Pollen performance, cell number, and physiological state in the early-divergent angiosperm Annona cherimola Mill. (Annonaceae) are related to environmental conditions during the final stages of pollen development

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Abstract Pollen performance is an important determinant for fertilization success, but high variability in pollen behavior both between and within species occurs in different years and under varying environmental conditions. *Annona cherimola*, an early-divergent angiosperm, is a species that releases a variable ratio of bicellular and tricellular hydrated pollen at anther dehiscence depending on temperature. The presence of both bi- and tricellular types of pollen is an uncommon characteristic in angiosperms and makes *Annona cherimola* an interesting model to study the effect of varying environmental conditions on subsequent pollen performance during the final stages of pollen development. In this work, we study the influence of changes in temperature and humidity during the final stages of pollen development on subsequent pollen performance, evaluating pollen germination, presence of carbohydrates, number of nuclei, and water content. At 25 ºC, which is the average field temperature during the flowering period of this species, pollen had a viability of 60–70 %, starch hydrolyzed just prior to shedding, and pollen mitosis II was taking place, resulting in a mixture of bi- and tricellular pollen. This activity may be related to the pollen retaining 70 % water content at shedding. Temperatures above 30 ºC resulted in a decrease in pollen germination, whereas lower temperatures did not have a clear influence on pollen germination, although they did have a clear effect on starch hydrolysis. On the other hand, slightly higher dehydration accelerated mitosis II, whereas strong dehydration arrested starch hydrolysis and reduced pollen germination. These results show a significant influence of environmental conditions on myriad pollen characteristics during the final stages of pollen development modifying subsequent pollen behavior and contributing to our understanding of the variability observed in pollen tube performance.

Keywords Annona cherimola, Cherimoya, Humidity, Pollen performance, Temperature

Introduction

Pollen performance is one of the most important parameters for fertilization success in plants, with a high variability between and within species from year to year and with respect to differing environmental conditions (Delph et al. 1997). These environmental conditions include temperature (Aloni et al. 2001; Hedhly et al. 2005, 2009; Hedhly 2011; Johannsen and Stephenson 1998; Ledesma and Sugiyama 2005; Prasad et al. 1999, 2008, 2011; Rao et al. 1992; Snider et al. 2009), water stress (Saini 1997), nutrient availability (Havens et al. 1995; Lau and Stephenson 1993; Poulton et al. 2001a, b, 2002; Travers 1999; Young and Stanton 1990), UV radiation (Demchik and Day 1996), light intensity and quality (Galen and Stanton 2003), and CO_2 concentration (Marshall et al. 2010). While the prevailing environmental conditions do have an influence on pollen germination and pollen tube growth, there is little information on how factors affecting pollen development may affect the subsequent pollen performance.

Pollen development is a highly regulated and conserved process in angiosperms that takes place in the anther (Borg et al. 2009; Ma 2005; Pacini 2010; Scott et al. 2006). In angiosperm microsporogenesis, the sporogenous cells of the anthers produce microsporocytes or pollen mother cells that each undergoes two successive divisions during meiosis to form a tetrad of haploid microspores. Each unicellular microspore undergoes an asymmetric division (pollen mitosis I), resulting in a pollen grain with a larger vegetative cell that hosts a smaller generative cell. The latter will go through a symmetrical mitotic division (pollen mitosis II) to produce two sperm cells that will be delivered into the embryo sac of the ovule during double fertilization. While the overall process is highly conserved, the time when pollen mitosis II and starch hydrolysis, the final steps of pollen development, take place differs among species, resulting in different types of mature pollen with different numbers of nuclei or water and carbohydrate content. Thus, released pollen can be either bicellular or tricellular, depending on the timing of pollen mitosis II. In most (70 %) of the angiosperm species (Brewbaker 1967) and in all extant non-flowering seed plants (Friedman 1999), pollen mitosis II takes place after pollen germination within the elongating pollen tube, and consequently, pollen is released from the anthers in a bicellular stage, whereas in the remaining 30 % of angiosperm species, mitosis II takes place before pollen germination, resulting in the release of tricellular pollen at anther dehiscence. However, the presence of both bicellular and tricellular pollen grains in the same anther at anther dehiscence is unusual but has been documented in a few species, including A. cherimola (Lora et al. 2009a).

