PREFERENTIAL REPRODUCTION MODE OF HERMAPHRODITE PAPAYA PLANT (*Carica papaya* L*; Caricaceae*)[1]

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ABSTRACT - This research was done to study the reproductive system of papaya hermaphrodite plant based on the histochemical nature of pollen grain, stigma receptivity, *in vivo* pollen grain germination and pollen:ovule ratio. In the histochemical analysis, pollen grains were stained by using Sudan IV and I_2KI solution; the stigma receptivity was assessed by alpha-naphthtyl acetate solution in closed and opened flowers. Pollen germination and pollen tube growing were examined in flower buds near anthesis with 0.1% aniline blue. To estimate the pollen:ovule ratio , anthers from each flower bud were dissected and all pollen grains were counted; ovules were dissected from ovaries and were counted under stereomicroscope. The results indicated that papaya pollen grains are of lipidic nature; the stigmas were receptive before the opening and until 48 hours after opening; the pollen grains germinated and emitted polinic tube before flower opening and the pollen:ovule ratio indicated the predominance of autogamous reproductive system. These results indicate that hermaphrodite papaya trees is preferentially of optional autogamous with cleistogamy.

Index terms: *Carica papaya*, cleistogamy, autogamy, pollen grain histochemistry, P:O ratio, stigma receptivity.

MODO DE REPRODUÇÃO PREFERENCIAL DE PLANTAS HERMAFRODITAS DE MAMOEIRO (*Carica papaya* L*; Caricaceae)*

RESUMO - Esta pesquisa teve o objetivo de estudar o sistema reprodutivo preferencial de plantas hermafroditas de mamoeiro, com base na natureza histoquímica dos grãos de pólen, receptividade do estigma, teste de germinação *in vivo* do grão de pólen e razão pólen : óvulo. Na análise histoquímica dos grãos de pólen, foram utilizados os corantes Sudan IV e solução de I_2KI; a receptividade do estigma foi avaliada com a solução de acetato de alfa-naftil em flores abertas e fechadas. A germinação e o crescimento do tubo polínico *in vivo* foram avaliados em flores fechadas, utilizando solução de azul de anilina a 0,1%. Para estimar a razão pólen:óvulo, anteras de cada flor foram dissecadas, e os grãos de pólen foram contados; ovários foram dissecados, e os óvulos foram contados sob estereomicroscópio. Os resultados indicaram que os grãos de pólen do mamoeiro são de natureza lipídica; os estigmas estavam receptivos antes da abertura e até 48 h após a abertura; os grãos de pólen germinaram e emitiram tubo polínico antes da abertura floral, e a relação pólen:óvulo, indicou predominância do sistema reprodutivo autógamo. Esses resultados indicaram que o modo de reprodução preferencial do mamoeiro hermafrodita é autógamo facultativo com cleistogamia.

Termos para indexação: *Carica papaya*, cleistogamia, autogamia, histoquímica de grãos de pólen, relação P : O, receptividade de estigma.

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INTRODUCTION

Species can be classified by their reproduction mode as autogamous (obligatory or facultative) or allogamous (obligatory or facultative), depending on the pollination mode. Self-pollination can be favored by cleistogamy, chasmogamy and self-compatibility, while cross pollination can be favored by dichogamy, herkogamy and self-incompatibility (Frankel & Galun, 1977).

The cultivated papaya (*Carica papaya* L.) can be dioecious (male and female flowers on separate plants) or gynodioecious with hermaphrodite plants (hermaphrodite flower) and with female plants (female flower). In Brazil only hermaphrodite plants are commercially cultivated. The hermaphrodite plant has perfect flowers with 10 heteronomous stamina which compose the androecium and an ovary with five partite-stigmas. The hermaphrodite papaya plant can be either autogamous or allogamous.

According to Allard (1960), for the efficient conduction of a breeding program the reproduction system and the rate of natural crossing should be determined for the environment in which the breeding program will be carried out. There are various methodologies that can be used for this purpose, such as the pollen grain:ovule (P:O) ratio (Cruden, 1977), for instance, which predicts the reproductive system of the plant, better than the others which use flower size and morphology. P:O ratio is widely used to predict the reproduction mode of plant species (Wang et al. 2004). Histochemical nature of the pollen grains, whether lipidic or starchy, and the pollinator's behavior (Dafni, 1992) are factors that may be related to the reproduction mode.

