ORIGINAL ARTICLE

Antheridial dehiscence in ferns

S. Muccifora · L. M. Bellani

Abstract We investigated the mechanism of antheridial dehiscence in ferns for the first time using fluorescence microscopy as well as scanning and transmission electron microscopy. The mechanism leading to antheridial dehiscence in *Polystichum setiferum*, *Asplenium trichomanes* and *A. onopteris* was found to depend on the different cellulose contents of the inner and outer walls of the ring cells detected with calcofluor white stain and the Thiéry test. The extremely low cellulose content of the ring cell walls facing spermatozoids made them less mechanically resilient than external wall cells. When the ring cells absorbed water they expanded only into the antheridial cavity, pushing the gametes against the cap cell, which detached from the ring cell below and enabled spermatozoid release. The newly released spermatozoids were spherical bodies covered in cellulose fibrils. The significance of cellulose fibrils could be to isolate the gametes from each other, to reinforce the electron transparent material and to protect the gamete from pressure created by the ring cells during release.

Keywords *Polystichum setiferum* ·
Asplenium trichomanes · *Asplenium onopteris* ·
Antheridial dehiscence · Sperm release ·
Fluorescence microscopy · TEM

Introduction

Polystichum setiferum (Forssk.) T. Moore ex Woynar (Dryopteridaceae), *Asplenium trichomanes* (L.) and

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A. onopteris (L.) (Aspleniaceae) are homosporous ferns that are common in several European countries (Tutin et al. 1993; Smith et al. 2006). They are often found in hedges, fissures of cliff faces and steep stream banks (Ferrarini et al. 1986).

The spermatogenesis and mature spermatozoids of *A. trichomanes* (Muccifora et al. 1996; Gori et al. 1997) and the mature spermatozoids of *A. onopteris* (Muccifora and Gori 2005) have been the subject of previous studies. The literature on the mechanism by which spermatozoids are released from the antheridia of ferns is mostly old material limited to the opening modes of the cap cell in a few species, i.e. complete detachment or detachment of only one side of the cap cell, or limited laceration of the cap cell (Hartman 1931; Atkinson and Stokey 1973; Elmore and Adams 1976; Nester 1985; Tigerschiöld 1989, 2008; Kotenko 1990; Muccifora et al. 2000; Muñiz-Díaz de Leon et al. 2008).

In this study we examined the mechanisms causing antheridial dehiscence in *P. setiferum*, *A. trichomanes* and *A. onopteris* by light microscopy and electron microscopy (transmission and scanning electron microscopy, TEM and SEM) to obtain an insight into this relatively neglected topic.

Materials and methods

Gametophyte cultures

P. setiferum (Forssk.) T. Moore ex Woynar, *A. trichomanes* (L.) and *A. onopteris* (L.) spores were collected near Siena (Italy) in spring 2008. They were sterilized, sown and cultured as previously described (Gori et al. 1997).

Transmission electron microscopy

Mature gametophytes were fixed in Karnovsky solution and osmium tetroxide, dehydrated and embedded in a mixture of Epon 812-Araldite A/M as previously described (Gori et al. 1997). Thin sections were stained with uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963). Polysaccharides (1,4-linked polysaccharide) were detected by the periodic acid-thiocarbohydrazide-silver proteinate method (Thiéry 1967; Muccifora et al. 2000). The thio-carbohydrazide treatments lasted 30–40 min. Sections were viewed in a Philips CM 10 electron microscope at 80 kV.

Light and fluorescent microscopy

Mature gametophytes were frozen and sectioned using an AMES LAB-TEK cryostat. Sections of about 5 μm thickness were stained with calcofluor white M2R (Hughes and McCully 1975; Ruzin 1999) for β-linked glucans, double-stained with aniline blue (O'Brien and McCully 1981) for callose and 4′,6-diamidino-2-phenylindole (DAPI) for nuclei, and double-stained with calcofluor white M2R and DAPI.

Mature gametophytes were placed on glass slides and soaked with distilled water (pH 7.0) to induce spermatozoid release.

