ARTÍCULOS

Botanica Complutensis
ISSN-e 1988-2874

EDICIONES COMPLUTENSE

https://dx.doi.org/10.5209/bocm.73313

Study of the Gametophytic phase of *Diplazium caudatum* (Athyriaceae, Polypodiopsida) from Spain

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Abstract. The gametophytes of the Athyriaceae are not very well known, such as *Diplazium caudatum*. The aim of this work is to analyze the gametophytic phase of this species, which includes germination study, morphological development and reproductive phase. Spores belonging to two different sporophytes from La Gomera, Spain, were mixed and sown in multisporic cultures. Plates have been cultured in chambers with nutritive agar at 25ºC and 12 hours photoperiod. The germination was registered every three days, and the main vegetative and reproductive development was checked throughout the observational period. The germination rate reached a maximum of 58%. The spore germination followed a *Vittaria* pattern meanwhile prothallial development followed an *Adiantum* type. Regarding sexual expression, all gametophytes developed archegonia and later some of them developed antheridia becoming bisexual.
Keywords: development; fern; gametophyte; morphology; reproduction.

[es] Estudio de la fase gametofítica de *Diplazium caudatum* (Athyriaceae, Polypodiopsida) en España

Resumen. Los gametofitos de las Athyriaceae no son muy conocidos, tal es el caso de *Diplazium caudatum*. El objetivo de este trabajo es analizar la fase gametofítica de esta especie, que incluye el estudio de la germinación, desarrollo morfológico y fase reproductiva. Las esporas pertenecientes a dos esporofitos diferentes de La Gomera (España) han sido mezcladas y sembradas en placas de cultivo. Éstas se han cultivado en cámaras con agar nutritivo a 25ºC y 12 horas de fotoperíodo. La germinación se registró cada tres días y se comprobó el principal desarrollo vegetativo y reproductivo durante todo el período de observación. La tasa de germinación alcanzó un máximo del 58%. La germinación de las esporas siguió un patrón de tipo *Vittaria*, mientras que el desarrollo del prótalo siguió un patrón de tipo *Adiantum*. En cuanto a la expresión sexual, todos ellos desarrollaron arquegonios, y posteriormente una pequeña parte desarrolló anteridios convirtiéndose en bisexuales.
Palabras clave: desarrollo; helecho; gametofito; morfología; reproducción.

Introduction

Leptosporangiate ferns are the second most diverse group of plants after angiosperms, with about 10,000 species (Baker & Wolf 2010).

These plants are particularly interesting because of their reproductive cycle, which consists of two independent generations: the gametophyte (haploid) and the sporophyte (diploid). Moreover, these two generations present radically different structures: the gametophyte typically consists of a tallophytic structure without vascular tissues, while the sporophyte has a complex vascular structure. This marked structural difference of individuals in the same reproductive cycle is unique in the current plant world. (Page 2002).

However, only few studies of the gametophytic phase in ferns have been made, probably because it is a phase that takes place in a short period of time and with a little structural complexity in comparison with the sporophyte. It was not until the 1960s and 1970s when the first studies on the morphological and reproductive features with more detail were released (e.g. Atkinson 1973, Atkinson & Stokey 1964; Nayar & Kaur 1968, 1969, 1971; Whittier 1970).

The morphology of the gametophyte is highly variable, and it is even possible to identify different taxa in relation to it. Each species is adapted to different environmental conditions which influences their chances of survival. (Farrar et al. 2008; Lindsay & Dyer 1990; Raghavan 1989).

The genus *Diplazium* Sw. (Athyriaceae) consists of ca. 400 species that are distributed worldwide, appearing mostly in the south-central area of America, in the region of Indonesia and Japan (Kramer 1990;

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PPGI 2016). Only two species are represented on the European continent: *Diplazium sibiricum* (Turcz. ex Kunze) Sa. Kurata (northwestern Europe) and *Diplazium caudatum* (Cav.) Jermy (Macaronesian region and Iberian Peninsula) (Ormonde 1986; Salvo Tierra 1990).

