

Gametophytic phase of the Indonesian ferns *Amblovenatum immersum* (Blume) Mazumdar and *Christella subpubescens* (Blume) Holttum (Thelypteridaceae)

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Abstract. The gametophytic generation of *Amblovenatum immersum* and *Christella subpubescens* (Thelypteridaceae), including spore germination, morphological development of the gametophytes, major vegetative features and reproduction strategies, was studied. For both species, the spore germination was of the *Vittaria* type and the developmental pattern was of the *Drynaria* type. Adult gametophytes were cordate and hairy, with unicellular and secretory hairs located in the margins and both the ventral and dorsal surfaces of the prothalli. *C. subpubescens* has another type of acicular hairs only in the margin of the prothallus. Gametangia were of the normal type described for leptosporangiate ferns. In *A. immersum* all the gametophytes were female. In *C. subpubescens* the gametophytes produced at first instance female gametangia and then became bisexual with time. Antheridiogen activity was observed in both species, suggested by the presence of small young ameristic gametophytes with antheridia surrounding well-developed female ones.

Keywords: *Amblovenatum immersum*, *Christella subpubescens*, development, gametophyte, morphology, sex expression, spore.

[es] Fase gametofítica de los helechos indonesios *Amblovenatum immersum* (Blume) Mazumdar y *Christella subpubescens* (Blume) Holttum (Thelypteridaceae)

Resumen. Se estudió la fase gametofítica de las especies *Amblovenatum immersum* y *Christella subpubescens* (Thelypteridaceae) incluyendo la germinación, el desarrollo morfológico, características vegetativas y estrategias reproductivas. El modelo de germinación para ambas especies se ajusta al tipo *Vittaria*, mientras que el modelo de desarrollo del gametófito corresponde con el tipo *Drynaria*. Los gametófitos adultos eran cordados, con multitud de pelos unicelulares y secretores tanto por el margen, como por ambas caras del protalo. Además, en *C. subpubescens* se detectaron otro tipo de pelos aciculares, únicamente en el margen. Los gametangios presentaban el aspecto típico de los helechos leptosporangiatos. En *A. immersum* todos los gametófitos se desarrollaron como femeninos. En *C. subpubescens* los gametófitos primeramente son femeninos, con el paso del tiempo se vuelven bisexuales. Se detectó actividad de anteridiógeno en ambas especies, ya que después del desarrollo de los gametófitos femeninos aparecieron en los alrededores protalos masculinos en fases muy tempranas de desarrollo (con ausencia de meristemo), pero sexualmente maduros.

Palabras clave: *Amblovenatum immersum*, *Christella subpubescens*, desarrollo, desarrollo sexual, gametófito, morfología, espora.

Introduction

The Thelypteridaceae Ching ex Pic. Serm. is one of the largest cosmopolitan fern families, with a tropical and subtropical distribution, reaching its greatest diversification in Malaysia (Tigerschiöld 1989). With approximately 900-1000 species (He & Zhang 2012), it is one of the most diverse fern families. There is some controversy in the number of genera in which

it is divided since it varies between 5 and 30 (Smith *et al.* 2006). According to the most recent classification it contains 27 genera (PPGI 2016). Currently, the classification of the family remains quite controversial due to the high degree of convergence and parallel evolution that its genera present, especially in the morphological characters. In addition, there are still unsolved genera such as *Christella* H. Lév., which

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is polyphyletic according to the molecular studies by He & Zhang (2012) and Almeida *et al.* (2016).

Within the family we can find genera such as *Amblovenatum* J.P.Roux and *Christella*, among others, which have been used in the work we have carried out. Traditionally they were enclosed in *Cyclosorus* Link *sensu lato*, which was later segregated into several smaller genera (Smith & Cranfill 2002; He & Zhang 2012; Almeida *et al.* 2016.). *Amblovenatum* is a monophyletic genus with 12 species and *Christella* is a non-monophyletic genus with about 70 species, taxonomically complex and yet to be researched and resolved phylogenetically (Almeida *et al.* 2016). Both genera are highly related phylogenetically and morphologically which makes them difficult to differentiate in some cases (Field 2020), especially through some characters such as spores.