Observations were made using a Leica DM MB light microscope equipped for fluorescence with an excitation filter at 330–380 nm and a barrier filter at 400 nm.

Scanning electron microscopy

Mature gametophytes were floated on drops of distilled water on coverslips coated with polylysine (poly-L-lysine hydrobromide MW 150,000–300,000 Da; Sigma). After 3, 5, 10 and 15 min the coverslips bearing the gametophytes were fixed for 1 h in Karnovsky solution, washed in 0.1 M cacodylate buffer (pH 7.2), dehydrated, critical point dried, coated with gold and viewed in a Philips XL 20 scanning electron microscope.

Results

The antheridia of *P. setiferum* (Forssk.) T. Moore ex Woynar, *A. trichomanes* (L.) and *A. onopteris* (L.) were spherical bodies with diameters up to 33 μm (Fig. 1a). They consisted of three sterile jacket cells (a cap cell and two ring cells) that formed the container for 32 tightly packed spermatozoids (Fig. 1a, b). When viewed by TEM, the sterile cells showed a few cytoplasmic organelles and

large vacuoles (Fig. 1b); the vacuole content of the ring cells was more osmiophilic than that of the cap cell (Fig. 1b). The part of their walls facing the antheridial cavity was thinner and more electron-transparent than the part facing outward (Fig. 1b). Moreover, the inner part of the cap cell wall adjacent to the spermatozoids was the thinnest of all and poorly structured (Fig. 1b). Mature unreleased spermatozoids of all three species were embedded in electron-transparent material deposited in the first stages of spermatogenesis, before the start of cellular morphogenesis, when nuclei and protoplasts had round profiles (Fig. 1b, c). This material was surrounded by an electron-opaque line, presumably the original spermato-cyte wall (Fig. 1b). In the antheridial cavity spermatozoids were embedded in weakly electron-opaque material (Fig. 1b).

The antheridia of all three species were double-stained with aniline blue and DAPI at different stages of sper-matogenesis and viewed under a fluorescence microscope (Fig. 1d, e). The fluorescence of aniline blue was evident between spermatozoids, corresponding to the weakly electron-opaque material (Fig. 1d, e). DAPI showed the nuclear form and the degree of condensation of chromatin indicating the stage of spermatogenesis. The fluorescence of aniline blue decreased as spermatogenesis proceeded, vanishing once the nucleus was coiled, as in the mature gamete (data not shown). Mature antheridia stained with calcofluor white or double-stained with calcofluor white and DAPI showed thin fluorescent lines around the sper-matozoids and a strong fluorescent line on the external edge of the antheridium (Fig. 1f, g); the inner wall part of antheridial cells was weakly visible (Fig. 1f).

After the Thiéry test involving treatment for 30–40 min with thiocarbohydrazine (TCH), metallic silver precipitate strongly marked spermatozoid walls around electron-transparent material and the external edge of the antheridia (Fig. 2a, b). Antheridial cell walls facing spermatozoids were weakly marked by metallic silver precipitate and no precipitate was observed on the inner part of the cap cell walls adjacent to spermatozoids (Fig. 2a, b).

Mature antheridia stained with ruthenium red showed a red line on the inner walls of the antheridial cells and between spermatozoids coinciding with the spermatozoid walls (data not shown).

Antheridial dehiscence occurred when mature antheri-dia contacted a water film (Fig. 2c–f). The sterile jacket cells absorbed water and expanded into the antheridial cavity, pushing the spermatozoid mass against the cap cell and detaching it from the ring cell completely or on one side (Fig. 2e, f). The gametes came out one at a time and remained motionless on the outer surface of the anther-idium for some minutes (Fig. 2d). In fresh specimens they were enveloped in translucent material (Fig. 2d, e). SEM