Diplazium caudatum is a fern that is characterized by a creeping rhizome of up to 40 cm in length and fronds (leaf) of variable size, (60)100-220(240) cm long. The petiole (or stipe) is shorter than the blade, shiny black at the bottom (Ormonde 1986; Salvo Tierra 1990) (Fig. 1).The sporangia are typical of leptosporangiate species, with an arc of approximately 15 cells.

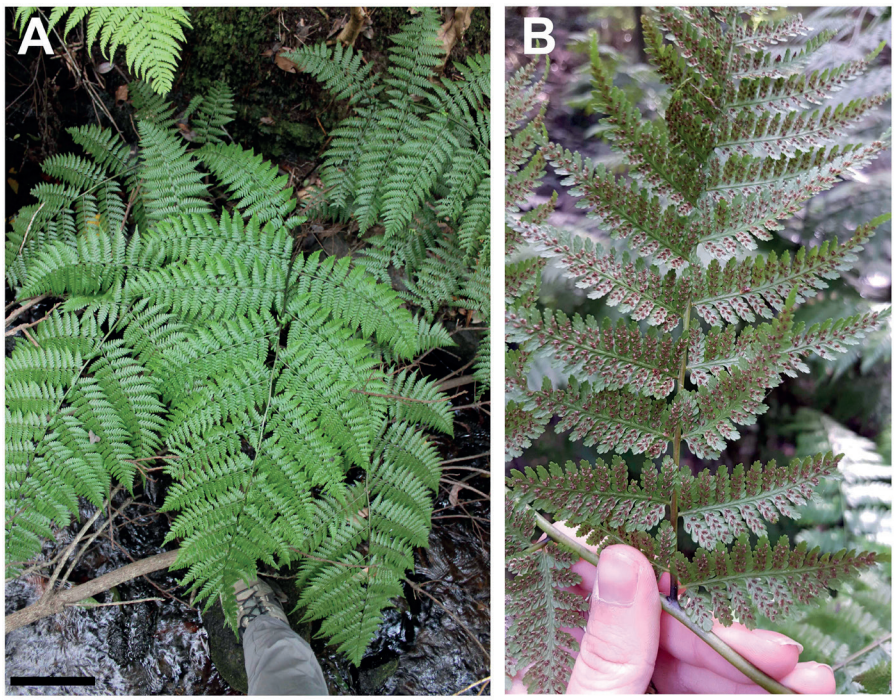

Figure 1. *Diplazium caudatum* plant in the field. A. Fronds; B. Detail of the abaxial surface of the pinna with sori. Detail of the abaxial surface of a pinna bearing sori. (La Gomera Island, December 2017). Bar = 12 cm in A; 2 cm in B.

The spores of this genus are monolete, as is usual in the family (Ormonde 1986). Regarding the location, *Diplazium caudatum* is mostly found in the Macaronesian area and in the southern region of the Iberian Peninsula (Fig. 2).

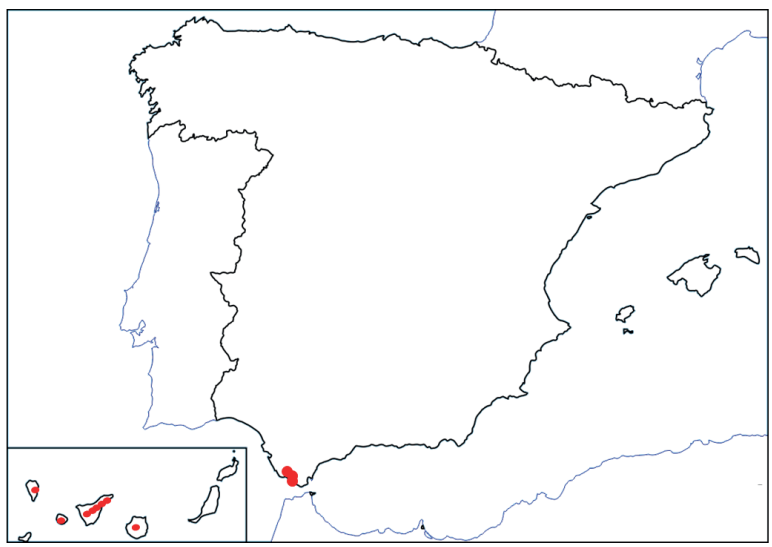

Figure 2. Distribution of the species *Diplazium caudatum* in the Iberian Peninsula and Canary Islands (Anthos 2020).

