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Spores germination and gametophytes of *Alsophila odonelliana* (Cyatheaceae) in different sterile media

Germinación de esporas y gametofitos de *Alsophila odonelliana* (Cyatheaceae) en diferentes medios estériles

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Abstract. The tree fern *Alsophila odonelliana* is found from southern Bolivia to northwestern Argentina, in the Tucuman-Bolivian forests. Spore germination was tested in three sterile nutritive media: Dyer (D), Murashige and Skoog (MS) and Knudson (K). We also evaluated the effect on gametophyte development of the addition of two growth regulators, BAP and 2,4-D to the culture medium Dyer; 2,4-D was evaluated using two concentrations: 0.1 and 2.0 mg/L, and BAP using three concentrations, 0.01; 0.1 and 1.0 mg/L. We found no significant differences in the germination of spores among all three media. We noted that supplementing media with BAP affected the longitudinal growth of the filamentous gametophytes, and the proliferation of ramifications from early stages. Supplementation with 2,4-D led to the development of short filamentous gametophytes and ameristic laminar forms. Both regulators affected the structure of male gametangia. There was not 2,4-D somatic embryogenesis as in other plants.

Keywords: Tree ferns; Spores; Germination; BAP; 2,4-D.

Resumen. El helecho arbóreo *Alsophila odonelliana* crece desde el sur de Bolivia hasta el Noroeste de Argentina, en la selva tucumano-boliviana. Se evalúa la germinación de las esporas en tres medios nutritivos estériles, Dyer (D) Murashige & Skoog (MS) y Knudson (K), y el efecto sobre el desarrollo gametofítico de la adición al medio de cultivo Dyer, de dos reguladores de crecimiento, BAP y 2,4 D. El efecto del 2,4-D fue evaluado en dos concentraciones: 0,1 y 2,0 mg/L, y para BAP se utilizaron tres concentraciones, 0,01; 0,1 y 1,0 mg/L. No se encuentran diferencias significativas en la germinación de las esporas en los tres medios de cultivo. Se observa que el suplemento del 2,4 D conduce al desarrollo de gametofitos filamentosos cortos y formas laminares amerísticas; el suplemento de BAP afecta el crecimiento en longitud de los gametofitos filamentosos y la proliferación de ramificaciones desde etapas tempranas. Ambos reguladores de crecimiento afectan la estructura de los gametangios masculinos. El efecto del 2,4 D no produce embriogénesis somática como en otras plantas.

Palabras clave: Helechos arbóreos; Esporas; Germinación; BAP; 2,4-D.

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INTRODUCTION

Cyathaeceae is a family of tree ferns with approximately 500 species (Korall et al., 2006), growing in mountainous regions of the Pelotropic and Neotropics. *Alsophila odonelliana* (Alston) Lehnert is found in southern Bolivia and Northwestern Argentina (Lehnert, 2005) in the Tucuman-Bolivian forests, where it grows in humid ravines, on gullies, margins of rivers, etc.

Sexual reproduction in Cyatheaceae has been confirmed by Conant (1990), Khare et al. (2005), Cheng et al. (2008), and Chen et al. (2008). However, during three years we have tried to grow *Alsophila odonelliana* sporophytes on *in vitro* cultures, unsuccessfully. The lack of positive results prompted us to experiment different culture media to assess spore germination, and the response of gametophytes to growth regulators.

In studies focusing on the gametophytic phase of ferns in sterile culture, media with changing proportions of different macro and micronutrients were tried. Frequently used nutrient media for growing fern spores are Dyer (1979), Knudson modified by Steeves et al. (1955), Thompson (Klekowski, 1969) and Murashige & Skoog (1962) (Reyes Jaramillo & Pérez García, 1994; Saifullah, 2008; Narváez, 2008; Martínez, 2010; Gabriel & Galán, 2011). Dykeman & Cumming (1985) used *in vitro* cultures to obtain seedlings from explants of rhizomes and leaves; they achieved the propagation of *Matteuccia struthiopteris* (L.) Todaro from rhizome buds in Murashige & Skoog (1962) culture medium. The addition of growth regulators to culture media favors cellular multiplication and differentiation; thus, they have used them to promote the formation of somatic embryos in the propagation of ferns and other vascular plants (Marconi & Radice, 1997; Hedge et al., 2006; Garro-Monge et al., 2008; Alam & Khaleque, 2010).

