

## The progamic phase of an early-divergent angiosperm, *Annona cherimola* (Annonaceae)

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• **Background and Aims** Recent studies of reproductive biology in ancient angiosperm lineages are beginning to shed light on the early evolution of flowering plants, but comparative studies are restricted by fragmented and meagre species representation in these angiosperm clades. In the present study, the progamic phase, from pollination to fertilization, is characterized in *Annona cherimola*, which is a member of the Annonaceae, the largest extant family among early-divergent angiosperms. Beside interest due to its phylogenetic position, this species is also an ancient crop with a clear niche for expansion in subtropical climates.

• **Methods** The kinetics of the reproductive process was established following controlled pollinations and sequential fixation. Gynoecium anatomy, pollen tube pathway, embryo sac and early post-fertilization events were characterized histochemically.

• **Key Results** A plesiomorphic gynoecium with a semi-open carpel shows a continuous secretory papillar surface along the carpel margins, which run from the stigma down to the obturator in the ovary. The pollen grains germinate in the stigma and compete in the stigma-style interface to reach the narrow secretory area that lines the margins of the semi-open stylar canal and is able to host just one to three pollen tubes. The embryo sac has eight nuclei and is well provisioned with large starch grains that are used during early cellular endosperm development.

• **Conclusions** A plesiomorphic simple gynoecium hosts a simple pollen–pistil interaction, based on a support–control system of pollen tube growth. Support is provided through basipetal secretory activity in the cells that line the pollen tube pathway. Spatial constraints, favouring pollen tube competition, are mediated by a dramatic reduction in the secretory surface available for pollen tube growth at the stigma–style interface. This extramural pollen tube competition contrasts with the intrastylar competition predominant in more recently derived lineages of angiosperms.

**Key words:** *Annona cherimola*, Annonaceae, embryo sac, endosperm, Magnoliid, ovule, pollen–pistil interaction, pollen tube.

### INTRODUCTION

Study of the reproductive biology of basal and early-divergent angiosperms has experienced a renaissance in recent years and is providing valuable information on evolutionary trends in flowering plants (Friedman and Ryerson, 2009; Rudall *et al.*, 2009). Although much remains to be discovered about the basic features of the sexual process in angiosperms, a new understanding of its evolutionary developmental biology is beginning to emerge. Most angiosperms conform to a defined suite of reproductive characteristics but new data derived from studies on ancient extant flowering plant lineages reveal that the reproductive features of the first flowering plants differed significantly from those shown by the majority of extant flowering plants (Friedman and Williams, 2004). Recent work is revealing unique features in the female gametophyte of ancient extant angiosperms, such as the egg cell apparatus (Friedman, 2006), the endosperm (Friedman *et al.*, 2008) and the provisioning of ovular resources (Friedman, 2008; Rudall *et al.*, 2008). The study of pollen development and evolution is also emerging as a powerful field in understanding the evolution of reproductive

characters (Rudall and Bateman, 2007; Lora *et al.*, 2009a). Although information on the male and female side is accumulating in ancient lineages of extant angiosperms, there is still much to be learned about the interplay between the two: pollen–pistil interaction.

The progamic phase, the period of pollen tube growth through the pistil that elapses from pollination to fertilization and gamete fusion (Linskens, 1975; Williams, 2009), provides an opportunity for pollen–pistil interaction, which is emerging as a powerful strategy regulating mating in flowering plants (Herrero and Hormaza, 1996; Herrero, 2000, 2003; de Graaf *et al.*, 2001; Rea and Nasrallah, 2008). The molecular mechanisms involved in this signalling are being deciphered (Escobar-Restrepo *et al.*, 2007; Higashiyama and Hamamura, 2008; Hiscock and Allen, 2008), although a comprehensive view on the events and their implications remains to be made. Recent work in *Amborella*, sister to all extant angiosperms, sets the baseline for understanding the evolution of pollen–pistil interaction (Williams, 2009); in this sense, the study of the progamic phase in ancient angiosperms, which

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has been performed in a number of species belonging to ancient flowering plant lineages (Vithanage, 1984; Orban and Bouharmont, 1995; Pontieri and Sage, 1999; Thien *et al.*, 2003; Sage and Sampson, 2003; Koehl *et al.*, 2004; Hristova *et al.*, 2005; Lyew *et al.*, 2007), may prove a highly valuable tool to track the evolution of this process (Hiscock and Allen, 2008). Also, the evolution and function of the transmitting tissue in extant representatives of early-divergent angiosperm lineages has been recently explored (Sage *et al.*, 2009).

However, one of the main limiting factors in studying developmental processes in early angiosperms and placing them in a phylogenetic framework is that most ancestral angiosperm lineages have arrived at the present time in a highly fragmented way. Early-divergent angiosperms have a very meagre representation, with some lineages represented by only one or very few species. Annonaceae is the largest living family in the early-divergent angiosperm clade magnoliids (APG II, 2003; Soltis *et al.*, 2005), including about 130 genera and 2300 species with a worldwide distribution (Chatrou *et al.*, 2004). Besides interest in the family regarding questions on early angiosperm evolution, some of the species in the Annonaceae, such as cherimoya (*Annona cherimola*), sugar apple (*A. squamosa*) or soursop (*A. muricata*), have been used as a food source by pre-Columbian cultures in South America (Popenoe, 1989), and they now have a clear niche for expansion in developing countries with subtropical climates. However, despite the importance for both basic and applied studies, very little is known of the reproductive biology of this family.

Most papers on the reproductive biology in the Annonaceae have focused on descriptive studies of the flowers (Norman *et al.*, 1986, 1992; Carvalho and Weber, 2000; Norman, 2003; Kiill and Costa, 2003), embryology (Svoma, 1998a) and flower development (Decraene and Smets, 1990; Leins and Erbar, 1996). Studies on the pollen tube pathway are limited and restricted just to pollen germination in the stigma (Vithanage, 1984). Recently, pollen development and release in groups in *A. cherimola* have been studied (Lora *et al.*, 2009b), and the coexistence of bi- and tricellular pollen at anther dehiscence in this species has been shown, contributing to the understanding of the heterochronic shift from bicellular to tricellular pollen (Lora *et al.*, 2009a).

Here, the progamic phase and early embryo development is characterized in the early-divergent angiosperm *A. cherimola*. The examination of pistil anatomy reveals features showing a simple pollen–pistil interaction that are likely to be plesiomorphic for all angiosperms.