Two key processes that take place during pollen development in the anther are carbohydrate metabolism and dehydration. Carbohydrate storage reserves are very important in the protection of pollen against desiccation by maintaining the internal turgor pressure (Franchi et al. 1996), and developing pollen grains constitute a major sink for carbohydrates that reach the anther (Castro and Clement 2007). Starch is synthesized in amyloplasts during pollen development (Clement and Pacini 2001) and can be totally or partially hydrolyzed, resulting in two groups of species according to the starch content of mature pollen: species with starchless pollen and species with starchy pollen (Baker and Baker 1979). In starchless pollen, carbohydrates are stored in the cytoplasm as soluble products— mainly sucrose—and insoluble carbohydrates (Franchi et al. 1996; Speranza et al. 1997). Besides differences in starch content, pollen can also be released with a different hydration status. As a result of dehydration, pollen grains are generally shed as partially dehydrated (Stanley and Linkens 1974), although some species release partially hydrated pollen grains (Nepi et al. 2001; Pacini 2010; Pacini et al. 2006). Carbohydrates, water content, and pollen viability and longevity are closely related (Nepi et al. 2001; Pacini et al. 2006). In general, pollen with low sucrose content is released as partially hydrated (Nepi et al. 2001), and bicellular pollen is often associated with starchless pollen. Thus, according to Baker and Baker (1979) and Brewbaker (1967), 72 % of plant families with starchless pollen show bicellular pollen, 16 % produce tricellular pollen, and the rest (11 %) show bicellular and tricellular pollen. Carbohydrate storage reserves, where sucrose is formed by starch hydrolysis, are important for an adequate pollen grain hydration and also for pollen germination
in the stigma, since they provide the reserves for the early stages of pollen tube growth (Herrero and Dickinson 1981). However, little attention has been focused on whether differences in pollen grain reserves may affect the subsequent pollen performance and how environmental conditions during the final steps of pollen development may have a bearing on the subsequent pollen viability and performance.

Annona cherimola belongs to the early-divergent Annonaceae, the most diverse family within the Magnoliales with more than 2,400 species (Couvreur et al. 2011), 900 of which are found in the neotropics (Chatrou et al. 2004). Some species of the family such as cherimoya (*A. cherimola*), sugar apple (*A. squamosa*), or soursop (*A. muricata*) were already being used as a food source by pre-Columbian cultures in Central and South America (Popenoe 1989). Currently, cherimoya is an underutilized fruit crop, although its excellent organoleptic qualities provide it with a clear niche for expansion in developing countries with subtropical climates.

Cherimoya shows protogynous dichogamy (Wester 1910), a common trait in Annonaceae (Gottsberger 1999) and in other early-divergent angiosperm lineages with hermaphrodite flowers (Endress 2010), where female and male structures do not mature simultaneously, generally preventing self-fertilization in the same flower. Moreover, flowers of the same genotype are synchronized, and consequently, transfer of pollen between different flowers of the same genotype is also hindered (Lora et al. 2010). The cycle of the flower is completed in 2 days. On the first day of the cycle, the flower is in preanthesis with the petals tightly closed; the flower then passes to the female stage around midday with the petals completely open. This phase lasts for about 30 h, and on the second day, the flower switches to the male stage, when the anthers dehisce, around 17:00–18:00 hours under the growing conditions in southern Spain. After anther dehiscence, the stigma rapidly dries up, losing receptivity. Environmental conditions significantly influence stigmatic receptivity, which is extended with increasing humidity and decreasing temperatures and shortened with decreasing humidity and increasing temperatures (George et al. 1989; Lora et al. 2011). The lack of overlap between male and female stages in cherimoya, together with the fact that the crop was expanded from its areas of origin without its natural agent of pollination, results in the need for hand pollination for commercial production in all countries where the crop has been introduced (Schroeder 1971; Soria et al. 1990). However, erratic yields are frequently following hand pollination, indicating that differences in pollen management can play a key role in fertilization success and, consequently, fruit yield.

