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The Pore of the Leaf Cavity of *Azolla*: Interspecific Morphological Differences and Continuity between the Cavity Envelopes

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Abstract

Interspecific morphological differences between mature leaf cavity pores of the different *Azolla* species were investigated by light and low temperature scanning electron microscopy. Results indicated that the pore morphology constitutes a novel taxonomic criterion allowing us to separate the *Azolla* and *Rhizosperma* sections. Scanning documents also gave further insight into the pore function: it ensures gaseous exchanges between the environment and the cavity, defends the cavity from external ingress and contributes to the maintenance of the endosymbionts within this cavity. Transmission electron microscopy of the cavity in the pore region indicated that the previously described one-layered inner envelope and three-layered outer envelope, both limiting the mucilage containing the endosymbionts, are not separate entities: they join together at the pore periphery and the inner envelope is actually constitutive of one of the layers of the outer envelope. Our observations allowed us to explain for the first time the possible origin and nature of each of the three constitutive layers of the latter envelope.

Keywords: *Azolla*, leaf pore, cell wall projections, taxonomy, mucilage, envelopes

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1. Introduction

The *Azolla-Anabaena* symbiosis has attracted attention for the last decades for two main reasons: its multiple utilisation in agriculture, especially in integrated farming systems including rice, vegetables, poultry, pigs and fish, but also for the remarkable complexity of the relations between the two partners (e.g. Lejeune et al., 1999). Symbiotic exchanges between the fern *Azolla* and the nitrogen-fixing cyanobacterium *Anabaena azollae* Strasb. occur in a cavity of the upper leaf lobe of the fern. This cavity harbours the prokaryotic partner within a peripheral mucilaginous region (Schaede, 1947) surrounding a gaseous central region (Peters and Meeks, 1989). This mucilaginous matrix has been described as limited by two structures called the outer and inner envelopes whose relationship, origins and compositions remain uncertain. The outer envelope was detected by Peters (1976) who, by enzymatic digestion of the plant tissues, incidentally obtained not dispersed *Anabaena* filaments but what he named 'algal packets' (Peters et al., 1978) of *Anabaena* enclosed in an envelope. Uheda and Kitoh (1991) showed that this outer envelope has a tripartite ultrastructure. In contrast the inner envelope, first described by Nierzwicki-Bauer et al. (1989), separates the mucilage from the gaseous central region and lacks a tripartite ultrastructure.

Recently we demonstrated that the leaf cavity pore, connecting the cavity in which the symbionts reside with the external environment, remains open in all developmental stages of the leaf (Veys et al., 1999). We showed that the morphology of the pore of *A. filiculoides* is a truncated cone, protruding into the cavity, limited by a special type of elongated trichomes that we called teat-cells, forming a system of baffles probably acting as a mechanical filter. Ultrastructural analysis of these cells revealed their secretory function. Teat-cells produce on their cell wall surface numerous projections composed of proteins, pectin and callose and we suggested they might play a defensive role. Although we compared the ontogeny of the pore on three different *Azolla* species, the morphological differences that could be found between mature pores of the different *Azolla* species as well as their potential taxonomic significance, if any, were not investigated at that time.

In the present paper we compare the morphology of the mature pore in different *Azolla* strains representing the seven species currently recognized and separated by taxonomists into two sections: *Azolla* and *Rhizosperma* (Lumpkin and Plucknett, 1982), even if this classification is controversial. In addition, since we have demonstrated the persistence of the pore and considered its possible role in exchanges between the leaf cavity and the external environment (Veys et al., 1999), we now try to confirm the presence or absence of mucilaginous material that could clog it. Finally we examine in the pore region

the relation between what has been described as the outer and inner envelopes of the leaf cavity mucilage in order to elucidate their possible relationship, constitution and origin.

2. Materials and Methods

Plant material

The *Azolla* strains used in this study belong to the collection of the Laboratory of Plant Biology, Catholic University of Louvain (Van Hove et al., 1987). These were (1) for the *Azolla* section: *A. filiculoides* (ADUL-81FI, -173FI, -430FI and -724FI), *A. rubra* (ADUL-200RU and -273RU), *A. caroliniana* (ADUL-269CA and -519CA), *A. mexicana* (ADUL-222ME) and *A. microphylla* (ADUL-69MI); (2) for the *Rhizosperma* section: *A. pinnata* var. *pinnata* (ADUL-136PP, -203PP and -261PP), *A. pinnata* var. *imbricata* (ADUL-379PI and -401PI) and *A. nilotica* (ADUL-82NI). Two *Anabaena*-free strains of *A. filiculoides* (ADUL-173FI.AF) and *A. mexicana* (ADUL-222ME.AF) were also included. Culture conditions were as described by Van Hove et al. (1987) except for strain ADUL-724FI which was cultivated in field conditions where it matured into a typical erect morphology with elongated internodes.