Ferns are characterized by a cycle with two distinct and independent phases, gametophyte and sporophyte. The gametophytic phase is the most unknown, despite being fundamental for the survival and subsequent distribution of the sporophytic phase. Nevertheless, studies on gametophytes are quite scarce, which supposes a lack of information related to this phase and therefore of the life cycle of ferns and how it can be affected. For this reason, it is important to increase the knowledge of this phase by means of different investigations on the morphological and sexual development of the gametophytes.

The morphological and sexual characters of gametophytes have been traditionally studied (Atkinson & Stokey 1964; Nayar & Kaur 1971; Atkinson 1973; Raghavan 1989), in particular in the family Thelypteridaceae, works have been made mainly on the genera *Thelypteris* Schmidel and *Macrothelypteris* (H. Itô) Ching (Atkinson 1971; Tigerschiöld 1989; Watkins & Farrar 2005; de León *et al.* 2008; Mishra *et al.* 2019), but also from other genera of the family such as *Amauropelta* Kunze *Pneumatopteris* Nakai, *Sphaerostephanos* J.Sm. (Tigerschiöld 1989) Despite all these descriptive works on gametophytes in general and in the family Thelypteridaceae, the gametophytes of *Amblovenatum immersum* and *Christella subpubescens* are unknown to science.

The study species are *Amblovenatum immersum* (Blume) Mazumdar, which is distributed in China, Malesia (the botanical and biogeographical region (Johns 1995)), New Hebrides, New Caledonia, and Loyalty Islands (Holttum 1977) and *Christella subpubescens* (Blume) Holttum, with distribution in India to southern China; throughout Malesia; Thailand and Vietnam; New Hebrides; Fiji and Samoa (Holttum 1976). The aim of the work is to study the spore germination, the morphological development of the prothallus, the morphology of the adult gametophyte and the sexual expression of *A. immersum* and *C. subpubescens*.

Materials and methods

Plant materials

Sporophytes of *Amblovenatum immersum* and *Christella subpubescens* were collected at the following two locations respectively: **1.** Indonesia, Bali, Candikuning, Tabanan, around the botanical garden, -8.269900° 115.156988°, undergrowth, 1320 m, *Gabriel y Galán s.n.*, 26/08/2018 (MACB). **2.** Indonesia, Bali, Ubud, near Camphuan Ridge Walk, -8.501118° 115.262472°, rice field edge, 270 m, *Gabriel y Galán s.n.*, 25/08/2018 (MACB). The material was identified following the Flora Malesiana (Holttum 1982).

Spore cultures and morphological observations

The spores were sown by shacking one single fertile pinnae of each species on a weigh paper, and placing the obtained spores in a 6 cm Ø Petri dishes with mineral agar (Dyer 1979). The sowing of each sample was replicated four times on three plates for each replica, in such a way that finally a total of twelve plates per species were planted and kept in a culture chamber at a temperature of $22 \pm 2^\circ$ C under fluorescent light with a photoperiod of 12-h light, 12-h dark cycle. The germination percentage was recorded by counting under microscope 100 spores randomly selected every three days until the values were stabilized. Spores were considered as germinated when a first rhizoid was clearly emerged (Gabriel y Galán & Prada 2010). *In vivo* observations of the morphological development and reproductive structures of gametophytes were made using a Leica stereomicroscope (Stereozoom S9i model), and photographed and measured with the software Leica Application Suite version 4.12.0. Finally micro characters observations were made using a Nikon microscope (Labophot-2 light model; Eclipse Ci-L model) and photographed and measured with a Nikon Colpix MDC camera and the software NIS-Elements F version 4.00.00.

Results

Amblovenatum immersum

Germination started about 9 days after sowing, when few spores (ca. 15%) showed a first rhizoid. The maximum germination percentage reached was relatively low, ca. 51% of the spores (Fig. 1). This maximum percentage was achieved ca. 45 days after sowing.

The spores of *A. immersum* were bilateral, monolete, dark brown, with an irregular ridged perispore (25 x 42µm) (Fig. 2A&B). Eleven days after the spores germinated, the first rhizoid cells and the first prothallic cells were formed (Fig. 2A&B). After 13-15 days short filaments of 3-5 cells (84-100 µm) appeared in the plates (Fig. 2C&D). The apical cell undergoes a longitudinal division that gives rise to

two apical daughter cells (Fig. 2E). These daughter cells divided longitudinally repeatedly to form a long filament of cells that were very abundant in the plates around day 18 after sowing (Fig. 2F). After the longitudinal divisions the spatulate phase was formed, which presented a multicellular meristem at the apex of the cell lamina around day 21 (Fig. 2G).

