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ORIGINAL ARTICLE

Abnormal spore morphology and wall ultrastructure in *Anemia tomentosa* var. *anthriscifolia* and *A. tomentosa* var. *tomentosa* (Anemiaceae)

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Abstract The spores of *Anemia tomentosa* var. *anthriscifolia* and *A. tomentosa* var. *tomentosa* were studied focusing the attention on their abnormalities. The study was based on fresh and herbarium material and the spores were examined with light, scanning and transmission electron microscopy. Normal, abnormal and abortive spores were observed in both taxa. The normal spores were trilete, triangular in polar view, and the ornamentation consisted of parallel ridges separated by narrow and smooth grooves. The spores were observed in monads, dyads, triads and tetrads. The abnormal spores were monolete, trilete, tetralete or alete with great variations in size. In fact, some spores were almost double the size of the normal ones. Some differences were also found in the ornamentation of the spores. Aborted and not completely developed spores were also observed in the specimens. The wall ultrastructure of the taxa was studied for the first time. The exospore was two-layered with numerous cavities inside its structure, and the perispore was also two-layered. The results revealed that the sporoderm ultrastructure of both normal and abnormal spores of the taxa analyzed was very similar.

Keywords *Anemia* · Anemiaceae · Spores · Abnormalities · Morphology · Ultrastructure

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Introduction

The genus *Anemia* Sw. is the only one of the family Anemiaceae, and includes about 120 species. Most of them occur in Latin America, ten in Africa and one in southern India. The highest concentration of species is in Brazil and the second highest in Mexico (Mickel and Smith 2004; Smith et al. 2006).

Anemia is characterized by having rhizomes with dark or orange hairs, its fronds are hemidimorphic, rarely wholly dimorphic, and sporangia with subapical annulus restricted to the erect, lowermost pair of pinnae (Mickel and Smith 2004).

Anemia tomentosa (Savigny) Sw. has three varieties: *A. tomentosa* (Savigny) Sw. var. *anthriscifolia* (Schrad.) Mickel, *A. tomentosa* (Savigny) Sw. var. *mexicana* (C. Presl) Mickel and *A. tomentosa* var. *tomentosa*.

The species have a disjunt distribution: *A. tomentosa* var. *mexicana* grows in Mexico, Colombia and Venezuela. *A. tomentosa* var. *anthriscifolia* occurs in Brazil, Bolivia, Paraguay and Argentina. *A. tomentosa* var. *tomentosa* grows in Brazil, Paraguay, Uruguay and Argentina. Of the several Latin American localities, Argentina corresponds to the southern limit in the distribution of the genus.

The spores of these taxa were studied by Hill (1977); he analysed material from Brazil with scanning electron microscopy (SEM).

In a monographic study of *Anemia* subg. *Coptophyllum*, Mickel (1962) studied the different spores of *A. tomentosa* by light microscopy (LM). In *A. tomentosa* var. *anthriscifolia* and *A. tomentosa* var. *tomentosa*, the author observed variations in spore size, the presence of large spheres accompanied by many abortive spores as well as miscellaneous particles. Moreover, *A. tomentosa* var. *mexicana* was reported as having regular and uniform spores.

De la Sota and Mickel (1968) mentioned the presence of spores with irregular shape and variable dimensions, frequently aborted in *A. tomentosa* var. *tomentosa* and *A. tomentosa* var. *anthriscifolia*.

Tryon and Tryon (1982) and Tryon and Lugardon (1991) carried out several palynological studies on some *Anemia* taxa with SEM and transmission electron microscopy (TEM). Nevertheless, none of these authors mentioned the presence of abnormal spores.

In later works, some abnormalities like aborted, immature or spores packed in tetrads were reported in both taxa by Ramos Giacosa et al. (2012).

The presence of abnormal and abortive spores was also reported in other genera of ferns. Wagner and Chen (1965) mentioned abortive spores as a detection of hybrids in *Dryopteris*. In the same genus, Quintanilla and Escudero (2006) studied spore abortion in relation to polyploidy and spore germination. Morbelli (1974) reported the presence of some spore abnormalities in *Blechnum* hybrids. Hickok and Klekowski (1973) mentioned cases of spore abortion in *Ceratopteris* hybrids, while Pryer and Britton (1983) dealt with this same topic in *Gymnocarpium*, and Nakato et al. (2012) in *Thelypteris*.

As for *Anemia*, there are no detailed studies regarding spore abnormalities yet, and therefore, many aspects of their nature are still unknown. So far, analysis of these spores with SEM has not been carried out and the wall ultrastructure of these abnormal spores is completely unknown.