Fig. 1 a Light micrograph showing an antheridium. **b, c** Transmission electron micrographs showing (**b**) a mature antheridium and (**c**) an antheridium in the first stages of spermatogenesis. An electron opaque line (*arrows*) surrounds electron-transparent material (*asterisks*) embedding the spermatozoids. *Stars* indicate weakly electron-opaque material. **d, e** Antheridia double-stained with DAPI and aniline blue; note (*c*) callose fluorescence between the spermatozoids (*arrows*). **f** Mature antheridium stained with calcofluor white showing a strong fluorescent line on the external edge of the antheridium (*arrow*) and a thin line around the spermatozoids (*arrowheads*). Note the inner wall of antheridial cells (*double arrows*). **g** Spermatozoid stained with DAPI and calcofluor white showing a coiled nucleus and fluorescent line around the spermatozoid (*arrowhead*). *c* callose, *cc* cap cell, *rc* ring cell, *s* spermatozoids, *n* nucleus, *v* vacuole; *scale bars* **a** 5 μm, **b–g** 2 μm
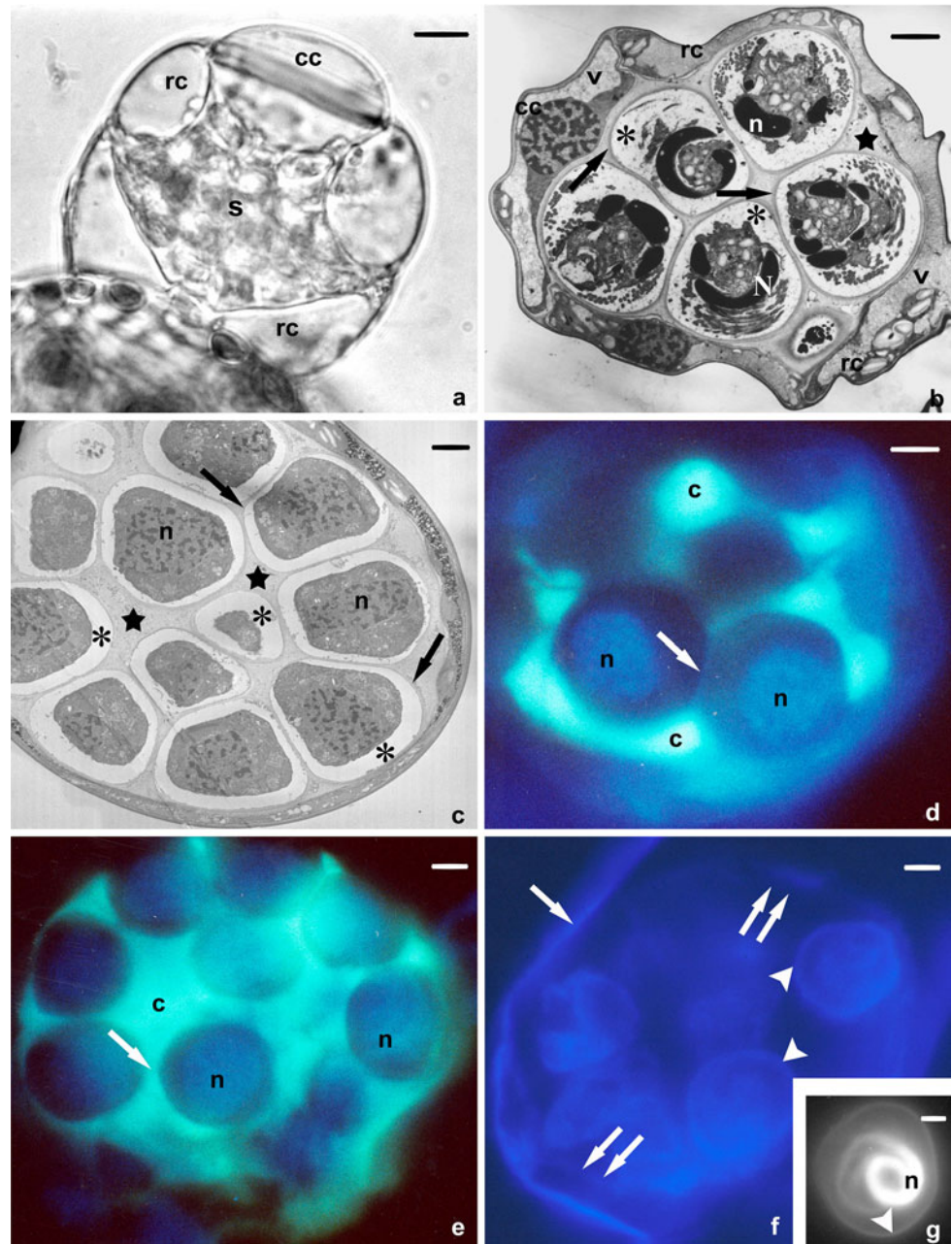

