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Flower anatomy related to blooming development of *Berberis microphylla* G. Forst (*Berberidaceae*)

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Abstract: *Berberis microphylla* G. Forst. is a Patagonian native shrub commonly named "calafate", which has a growing economic potential due to its dark blue berries that are consumed fresh, as jams and preserves, and are used for the production of soft drinks and ice cream. Moreover, the fruits have a high content of carbohydrates, phenols and antioxidants. The objective of this work was to show the changes observed in the flower from the emergence in relation to the floral phases and the importance that they have on pollination and fertilization. During the anthesis, the nectar is excreted inside and outside of the petal through the epidermis of the secretory tissue. The epidermis of the stigma is papillae with cells of greater length in the periphery of this structure simulating an additional ring. Secretory tissue is also present on the area of the fusion carpel. During anthesis, the epidermis glands of the stigma showed active secretion and these conditions favor pollen grain germination. Germinated pollen grains were observed after 12 hours of pollination and ten days later the pollen tube reached the ovule area. Pollen tube grew surrounded the ovules and probably some of them already accomplished the fertilization.

1. Introduction

Berberis microphylla G. Forst. is a Patagonian native shrub commonly named "calafate", with a large distribution from Neuquén (37° SL) to Tierra del Fuego (54° 8´ SL) (Orsi, 1984). This species has a growing economic potential due to the production of fruits as a non-timber forest product (Tacón Clavaín, 2004). In fact, its dark blue berries are consumed fresh, as jams and preserves, and are used for the production of soft drinks and ice cream. Moreover, the fruits have a high content of carbohydrates, phenols and antioxidants (Arena and Curvetto, 2008; Arena *et al*., 2011, 2012, 2013 b). The genetic and morphological analysis of spontaneous accessions in natural populations of *B. microphylla* grown on Tierra del Fuego (Giordani *et al*., 2016), as well as the changes in form and leaf anatomy due to weather conditions (Radice and Arena, 2015) were recently studied.

The study of flower anatomy related to blooming is an important step for programs of genetic resource conservation and improvement, complementing basic studies of floral biology (de Castro Nunes *et al*., 2012; Wetzstein *et al*., 2014). Flower structure and floral biology was well described by Arena *et al*. (2011), like the phenological stages (Arena *et al*., 2013 a) and flower bud differentiation (Arena and Radice, 2014). More recently, a comprehensive study of pollen grain was published (Radice and Arena, 2016 a).

Nevertheless, pollination was not clear until now. Fertilization of Patagonian *Berberis* has been classified as cross-pollination by Orsi (1984). However, Hegi (1958) and Romeo *et al*. (2005) referred the *Berberis* species as autogamous due to the absence of visiting insects together with extreme climatic conditions prevalent in most areas of Patagonia. However, during flowering, the activity of different *Syrphidae* was observed (Radice *et al*., 2016). On the other hand, floral movements have been appointed as mechanisms to facilitate self-pollination (Darwin, 1862). Stamen movement has been documented in a few plant families, among them *Berberidaceae* (Lechowski and Bialczyk, 1992). However, results of controlled treatments of self- and cross-pollination compared with those of open-pollination performed during three different periods in *B. microphylla* do

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not support this hypothesis (Radice and Arena, 2016 b). Thus, self-pollination resulted only in pollen germination on the stigmas but the pollen tubes were not able to reach the ovules. According to these antecedents, anatomical studies are necessary for a better understanding of the physiological processes during floral biology and pollination that interact with *Syrphidae* activity. The objective of this work was to show the changes observed in the flower from its emergence in relation to the floral phases and the importance that they have on pollination and fertilization.

2. Materials and Methods

Plant material

Flowers of *Berberis microphylla* G. Forst. (*n = 20*) on growth stages ranging from 53 to 68 on the BBCH scale proposed for *B. buxifolia* (Arena *et al*., 2013 a) were collected on plants grown near Ushuaia city, Tierra del Fuego (54° 48' SL, 68° 19' WL and 30 m asl), and were fixed in FAA (formaldehyde, 100 mL; ethyl alcohol, 500 mL; acetic acid, 50 mL; distilled water, 350 mL).

Light microscopy

Button flowers were dehydrated in an ethanol series and embedded in Spurr's resin. Thin sections (75-90 nm thick) were stained with uranyl acetate and lead citrate.