The aim of this study is to analyze the spores of *A. tomentosa* var. *antrhiscifolia* and *A. tomentosa* var. *tomentosa* with LM, SEM and TEM to reveal the variations and abnormalities of the spores within these taxa, and to establish if these variations are also present at ultrastructural level.

Materials and methods

Spores were collected from plants growing in the field and also obtained from herbarium specimens from the following Institutions: Instituto Miguel Lillo, Tucumán (LIL), División Plantas Vasculares, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata (LP) and Instituto de Botánica Darwinion (SI).

The spores were studied using LM, SEM and TEM. For LM, the spores were treated with hot 3 % sodium carbonate for 2 min in order to preserve the perispore (Morbelli 1980) and acetolyzed according to the method of Erdtman (1960).

For SEM, the material without treatment was transferred to acetate plates and after drying they were coated with gold.

For studies with TEM, normal spores and alete with spherical shape abnormal spores were analyzed. The material was hydrated following the technique by Rowley and Nilsson (1972), using phosphate buffer and alcian blue (AB). Then, the material was fixed with Glutaraldehyde + 1 % Alcian Blue in phosphate buffer for 12 h and post-fixed with 1 % OsO4 in water plus 1 % Alcian Blue. The spores were dehydrated in an acetone series and then embedded in spurr soft mixture. 3 µm thick sections were stained with toluidine blue and observed with LM. Ultrathin sections were stained with 1 % uranyl acetate for 15 min followed by lead citrate for 3 min.

The observations were made with an Olympus BH2, a JEOL JSMT-100 SEM, and a Zeiss T-109 TEM.

Results

Morphology of normal spores

In both taxa, the spores are trilete, triangular, with convex sides in polar view and prominent angles. In equatorial view, the proximal face is convex and the distal face is hemispheric (Figs. 1, 2).

In *A. tomentosa* var. *anthriscifolia*, the equatorial diameter is 63–86 µm, and the polar diameter is 53–75 µm. The laesurae are 31–41 µm long. In *A. tomentosa* var. *tomentosa*, the equatorial diameter is 77–115 µm, and the polar diameter is 72–108 µm. The laesurae are 35–51 µm long.

The ornamentation of the spores in both taxa consists of parallel ridges 3–7.5 µm wide, which are separated by narrow and smooth grooves of 0.8–2.5 µm width. The ridges are fused near the angles of the spores and they form prominent and thickened angles (Figs. 1c, 2c). Spines of 0.7–2.3 height can be observed on the ridge surfaces. They are frequently ramified and have acute or rounded apices. Abundant perforations of variable sizes are also present on the perispore surface (Figs. 1d, 2d).

The laesurae have the same ornamentation as the ridge surfaces (Figs. 1a, 2a). A few globules are seen on the perispore surface (Fig. 1b, d).

Morphology of abnormal spores

In *A. tomentosa* var. *anthriscifolia* and *A. tomentosa* var. *tomentosa*, there are several abnormalities in the spores, as well as differences in their size and shape.

Spores packed in dyads (Figs. 3e, 4d), triads (Fig. 3f) and tetrads (Fig. 4e, f) were found in the material analyzed. A few of them seem to be normal in size and shape, but generally have some alterations such as variations in size in

