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RESEARCH PAPER

Pistil anatomy and pollen tube development in *Polygala vayredae* Costa (Polygalaceae)

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ABSTRACT

Low seed ovule ratios have been observed in natural populations of *Polygala vayredae* Costa, a narrowly endemic species from the oriental pre-Pyrenees. To evaluate physical and nutritional constraints and pollen tube attrition in this endemic species, stigma and style anatomy, as well as pollen tube development along the pistil were investigated using light and fluorescence microscopy. The structural morphology of the stigmatic region was also examined with scanning electron microscopy. Pollen grains that reached the stigmatic papillae came into contact with a lipid-rich exudate and germinated easily. Although a large number of pollen grains reach the stigmatic papillae, few pollen tubes were able to grow into the style towards the ovary. The style was hollow, with the stylar channel beginning a few cells below the stigmatic papillae. Initially, the stylar channel area was small compared to other levels of the style, and was surrounded by lipid-rich, highly metabolic active cells. Furthermore, lipid-rich mucilage was detected inside the stylar channel. At subsequent style levels towards the ovary, no major reserves were detected histochemically. The reduced intercellular spaces below the stigmatic papillae and the reduced area of the stylar channel at its commencement are suggested to physically constrain the number of pollen tubes that can develop. In subsequent levels of the style, the stylar channel could physically support a larger number of pollen tubes, but the lack of nutritional reserves cannot be disregarded as a cause of pollen tube attrition. Finally, the number of pollen tubes entering the ovary was greater than the number of ovules, suggesting that interactions occurring at this level play a major role in the final reproductive outcome in this species.

INTRODUCTION

After pollination, the success of pollen grains in ovule fertilisation depends on an array of complex physical and physiological interactions between the pistil and the number and origin of pollen tubes developing in the style (Knox 1984; Cruzan 1993; Mitchell & Marshall 1995; Hormaza & Herrero 1996).

The pistil is suited to support the germination of pollen grains and subsequent pollen tube growth. The stigmatic papillae supply an environment for pollen germination (*e.g.* Goldman *et al.* 1994 and references therein; Edlund *et al.* 2004) and the style provides the nutrition required for pollen tube development (*e.g.* Herrero & Dickinson 1979; Wang *et al.* 1993), while the ovary seems to be involved in directional signalling for pollen tube growth (*e.g.* Ray *et al.* 1997; Cheung & Wu 2001). However, during the progamic phase, the pistil may also impose constraints on pollen tube development. In the pollen tube pathway, a progressive reduction in the number of pollen tubes travelling along the style (*i.e.* pollen tube attrition) has been observed in several plant species (*e.g.* Cruzan 1986, 1989; Herscovitch & Martin 1990; Herrero 1992; Smith-Huerta 1997; Erbar 2003). After an initial period of autotrophic growth, pollen tube development becomes heterotrophic (Herrero & Dickinson 1981; Mulcahy & Mulcahy 1983 in Hormaza & Herrero 1996), therefore the reduction in number of pollen tubes could

be due to physical and/or physiological restrictions, resulting from a progressive decrease in the space and/or reserves available for pollen tube growth along the style (*e.g.* Heslop-Harrison *et al.* 1985; Lord & Kohorn 1986; Cruzan 1986, 1989; Herrero 1992). Alternatively, during the progamic phase, pollen tube attrition could result from close interactions among the male gametophytes (through differences in pollen competitive ability) and between the male gametophytes and the female tissues (through pollen–pistil genetic interactions). This series of events will determine which male gametophytes, among all the possible candidates, reach and fertilise the ovules, thus determining the final reproductive outcome for the plant.

The genus *Polygala* L. (Polygalaceae) comprises about 725 species distributed throughout the world. Species in *Polygala* present marked patterns of regional endemism and, according to the IUCN and WWF, they are well represented in diversification centres. Thus, *Polygala* is an important genus for conservation. Although its floral anatomy has been extensively studied for taxonomic and phylogenetic purposes (Mukherjee 1961; Dube 1962; Milby 1976; Eriksen 1993; Prenner 2004), there have been few studies on its pollination ecology (Brantjes & van der Pijl 1980; Norderhaug 1995; Weekley & Brothers 2006; Castro *et al.* 2008a) and on how anatomical and morphological features relate to floral functioning and reproductive outcome (Venkatesh 1956; Westerkamp & Weber 1997; Castro *et al.* 2008b).

In previous studies on the endangered *Polygala vayredae* Costa, we observed a reduced seed ovule ratio resulting in low offspring production. An external reduction in the diameter of the style (from the apical to basal portion) has also been observed (Castro *et al.* 2008a). Considering the importance of pre-fertilisation events in seed production and constraints imposed by the pistil in other species, the objectives of the present study were: (i) to describe the structural morphology of the bi-lobed stigma and provide a detailed histochemical and anatomical characterisation of the stigma and style of *P vayredae*; and (ii) to quantify the rate of pollen tube attrition and determine the relationship between pollen load and the number of pollen tubes growing down the style and into the ovary. The study attempts to elucidate the role that the structure and anatomy of the pistil plays in determining pollen tube attrition rates in *P. vayredae*, to provide insights into pre-fertilisation factors that limit seed production in this endangered species.

MATERIALS AND METHODS

Study species

Polygala vayredae Costa (Polygalaceae) is an endangered, narrowly endemic species from the oriental pre-Pyrenees and only occurs in an area of approximately 12 km^2. It has attractive flag blossoms (superficially similar to those of Fabaceae) and strictly depends on pollination vectors to produce seeds because it lacks the ability to self-fertilise and has a self-incompatibility system that blocks self-pollen tube development at the stigmatic papillae (Castro *et al.* 2008a). The pistil contains two fused carpels, with a superior, bilocular ovary with one pendulous ovule per locule, a hollow style, and a stigmatic area divided into two lobes that have a specialised mechanism for secondary pollen presentation (for illustrations see Castro *et al.* 2008b). The pistil is sheltered inside the corolla, and the stigmatic region is exposed to insect pollinators by the downward movement of the keel, activated by the insect weight (Castro *et al.* 2008b).

