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DISTINCTIVE PATTERNS OF CALLOSE DEPOSITION PROVIDE AN EARLY SIGN OF OVULE ABORTION[1] IN THE ANDROMONOECIOUS SHRUB *CAESALPINIA GILLIESII*

A. CALVIÑO [2] y C. CARRIZO GARCÍA [2]

Summary: Ovule abortion may lead to the partial or complete reduction of the number of functional ovules in the ovary and then is the primary factor that determines the sexual expression of the plant. In different species, ovule abortion was associated with the presence of callose in advanced ovules. Based on these observations, we studied the pattern of ovule development and callose deposition in both perfect and staminate flowers of the andromonoecious shrub *Caesalpinia gilliesii*. The obtained results showed that all ovules reached the 8-nucleate stage of embryo sac development and exhibited callose deposition at advanced stages. Nevertheless, from the 2-nucleate stage, a distinct spatial pattern of callose deposition within the ovule was observed between perfect and staminate flowers. Differences in callose deposition suggest that not callose *per se* but its distribution pattern in the ovule tissues is the main factor that triggers ovule isolation and abortion and leads to flowers with non-functional pistils in this species. Furthermore, previous observed differential growth rates between perfect and staminate flowers support for resource depletion as the likely underlying mechanism of ovule fate in *C. gilliesii.*

Key words: *Caesalpinia gilliesii*, ovule sterility, callose, nucellus degeneration, sexual expression.

Resumen: Patrones distintivos de deposición de calosa proveen evidencia temprana de aborto de óvulos en el arbusto andromonoico *Caesalpinia gilliesii*. El aborto de óvulos puede llevar a la reducción parcial o completa de óvulos fértiles en el ovario, pudiendo ser entonces un factor primario que determine la expresión sexual de toda la planta. En diferentes especies de plantas, el aborto de los óvulos ha sido asociado con la presencia de calosa en estadios avanzados del desarrollo de los óvulos. Basado en estas observaciones, se estudiaron el desarrollo de los óvulos y el patrón de deposición de calosa tanto en flores perfectas como estaminadas en el arbusto andromonoico *C. gilliesii*. Los resultados obtenidos muestran que los sacos embrionarios alcanzan el estadio de desarrollo 8-nucleado en todos los óvulos, y que presentan deposición de calosa en los estadios avanzados. Sin embargo, desde el estadio 2-nucleado, se observó un patrón espacial distintivo de deposición de calosa dentro del óvulo entre las flores perfectas y estaminadas. Las diferencias en la deposición de calosa sugieren que no sería la calosa *per se* sino su patrón de distribución en los tejidos de los óvulos el principal factor desencadenante del aislamiento del óvulo y su aborto, y por ende de la pérdida de la funcionalidad del gineceo en algunas flores de la especie. Además, observaciones previas de las tasas de crecimiento diferencial entre flores perfectas y estaminadas sugieren que el mecanismo subyacente al aborto de óvulos en *C. gilliesii* estaría vinculado al agotamiento de recursos en la inflorescencia.

Palabras clave: *Caesalpinia gilliesii*, óvulos estériles, calosa, degeneración de la nucela, expresión sexual.

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[1] In a strict sense, ovule abortion indicates a developmental interruption of the ovule that leads to a non-functional structure. There is, however, discrepancy on when the function of the ovule is completed. Considering that the final function of the ovule ends with the production of mother megaspore cells further developmental interruption may not be considered as abortion. Nevertheless, in order to compare our results with those of other studies on this matter, we need to adopt a broader sense. Therefore, ovule abortion refers here to ovule fate in any developmental stage.

[2] Instituto Multidisciplinario de Biología Vegetal (IMBIV-CONICET). CC 495 CP X5000JJC. Tel-Fax 00543514332104. Córdoba, Argentina. Corresponding author: anacalv@imbiv.unc.edu.ar