showed that newly released spermatozoids were spherical bodies about 6–7 μm in diameter coated with fibrillar material (Fig. 2g) or filled with wrinkled material (Fig. 2i), some appearing partially fibrillar and partially wrinkled (Fig. 2g, h). After remaining immobile for some minutes, they suddenly lost their translucent envelope and began to swim. At the end of spermatozoid release, the empty antheridia did not show any change in size but the ring cells, especially the lower one, were swollen and occluded the antheridial cavity (Fig. 2e, f). Figure 3 shows the steps of antheridium opening and spermatozoid release.

Discussion

The electron-transparent material embedding the spermatozoids of *P. setiferum* (Forssk.) T. Moore ex Woynar, was similar to that of *A. trichomanes* (L.) (Gori et al. 1997) and *A. onopteris* (L.) (Muccifora and Gori 2005), and to that surrounding all fern spermatozoids so far described (Kotenko 1990; Muccifora et al. 2000). Its nature has not yet been determined. The absence of calcofluor white fluorescence of this material excludes the presence of hemicellulose, as suggested by Cave and Bell (1973). Nor is this material stained by ruthenium red (data not shown)

Fig. 2 a, **b** Sections of antheridia and spermatozoids after Thiéry's test. Metallic silver precipitate strongly marks parts of the outer wall of sterile jacket cells (*double arrows*) and weakly marks parts of the inner wall (*arrowheads*); the inner wall of the cap cell is devoid of metallic silver precipitate (*arrow*). Metallic silver precipitate marks the wall around electron-transparent material (*double arrowheads*). **c–f** Light microscope sequence of spermatozoid release. The spermatozoids (*arrows*) came out one at a time and remain motionless on the outer surface of the antheridium. Note the lower ring cell occluding the cavity of the empty antheridium. **g–i** Scanning electron micrographs of newly released spermatozoids coated with fibrillar material (*arrows*) or full of wrinkled material (*arrowheads*) or showing partially fibrillar and partially wrinkled material. *a* starch grain, *cc* cap cell, *rc* ring cell, *n* nucleus; scale bars **a** 2 μm, **b**, **g**, **i** 1 μm, **c–f** 5 μm, **h** 0.5 μm
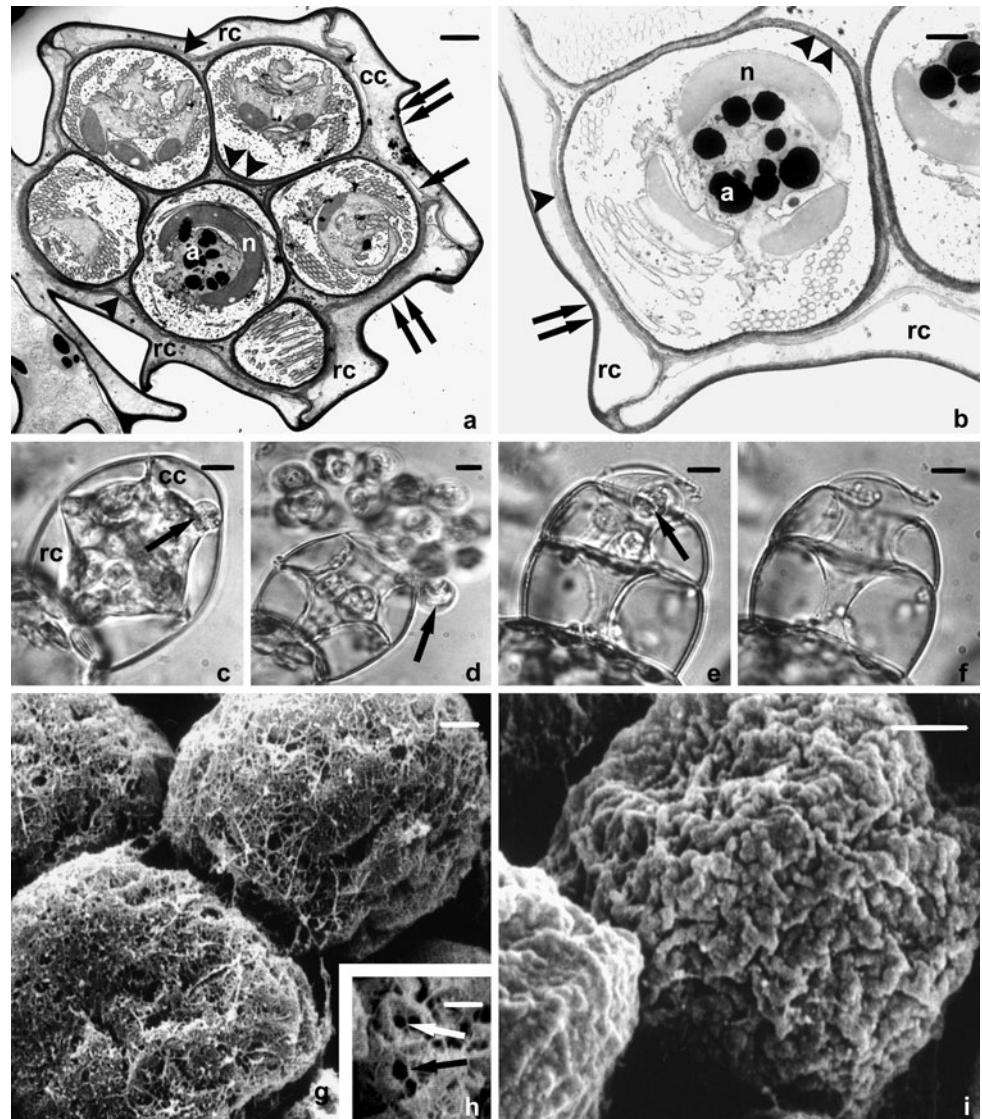