Fluorescent microscopy

Flowers fixed in FAA were washed with distilled water and softened with NaOH (8N) as described by Martin (1959). Then, they were stained with aniline blue to study pollen tube growth. Squash material was observed by a Leica microscope (DM 2500) with DAPI filter.

Scanning electron microscopy (SEM)

Button flowers fixed in FAA were dehydrated in an ethanol series and critical point drying technique was employed. Samples were sputter coated with 20 nm gold and observed with a Philips XL 30 SEM.

Ovules and seeds relation

The number of ovules on button flowers (*n= 130*) and the number of seeds on formed fruits (*n=100*) were counted.

3. Results

Pistil of *B. microphylla* is similar to a bottle (Fig. 1A). Flowers before anthesis (growth stage 54) showed anthers with microspores at an advanced stage of development and underdeveloped ovules (Figs. 1A-D). In effect, microsporangia contain tapetal cells metabolized, i.e. a thick portion was deposited on the wall of the microspore and inside it is possible to observe vegetative and generative cells that are surrounded by a thin delicate wall (Fig. 1D). On the other hand, ovules are rudimentary with an active cellular proliferation on the nucellus and integuments (Fig. 1C).

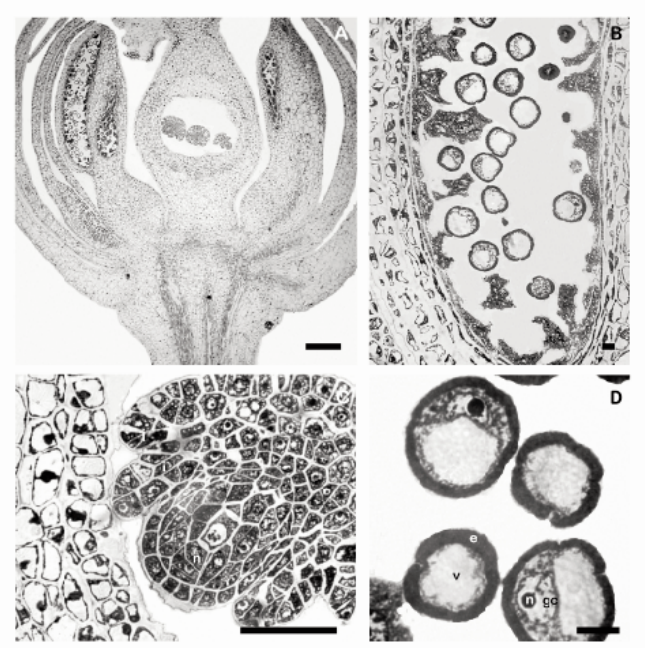

Fig. 1 - Bottom flower of *B. microphylla* G. Forst (light micrograph). A, longitudinal section of a flower on stage 54. B, anther with mature pollen grains; C, rudimentary ovule with nucellus (n) and integuments (i) in development; D, detail of pollen grain with exine (e), Vacuole (v) and generative cell (gc) contained into the vegetative cell; n= nucleus. Bars: A = 200 µm; B, D = 10 µm; C = 50 µm.

The epidermis of the stigma is covered by secretory cells (Figs. 2 A, C, E) and the short style is also recovered by glands (Fig. 2B). Petals have two thick nectaries on the basal position (Fig. 3A). Both petal epidermises are also formed by glandular cells (Figs. 3B, D, E, F). On a later flower phase (growth stage 59), the epidermis and nectariferous cells of the nectar have dense cytoplasm and conspicuous nucleus (Figs. 3C). Nectar is abundant in the intercellular spaces (Fig. 3C). Flowers on the anthesis phase (growth stage 60) showed nectariferous cells with a gradual diminution of staining density (Fig. 3G). Total production of nectar per flower is poor; it is secreted through the gland cells that are present on the epidermis of the nectaries (Figs. 3C, G) and two epider-