The activity of this meristematic area gave rise to a pre-cordate prothallus, 25 after germination. This prothallus showed a multitude of unicellular secretory hairs on the margin and the adaxial and abaxial faces (Fig. 2I). Gametophytes needed 5 days more to acquire their definitive pre-sexual shape and size. Adult gametophytes were completely symmetrical, cordate and hairy (Fig. 3A). Marginal hairs were located all around the gametophyte, and on both the ventral and dorsal surface of the prothallial lamina. These marginal and surface hairs were morphologically similar.

The reproductive structures were detected 30 days after germination; archegonia were the first to appear (Fig. 3B). They were formed in the central basal area of the ventral face and the notch. Most of the gametophytes in the plates were female as they presented female reproductive structures (archegonia) and over time (35 days after germination) the antheridia were observed, in this case they were found in a lot of small and irregular gametophytes close to adult and female gametophytes (Fig. 3C). In the majority of cases, the gametophytes presented early growth stages without meristem or with a not well-defined meristem, but they were sexually mature because they presented antheridia. The prothalli were therefore unisexual throughout the observation period. No sporophytes were formed in the cultures.

Christella subpubescens

Germination started around day 9 after sowing when there were approximately ca. 21% spores germinated. The maximum percentage of germination was ca. 83% (Fig. 4) 30 days after sowing.

The spores of *C. subpubescens* were bilateral, monolet, dark brown, with striated or tubercular perispore and very similar to the spores of *A. immersum*, 23 x 40 µm (Fig. 5A&B). Thirteen days after sowing, the first prothallial cells appeared (Fig. 5B). Around the 15th and 18th day after sowing, short filaments of 3 cells were observed in the plates (Fig. 5C). The apical cell had a longitudinal division that gave rise to two daughter cells. In the following days, these daughter cells divided longitudinally repeatedly resulting in the spatulate phase, which is characterized by a pluricellular meristem at the apex of the cell lamina, this took place on day 22 (Fig. 5E).

Cellular development in the meristematic zone gave rise to the pre-cordate prothallus 24 days after sowing. The prothallus had a multitude of unicellular secretory hairs along the margin as well as on the ventral and dorsal sides (Fig. 5H) as occurred in the gametophytes of *A. immersum*. Six days later the

prothallia acquired the shape and size of pre-sexual adults. The unicellular secretory, marginal and surface hairs had the same morphology. In addition, in *C. subpubescens* other types of hair were observed, in this case they only appeared in the margin between the glandular hairs and in a more scarce form than the secretory trichomes. They were elongated, acicular, non-secretory and unicellular (Fig. 6D).

Sexual activity was detected 35 days after germination when the archegonia appeared, later (ca. 40 days after germination) some gametophytes began to produce antheridia becoming bisexual (Fig. 6A). The archegonia were located in the notch near the apex, while the antheridia were located in the vicinity of the archegonia but more towards the ventral and near the rhizoids of the gametophyte. After the appearance of the archegonia, small and irregularly shaped male gametophytes could be observed close to the adult bisexual gametophytes (Fig. 6B&C). Male gametophytes were less abundant than in the *A. immersum* plates. No sporophytes were formed in the cultures.

Discussion

Our observations of the spores of *A. immersum* and *C. subpubescens* are consistent with previous descriptions for both genera (Nayar & Kaur 1971; Holttum 1976, 1977; Smith 1990) on size, shape and ornamentation. The spores of both species germinated around the ninth day, which is within the average germination time of fern spores without photosynthetic pigments (Gabriel y Galán & Prada 2010) and corresponds to the germination times of spores from the same family (Sen 1981).