which excludes the presence of pectins. It is reasonable to suppose that the material is a polysaccharide with a gel consistency (Cave and Bell 1973; Muccifora et al. 2000) that adapts readily to the shape of the gametes and to growth of the flagella (Muccifora et al. 2000).

The striking fluorescence of aniline blue indicated that the weakly electron-opaque material embedding spermatozoids in the antheridial cavity of all three species was callose, as observed in *Ceratopteris thalictroides* (Cave and Bell 1973). The colocation of callose around fern spermatocytes recalls the callose deposited around microsporocytes in Angiosperms during pollen development (Pacini 2010; Gori 1982; Gori and Ferri 1982). The formation of a callose wall during microsporogenesis is believed to be an event necessary to isolate archesporial tissue from somatic tissue (Heslop-Harrison 1966), to protect the sporocytes from the effects of hormones in the surrounding tissues (Godwin 1968), to maintain cell

autonomy and to isolate the sporocytes from the influence of adjoining cells (Teng et al. 2005). All these aspects justify the presence of callose around fern spermatocytes. The decreasing fluorescence of aniline blue during spermatogenesis, reaching zero in mature spermatozoids, and the absence of fluorescence of calcofluor white in mature antheridia suggests that the weakly electron-opaque material is reabsorbed or newly elaborated, like the callose deposited around microspores (Pacini 2010; Gori and Ferri 1982; Teng et al. 2005).