Little is known about the gametophyte of Athyriaceae, although it has been studied in some species and morphological differences have been observed throughout the group. In all the cases studied, a germination of the *Vittaria* type and the development of the *Adiantum* type gametophyte were observed (Atkinson 1967; Guo et al. 2010; Nayar & Kaur 1971).

The prothallus may present trichomes, as we can see in *Diplazium stellatopilosum* (Brause) Holtt , or be naked, as in *Diplazium pin-faense* Ching (Guo et al. 2010; Nayar & Kaur 1971). This indument can be located in the prothallus margin cells or on the surface of the lamina. Their function is varied: they provide protection from environmental conditions such as droughts or intense light exposure; they are also often associated with the protection of sexual organs, particularly archegonia (Atkinson 1967).

Diplazium caudatum is considered an endangered species, inhabits in deep ravines with high atmospheric humidity. Its distribution area is very restricted, with scarce populations and a very small number of individuals, most of them are located in the Canary Islands (Delgado & Plaza, 2006; Moreno et al. 2019; Salazar-Medias et al. 2019). It is known that the events that take place during the gametophitic phase in ferns are of great importance, as they determine the distribution of subsequent sporophytes and the evolution of species (Banks 1999; Quintanilla et al. 2007; Mehltreter et al. 2010). In fact, studies suggest that in general gametophytes develop even more characters for adaptation in adverse environments than sporophytes (Ebihara et al. 2013; Farrar 1998; Pittermann et al. 2013; Sato & Sakai 1979; Watkins et al. 2007). We believe, therefore, that knowing the details of this phase in *Diplazium caudatum* can be advantageous when planning future conservation measures, which has already been proposed for other species in this genus (Golamaully et al. 2015).

In this work we aim to study the germination of *Diplazium caudatum* spores as well as to describe its gametophytic development and morphology of the prothallus, unknown so far.

Material and methods

Spores from two different sporophytes were obtained from the following population: *Diplazium caudatum,* Spain, Canary Islands, province of Tenerife, La Gomera, Barranco del Cedro, *S. Molino s.n.*, 12-2017 (MACB). This ravine is located within the Garajonay National Park, at this location *D. caudatum* grows forming rows in the understory of the laurisilva forests.

The spores were obtained 2 weeks after the collection. They were extracted by exposing the fronds to a mild heat for one night. The cultivation protocol follows the guidelines of other similar works (Gabriel y Galán et al. 2008; Seral et al. 2016), with some modifications regarding the photoperiod. The spores were mixed to avoid possible individual differences. Multispore cultures were made in 6 cm diameter petri dishes on mineral agar with an approximate density of 30 spores per cm² (Dyer 1979). This culture was replicated six times, the plates were introduced at 25°C with a photoperiod of 12 hours and a light intensity of 30±45 μmol m^{-2} s^{-1}.

The spores were considered to have germinated when the first obvious rhizoid emerged (Gabriel y Galán & Prada 2010). To measure the changes in germination, the number of spores germinated was counted randomly out of a total of 100 in each plate until the germination percentage was stabilized. Ten spore measurements/plate were taken without considering the perispore, as this can be very variable.

For the morphological and reproductive study, measurements of the gametophytes were made (expressed as mean values width x length), as well as the length of the rhizoids and trichomes.

Every three days the plates were observed with a Nikon LaboPhot-2 microscope and we took pictures on *in vivo* gametophytes slides with a Nikon Coolpix MDC camera.

For detailed observation of the reproductive and vegetative characters of the gametophytes, they were mounted on optical microscope slides without treatment 38 days after sowing, when they were already in the mature phase.

Results

Germination began five days after sowing, with an average germination rate of 6%. Two days later, the average germination rate increased slightly (16%). Five days later the value increased twice (around 30%). Eight days later a value of 50% was reached and finally, 27 days after sowing, the maximum percentage of germination rate of the experiment was reached (58%). The dynamics of germination can be seen in Fig. 3.