## MATERIALS AND METHODS

### *Plant material*

*Annona cherimola* shows protogynous dichogamy (Schroeder, 1971). Flowers are hermaphroditic, but female and male structures do not mature simultaneously, hindering self-fertilization in the same flower. Moreover, most flowers of the same genotype are synchronized and, consequently, transfer of pollen between different flowers of the same genotype is also difficult. The flower cycle is completed in 2 d; in the morning of

the first day the flower is in preanthesis with the petals tightly closed. Around midday the flower passes to the female stage, petals slightly widen apart and the stigma is receptive. After approximately 30 h, flowers switch to the male stage. Anthers dehisce at approx. 1700–1800 h under the environmental conditions used herein. Concomitantly with anther dehiscence, the petals widen apart and the stigma dries up and loses receptivity.

Adult trees of *A. cherimola* ‘Campas’ located in a field cultivar collection at the EE La Mayora-CSIC, Málaga, Spain, were used in these experiments. Flowers were fixed along the flowering period, during two consecutive years.

### *Pollination procedures*

Anthers and pollen were collected from flowers just before anther dehiscence, stored at 4 °C and used for hand pollination the following day. Fifteen flowers per day were pollinated at 0900 h on the first day of the flower cycle and the floral tube was then plugged with cotton to prevent further unwanted pollination. To evaluate developmental changes independent of pollination, similarly treated flowers were left unpollinated. The gynoecia of 15 pollinated and 15 unpollinated flowers were weighed and fixed daily from preanthesis (1 d before anthesis) to 3 weeks following hand pollination.

An additional group of flowers were collected in the field, placed in water in the laboratory at room temperature and pollinated at 0900 h on the first day of the flower cycle to study pollen tube growth. Regression analyses were used to describe the relationships between days after anthesis and pistil weight. Several regression models were tested and third-order polynomial regression was selected.

### *Microscopic preparations*

Pollen tube growth was documented using squashed preparations of pistils from hand-pollinated flowers kept in water at room temperature. For this purpose, pistils were fixed in formalin–acetic acid–alcohol (FAA) 3, 6 and 9 h after pollination. Pistils were water washed and placed in 1 M NaOH for 1 h to soften the tissues. Individual pistils were dissected and squashed preparations were stained with 0.1 % aniline blue in PO<sub>4</sub>K<sub>3</sub> (Currier, 1957; Linskens and Esser, 1957).

Following hand pollination in the field, pistils were also sequentially fixed. Eight flowers per day were collected at 0900 h and fixed in FAA, dehydrated in a graded ethanol series, and then embedded in paraffin wax. Seven flowers per day were fixed in 2.5 % glutaraldehyde in 0.03 M phosphate buffer (Sabatini *et al.*, 1963). Finally, three flowers per day were fixed for 24 h in 3 : 1 (V1/V2) ethanol/acetic acid and transferred to 75 % ethanol for storage at 4 °C following the method of Williams *et al.* (1999). These flowers and those fixed in 2.5 % glutaraldehyde were dehydrated in a graded ethanol series and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) resin.

To observe pollen tubes and callose, squashed preparations and paraffin-embedded material sectioned at 10 µm were stained with 0.1 % aniline blue in 0.1 N PO<sub>4</sub>K<sub>3</sub> (Currier, 1957; Linskens and Esser, 1957). Sections were also stained with 0.01 % acridine orange in 0.03 M phosphate buffer to

observe DNA and RNA (Nicholas *et al.*, 1986; Dudley *et al.*, 1987), with 0.01 % auramine O in 0.05 M phosphate buffer to observe cutine and suberine (Heslop-Harrison, 1977) and with 0.07 % calcofluor in water for cellulose (Hughes and McCully, 1975). For general histological examination, paraffin-embedded material was stained with a mixed staining in the following order: 0.1 % aniline blue in 0.1 N  $\text{PO}_4\text{K}_3$ , 0.01 % acridine orange in water, 0.01 % auramine O in water and 0.01 % calcofluor in water, for 10 min each at 40 °C to accelerate staining and drying. Others sections were also stained with safranin, crystal violet and green light according to Gerlach (1969). Resin-embedded material fixed in glutaraldehyde was sectioned at 2  $\mu\text{m}$  and stained with periodic acid-Shiff's reagent (PAS) followed by 0.2 % toluidine blue in water (Feder and O'Brien, 1968) to observe insoluble carbohydrates and nuclei. Resin-embedded material in glutaraldehyde was also stained with iodine potassium iodide (IKI) for starch (Johansen, 1940). Resin-embedded material fixed with 3 : 1 (V1/V2) ethanol/acetic acid was sectioned at 5  $\mu\text{m}$  and stained with a solution of 0.25 mg  $\text{mL}^{-1}$  4',6-diamidino-2-phenylindole (DAPI) and 0.1 mg  $\text{mL}^{-1}$  *p*-phenylenediamine (added to reduce fading) in 0.05 M Tris (pH 7.2) for 1 h at room temperature in a light-free environment (Williams *et al.*, 1999). Preparations were observed under an epifluorescence Leica DM LB2 microscope with 340–380-nm bandpass and 425-nm longpass filters for DAPI. Pollen tube growth rates were calculated as the length of the longest pollen tube divided by the time elapsed from pollination.

## RESULTS

### *Gynoecium anatomy and pollen tube growth*

The average number of carpels in the gynoecium of *A. cherimola* was  $90.4 \pm 5.4$  ( $n = 5$  flowers), and these were fused to form a syncarp occupying the centre of a conical receptacle. Each of the carpels had a single anatropous ovule that could develop into a single seed. The androecium was located below the gynoecium forming a helicoidal structure with up to 200 stamens. The pistil had a large stigma and a relatively short style with partial postgenital fusion at the periphery of the innermost side (Fig. 1A), forming an open stylar canal. The closing area of this canal was covered in a zip-like fashion by unicellular secretory papillae that resulted in a continuous secretory carpet starting in the stigma and going all the way through the style down to the ovary (Fig. 1B). Idioblastic solitary oil cells (Fig. 1C), thick-walled sclereid cells (Fig. 1D) and tanniferous cells were observed along the cortical tissue. Tanniferous cells were also observed in the ovular epidermis.