INTRODUCTION

Ovule abortion may occur in certain ovules of the ovary and therefore the female function of the flower is maintained by fertile ovules, or it can affect all ovules within the ovary leading to a flower with non-functional pistils. Histological observations on diverse taxa reveal different features linked with ovule abortion in flowers, and particularly, with the involvement of callose in ovule abortion in both monoecious and dioecious species (Pimienta & Polito, 1982; Rodrigo & Herrero, 1998; Rosellini *et al.*, 1998, 2003; Teng *et al.*, 2006). As a general rule, callose is present during meiosis of the megaspore mother cell (MMC), and later disappears as non-functional megaspores degenerate and the surviving megaspore divides (Rodkiewicz, 1970; Williams *et al.*, 1984 and references therein). Nevertheless, the failure of callose dissolution after MMC meiosis or the presence of callose in advanced ovules is considered abnormal, and related to ovule sterility (Pimienta & Polito, 1982; Vyshniakova, 1991; Rodrigo & Herrero, 1998; Rosellini *et al.*, 2003; Sun *et al.* 2004).

The influence of callose on ovule abortion was studied in staminate flowers of dioecious plants (Rosellini *et al.*, 1998, 2003), and in perfect flowers of other species that abort either one (Pimienta & Polito, 1982; Rodrigo & Herrero, 1998) or all ovules in the ovary (Teng *et al.*, 2006). In those species, callose appears associated with an interruption of ovule nutrition that leads to ovule abortion by starvation (Pimienta & Polito, 1982; Rodrigo & Herrero, 1998; Rosellini *et al.*, 1998, 2003). Changes in the intraovular reserves of the ovule seem to trigger callose deposition and subsequent abortion, suggesting the influence of resource constraints in ovule abortion (Pimienta & Polito, 1982; Rodrigo & Herrero, 1998).

Caesalpinia gilliesii (Hook.) Dietr. (Fabaceae) is an andromonoecious shrub that produces inflorescences comprising entirely staminate (i.e., with non-functional pistils) or perfect flowers, in addition to others with a mixed array of both flowers types (Cocucci *et al.*, 1992; Jausoro & Galetto, 2001). Factors that can be related to a lower resource availability, such as thinner stem diameter and lower floral growth rates, have been observed in inflorescences of *C. gilliesii* that produce staminate flowers only (Calviño & Carrizo García, 2005). Hence, like in other andromonoecious species (e.g., Diggle, 1991a; Gibbs *et al.*, 1999), the arrest of ovules in staminate flowers of *C. gilliesii* can be attributed to resources shortage within the inflorescence (Calviño & Carrizo García, 2005). Because callose has been repeatedly observed in abortive ovules and it was linked to resource depletion on species with different reproductive systems, the study of callose deposition on ovules of *C. gilliesii* may help to elucidate the factors involved in the arrest of ovules and in the expression of female sterility in certain flowers.

Here, the comparative pattern of ovule development and callose deposition was analyzed in staminate and perfect flowers of the andromonoecious species *C. gilliesii*. The aims of this study were to identify the stage of ovule abortion and address if callose deposition within the ovule could account for ovule abortion in staminate flowers in *C. gilliesii*. The results are interpreted in relation to previous research on this topic.

MATERIALS AND METHODS

Staminate flowers of *C. gilliesii* have an atrophied pistil (Jausoro & Galetto, 2000) which is generally 7-10 times shorter than that of perfect flowers, and contain smaller, papery ovules (Calviño & Carrizo García, 2005). Sexual dimorphism of *C. gilliesii* flowers relies on pistil and ovule size differences, since allocation to staminate structures (i.e, pollen size or number) did not differ between flower morphs (Calviño & Carrizo García, 2005).

Based on a previous allometric study we were able to distinguish inflorescences with staminate flowers only (i.e. those where the pistil growth is interrupted), from those with perfect flowers (Calviño & Carrizo García, 2005). Then, buds of staminate and perfect inflorescences provided a sequence of developmental stages for staminate and perfect flowers.

Whole inflorescences bearing buds and flowers of varying developmental stages were sampled from a natural population of *C. gilliesii* in Cordoba Province, Argentina, during September 2003. The inflorescences were fixed in ethanol-acetic acid (3:1) for 24 h and stored in ethanol 70%. A total of 50 flowers and buds from 7 female sterile inflorescences and 20 from 3 perfect inflorescences were observed. Ovaries from flowers and buds of every inflorescence were separated and ordered in a developmental sequence according to their position along the inflorescence.