Fixation of plant material

Azolla material was fixed for 2 h at 20°C in a solution of 2.5% glutaraldehyde - 3.7% paraformaldehyde in 0.05 M cacodylate buffer (pH 6.8) with 5 mM $CaCl_2$. Samples were rinsed with the same 0.05 M cacodylate buffer (pH 6.8) with 5 mM $CaCl_2$, dehydrated in 90% ethanol and embedded in a hydroxyethylmethacrylate resin (Technovit 7100, Kulzer, Germany). We also used a combined glutaraldehyde - paraformaldehyde - osmium tetroxide fixation modified from Calvert et al. (1985) and Roland and Vian (1991) as follows: isolated leaves were prefixed for 2 h at 20°C in a solution of 2.5% glutaraldehyde - 3.7% paraformaldehyde in 0.05 M cacodylate buffer (pH 6.8) with 5 mM $CaCl_2$. Samples were then postfixed for 30 min at 0°C in 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.2). After a graded serial dehydration in ethanol, samples were embedded in a low viscosity resin (Spurr, 1969).

Semi-thin sections of about 2 µm were cut with glass knives and, after staining by Toluidine blue (0,1% w/v in 0,1% $Na_2B_4O_7$), mounted in immersion oil.

To verify the presence or absence of projections on the teat-cell surface, entire fresh plants, or plants fixed in 70% ethanol for 24 h, were carefully dissected under a stereo microscope in order to isolate the adaxial surface of the upper

leaf lobe presenting the pore region. After lactophenol clearing (Gerlach, 1969) these fragments were observed by Nomarski interferential contrast microscopy.

Light microscopy (LM)

Observations in Nomarski interferential contrast and light microscopy were realised on a Polyvar microscope (Reichert-Jung, Germany). Micrographs were recorded on black/white Kodak T-MAX 100 (Iso 100/21°) films.

Transmission electron microscopy (TEM)

Spurr's resin embedded leaves were trimmed and sectioned with a diamond knife. Ultra-thin sections were stained by 2% uranyl acetate followed by lead citrate (Reynolds, 1963) for 3 min in each solution. Sections were examined in a Jeol JEM-100SX transmission electron microscope operating at 80 kV. Micrographs were recorded on Agfa Scientia EM film.

Low-temperature scanning electron microscopy (LTSEM)

LTSEM observations were realised on a Philips XL-20 scanning electron microscope. Fresh *Azolla* leaves were mounted on a shrouded specimen stub using a cryo-adhesive and immediately cryofixed in liquid nitrogen. Fracturing and gold coating of the samples were performed in the specimen chamber of the microscope.

'Algal packets'

'Algal packets' (Peters et al., 1978) from *A. filiculoides* were obtained by maceration in a mixture of cellulase Onozuka RS (Yakult Honsha, Japan) and pectolyase Y-23 (Kikkoman, Japan) as described by de Roissart et al. (1994). They were thereafter fixed by the above mentioned glutaraldehyde - paraformaldehyde - osmium tetroxide fixation, embedded in Spurr's resin and prepared for TEM.

3. Results

Interspecific morphological differences of mature pores

A comparative study of the pore morphology realised on the different strains of *A. filiculoides, A. caroliniana, A. mexicana, A. microphylla* and *A. rubra,* all belonging to section *Azolla,* revealed identical structural features.