The length of the pollen tube pathway from the stigma to the ovule was  $1832.4 \mu\text{m}$  ( $\pm 132.5$ ,  $n = 10$ ). Pollen grains germinated in the stigma and the pollen tubes grew on the stigmatic surface towards the suture line (Fig. 1E) to penetrate in the style. The stigmatic suture line formed a furrow along the entire stigma continuous with the style secretory area of the semi-open canal (Fig. 1F). Germination occurred rapidly and 1 h after pollination pollen grains had germinated and grew freely in the stigma. An average of 22.5 pollen grains

( $\pm 15.2$ ,  $n = 70$  stigmas) were recorded per stigma with an average pollen germination of 43.6 % ( $\pm 8.4$  %,  $n = 70$  stigmas). However, a drastic reduction in the number of pollen tubes occurred at the stigma–style interface (Fig. 1F), and only one to three pollen tubes were observed in the style. This reduction is related to the receptive surface available for pollen tube growth. Whereas the stigma was formed by a wide papillar surface  $470.1 \mu\text{m}$  ( $\pm 78$ ,  $n = 10$ ) in length and  $307 \mu\text{m}$  ( $\pm 69$ ,  $n = 10$ ) in width, the receptive surface in this area was restricted to the narrow stylar semi-closed margins that were lined by secretory papillae continuous with those of the stigma. This stigma–style interface was able to lodge very few pollen tubes which, in their way through the style, stick to this narrow receptive surface leaving the rest of the non-receptive stylar canal empty. Pollen tubes were first seen growing in the style 2–3 h after pollination, and travelled over  $836.3 \mu\text{m}$  ( $\pm 77.3$ ,  $n = 10$ ) to reach the ovary locule (Fig. 1G) some 4–6 h after pollination, with an average pollen tube growth rate in the style of  $280 \mu\text{m h}^{-1}$ . At the ovary locule, the pollen tubes grew over the obturator and penetrated the hood-shaped ovule after traversing a distance of  $526 \mu\text{m}$  ( $\pm 86$ ,  $n = 10$ ). A single pollen tube was observed penetrating each ovule (Fig. 1H) and the first fertilized ovules were observed 1 d after pollination.

### *Embryo sac and fertilization*

*Annona cherimola* has an anatropous, bitegmic and crassinucellate ovule, with an endostomal micropyle formed by the inner integument that protrudes over the external integument (Fig. 2A). The outer integument is vascularized. At anthesis, the embryo sac is mature and shows the Polygonum-type structure with seven cells and eight nuclei, three at the micropylar end [the two synergids (Fig. 2A) and the egg cell (Fig. 2B)], three at the chalazal end [the three antipodal cells (Fig. 2C)] and two polar nuclei in the centre (Fig. 2D), which are not fused at anthesis.

The embryo sac contains large starch grains distributed in the central cell (Fig. 3A). These starch grains are much larger than the standard starch grains located in the sporophytic tissues of the ovule. They react both to PAS and IKI stains. Starch accumulates around the egg cell (Fig. 3B), and also around the two polar nuclei (Fig. 3C) that fuse close to the time of fertilization. Both synergids have a large vacuole at the top of the cell (antipodal side) and the cytoplasm and the nucleus are located at the base (micropyle side) (Fig. 3D) where a filiform apparatus is developed (Fig. 3E). Starch disappears following fertilization concomitant with endosperm development. Endosperm is cellular and starts to develop 3 d after pollination. It has a bipolar nature where the first division produces a large cell in the micropylar pole and a smaller cell in the chalazal pole. Starch grains accumulate in the chalazal pole cell (Fig. 3F) and decrease with endosperm enlargement (Fig. 3G), disappearing 3 weeks after pollination concomitant with further endosperm cellular division. After fertilization, a zygote develops and the first cell division could be seen 8 d after pollination when the endosperm already has four cells (Fig. 3H).