The germination pattern for both species corresponds to the *Vittaria* type (Nayar & Kaur 1968, 1971), which is characterized by the first rhizoid and the first prothallial cell emerging perpendicularly one to each other. This is one of the most typical germination patterns in leptosporangiate ferns (Nayar & Kaur 1968, 1971) and has already been reported for other species in the family Thelypteridaceae (Huckaby & Raghavan 1981; Sen 1981; de León *et al.* 2008; Mishra *et al.* 2019). The process of development of the gametophyte corresponds to the *Drynaria* type (Nayar & Kaur 1969, 1971), in which an apical meristematic cell being differentiated after a spatulate nonmeristic thallus is formed by divisions of the anterior cell of the germ filament. This model of gametophyte development has already been cited by other authors for the genus *Thelypteris* (Pérez-García *et al.* 1994). By contrast, other studies in the same genus have reported the *Aspidium* type as prothallial development (Pérez-García & Mendoza-Ruiz 2004; de León *et al.* 2008; Mishra *et al.* 2019).

The gametophytes of both species have a lot of hairs on the surface of both sides and on the margin of the prothallus, most of which are secretory and unicellular. The presence of this type of glandular hair

has already been reported by other authors (Nayar & Kaur 1971; Sen 1981; Tigerschiöld 1989, 1990) and is one of the typical characteristics of gametophytes from the family Thelypteridaceae (Atkinson 1971; Nayar & Kaur 1971; Smith 1990; Pérez-García *et al.* 1994). The unicellular glandular hairs of the gametophytic phase have, in many cases, the same morphology as those of the sporophytic phase in both genera (Holttum, 1976, 1977).

In addition, other types of hairs have been observed in the species of the genus *Christella*, these hairs are again unicellular but acicular in appearance, long and narrow. Again, our observations agree with those of other authors (Nayar & Kaur 1971; Sen 1981) who observed another type of trichomes less common among ferns, which are restricted to the families Grammitidaceae and Thelypteridaceae (Nayar & Kaur 1971). Within the family Thelypteridaceae only some genera present them (Tigerschiöld 1989, 1990).

Tigerschiöld (1990) points out that the presence of acicular hairs is quite noticeable for the genus *Christella*, finding this type of trichomes in 4 of the 5 *Christella* species she studied. These acicular hairs appear in very advanced phases of the development of the gametophyte (Nayar & Kaur 1971; Sen 1981). In our case the presence of this type of hairs was noticed in gametophytes with more than 80 days since sowing.

The presence of secretory hairs and their length is an interesting character for gametophytes. Tigerschiöld's study (1990) found significant differences in the length of the secretory glands to differentiate species of some genera of Thelypteridaceae; and together with other morphological characters of the gametophytic phase they could be interesting for the identification of species. Although both glandular and acicular hairs can be pluricellular with 2 or 3 cells, even in the case of acicular hairs they can be septate, this is not the most common characteristic of homosporous ferns (Nayar & Kaur 1971; Sen 1981). In our observations, no such cases have been found.

As for the sexual organs, archegonia have the typical appearance of leptosporangiate ferns (de León *et al.* 2008). The antheridia have a distribution close to the notch, which had already been shown in other works (Tigerschiöld 1989, 1990; Mishra *et al.* 2019). In the case of *C. subpubescens*, the antheridia, on the one hand, developed in gametophytes that already had archegonia, meaning that they are bisexual prothallus; on the other hand, amorphous gametophytes were observed, small with antheridia from day 45 after germination. In the case of *A. immersum* the antheridia developed on gametophytes close to the female ones and in all cases they were in an early stage of development and presented an irregular and immature aspect. This suggests the presence of antheridiogen in both species.

Antheridiogen was first identified by Döpp (1950) in *Pteridium aquilinum*, and is able to affect the

gametangial development in gametophytes (Schneller *et al.* 1990; Yatskievych 1993; Banks 1999). These are pheromones released into the environment by sexually mature female or bisexual gametophytes (Banks 1999) and promote maleness on the nearby presexual gametophytes. Although not all species can produce or respond to antheridiogens, the family Thelypteridaceae is one of the families that respond and produce antheridiogens (Raghavan 1989; Hornykh *et al.* 2020) along with others such as Blechnaceae, Dryopteridaceae or Polypodiaceae (Yatskievych 1993; Hornykh *et al.* 2020). The antheridiogen suppresses the early developmental functions of gametophytes, which is why they are small, asymmetrical in appearance, or in some cases lack meristem (Banks 1999), promoting the rapid differentiation of gametophyte cells into antheridia. The function attributed to the antheridiogen is to promote crossing in pteridophytes, through the ontogenetic control of the formation of gametangia in gametophyte populations (Schneller *et al.* 1990; Yatskievych 1993; Hornykh *et al.* 2020). The observations carried out on male gametophytes are consistent with those reported by other studies, and supports the idea of the presence of antheridiogens in the species *A. immersum* and *C. subpubescens*.