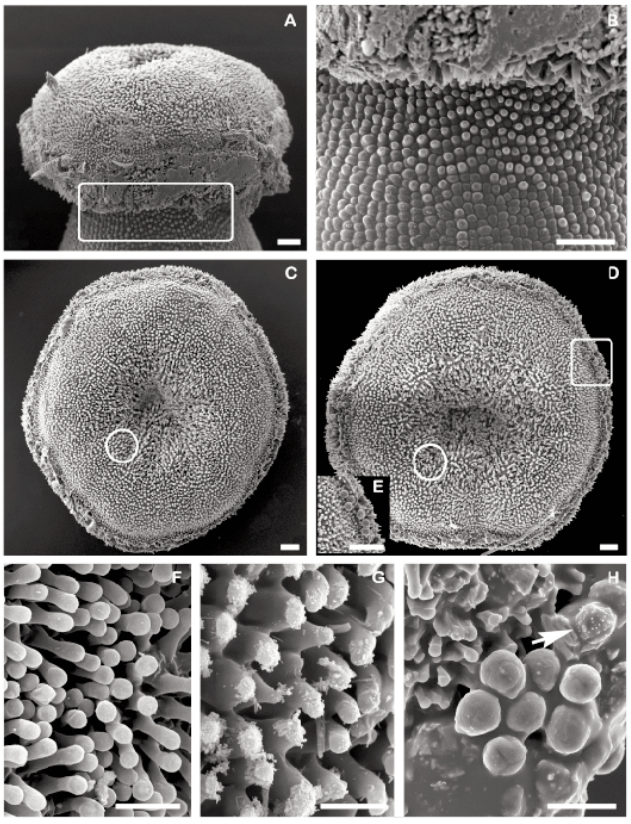

Fig. 2 - SEM micrograph of the pistil of *B. microphylla* G. Forst. A, stigma of a flower on stage 59; B, detail of box in the picture A, periphery of the upper end of the ovary with glands; C, stigma front view shown in A; D, stigma front view shown of a flower on stage 60; E, detail of box in the picture D, long hairs peripheral stigma with pollen grains attached; F, detail of circle in the picture C, epidermal cells of stigma; G, detail of circle in the picture D, active secretions glands; H, mixture of pollen grains wrapped in stigmatic mucilage, arrow points to a grain of foreign pollen. Bars: A-E =100 µm; F-H= 50 µm.

mal layers of petals (Figs. 3E,F). Nectar is exuded through the cuticle with rupture of its outer layer. Greater presence of vacuoles is observed in both nectar tissues (Fig. 3G).

Flowers on growth stage 59 showed a monocarpellary pistil with a clavate shaped stigma (Fig. 4A). Epidermis of the stigma is papillae (Figs. 4B, C, E) with cells of greater length in the periphery of this structure simulating an additional ring (Fig. 4E). These cells secrete a sticky substance that keeps always the stigma hydrated during the anthesis phase (Fig. 2F). Secretory tissue is also present on the area of the fusion carpel (Figs. 4B, D). On the other hand, stigmas on growth stage 59 are receptive, i.e. they can promote pollen germination while stigma on less developed growth phases is not receptive (Fig. 2E).

Flowers on anthesis phase (growth stage 60)

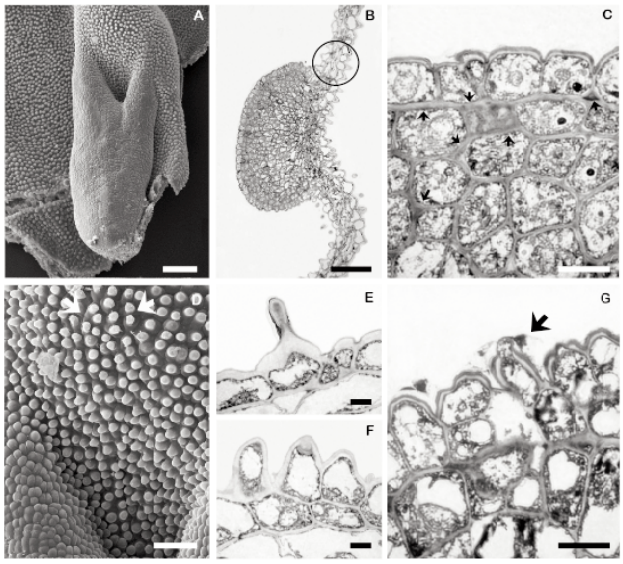

Fig. 3 - Nectary of *B. microphylla* G. Forst. A-D SEM micrograph. B-C, E-G light micrograph. A, nectary view at the base of petal; B, section of a petal and a nectariferous area; C, detail of tissue of nectariferous area of a flower on stage 59; intercellular space with nectar (arrows); D, detail of circle in the picture A, epidermis with glands, arrows show secretory glands. E, detail of circle in the picture B, petal outer epidermis; F, detail of circle in the picture B, petal inner epidermis; G, detail of tissue of nectariferous area of a flower on stage 60, arrow indicates an epidermal cell in active secretion. Bars: A-B= 100 µm; C= 10 µm; D= 50 µm; E-G= 10 µm.