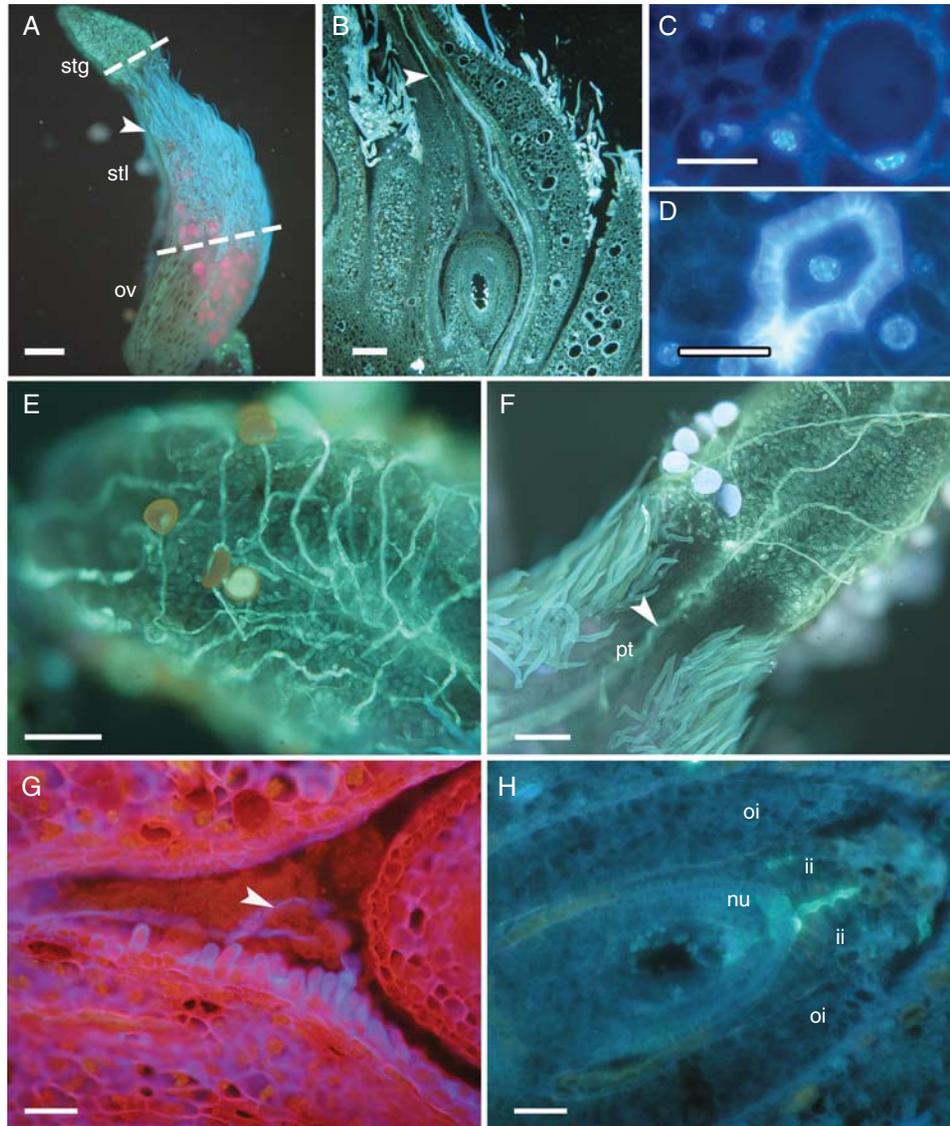


FIG. 1 Gynoecium anatomy and pollen tube growth in *Annona cherimola*. (A) Pistil showing the stigma (stg), short style (stl) and ovary (ov) with partial post-genital fusion at the periphery of the innermost side (arrowhead). (B) Longitudinal section of the pistil showing pollen tube growth (arrowhead) through the short open styler canal that leads to an anatropous ovule. (C) Oil cells. (D) Thick-walled sclereid cells. (E) Pollen tubes growing on the stigma towards the stigmatic furrow that leads to the styler canal. (F) Stigma–style interface, with the stigmatic furrow leading to the narrow receptive closing margins of the styler canal (arrowhead) and pollen tube growing through (pt). (G) Pollen tube (arrowhead) reaching the locule over the continuous papillar secretory zone. (H) Pollen tube growing through the micropyle formed by the inner integument (ii) that protrudes over a hood-shaped outer integument (oi), and reaching the nucellus (nu) 24 h after pollination. (A) Whole mount of a dissected pistil stained with aniline blue. (B, H) Aniline blue staining of a 10- $\mu$ m paraffin section. (C, D) DAPI staining of a 5- $\mu$ m resin section. (E, F) Aniline blue staining of squashed preparation. (G) Mixed staining of a 10- $\mu$ m paraffin section. Scale bars: (A) 200  $\mu$ m; (B) 200  $\mu$ m; (C) 20  $\mu$ m; (D) 20  $\mu$ m; (E) 20  $\mu$ m; (F) 100  $\mu$ m; (G) 20  $\mu$ m; (H) 20  $\mu$ m.

#### Changes in the pistil

The pollen tube pathway is lined by secretory papillae that form a continuous carpet from the stigma down to the placenta facing the ovule endostome. The papillae in the placenta resemble an obturator as they form a protuberance towards the ovule entrance. Cytologically, these papillae are similar to those of the stigma and style (Fig. 4A–C) although a basipetal maturation sequence can be seen from the stigma down to the obturator. The papillae in the less mature areas are rich in starch and show no secretion. As papillae mature, starch disappears concomitant with the production of a

secretion on the surface of the papillae. Thus, although at pre-anthesis the stigma does not appear to contain starch (Fig. 4A), starch is still present in the style (Fig. 4B) where a secretion is being produced. At the obturator (Fig. 4C) starch is far more conspicuous and the secretion is not apparent at this time.

The secretion of the papillae is present along the whole pollen tube pathway from anthesis to 6 d later. This secretion stains heavily with PAS and with toluidine blue (Fig. 4B). The papillae and secretion are present just in the outermost side of the semi-open styler canal (Fig. 4D). Before anthesis, the unicellular secretory papillae are rich in starch reserves in the style (Fig. 4B, E). One day after anthesis, starch disappears and the

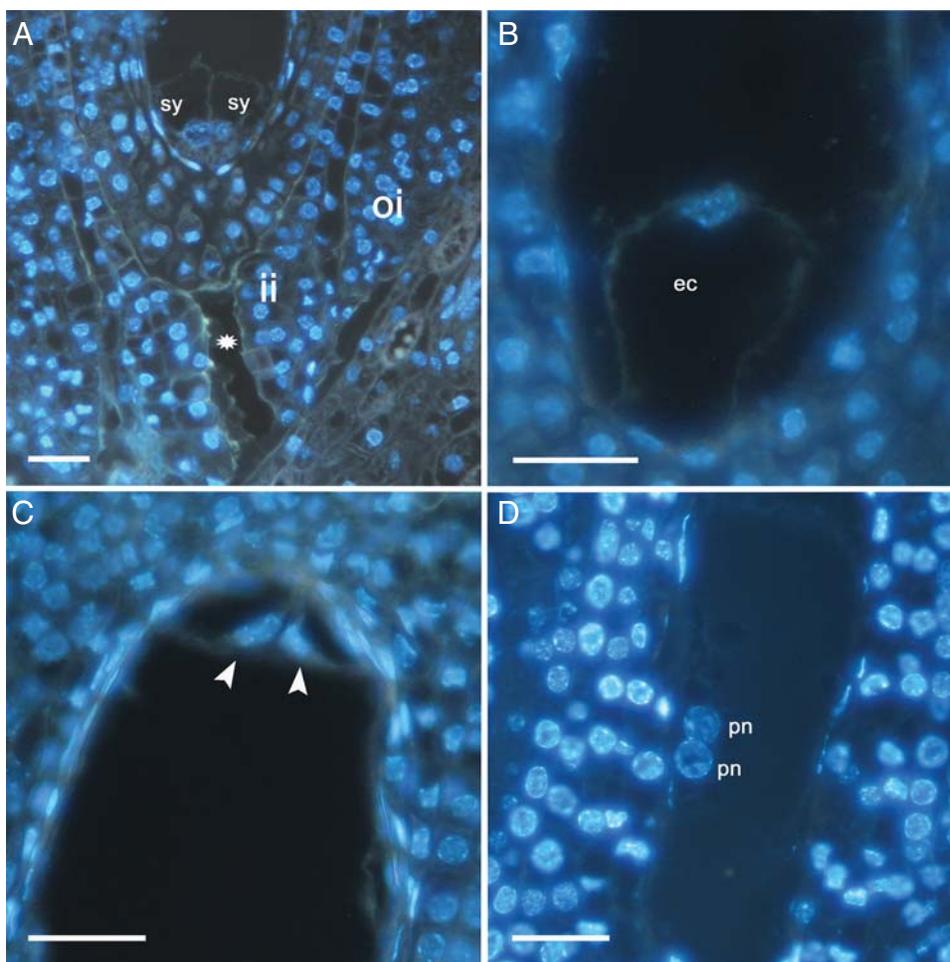


FIG. 2 Embryo sac in *Annona cherimola*. (A) Ovule showing the micropyle (asterisk) formed by the inner integument (ii), the shorter outer integument (oi) and the embryo sac with two synergids (sy). (B) Egg cell (ec). (C) Two of the three antipodal cells (arrowheads). (D) Two polar nuclei (pn). DAPI staining of 5- $\mu\text{m}$  resin sections. Scale bars = 20  $\mu\text{m}$ .

secretion increases (Fig. 4F) concomitant with pollen tube passage. The same situation can be seen in the obturator where starch is conspicuous before anthesis (Fig. 4G) but disappears as secretion increases 1 d later (Fig. 4H). This process does not seem to be triggered by the pollen tube passage, but appears to be developmentally regulated, as it occurs in the same way and at the same time in unpollinated flowers.