The addition of growth regulators to sterile culture media for the study of fern gametophytes is not a usual practice. The results of these treatments show that 2,4-Dichlorophenoxiacetic ammonium (2,4-D) inhibits the growth of gametophytes in *Pteris longifolia* L. (Strickler, 1946); gibberellin and benzylaminopurine (BAP) accelerate germination of stored spores of *Pteris inermis* (Rosenst.) de la Sota (Tanco et al. 2009). Menéndez et al. (2009) found that exogenous and endogenous cytokinins of female gametophytes of *Blechnum spicant* (L.) Roth in the culture media induce the formation of male gametophytes in this species; Furelli & García (1987) obtained calli of *Pteris cretica* L. from explants of young leaves by adding 2 mg/L 2,4-D and 2 mg/L BAP to the culture medium.

The aims of this work were to evaluate (1) the germination of *A. odonelliana* spores in three culture media, Dyer (D), Murashige & Skoog (MS) and Knudson (K) with the purpose of determining which of these three substrates is the optimal, and (2) the effect of the addition of two growth regulators (BAP and 2,4-D) to the culture medium, on the development of the gametophytic phase of this fern.

We hope to provide information on the action of BAP cytokinin and 2,4-D auxin on the gametophyte development of *A. odonelliana,* and the use of different culture media in the germination of fern spores, as a practical contribution for fern conservation projects.

MATERIALS AND METHODS

The plant material used in this study was collected from Northwestern Argentina: Salta, Dpto. Santa Victoria, Parque Nacional Baritú [Martínez et al. (1860, 1862, 1864); 11-08-2009].

Spores were collected after natural dehiscence of sporangia in stove at 30˚C, and stored for 16 months. The dimensions of the spores were calculated from a 50-spore sample from mature sporangia. Equatorial and polar diameters were measured and standard deviations (SD) calculated.

For *in vitro* cultures, spores were cold-stored at 4 ± 2˚C, sterilized using 5% sodium hypochlorite plus 2 drops of Tween for 2 minutes under continuous stirring, and then washed three times with sterile distilled water.

The germination of spores is a gradual process taking several weeks according to the species; therefore, we made observations at 60 and 120 days to compare and evaluate the germination progress. The emission of a rhizoid was considered a positive germination.

In order to study the effect of different culture media on the germination of spores, we used three nutritive media, Dyer (D), Knudson (K) and Murashige & Skoog (MS), solidified through the addition of 7 g/L Britania agar. Cultures were kept in a growth chamber at 25 ± 3 ˚C under a 12:12 L/D photoperiod.

We evaluated the effect of growth regulators on the morphogenesis of gametophytes using Dyer medium supplemented with 2,4-D in two concentrations: 0.1 and 2.0 mg/L, and BAP in three concentrations, 0.01, 0.1 and 1.0 mg/L. We employed a completely randomized factorial design. One of the factors studied was each medium, with growth regulators at their respective concentrations. Gametophytes were grown in 12 flasks, 5 cm high by 4 cm diameter, for each treatment. The other study factor was the time for spore germination. Cultures were maintained during twenty months.

The variable analyzed was the percentage of germination. For each observation four quadrants were considered per flask, and the records were the average of ten counts per quadrant. The effect of the culture medium and the time of germination, as well as their interactions, were analyzed using ANOVA, and the significance level was set at 5%. Data were analyzed using InfoStat ver. 2008.

For photographing under a light microscope, gametophytes were dyed with aceto-carmine chloral hydrate (Edwards & Miller, 1972) and mounted in water; microphotography samples were fixed with glutaraldehyde in phosphate solution. All

observations, illustrations and photographs were made using a Hitachi S-3000 and a Standard 16 Zeiss light microscopes, and a JEOL JSM 6480LL Scanning Electron Microscope, property of Universidad Nacional de Salta (Argentina).

RESULTS

Palinological data. Spores are brown, trilete, tetrahedral, subtriangular in polar view, convex-hemispherical in equatorial view, with crested ornamentation. The equatorial diameter was 68.25, SD=9.03 μm and the polar diameter was 53.0, SD=5.91 μm (Fig. 1).

From a 300-spore sample, we found that 94% were viable, whereas the remaining 6% lacked cellular content or were very small-sized.

