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Histological analysis of pollen-pistil interactions in sour passion fruit plants *(Passiflora edulis* Sims)

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Key words: flower, pollen tube, pollination, stigma

ABSTRACT: The success of sexual plant reproduction is directly influenced by specific interactions between the pollen and pistil. Light, fluorescence and scanning electron microscopy techniques were used to evaluate the steps of pollination in sour passion fruit plants (*Passiflora edulis* Sims). In the compatible interaction, pollen tubes grow through stigma projections towards the ovary. The pollen grain surface was found to be spheroidal and to consist of heteroreticulate exine with six colpi. Furthermore, analysis *in vivo* of pollen-pistil interactions indicated that stigmas of flowers 24 hours before anthesis are unable to discriminate compatible (genetically unrelated) and incompatible (genetically related) pollen grains. Taken together, these results provide insight into the cellular mechanisms underlying pollination in passion fruit plants.

Introduction

Sour passion fruit or 'maracujá' (*Passiflora edulis* Sims) is of great interest to fruit producers, due to its rapid production rates in comparison with other fruits and its demand in the market, both for fresh consumption and industrial processing. The species is highly dependent on cross-pollination for fruit set, due to the presence of self-incompatibility, a mechanism that favors outcrossing and thus promotes genetic variability within the species (Bruckner *et al*., 1995; Higashiyama, 2010). The process of self-incompatibility allows female reproductive cells to discriminate between genetically related (incompatible) and genetically unrelated (compatible) pollen, and specifically reject incompatible pollen (Rea and Nasrallah, 2008).

Since reproduction is a critical step in the life cycle of all organisms, there is great interest in determining how this process is controlled. In angiosperms, one of the most important stages in the reproductive process is pollination (Piechowski *et al*., 2009; Jha and Dick, 2010); however, there are several steps before and after this event that determine reproductive success. Pollination and fertilization occur in the flowers of angiosperms; among the various organs that constitute the flower, the stamen and pistil are directly involved in plant sexual reproduction. Pollen, the male gametophyte, is surrounded by a coat named the sporoderm, which consists of the intine (the inner layer, which is composed of cellulose, pectin, and protein) and the exine (the outer layer, which is synthesized and secreted by the tapetal tissue of the anther and is composed mainly by sporopol-

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lenin). Pollen coat proteins interact with glycoproteins present in the pistil (Zhang *et al*., 2008).

During compatible interactions, after pollen has been deposited on and adheres to the stigma, the pollen hydrates and germinates by emitting the pollen tube, which penetrates the cuticle of the stigma cells and grows through the extracellular matrix of the transmission tissue of the style. The process culminates in the discharge of male gametes into the embryo sac. In *Passiflora* the embryo sac is the Polygonum type, as described for about 70% of the angiosperm species (Souza *et al*., 2002; Garcia *et al*., 2003). In this pattern, each pollen tube bears two sperm cells, one of which will fuse to the egg cell, and the other to the two polar nuclei of the central cell to generate the triploid endosperm (Hiscock and Allen, 2008; Gotelli *et al*., 2010).

In the angiosperms, the male gametophytes are released from the anther as dehydrated pollen grains, whose degree of dehydration varies depending on the species. It is estimated that the percentage of water present in the pollen during anthesis varies from 15 to 35% (Dumas and Knox, 1983). The hydration needed for pollen germination to occur usually takes place after the pollen interacts with a stigma of the same species.

The stigma surface is classified as being wet or dry, depending on the amount of secretion in this region (Heslop-Harrison *et al*., 1995; Heslop-Harrison and Shivanna, 1977). Theses secretions are directly related to pollination success, since absorption of water is an essential factor for pollen germination and pollen tube penetration of the stigma (Goldman *et al*., 1994). Reproductive biology studies of the sour passion fruit flower revealed that the stigma is dry and the style solid (Rêgo *et al*., 2000; Souza *et al.,* 2006). These characteristics may be related to the sporophytic self-incompatibility mechanisms described for this species.

In species containing dry stigmas, regulated pollen hydration provides an effective early barrier to incompatible pollination (Edlund *et al*., 2004). Regulated pollen hydration is active in self-incompatible crosses (Sarker *et al.,* 1988) and in crosses between species (Lewis and Crowe, 1958; Hulskamp *et al*., 1995). Remarkably, the stigma can hydrate a compatible pollen grain while restricting the hydration of incompatible pollen on the same papillus (Dickinson, 1995).