The stigma receptivity is also another factor that should be considerated when establishing the reproduction mode of a crop that is being studied. According to Galen et al. (1987), the receptivity of the stigma is a very important stage in the flower maturation, which can influence the fertilization rate. The flower stage, period of the day, and presence or absence of exudate on the stigma are factors that can influence the stigma receptivity (Dumas et al. 1984).

The reproduction mode and the natural pollination rate can be considered well-known for most species and according to Oliveira & Lima (2000) this knowledge is very important not only for breeding but also to commercial exploration of the plant. There are few data about reproduction mode of papaya cultivars in Brazil.

The objective of the present study was to verify the reproduction mode of some papaya cultivars planted in Brazil, based on pollen grain histochemistry, pollen grain:ovule ratio (P:O) as well as stigma receptivity and pollen grain germination *in vivo*.

MATERIALS AND METHODS

The genetic materials used in this research were the *Carica papaya* 'Golden' (Solo) and 'Tainung 01' (Formosa) cultivars. The materials were cultivated in the field, in double rows spaced by 3.6 x 2.0 x 2.0 m and irrigated with localized irrigation. The crop management was done according to Marin et al. (1995). Four months after transplanting, the plants were classified by sex and only the hermaphrodite plants were kept in the field.

Pollen grain histochemistry

Ten flower buds per plant per cultivar, at anthesis, were fixed in 70% ethanol solution, kept in the refrigerator at 4°C. Two anthers per flower per cultivar were squashed in a slide with a drop of iodine-potassium iodide (I_2KI) solution (Johansen, 1940) and other two anthers from the same flower were squashed in a drop of Sudan IV solution (Vaissière, 1991). The slides were observed with optical microscope Olympus BX 60, USA. It was counted 250 pollen grains per slide, totalizing 5,000 pollen grains per cultivar by staining methodology. A dark bluish-black, or purple colour, in some cases, indicates the presence of starch (stained with I_2KI) and a red color indicates the presence of lipids (stained with Sudan IV) (Dafni, 1992).

Pollen grain germination in vivo test

In order to verify if pollen grains are viable and mature before flower opening, flower buds at anthesis were randomly collected, and the pistils were fixed in FAA (37% formalin, acetic acid and 30% ethylic alcohol, on the ratio of 5:5:90), according to (Dafni, 1992). After 24 hours the pistils were immersed in 0.5 M sodium hydroxide for 12 hours for softening. The pistils were then washed in tap water to remove the sodium hydroxide, and immersed in 0.1% aniline blue diluted in potassium acetate buffer according to Martin (1959), for four hours. After this preparation, the pistils were cut, with a scalpel, in three parts: stigma, style and ovary. Each part was squashed on a slide, mounted on one drop of potassium acetate buffer, and observed under a fluorescent microscope Olympus BX 60, USA, using an ultraviolet with exciting filter, 370nm, and

emission filter, 509nm. It was collected 20 flower buds per cultivar and 60 portions were observed for each cultivar.

Stigma receptivity

Two methodologies were used to evaluate stigma receptivity: one with flower at anthesis and other with flower buds two days before anthesis. For both methodologies, 20 flower buds per cultivar were identified and protected with paper bags. In the next day, opened flowers were collected from 8 A.M. to 4 P.M., with a two-hour interval. In order to observe the period of stigma receptivity, flowers were collected 24 and 48 hours after flower opening. At each collecting time, the stigmas were excised and immersed directly and immediately in alpha-naphthyl acetate stain, according to Pearse (1972), for five minutes. The stigmas were observed under a stereomicroscope (Nikon SM 7800, USA). Receptive stigmas presented black color after staining (Dafni, 1992).

Pollen:ovule ratio (P:O)

The pollen:ovule ratio (P:O) was estimated according to Cruden (1977) and Dafni (1992). Flower buds at anthesis were fixed in 70% ethanol solution and kept at 4°C. One anther was sliced and transferred to a calibrated microtube containing 1 ml of a compound solution of 0.9 ml 70% ethanol solution, three drops of 0.5% methylene blue solution, 4 drops of Triton-X detergent. The suspension was stirred in a vortex mixer for 60s at 2,000 rpm; 1µl sample of suspended solution was dropped on the slide. It was prepared ten slides per anther and the pollen grain numbers were counted under the optical microscope.