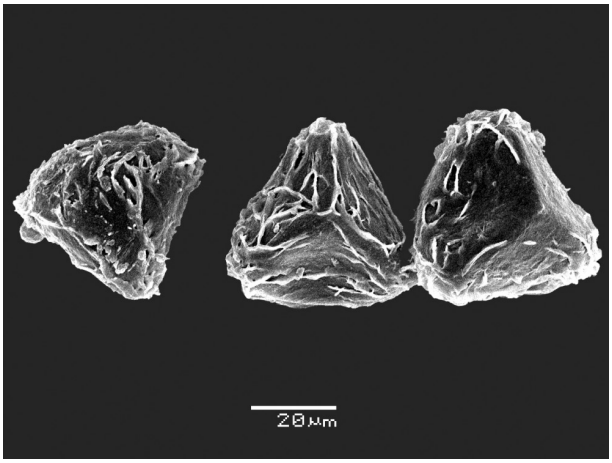

Fig. 1. Spores of *Alsophila odonelliana*, two spores in proximal view (right) and one in distal view (left), exhibiting ornamentation (x 1000).
Fig. 1. Esporas de *Alsophila odonelliana*, dos en vista proximal (derecha) y una en vista distal (izquierda), mostrando ornamentación (x 1000).

Effect of the culture medium on the germination of spores. The germination of spores began 9-12 days after sowing. Although sixty days after sowing we found a 26% germination in MS medium, 34% in D and 43% in K, after 120 days we found that all three media had the same effect on the germination of spores, with differences of less than 9% among them (Fig 2).

At the end of the experiment, no significant differences among the culture media Dyer (D), Murashige & Skoog (MS) and Knudson (K) were found.

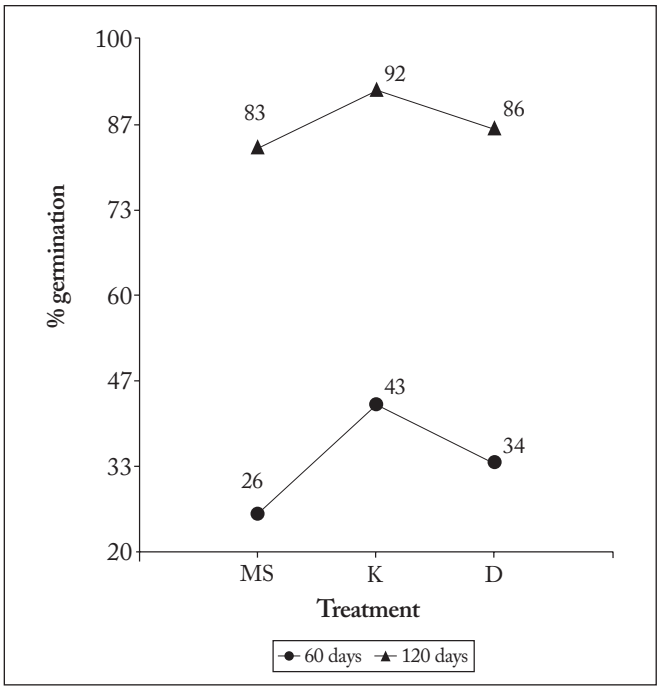

Fig. 2. Percentage of germination of *Alsophila odonelliana* spores in MS, K and D culture media without growth regulators. The curves correspond to records obtained at 60 and 120 days after sowing.
Fig. 2. Porcentajes de germinación de las esporas de *Alsophila odonelliana* en medio de cultivo MS, K y D sin reguladores de crecimiento. Las curvas corresponden a los resultados obtenidos a los 60 y 120 días después de la siembra.

