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Gametophytic phase of *Doryopteris triphylla* (*Pteridaceae,* Polypodiopsida)

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Abstract. Gametophytes of the *Pteridaceae* are moderately well known but there is still a quite large set of species to describe. Among these is *Doryopteris triphylla*, a member of the cheilanthoid clade (subgroup hemionitids). The main objective of this work is to describe the gametophyte of *D. triphylla*. In vitro cultures were prepared with spores coming from various sporophytes of different locations. In vivo observations were done periodically to monitor developmental critical events and reproductive activity. Germination followed the *Vittaria* pattern and the developmental processes adjusted to the *Adiantum* type. Adult cordate gametophytes produced normal sexual organs in unisexual prothalli. *Doryopteris triphylla* gametophytes exhibited somewhat peculiar features: the apical cell divided longitudinally, the first meristematic cell was rectangular, and archegoniate prothalli appeared much before than antheridiate ones.

Keywords: development; fern; morphology; reproduction.

Gametófito de *Doryopteris triphylla* (*Pteridaceae,* Polypodiopsida)

Resumen. Los gametófitos entre las *Pteridaceae* están bastante bien conocidos, si bien hay aún un buen número de especies por describir, entr las que se encuentra *Doryopteris triphylla*, un miembro del clado de los Cheilanthoides. El principal objetivo de este trabajo es describir la fase gametofítica de *D. triphylla*. Para ello, se sembraron esporas de diferentes esporófitos de varias localidades diferentes, en cultivos multispóricos. A lo largo del desarrollo de los protalos se realizaron observaciones in vivo de los principales eventos de desarrollo y reproducción. La germinación sigue el modelo *Vittaria* y el subsiguiente proceso de desarrollo se ajusta al tipo *Adiantum*. Los gametófitos adultos cordados producen órganos sexuales normales en protalos unisexuales. Los gametófitos de *D. triphylla* exhiben algunas peculiaridades, como por ejemplo la división longitudinal de la célula apical, la forma rectangular de la primera célula meristemática y el hecho de que los protalos arquegoniados aparecen mucho tiempo antes que los anteridiados.

Palabras clave: desarrollo; helecho; morfología; reproducción.

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Introduction

The genus *Doryopteris* J. Sm. is a member of the cheilanthoid clade of the *Pteridaceae*, and has been treated within the hemionitid subgroup by several authors (Schuettpelz *et al.* 2007). The genus is taxonomically complex, and has been object of several research in order to clearly identify its boundaries with respect to other related genera (Windham *et al.*

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2009), undoubtely stating the non-monophyly of the genus. The most recent review, based both on molecular and morphological data, has segregated many species in several other genera (Yesilyurt *et al.* 2015), leaving within *Doryopteris* a group of about 25 species. These species are mostly distributed in the Southern Hemisphere, with particularly important spots in South America, Hawaii and South Africa.

The species subject of this study, *D. triphylla* (Lam.) Christ, is distributed exclusively in South America: Argentina, Brazil, Bolivia, Paraguay and Uruguay, inhabiting pastures and rock crevices. It has been sometimes segregated to the monotypic genus *Cassebeera* Kaulf. (de la Sota & Giudice 2004), which is not considered in the modern circunscription (Yesilyurt *et al.* 2015).

Gametophytic characters have long been evaluated in fern systematics (Atkinson & Stokey 1964, Nayar & Kaur 1971, Atkinson 1973). This fact continues to our days, particularly with the Pteridaceae, in which many studies focusing gametophytes are aimed to clarify the complex generic delimitation of the family (Rothfels *et al.* 2008, Gabriel y Galán & Migliaro 2011, Sigel *et al.* 2011, Gabriel y Galán & Prada 2012, Johnson *et al.* 2012). In spite of all the effort made to describe gametophytes of ferns in general and the Pteridaceae in particular, the haploid phase of *D. triphylla* is not known yet, except for some works related to the spore (Tryon 1942, Tryon & Lugardon 1991). The gametophytes of some other *Doryopteris* species have been previously observed (Nayar 1960, Nayar & Kaur 1969, Zamora *et al.* 1992).