Longitudinal sections through pores of these species showed that four – rarely three – tiers of teat-cells bound them (Fig. 1A and Veys et al., 1999). As also revealed by LTSEM, superficial teat-cells conspicuously point and extend outwards the adaxial surface of the leaf (Fig. 2A). The inner margin of the pore, presenting the deep-seated smaller teat-cells, clearly protrudes into the leaf cavity (Fig. 1A). LTSEM front views of the adaxial surface of the leaf showed that the pore is circular, or slightly elliptic (Fig. 3A). The distance between the pore and the leaf axil has a mean value of 92 µm (Table 1). This value is independent of the gross morphology as shown by the comparison of *A. filiculoides* grown in lab conditions where they have a compact, horizontal morphology and in field conditions where they grow erect with elongated internodes. Mature pores of these species always present cell wall projections on the teat-cells. These projections are found at the basal part of the teat-cells as previously reported (Veys et al., 1999) and are not produced by the superficial teat-cells. When observed by LTSEM they appear as droplet-like exudates with their surface free from any wax deposits or crystals (Fig. 4).

Table 1. Distance between the pore and the leaf axil

Section	Species	Strain	N	Distance (µm)		
				M	STD-error	MW
Azolla	*A. filiculoides*	ADUL-173FI	12	86	4.13	
		ADUL-430FI	6	95	4.49	
		ADUL-724FI*	12	97	5.36	
	A. rubra	ADUL-273RU	12	94	5.02	
		ADUL-200RU	7	99	5.45	92
	A. caroliniana	ADUL-519CA	12	101	3.06	
		ADUL-269CA	7	92	7.2	
	A. mexicana	ADUL-222ME	12	83	3.66	
	A. microphylla	ADUL-69MI	12	86	2.84	
Rhizosperma	*A. pinnata*	ADUL-379PI	12	221	5.85	
	var. *imbricata*	ADUL-401PI	7	192	9.67	
	A. pinnata	ADUL-136PP	12	240	10.14	220
	var. *pinnata*	ADUL-203PP	7	213	10.02	
		ADUL-261PP	7	216	9.45	
	A. nilotica	ADUL-82NI	12	349	16.14	

Note: Counts on 15–20th leaves, 1 leaf per plant. N: number of counts; M: mean; STD-error: standard error; MW: weighted mean; *: grown in field conditions.

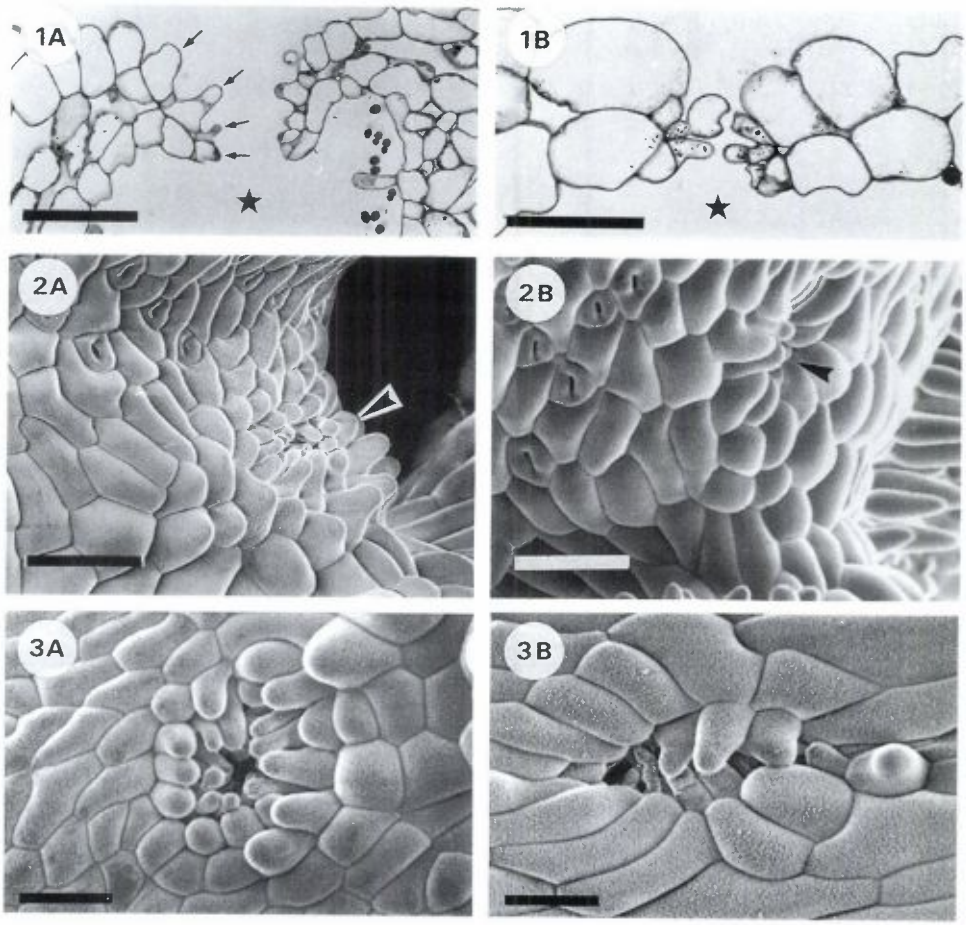
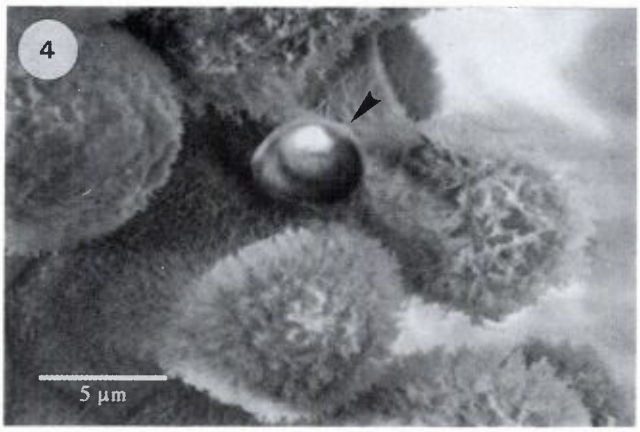