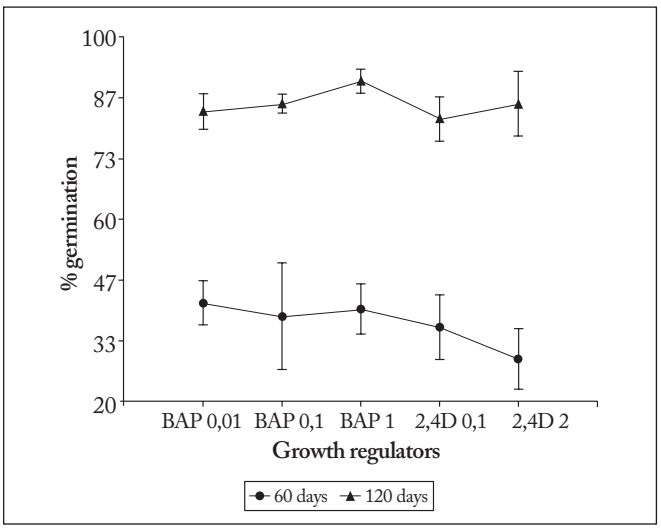

Fig. 3. Percentage of germination of *Alsophila odonelliana* spores in Dyer culture medium with growth regulators. Curves correspond to records obtained at 60 and 120 days after sowing with BAP in three concentrations and 2,4 D in two concentrations.
Fig. 3. Porcentaje de germinación de las esporas de *Alsophila odonelliana* en medio de cultivo Dyer con reguladores de crecimiento. Las curvas corresponden a los resultados obtenidos a los 60 y 120 días después de la siembra en BAP con tres concentraciones y en 2,4 D con dos concentraciones.