Recent studies on the final stages of pollen development in *A. cherimola* have shown a rapid and active pollen development prior to pollen shedding, resulting in the presence of bi- and tricellular pollen at anther dehiscence. The presence of both types of pollen is an uncommon characteristic in angiosperms and makes *A. cherimola* an excellent model to study final pollen development stages. The fact that the ratio between bicellular and tricellular pollen was greatly influenced by temperature (Lora et al. 2009a) led us to suspect that the prevailing environmental conditions during the last stages of pollen development could play a major part on subsequent pollen performance. In this work, we study the influence of two variable environmental factors, temperature and humidity, in those stages and in subsequent pollen performance after anther dehiscence.

Materials and methods

Plant material

Adult trees of *A. cherimola* cv. Campas located in a field cultivar collection at the EE La Mayora-CSIC, Málaga, Spain, were used in experiments performed for 3 years, during the flowering period from June to September.

Temperature treatments

The effect of temperature on pollen germination was studied during the flowering period from June to September in natural conditions in the field. Flowers in the female stage were collected from the field two or three times a week from June to September, at 16:00 hours, approximately half an hour before they entered the male stage, and were brought to the laboratory to evaluate pollen germination in vitro. Temperature in the field for the time of the experiment was monitored at hourly intervals with a data logger.

The effect of temperature during the final phases of pollen development was also studied in growth chambers under controlled conditions. Flowers were collected at preanthesis at 9:00 hours and put in 50-ml Falcon tubes with water in growth chambers at 15, 25, 30, and 35 ºC, at 60–70 % relative humidity, and 15-h photoperiod until the time of anther dehiscence. Then, in vitro pollen germination was evaluated at 15, 25, 30, and 35 ºC using the germination medium described below. Cytological studies were also performed in flowers collected at preanthesis at 9:00 a.m. and maintained at 15, 25, 30, and 35 ºC up to anther dehiscence.

For the two experiments, three flowers per pollinating time were collected.

Final pollen handling and moisture content

To evaluate the influence of the hydration status during final pollen development on mature pollen, pollen development and pollen germination were evaluated in four treatments, two with anthers detached from the flowers and two with anthers left in the flowers. Anthers from flowers collected from trees at 9:00 hours on the first day of the flower cycle were separated from the flowers and either desiccated with silica gel or left at room temperature at 60–70 % relative humidity. In the two other treatments, anthers were left in the flowers, and these were either kept at room temperature in the laboratory or left in the trees under field conditions. To evaluate a possible effect of the time of flower

collection, in the four treatments, three flowers were sampled at different times: at 14:00 and 19:00 hours on the first day and at 9:00, 14:00, 16:00, and 18:00 hours on the second day of the flower cycle. In the pollen from these flowers, moisture content, the presence of starch grains, number of nuclei, and pollen germination were evaluated. The moisture content of the pollen was determined by drying the pollen in an oven at 80 ºC for 3 days and comparing the weight of fresh and dried pollen.

Microscopic preparations

Anthers were fixed in 2.5 % glutaraldehyde in 0.03 M phosphate buffer (Sabatini et al. 1963), dehydrated in an ethanol series, embedded in Technovit 7100 (Kulzer), and sectioned at 2 lm. A total of 100–200 pollen grains were observed following staining with iodine potassium iodide (IKI) solution (2 % potassium iodide and 0.2 % iodide in water) for 5–10 min at room temperature (Johansen 1940). Starch grains and other insoluble carbohydrates were observed with periodic acid-Schiff's reagent (PAS). Sections were kept in 0.5 % periodic acid in water for 2 h, washed with water (3, 9, 10 min), and maintained in PAS in the dark for 2 h, followed by washing in water (3, 9, 10 min) and mounted with Entellan (Merck). Sections were also stained with a combined stain of PAS and toluidine blue for general histological observation (Feder and O'Brien 1968). To study the number of nuclei, 200–400 fresh pollen grains were stained with propidium iodide (PI) (5 lg/ml in water). Observations were performed under a Leica DM LB2 microscope with epifluorescent UV with an excitation filter of 515–560 nm and a LP barrier filter of 590 nm.