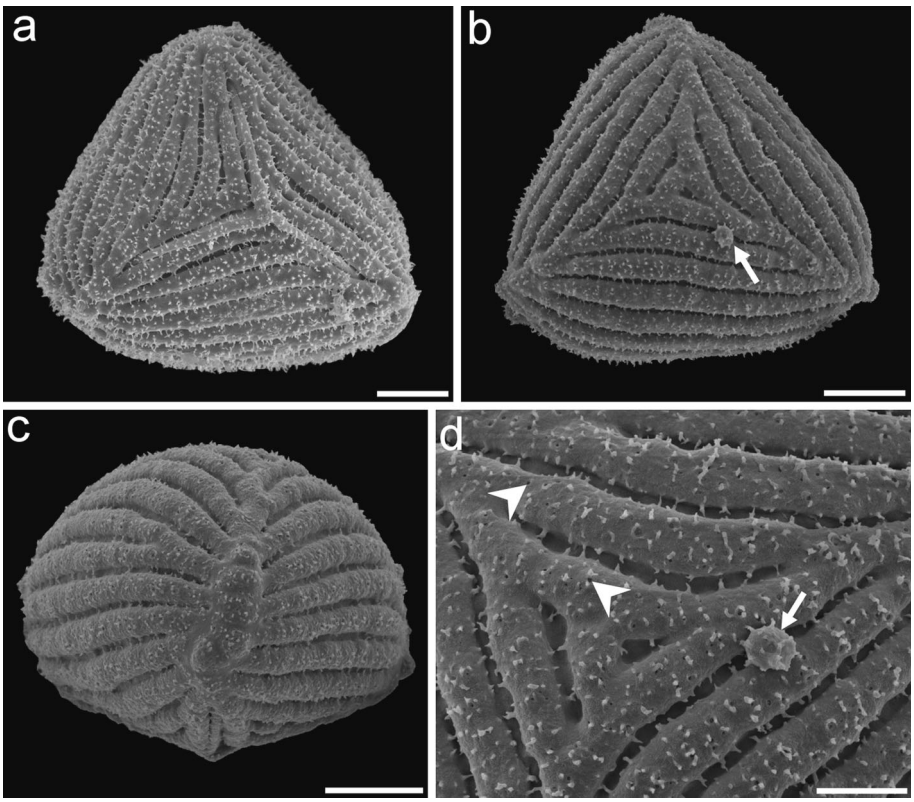

Fig. 1 Normal spores of *Anemia tomentosa* var. *anthriscifolia* with SEM. **a** Proximal view of a triangular spore with convex sides. **b** Distal view of a spore. The ornamentation is composed of wide parallel ridges separated by narrow and smooth grooves. A globule is observed on the spore surface (*arrow*). **c** Equatorial view. The ridges are fused near the angles of the spores and they form prominent and thickened angles. **d** Detail of the perispore surface. On the ridges surface, the spines have acute or rounded apices. Abundant perforations (*arrowheads*) are also evident. A globule (*arrow*) has the same ornamentation as the perispore. *Scale bars* **a**–**c**: 20 μm, **d**: 10 μm

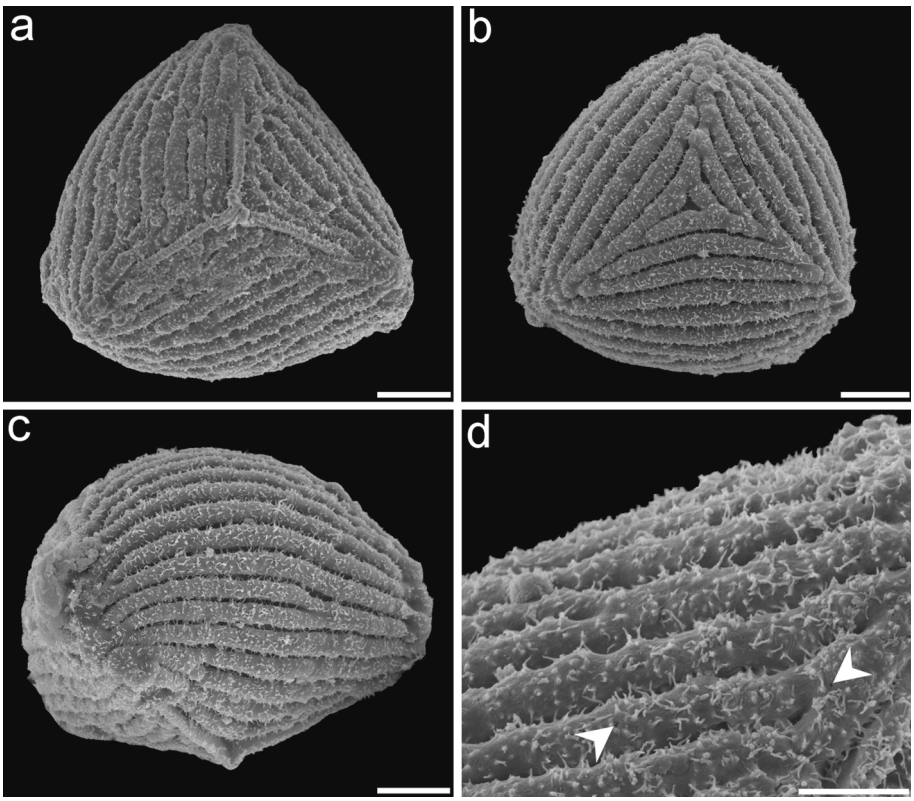

Fig. 2 Normal spores of *Anemia tomentosa* var. *tomentosa* with SEM. **a** Proximal view of a triangular spore with convex sides. **b** Distal view of a spore. The ornamentation is composed of wide parallel ridges separated by narrow and smooth grooves. **c** Equatorial view. The proximal face is convex and the distal face is hemispheric. **d** Detail of the perispore. The ridges surfaces are densely covered by spines. They have acute or rounded apices and could be ramified. Some perforations are also visible (*arrowheads*). *Scale bars* **a**–**c**: 20 μm, **d**: 10 μm