All the flower samples of *P. vayredae* were collected from the Colldecarrera population, La Garrotxa (Catalunya, Spain, UTM DG57), during the spring of 2005.

Structure of the stigmatic region

Mature flowers were collected and fixed in 2.5% glutaraldehyde (prepared in a 1.25% piperazine-*N*,*N*′-bis-2-ethanesulfonic acid (PIPES) buffer, pH 7.4). The structure of the stigmatic region was studied *via* scanning electron microscopy (SEM). Samples were dehydrated through successive aqueous ethanol solutions of increasing concentration (50–100%), and then passed through successive amyloacetate-ethanol solutions (1:3, 2:2, 3:1). Finally, samples were put through a critical point dryer, mounted on metallic stubs and coated with a gold/palladium film in a sputter chamber. Samples were observed with a Jeol JSM 6700F scanning electron microscope (JEOL USA, Inc., Peabody, MA, USA), operating at 12 kV.

Stigma and style anatomy

Mature, unpollinated flowers from five distinct individuals were collected and fixed in 2.5% glutaraldehyde. For light microscopy, samples were washed in PIPES buffer, immersed in 1.0% osmium tetroxide for 1 h, and again washed in PIPES buffer. Samples were dehydrated through an acetone series (50–100%) and then embedded in graded low-viscosity epoxy resin (Embed-812). The following semi-thin sections (0.5–1.5 μm) were obtained using a glass knife: stigmatic papillae longitudinal and cross sections, sections below the stigmatic papillae (0), sections below the stigmatic region (1), and sections at the apex (2), middle (3) and base of the style (4) (Fig. 1; the latter five positions are termed levels 0, 1, 2, 3 and 4, respectively). Sections were stained with periodic acid-Schiff (PAS) for starch detection, with 0.3% Sudan black (in 70% ethanol) for lipid detection, with 1% bromophenol blue (in 95% ethanol saturated with 10% $HgCl_2$) for protein detection, and with 0.1% toluidine blue (in 1:1 1% borax and 1% Azur II) for general staining. Stained sections were observed under a light microscope and photomicrographs were taken using a Leica DC200 digital camera (Leica Microsystems AG, Wetzlar, Germany).

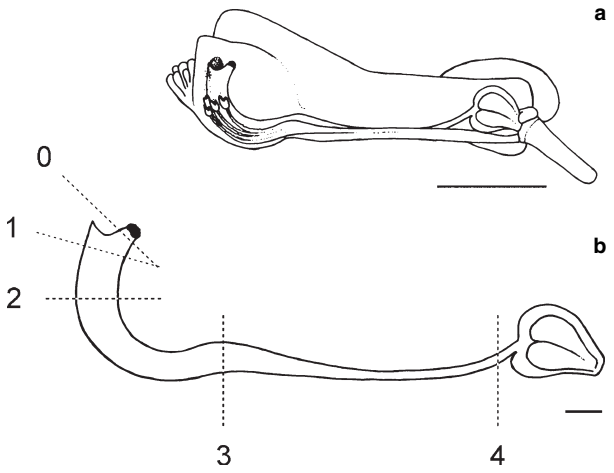


Fig. 1. Schematic illustrations of the flower, androecium and gynoecium of *Polygala vayredae*. a: Opened flower showing the stamens and the pistil disposition inside the corolla. b: Detailed illustration of pistil with studied sections highlighted. Studied sections: 0 – below stigmatic papillae, 1 – below the stigmatic region, 2 – beginning of the style, 3 – middle of the style and 4 – base of the style. Bars: a = 5 mm; b = 1 mm.

Morphometric analyses were performed on photographs using ImageTool (v.3.0 for Windows, University of Texas Health Science Center, San Antonio, TX, USA). At the five levels in the style, the following structures were assessed: parenchyma tissue, vascular regions, style channel and intercellular spaces. Intercellular spaces were also evaluated just below the stigmatic papillae. Because the stylar channel is not well delimited at level 0, the area of the intercellular spaces in a 50-μm radius was added to the channel area. Except for the proportion of intercellular spaces, differences among levels were evaluated with a Kruskal–Wallis one-way ANOVA on ranks, followed by a Tukey test for all pair-wise multiple comparisons. The proportion of intercellular spaces was analysed using a one-way ANOVA.

Pollen tube development along the style

Two sets of mature flowers from distinct individuals (separated by at least 5 m) were randomly collected and harvested into 70% ethanol: (i) flowers with open pollination (n = 87) and (ii) flowers with open pollination that were previously pollinated with a fresh mixture of outcrossed pollen (outcrossed pollen was collected from at least 10 distinct plants) (n = 30). While the first set of flowers enabled the observation of pollen tube development under natural conditions, the second set enabled the observation of pollen tube development after xenogamous pollination. In the laboratory, pistils were softened with 8 M sodium hydroxide for 4 h, stained overnight with 0.05% aniline blue, and then mounted in a drop of 50% glycerine (Dafni *et al.* 2005). Samples were observed with a Nikon Eclipse 80i epifluorescence microscope equipped with a UV-2A filter cube (330–380 nm excitation; Nikon Instruments, Inc., Kanagawa, Japan), and the following parameters were assessed: total number of delivered pollen grains, number of germinated pollen grains, number of pollen tubes at the apex, middle and base of the style, number of ovules with pollen tubes at the micropyle, and diameter of pollen tubes. Pollen viability was assessed through the proportion of germinated pollen grains over the stigmatic papillae for each observed flower (n = 117). To confirm where pollen tube development occurs (in the style channel or in the transmitting tissue), sections of the style were prepared, stained with aniline blue and observed under an epifluorescence microscope. Pollen tube development along the style was analysed with a two-way ANOVA followed by a Tukey test for all pair-wise multiple comparisons.