Due to the presence of abundant stiff hairs on the ovary surface, pistils were pre-treated with HCl 20% in distilled water for 16-20 hours; ovaries were then washed with distilled water, dehydrated in an ethanol-xylol series, and embedded in Paraplast (Sigma, USA). Serial cross-sections of 6-10 µm were stained with Activated Haematoxylin (Biopur, Argentina) for light microscope observations. A subset of sectioned ovules of different developmental stages was stained with Aniline Blue (0.1%) in potassium acetate to detect the presence of callose under UV light (Martin, 1959) in a Zeiss Axiophot fluorescence microscope at λ 365 nm. These ovules were subsequently stained with Activated Haematoxylin and observed under a light microscope.

RESULTS

Ovule development in perfect flowers

The fully developed ovules of *C. gilliesii* are anatropous, crassinucellate, and bitegmic, with a zig-zag micropyle. The embryo sac follows the *Polygonum*-type of development, with the mature embryo sac comprising two synergids, the egg cell, the central cell and three small antipodal cells at the chalazal end (Fig. 1 A). Callose was not detected around the megaspore mother cell (MMC), but there was a group of nucellar cells at the chalazal end with callose deposited in their walls (Fig. 1 B). The presence of callose was also observed post meiosis in the wall plates that separate the four megaspores (Fig. 1 C). These callose plates disappear after the degeneration of the three non-functional megaspores at the micropylar end. Nevertheless, and although with only a weakly defined fluorescent reaction, callose at the chalazal end persisted throughout embryo sac development (Figs. 1 C-G). The functional megaspore divided normally, and the 2-nucleate, 4-nucleate and finally the 8-nucleate embryo sac stages were observed (Fig. 1 G). Callose was not evident elsewhere in the ovules (Figs. 1 B-G).

Ovule development in staminate flowers

Ovules from staminate inflorescences are also bitegmic, with a zig-zag micropyle. Meiosis of the MMC occurs normally (Fig. 2 A, B), and the callose plates that separate the products of meiosis disappeared later as in the ovule of perfect flowers. Subsequent development of the embryo sac followed the same steps observed in fertile ovules (Figs. 2 C, E, G), but no nucellar cells staining for callose were observed at the chalazal end (Figs. 2 B, D, F, H). In contrast to fertile ovules, the presence of callose was evident from the 2-nucleate stage (Fig. 2 D) onwards in the innermost part of the nucellus, in cells that were in contact with the embryo sac. This callose was hardly appreciable at the 2-nucleate stage (Fig. 2 D), but staining became gradually stronger in the subsequent stages (Figs. 2 F, H, I) until it completely occupied the nucellar cells around the embryo sac (Figs. 2 H-J). Despite this progressive deposition of callose in the surrounding nucellus, the 8-nucleate stage was completed (Fig. 2 G), and the synergids, egg cell, central cell, and antipodals were differentiated. Evident signs of ovule abortion were observed later: the nucellus and inner integument degenerated, and the integuments separated from each other, so that the embryo sac became spatially isolated (Figs. 2 J, K). Thereafter, the ovule acquired a necrotic appearance (Figs. 2 J, K) and the embryo sac structure became disorganized. At flowering, only remnants of the embryo sac were observed (Figs. 2 K, L).The timing of abortion was uniform among the ovules and flower buds observed.

DISCUSSION

The arrest of a floral sexual organ can occur at any stage of development (Dellaporta & Calderon-Urrea, 1993). In the andromonoecious *Solanum* species studied, the time of ovule abortion is variable even within a species and it ranges from the MMC to the 8-nucleate stage (Martin, 1972; Dulberger *et al*., 1981; Diggle, 1991*a*, *b*). Ovules of *Caesalpinia gilliesii* follow the *Polygonum*-type of development, and the characteristic 8-nucleated embryo sac is reached in both fertile and sterile ovules. Ovule abortion in staminate flowers is only evident later, and this was uniform among all ovules observed either within the same ovary, or in different ovaries. Furthermore, all abortive ovules exhibit a consistent pattern of callose deposition that differed from that observed in fertile ovules.