The aim of this work is the study of the gametophytic phase of *D. triphylla*, which comprises the observation of the spore and its germination, the morphological development of the gametophyte and the reproduction.

Material and Methods

Plant materials. Several different sporophytes were collected in the following two localities. 1. Argentina, Buenos Aires Province: Azul, estancia San Javier-Los Angeles, 5 km from Monasterio de Trapa to Pablo Acosta, 200 m, Prada *et al.* 2012-1. 2. Argentina, Buenos Aires Province: Tandil, La Cascada, pr. Tandil, 350 m, Prada *et al.* 2012-4.

Spore cultures and morphological observations. Given the high incidence of apogamous processes in the family *Pteridaceae* (Huang *et al.* 2011), we counted the number of mature spores in four sporangia of different sporophytes in order to detect a deviation from the normal production. Spore size was measured in 120 spores, randomly selected, from four different sporophytes (30 each). Size is expressed in mean values (polar × equatorial lengths).

Spores from four different sporophytes of each location were mixed and sowed in petri plates six cm in diameter with mineral agar medium (Dyer 1979). We sowed five plates for each location, for a total of 10 plates. Spores were cultured in a chamber at 20 ± 2°C and a 16 hours light photoperiod (daylight fluorescent tubes, photon irradiance 30-45 μmol m^{-2} s^{-1} in the 400-700 nm region). Germination percentage was recorded daily, by observing 100 spores randomly selected in each plate, until the percentage reached its maximum. Spores were considered as germinated when a first rhizoid was clearly emerged (Gabriel y Galán & Prada 2010b). Morphological development and reproductive structures were observed by *in vivo* preparations, using a Nikon LaboPhot-2 light microscope and a Nikon Coolpix MDC camera. Sizes of gametophytes at different stages are given as mean values (length × width) of at least 10 measurements.

Results

The number of spores per sporangium was of 64 in all cases. The first germinated spores were detected seven days after sowing; three days later the percentage of germinated spores was of 43%; 13 days after sowing the spores achieved a maximum germination of 80% (Fig. 1). The spores of *D. triphylla* were trilete, non-chlorophyllous, amber in colour, with slightly rugose perispore, and nearly rounded in shape, with little variation in size, 32.9 ± 1.9 × 35.2 ± 2.4 μm (Fig. 2A).

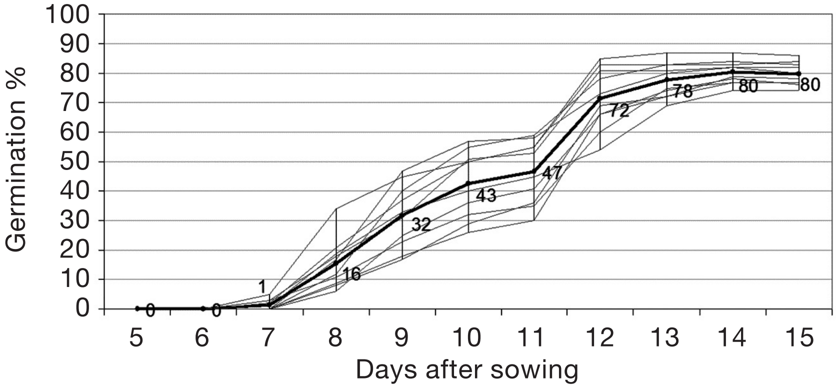

Figure 1. Changes in germination percentages of *D. triphylla* over the observational period. Light grey lines express the germination behavior of each of the 10 individual plates observed. Black line expresses the medium values for all the plates.