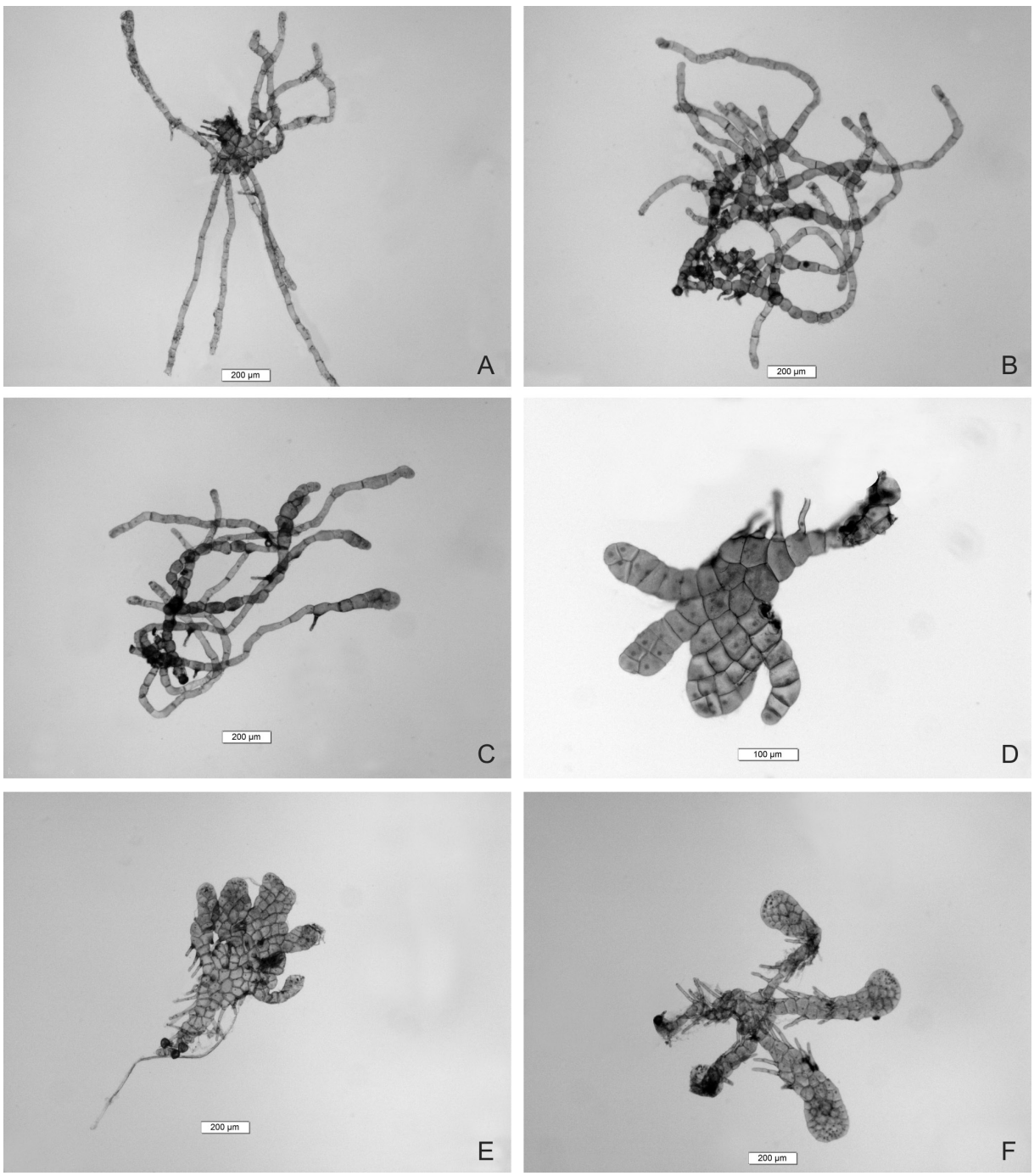

Fig. 4. Young gametophytes of *Alsophila odonelliana*. (A) ramified filamentous gametophyte in D medium with 0.1 mg/L BAP, 35 days after germination of spores (x 4); (B) filamentous gametophyte in medium with 0.1 mg/L BAP at 45 days (x 4); (C) filamentous gametophyte in medium with 1 mg/L BAP at 45 days (x 4); (D-E) laminar gametophytes in medium with 2 mg/L 2,4 D at 45 days (x 10, x 4); (F) laminar gametophytes in medium with 0.01 mg/L BAP at 45 days (x 4).

Fig. 4. Gametofitos jóvenes de *Alsophila odonelliana*. (A) gametofito filamentoso ramificado en medio D con 0,1 mg/L BAP a los 35 días desde la germinación de las esporas (x 4); (B) gametofito filamentoso en medio con 0,1 mg/L de BAP a los 45 días (x 4); (C) gametofito filamentoso en medio con 1 mg/L BAP a los 45 días (x 4); (D-E) gametofitos laminares en medio con 2 mg/L 2,4 D a los 45 días (x 10, x 4); (F) gametofitos laminares en medio con 0,01 mg/L BAP a los 45 días (x 4).

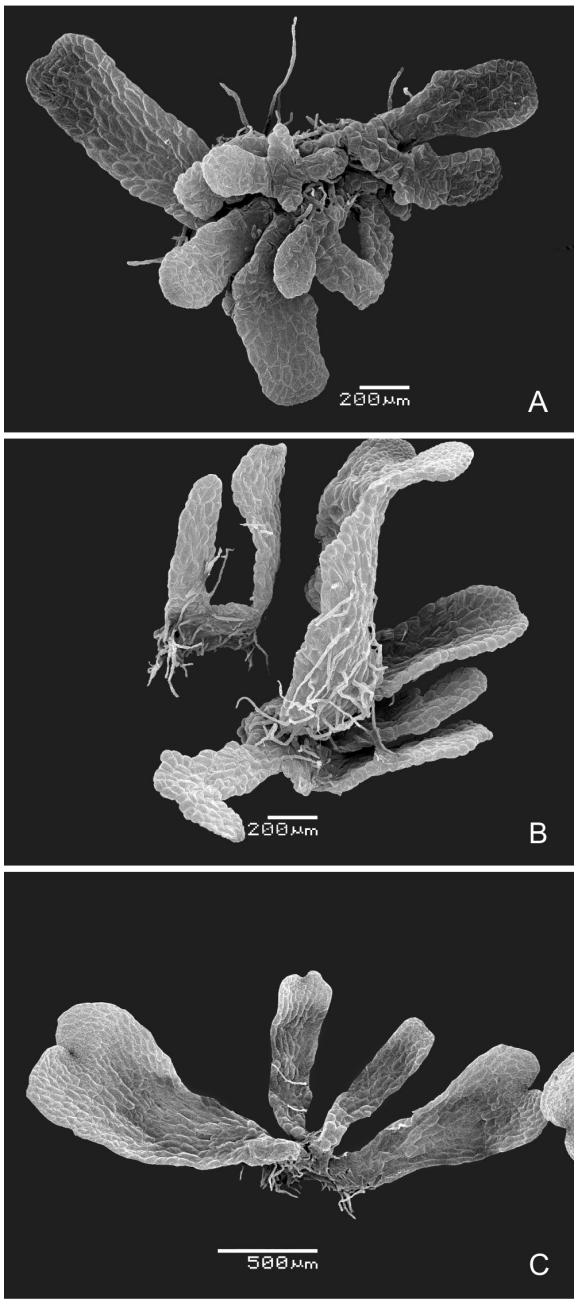

Fig. 5. Laminar gametophytes of *Alsophila odonelliana* in BAP-supplemented medium. (A) colony of gametophytes developing in medium with 0.1 mg/L BAP 50 days after germination of spores (x 60); (B) laminar gametophytes without notch developing in medium with 0.01 mg/L BAP at 60 days (x 55); (C) four gametophytes with marked notch in medium with 1 mg/L BAP at 70 days (x 45).
Fig. 5. Gametofitos laminares of *Alsophila odonelliana* en medio suplementado con BAP. (A) colonia de gametofitos desarrollándose en medio con 0,1 mg/L BAP a los 50 días desde la germinación de las esporas (x 60); (B) gametofitos laminares sin escotadura desarrollándose en medio con 0,01 mg/L BAP a los 60 días (x 55); (C) cuatro gametofitos con escotadura marcada en medio con 1 mg/L BAP a los 70 días (x 45).