As usually observed in several species, callose

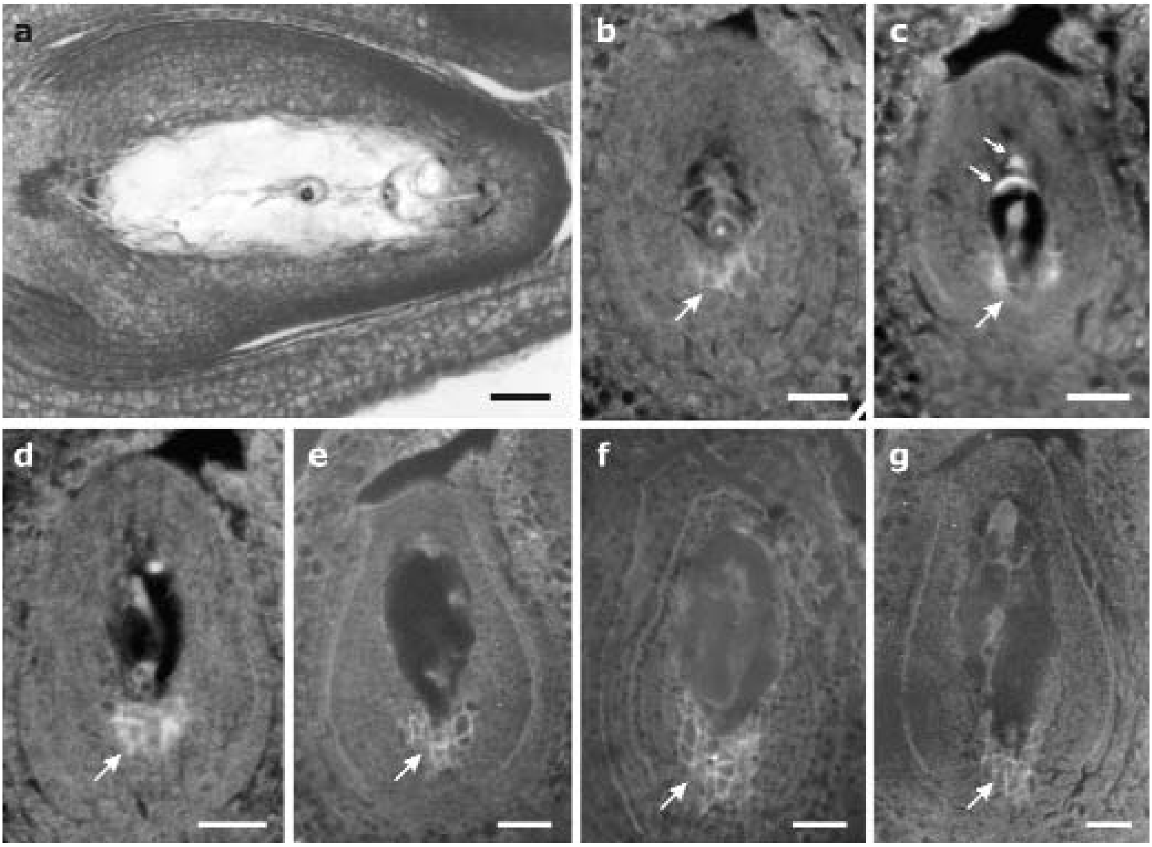

Fig. 1. Ovule development in perfect inflorescences of *Caesalpinia gilliesii*. **A:** mature embryo sac from flower stage. **B-G:** stages of embryo sac development. **B:** megaspore mother cell. **C:** functional megaspore definition and degeneration of non-functional megaspores. **D:** two-nucleated embryo sac. **E:** four-nucleated embryo sac. **F:** eight-nucleated embryo sac. **G:** mature embryo sac from an advanced flower bud. Observe the presence of callose in the nucellar cells of the chalazal end (arrows) in all the stages (B-G), and the callose plates (small arrows; only two observable) between megaspores in C. **A:** bright field, stained with Activated Haematoxylin. **B-G:** fluorescence light, stained with Aniline Blue. Bars. **A-G:** 40 µm.

plates between megaspores disappear in both fertile and sterile ovules of *C. gilliesii*. Nevertheless, callose appears again at more advanced stages of ovule development. In abortive ovules, callose exhibited a progressive deposition in the inner cells of the nucellus from the 2-nucleate stage embryo sac development onwards, but in fertile ovules callose was only observed at the chalazal end. This region, sometimes referred to as the hypostase, has been assumed to regulate nutrients and/or water transport to the ovule (Newcomb, 1973; Boesewinkel & Bouman, 1984; Tiwari, 1984; but see Kapil, 1974). Furthermore, it has been suggested that callose deposition in cells of the chalazal end could block the translocation of substances into the ovule, leading to its starvation (Pimienta & Polito, 1982; Rodrigo & Herrero, 1998). In the fertile ovules of *C. gilliesii* nevertheless, callose was consistently observed at the chalazal end and hence, embryo sac nutrition do not strictly depends from these cells in this species. In addition, degeneration of embryo sac and integuments in ovules of *C. gilliesii* female sterile flowers, was observed only after it was completely surrounded by a callose layer of nucellar cells indicating that callose would not provoke embryo sac abortion until a complete ‘barrier’ was formed.