In vitro pollen germination

To evaluate in vitro pollen germination, pollen was maintained with the dehisced anthers as reported previously (Rosell et al. 1999); the anthers were hydrated by placing them in a glass vial placed in a covered tray with wet filter paper for 60 min at room temperature. Then, approximately 0.02 g of pollen with anthers was placed on a 35-mm Petri dish with 1–2 ml of liquid germination medium at room temperature (Lora et al. 2006). Pollen was considered germinated when the length of the tube was longer than the grain diameter. Data were collected from four Petri dishes, each containing at least 200 pollen grains. The pollen germination medium consisted of 8 % sucrose, 200 mg/l $MgSO_4 7H_2O$, 250 mg/l $Ca(NO_3)_2 4H_2O$, 100 mg/l KNO_3 and 100 mg/l H_3BO_3 (Lora et al. 2006).

Statistical analyses

Percentage data were normalized by arcsine-square root transformation prior to analysis. The association between pollen germination and differing field temperatures was tested using Pearson correlation analysis. An analysis of variance by the general linear model procedure was performed to study the effect of temperature either during the final pollen development stages or on the percentage of in vitro pollen germination. Since the analysis of variance in the interaction was in the limit of significance ($F_{9,34} = 1.935$ $p = 0.08$), Duncan's multiple range tests were used for mean separations for the effect of temperature during final pollen development and pollen germination. Statistical analyses were performed with SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA).

Results

Influence of temperature on pollen germination and starch metabolism

From the end of June to mid-September—the cherimoya flowering season—average daily temperatures in the field ranged from 21 to 29 ºC. The percentage of in vitro pollen germination during the same period was highly variable, ranging from 27 to 78 % (Fig. 1). Pollen germination showed a significant inverse correlation with the average temperatures two (Pearson's correlation coefficient, -0.443 p\0.009) and one (Pearson's correlation coefficient, -0.668 p\0.0001) days prior to pollen shedding. Thus, the percentage of pollen germination was higher under lower average temperatures and decreased at higher temperatures. Two days before anther dehiscence, the flowers were still closed, and one day before anther dehiscence, the flowers were in the female stage in the first day of the flower cycle and pollen was still not totally developed.

To evaluate with more detail the implications of temperature during pollen development on pollen performance, flowers were placed in controlled environmental chambers at 15, 25, 30, and 35 ºC one day prior to pollen shedding. The pollen collected from those flowers was germinated at all those temperatures. The analysis of variance revealed a significant effect of temperature during pollen development ($F_{3,34} = 16.214$ p\0.0001) and during pollen germination ($F_{3,34} = 17.387$ p\0.0001) on the percentage of pollen that successfully germinated. The highest percentage of pollen germination was

observed in pollen obtained from flowers maintained at 25 ºC and germinated at the same temperature (Fig. 2). But a significant decrease in pollen germination was observed in pollen obtained from flowers maintained at 30 or 35 ºC, and also in pollen germinated at 35 or 15 ºC

The influence of temperature on starch metabolism during the final stages of pollen development was also evaluated with cytological studies. The results showed that starch grains were present at anther dehiscence in pollen developed at 15 ºC (Fig. 3a), but starch grains were hydrolyzed in pollen developed at 25 ºC (Fig. 3b) or higher temperatures.

Influence of water content on pollen development and pollen germination

In order to evaluate the influence of water content on final pollen development, undehisced anthers separated from the flowers were desiccated with silica gel or kept at room temperature; in both the cases, no pollen germination was observed (Fig. 4). In detached flowers, the process appears somehow altered with the highest germination rate (53 %) at predehiscence. However, as it might be expected, under field conditions pollen germination showed the highest germination rate at anther dehiscence (64 %).

