Effects of Temperature on Cherimoya Reproductive Organs

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Contents

Chapter 1

General Introduction

Botany and Economic Importance

The Annonaceae family consists of approximately 100 genera (USDA GRIN, 2014), mainly distributed in subtropical and tropical zones. All species possess dichogamous, hermaphroditic flowers pollinated by entomophily (e.g. Wester, 1910).

The flowering behavior of cherimoya (*Annona cherimola* Mill.), namely, protogynous dichogamy, is shown in Fig. 1. The pistil becomes receptive within 3 days before anthesis when the tips of three petals start to separate slightly (Fig. 1.A). The aperture of the separating petals then increases gradually to approximately 30° (Figs. 1.B–C), and the pistil remains receptive until around noon on the day of anthesis. Anthesis occurs in the late afternoon, the petals opening completely in a short time (Fig. 1.D) and coinciding with anther dehiscence and pollen shedding. Natural pollination succeeds when insects move from the male flower, carrying shed pollen to another flower in the previous female state.

The genus *Annona* includes approximately 100 species indigenous to

Central-Southern America, characterized by edible syncarpous fruit containing tens to hundreds of seeds. The edible component is the sweet or sour pulp, which is used for fresh consumption and processing. Several *Annona* species such as cherimoya, sugar apple (*A. squamosa* L.), atemoya (*A. cherimola* × *A. squamosa*), soursop (*A. muricata* L.), and bullock's heart (*A. reticulata* L.) are commercially cultivated in many other subtropical and tropical zones including Africa, Australia, California, Florida, Southeast Asia, and Southern Europe.

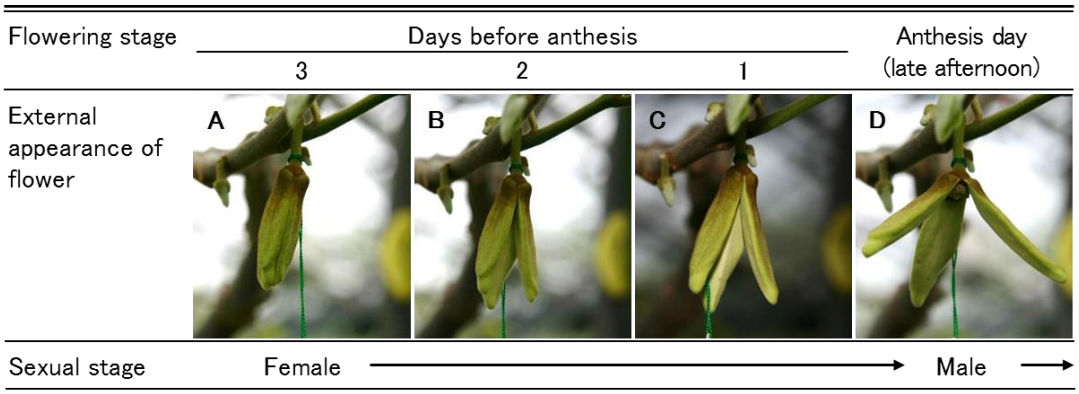

Fig. 1 Time course of a cherimoya dichogamous hermaphrodite showing protogyny. The external appearance is correlated with the sexual stage. The female stage starts within 3 days before anthesis; the petal tip beginning to separate slightly (A). This stage continues, he petal tip aperture becoming gradually wider within the final 2 days prior to anthesis (B–C). Finally, in the late afternoon on the day of anthesis, the flower attains anthesis and the sexual stage changes from female to male, the petals opening completely (D). This coincides with anther dehiscence and pollen shedding.

Cherimoya is the most commercially important member of the family due to the excellent quality of its fruit, particularly its aromatic, sweet, and subtly sour flavor. The origin of cherimoya is the Inter-Andean valleys spreading across Ecuador, Colombia and Bolivia, where the climate is always cool (17–20ºC; Morton,

1987). Currently, cherimoya is cultivated widely in subtropical to warm-temperate zones such as California in the USA, Chile, Israel, Italy, Japan, New Zealand, Peru, Queensland (Australia), South Africa, and Spain. Cultivation in lowland tropics, however, has not been successful (Morton, 1987) because of its reproductive weakness under tropical high temperature conditions (e.g. Higuchi *et al*., 1998).

