena* filaments is evident (asterisks). Fig. 3a shows a magnification of a simple hair in the cavity.

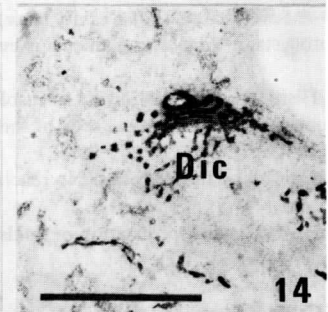
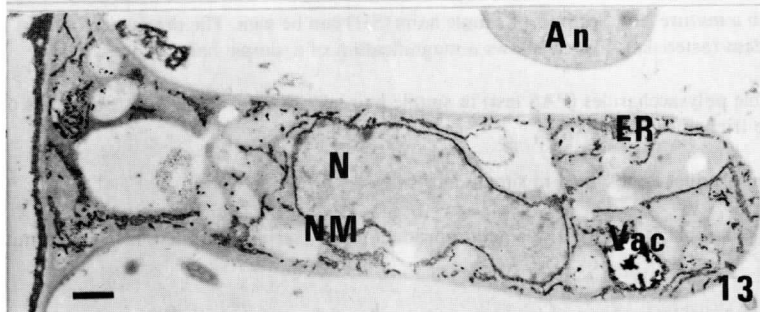
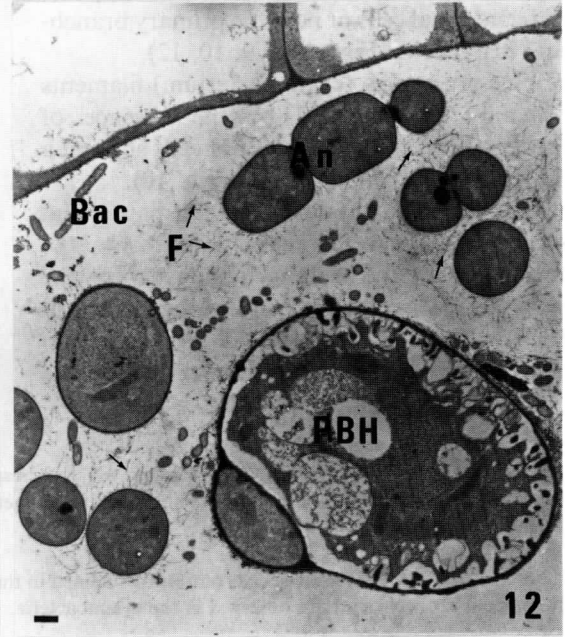
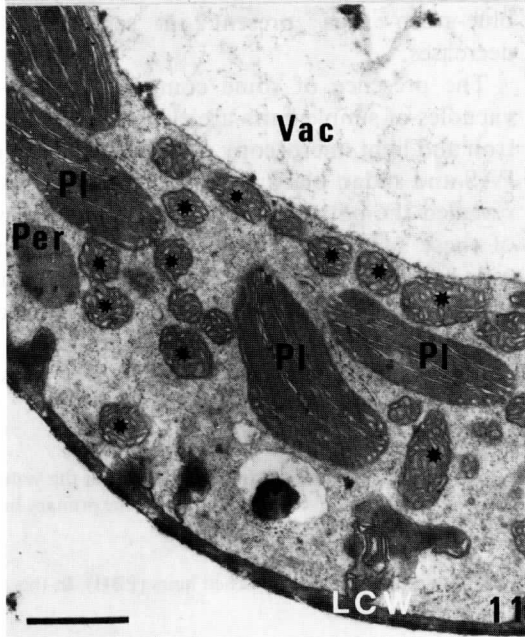
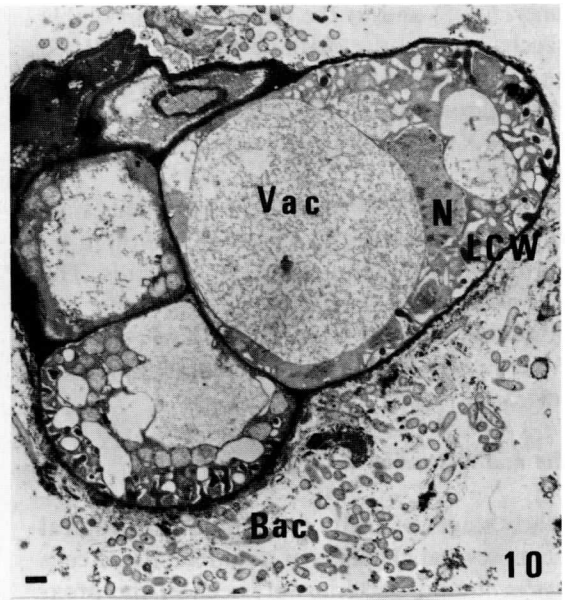
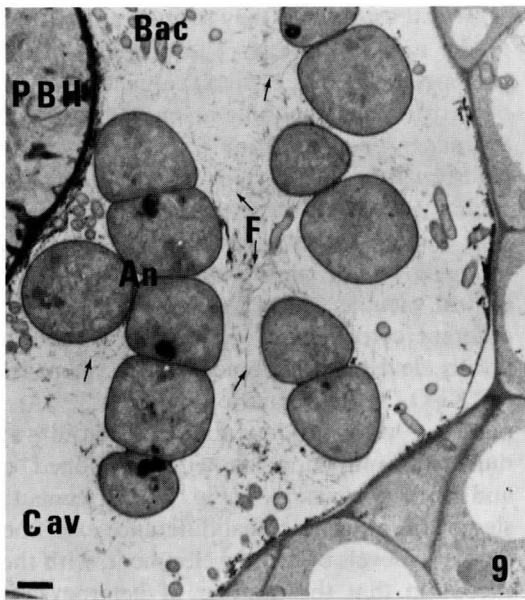
Fig. 5. Histochemical localization of insoluble polysaccharides (PAS test) in simple hair cells (SH). The reaction product is observed in vacuole sap of the terminal cells of these trichomes (asterisk).