In an effort to improve our understanding of the cell and tissue interactions post-pollination, we investigated the reactions that occur in pollen-pistil interactions in sour passion fruit plants at the anatomical and ultrastructural level.

Material and Methods

Plant Material

Sour passion fruit plants were grown at an experimental field established in the Centro de Ciências e Tecnologias Agropecuárias of Universidade Estadual do Norte Fluminense Darcy Ribeiro, located at the Escola Agrícola Antônio Sarlo, the city of Campos do Goytacazes, Northern State Rio de Janeiro, Southeastern Brazil, with latitude 21° 45', longitude 41º 20' W and altitude 11 m. The experiment was conducted from September to June in the years 2007 and 2008. Compatible (genetically unrelated) and incompatible (genetically related) pollen tube samples were obtained from hand-pollinated plants. If the ovary was swollen and still attached to the stalk 48 h after pollination, the cross was considered successful and compatible. Otherwise, the cross combination was considered to be self-incompatible.

Light Microscopy

Thirty samples of stigmas subjected to compatible or incompatible pollination were fixed for 2 h in an aqueous solution containing 2.5% glutaraldehyde, 4% formaldehyde, and 0.05 M cacodylate buffer (pH 7.2). Subsequently, the samples were post-fixed for 2 h in a solution of 1% osmium tetroxide diluted in 0.05 M cacodylate buffer (pH 7.2) at room temperature. The samples were then dehydrated in an ascending series of acetone solutions, for 1 h at each step, and the material was infiltrated and embedded in epoxy resin (Polybed®). Semi-thin transverse sections (1.0 μm) of the stigma tissue were stained with toluidine blue (0.1% aqueous solution) (Martin, 1959), and the slides were sealed with Entellan®. The sections were observed using an Olympus BX 60 microscope and images were captured using Image Pro-Plus software®.

Scanning Electron Microscopy

For scanning electron microscopy studies, all of the acetone in samples subjected to the fixation, post-fixation, and dehydration steps described above was replaced with liquid CO_2 under high pressure conditions (up to the critical point for CO_2), using the Bal-Tec CPD 030 Critical Point Dryer. The fragments were fixed on stubs, coated with a thin gold layer of 20 nm, and observed with a ZEISS-DSEM 962 scanning electron microscope operating at 25 kV.