Although at preanthesis callose is not apparent in the papillae secretory cells, callose layering starts in the papillae 1 d after pollination (Fig. 5A) and is also present in the obturator (Fig. 5B). This callose layering occurs in a similar way in pollinated (Fig. 5B) and unpollinated flowers (Fig. 5C). However, callose layering in the nucellus at the base of the embryo sac appears 3 d after pollination only in pollinated flowers (Fig. 5D). By contrast, callose layering in the vascular bundles is only observed in ovules of unpollinated flowers 1 d after anthesis (Fig. 5E). During ovary development, callose also appears in the cell plates forming the walls of the cellular endosperm (Fig. 5F, G).

Gynoecium weight increases slowly and is similar in pollinated and unpollinated flowers during the first 4 d after anthesis. Six days after anthesis differences can be observed among

pollinated and unpollinated flowers; thus, whereas unpollinated flowers start to drop, pollinated flowers experience rapid growth (Fig. 6). A similar pattern was observed for pollinated and unpollinated flowers in the two years of observations.

## DISCUSSION

*Annona cherimola* shows a simple and plesiomorphic pistil, with a short style and a semi-open continuous secretory carpel, which supports a simple pollen–pistil interaction. Interestingly, this interaction exhibits a support–constrain strategy that is prevalent in phylogenetically derived angiosperm species (Herrero and Hormaza, 1996) although in the former case it takes place in the stigma rather than the style.

### *Pistil support to pollen tube growth*

*Annona cherimola* has a wet stigma like other closely related species in this genus (Heslop-Harrison and Shivanna, 1977; Vithanage, 1984). In *A. cherimola* the common secretory papillar carpet that covers the stigma, style and ovary along the semi-suture line provides a substrate for pollen tube

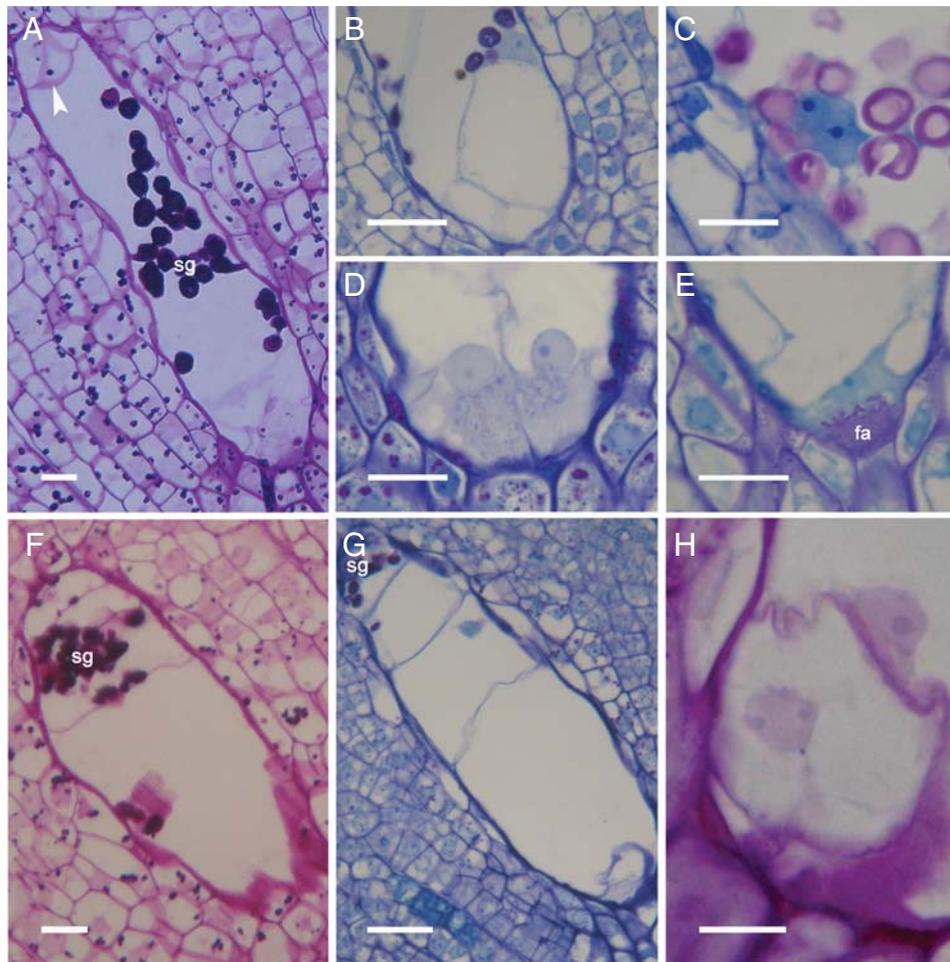


FIG. 3 Embryo sac and endosperm development in *Annona cherimola*. (A) Embryo sac of flower in preanthesis with starch grains (sg) around the two polar nuclei and showing the three antipodal cells (arrowhead). The difference between the standard starch grains (sg) located in the sporophytic tissues of the ovule and the big starch grains in the female gametophyte is apparent. (B) Egg cell with a large vacuole at the base of the cell. (C) Two polar nuclei surrounded by large starch grains. (D) Two synergid cells with nucleus and cytoplasm at the micropylar end and a large vacuole at the top of the cell. (E) Filiform apparatus (fa) of a synergid cell. (F) Starch grains (sg) accumulating in the chalazal pole of the embryo sac in a fertilized ovule four days after pollination. (G) Cellular endosperm 8 d after pollination with starch (sg) accumulated at the cell of the chalazal end. (H) Zygote first division 8 d after pollination. PAS (A, F, H) and PAS and toluidine blue (B–E, G) staining of a 2- $\mu$ m resin section. Scale bars: (A) 20  $\mu$ m; (B) 10  $\mu$ m; (C) 10  $\mu$ m; (D) 10  $\mu$ m; (E) 10  $\mu$ m; (F) 20  $\mu$ m; (G) 20  $\mu$ m; (H) 10  $\mu$ m.

growth. Secretion along the pollen tube pathway has also been reported in other members of ancient angiosperm clades such as *Trimenia moorei* (Bernhardt *et al.*, 2003), *Illicium floridanum* (Koehl, 2002, cited by Bernhardt *et al.*, 2003), *Amborella trichopoda* (Thien *et al.*, 2003), *Saururus cernuus* (Pontieri and Sage, 1999), *Pseudowintera axillaries* (Sage and Sampson, 2003) and *Kadsura longipedunculata* (Lyew *et al.*, 2007), and the substance involved appears to be composed of arabinogalactan and arabinogalactan-proteins (Sage *et al.*, 2009). Although dry stigmas are considered as plesiomorphic in flowering plants (Thien *et al.*, 2009) both dry and wet stigmas can be found in taxa of the ANITA and magnoliid clades (Thien *et al.*, 2009). Molecular studies on wet and dry stigmas and their implications in pollen–pistil interaction have been performed only in a limited number of evolutionarily-derived angiosperm taxa and, consequently, there is a need for more studies on this topic among early-divergent angiosperm taxa (Hiscock and Allen, 2008).