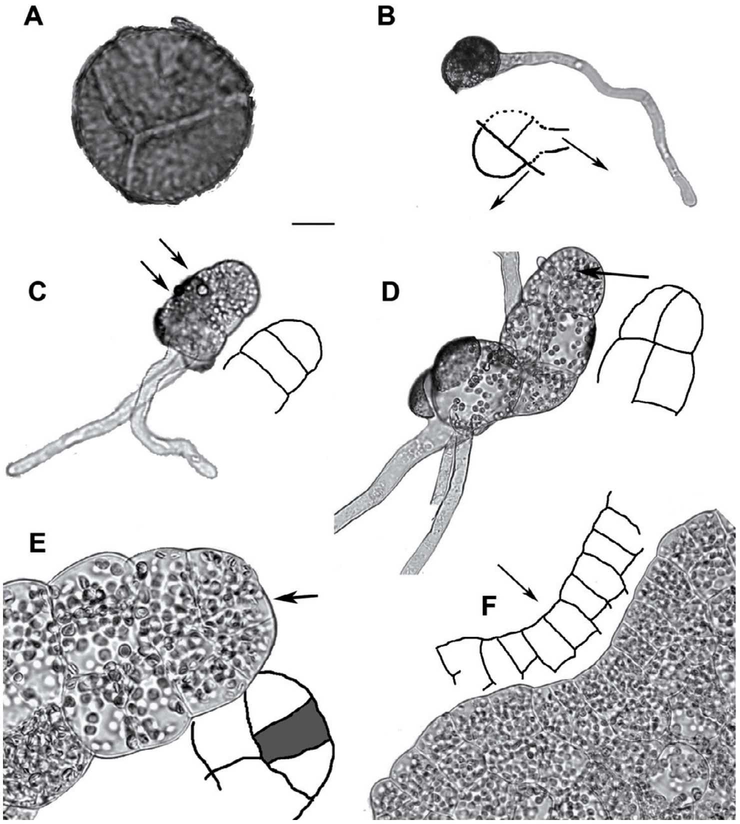

Figure 2. Major events in the vegetative development of *D. triphylla* gametophyte, with representative simplified schemes. **A:** spore in proximal view, showing the laesura. **B:** first stage of germination, with one rhizoid and a perpendicular first prothallial cell, 11 days. **C:** uniseriate filament of three prothallial cells; arrows point out the transversal divisions occurred, 14 days. **D:** origin of the planar stage, with longitudinal divisions (arrow), 17 days. **E:** development of an apical meristematic cell (arrow and shadow), 20 days. **F:** beginning of the cordate shape, with the establishment of a well-developed apical meristem (arrow), 26 days. Bar = 7.5 µm in A; 35 µm in B, C; 20 µm in D, F; 10 µm in E. Days are measured from sowing.

Spore germination was characterized by the emergence, at first, of a unicellular, hyaline rhizoid. 9-12 days after sowing, the first prothallial cell appeared, in a plane perpendicular to the rhizoid, showing abundant chloroplasts (Fig. 2B). This prothallial cell transversely divided several times, to form a short-celled uniseriate filament, of not more of six cells and a size of 90 ± 5 × 55 ± 3 µm. Those filaments were abundant in the cultures around 14 days after sowing (Fig. 2C). This was an ephemeral phase, as it quickly underwent longitudinal divisions in all the cells, including the apical one, arising bidimensional prothalli around 12 days after germination (Fig. 2D). About 22 days after sowing, c. 65% of gametophytes reached this bidimensional condition, which measured 120 ± 9 × 60 ± 3 µm. A meristematic, more or less rectangular cell was developed in the apex of the prothalli (Fig. 2E), the activity of which resulted in the acquisition of a spathulate shape. Finally, an apical, conspicuous multicellular meristem was developed (Fig. 2F) leading to the adult stage, about 30 days after sowing. At this time, almost 50% of gametophytes were cordate or slightly asymmetric in shape (Fig. 3A), with a mean size of 680 ± 15 × 340 ± 9 µm, and a pronounced meristematic notch. By the end of the observational period (which extended over more than 60 days), some individuals seemed to culminate its development in a diffuse way, leading to adult gametophytes with irregular or highly asymmetric cordate shapes. We want to point out two other vegetative features: first, no marginal or superficial hairs have been observed on the gametophytes of *D. triphylla*; second, the rhizoids in adult stages presented a spatulate apex (Fig. 3B).