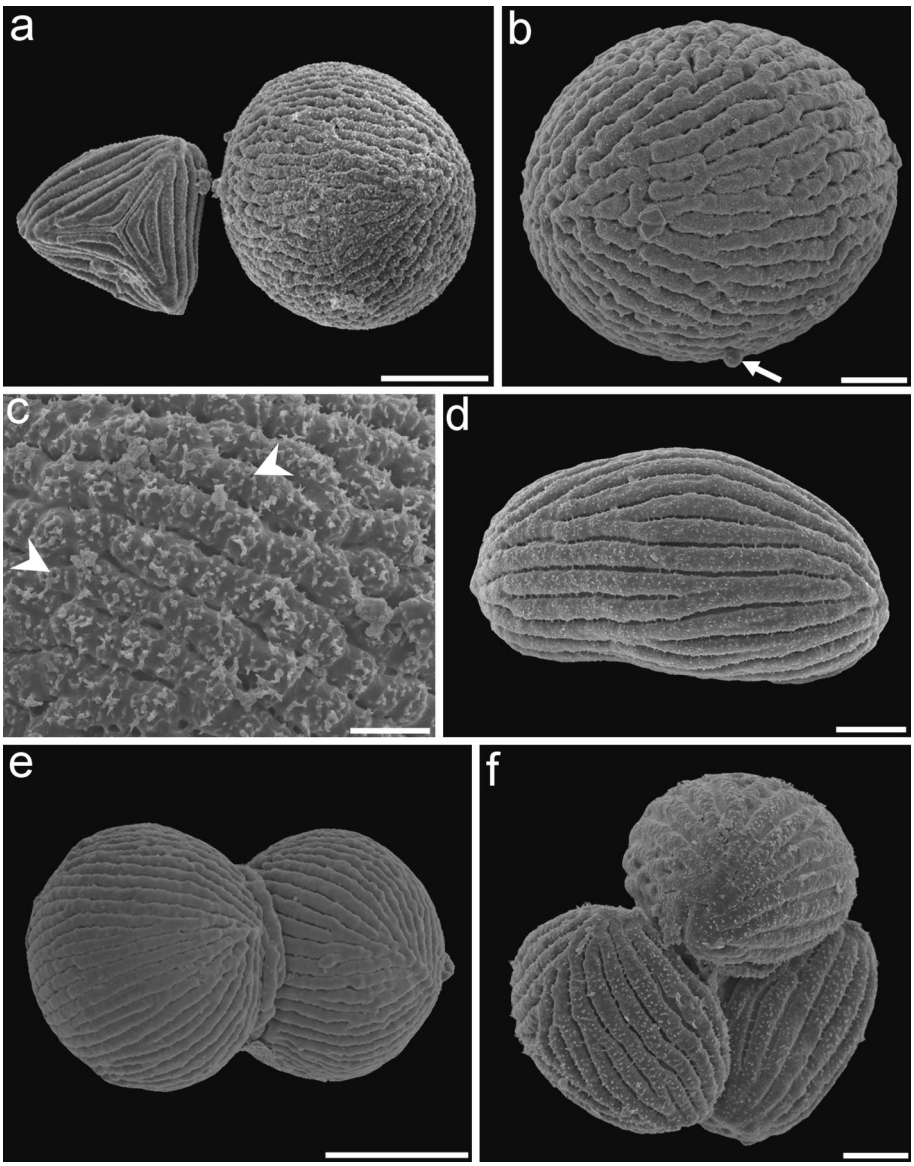

Fig. 3 Abnormal spores of *Anemia tomentosa* var. *anthriscifolia* with SEM. **a** A normal trilete spore (left) with a big alete spore with a spherical shape (right). **b** Big alete spore. The exospore is composed of parallel ridges frequently fused or dichotomized. A globule is observed on the surface (*arrow*). **c** Detail of the alete spore ornamentation. Spines of variable sizes and perforations (*arrowheads*) of the perispore are observed. **d** Monolete spore with bifurcated ridges. **e** Dyad. The spores are strongly attached to each other. The ridges are smooth and have no spines. **f** Triad. The ornamentation of the spores is similar to that of the normal ones. *Scale bars* **a**: 50 μm, **b**, **d**, **f**: 20 μm, **c**: 10 μm **e**: 50 μm

all the spores that constitute the tetrad (Fig. 4f), or one spore of the tetrad being bigger than the others or aborted (Fig. 4e).