To examine the causes of these differences in pollen germination, we performed cytological studies with special attention to the number of nuclei and starch reserves, relating those observations to the water content of pollen. In pollen from flowers collected from the field, starch was present in the first day of the flower cycle and disappeared at anther dehiscence (Lora et al. 2009b). A similar pattern was observed in pollen from flowers kept at room temperature (Fig. 5a, b) with a reduction in the number of starch grains, resulting in a near absence of starch grains in pollen from male-stage flowers (Fig. 5b). However, starch remained at anther dehiscence in pollen from detached anthers, both in the pollen released and in the pollen that still remained in the anther (Fig. 5c), in which pollen development appeared to be halted and where the tapetum was also not completely degenerated.

Annona cherimola shows both bi- and tricellular pollen at anther dehiscence under field conditions (Lora et al. 2009a). However, pollen from anthers separated from flowers and desiccated at room temperature showed only bicellular pollen, and the second mitotic division was only produced when the anthers remained in flowers kept at room temperature or left in the tree in the field (Fig. 6). This mitotic division occurred on the second day of the flower cycle following the same pattern as pollen from flowers maintained under field conditions, but it occurred faster in detached flowers kept at room temperature. In these cases, 47 % of tricellular pollen was observed at 9:00 hours (Fig. 6), whereas flowers under field conditions showed only 3 % of tricellular pollen at the same time (Fig. 6). Interestingly, 71 % of ungerminated pollen was bicellular at anther dehiscence under field conditions (Lora et al. 2009a).

Pollen water content decreased rapidly from 69 % at preanthesis to 3 % in anthers left to dry with silica gel and to 5 % in those left at room temperature (Fig. 7). However, pollen from flowers kept under field conditions or in detached flowers maintained at room temperature retained a high water content, although it decreased more rapidly in anthers from detached flowers left at room temperature (Fig. 7). Pollen water content in the field at anther dehiscence was 60 ± 2.4 %, and consequently, cherimoya can be classified as a species that releases hydrated pollen, according to Nepi et al. (2001).

Discussion

Rapid and conspicuous changes were observed in the cherimoya pollen grains during the final stages of pollen development. These changes include metabolism of starch reserves resulting in starchless pollen and the second mitosis of the generative nucleus that takes place at the end of pollen development, resulting in a mixture of bi- and tricellular pollen at anther dehiscence (Lora et al. 2009a). These processes, occurring so close to the time of pollen release, can be explained by the fact that cherimoya pollen is shed in a hydrated stage that maintains continuous cell activity. But this hydrated stage also gives pollen a vulnerability to the prevailing environmental conditions, most notably temperature and humidity.

Temperature influences pollen germination and starch hydrolysis

Temperature during the final stages of pollen development and during pollen germination had a clear effect on pollen performance. An increase in temperature resulted in a decrease in pollen germination (Figs. 1, 2). Similar results were obtained in the field and in the laboratory. Thus, lower pollen germination was observed with high temperatures in the field during the flowering season. These results are in line with those reported by Rosell et al. (1999), who observed optimum cherimoya pollen germination in the range between 20 and 25 ºC. A similar trend was obtained with flowers kept under

controlled conditions in growth chambers. In this case, the highest percentage of pollen germination was obtained in flowers where the last pollen developmental stages and pollen germination took place at 25 ºC. However, temperatures of 35 ºC, either during the final stages of pollen development or during pollen germination, resulted in significantly lower rates of pollen germination. The effects of temperature on pollen performance have been reported in several other species (Hedhly 2011; Hedhly et al. 2005, 2009; Johannsen and Stephenson 1998; Ledesma and Sugiyama 2005; Prasad et al. 1999, 2008, 2011; Rao et al. 1992), and recent studies in rice show that the pollen transcriptional profile is also affected by temperature (Endo et al. 2009). Most of these studies have examined the whole pollen developmental process. However, our results show a significant effect of high temperatures during the final stages of pollen development, found to occur 13 days after meiosis (Lora et al. 2009a).