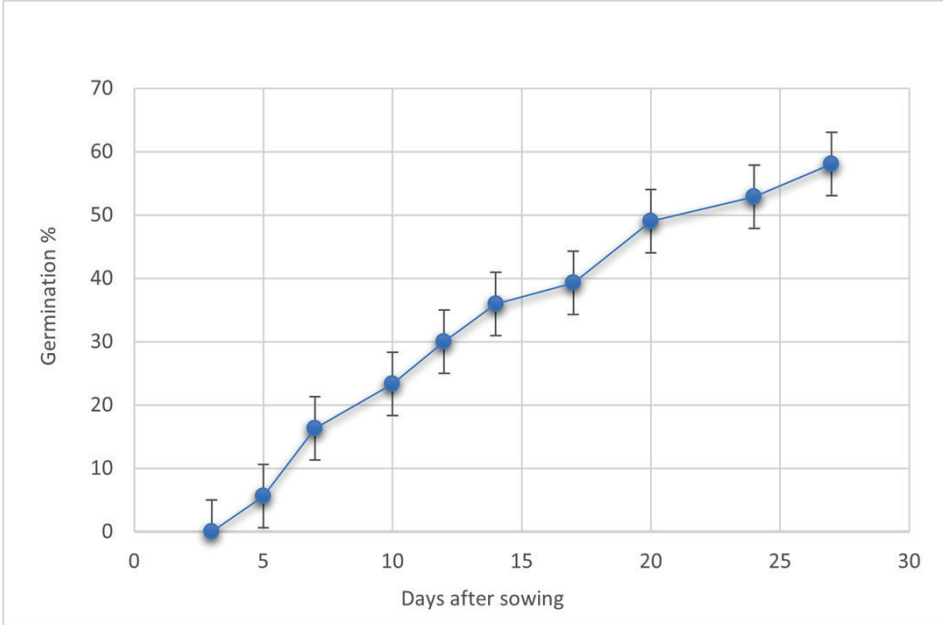

Figure 3. Graphic of spore germination rate in *Diplazium caudatum*.

The spores of *D. caudatum* are monolete, with bilateral symmetry and elliptical or oblong contour. Its length is 20 and its width 18 µm, with a smooth exospore, the perisporium is formed by numerous walls delimiting circular areolae. Spores present a single laesura through which the first rhizoid emerges (Fig. 4A). The spore germination was of the *Vittaria* type, since the first cell that appears emerges perpendicular to the first rhizoid and to the polar axis.

Seven days after germination, gametophytes in uniserial filamentous phase were observed, consisting of 2 cells, one of them being the prothallic cell (18µm in length, perpendicular to the rhizoid), with a rhizoid in perpendicular disposition to the filament and the polar axis of the spore (Fig. 4B).

Ten days after sowing, gametophytes in spatulate phase were observed in which three rhizoids were detected, one growing in a direction perpendicular to the filament other in a longitudinal direction and the last one was starting to emerge from it (Fig. 4C). The filaments were at this moment approximately 150 µm, 300 µm and 40 µm long from the base of the gametophyte to the most apical region.

The prothallus follows an *Adiantum* type of development, with a tendency to delay the growth of the meristematic cell. It was possible to observe the appearance of the first meristematic cell 24 days after sowing, from which the contiguous cells develop on both sides of it to give rise to definitive cordate morphology (Fig. 4D).

The first young gametophytes in the cordate phase had a width of 250 x 100 µm long from the notch to the base, with a multicellular meristem (Fig. 4E). The gametophytes with adult cordate morphology had a width of about 270 x 275 µm in length from the base to the notch (Fig. 4F).