The ovaries were longitudinally cut, the ovules were loosened from the placenta and spread in a slide with a drop of lactophenol-cotton blue solution (Ornduff, 1969) and then they were counted under a stereomicroscope Nikon SM 7800, USA. Five flower buds per cultivar and four anthers per flower bud were used, totalizing four anthers per ovary. Thus, 40 slides were made per flower bud totalizing 200 slides for each cultivar, considering the anthers. The P:O ratio was calculated for each cultivar and it is expressed as the mean number of pollen grains per flower divided by the average number of ovules. The P:O ratio for each cultivar was estimated based on the average number of pollen grains and ovules. The means of pollen grains per flower, number of ovules per flower and P:O ratio, from Golden and Tainung 01 cultivar, were compared by the *t* test at the level of 5% probability. The statistical analysis was performed using the software Genes (Cruz, 2001).

RESULTS

Pollen grain histochemistry

The pollen grains of both cultivars reacted positively with Sudan IV and presented red colored pollen grains (Fig. 1A) indicating that the histochemical nature of the pollen grains is lipidic. The pollen grains did not react to the I_2KI solution and presented yellowish color (Fig. 1B). Lipid droplets were observed adhering to the pollen grain walls (Figs 1C and 1D), suggesting the presence of pollenkitt in the papaya pollen grains.

In vivo pollen grain germination test

Pollen grain germination with pollen tube emission on the stigma and pollen tube growth in the style of flower buds were observed in all analyzed pistils analyzed (Fig. 2). Pollen grains were observed germinating on the stigma (Fig. 2A and 2B) and growing in the style towards to ovary (Fig. 2C); but the presence of pollen tubes in the ovary (Fig. 2D) was only observed in 60% of the pistils in the Golden cultivar, and in 80% of the pistils in the Tainung 01 cultivar.

Stigma receptivity

It was observed that all the stigmas from opened flowers were receptive since they reacted positively to the alpha-naphthyl acetate solution, staining their surface with black color. The stigmas were receptive to pollen grains at 8 A.M. (Fig. 3A) and remained receptive until 48 hours after flower opening (Fig. 3B). The stigma receptivity in flower bud was also assessed by the receptivity reaction with alpha-naphthyl, and the results indicated that the stigma was receptive before flower bud opening (Figs 3C and 3D).

Pollen:ovule ratio (P:O)

The P:O ratio observed in this study was 233.27; the average number of pollen grains per flower was 115,000 and the average number of ovules was 493. Based on P:O ratio, papaya can be classified as a facultative self-pollinating species, that is, self-pollinating with a low cross pollination rate (Cruden, 1977).

The estimative number of pollen grains per flower in Tainung 01 cultivar (mean =121,950) was higher than the estimative number of pollen grains per flower in Golden cultivar (mean=108,050). On the other hand, the mean number of ovules in Golden cultivar (mean =

525) is higher than the mean number of ovules observed in the Tainung 01 cultivar (mean = 461). The P:O ratio estimated for the Tainung 01 was higher (mean=264.50) than the P:O ratio for Golden cultivar (mean=205.81). The *t* test ($p<0.05$) showed that there was significant difference between the Golden and Tainung 01 cultivars for the number of pollen grains per flower, number of ovules/flower and number of pollen grains for each ovule (P:O ratio).

DISCUSSION

Pollen grain histochemistry

The papaya pollen grain histochemical nature is lipidic. However, the cytoplasmic staining reaction was not uniform in the pollen grains. Rodrigues-Garcia et al. (2003) observed that the lipid bodies did not show a specific location in the olive tree (*Olea europaea* L.) pollen grains and the lipid bodies moved towards the pollen tube only at the moment of pollen grain germination. Several species, including *Gossypium hirsutum* (Wetzel & Jensen, 1992), *Brassica napus* (Charzynska et al. 1989), and *Tradescantia bracteata* (Mepham & Lane, 1970) present lipidic-type pollen grains.