Production of this secretion is already seen before flower opening and reaches a maximum level 1 d after anthesis, concomitant with pollen tube passage. However, the production of secretion is independent of pollination as it occurs in the same way and at the same time in pollinated and unpollinated flowers. This contrasts with higher angiosperms, in which pollen tube growth in the style triggers starch degradation (Herrero and Dickinson, 1979; Gonzalez *et al.*, 1996). Interestingly, however, this production of secretion is very similar to the behaviour of the obturator in which secretion occurs at a particular time of development independent of pollination (Herrero and Arbeloa, 1989; Arbeloa and Herrero, 1991). In *A. cherimola* a primitive obturator, formed by the protuberance of the placenta, continuous with and with the same cytohistological features as the secretory papillae, appears to be present in the ovary. Similar structures have been described in other ancient angiosperm lineages, such as in species of the Magnoliaceae that show a funicular

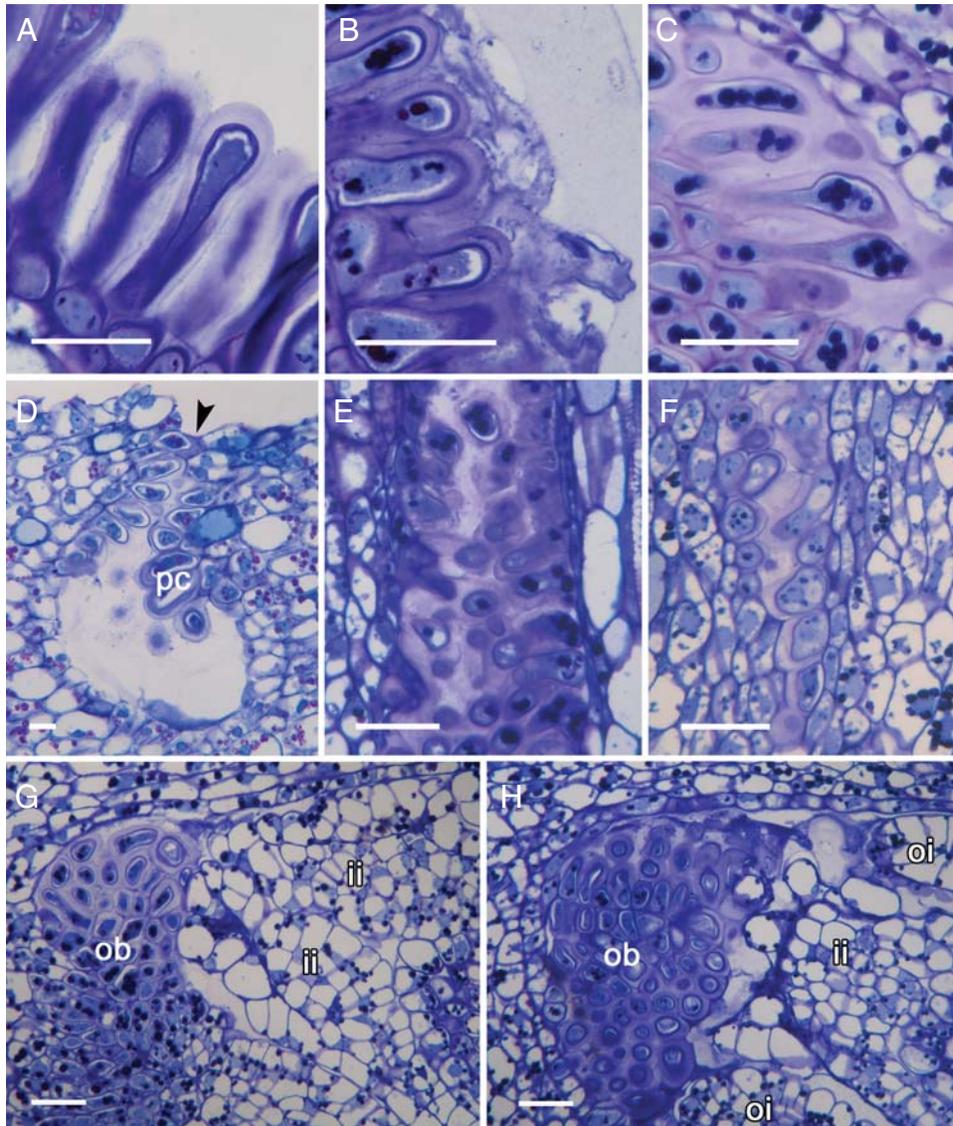


FIG. 4 Secretion along the pollen tube pathway in *Annona cherimola*. Longitudinal section of secretory papillae of the (A) stigma, (B) style and (C) obturator in preanthesis with the same cytohistological features although with differences in maturation and starch content: while at the stigma (A) starch has already disappeared, in the style (B) starch is still present and a rich secretion is apparent; and the obturator (C) is still full of starch. (D) Transverse section of the style showing a semi-open styler canal (arrowhead) lined only in the outermost side with papillar cells (pc) with secretion. Styler longitudinal section in the outermost papillar secretory zone at preanthesis (E), showing starch grains. Same area, 1 d after pollination (F), shows less starch in the cells and secretion. Transverse section of obturator (ob) cells at preanthesis (G) with starch; (ii: inner integument) and 1 d after pollination (H) with little starch and abundant secretion (ii: inner integument; oi: outer integument). PAS and toluidine blue staining of 2- $\mu$ m resin sections. Scale bars = 20  $\mu$ m.

outgrowth with papillose cells (Matsui *et al.*, 1993; Umeda *et al.*, 1994), in Schisandraceae (Lyew *et al.*, 2007), Lauraceae (Sedgley and Annells, 1981) and in the monocot *Ornithogalum caudatum* (Tilton and Horner, 1980). In *A. cherimola* the fact that secretion is present right from anthesis at pollination provides an adequate substrate for rapid pollen tube growth. This contrasts with longer times for pollen tube growth reported in other species, which are related to waiting times in order to reach the phase where secretion is produced in the pistilar structures (Herrero and Arbeloa, 1989; Herrero, 2000, 2003).