Assessment of pollen grain number and germination

To understand whether the number of pollen tubes growing in the style was dependent upon the number of pollen grains that reached the stigmatic papillae, the set of open-pollinated flowers (n = 87) was categorised according to their stigma pollen loads (0–19, 20–39, 40–59, 60–79, 80–99, 100–199 and >200 pollen grains). The number of pollen tubes (at the apex of the style) was represented as a function of the number of pollen grains over the stigma. Differences among categories were assessed using a one-way ANOVA followed by a Tukey test for all pair-wise multiple comparisons. GLM procedures were used because of unbalanced data.

RESULTS

Structure of the stigmatic region

SEM permitted observation of the structural morphology of the *P. vayredae* stigmatic region (Fig. 2). In this species, the stigmatic area is divided in two lobes: one fertile anterior lobe with wet stigmatic papillae, and one sterile posterior lobe. The posterior lobe is basket-shaped and is specially adapted to pollen presentation (*i.e.* it is the structure responsible for presenting the pollen to pollinators; Fig. 2a). The stigmatic papillae on the anterior surface were slightly elevated with respect to the sterile branch (Fig. 2b) and conspicuous exudate production was detected (Fig. 2d). Germinated pollen grains and pollen tube penetration in the stigmatic papillae were observed (Fig. 2c). The morphology of the epidermis was smooth in the branch where stigmatic papillae are elevated (Fig. 2b) but was highly ornate on the pollen presenter (*i.e.* sterile branch), where pollen is released from the anthers to be presented to pollinators (Fig. 2e).

Stigma and style anatomy

Histochemical and anatomical sections of the stigma and style are presented in Figs 3 and 4, and the main features

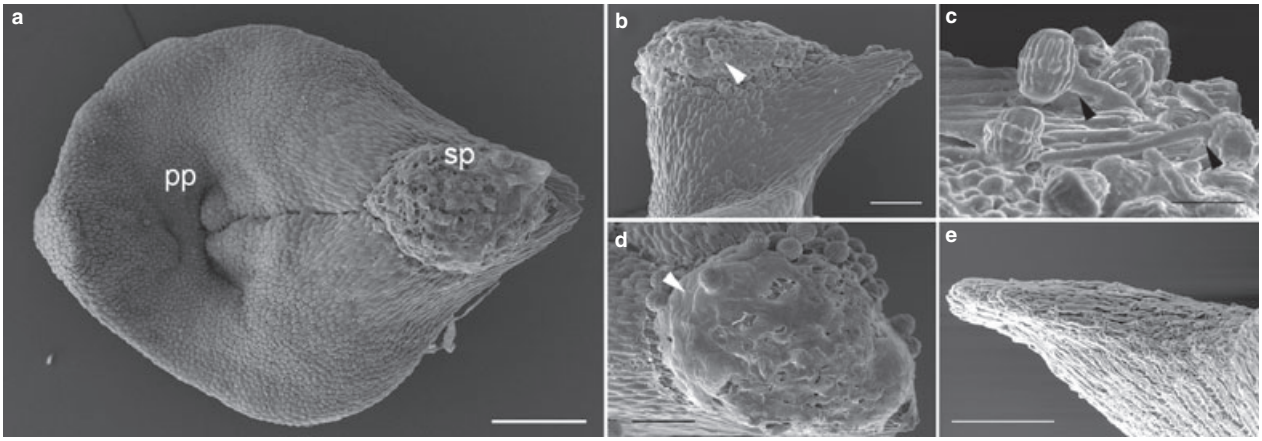


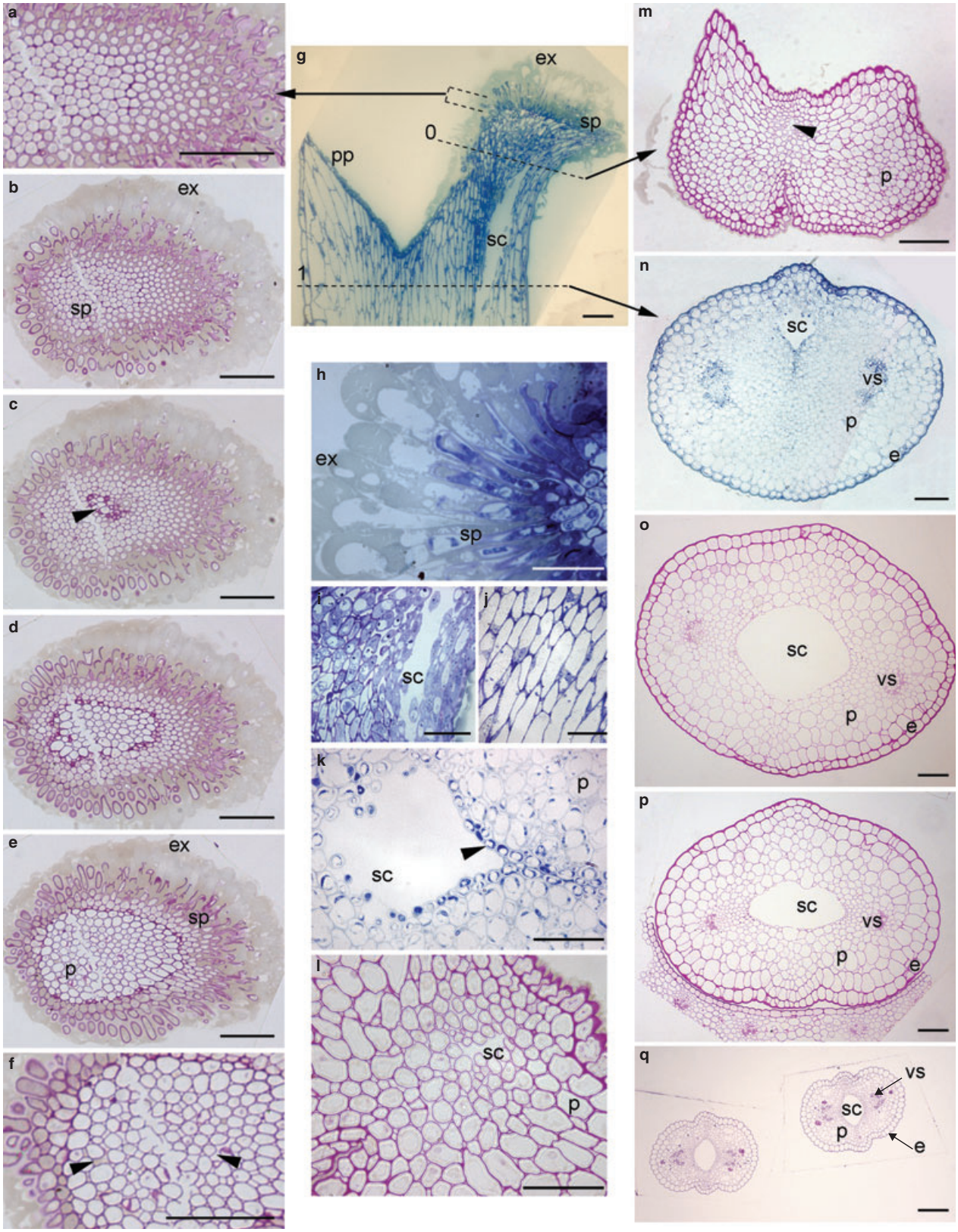
Fig. 2. Structural morphology of *Polygala vayredae* stigmatic region under SEM. a: Stigmatic region, view from above; b: stigmatic papillae (arrow), lateral view; c: pollen grain germination (arrows); d: stigmatic papillae with conspicuous exudate production (arrow), view from above; e: extremity of pollen presenter. Legend: pp – pollen presenter, sp – stigmatic papillae. Bars: a–b = 200 μm; c = 50 μm; d–e = 100 μm.