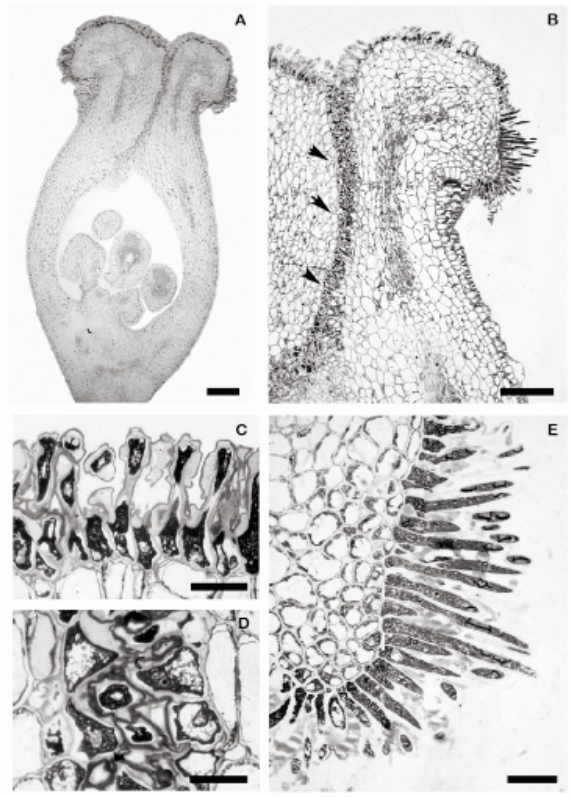

Fig. 4 - Light micrograph of the pistil of *B. microphylla* G. Forst. A, longitudinal section of a flower on stage 59.B, view of stigma and the upper end of the ovary; C, glandular cells of the flat part of stigma; D, detail of glandular cells of fusion area of the carpel; E, detail of hairs surrounding the stigma. Bars: A= 100 µm; B= 200 µm; C-E= 50 µm.

showed mature pollen grain with a well-formed external wall and the cytoplasm of the vegetative cell rich of starch (Fig. 5E). In this phase anthers are dehiscent. On the contrary, ovules are externally coated by the integuments and attached to the ovary by the funiculus on basal placentation (Fig. 5D). Internally, the ovules present some delay in comparison to the pollen grain development. Pollinated flowers show ovules with the embryo sac with egg cell, synergists, antipodes and polar nuclei cells developed (Figs. 5 A-C); nevertheless, no pollinated pistils show ovules with megaspore mother cells without development.

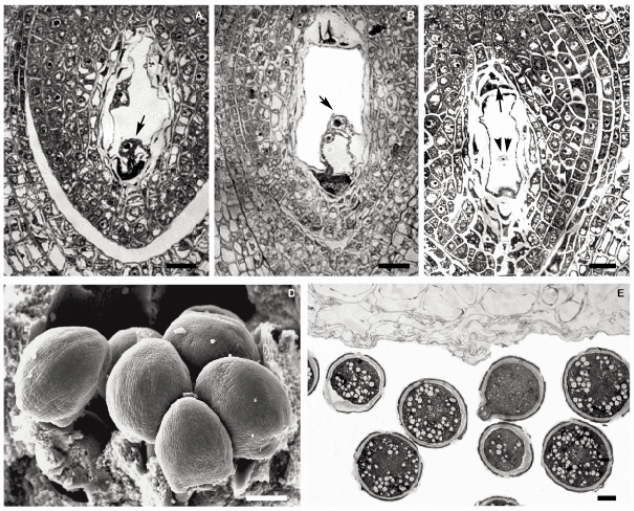

Fig. 5 - Details of the ovary and pollen grains of a flower on anthesis stage of *B.* G. Forst. A-C, embryo sac with egg cell (A, arrow), synergids (B, arrow) and antipodes (C, arrow) and polar nuclei (C, arrows head); D, SEM micrograph of ovules on the basal insertion of ovary; E, mature pollen grains with cytoplasm rich in starch grains. Bars: A-C= 200 µm; D= 100 µm; E= 10 µm.