Following the production of secretion, callose is layered in the papillar secretory structures in the same way reported

in the obturator of peach (Arbeloa and Herrero, 1987), perhaps protecting this area and fulfilling what has been considered as a prophylactic role (Heslop-Harrison, 1999, 2000).

#### *Pistil constraint to pollen tube growth*

Pollen tube growth proceeds rapidly and, within 1 d of pollination, the pollen tubes reach the hood-shaped ovule, which has been considered as a plesiomorphic trait in angiosperms (Soltis *et al.*, 2005). Relatively rapid pollen tube growth has also been found in other members of ancient angiosperm clades (Bernhardt *et al.*, 2003; Sage and Sampson, 2003;

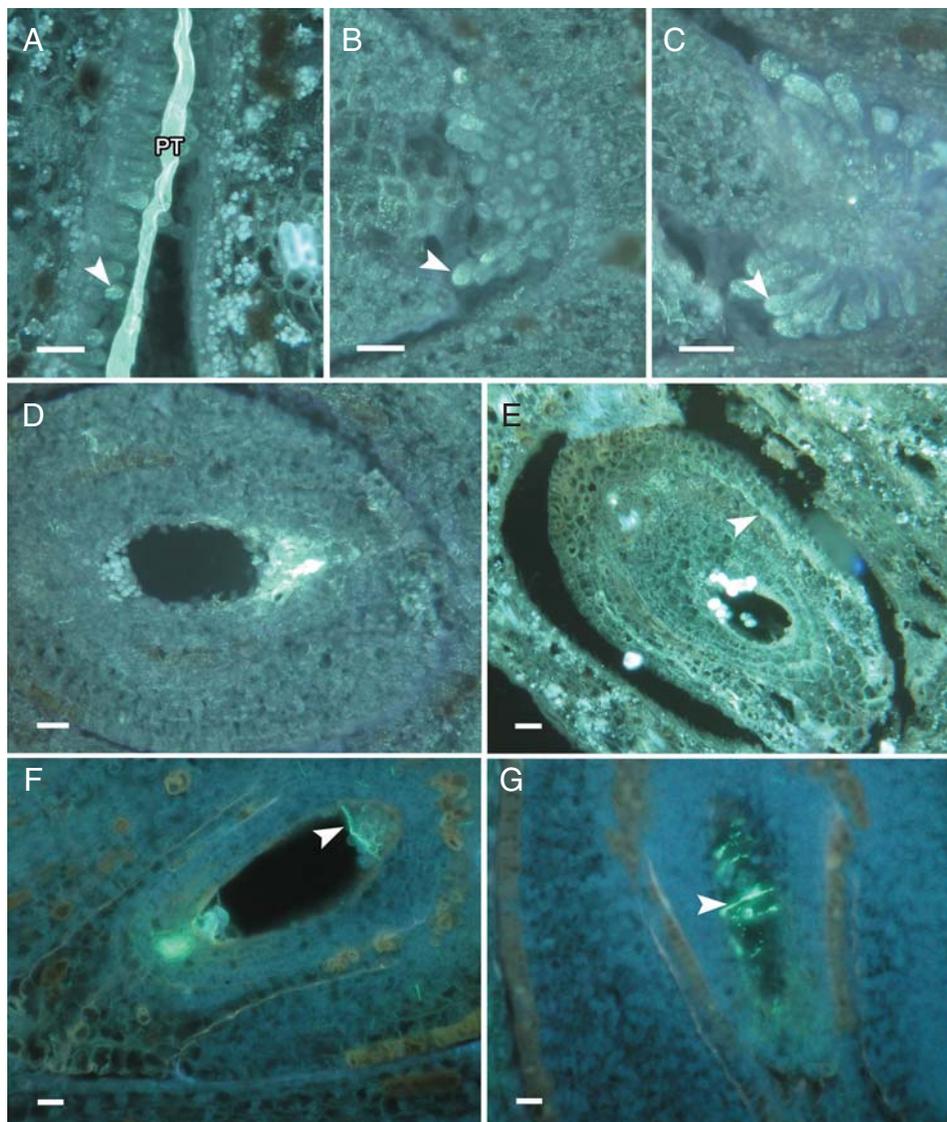


FIG. 5 Callose layering along the pollen tube pathway and during early endosperm development in *Annona cherimola*. Callose 1 d after pollination in papillar cells (arrowhead) of style (A) and obturator (arrowhead) of a pollinated (B) and an unpollinated (C) flower. (D) Callose in the nucellus micropylar pole 3 d after anthesis. (E) Deposition of callose in vascular bundles of the ovule (arrowhead) of unpollinated flowers 1 d after pollination. Callose in the cell plates (arrowhead) forming the walls of the cellular endosperm, 4 d after pollination (F) and 8 d after pollination (G). Aniline blue staining of 10- $\mu\text{m}$  paraffin sections. Scale bars = 20  $\mu\text{m}$ .

Koehl *et al.*, 2004; Hristova *et al.*, 2005; Williams, 2008, 2009) and contrasts with the slow growth of pollen tubes in gymnosperms (Gelbart and Von Aderkas, 2002). Both the pollen tube growth rate of *A. cherimola* ( $480 \mu\text{m h}^{-1}$ ) and the length of the pollen tube pathway (1.83 mm) are in the range described for basal grade angiosperms (Williams, 2008: approx.  $80\text{--}600 \mu\text{m h}^{-1}$  and  $<0.5$  to approx. 15 mm long). Although in some derived angiosperms delayed fertilization has also been recorded (Sogo and Tobe, 2005, 2006a, b), an evolutionary trend towards rapid pollen tube growth in seed plant pollen has been proposed (Pettitt, 1982; Williams, 2008) where the development of callose plugs in pollen tubes could have played a major role (Williams, 2008). Differences in timing also appear to be related to differences in maturation of the pistil (Herrero and Arbeloa, 1989; Sogo

and Tobe, 2005, 2006a, b) and to a requirement for male–female synchrony (Herrero, 2003).