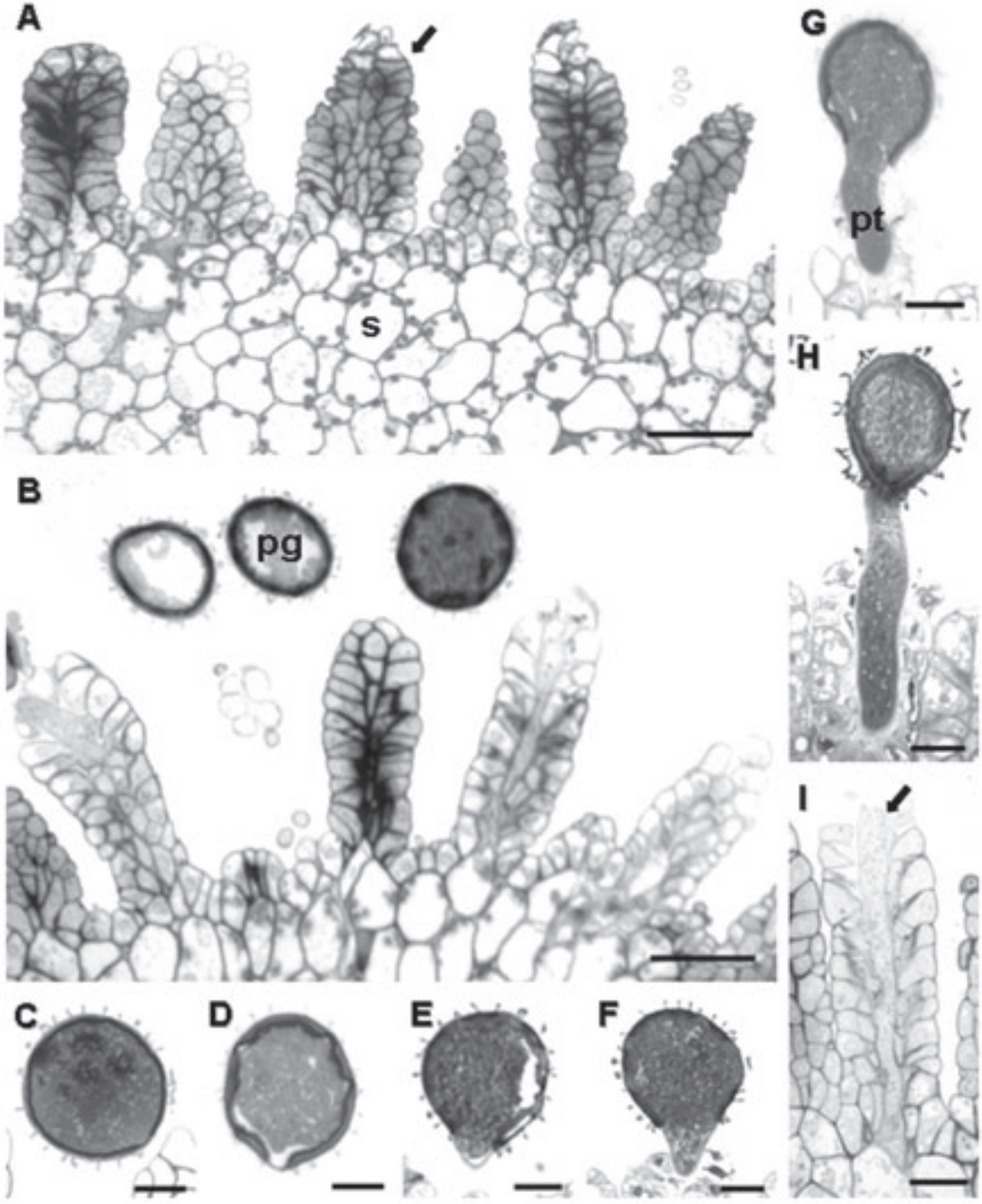

FIGURE 1. Optical microscopy of pollen-stigma interactions in sour passion fruit. **A.** Cross-section of an unpollinated stigma. The arrow indicates the stigmatic projections. **B.** Section through the stigma after pollination. **C.** Pollen grain 5 min after compatible pollination. **D.** Pollen grain 10 min after compatible pollination. **E.** Pollen grain 15 min after compatible pollination. **F.** Pollen grain 20 min after compatible pollination. **G.** Pollen grain 1 h after compatible pollination. **H.** Pollen grain 2 h after compatible pollination. **I.** Pollen grain 3 h after compatible pollination. The arrow indicates the pollen tube within the stigma. Symbols: s, stigma; pg, pollen grain; pt, pollen tube. Bars: A,B, 50 μm; C-I, 30 μm.

Fluorescence Microscopy

Flowers 24 h before anthesis and pistils of flowers at anthesis were collected 1, 12 and 24 h after hand self- and cross-pollinations, and were fixed in 70% FAA (formalin, alcohol, acetic acid). Samples were softened in 1 M sodium hydroxide (NaOH) solution for 8 h, washed with tap water, and stained with 1% aniline blue (Currier, 1957). The stained tissues were positioned on slides and analyzed by fluorescence microscopy using an Olympus BX 60 microscope fitted with a UV filter.

To evaluate pollen tube growth through histological sections, samples were fixed and washed as described above. The stigmas were then dehydrated in an ascending series of alcohol, for 1 h at each step. Infiltration was performed by gradually replacing the alcohol solution with Leica Historesin infiltration medium. Once polymerized, thin slices (4 μm thick) of sample were sectioned using a rotary microtome (Slee Mainz Cut 4050) and steel knife. The sections were transferred to glass slides, stained, and analyzed as described above.

Results

Compatible interaction between the stigma and pollen grains of sour passion fruit plants

The stigmatic tissue of passion fruit plants is not composed of papillae, but of stigma projections that consist of epidermal and subepidermal cells (Fig. 1A). Pollination occurred with the deposition of pollen on or near the apex of the stigma projections (Fig. 1B). During the compatible (genetically unrelated) interaction, the pollen grain hydrated and germinated after adherence to the stigma (Fig. 1C-E). The grain emitted a pollen tube that traveled through the transmitting tissue of the stigma (Fig. 1F-I), in search of the ovary. One hour after pollination, pollen tubes were observed to penetrate and grow within the stigma (Fig. 1G). The pollen tubes grew through the intercellular spaces of a stigma projection, and not in the gaps between stigma projections (Fig.1G-I). The morphological data presented here indicate that the stigmatic cu-