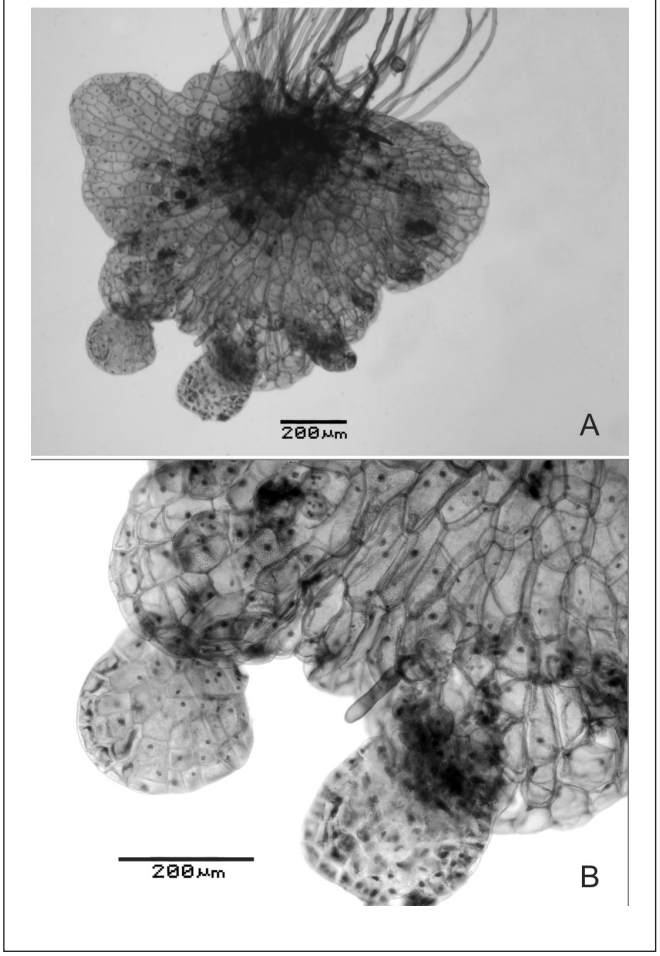

Fig. 6. Mature gametophytes of *Alsophila odonelliana* with submarginal proliferations. (A) two proliferations in ameristic gametophyte; (B) detail of proliferations.
Fig. 6. Gametofitos maduros de *Alsophila odonelliana* con proliferaciones submarginales. (A) dos proliferaciones en gametofito amerístico; (B) detalle de las proliferaciones.

The addition of growth regulators to the culture media did not affect the germination of spores. None of the growth regulators in any of the study concentrations induced a significantly different average percentage of germination ($p=0.73$). The interaction between growth regulators and time was also not significant ($p=0.83$). However, the ANOVA showed that the average percentage of germination was significantly higher at 120 days ($p<0.0001$) (Fig 3).

Effect of growth regulators on the gametophytic morphogenesis. The qualitative results showed different effects of the growth regulators on the gametophytes.

2,4 D Effect: *Filamentous phase*: at 0.1 mg/L, 6-8 cells long filaments with 2-3 ramifications, whereas at 2 mg/L, 3-4 cells short filaments developed.