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Figures

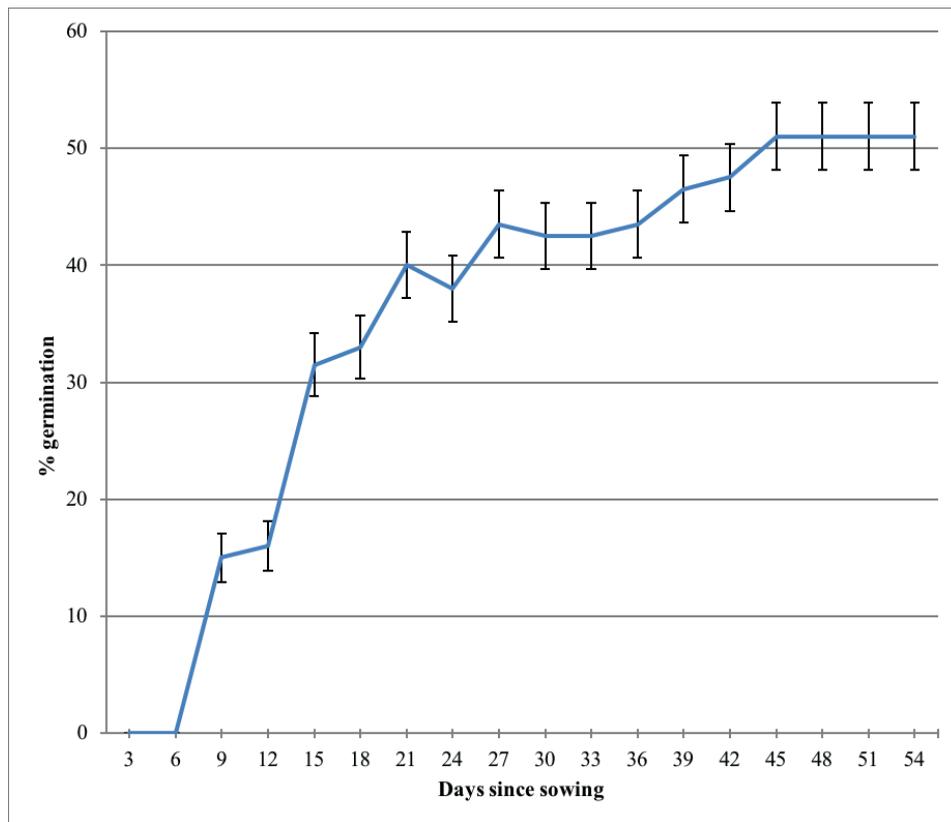


Fig. 1. Germination kinetics of the *A. immersum* spores corresponding to the mean of all the plates.