Fig. 6. Control of the PAS test. No reaction product is observed in simple hair cells.

Fig. 7. Histochemical localization of lipids (sudan black B test). The reaction product is only seen in the vacuole of the terminal cells of simple hairs (asterisk).

Fig. 8. Control of the sudan black B test. No reaction is observed in the terminal cells of the simple hair (SH).

In Figures 1–8 bar represents 20 μm .



or if, on the contrary, different types of transfer hairs exist with specialized metabolic functions. These compounds may be involved in the protection against foreign organisms outside the symbiotic partners. Further research is needed to clarify this matter.

One of the more interesting features of this association is the existence of a mucilaginous fibrillar network, revealed by ruthenium red and present in all stages of leaf development, which fills the cavity and in which the blue-green algae and bacteria are immersed (Figs. 9, 12). This technique shows the polysaccharadic nature of the *Anabaena* mucilaginous cell wall and agrees with the results of Bergman *et al.*, (1985) in the free-living species *Anabaena cylindrica*. The mucilaginous fibrillar network observed in the *Azolla* cavity may play a part in the recognition process between the fern cells, cyanobacteria and bacteria. The presence of this mucilaginous network in the first steps of cavity formation, strongly suggests that its formation is not dependent on the fern, but related to the metabolic activity of the blue-green algae or the bacteria. These data support the hypothesis formulated by Duckett *et al.*, (1975) in *Anabaena*-free *Azolla*, that any mucilaginous compounds were formed in the cavity of the fern, which indicates that these substances are produced by the *Anabaena* cells.

Numerous bacteria with a typical coryneform or coccal morphology, probably of the genus *Arthrobacter* (Forni, 1987, personal communication; Wallace and Gates, 1986), are present in all stages of leaf development, as a member of the prokaryotic colony, in close association with the transfer hairs and in the proximity of *Azolla* epidermal

cells. The metabolic function of these bacteria in the symbiosis is unknown.

In conclusion, we suggest that a bacterium, probably *Arthrobacter*, is the third permanent partner of the symbiosis. The bacteria are present at all stages of leaf development in close association with the primary branched hairs, simple hairs or *Azolla* epidermal cells, following a location pattern identical to that of *Anabaena*.

A mucilaginous fibrillar network revealed by ruthenium red and present at all stages of leaf development, fills the cavity in which blue-green algae and bacteria are contained. This network may play a part in the recognition process between the three partners of the symbiosis.

The apical cells of simple hairs accumulate substances of lipophilic and polysaccharidic nature. The function of these compounds is unknown, but we suggest that they may be involved in the defence against foreign organisms other than the symbiotic partners.

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Figs. 9 and 12. Ultrastructural aspects of the early stages of cavity formation. The *Anabaena* filaments (An) and bacteria (Bac) are in close proximity with the primary branched hair (PBH). A fibrillar network (F), revealed by ruthenium red (arrows) can be observed, surrounding the *Anabaena* cells and bacteria.

Fig. 10. Section of a primary branched hair. The cells of this hair present a labyrinthine cell wall (LCW) and are highly vacuolated (Vac). A great number of bacteria (Bac) are in close association with this hair.

Fig. 11. Detail of a simple hair cell. These cells also show a transfer cell morphology. A great number of mitochondria (asterisks) and a few plastids (Pl) are observed. The number of peroxisome (Per) sections are, on the contrary, very low. These cells show, frequently, a large vacuole (Vac) containing fibrillar material and electron-dense deposits.

Figs. 13–14. Aspects of a simple hair cell treated with the $KI - OsO_4$ mixture. The reactivity is observed in the endoplasmic reticulum (ER), dictyosome (Dic), nuclear membrane (NM) and in the sap of some vacuoles (Vac).

In figures 9 to 14 bar represents 1 μm .

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