The presence of pollenkitt in pollen grains suggests that the insects may act as pollinator agents in papaya. Pollenkitt is a colloidal substance present on the pollen grain surface that contains volatile compounds attractive to pollinators (Troll, 1928); the pollenkitt is highly hydrophobic, homogeneous, surrounds the mature pollen grain exine, and contains lipids, carotenoids, flavonoids and fatty acids, all secreted by the tapetum cells (Stanley & Linskins, 1974). According to Lush (1999), the lipids, due to hydrophobic nature, restrict water loss in the stigma and pollen grains. Thus, when the stigma is dry, as the papaya tree (Parés et al. 2002), it requires substances in the pollen grain to moisturize it during the pollen grain and stigma interaction (Rodríguez-Garcia et al. 2003). Considering that papaya stigma is dry, the presence of pollenkitt can be expected.

According to Baker & Baker (1983) starchy pollen grains are generally considered to be characteristics of species that are self-pollinated, wind pollinated, pollinated by Lepidoptera or by birds, while lipidic pollen grains are characteristics that have species of bee-pollination and fly-pollination. Storey (1969) reported that pollination in the papaya is apparently widely carried out by wind, but this author believes that insects also play some role. Frankel & Galun (1977) reported that the main pollinating agents of the papaya are wind and insects. Since the papaya tree can be female, male or hermaphrodite it seems normal that the pollen can be carried out by wind and/or insects to ensure the pollination and fruit development.

In vivo pollen grain germination test

The results indicated that cleistogamy might occur in the papaya tree, which enables self-pollination. Rodríguez et al. (1990) working with other Solo cultivars, also observed the occurrence of cleistogamy in papaya. Cleistogamy is a common phenomenon in cultivated and homogamous-type plants, that is, the pollen grains and the stigma are receptive at the same time when the flower is still closed (Frankel & Galun, 1977).

Our results agree with those reports in which anthers dehiscence and pollen grains are viable even while the flowers are closed. According to Parés et al. (2002), anthers in the Cartagena Amarilla cultivar were dehiscent two days before flower opening. Couto & Nacif (1999) working with papaya also reported the presence of viable pollen grains in the pre-anthesis and that the pollen viability was maintained for some time afterwards.

Stigma receptivity

The results showed that stigmas are receptive before flower opening and this condition remains until 48 hours after the anthesis. Parés et al. (2002) and Rodrigues et al. (1990) also observed that the papaya stigma is receptive before flower opening and Couto & Nacif (1999) observed that the stigmas are receptive before and after anthesis in hermaphrodite and female papaya flowers, but a higher receptivity was detected shortly after anthesis.

Parés et al. (2002) reported that the papaya flower stigmas remain receptive for three days after anthesis, and the best results were obtained with artificial pollination carried out in the first 48 hours after flower opening. In this study, 48 hours after opening the stigmas showed black/brown sectors indicating that a degenerative process was probably starting; so, it is interesting to do crosses until 48 hours and no more than that.

Pollen:ovule ratio (P:O)

Based on our data, Tainung 01 cultivar can be classified as facultative self-pollinated as well as facultative cross pollinated, while Golden cultivar is classified as facultative self-pollinating. According to Allard (1960), varieties within the same species can show great differences in the proportions of natural crosses and the crossing rate for each variety can also

be highly influenced by changes in the environment. The Golden and Tainung 01 cultivars significantly differed, but due to their reproductive tendency, these plants were classified as self-pollinated species based on Cruden (1977). The Tainung 01 cultivar is in the range that includes facultative self-pollination or in the range that includes facultative cross pollination, thus it can present a higher cross pollination rate than the Golden cultivar. It was observed that in hermaphrodite plants of the Improved Sunrise Solo 72/12 (Solo) and JS 12 (Formosa) cultivars, the percentage of seeds derived from self-pollination was greater in plants in the Solo group when compared with the Formosa group (data not shown). So, papaya can be classified as a facultative self-pollinating species, that is, self-pollinating with a low cross pollination rate (Cruden, 1977).

Pollen:ovule ratio reflects the probability of the pollen grains reaching the stigma, resulting in maximum seed production. More efficient pollen grain transference will result in a lower P:O ratio. Thus, cleistogamous plants should have a low P:O ratio and self-pollinating plants should have a lower P:O ratio than cross pollinating plants; in this way the P:O ratio is correlated with the reproductive system of the plant (Cruden, 1977). The P:O ratio has been used to determine the preferential reproduction mode in different species including *Draba reptans* (Cruden, 1977), *Zingiberaceae* (Wang et al. 2004) and *Lactoris fernandeziana* (Bernadello et al. 1999). According to Cruden (1977) the facultative cross-pollinating species are self-compatible and protogynous or homogamous and, although some species require pollinators, most are self-pollinated when the flowers are closed. Self-pollinating species tend to get self-pollinated before or during flower opening, and the stigma is receptive to potential pollinators after self-pollination has taken place.