Around 36 days after sowing, reproductive activity was observed with the presence of female gametangia in about 60% of adult cordate gametophytes (Fig. 3C). Archegonia were always produced immediately under the notch. The neck of these gametangia was ma-

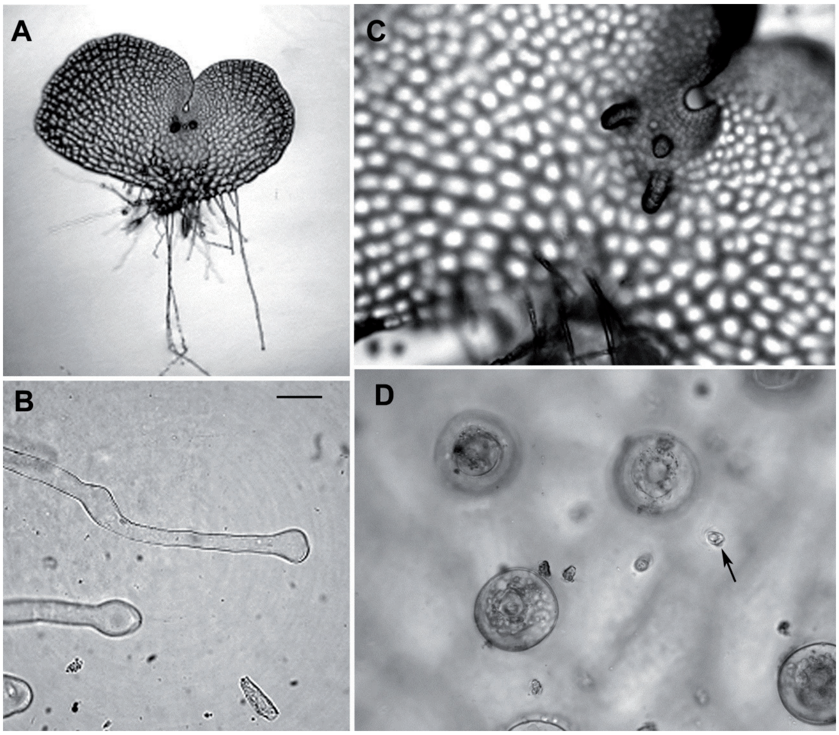

Figure 3. Features of the adult gametophyte of *D. triphylla*. **A:** sexual cordate gametophyte, 35 days. **B:** Detail of rhizoid apices, 35 days. **C:** Archegonia, 36 days. **D:** antheridia, with released sperm (arrow), 47 days. Bar = 175 µm in A; 30 µm in B, C; 20 µm in D. Days are measured from sowing.

de up by four rows of five cells in length (Fig. 3D). We never detected archegonia in the irregular individuals. Antheridia appeared 46-51 days after sowing, in both irregular and cordate individuals with no archegonia, i.e. the prothalli were unisexual, and they maintained so throughout the observational period. Antheridia were typical of leptosporangiates; when mature, they produced and released sperm cells (Fig. 3D), which were observed swimming in the in vivo microscope mountings. No sporophytes were formed in the cultures.

Discussion

Our observations on the spores of *D. triphylla* completely agree with previous studies (Tryon & Lugardon 1991; de la Sota & Giudice 2004), in size, shape and ornamentation. The germination of this species is of the *Vittaria* type (Nayar & Kaur 1968), as the first prothallial cell appears by means of a division that occurs perpendicular to the first rhizoid. This is one of the most common patterns of germination within leptosporangiates, and it has been cited as usual in the *Pteridaceae* (Nayar & Kaur 1969, 1971). Time from sowing to first germination and germination percentages reached in our cultures are in normal values fore the leptosporangiates with non-chlorophyllous spores, considering the age of spores at sowing (Courbet 1963, Lloyd & Klekowski 1970, Dyer 1979, Sheffield 1996, Gabriel y Galán & Prada 2010b).