Several pollen grains germinate on the stigma, but only one pollen tube reaches the ovule and achieves fertilization. Although pollen grains germinate freely at the stigma and direct their growth towards the semi-open suture line, a clear restriction and reduction in the number of pollen tubes occur at this point of entrance in the short stylar canal. Only 1–3 pollen tubes penetrate the style. This reduction in the number of pollen tubes may be related to the limited space available with only a narrow papillar secretory area that paves, along the semi-open suture line, the carpel margins. Pollen competition and selection appears to be a common feature shared by most angiosperms (Mulcahy, 1979; Hormaza and Herrero, 1992, 1996) and it is usually reflected

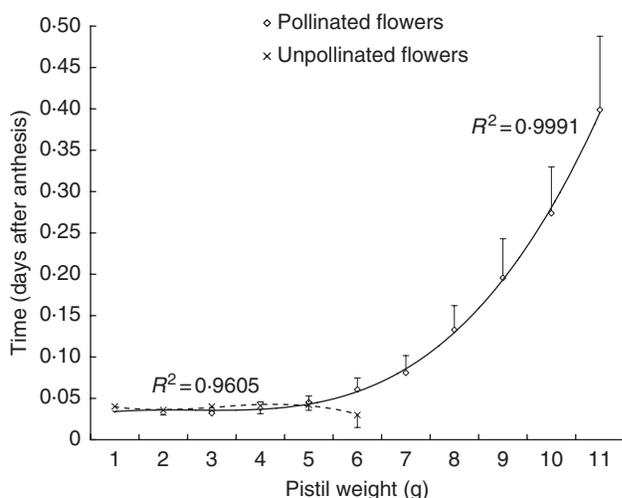


FIG. 6 Mean pistil weight after anthesis in pollinated and unpollinated flowers of *Annona cherimola*. Bars indicate s.d.

by a reduction in the number of pollen tubes that continue to grow in the style (Sedgley, 1977; Cruzan, 1990; Hormaza and Herrero, 1996). However, results herein show that in *A. cherimola* the main restriction point appears at the stigma–style interface. This behaviour should be investigated in other early-divergent angiosperms, but interesting recent work in *Amborella trichopoda* (Williams, 2009) shows a very similar behaviour. *A. cherimola* has a semi-open stylar canal similar to that described in *Amborella trichopoda* (Williams, 2009) and in *A. cherimola* only the carpel margins are layered with secretory papillae, paving a narrow way for the few pollen tubes growing in the style. A semi-open stylar canal is a common feature found in other ancient angiosperms (Endress and Igersheim, 2000) and it would be interesting to evaluate if papillar secretory cells restricted to the margins also provide a similar pollen restriction mechanism, in contrast to the typical pollen tube attrition recorded in the style in evolutionarily derived angiosperms. If this is so, during angiosperm evolution, the arena for pollen competition would have changed from the stigma to within the style and, consequently, pollen competition in the style could be considered an innovation in evolutionarily derived clades of flowering plants.

#### Post-fertilization events

Three days after pollination callose is layered in the nucellus under the embryo sac micropylar pole only in the ovules of pollinated flowers that appear to have been fertilized. By contrast, in ovules of unpollinated flowers, deposition of callose in vascular bundles was observed 6 d after pollination, suggesting impending ovule abortion. This has been shown in other species and explained in terms of blockage of metabolite translocation (Pimienta and Polito, 1982; Herrero and Arbeloa, 1989; Rodrigo and Herrero, 1998).

The presence of starch grains has been reported in mature embryo sacs in some ancient angiosperm lineages (Cook, 1902; Kimoto and Tobe, 2001; Friedman, 2008), including species in the Annonaceae (reviewed in Svoma, 1998b), and

also in higher angiosperms (Evans, 1919; Maheshwari, 1950). However, the abundance and large size of the starch grains observed in the present study are striking. Recent results in Hydatellaceae (Friedman, 2008; Rudall *et al.*, 2008), a family recently recognized among early-divergent extant angiosperms (Saarela *et al.*, 2007), show a maternal seed-provisioning strategy similar to that observed in gymnosperms. The provision of starch grains reported here in the embryo sac before fertilization in *A. cherimola* could respond to a similar plesiomorphic strategy. Through a different accumulation pattern, the accumulation of starch reserves in either the sporophytic or the gametophytic tissue would constitute an accumulation of reserves before fertilization to support early post-fertilization processes.

Although zygote cell division does not start until 8 d after pollination, endosperm cell division starts 3 d after pollination. Division of the endosperm is bipolar, giving rise to a large cell close to the zygote and a small cell full of starch at the chalazal end. This situation persists during the first endosperm divisions. Although this behaviour in relation to starch accumulation has not been reported previously, a bipolar endosperm cellular division has been shown in several species of the Winteraceae (a sister group to Annonaceae in the Magnoliales) such as *Drimys winteri* (Bhandari and Venkatar, 1968; Floyd and Friedman, 2000), *Pseudowintera axillaries* (Sampson, 1963, cited in Bhandari and Venkatar, 1968) and *Zygogynum bailloni* (Swamy, 1952). The presence of a similar cellular endosperm with unequal division has also been reported in other ancient angiosperms (Floyd and Friedman, 2000; Tobe *et al.*, 2000) and seems to be a plesiomorphic feature in angiosperms. The prominence of endosperm development in *A. cherimola* contrasts with an underdeveloped embryo that in the mature seed is embedded in abundant ruminant endosperm, similar to other species of the Annonaceae (Corner, 1949; Svoma, 1998b), where this slight embryo development has been postulated as an ancestral feature (Hayat, 1963; Finch-Savage and Leubner-Metzger, 2006).

#### CONCLUDING REMARKS

Flowers of *A. cherimola* have a number of ancestral characteristics of angiosperms such as the semi-open simple carpel, hood-shaped ovule, cellular endosperm and seed type with a rudimentary embryo. However, this primitive carpel hosts a support–constrain strategy for pollen tube growth conserved in phylogenetically derived angiosperm lineages. Support is provided by the continuous secretory papillar carpet that paves the pollen tube pathway and that provides evidence for a common ontogenetic origin for this tissue as well as for a conserved basipetal maturation that encompasses pollen tube growth. Constraint and restriction in the number of pollen tubes occurs at the stigma–style interface and is mediated by a dramatic reduction in the secretory surface available for pollen tube growth from the stigma to the margins of the semi-open stylar canal. It will be of interest to evaluate in other ancient lineages of angiosperms with a similar pistil anatomy if this extramural pollen competition is conserved as compared with the stylar intramural competition in modern angiosperms.

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