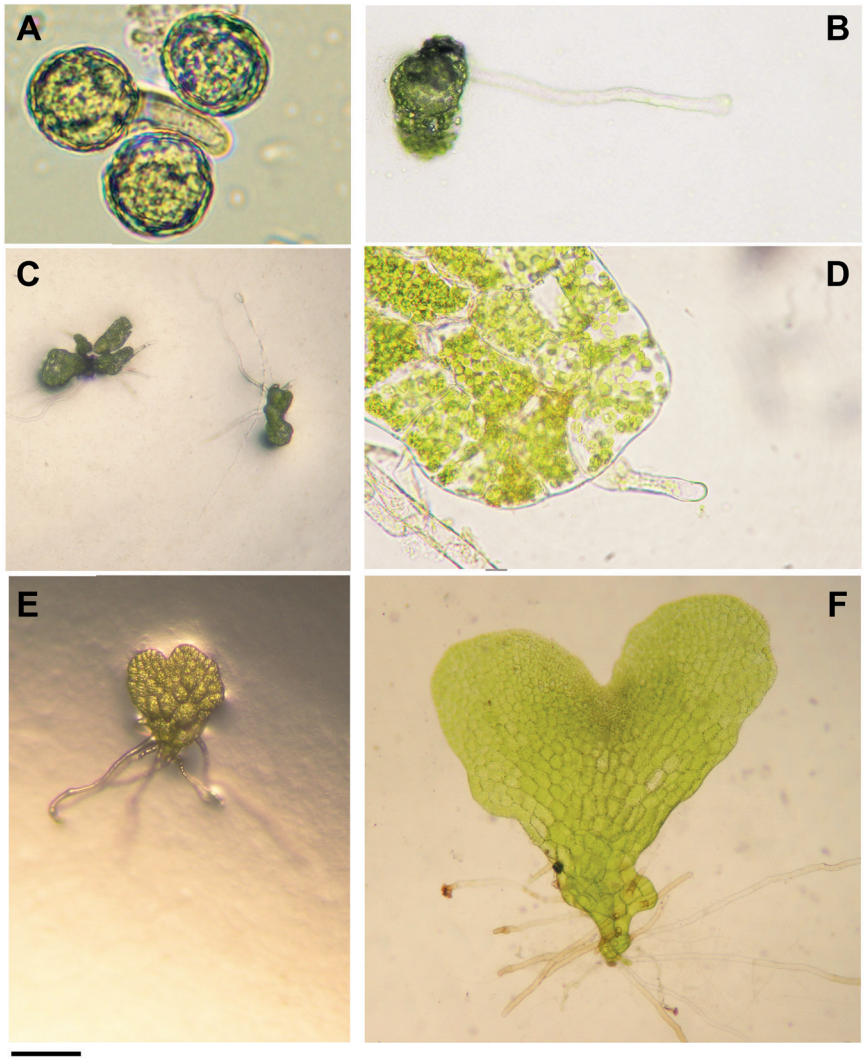

Figure 4. Representative phases of the gametophytic development of *Diplazium caudatum*. A. Spore germination; B. Filamentous phase with the first rhizoid; C. Spatular phase; D. Detail of the first meristematic cell; E. Beginning of the cordate phase; F. Presexual cordate phase. Bar = 10 µm A and D; 12 µm in B; 85 µm in C, E and F.

In the preparations made 38 days after sowing with adult gametophytes, we observed unisexual prothallia with archegonia (Figs. 5A & B). During that period, some of the unisexual females have developed antheridium becoming bisexuals.

Nevertheless, the gametophytes originated vegetative proliferations of premature development in a spatular phase. These structures have been observed in a variable number (2-5) in both types of sexual gametophytes (female and bisexual) (Fig. 5C), showing transparent colour. These proliferations had a variable width between 195 µm and a length of approximately 500 µm.

As for the vegetative characters, 75 days after sowing, trichomes were observed in most of the adult gametophytes. They appeared both at the margins of the gametophyte and in the middle of the lamina and were approximately 45 µm long (Fig. 5D & E).

The rhizoids emerged from the most basal and even pleural areas of the gametophyte (Fig. 5F).