Figure 1. LM micrographs. (A) Longitudinal section through a pore of *A. filiculoides* showing the four tiers of teat-cells (arrows) and the invagination of the pore inner margin towards the leaf cavity (star). (B) Longitudinal section of a pore of *A. pinnata* var. *pinnata*. The leaf cavity is indicated by the star. Bars = 50 µm.

Figure 2. LTSEM micrographs. (A) Adaxial surface of the upper leaf lobe of *A. filiculoides* showing the pore (arrowhead) and its protruding superficial teat-cells. (B) Same view as in (A) but on *A. pinnata* var. *pinnata*. The pore (arrowhead) does not present protruding teat-cells. Bars = 100 µm.

Figure 3. LTSEM micrographs. (A) Front view of the circular pore of *A. filiculoides*. (B) Front view of the slit-elongated pore of *A. pinnata* var. *pinnata*. Bars = 50 µm.

Teat-cell projections were also observed in the two *Anabaena*-free strains of *A. filiculoides* and *A. mexicana* investigated in this study. Projections were never observed on any other cell type of the upper leaf lobe.



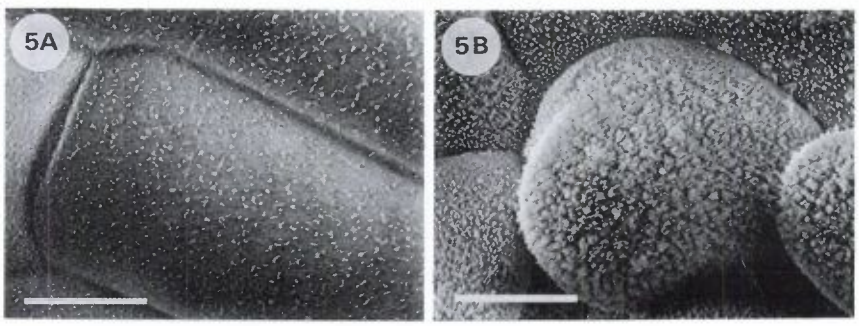


Figure 4. LTSEM micrograph of a fractured pore of *A. filiculoides* showing a cell wall projection (arrowhead) devoid of wax deposit on the teat-cell surface which is covered with abundant wax crystals. Bar = 5 µm.

Figure 5. LTSEM micrographs showing the difference of density of the wax deposits on adaxial epidermal cell surface (A) and the teat-cell surface (B) in *A. nilotica*. Bars = 20 µm.