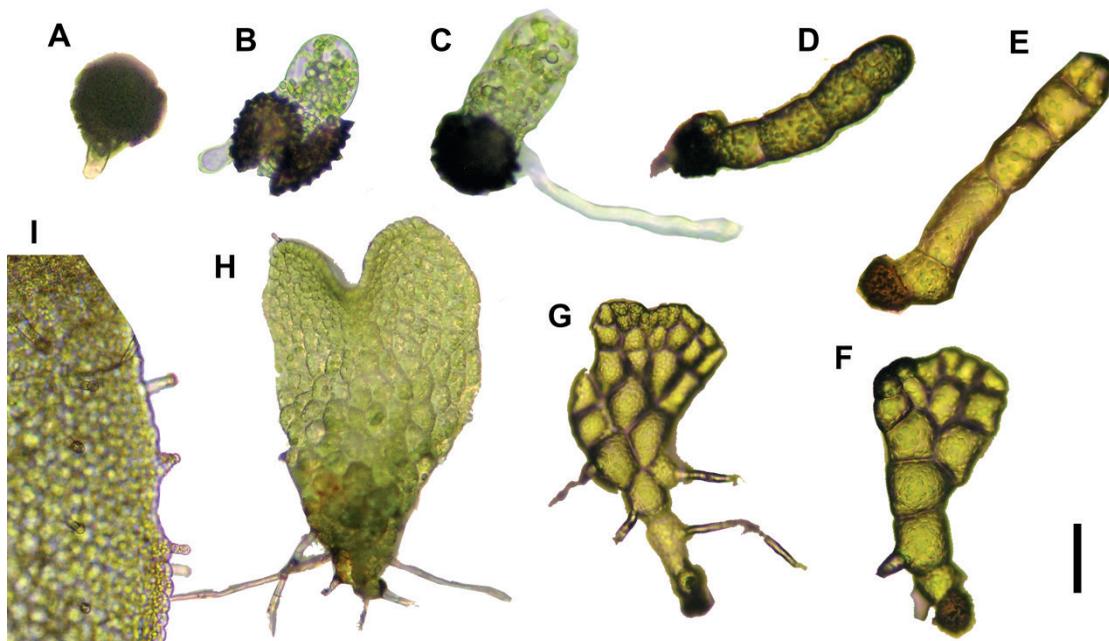


Fig. 2. Main phases in the development of the gamethophyte of *A. immersum*. A) Germinated spore, with a first rhizoid (9 days). B) First prothallic cells (11 days). C) 3-cell filament (13 days). D) 5-cell filament (15 days). E) Longitudinal apical division (18 days). F) Spatulate phase, with a first apical meristematic cell (20 days). G) Spatulate phase, with the pluricellular meristem developed (21 days). H) Beginning of the presexual adult phase, with well-developed wings and indument (25 days). I) Detail of indument, marginal and superficial glandular hairs (25 days). Bar = 28 μ m in A-C; 25 μ m in D, E; 41 μ m in F, G; 45 μ m in H, I.

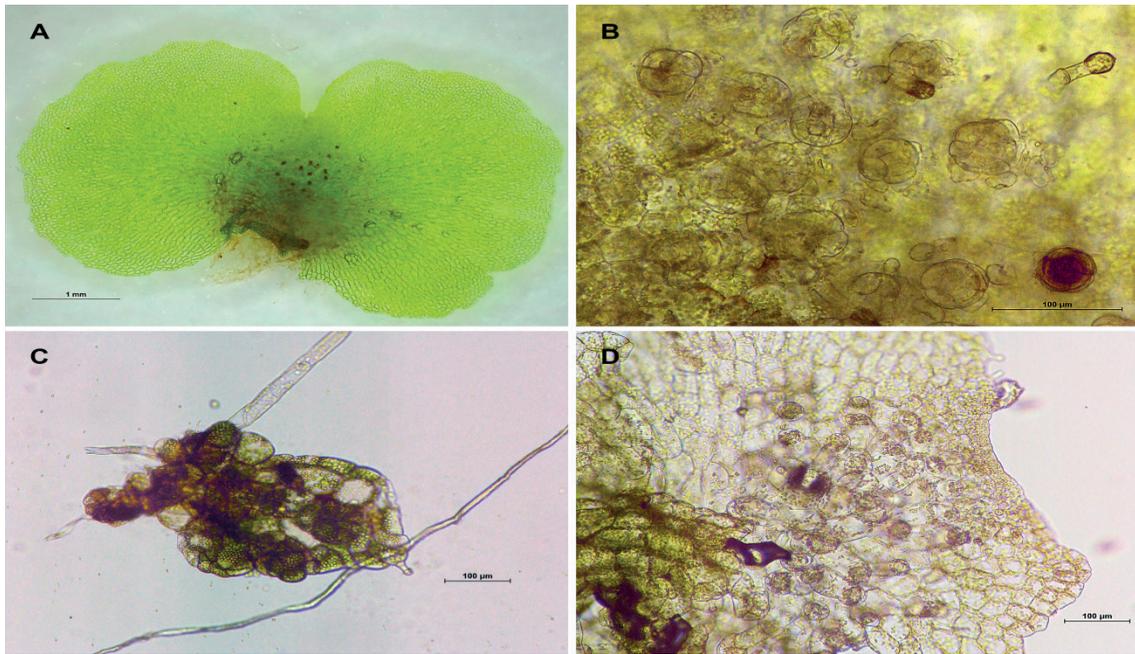


Fig. 3. Reproductive structures in the gametophyte of *A. immersum*. A) Female gametophyte, cordate, with abundant archegonia in the central area under the notch (30 days). B) Detail of the archegonia (30 days). C) Small and irregular male gametophyte, with numerous antheridia (35 days). D) Detail of the antheridia (35 days).