This differential pattern of callose deposition between fertile and sterile ovules was previously observed but on separate species. A punctual callose deposition is frequently observed in different regions of fertile ovules such the mycropilar end (Bittencourt & Mariath, 2002), the filiform apparatus and the hypostase (Williams *et al.*, 1984; Punwani & Drewss, 2008), and in the chalazal region of the protruding embryo sac (Tiwari, 1978). On the other

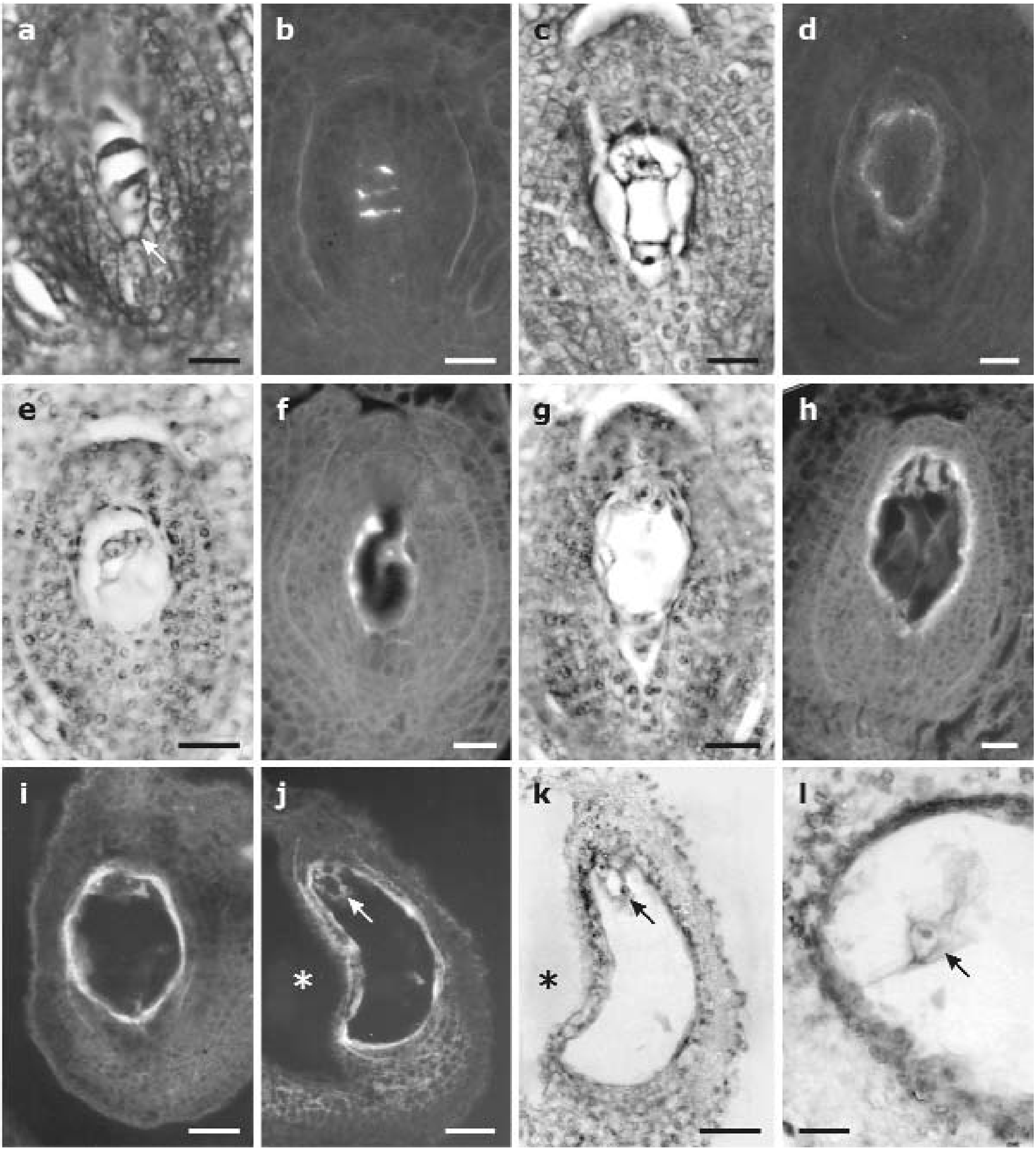

Fig. 2. Ovule development in staminate inflorescences of *Caesalpinia gilliesii*. **A, B:** functional megaspore differentiation and degeneration of non-functional megaspores. Observe the callose plates between megaspores in B. **C, D:** two-nucleated embryo sac. **E, F:** four-nucleated embryo sac. **G:** eight-nucleated embryo sac (only six nuclei are observable in this section). **H:** mature embryo sac. **I-L:** abortive ovules. **D, F, H, I, J:** increasing amounts of callose deposited in the inner surface of the nucellus, surrounding the embryo sac. **I, J:** callose around the embryo sac; nucellus, integuments, and embryo sac degenerated. **J, K:** separated integuments (asterisk in the space formed) and the rests of the degenerated embryo sac (arrow). **L:** isolated nucleus of a degenerated embryo sac (arrow). **A, C, E, G, K, L:** bright field, stained with Activated Haematoxylin. **B, D, F, H, I, J:** fluorescence light, stained with Aniline Blue. Bars. **A-D, F, H:** 25 µm. **E, G, L:** 15 µm. **I-K:** 50 µm.

hand, in abortive ovules of *Prunus* spp. callose first appears at the chalazal end but then progress until it completely surrounds the embryo sac (Pimienta & Polito, 1982; Rodrigo & Herrero, 1998). In addition, callose entirely surrounds the embryo sac of abortive ovules in female sterile *Medicago* cultivars

(Rosellini *et al*., 1998), and in staminate flowers of the dioecious vine *Vitis vinifera* (Caporalli *et al*., 2003), suggesting that the influential factor in ovule abortion is the spatial distribution of this substance around the embryo sac.