are given in Table 1. In sequential cross sections of the stigmatic papillae (from the papillae downwards; Fig. 3a–f), a tight disposition of the cells with prominent exudate production was observed (Fig. 3a,b). The receptive region of the stigma was formed by a unicellular layer of densely packed cells (Fig. 3g–h). This cell layer produced abundant mucilage that was mostly composed of lipids (Fig. 4a,b), and is the site where pollen grains land, stuck and germinated (Fig. 4a). The secretion and papillae cell contents were PAS-negative, while the cell walls were PAS-positive (*e.g.* Fig. 3a–f). No proteins were detected at this level.

Below these papillae (Fig. 3c–f) was a parenchyma tissue characterised by small intercellular spaces (26.2 ± 31.82 μm^2; Fig. 3f) with a total area of occupation (5.8 ± 1.57%) significantly larger than the one observed in the parenchyma tissue of adjacent levels (*i.e.* levels 0 and 1; $F = 142.4$, $P < 0.001$; Table 1). Longitudinal sections of the stigmatic region revealed that the hollow pistil is a closed structure and that the stylar channel starts near the stigmatic papillae (86.7 ± 7.64 μm below) inside the receptive branch (Fig. 3g). In this area, the parenchyma cells had dense cytoplasm (Fig. 3g,i), an accumulation of lipid reserves (strongly stained with Sudan black; Fig. 4b,c), but no detectable carbohydrates or proteins. On the contrary, parenchyma cells at lower levels (levels 1–4) were larger, more vacuolated and depleted of reserves (Figs 3j, 4e). On the adaxial surface of the stylar channel apex, a cell layer of the epidermal type was observed (Fig. 3k) and, although less conspicuous, lipid mucilage was detected inside the channel at levels 0 and 1 (Fig. 4d). Throughout the remaining levels of the style (levels 2–4), the cells surrounding the wall of the channel did not have visible cell content differences when compared to the remaining parenchyma. Moreover, no carbohydrate (Fig. 3o–q), protein, lipid or mucilage production was detected at these levels (levels 2–4; Fig. 4e). The cells surrounding the wall of the channel were loosely arranged, mainly at its apex (on the adaxial surface; Fig. 3k), and only formed a well-defined epidermis at the base of the channel.

Morphometric analysis allowed us to quantitatively characterise stylar anatomical sections (Table 1). Below the stigmatic papillae, the parenchyma had intercellular spaces that were irregularly distributed (Fig. 3d–f). From this point to level 0, the intercellular spaces became organised in the centre where, finally, the stylar channel appeared, while the surrounding parenchyma became highly compact (with few intercellular spaces; Table 1, Fig. 3l,m). After this stage, the intercellular spaces significantly increased along the style ($F = 142.4$, $P < 0.001$; Table 1). The stylar channel area was smaller at the recep-