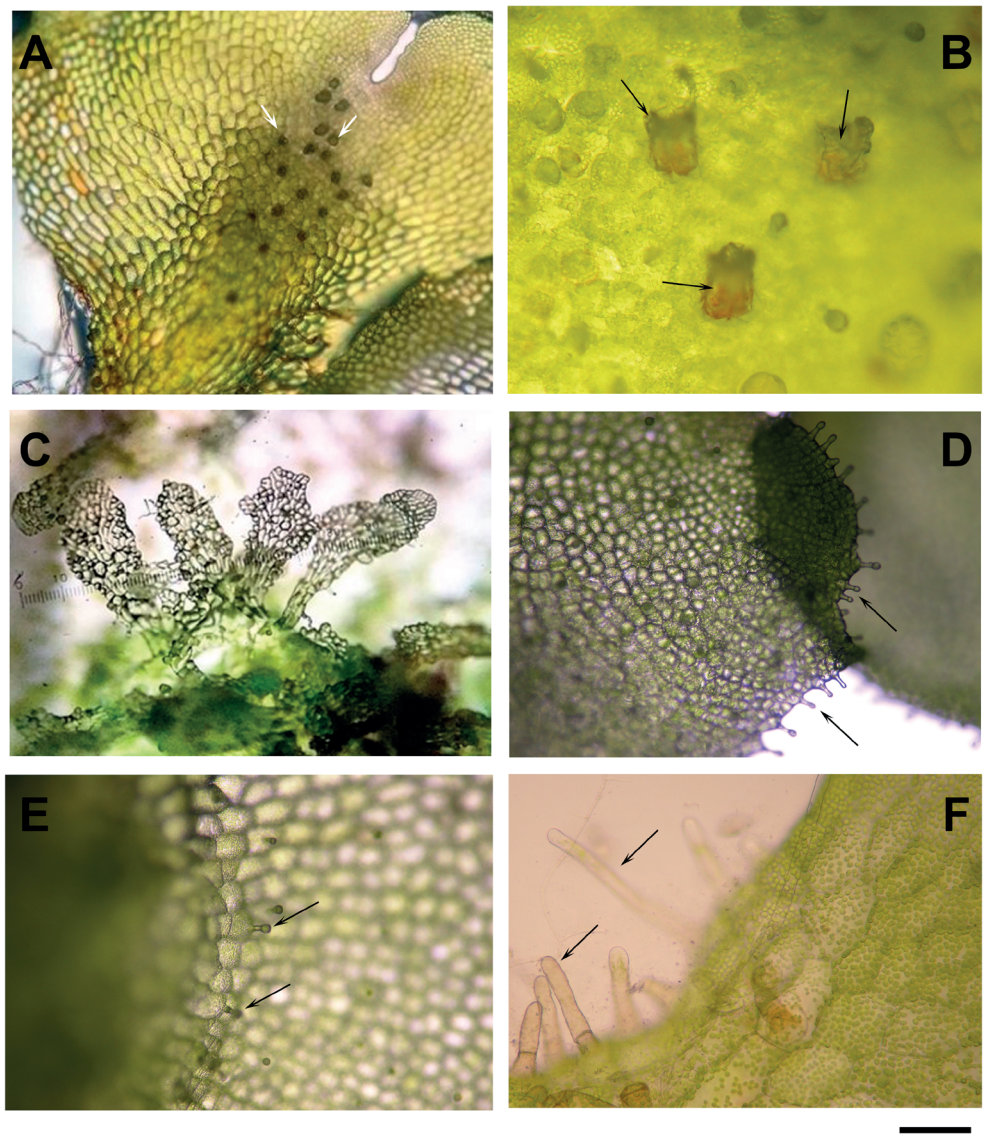

Figure 5. Morphological and reproductive characters in the gametophyte of *Diplazium caudatum*. A. Mature gametophyte with archegonia (arrows); B. Detail of archegonia (arrows); C. Vegetative proliferations of a gametophyte; D & E. Detail of the trichomes (arrows); F. Detail of the rhizoids (arrows). Bar = 85 µm in A and C; 46 µm in B and F; 198 µm in D; 495 µm in E.

Discussion

The spores observed for *D. caudatum* coincide with what was previously known for the species in terms of size and ornamentation (Ormonde 1986; Sánchez-Velázquez 1996). Such smooth or slightly rough ornamentation has, in fact, been observed as a constant character for the entire genus (Chiu et al. 2006; Guo et al. 2010; Liu et al. 2000; Norman et al. 2015).

Vittaria type of growth and *Adiantum* type of prothallic development has already been observed in other species of the same genus in previous studies, so it seems to be a common character in the genus, as well as the cordate shape of the gametophyte (Chiou et al. 2006; Dai et al. 2007; Nayar & Kaur 1971).