In these groups of spores, the ornamentation is the same as in the normal ones, but smooth ridges without spines were also observed (Fig. 3e).

In both taxa, monolete spores were noticed (Fig. 3d). These spores have a similar ornamentation to that of the normal ones consisting of parallel ridges separated by narrow and smooth grooves. The ridges are often bifurcated and their surfaces are covered by spines.

Other abnormal spores frequently found in these specimens have a spherical shape and are larger than the others (Figs. 3a, b, 4a). They are 114–155 μm in diameter and they lack the laesurae (alete spores). These spores have a variable exospore and perispore ornamentation. The exospore is composed of ridges that may be parallel or present an irregular shape, frequently fused with one another or dichotomized. In some cases, the ridges are shorter and more fused, forming an irregular ornamentation. The perispore is also variable since spines of different sizes and densities are observed. As it occurs with normal spores, abundant perforations are visible on the perispore surface (Figs. 3c, 4b).

Many abortive spores are usually observed. They are irregular in shape, not completely developed, and some of them are darker or colorless. They can be found together forming an irregular mass of spores.

In *A. tomentosa* var. *tomentosa*, a few tetralete spores are also identified. They have a rectangular shape in polar view and a similar ornamentation to the trilete spores (Fig. 4c).

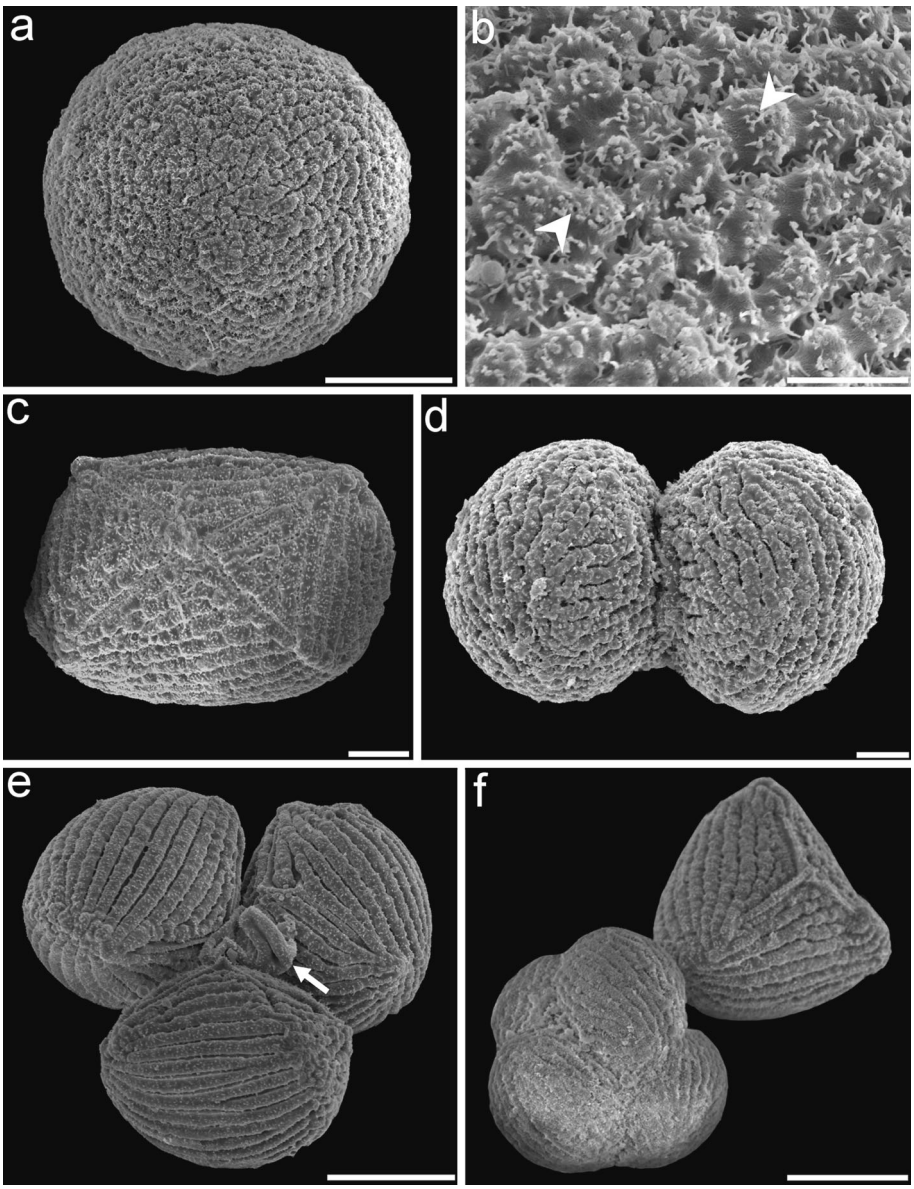

Fig. 4 Abnormal spores of *Anemia tomentosa* var. *tomentosa* with SEM. **a** Big alete spore **b**. Detail of the ornamentation. The ridges are short, with an irregular shape and frequently fused. On the ridge surfaces, spines of variable sizes and shapes are observed. Abundant perforations (*arrowheads*) are seen. **c** Tetralete spore with rectangular shape. **d** Dyad. The spores have a similar ornamentation to the normal spores of the taxa. **e** Tetrad with one, aborted spore (*arrow*). **f** Normal spore and a tetrad of spore. *Scale bars* **a**, **e**, **f**: 50 μm, **b**: 10 μm, **c**, **d**: 20 μm

Ultrastructure

The normal and abnormal spores of both taxa have a 2.4–6.2 μm thick exospore composing of two layers: an inner layer of 1.2–1.4 μm thickness and an outer 1.2–5 μm thick layer that forms the ridges. The outer layer of the exospore has numerous cavities of irregular shape and size. These cavities are especially abundant in the central part of the ridges and form a boundary between the two layers of the exospore (Fig. 5a, b, d).

Radial channels with a dark content are mainly observed in the inner exospore layer (Fig. 5d).

The perispore measures 2.4–7 μm thick and is composed of two layers: P1 and P2. The inner layer (P1) is 0.1–0.3 μm thick and has three strata. An inner stratum which measures 40–70 nm thick is strongly adhered to the exospore. The 60–200 nm thick middle stratum is made up of small portions of membranes (scales) which are variable in size and density depending on the area of the spore analyzed. The outer stratum is 30–50 nm thick and sometimes difficult to differentiate (Fig. 5c, e).

The outer layer (P2) is 0.1–0.6 μm thick and less contrasted than P1. This layer forms the surface ornamentation of the spore constituted of spines with different sizes (Fig. 5c, e).

Scarce globules with an inner portion which has the same structure and contrast as the exospore, and an outer layer with the same structure as the perispore, can be observed on the perispore surface (Fig. 5f, g).