Fig. 3. Anatomical and histochemical characterisation of the stigmatic region and style of *Polygala vayredae* stained with PAS (pink) and toluidine blue (blue). a–e: Sequential cross sections of the stigmatic papillae showing its base (arrow) and parenchyma cells below; f: cross section just below stigmatic papillae showing the intercellular spaces in the parenchyma (arrows); g: longitudinal section of stigmatic region showing the pollen presenter, the stigmatic papillae, the stylar channel and an active region below stigmatic papillae; h: longitudinal section of the unicellular layer of stigmatic papillae; i and j: cross section of the receptive branch in detail, at the apex of the stylar channel and 200 μm below, respectively; k: detail of the stylar channel below the stigmatic region showing a cell layer of epidermal-type cells on the abaxial surface (arrow); l: cross section at the beginning of the stylar channel showing several spaces around it; m: cross section of receptive branch (level 0) at the beginning of the stylar channel (arrow); n–q: cross sections below stigmatic region and along the style (levels 1, 2, 3 and 4, respectively) represented at the same scale. Legend: e – epidermis, ex – exudate, p – parenchyma tissue, pp – pollen presenter, sc – stylar channel, sp – stigmatic papillae and vs – vascular strand. Bars: a–g, m–q = 100 μm; h–l = 50 μm.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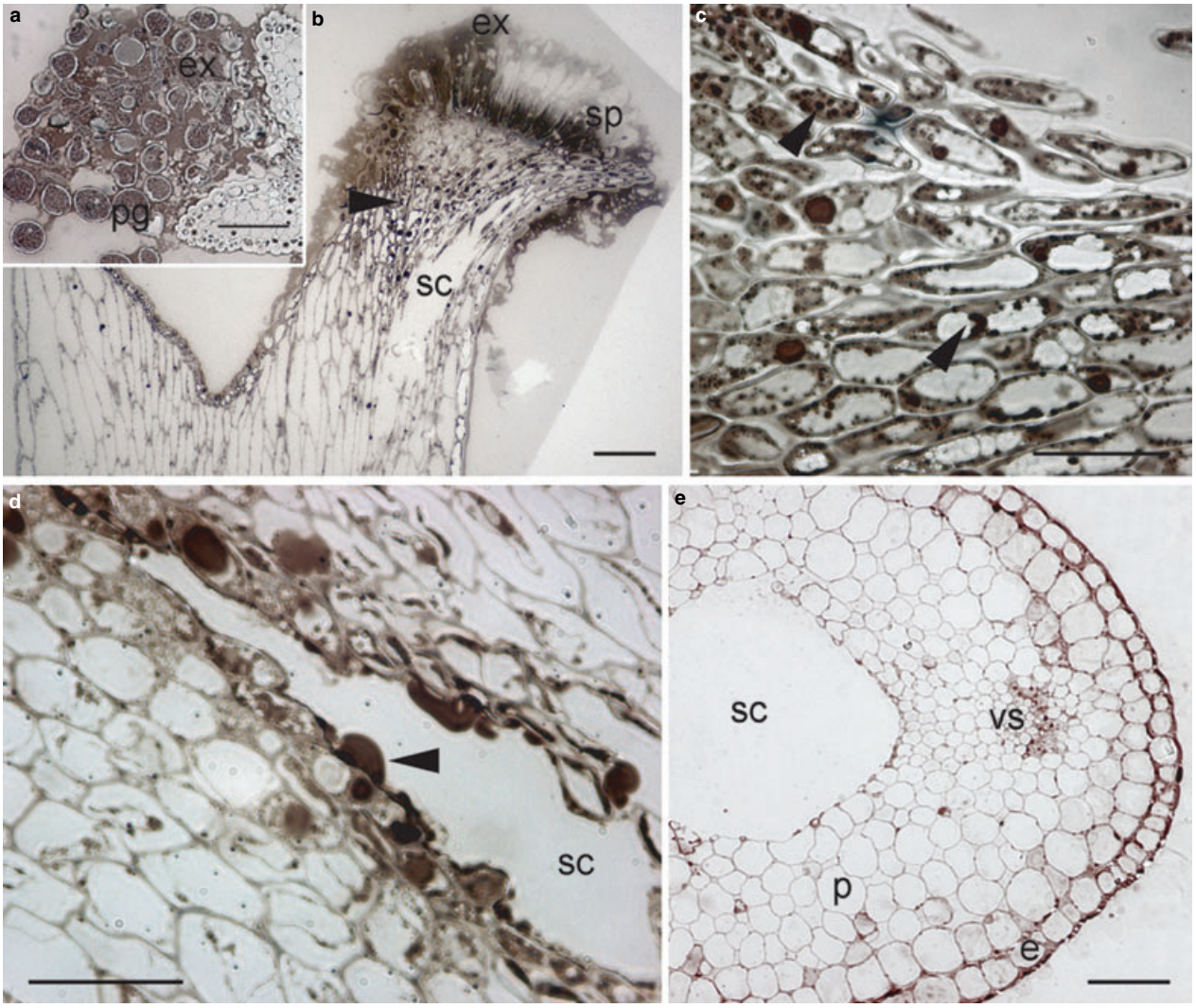

Fig. 4. Anatomical and histochemical characterisation of the stigmatic region and style of *Polygala vayredae* stained with Sudan black. a: Stigmatic exudate with adhered pollen grains showing its lipid-rich composition; b: longitudinal section of stigmatic region showing the lipid-rich exudates over the stigmatic papillae, and lipid accumulation in the parenchyma of the receptive branch (arrow); c: detail of the receptive branch parenchyma showing lipid accumulation (arrows); d: longitudinal section of the stylar channel showing lipid-rich secretions (arrow) and active cell layer of the epidermal type; e: cross sections at the beginning of the style (level 2). Legend: e – epidermis, ex – exudate, p – parenchyma tissue, pg – pollen grains, sc – stylar channel, sp – stigmatic papillae and vs – vascular strand. Bars: a–b, e = 100 μm; c–d = 50 μm.

tive branch (level 0), increased until the middle of the style (up to level 2), and then was reduced drastically to areas similar to those of the upper style levels ($H = 50.0$, $P < 0.001$; Table 1; Fig. 3m,n–q, with the latter represented at the same scale). By considering the area of the stylar channel below the stigmatic papillae (Table 1), and the mean area occupied by the pollen tubes (114.2 ± 98.76 μm^2, see below), one can estimate that, at this point (level 0), the stylar channel could physically support the simultaneous growth of only about six pollen tubes. In subsequent style levels (levels 1–4), physically, the channel can potentially support a larger number of pollen tubes. The style area was smaller in the receptive branch (level 0), enlarged at level 1, 2 and 3, and was finally significantly reduced from the middle (level 3) to the base of the style (level 4) ($H = 50.1$, $P < 0.001$; Table 1; Fig. 3m,n–q, with the latter represented at the same scale). Conversely, vascular regions were larger at the beginning of the style (level 0), after which they were significantly reduced until the base ($H = 44.2$, $P < 0.001$; Table 1).