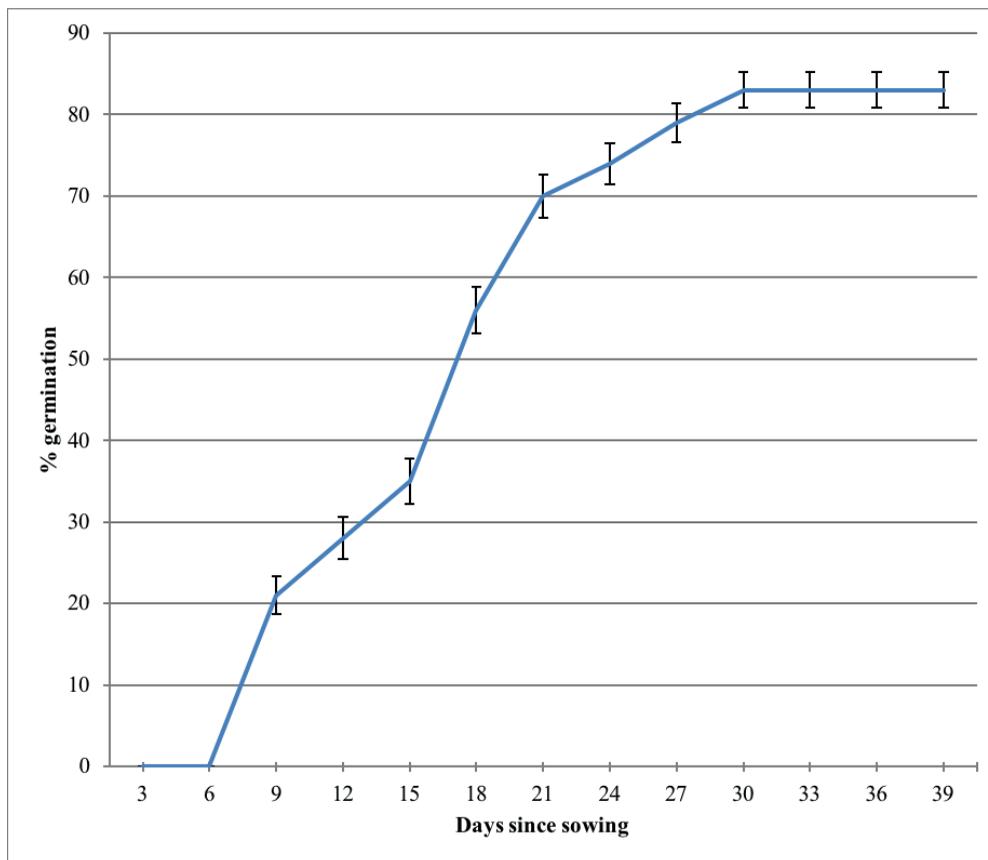


Fig. 4. Germination kinetics of the *C. subpubescens* spores corresponding to the mean of all the plates.