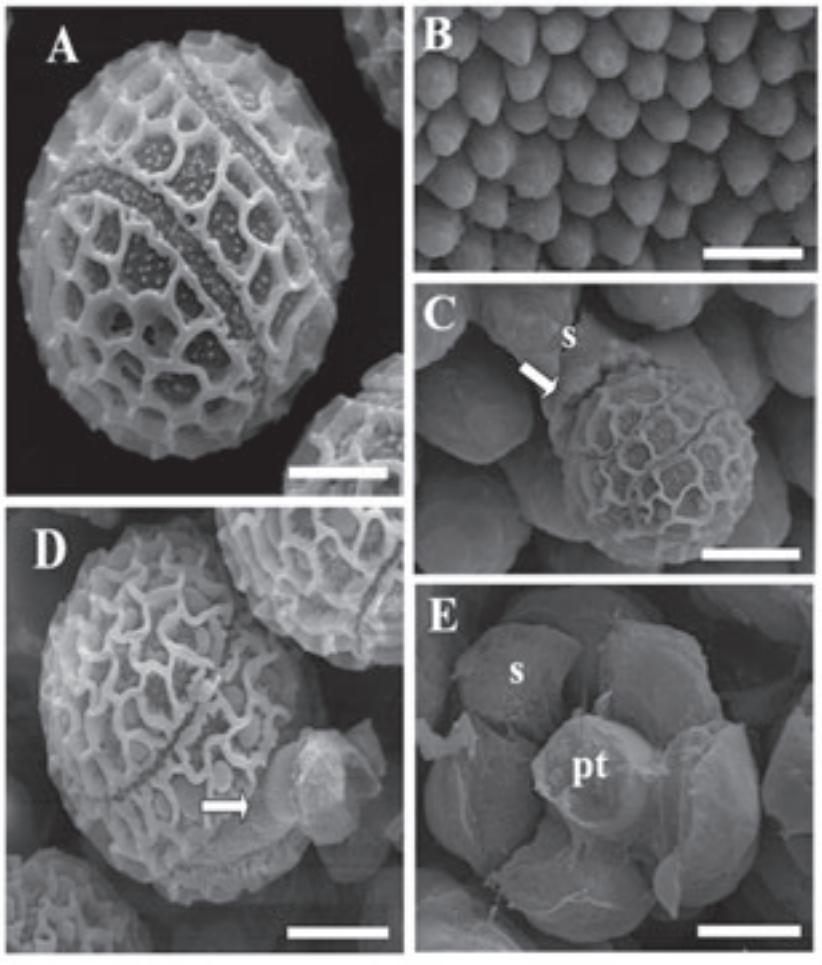

FIGURE 2. Scanning electron micrographs of the pollen grains, stigma, and pollen tube of sour passion fruit. **A.** Pollen grain surface. **B.** Stigma surface showing the stigma projections. **C.** Detail of compatible pollen grain on the stigma. Note the structural change of the stigma. **D.** Pollen tube formed at pollen grain aperture. The arrow indicates the opening of the colpi. **E.** Pollen tube detached from pollen grain, inserted into the stigma projection, among stigma cells. Symbols: s, stigma; pt, pollen tube. Bars: A-C, E, 20 μm; D, 10 μm.