The cherimoya global cultivation area in 1994 was estimated to be 13,500 ha, with the total annual production reaching 81,000 t (Pinto, 2005). Production in major cultivation areas was estimated to be 29,000 t in Spain and 14,000 t in Peru. In Japan, cherimoya is largely imported, with the annual volume of imported fruit from California, Chile, and so on estimated to be several tens of tons. The normal market price of imported cherimoya is around ¥2,000/kg. Domestic cultivation was started in 1990s under greenhouse conditions; however, the cultivation area remains limited at approximately 1 ha, producing about 2 t in 2004 (MAFF Japan, 2011). Despite the low domestic production, the market price of domestic cherimoya in Japan is higher than ¥2,000/kg, exceeding ¥5,000/kg for high-quality, symmetrical, large unblemished fruits, which are often purchased as formal gifts.

Intensive Cultivation

The favorable climate for cherimoya cultivation is generally within 18–25ºC during the summer and 5–18ºC during the winter (Morton, 1987). To produce cherimoya commercially, growers practice intensive cultivation methods, as described below.

Cherimoya is propagated mostly by grafting or budding, and is difficult by cutting; grafting has a success rate of more than 70% (Nakasone and Paull, 1998; Fuentes, 1999). After planting the grafted trees, 2–3 years are usually needed to attain commercially feasible production. Many good commercial cultivars have been

bred in cherimoya-introducing areas; major cultivars include ‘Bays’, ‘Big Sister’, ‘Pierce’, and ‘White’ from California, ‘Concha Lisa’ and ‘Bronceada’ from Chile (Morton, 1987), and ‘Fino de Jete’ and ‘Campas’ from Spain (Sarasola, 1960).

Cherimoya tree shape is easily trained, generally into an open vase or pyramidal shape (Cautín *et al*., 1999; Farré *et al*., 1999). Dormant buds on the leaf axil are covered by the petioles until defoliating, with flower buds on a 4-week-old leaf axil found to be already differentiated (Higuchi and Utsunomiya, 1999). Thus, defoliation induces vegetative shooting together with flower bud formation. Growers can therefore control both vegetative and reproductive growth easily by defoliating and pinching, although vigorous branches result in fewer flowers (Cautín *et al*., 1999). V-shape straight-lined training is mainly conducted in Japanese greenhouse cultivation to facilitate hand-pollination activity (Yonemoto, 2009). Fertilizer (N:P:K = 10:8:6) is recommended at an amount of approximately 700 g/tree/year up to the second year after planting (Morton, 1987). Fertilizer formula is then changed to N:P:K = 6:10:8 and the amount of application increased from 0.45 to 2.27 kg/tree/year.

Pollination is conducted naturally by small insects such as beetles (Coleoptera Nitidulidae) (e.g. Gazit *et al*., 1982) and flower bugs (Hemiptera Anthocoridae) (e.g. Farré *et al*., 1999). Insect pollination, however, often results in unstable fruit set and the fruit shape tends to be inferior. To obtain satisfactory fruit set, hand-pollination is therefore widely conducted to supplement this unstable natural pollination. Because of the syncarpous nature of the flowers, cherimoya fruit quality focuses on symmetry, which depends mainly on pollination quality. Pollination methods are largely concerned with obtaining reliable fruit set, as described in detail in the next section.

Fruits are harvested before natural ripening on the trees in order to prevent dropping damage and to prolong the shelf life. The optimum harvest time is indicated by the point when the peel color turns from deep green to yellowish light

green, or when an adequate number of days have passed since pollination. The day requirement for harvest varies by cultivar and climate conditions from 4–6 months in California (e.g. Schroeder, 1941), Japan (e.g. Higuchi *et al.*, 1998), and Spain (e.g. Soler and Cuevas, 2008), to 8 months in Chile (e.g. Saavedra, 1979).

Post-harvest ripening is conducted for several days to allow softening, because of the climacteric characteristics (Biale *et al.*, 1954). Favorable temperature conditions for ripening is around 20ºC (e.g. Fúster and Préstamo, 1980; Yonemoto *et al.*, 2002), with post-harvest storage reportedly effective at 10ºC for 2–3 weeks. Temperatures lower than 8ºC have been shown to disable fruit ripening (Alique *et al.*, 1994). Shelf life under such conditions can be prolonged by controlling atmospheric O_2 at 5% up until 7 weeks after harvest (Palma *et al.*, 1993).

Pollination Practices that Produce High-quality Fruit

Many entomophilic pollination methods have been tested to mitigate the laborious hand-pollination practices. In Spain, the population numbers *Orius* (Anthocoridae) spp. are reportedly higher in plots intercropped with corn (Farré *et al.*, 1999), and in Italy, pollination by releasing *Orius* spp. has also been reported (e.g. Caleca *et al.*, 1998). *Carpophilus* and other genera (Nitidulidae) have been tested as insect pollinators in Queensland (George *et al.*, 1989) and Israel (Gazit *et al.*, 1982). Recently in Japan, a new pollination method employing mass-propagation by nitidulid beetles such as *Carpophilus* was reported (e.g. Higuchi *et al.*, 2014), although it was not economically feasible. Thus, human hand-pollination remains indispensable, and so this practice needs to be improved to ensure stable production of symmetrically large fruits.