Pollen is deposited on the stigma mainly between the secretory cells that surround this structure (Figs. 2D, G). Germinated pollen grains were observed after 12 hours from pollination (Fig. 6B). After 24 hours pollen tube crosses the stigma (Fig. 6A) and ten days later the pollen tube reached the ovule area. Pollen tube grew surrounded the ovules and probably some of them accomplished the fertilization (Figs. 7A, B).

Subsequently seed growth is observed (Fig. 7C) but this process is not given in all the ovules. In effect, on a selected natural population the ovules and seeds were counted, and an average of 8.95 (ranged from 6.9 to 10.0) and 5.28 (ranged from 3.3 to 7.2), respectively, were registered, i.e. 40.97% of all ovules produced aborted.

4. Discussion and Conclusions

Flowering plants are associated with a broad spectrum of animal pollinators, among these bees constitute an important but not exclusive one (Dötterl and Vereecken, 2010). In effect, it has already been demonstrated that *B. microphylla* is not self-fertile so it depends on insect pollination (Radice and Arena, 2016 b). Tierra del Fuego (Argentina) offers an extreme climatic situation where the bees cannot prosper; accordingly calafate flowers are visited by different syrphids (Radice *et al.,* 2016). Pollination by insects, including flies, is commonly a mutualistic interaction, in which both the plant and the insect benefit; thus, anatomical organization and pollination strategies developed on the flowers must be adapted to the environmental conditions.

Calafate shrubs bloom with abundant yellow flowers that produce aromatic nectar (Radice *et al.,* 2016), that exudates inside the flower as well as the outside through the petals mainly in their insertion

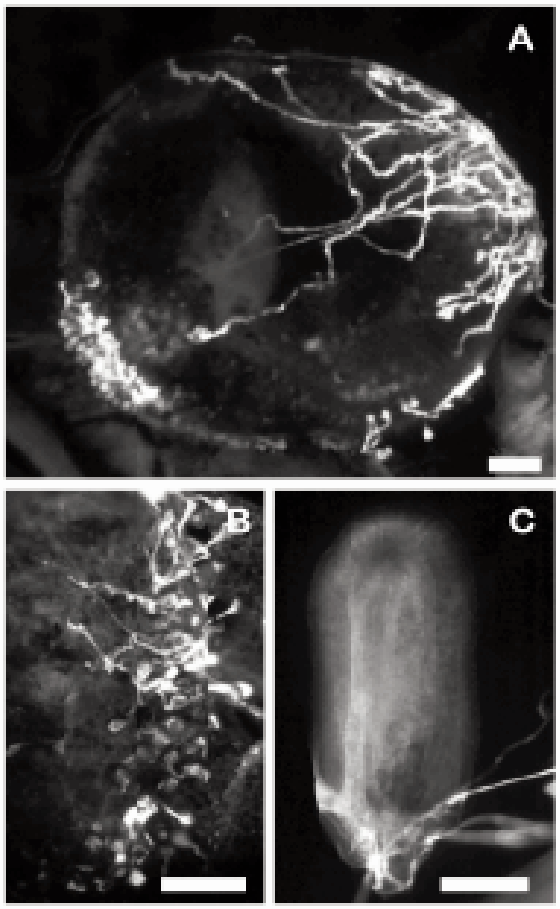

Fig. 6 - Fluorescent light micrograph of pollen tube germinated on pistils of *B. microphylla* G. Forst. A, view of stigma with pollen grain germinated and pollen tubes inserted in the ovary; B, pollen tubes on the first stage of growth; C, pollen tube penetrating the ovule. Bars: A-C= 100 µm.