Temperature also had an effect on starch hydrolysis. While starch hydrolysis occurred normally at temperatures higher than 20 ºC, in flowers kept at 15 ºC, amylolysis was arrested, resulting in the release of starchy pollen at anther dehiscence (Fig. 3). Several reports have shown that temperature during pollen development can influence starch content. For instance, Pelargonium pollen shows less starch at 25 and 30 ºC than at 15 ºC (Sears 1926), and the same species shows starchy pollen when grown in higher latitudes compared to starchless pollen in lower latitudes (for review see Baker and Baker 1979). Some palm species show seasonal variations with starchless pollen in winter and starchy pollen in summer (Zona 2001). Studies in Sorghum also show the effect of temperature on starch hydrolysis, with the production of starchless pollen and considerably reduced pollen germination at high temperatures (Jain et al. 2007). In tomato, Pressman et al. (2002) observed a decrease in starch concentration in the final stages of pollen development at elevated temperatures and hypothesized that this could explain the observed decrease in pollen viability. In fact, heat-tolerant tomato cultivars do not show that decrease (Firon et al. 2006). Moreover, studies in rice (Kong et al. 2007) suggest that reduced energy supply and sugar levels may lead to pollen sterility. However, in cherimoya, differences in starch content at 15 and 25 ºC are not reflected in significant differences in pollen germination. Similarly, the differences in pollen germination at 25 and 35 ºC (Lora et al. 2009a) are not reflected in differences in starch content since starchless pollen is present at both temperatures. However, the fact that sucrose was added to our germination medium may mediate this response, and the evaluation of pollen viability in vivo would clarify this point. Recent reports in tomato show no significant differences in the viability of pollen with different carbohydrate content (Carrizo Garcı´a et al. 2010), probably reflecting some flexibility in pollen metabolism within a certain range of carbohydrate content. However, studies in *Parietaria judaica*, in which starchy pollen and starchless pollen are present at anther dehiscence, show differences in in vitro pollen germination at different sucrose concentrations, indicating that the type of carbohydrate present at anther dehiscence is critical for pollen maturation (Franchi et al. 2007). Overall, the results obtained in our study indicate that temperatures higher than 30 ºC resulted in a decrease in pollen germination, whereas lower temperatures did not have a clear effect on pollen germination, though they clearly affected starch hydrolysis.

Pollen hydration is a key factor for final pollen development

Pollen hydration at anther dehiscence is a key factor that affects pollen survival during the pollination process. In this work, we evaluated changes in water content during the final stages of pollen development and related them to starch hydrolysis, mitosis II, and pollen germination.

No pollen germination was observed in pollen from detached anthers either maintained at room temperature or completely desiccated (Fig. 4). Moreover, the pollen obtained from detached anthers is starchy (Fig. 5), and no tricellular pollen was observed under those conditions. All this suggests that metabolic activity is arrested in pollen from detached anthers and, consequently, mitosis II and starch hydrolysis do not take place. Starch hydrolysis is related to protection against desiccation (Speranza et al. 1997), and recent studies have demonstrated the association between the maintenance of pollen viability and the maintenance of low moisture content and high levels of sucrose and total insoluble cytoplasmic carbohydrates (Guarnieri et al. 2006; Nepi et al. 2010). In fact, at anther dehiscence, bicellular pollen usually shows lower metabolic activity and lower water content than tricellular pollen, which allows for better storage of bicellular pollen at low temperatures (Barnabas and Kovacs 1997). Thus, starch hydrolysis is necessary to protect pollen grains against a strong desiccation, as was reflected in this study by the lack of germination in pollen from anthers either left to dry with silica gel or left at room temperature.

A similar developmental pattern is seen between pollen from anthers in detached flowers kept at room temperature and pollen from flowers left in the tree in the field. However, in the detached flowers, the process appears accelerated as compared to those under field conditions. This may be related to a higher water content decrease in flowers maintained at room temperature (Fig. 7). Acceleration of cell activity is reflected in higher pollen viability at predehiscence and also in a faster pace of mitosis II with a

higher percentage of tricellular pollen at anther dehiscence (Fig. 6) (Lora et al. 2009a). Both bi- and tricellular pollen are capable of germination (Lora et al. 2009a), although further work is required to elucidate whether both pollen types have the same likelihood of fertilization.