Considering the flower biology, Westergaard (1958) proposed that in the papaya not only cross pollinating but also self-pollination could be considered. A good indication of self-pollination is the endogamic effect. If endogamy can occur without adverse effects, the species is probably self-pollinating (Allard, 1960). According to Storey (1941), the papaya can be self-pollinating without losing vigor. Kim et al. (2002) and Manshardt (2002) agreed that the predominant reproductive system in papaya is self-pollination. These results corroborate our results, that is, the papaya can be classified as a facultative self-pollinating species.

Based on the results, it can be concluded that papaya pollen grains are of lipidic nature and papaya hermaphrodite plants are cleistogamous, and the preferential mode of reproduction is facultative self-pollination. All those data are important for papaya breeding programs.

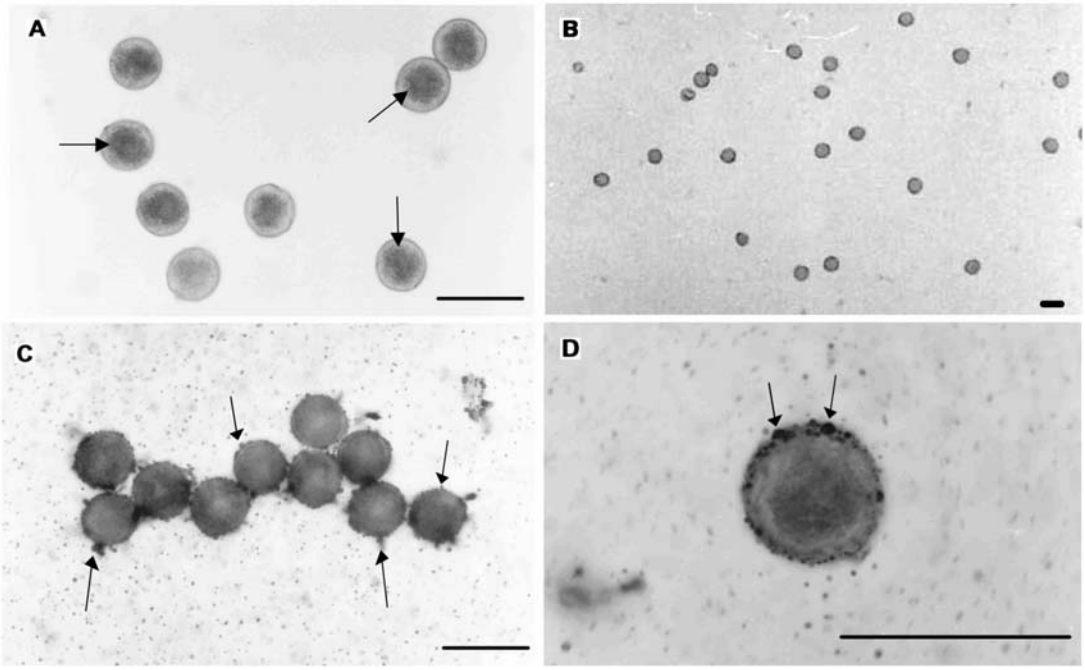

FIGURE 1 - Histochemistry of papaya pollen grain. A) Pollen grains stained with Sudan IV showing reaction (arrows) with the stain. B) Pollen grains I_2KI solution showing no reaction with the stain. C) Pollen grains showing pollenkitt (arrows) around them. D) Detail of pollenkitt (arrows) around pollen grain. Scale = 50µm.