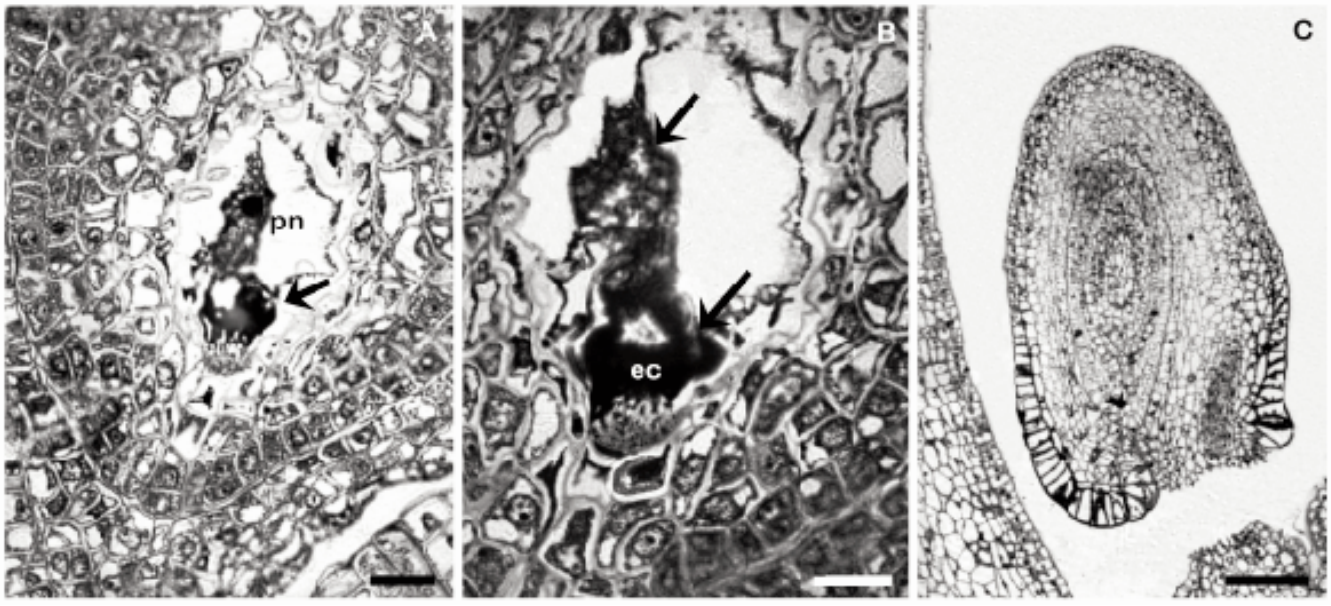

Fig. 7 - Fertility of *B. microphylla* G. Forst. A-B, time of discharge of sperm nuclei (arrows) into the egg cell (ec) and polar nuclei (pn), C, growing seed. Bars: A-B= 200 µm; C= 100 µm.

area. This particularity is very important because the fluorescent emission of nectar attracts pollinating insects. *B. microphylla* have perigonial nectaries type "1a" according to the topographic classification of Fahn (1982). Unlike what was found on the nectar tissue of *Berberis corymbosa* by Bernardello *et al*. (2000), no stomata were found on this species either on histological sections or through SEM observations. In effect, nectar is exuded through the epidermis as cited by Bernardello (2007). *Berberis* produce small amounts of nectar per flower; effectively it was registered less than 1 µl on *B. corymbosa* (Bernardello *et al*., 2000) and 1.57 µl on *B. microphylla* (Radice *al*., 2016). Nectar concentration was considered as intermediate for *B. buxifolia* (31.2±14.8%) (Chalcoff *et al*., 2006). This result is in coincidence with Radice *et al*. (2016) who measured 36.28 °Brix in nectar of the *Berberis* population studied.

Stigma epidermis is covered by hairs that secrete a stigmatic fluid to promote pollen germination. Surface hairs on the stigma can be seen in others species like *Papaver rhoeas*, or *Lupinus luteus* (Fahn, 1982). Once overcome the stigma, the pollen tubes grow through the carpel wall. In effect the margin of the carpel is covered by glands that nourish the germinated pollen. The *Berberidaceae* are generally considered to have originated in some part of the ranalian complex (Chapman, 1936), i.e., it is belonging to the group of the oldest dicotyledons which is confirmed by its structure devoid of carpelar style (Fahn, 1982).

There are three important elements to attract the pollinating insect on *B. microphylla* such as color, scent and nectar. It has long been known that bees utilize not only visual but also olfactory flower cues for finding suitable host plants (Dötterl and Vereecken, 2010). This pollination strategies present in calafate could be useful in other growth areas of the species. On the other hand, young flies in the presence of generic floral scent respond more strongly to a uniformly yellow cue than to any other uniform color cue (green, white, black, blue, red) except for ultraviolet (Brodie *et al*., 2015). Nectar is the most commonly sought reward by flower-visiting flies (Woodcock *et al*., 2014) because carbohydrates contained in the nectar provide short-term energy supply. So the abundant number of yellow flowers plus the fluorescent emitted cue by nectar must constitute a strong attraction for syrphids.

Acknowledgements

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