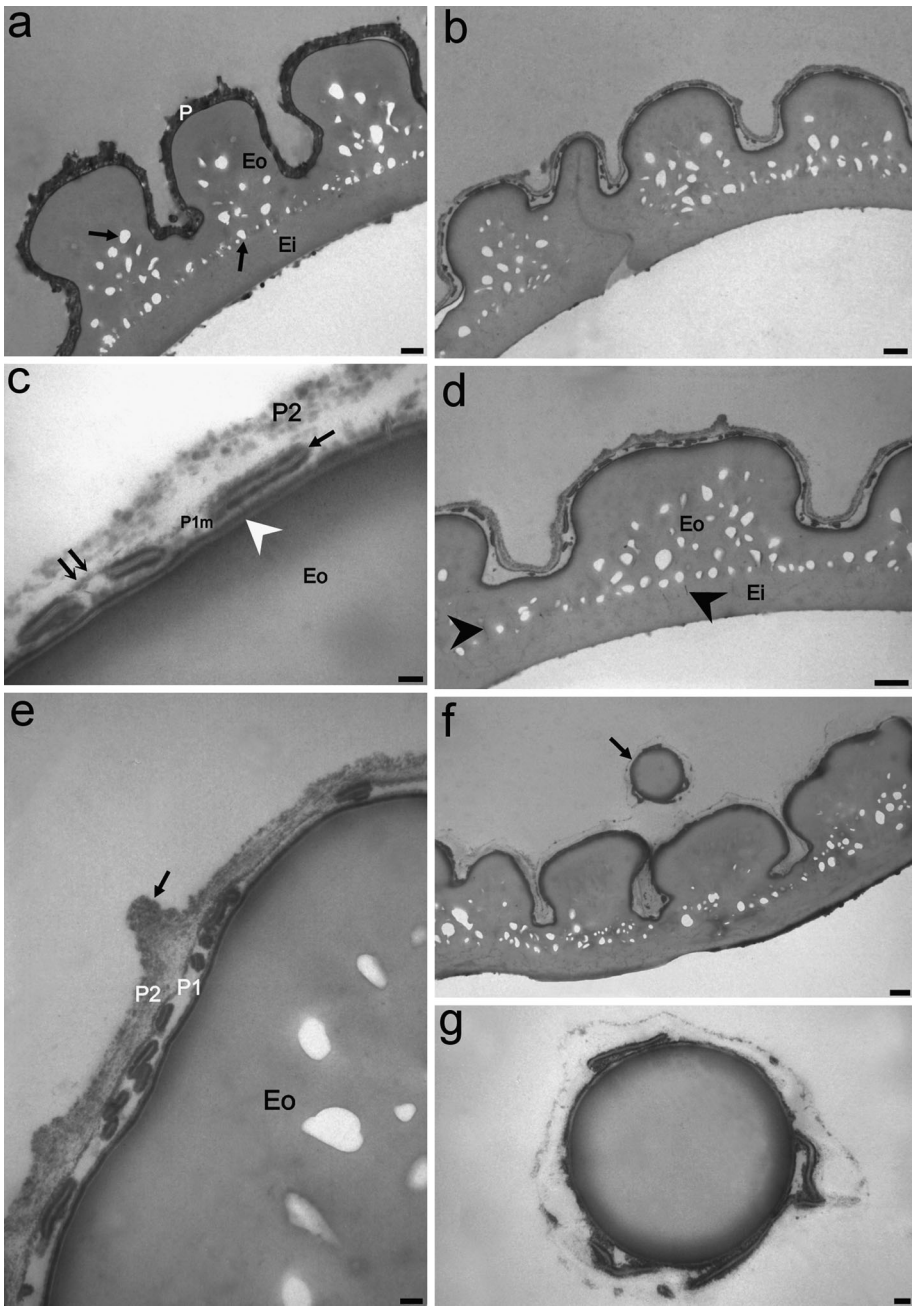

Fig. 5 Spores of *Anemia tomentosa* var. *anthriscifolia* and *A. tomentosa* var. *tomentosa* with TEM. **a** Normal spore of *A. tomentosa* var. *tomentosa*. The exospore is composed of two layers: an inner layer (Ei) and an outer layer (Eo) with numerous cavities (*arrows*). The perispore (P) is more contrasted than the exospore. **b**, **c** Normal spores of *A. tomentosa var. anthriscifolia*. **b** Section through a laesura. The cavities of the outer exospore layer are significantly abundant in the central part of the ridges. **c** The perispore is two-layered: P1 and P2. The inner layer (P1) has three strata: the inner stratum (*arrowhead*) is strongly adhered to the exospore. The middle stratum (P1 m) is composed of small portions of scales (*arrow*) and the outer stratum is thin and, at times, difficult to differentiate (*double arrow*). P2 layer forms the perispore ornamentation. **d-g** Abnormal spores of *A. tomentosa* var. *anthriscifolia*. **d**, **e** The exospore is two-layered: Ei and Eo. Radial channels with dark content are observed (*arrowheads*). The perispore is two-layered (P1 and P2) with three strata like the normal spores. P2 layer forms the spines (*arrow*) of the perispore. **f** A globule (*arrow*) is observed, as it can be seen in the surface of the spore in Fig. 3b. Its centre has a similar structure and contrast to the exospore. **g** Detail of the globule. Several scales of the perispore P1 layer are visible and distributed tangentially. *Scale bars* **a**, **b**, **d**, **f** 1 μm, **c** 100 nm, **e**, **g** 0.2 μm

Discussion and conclusion