Our results show that temperature and water content during the final stages of pollen development have an influence on subsequent pollen germination and starch content. Pollen water content is correlated with starch hydrolysis, mitosis II, and pollen germination. While a slight dehydration accelerated mitosis II, strong dehydration arrested starch hydrolysis and reduced pollen germination. Thus, pollen water content shows a key role in modulating cell activity.

Cherimoya pollen is shed in a partially hydrated stage that facilitates the release of two types of pollen, bicellular and tricellular (Lora et al. 2009a), with continuous cell activity as shown in starch metabolism and mitosis II. Temperature likely affects the water content of pollen directly and could induce phenotypic plasticity in the tricellular/ bicellular pollen ratio and starch hydrolysis that is reflected in pollen germination. The results obtained here are of relevance both from an applied point of view, in order to design strategies to optimize pollen collection in species where pollen is shed partially hydrated, and from a basic point of view to understand the effect of environmental conditions during final pollen development on subsequent pollen performance.

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Fig. 1 Pollen germination and average temperatures during the cherimoya flowering period from June to September

Fig. 2 Cherimoya pollen germination (%) at different temperatures after pollen development at 15, 25, 30, and 35 ºC. Mean followed by different letters at each temperature during pollen germination are significantly different (p B 0.05) by Duncan's multiple range test. Error bars represent the standard deviation (SD) of the mean

Fig. 3 Influence of temperature on starch metabolism during pollen development. Pollen at anther dehiscence from flowers developed at 15 ºC, showing starch (a), and at 25 ºC, lacking starch (b). Pollen grains were stained with PAS and toluidine blue. Starch grains are indicated by an *arrow. Scale bars* 20 lm

Fig. 4 Percentage of cherimoya pollen germination (mean ± SD) during the flower cycle from detached anthers desiccated or maintained at room temperature and from flowers from the field or maintained at room temperature. F1 female stage on the first day of the flower cycle. F2 female stage on the second day of the flower cycle. M male stage on the second day of the flower cycle. Anther dehiscence occurred at 17:00–18:00 hours, on the second day of the flower cycle (arrow). Mean followed by different letters are significantly different (p B 0.05) by Duncan's multiple range test ($F_{20,57}$ = 84.440 p\0.0001)

Fig. 5 Influence of water content on starch reserves. Pollen from flowers maintained at room temperature at 9:00 hours the first day of the flower cycle showing starch grains (a) and at anther dehiscence that are devoid of starch (b). Pollen in the anthers detached from flowers and kept at room temperature at anther dehiscence maintained starch (c). Pollen grains were stained with PAS and toluidine blue. Starch grains (arrow), t tapetum. Scale bars 20 lm

Fig. 6 Percentage of tricellular pollen (mean ± SD) during the flower cycle in the field and in detached flowers kept at room temperature. F1 female stage on the first day of the flower cycle. F2 female stage on the second day of the flower cycle. M male stage on the second day of the flower cycle. Anther dehiscence occurred at 17:00–18:00 hours, on the second day of the cycle (arrow) in both circumstances. Mean followed by the same letter are not significantly different (p B 0.05) by Duncan's multiple range test ($F_{11,32}$ = 315.017 p\0.0001)

Fig. 7 Water content (mean ± SD) of pollen during the flower cycle in anthers desiccated or kept at room temperature (RT), and in detached flowers kept at room temperature (RT) and flowers left in the tree in the field. F1 female stage on the first day of the flower cycle. F2 Female stage on the second day of the flower cycle. M male stage on the second day of the flower cycle. Anther dehiscence occurred at 17:00–18:00 hours of the second day of the cycle (arrow). Mean followed by the same letter within a treatment are not significantly different (p B 0.05) by Duncan's multiple range test ($F_{20,45}$ = 51,522 p\0.0001)

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