Deft hand-pollination immediately after anthesis is recommended (e.g.

Schroeder, 1943) because of the brittle nature of the pollen (e.g. Rosell *et al*., 2006). Pollen handling practices that maintain high pollen vigor and facilitate hand-pollination are therefore used. For example, a small glass or plastic vial (*cf*. Schroeder, 1943) is used to carry the pollen, thus excluding the effects of the worker's body heat. Hand-pollination is then conducted using a small paint brush. In addition to this, the Japanese persimmon pollination gun combined with pollen-diluting powder such as *Lycopodium* spores has also been used successfully in California (George *et al*., 1987).

No self- or cross-sterility is observed among cultivars (Schroeder, 1947), and hand-pollination can obtain almost 100% fruit set on a good day; pollen germination is reportedly promoted at temperatures of 22–25ºC (Rosell *et al*., 1999; Yonemoto *et al*., 1999). Later in the warm season, flowers tend to produce larger fruits (Richardson and Anderson, 1996; Soler and Cuevas, 2008).

Nevertheless, George *et al*. (1987) noted that fruit set by hand-pollination decreased on hot days with low humidity (<70% relative humidity [RH]) in addition to wet or excessively humid days in Queensland. Fruit set is also reportedly low in cool, early spring weather in warm-temperate zones in California (Schroeder, 1941) and Japan (Yonemoto and Nakao, 1993). In spring, pollen immaturity has also been observed in Chile (Saavedra, 1977), while in Spain, flowers tend to produce asymmetrical fruits (Matsuda, 2013). Thus, cherimoya reproductive organs seem to be very sensitive to meteorological factors such as temperature and humidity, despite large diurnal and seasonal environmental variation in subtropical to warm-temperate zones. Proper management must therefore be established.

Reliable fruit set requires application of vigorous pollen. Empirically, a pollen germination percentage of over 30% is necessary for practical hand-pollination of fruit trees. However, Rosell *et al*. (2006) reported that the germination percentage of cherimoya pollen decreased to about one-third by 2 h

after anthesis in mid-summer, and empirically, the pollen sometimes barely germinated, even several hours after anthesis. Pollen storage is reportedly effective for a few days in a refrigerator (4ºC) and for a few weeks under cryostorage (-196ºC) (Lora *et al*., 2006): the pollen has a germination percentage of about 60% before storage and >30% after storage for these periods. For longer storage periods, however, germination decreased to an unreliable level for practical pollination.

Pre-pollination environmental management must be established in order to obtain pollen that is as vigorous as possible. Lora *et al*. (2012) indicated that the pre-anthesis temperature from 1 day before anthesis affected germinability of shed pollen; highest rates observed at 25ºC, with a remarkable decrease at 35ºC. Whether pre-anthesis temperature affects pollen vigor should therefore be investigated separately with respect to day and night effects.

Night heating is the most practical management option under greenhouse cultivation because the daytime temperatures even in cool spring can often reach 20–25ºC, which is as warm as following seasons. Determining the critical low night temperature at pre-anthesis which reduces pollen vigor could help mitigate decreases in germinability during cool spring through the use of adequate heating within the greenhouse.

Daytime management of anthesis can also help maintain high pollen vigor during pollination. Koura *et al*. (2001) pointed out that temperatures higher than 35ºC even in the several hours before anthesis decreased pollen germinability. However, sufficient management has yet to be established. In daytime, both the temperature and the humidity change drastically, unlike at night. Favorable temperature and humidity conditions must therefore be clarified.

Reliable fruit set also requires vigorous pollen-tube elongation supported by high pistil receptivity, and subsequent steady fertilization. The detrimental effects of high temperatures have been studied in both male and female organs. Day/night

temperatures of 30/25ºC were shown to negatively affect the development of reproductive organs resulting in low fruit set (Higuchi *et al.*, 1998). Moreover, high temperature conditions of over 30ºC reportedly depress pollen germination (Rosell *et al.*, 1999) and shorten stigmatic receptivity (Lora *et al.*, 2011). Such high temperatures in mid-summer have also been shown to result in fewer flowers (George and Nissen, 1987; Higuchi and Utsunomiya, 1999).

On the contrary, little is known about the effects of low temperatures on the development of reproductive organs, pollen-tube growth after pollination, and subsequent fertilization and fruit set, though pollen germination *in vitro* was reportedly inhibited by low temperatures below 10ºC (e.g. Yonemoto *et al.*, 1999). Accordingly, resolution of the critical low and high temperatures that negatively affect the development of reproductive organs, pollen-tube growth, fertilization, and fruit set would help support cultivation management by suggesting appropriate heating protocols, effective pollination techniques, and feasible labor allocations.