Similar analyses were performed on *A. pinnata* and *A. nilotica,* belonging to section *Rhizosperma*. In the two varieties of *A. pinnata* the pore is elongated like a slit in the longitudinal direction of the upper leaf lobe (Fig. 3B) and is limited by only two – sometimes three – tiers of teat-cells (Fig. 1B). Its superficial teat-cells do not obviously extend outwards the adaxial surface of the leaf (Fig. 2B) and its inner margin does not protrude into the cavity (Fig. 1B) as in the *Azolla* section. Moreover, the distance between the pore and the leaf axil is approximately two and a half times greater than in the *Azolla* section (mean value of 220 µm) (Table 1). *A. nilotica* presents the same morphological features as the two *A. pinnata* varieties with the exception of a larger pore - leaf axil distance (mean value of 349 µm) (Table 1). In our culture

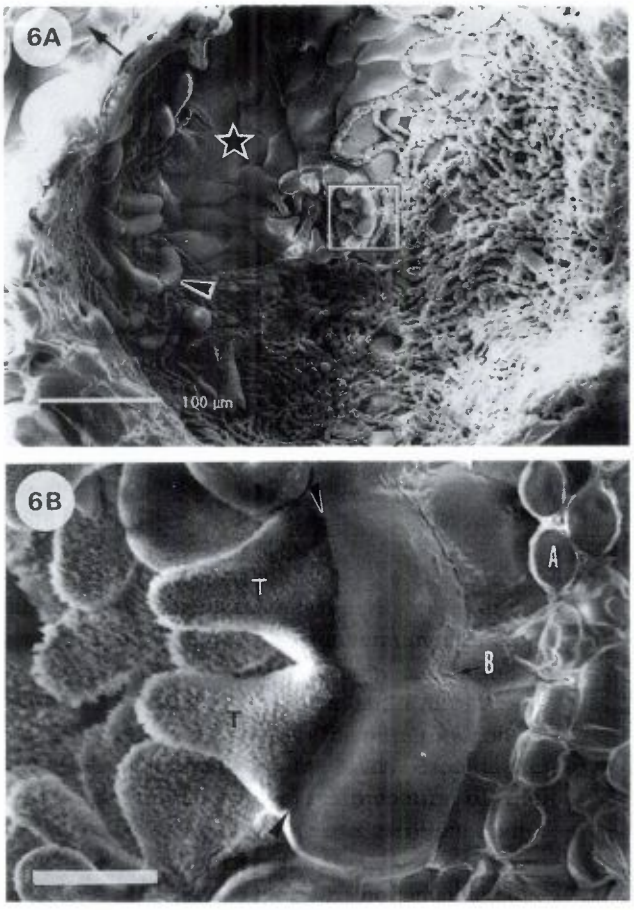


Figure 6. LTSEM micrographs. (A) Internal view of the cavity showing the pore region in *A. filiculoides*. The star indicates the region of the cavity free from *Anabaena* and transfer trichomes (arrowhead). The arrow indicates the leaf base. Bar = 100 µm. (B) Detailed view of the area indicated by a rectangle in (A) showing deep-seated teat-cells (T) covered with a waxy coating and protruding into the cavity. The thin mucilage of the cavity, through which *Anabaena* (A) and other bacteria (B) are visible, stops at the base of these cells (between the two arrowheads). Bar = 10 µm.

conditions projections on the teat-cells were never observed in the *Rhizosperma* section.

For all *Azolla* species of both sections, LTSEM study of the pore region showed that, with few exceptions, the wax deposits on the teat-cells, and