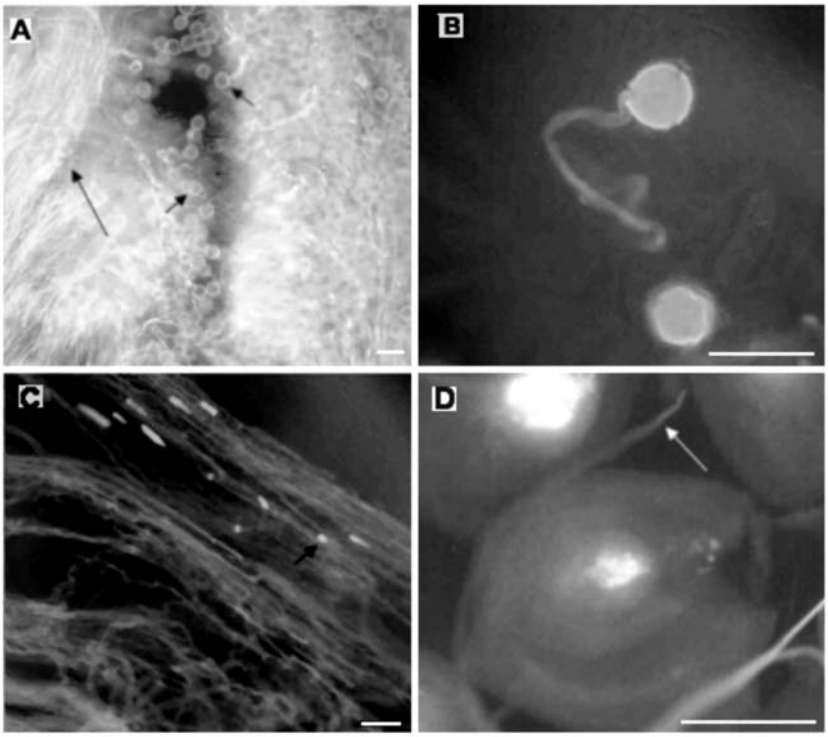

FIGURE 2 - Pollen grain germination in closed flower bud. (A) Pollen grain (small arrow) and pollen grain tube (large arrow) on the stigma surface. (B) Detail of germinated pollen grain on the stigma surface. (C) Pollen grain tube growing in the style showing callose plug (arrow). (D). Pollen grain tube down in the ovary. Scale bar = 50 μm.

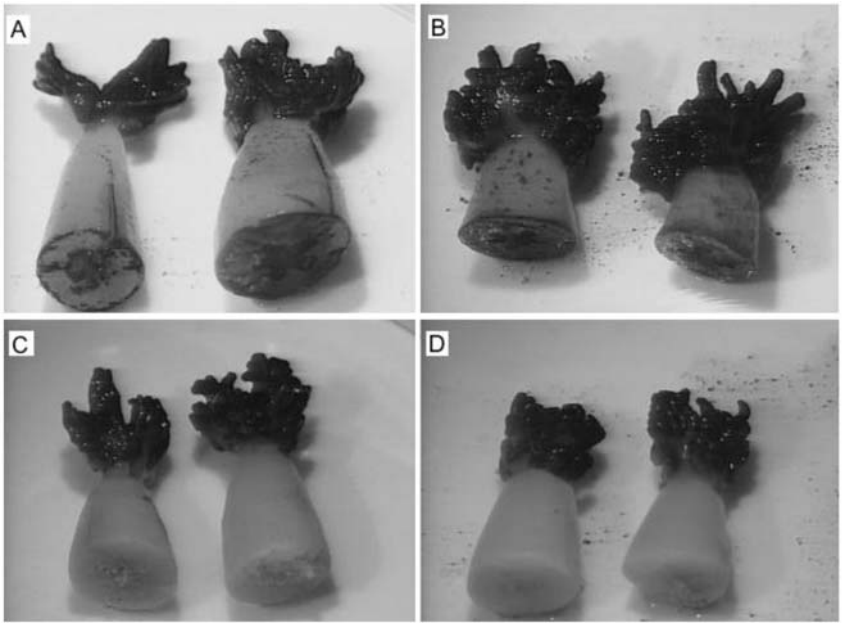

FIGURE 3 - Stigma receptivity of papaya hermaphrodite flower stained with alpha-naphthyl. A) Stigma receptivity on Tainung 01 cultivar on fresh opened flower collected at 8 A.M. (left) and 48 hours after flower opening (right); B)Stigma receptivity on Golden cultivar on fresh opened flower collected at 8 A.M. (left) and 48 hours after flower opening (right); C and D)Stigma receptivity on Tainung 01 cultivar (C) and in Golden cultivar (D) before flower opening. Magnification = 15x.

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