The present work reveals some important details of the morphological variations and ultrastructural features of the spores of *A. tomentosa* var. *anthriscifolia* and *A. tomentosa* var. *tomentosa*.

Inside the sporangia of *A. tomentosa* var. *anthriscifolia* and *A. tomentosa* var. *tomentosa*, normal and a variety of abnormal spores are formed. The abnormalities include the presence of monolete, tetralete and alete spores with great variations in size. In addition, aborted and not completely developed spores are commonly found in the specimens analyzed.

The alete spores have a spherical shape and are the biggest, almost double the size of the normal spores. There are also significant variations in the exospore and perispore ornamentation. In a few spores, the ridges are parallel but, in most cases, they are arranged in a very irregular way. They are fused to one another forming a complex network and other spores are seen having short ridges fused and ramified.

Moreover, spines of variable sizes and densities are observed in the perispore ornamentation.

The monolete spores have a similar ornamentation to that of the normal spores; however, they frequently show bifurcated ridges. The tetralete spores are scarce and the ornamentation is also similar to the one in the normal spores.

The presence of big spores was also cited by Mickel (1962), who mentioned the presence of giant spheres, accompanied by many abortive spores and miscellaneous particles.

Another aspect to take into account is the recurrent presence of spores packed in dyads, triads and tetrads. Some tetrads seem to be normal spores packed together, but others vary in size, and at least one of the spores seems to be aborted.