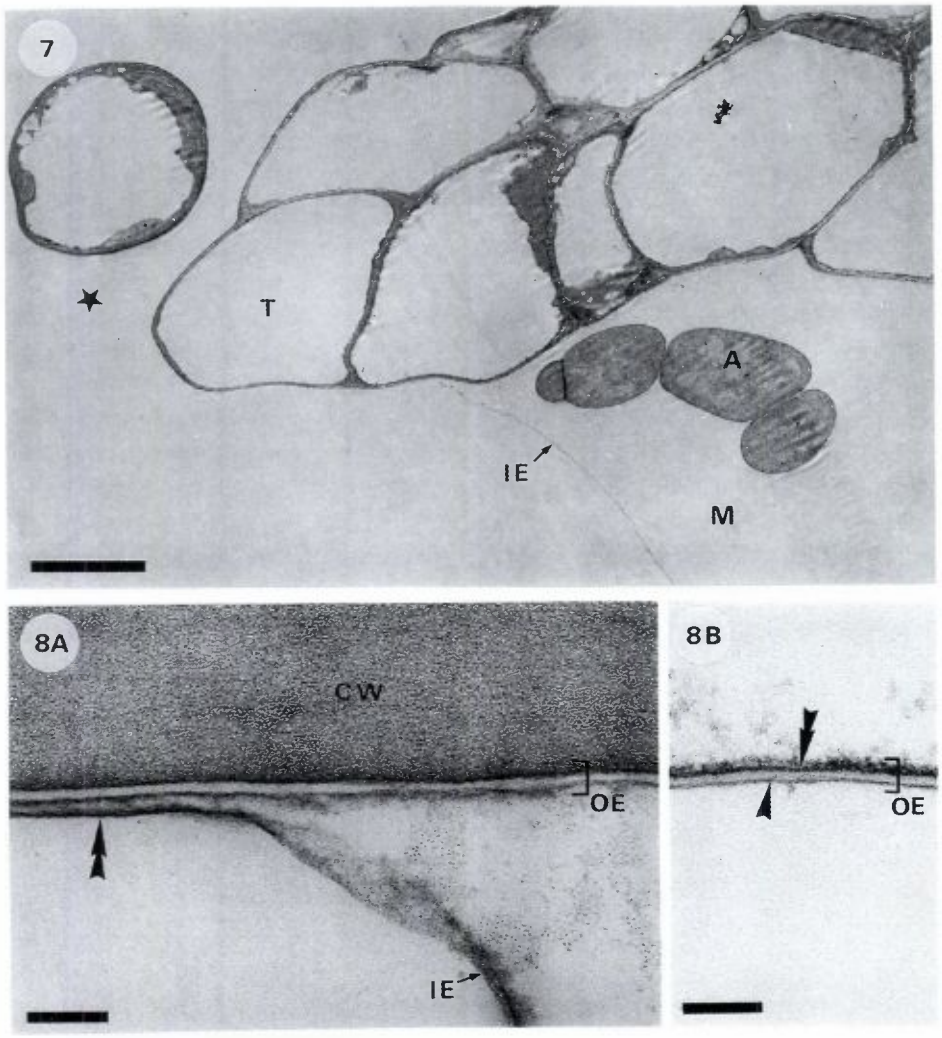


Figure 7. TEM micrograph of the pore region in *A. filiculoides*. The inner envelope (IE) limiting the mucilaginous peripheral region (M) containing the *Anabaena* (A) joins the margin of the pore nearby the deep-seated teat-cells (T). The star indicates the lumen of the pore. Bar = 5 μm.

Figure 8. TEM micrographs. (A) Joining zone of the inner (IE) and outer (OE) envelopes in the pore region observed *in situ*. The three-layered ultrastructure of the outer envelope is indicated by (]). Further towards the pore both envelopes run parallel (double arrowhead). CW indicates the cell wall of an epidermal cell lining the cavity. (B) Outer envelope (OE) observed on 'algal packets'. The three-layered ultrastructure corresponds to the outermost opaque layer (double arrowhead) and the innermost opaque layer (arrowhead) separated by a middle electron transparent layer. Note the thicker and rougher aspect of the outermost layer that corresponds to the cuticle (compare (A) and (B)). Bars = 100 nm.