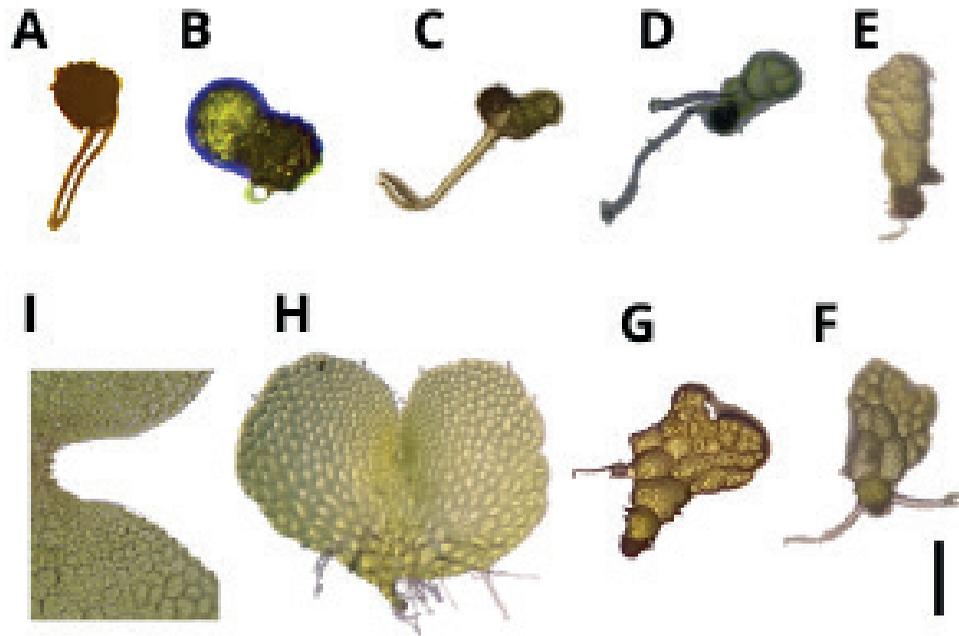


Fig. 5. Main phases in the development of the gametophyte of *C. subpubescens*. A) Germinated spore, with a first rhizoid (9 days). B) First prothallial cells (13 days). C) 3-cell filament (15 days). D) Longitudinal apical division (20 days). E) Spatulate phase, with a first apical meristematic cell (22 days). F) Spatulate phase, with the pluricellular meristem developed (24 days). G) Beginning of the presexual adult phase, with patent wings and indument (24 days). H) Presexual adult (30 days). I) Detail of notch (30 days). Bar = 28 μm in A; 50 μm in C; 100 μm in C, D, I; 105 μm in E, F; 270 μm in H.

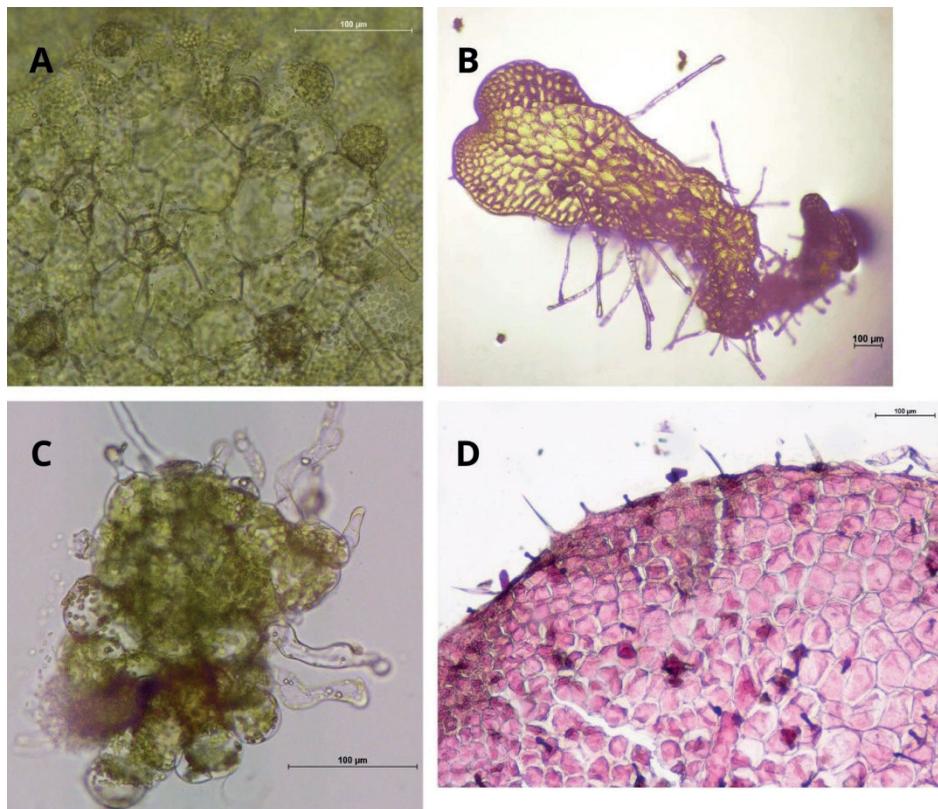


Fig. 6. Reproductive structures in the gametophyte of *C. subpubescens*. A) Detail of the archegonia and antheridia (40 days). B) Male gametophyte in early stage of development but sexually mature (45 days). C) Small and irregular male gametophyte, with numerous antheridia (45 days). D) Detail of the margin with glandular and acicular hairs (80 days).