Pollen tube development along the style

Although highly variable, the number of pollen grains deposited by pollinators on the stigma (67 ± 79.0 pollen

Table 1. Description of the main anatomical features of the *Polygala vayredae* style.

level	total area (μm^2)	parenchyma (μm^2)	stylar channel (μm^2)	vascular boundaries (μm^2)	intercellular spaces (%)
below stigmatic papillae	–	–	–	–	5.8 ± 1.57[a]
style level 0	160,070.1 ± 4,855.36[a]	127,150.8 ± 3,413.08[a]	738.0 ± 90.81[a]	–	0.9 ± 0.22[b]
style level 1	464,756.1 ± 20,305.70[b]	367,686.6 ± 18,586.59[bc]	7,778.7 ± 378.06[b]	25,990.5 ± 1,411.76[a]	1.1 ± 1.32[b]
style level 2	675,080.4 ± 94,844.87[c]	490,165.7 ± 59,566.57[c]	68,479.2 ± 21,915.09[c]	13,395.6 ± 2,320.75[b]	4.7 ± 0.68[a]
style level 3	603,711.4 ± 129,073.02[c]	432,102.3 ± 88,470.71[c]	53,624.5 ± 21,914.19[c]	14,841.3 ± 7,395.56[b]	4.9 ± 0.60[a]
style level 4	88,423.9 ± 18,254.87[a]	53,577.7 ± 10,592.27[a]	7,684.9 ± 1,915.49[b]	5,342.3 ± 709.34[c]	15.4 ± 0.36[c]
comparison test	H = 50.1***	H = 63.9***	H = 64.4***	H = 44.2***	F = 142.4***

Studied style levels: 0 – beginning of the stylar channel, 1 – below the stigmatic region, 2 – beginning of the style, 3 – middle of the style, and 4 – base of the style (for details see Fig. 1). Values are given as mean and standard deviation of the mean. Differences among levels were evaluated with a Kruskal–Wallis one-way ANOVA on ranks, followed by a Tukey test for all pair-wise multiple comparisons, except for the proportion of intercellular spaces, where a one-way ANOVA was applied. Different letters indicate significant differences at $P < 0.05$. ***$P < 0.001$.

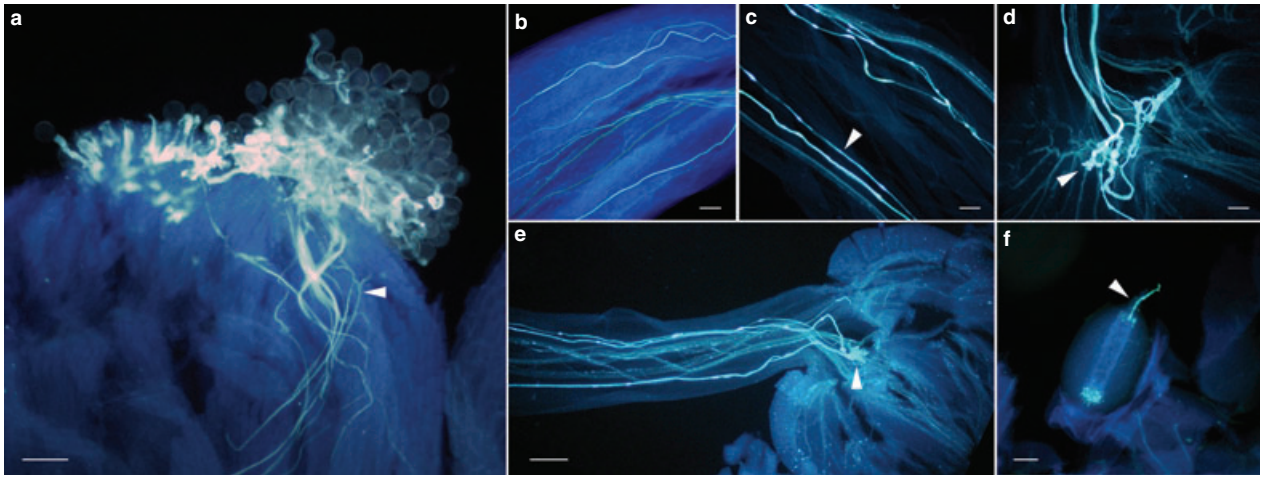

Fig. 5. Pollen tube development along *Polygala vayredae* style after open pollination, viewed under fluorescence microscopy. a: Pollen grains over the stigmatic papillae and pollen tube growth below them (arrow); b: pollen tube growth at the apical level of the style; c: pollen tube growth in the middle level of the style (arrow – orderly growing of the pollen tubes); d: pollen tube behaviour at the entrance of the ovary (arrow – erratic behaviour of the pollen tubes); e: pollen tube growth at the end of the style and entrance of the ovary (arrow – erratic behaviour of the pollen tubes); f: pollen tube in the micropyle (arrow). Bars: a–d, f = 100 μm; e = 200 μm.

Table 2. Pollen tube development along the style in *Polygala vayredae* flowers after open pollination and xenogamous pollination.

		no. of pollen tubes along the style			micropyle with pollen tube (%)
treatment	n	Apex	Middle	Base	
open pollination	87	12 ± 5.9	9 ± 5.1	8 ± 5.0	53.4[a]
xenogamous pollination	30	11 ± 5.6	9 ± 5.5	7 ± 4.4	65.4[a]

Number of pollen tubes along the style is given as mean and standard deviation of the mean. Micropyle with pollen tube is given as a proportion of the total number of ovules with a pollen tube at the micropyle and differences between treatments analysed with a z-test.
[a] No statistically significant differences at $P < 0.05$.

grains) was usually larger than that required to fertilise the two ovules enclosed in the ovary. In general, all pollen grains that reached the stigma germinated (98.4 ± 2.87% of pollen grains, n = 117 stigmas from control and hand-pollinations) and the pollen tubes that were able to pass through the stigmatic papillae grew throughout the style (Fig. 5) inside the stylar channel. Pollen tubes had a mean diameter of 11.3 μm (mean area 114.2 μm^2) but were highly variable (SD = 4.32 μm). Their growth was very orderly throughout the style (Fig. 5b,c,e), but once they entered the ovary they often behaved erratically (Fig. 5d) and very few reached the micropyle (Fig. 5f).

No significant differences were observed in the number of pollen tubes growing in the style between open and xenogamous pollination (F = 0.458, P = 0.499; Table 2).