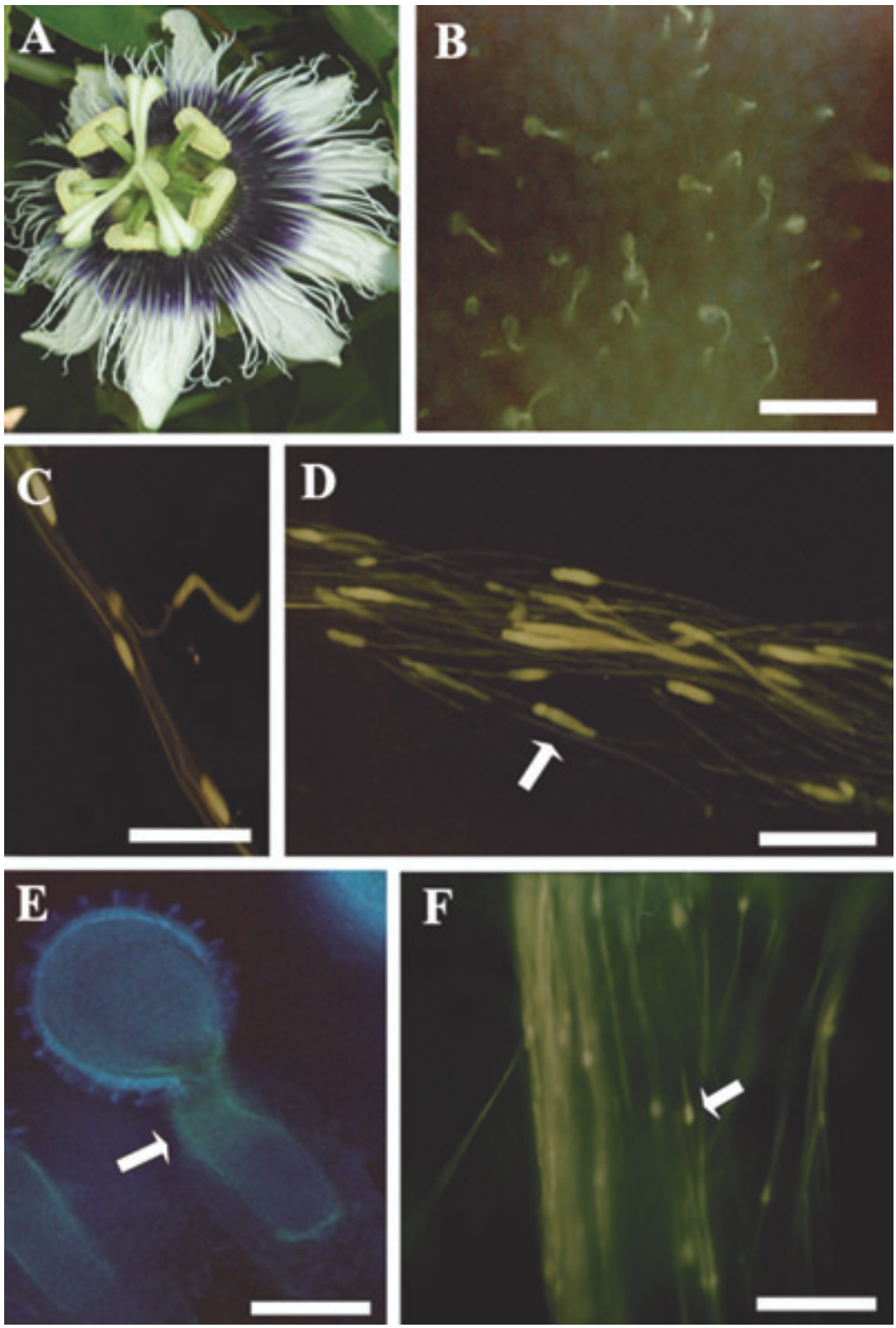

FIGURE 3. Fluorescence microscopy of the *in vivo* pollen-pistil interactions in sour passion fruit. **A.** Passion fruit flower. **B.** Pollen tubes growth over stigmatic surface 1 h after compatible pollination. **C.** Detail of pollen tube growth in the transmitting tissue of the style, 12 h after a compatible pollination. **D.** Pollen tubes at ovary 24 h after compatible pollination. The arrow indicates the callose plug in the elongating pollen tube. **E.** A compatible pollen grain emitting a pollen tube on a receptive stigma. The arrow indicates the callose present in pollen tubes. **F.** Pollen tubes expanding parallel to the style axis 12 h after incompatible pollination on the immature stigma. Arrows show the presence of callose buffers. Bars: B, F, 100 μm; C,D, 50 μm; E, 20 μm.

ticle of passion fruit plants does not rupture upon penetration by germinating pollen tubes, suggesting that an enzymatic process degrades the cuticle. Pollen tube growth can be relatively quick. In passion fruit, emerging pollen tubes could be observed as soon as 15 min after pollination (Fig. 1E).

External morphology of the pollen-pistil interactions

The sour passion fruit pollen grain had a spherical shape, 6-colpate, a furrowlike aperture, and a heteroreticulate exine coat (Fig. 2A). Prior to contact with pollen tubes, the stigma cells were turgid (Fig. 2B). However, regions of the stigma that made contact with pollen grains lost turgidity and underwent structural changes (Fig. 2C). When in contact with the stigmatic surface, the pollen grain germinated and emitted a pollen tube through one of its colpi (Fig. 2D). As already shown with the aid of optical microscopy (Fig. 1G), the pollen tube separated stigma cells inside the projection of the stigma 1 h after a compatible interaction (Fig. 2E).