The mechanism that allows antheridial dehiscence in *P. setiferum, A. trichomanes* and *A. onopteris* probably depends on the different polysaccharide compositions of the inner and external walls of the ring cells, as observed in *Phyllitis scolopendrium* (Muccifora et al. 2000). The fluorescent line around spermatozoids following staining with calcofluor white is a reliable indicator of *β*-linked glucans. Moreover, after 30–40 min of treatment with
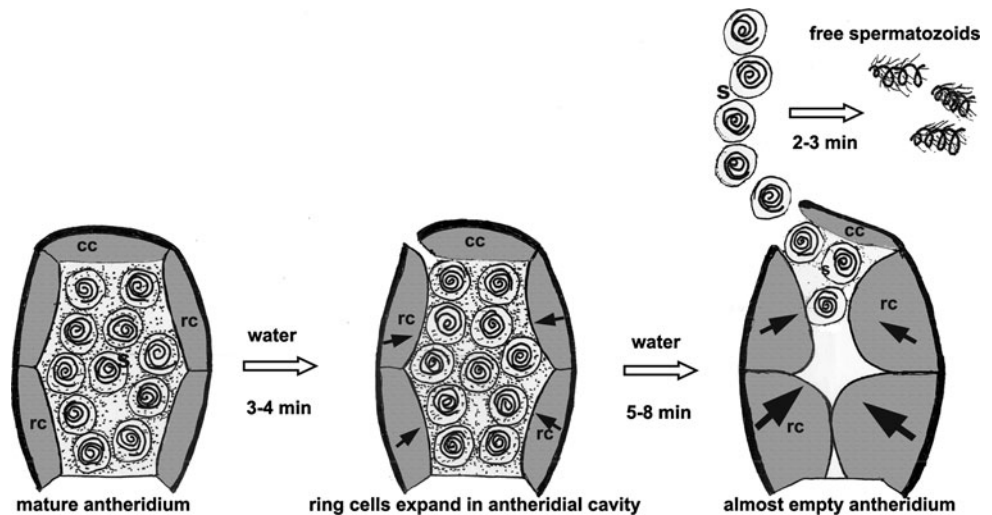
TCH, metallic silver precipitate marks stable thiocarbo-hydrazones formed after oxidation of 1,2-glycols, such as those in 1,4-linked polysaccharide (Thiéry 1967; Muccifora et al. 2000). This indicates that the external walls of the ring cells have a high cellulose content. The low cellulose content in the parts of the ring cell walls facing spermatozoids would make them less mechanically resilient, permitting massive swelling of the ring cells into the antheridial cavity. The thinness and weak structure of the inner wall of the cap cell presumably enables detach-ment or lifting of the cap cell and release of spermatozoids.

Hartman (1931) postulated an active role of antheridial cell walls and their different polysaccharide compositions in dehiscence in Polypodiaceae ferns but considered the primary cause of release to be swelling of the spermatog-enous mass and not the pressure of enlarging ring cells on the mass of spermatozoids, as observed in the present study in *P. setiferum, A. trichomanes* and *A. onopteris* and pre-viously in *P. scolopendrium* (Muccifora et al. 2000).

The fibrillar material enveloping the newly released spermatozoids observed by SEM is doubtless the cellulose of the original spermatocyte wall, as shown by calcofluor and fluorescent microscopy and by the Thiéry test and TEM. The cellulose wall presumably breaks down since no debris was evident. Degradation of the cellulose wall gradually reveals wrinkled underlying material, which was presumably the electron-transparent material; this dissolves rapidly releasing the gametes. The cellulose wall isolates the gametes from each other, reinforces the electron-transparent material and protects the gamete from the pressure created by the ring cell during release. The pres-ence of a wall with a high cellulose content around the spermatozoids excludes the possibility that the force causing antheridial dehiscence comes from swelling of spermatogenic cells after absorption of water, as observed

in some Polypodiaceae (Hartman 1931), certain Thelypte-ridaceae (Tigerschiöld 1989) and *Onoclea sensibilis* (Kotenko 1990), and provides support for the idea that the gametes are released due to pressure exerted on sperma-tozoids by expanding ring cells.

In conclusion, it is noteworthy that the features of the antheridial wall, the spermatozoid coatings and the mech-anisms causing spermatozoid release are common to the three species considered in the present study and appear similar to those reported in *P. scolopendrium* (Asplenia-ceae) (Muccifora et al. 2000). This is the first study to successfully correlate ultrastructure and cytochemical tests in investigating the structural features of antheridia and their involvement in spermatozoid release; it provides a new insight into fern antheridial dehiscence.

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