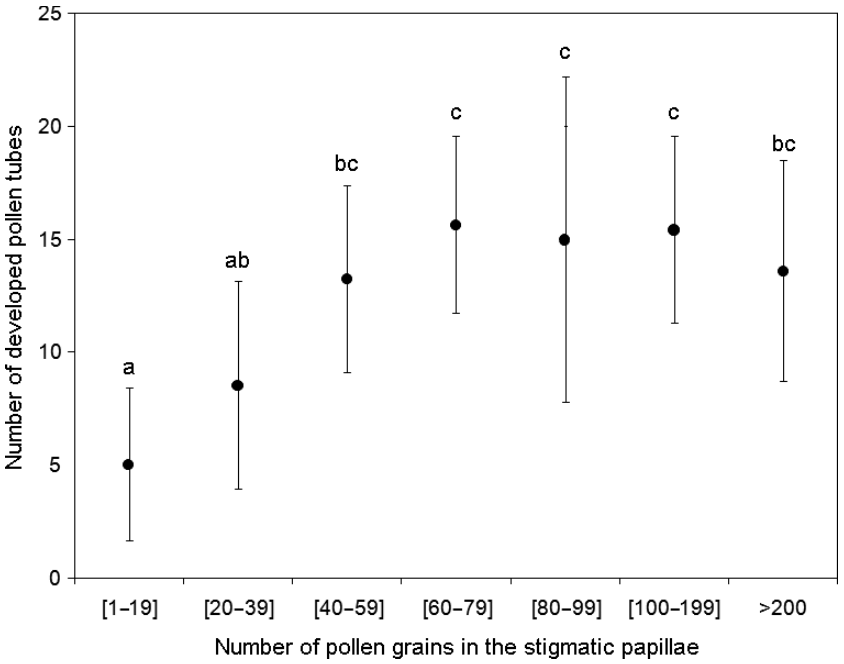

Fig. 6. Number of developed pollen tubes in the style below the stigmatic papillae (mean and standard deviation of the mean) as a function of the number of pollen grains on the stigmatic papillae of *Polygala vayredae* flowers. Different letters denote statistically significant differences at $P < 0.05$.

However, a progressive reduction in the number of tubes growing in the style was recorded ($F = 5.378$, $P = 0.005$; Table 2). Nevertheless, the number of pollen tubes at the entrance of the ovary was greater than the number of ovules (two ovules per flower). Regardless of the large number of pollen tubes entering the ovary, only about half of the ovules had a pollen tube at the entrance of the micropyle (53.4% after open pollination, and 65.4% after xenogamous pollination; Table 2).

A matter of pollen grain number?

The number of developed pollen tubes as a function of the pollen load received on the stigma is shown in Fig. 6. An increase in the number of pollen grains on the stigmatic papillae led to an increase in the number of developing pollen tubes until a plateau was reached. At this plateau level, more than 60 pollen grains did not significantly increase the number of pollen tubes ($F = 13.91$, $P < 0.001$).

DISCUSSION

In the present study, we demonstrated that several factors are involved in pollen tube development along the pistil of *P. vayredae*. First, strong interactions occur at the stigmatic papillae where lipid-rich exudates trigger pollen grains to germinate, and a self-incompatibility system blocks self-pollen tubes from passing through the stigmatic papillae. Second, physical constraints may restrain pollen tube development because of reduced intercellular spaces below the stigmatic papillae and reduced area at the apex of the stylar channel. This physical constraint introduces another factor, gametophyte competition, because only fast-growing pollen tubes will reach the stylar channel. Third, at more basal style levels, even though the channel can physically support a larger number of pollen tubes, a lack of reserves may restrain pollen tube development along the style. Finally, the number of pollen tubes entering the ovary is larger than the number of ovules, suggesting that genetic interactions play a major role at this level and are potentially the main factors determining the observed low seed–ovule ratios.

Stigmatic region

The stigmatic region of *P. vayredae* is morphologically similar to that of other species in the genus, with a pollen presenter in the form of a basket and a fertile branch with stigmatic papillae (*e.g. P. virgata* var. *virgata*, Krüger & Pretorius 1997; *P. myrtifolia*, Prenner 2004). In several *Polygala* species, including *P. vayredae*, pollen is presented to pollinators in the sterile branch (pollen presenter) rather than in the anthers. This is known as secondary pollen presentation and is believed to be a way to accurately deliver and receive pollen, thus increasing plant male and female fitness (Ladd 1994). In the present work, different epidermal ornamentations were observed in the stigmatic region of *P. vayredae*. These differences could be involved with self-pollen adhesion to the pollen presenter during secondary pollen relocation, and with minimisation of self-pollen adhesion near the stigmatic papillae to avoid papillae being clogged with self-pollen. Furthermore, elevation of the stigmatic papillae in the fertile branch was observed in *P. vayredae*. This separation has been described as a mechanism to minimise

self-interference and improve pollen reception in several species (Ladd 1994), including *P. vayredae* (Castro *et al.* 2008b).

The stigmatic papillae of *P. vayredae* are actively secreting cells producing an exudate that is very rich in lipids. Similar lipid-rich exudates have been described in *P. virgata* var. *virgata* (Krüger & Pretorius 1997), *Petunia* spp. (Wolters-Arts *et al.* 1998) and *Nicotiana sylvestris* (Kandasamy & Kristen 1987). Pectins and some proteins were also detected in the sigmatic exudate of *P. virgata* var. *virgata* (Krüger & Pretorius 1997). In *P. vayredae*, other compounds may also be present but may have been masked by the high abundance of lipids. Lipids play a key role in pollen–stigma interactions of several plant species (*e.g.* Wolters-Arts *et al.* 1998 and references therein; Edlund *et al.* 2004). Through selection of several exudate compounds, Wolters-Arts *et al.* (1998) observed that lipids were an essential factor for pollen tube penetration in the stigmatic papillae. These authors also proposed that, during pollen–stigma interactions, lipids were necessary to regulate water uptake, creating internal gradients involved in directional growth of the pollen tube. Nevertheless, the mechanisms by which this happens remain unclear (for a review see Edlund *et al.* 2004).

Pistil anatomy and physiology

Because pollen tube development is heterotrophic (both in species with solid and hollow styles), pistil structure and available reserves play a crucial role (Herrero & Hormaza 1996). *Polygala vayredae* presented progressive reductions in the transmitting tissue and in lipid accumulation along the style. In this species, the transmitting tissue immediately below the stigmatic papillae appears to physically restrict pollen tube development until the stylar channel is reached. At the top of the channel, pollen tube development can be further restrained, because the stylar channel and the surrounding intercellular spaces are very narrow. This physically limits the number of pollen tubes that can grow simultaneously. In subsequent levels of the style (levels 1–4), the channel would seem to be physically able to support a higher number of pollen tubes.