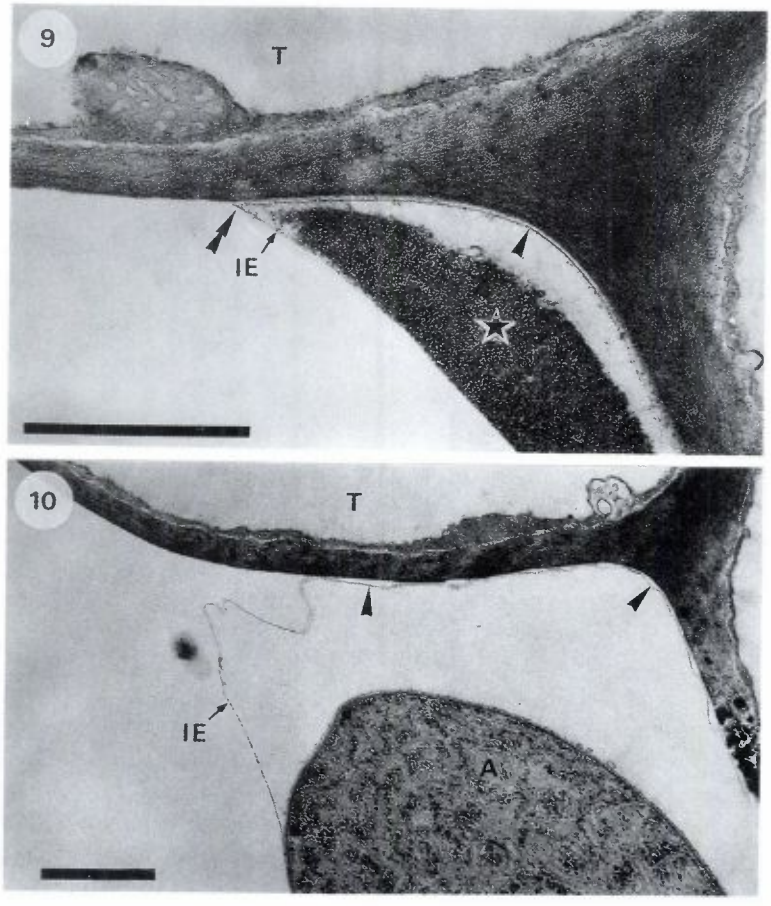


Figure 9. TEM micrograph. Detailed view of the mucilaginous region, with condensed material (star), limited by the inner envelope (IE) showing the continuity (double arrowhead) of this envelope with the innermost layer (arrowhead) of the outer envelope. T = Teat-cell. Bar = 1 µm.

Figure 10. TEM micrograph showing the inner envelope (IE) at the base of a deep-seated teat-cell (T). Folding of the envelope clearly shows that the inner envelope forms the innermost layer (arrowheads) of the outer envelope. A = *Anabaena* cell. Bar = 1 µm.

sometimes the immediate contiguous epidermal cells, are denser and composed of longer crystals than those on the surface of other adaxial epidermal cells (Figs. 5A and 5B). This is responsible for the typical fuzzy aspect of the teat-cell surface (Figs. 4 and 5B).

Mucilage and envelopes in the pore region

LTSEM observations of the pore, viewed from the external surface of the leaf as well as from the inside of the cavity, have been realised on *A. filiculoides*, *A. pinnata* var. *pinnata* and *A. nilotica*. They have all shown the absence of any clogging of the pore by mucilaginous or other material (Figs. 3A, 3B, 4 and 6A). Moreover, when observed from within the cavity, the pore region appears as an area with relatively few *Anabaena*; the region of the cavity closest to the leaf axil is even totally free of *Anabaena* and of transfer trichomes (Fig. 6A). Near the base of the deep-seated fuzzy teat-cells the mucilage, seen as an amorphous thin layer of slimy material embedding the *Anabaena* and other bacteria (Fig. 6B), covers the surrounding inner epidermal cells.

TEM micrographs at the vicinity of the pore showed that the peripheral mucilaginous region containing the *Anabaena* is separated from the central gaseous region of the cavity by the inner envelope described by Nierzwicki-Bauer et al. (1989) (Fig. 7). This envelope joins the margin of the pore close to the deep-seated teat-cells (Fig. 7). At a higher magnification of the same region, the outer envelope becomes visible and shows its three-layered (or tripartite) ultrastructure described by Uheda and Kitoh (1991), as compared to the inner envelope which is composed of only one film (Fig. 8A). This three-layered ultrastructure is identical in thickness and appearance to the one observed on envelopes limiting 'algal packets' obtained from enzymatic digestion of plant tissues (Fig. 8B). On such packets, the outermost opaque layer of the envelope is characterized by its thicker and less regular aspect as compared to the innermost opaque layer (Fig. 8B). As shown on Fig. 8A, the mucilage layer becomes very thin so that both envelopes approach each other and run parallel towards the pore. Some TEM documents revealed that at the proximity of the deep-seated teat-cells the inner envelope is continuous with the innermost layer of the outer envelope (Figs. 9 and 10). This clearly indicates that the innermost layer of the outer envelope corresponds to the inner envelope adhering to the cuticle of the cavity epidermis.

4. Discussion

The shape of the pore, the number of teat-cell tiers as well as the presence of excretory projections on them and the protrusion of the pore margin into the leaf cavity are similar in all the observed strains belonging to the *Azolla* section and clearly differentiate them from those of the *Rhizosperma* section. These morphological criteria reinforce, if needs be, the classical subdivision of the genus into two sections (Lumpkin and Plucknett, 1982). Moreover, the distance between the pore and the leaf axil constitutes a new character that further

separates *A. nilotica* from *A. pinnata*. This latter distinction is in line with recent data (Saunders and Fowler, 1993; Van Coppenolle et al., 1993), suggesting that the two species are more distant from each other than previously estimated.