In vivo pollen-pistil interaction in sour passion fruit

We investigated the pollen tube paths in compatible (genetically unrelated) and incompatible (genetically related) crosses using passion fruit flowers (Fig. 3A). The process of post-pollination events, starting from pollen deposition on the surface of the stigma (Fig. 3B). Pollen tubes subsequently grow into the pistil and converge into the transmitting tissue of the style where they form a dense bundle after 12 h (Fig. 3C). Callose plugs formed along the length of the pollen tubes (Fig. 3C-D). As the rate of pollen tube growth increased, so did the regularity of callose formation. The callose plugs isolate the less active regions of the cytoplasm from those of intense activity. During compatible interactions in passion fruit, the pollen tube reaches the ovary 24 h after pollination (Fig. 3D). Note that the callose was present in pollen tubes walls from the onset of germination (Fig. 3E; stained green, by aniline blue). Passion fruit plant is a self-sterile species. Once pollen grains come into contact with stigmatic papillae, only pollen grains recognized as compatible are accepted, thus allowing plants to ignore incompatible pollen. However, the stigmas of immature buds are self-compatible and only become self-incompatible 1 day before flower opening (Fig. 3F).

Discussion

Structural and cytochemical aspects of stigma, pollen grains, and details of pollen-pistil interactions following compatible pollination were investigated in sour passion fruit. According to the observations made in this work, the stigmatic tissue does not form papillae, the projection of the stigma is composed of several independent cells. The stigma of Passifloraceae flowers has been described in previous studies, Bernhard (1999) and Souza *et al*. (2006) named these stigma projections multicellular papillae. However, this characterization of the stigmatic surface of *Passiflora* was based only on the final stages of stigma development. Silvério and Mariath (2010) observed the formation of the stigmatic surface of *Passiflora elegans*, and concluded that the stigma of *Passiflora* consists of dermal and subdermal cells. They further maintained that the stigma should not be characterized in terms of colleters or papillae, but as stigma emergences. This view is corroborated by the results of our present study. Another important observation on the reproductive biology of passion fruit was revealed in this research, the pollen tube grows toward the ovary within the projections of the stigma and not between them. In compatible mating in most species (e.g., *Senecio squalidus* L.; Hiscock *et al*., 2002), the pollen tube grows between the stigma papillae and penetrates the stigma through the basal region of the papilla epidermis, where the cuticle is absent. Therefore, it is likely that the penetration of the pollen tube in *Senecio squalidus* L. occur physically and does not involve enzymatic processes for the degradation of the cuticle.

After 15 minutes of pollen-pistil interaction is possible to observe the early stages of germination of the pollen grain in passion fruit. In other species there are similar reports, such as *Ipomoea trifida*, a wild species related to sweet potato, pollen germination also occurs within 10 to 20 minutes of compatible pollinations (Kowyama *et al*., 2000). On the other hand, more than a month may elapse between pollen germination and fertilization in *Casuarina equisetifolia* (Sogo *et al.,* 2004). Extending the studies presented here, it was observed that as shown for other species, i.e., *Psychotria nuda* (Klein *et al*., 2009) and *Pinus densiflora* (Hiratsuka *et al*., 2002), the passage of pollen tubes through the stigma altered the structure of stigma cells.

According to the data presented, the pollen tube reaches the upper chamber of the ovary 24 h after pollination, however Rêgo *et al*. (2000) studying this species, observed fertilization occurring approximately

12 h after pollination. Ishihata (1991) and Ho and Shii (1986) reported that the majority of ovules were fertilized 18 and 24 h after pollination, respectively. Accordance with Heslop-Harrison (1975), such differences can be attributed to environmental factors, e.g., temperature, photoperiod, and relative humidity of the air.

Cytological studies indicated that the ability to discriminate between incompatible (genetically related) pollen and compatible (genetically unrelated) pollen, this self-incompatibility system, is affected by the developmental stage of the stigma. Pollinations performed on stigmas one day before the opening of the flower with pollen grains mature and incompatible showed that these cells can penetrate and reach the ovary. These results suggest that mature stigma emit signals that are responsible for the reaction of self-incompatibility. Thus, concluded that it is possible to perform pollination using self pollen, since the stigma is immature. Bruckner *et al*. (1995) performed self-pollination in flower buds of passion fruit and notified the 14.8% fruit set. These observations are consistent with those already described in *Brassica*, Nasrallah and Nasrallah (1993) observed that immature stigmas are unable to discriminate self-incompatible pollen from those compatible pollen. This developmental regulation allows the generation and maintenance of homozygous plants and production of inbred lines by bud pollination, key factors in the methodology of plant breeding.

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