From a nutritional standpoint, no major polysaccharide, lipid or protein accumulations were detected along the style of *P. vayredae*. However, at the beginning of the stylar channel, dense epidermal-type cells were highly metabolic and released lipid-rich mucilage into the channel lumen. Similar observations have been reported in other species with hollow styles, and this secretory layer of cells is known to produce several compounds (Knox 1984; Lord & Kohorn 1986; Scribailo & Barrett 1991; Cheung 1996) that are linked to pollen tube adhesion, nutrition and guidance (Sanders & Lord 1992; Cheung *et al.* 1995; Cheung 1996). Several experiments have demonstrated that such reserves can become depleted by the growing pollen tubes within the transmitting tissue, and other studies found incorporation of compounds from the transmitting tissue or exudate into the pollen tubes (Labarca & Loewus 1973; Herrero & Dickinson 1979; Gonzalez *et al.* 1996; de Graaf *et al.* 2003). This demonstrates the importance of these reserves in pollen tube development. Furthermore, stylar secretions can also be involved in assisting pollen tube development along the channel, as proposed by Sanders & Lord (1989). Contrary to observations in other species, where polysaccharide metabolism is involved in pollen tube nutrition (Labarca & Loewus 1973; Herrero & Dickinson 1979), *P. vayredae* only stained for lipid reserves. Although some insights have been provided by the present work, further studies are essential to completely clarify how pollen tube nutrition occurs in this species.

Pollen tube development along the pistil

The pistil appears to be designed to support pollen tube development and, at the same time, encourage pollen–pistil interactions (Cheung 1995; Ray *et al.* 1997; Cheung & Wu 2001; Herrero 2000, 2001). In *P. vayredae*, the stigmatic papillae appear to be the first barrier that could inhibit pollen tube development, due to self-incompatibility mechanisms (Castro *et al.* 2008a). Pollen grains had high germination, similar to observations in previous studies with this (Castro *et al.* 2008a) and other species (*e.g.* Smith & Gross 2002). This suggests that pollen grains are not selected at the germination level. However, because pollen loads are usually higher than the number of pollen tubes that the style can support, pollen competition may occur on the stigma. Similar observations have been reported, for example, in *Clarkia unguiculata* (Németh & Smith-Huerta 2003) and in *Cichorium intybus* (Erbar 2003).

After passing through the stigmatic papillae, pollen tubes penetrate the stylar tissue and reach the stylar channel. Like other species with hollow styles (Lord & Kohorn 1986; Leins & Erbar 2005; Reinhardt *et al.* 2007), pollen tube development in *P. vayredae* occurs inside the stylar channel. A slight decrease in the number of pollen tubes growing in the style was observed in this species. Pollen tube attrition has been described for several other species (*e.g.* Cruzan 1986, 1989; Herscovitch & Martin 1990; Herrero 1992; Plitmann 1993; Erbar 2003), sometimes even after different pollination regimes (Hormaza & Herrero 1996; Smith-Huerta 1997; Ortega *et al.* 2002). The 'quality' of the received pollen can also significantly affect pollen tube attrition (*e.g.* Cruzan 1989; Hormaza & Herrero 1996). This suggests that genetic interactions play a major role in the control of pollen tube growth along the pistil.

In *P. vayredae*, approximately 12 pollen tubes were observed entering the style. Generally, a larger number of pollen tubes (*ca.* eight) than the number of ovules (two) reach the ovary. Because an excess of pollen tubes enters the ovary, regardless of any physical or nutritive constraint along the style, the events occurring in the ovary will be of great importance to low seed ovule ratios. Indeed, in the present study, although the number of pollen tubes slightly diminished along the style, most of

them were inhibited at the entrance of the ovary. Pollen tube growth was very orderly throughout the style but, once at the ovary, it becomes erratic and pollen tubes were frequently unable to reach the ovules. The rather large dimensions of the stylar channel suggest that there is no or reduced competition for space among pollen tubes and little contact between them and the transmitting tissue, possibly resulting in a reduction of incompatible reactions along the style. Nevertheless, as the path straightens to the ovary, interactions may become stronger, possibly leading to the observed erratic behaviour.

Similar behaviours have been observed in other species, with pollen tubes being arrested at several levels of the style or suffering accelerations and decelerations in their growth near the entrance of the ovary (*e.g. Prunus* spp., Herrero 2000; *Myrica rubra*, Sogo & Tobe 2006). In some species, the main bottleneck seems to be in the upper section of the style (*e.g. Nicotiana glauca*, Cruzan 1986; *Pontederia sagittata*, Scribailo & Barrett 1991), which sometimes even results in a failure of the pollen tubes to penetrate the stigmatic papillae (*e.g. Medicago sativa*, Sayers & Murphy 1966). In others, a high rate of pollen tube attrition has been observed in the lower part of the pistil or at the entrance of the ovary (*e.g. Erythronium grandiflorum*, Cruzan 1989; *Tectona grandis*, Tangmitcharoen & Owens 1997). Finally, in some species, selection pressure can also occur along the entire length of the style (*e.g. Prunus avium*, Hormaza & Herrero 1996). In *P. vayredae,* one of the strongest interactions (among male gametophytes and/or between male gametophytes and female tissues) appears to take place at the entrance of the ovary. The available studies indicate that the ovary is another site where intraspecific and interspecific incompatibilities occur (*e.g.* Williams *et al.* 1982) and that it may even be involved in compatible mating selection (Herrero & Hormaza 1996). However, it is still not clear as to the extent of pollen–pistil interactions in determining which male gametophytes achieve fertilisation in compatible matings.

Additional studies involving visited and unvisited flowers and controlled pollination experiments with different competition regimes are needed to understand reserve allocation along the style and the dynamics of pollen tube development prior to fertilisation.

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