The influence of callose as an isolation mechanism has been observed in several plant species and on different developmental stages, nevertheless, it is not always related to the fate of the isolated structure. As an example, spatial pattern of callose distribution was important for the selection of the functional megaspore of some (Bittencourt & Mariath, 2002) but not all of the species studied (Moço & Mariath, 2003). Similarly, callose forms a continuous layer around the abortive ovule of *Prunus americana* (Rodrigo & Herrero, 1998) and the abortive zygotes of *Pissum sativum* (Briggs *et al*., 1997), but may also act as a helpful isolation mechanism of the normal zygote development in other plant species (Williams *et al*., 1984). Hence, spatial distribution *per se* does not necessarily account for ovule fate in all plant species.

In *C. gilliesii,* ovule abortion during staminate flower development was associated with a continuous callose layer that isolates the degenerating embryo, and thus this pattern may be considered an early sign of ovule abortion in this species. According to the available evidence for different plant species, however, other mechanisms should be involved in the final ovule fate. In this regard, it is known that plant reserves are compartmentalized at different levels of plant organization, from the ovule itself to the whole plant (Rodrigo & Herrero, 1998 and references therein), and that selective forces acting at higher morphological levels may drive the final sexual expression at lower modular levels (Cox, 1988). Therefore, because a lower resource availability seems important for staminate inflorescences in *C. gilliesii* (Calviño & Carrizo García, 2005), we expect that the observed pattern of callose deposition in abortive ovules is linked to a resource depletion mechanism. Nevertheless, further studies are necessary to identify the underlying mechanisms that trigger ovule abortion in *C. gilliesii*.

Finally, since selection of embryos may directly influence the quality of the remaining gametophytes (Bernasconi *et al*., 2004), future studies should also consider the direct implications of ovule fate in current reproductive costs, particularly in andromonocioues species, where ovule abortion is considered to be a less costly mechanism than aborting zygotes (Lloyd, 1980).

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