Germination began after five days. This was also observed by Norman et al. (2015) in *Diplazium esculentum* (Retz.) Sw., which they reported as an extraordinarily rapid growth compared to other genera such as *Asplenium* L. However, germination at five days, appearance of filamentous phase after one week and first cordate gametophytes around 25 days after sowing seems to be the usual dynamics of all species that have been cultivated in the genus *Diplazium* (Chiu et al. 2006; Guo et al. 2010; Norman et al. 2015). Only in the species *Diplazium proliferum* (Lam.) Thouars a much slower response was observed: 35 days (Golamaully et al. 2015), although it is not clear if this refers to the first germination or the first cordate gametophytes.

Although the presence of trichomes is characteristic of species with Aspidium-type development, whose prothalli have trichomes from the early stages, in species with other development patterns thichomes have been observed from the adult vegetative or sexual stage (Huang et al. 2001; Chambi &

Martinez 2020). In our study as for the appearance of trichomes, we can say that in the study species they appeared in the last phases of development, that is, when the prothallus had already reached the adult stage, with a remarkable frequency in most of the gametophytes observed. In other works of the same genus as in *Diplazium megaphyllum* (Baker) Christ or *Diplazium pinfaense* Ching the adult gametophytes were naked (Chiou et al. 2006; Guo et al. 2010). On the other hand, some species such as *Diplazium stellatopilosum* (Brause) Holttum do present trichomes in their adult phase and it is a species-specific character (Nayar & Kaur 1971). Momose (1967) described the morphology of the gametophytes of *Diplazium thunbergii* Nakai, which present marginal glandular hairs, similar to what is presented in *D. werckleanum* Christ. (Pacheco & Riba 2003).

In relation to the gametangia, archegonia mostly appeared in the central part of the gametophyte lamina, from the base to the notch, without the anomalies observed in other species of the genus (Chiou et al. 2006). The antheridia were typical of leptosporangiate ferns (Nayar & Kaur 1971).

The transparent colour of the vegetative proliferations possibly was caused by dehydration of the plate in which they were housed (Fig. 5C)

It is known that the mode of propagation (sexual or vegetative) of gametophytes changes according to the moisture and accessibility of nutrients in the microenvironment in which they grow (Atallah & Banks 2015; Bell 1992). We observed the formation of archegonia first, later some of them developed antheridia becoming bisexual. Nevertheless, sexual expression in *Diplazium* is highly variable. In fact, it has been previously observed that gametophytes of the genus *Diplazium* show plasticity with respect to reproductive efforts in stressful environments (Green & McCarthy 1999), as well as high tolerance to desiccation, frost and changes in their reproductive potential under stress (Green & McCarthy 1999; Sato 1982; Watkins et al. 2007). In a study with *Diplazium maximum* (D. Don) C. Chr., it was observed that with low levels of nutrients in the environment the gametophytes did not develop sporophytes, opting instead for asexual reproduction (Sareen et al. 2019).

Other studies have shown that the survival and germination rate of other *Diplazium* species increased by up to 95% by adding soil from the natural environment (Houser et al. 2016). Likewise, conservation studies suggest that it is advisable to use some type of prior sterilization in the spores, and that this way the success rate in terms of sporophyte formation increases (Golamaully et al. 2015).

In future research it would be interesting to cultivate with a lower density of spores, vary the photoperiod, humidity, temperature and light intensity, and/or substitute the substrate with another different from the one used in this work in order to make a comparison with those grown in mineral agar.

Conclusions

In this study we present a first approach to the study of the gametophytic phase of the species *Diplazium caudatum*. It has been observed that the dynamics of germination follows the times observed for other species of the genus. Likewise, we have observed a germination type *Vittaria* and a development type *Adiantum*.

We have described, for the first time, the morphology of the gametophytes of *D. caudatum*, showing that they present trichomes and the capacity to form vegetative outgrowths.

We have not observed sporophyte formation.

Acknowledgements

We want to thank our teacher and mentor José María Gabriel y Galán, with whom we started this project, for all his help and contributions without which this study and so many others would not have been possible.

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