Doryopteris triphylla follows a pattern of development that is easily ascribed to the *Adiantum* type (Nayar & Kaur 1969), in which a more or less obconical meristematic cell is established in early stages of development, the evolution of which lead to the acquisition of an apical multicellular meristem. Other species of *Doryopteris*, as *D. concolor* (Langsd. & Fisch.) Kuhn and *D. pedata* (L.) Fée, are also known to develop their gametophytes following this model (Nayar 1960). This developmental pattern is one of the most widespread in the family, along with the *Ceratopteris* model (Atkinson & Stokey 1964, Nayar & Kaur 1969). However, in many genera, an intermediate *Adiantum-Ceratopteris* way of development has been detected, in which an apical cell is formed but the meristem is lateral (Nayar & Bajpai 1964, Nayar & Kaur 1971, Gabriel y Galán & Migliaro 2011). It has been also stated that ameristic, irregular forms of development in these genera could be due to deviations from any of those *Adiantum* or *Ceratopteris* types, by means of inhibition of the initial meristematic cell (Gabriel y Galán & Migliaro 2011). This could be occurring in *D. triphylla*. The explanation for the existence of these odd morphologies that coexist with typical cordate individuals could be caused by several factors. First of all, many abiotic factors affect the development of fern gametophytes, including temperature, light intensity and quality, substrate composition, etc. (Raghavan 1989, Wada 2008). Though reasonable precautions were taken to minimize such factors, their possible influence cannot be ruled out. Second, fern gametophytes maintain several biotic interrelationships that are known to affect germination, development, size and sexual activity of individuals. That includes two aspects: on the one hand, intraspecific interactions in the form of pheromones, the antheridiogens, aimed basically to increase the effectiveness of sexual crossings (Greer & Curry 2004, Schneller 2008); in the other hand, interspecific alellopathy intended to inhibit or reduce the presence/growth of competitive species (Testo & Watkins 2013); this is achieved through a set of biomolecules, sometimes related with the antheridiogens. Nevertheless, our results suggest that neither system could be adduced to explain the irregular gametophytes in the current case: we can discard the existence of an antheridiogen system, as antheridia were observed only in well-developed, cordate gametophytes; and we can discard interspecific competence because our cultures were monospecific. Thus, we are more tempted to propose some kind of abiotic environmental influence, not excluding a mere individual genetic variability.

The velocity of the developmental process, from germination to gametangia formation, which occurred in roughly one month, is quick but falls within the normal range of variation for the leptosporangiates (Raghavan 1989, Banks 1999, Li *et al.* 2013). Furthermore, a quick development has been cited for the known gametophytes of the cheilanthoid clade (Windham & Yatskievych 2003), especially in association with apogamous taxa.

Rhizoids of *D. triphylla* have a spatulate apex when adult, character also observed in *D. concolor* (Zamora *et al.* 1992). But in a global perspective, this feature seems to be odd for the whole of the ferns, as only a few previous cases have been detected in the literature, for example, *Cheilanthes glauca* (Cav.) Mett. (Gabriel y Galán & Prada 2009), which is also a representative of the same clade of the family. Other species of *Cheilanthes*, as *Cheilanthes pilosa* Goldm., display also unusual morphologies in the rhizoids, with the capacity of branching the apices and producing sudden changes of direction (Gabriel y Galán & Prada 2010a). Our observations on *D. triphylla* abound in this sense. Rhizoids are structures that have been overlooked; so in our opinion is quite possible that these unexpected traits are more common than what is thought. In addition, research should be done to assess the relationships of these peculiar rhizoidal morphologies with variations of the substrates.

In *D. concolor* wall thickenings were detected in the cells of the wings (Nayar 1960). This is not the case of *D. triphylla*, whose cells present smooth and relatively thin walls.

The archegonia and antheridia observed in *D. triphylla* are typical for leptosporangiate ferns (Nayar & Kaur 1971). The development of apparently strict unisexual prothalli suggests that *D. triphylla* is favouring intergametophytic mating. The sexual expression of *D. triphylla* differs to that of other known species: in *D. pedata* and *D. concolor* antheridia has been observed prior to the adult cordate stage. Some authors stated that, in this species, fertilization occurred and normal sexual sporophytes were formed (Nayar 1960). Further observations on *D. concolor* from other geographical locations, showed that antheridia disappeared before the development of archegonia, and that the spores are formed in number of 32 per sporangium; the authors concluded that the sporophytes were of apogamous origin (Zamora *et al.* 1992). Although we have not observed sporophytes in our cultures, we can exclude the existence of apogamous processes in *D. triphylla* for three reasons: first, the number of spores per sporangium (64) indicate a normal sexual life cycle; second, we didn't see sign of apogamous sporophytes at any time after the emergence of the archegonia and until the end of the observational period; third, we have detected motile sperm with normal vitality. Thus, we conclude that *D. triphylla* is a normal sexual species that favor intergametophytic crossing within populations.

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