In a preceding paper (Veys et al., 1999) we suggested that the pore might act as a mechanical filter protecting the cavity from external ingress of foreign particles or microorganisms as well as from the escape of the endosymbionts, its teat-cells forming a system of baffles. In this respect the *Azolla* section seems to have developed a more elaborate filter than the *Rhizosperma* section, with a larger number of teat-cell tiers resulting in the protrusion of the inner margin of the pore into the cavity. Additionally, the teat-cell projections, which were observed only in the first section and are inborn to the plant and not related to the presence of *Anabaena*, could confer an additional, chemical, protection against invasion; a better knowledge of the composition of these excretions would nevertheless be necessary to support this hypothesis. At this point we have no indication whether these characteristics of the *Azolla* section could be related to the location of the pore which, in this section, is very close to the stem and therefore to the water surface.

The abundant waxy coating we observed on the teat-cells gives further indications about the pore function. By its hydrophobic properties it not only protects the cavity from capillary ingress of water but also prevents obstruction of the pore by the cavity mucilage, thereby also preventing the escape of the endosymbionts by mucilage outflow, while securing free gas exchanges between the cavity and the external atmosphere. On account of this the pore may be considered as a regulatory and maintenance element of the *Azolla-Anabaena* symbiotic association.

TEM observations on the envelopes in the pore region *in situ* and their comparative ultrastructural aspects on 'algal packets' further improve our knowledge of these structures. Previously two distinct unrelated structures, both termed envelope, were described: a three-layered outer envelope (Peters et al., 1978; Uheda, 1986; Uheda and Kitoh, 1991) and a one-layered inner envelope (Nierzwicki-Bauer et al., 1989). By observing these envelopes near the deep-seated teat-cells (Figs. 9 and 10), we could show that there is actually one single continuous envelope – i.e. the one-layered inner envelope – that limits the mucilage and probably corresponds to condensed mucilaginous material (Fig. 9). At the interface of the mucilage and the cavity epidermis this envelope is closely associated with the very thin cuticle. The three-layered ultrastructure observed (Figs. 8A and 8B) corresponds therefore to the envelope and the cuticle, apparently separated by a thin, electron transparent, layer. This also explains the three-layered ultrastructure of the outer envelope described by Uheda and Kitoh (1991) on 'algal packets' obtained by

digestion of the *Azolla* tissues with cellulase and pectolyase: the innermost electron opaque layer represents the envelope as just explained and the outermost electron opaque layer, usually irregular and rough in aspect, represents the very thin cuticle of the epidermis covering the cavity, which was not attacked by the enzymatic treatment. The cuticular composition of the outermost layer is consistent with the results of de Roissart et al. (1994) who gave microspectrometrical evidence for a cutinic composition of the surface of the 'algal packets'. What remains to be explained is the electron transparent layer situated in between the cuticle and the envelope. We suggest that it is artifactual, resulting from the fixation process which could have provoked the detachment of the two chemically distinct layers. This view is substantiated by reports (e.g. Goldberg and Escaig-Haye, 1986; Kadoya et al., 1997) on an analogous situation: the *lamina lucida* of the basement membrane of animal tissues. It has been demonstrated that this structure, characterized as an electron transparent layer between two electron opaque layers when conventional aldehyde fixation was used, was no longer visible after cryofixation or microwave-fixation that better conserve original structures and is therefore accepted as being artifactual.

Finally we noted that the *Anabaena* and the transfer trichomes are not evenly distributed within the cavity. Our LTSEM observations of an area of the cavity epidermis devoid of *Anabaena* and trichomes confirm those of Calvert and Peters (1981) except for its location which, according to these authors, is the lower distal part of the cavity whereas in our study it is consistently close to the leaf axil. Whatever its position, it appears that the presence of *Anabaena* is intimately linked to the trichomes and is probably related to the metabolic exchanges between the two partners of the symbiosis.

In conclusion our study of the pore strongly suggests a defensive and regulatory role in the *Azolla-Anabaena* symbiosis. Further studies need to focus on the ultrastructural modifications of the teat-cells during their differentiation and the relationship between the projections and the underlying cytoplasmic area. An insight into the physiological significance of these projections should be gained by investigating their composition and the factors affecting their formation.

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