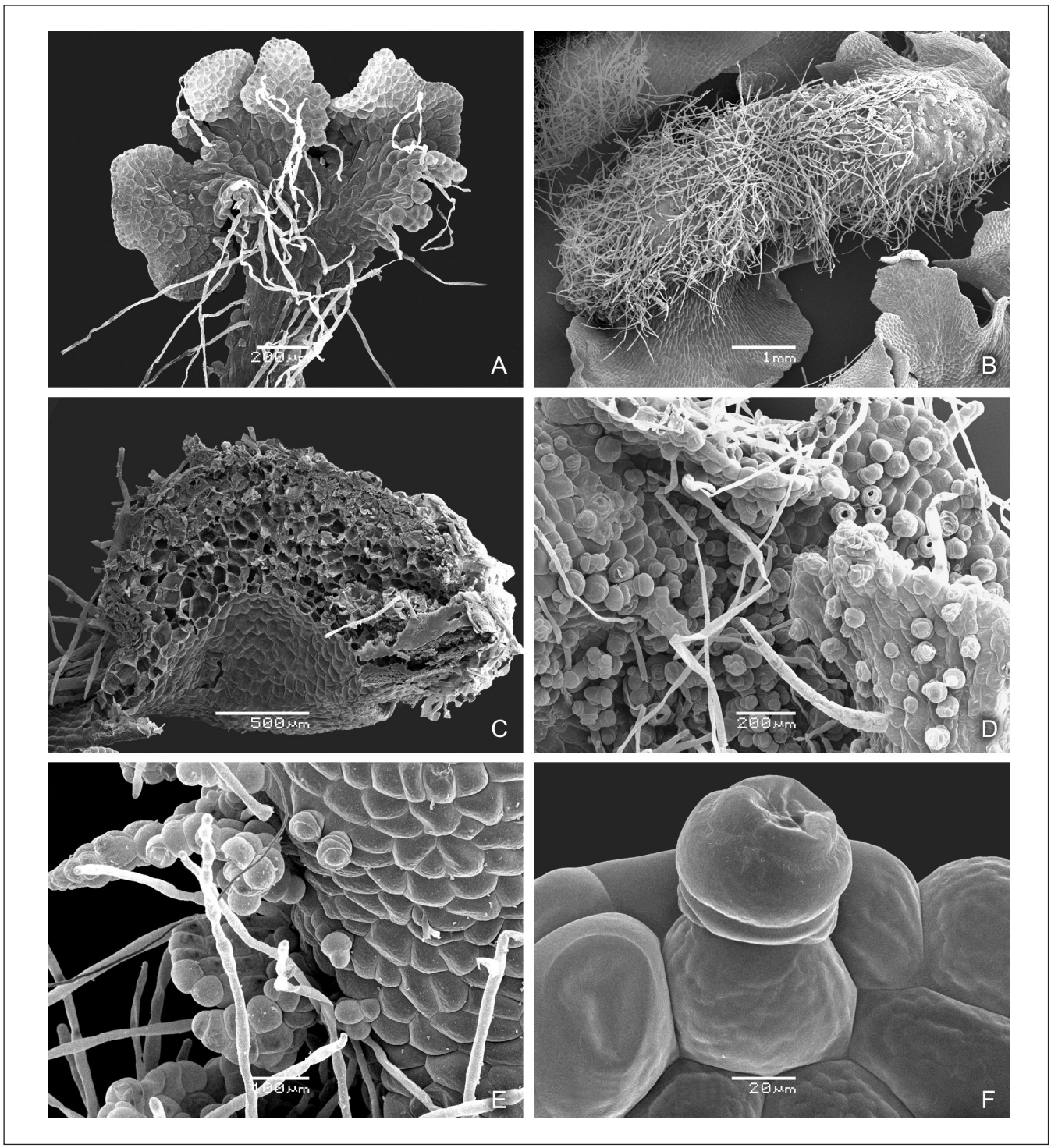

Fig. 7. Mature laminar gametophytes of *Alsophila odonelliana* developed in BAP-supplemented media. (A) gametophyte with lobule at the margin, six months after germination of spores (x 200); (B) gametophyte over a year old with prominent central ridge with antheridia (x 17); (C) transversal section of the central ridge showing pluristratified tissue (x 50); (D) detail of antheridia, some of them deformed (x 80); (E) antheridia deformed (x 40); (F) antheridium without deformations, in control media (x 700).

Fig. 7. Gametofitos laminares maduros de *Alsophila odonelliana* desarrollados en medios suplementados con BAP. (A) gametofito con lóbulos en el margen, seis meses después de la germinación de las esporas (x 200); (B) gametofito de más de un año con costilla central prominente con anteridios (x 17); (C) corte transversal de la costilla central mostrando tejido pluriestratificado (x 50); (D) detalle de anteridios, algunos deformados; (E) detalle de anteridios (x 40); (F) anteridio sin deformaciones, en medio control (x 700).

Laminar phase: growth was slow, and only 50% achieved chordate form. Some laminar forms emitted projections from the margin (Fig. 4 D, E). Most mature gametophytes, about 80% do not form notches, lost chlorophyll over time, turning pale to ocher in color, with few gametangia, and most by poor antheridia.