The spores that form the dyads are strongly adhered to one another, and some of them appear to be less ornamented than the normal ones.

Until now, the sporoderm ultrastructure of these taxa was unknown. The ultrastructural analysis was focused on the normal and the big alete abnormal spores. Such studies had revealed that there was no difference in the stratification and structure of the walls. Besides, *A. tomentosa* var. *antrhiscifolia* and *A. tomentosa* var. *tomentosa* share the same sporoderm ultrastructure. They have a different two-layered exospore with a great number of cavities of varying sizes, situated inside the ridges, and just a few of them between the ridges. The perispore is two layered (P1 and P2) with three strata of different structure.

Comparing the ultrastructural results obtained in the present work with the previous analysis carried out by Ramos Giacosa et al. (2012) in species with normal and uniform spores like *Anemia australis* and *A. simplicior*, it is evident that the sporoderm ultrastructure of the four taxa is very similar in terms of structure and stratification of the walls.

According to Mickel (1962), the spore abnormalities and abortions observed in *A. tomentosa* var. *anthriscifolia* could be explained by the hexaploidy of this taxon. The origins could be by a tetraploid and a diploid. The resulting triploid would have been sterile and a hexaploid could have been produced by allopolyploidy. Besides, this taxon has an apogamic reproduction.

In addition, hybridization in the genus *Anemia* has been reported (Mickel 1982).

Cytological research in several angiosperms has demonstrated that some meiotic abnormalities play an important role in the formation of dyads, triads, and sterile pollen of different sizes (Kumar and Singhal 2011).

Moreover, when Hickok and Klekowski (1973) studied the meiosis of the aquatic fern *Ceratopteris* suggested that the dyad spores of this genus are produced by a single division of the spore mother cell, followed by cytokinesis. This single nonreductional chromosomal division of the mother cells, results in an unequal distribution of chromosomes to daughter nuclei.

Following the same criteria, in a cytogenetic analysis of *Thelypteris decursive-pinnata*, Nakato et al. (2012) mentioned that in a sporangium, dyads, triads, and tetrads are simultaneously formed by different processes of meiosis. Dyads are formed by the lack of the second division, triads are formed by the second division in one of the two daughter cells, and tetrads are formed by two divisions of the spore mother cells. Although most mature spores are aborted because of unbalanced chromosome constitutions, fertile reduced and unreduced spores are produced at a low frequency.

Wagner et al. (1986) noted that the circular and big spores found in some fern hybrids are unreduced spores and may be originated by interruptions of meiosis of the spore mother cells.

Therefore, the big alete spores found in the material of *A. tomentosa* var. *anthriscifolia* and *A. tomentosa* var. *tomentosa* might be unreduced spores.

As revealed by the above-mentioned works, cytological features are essential to understand spore abnormalities. Although there are numerous investigations in the cytology of plants, most of them are focused on flower plants and thus, more research in ferns is needed.

Further studies regarding spore germination, viability of the spores and gametophyte development would also be useful to establish if these abnormalities play a role in the life cycle and the origin of the polyploidy of these taxa.

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Appendix

Voucher specimens. *Anemia tomentosa* var. *anthriscifolia*: Argentina: Prov. Catamarca, Schunck 9706 (LIL); Prov. Jujuy, El Carmen, Dique La Ciénaga, *Cabrera* et al. *14089* (LP); Prov. Misiones, Cainguás, Puerto Rico, *Montes 3993* (LP); Prov. Salta, Cafayate, Santa Teresa, *Lourteig 1041* (LIL); Capital, La Lagunilla, *Saravia Toledo 2152* (LP); Prov. Tucumán, Vipos, *Dinelli 832* (SI). Bolivia: Depto. Tarija, ruta Tarija- Villa Montes, *Krapovickas 19093* (LP). Paraguay: Depto. Cordillera, Ruta 2 km. 50, *Krapovickas* et al. *12479* (LP). *Anemia tomentosa* var. *tomentosa*: Argentina: Prov. Buenos Aires, Tornquist, Sierra de la Ventana, Reserva Integral "La Blanqueada", *Proyecto Ventania 15* (LP); Tandil, Cerro de las Animas, *Fabris & Schwabe 4750* (LP); La Cascada, *Cabrera & Torres 17.816* (LP); Prov. Misiones, San Javier, *Cabrera* et al. *322* (LP). Paraguay: Tobati, Schinini 24DA (LP). Uruguay: Depto. Maldonado, Piriápolis, Cerro San Antonio, *Castro 137* (LP).

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