BAP Effect: *Filamentous phase:* with 0.01 mg/L, short filaments of 4 cells were generated which ramified 2-5 times; with 0.1 mg/L, long filaments, with 6-25 cells, ramified 4-20 times (Fig. 4 A, B), and with 1 mg/L, filaments with 8-12 cells, ramified 3-10 times (Fig. 4 C).

Laminar phase: BAP produced a fast longitudinal development, each ramification of the filamentous gametophyte originated a laminar gametophyte, forming colonies of gametophytes (Fig. 4 F, 5 A-C). The mature gametophytes originate numerous submarginal proliferations that in turn developed other laminar gametophytes (Fig. 6). The maintenance of meristematic activity led to the formation of lobules in the wings (Fig. 7 A).

The mature gametophytes, in BAP, showed increased volume of the central ridge (Fig. 7 B-C), sometimes generating 1-2 ramifications, each emitting marginal proliferations. The lamellae of mature gametophytes exhibit a characteristic bright green color. Male gametophytes dominated over those bisexual and sterile ones. The antheridia evidence certain deformations compared to those originated in control media (Fig. 7 D-F). Male and female gametangia maintained their structure, although their cells noticeably increased in size, leading to deformations. Twenty months after sowing of spores, no calli or sporophytes were observed.

DISCUSSION

Spore germination in ferns is a process that extends through time according to the age of the spores, the time and conditions of storage (humidity, temperature, light, etc), and the taxonomic group they belong to (Lloyd & Klekowski, 1970; Smith & Robinson, 1975; Beri & Bir, 1993; Camloh, 1999). Spores of *Alsophila odonelliana* germinate 7-8 days after sowing (Martínez, 2011; unpublished data), whereas the spores used in this work, stored for 16 months, took longer to germinate, likely due to the biochemical changes produced during storage (Bery & Bir, 1993).

Germination time of the spores was relatively similar in all three nutritive media D, K and MS, despite MS contained a greater amount of micro and macronutrients than D and K. Sixty days after sowing, we observed a slightly higher percentage of germination in the K medium compared with D and MS, although the statistical analysis showed no significant differences; therefore, the amount of nutrients in the substrate would not be a limiting factor in the germination of *A. odonelliana* spores.

The formation of 1-2 ramifications in filamentous gametophytes and the production of buds in laminar gametophytes are frequent during the gametophytic development in the Cyatheaceae family in the absence of growth regulators (Conant, 1990; Chen et al., 2008). Our results indicate that 0.1 mg/L BAP massively activated cellular proliferation, which led to the formation of filamentous gametophytes of up to 20 cells which ramified 17-20 times. In concentrations of 1 mg/L, BAP caused less ramifications in filamentous gametophytes. Supplementing the culture media with BAP induced *A. odonelliana* to develop long filamentous gametophytes, and proliferation of ramifications from early developmental stages until the adult stage.

González-Santos (2009) noted that BAP possessed the property of maintaining the concentration of chlorophyll and total soluble proteins for longer periods in plants, so that the foliar senescence is decreased; therefore, this growth regulator is likely responsible for maintaining the amount of chlorophyll in the laminar gametophytes developing in media with BAP. BAP was efficient from a morphological viewpoint, since it induced massive proliferation of gametophytes from the ramifications or the production of vegetative seedlings.

Supplementing the culture medium with 2,4-D led to the formation of short filamentous gametophytes, which originated laminar gametophytes with certain peculiarities: ameristic forms, furcated gametophytes and decreases in pigmentation, particularly in those for which the concentration of this regulator was 2 mg/L. In higher plants such as *Cephaelis ipecacuanha* A. Richard and *Aloe vera* L., this growth regulator, at the same concentrations, induces the formation of calli for obtaining embryos (Roya & Sarkara, 1991; Routa et al., 2000); in *Alsophila odonelliana,* we did not observe formation of calli in the gametophytes.

None of the growth regulators, BAP or 2,4-D, induced cell differentiation in the pluristratified ridges of the mature gametophytes for the formation of calli, nor apogamic sporophytes in *A. odonelliana*, in this first experience. Further testing is needed, with more repetitions, a wider range of growth regulator concentrations and longer observation times, to determine whether the growth regulators fail to produce embryos of tree ferns from this methodology.

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