Objective of This Study

The thermal responses of the reproductive organs must be clarified in order to determine suitable temperature management for reliable fruit set. In Chapter 2, the morphogenic process of the reproductive organs is observed anatomically and correlated with the external appearance of the flower buds. In Chapter 3, observations of developmental changes in pollen starch are described and critical pre-anthesis night temperatures affecting pollen germinability determined. The favorable daytime temperature and humidity conditions for anthesis are also discussed. In Chapter 4, the effects of post-pollination temperatures on the time requirement of pollen-tube penetration into the embryo sac are examined, while in Chapter 5, the seasonal changes in pistil receptivity to pollen tubes in response to

atmospheric temperature fluctuations are described. Critical post-pollination night temperatures for fruit set are discussed in Chapter 6. Finally, the results of Chapters 3–6 are synthesized into recommendations for suitable temperature management during the flowering season.

Chapter 2

Morphogenesis of Reproductive Organs

Introduction

In cherimoya, morphogenesis of reproductive organs is still not correlated with the external appearance of flower buds. To research thermal response of the reproductive organs from not only physiological but also phenological aspects, the developmental process should be elucidated.

First, in this chapter, flower bud cross-sections at different developmental stages were observed to correlate the morphogenic process of the reproductive organs with the external appearance of flower buds.

Materials and Methods

Fourteen-year-old trees of potted ‘Big Sister’ cherimoya grown in a greenhouse (heating the minimum temperatures >10ºC during winter) at Kyoto University (35.0°N, 135.8°E) were used for observations of floral morphogenesis

until anthesis. To understand the process of reproductive organ morphogenesis and to correlate morphogenesis with changes in external appearance of flower buds, the buds were vertically sectioned and observed under an optical microscope in 2010. Flower buds at different stages were sampled and fixed in an FAA solution (37% formaldehyde solution: acetic acid: 50% ethyl alcohol = 1:1:18) from April to June, the middle of flowering season, and other flower buds at the same stage were tagged to estimate the day requirement to achieve anthesis. After rinsing the fixed buds with distilled water overnight, they were dehydrated in an alcohol series (30%, 50%, 70%, 80%, 85%, 90%, and 100% v/v ethanol, and 100% v/v butyl alcohol), and then embedded in paraffin wax and longitudinally sectioned at a 16 µm thickness. After de-waxing, the sections were stained with Mayer's acid haemalum solution (Avwioro, 2011). Then the stained sections were rinsed again with distilled water overnight, dehydrated in ethanol, phenol, and xylene, and mounted to create permanent preparations using NEW M·X (Matsunami Glass Ind., Ltd., Japan) medium.

Results

The developmental process in flower bud was observed weekly from 8 weeks before anthesis (Plate 2.1.A-H) to a day before anthesis (Plate 2.1.I). Embryo sacs developed according to the Polygonum-type (Maheshwari, 1950) process, as reported for Annonaceae (Svoma, 1998). The formation of petals and stamens was observed 8 weeks before anthesis (Plate 2.1.A-a). At 7 weeks before anthesis, pistils were observed (Plate 2.1.B-a). At 6 weeks before anthesis, locules were developing adjacent to the placenta (Plate 2.1.C-a). At 5 weeks before anthesis, ovules began to develop, and one-nucleate megaspore mother cells were enclosed with the integument (Plate 2.1.D-a). During this time, one-nucleate microspore mother cells

were observed in a row in the stamens (Plate 2.1.D-b). At 4 weeks before anthesis, megaspore mother cells were in the dyad stage in pistils (Plate 2.1.E-a); at 3 weeks, they were in the T-shaped tetrad stage (Plate 2.1.F-a). Around the same time, microspore mother cells at the tetrad stage were also observed in stamens (Plate 2.1.F-b). At 2 weeks before anthesis, embryo sacs were clearly recognized, as well as at the eight-nucleate stage of megagametogenesis when two groups of four nuclei were positioned separately in the embryo sacs (Plate 2.1.G-a). During this time, pollen tetrads were developing, and generative and vegetative nuclei were observed in the pollen grains (Plate 2.1.G-b). Finally, at 1 week before anthesis, the ovules had mature embryo sacs that contained two synergids and one egg cell nucleus at the micropylar side, two polar nuclei at the center, and three antipodal cells at the chalazal side (Plate 2.1.H-a), but little secretion was observed on the stigmatic papillae (Plate 2.1.H-b). However, plentiful secretions covered the stigmatic papillae at 1 day before anthesis.

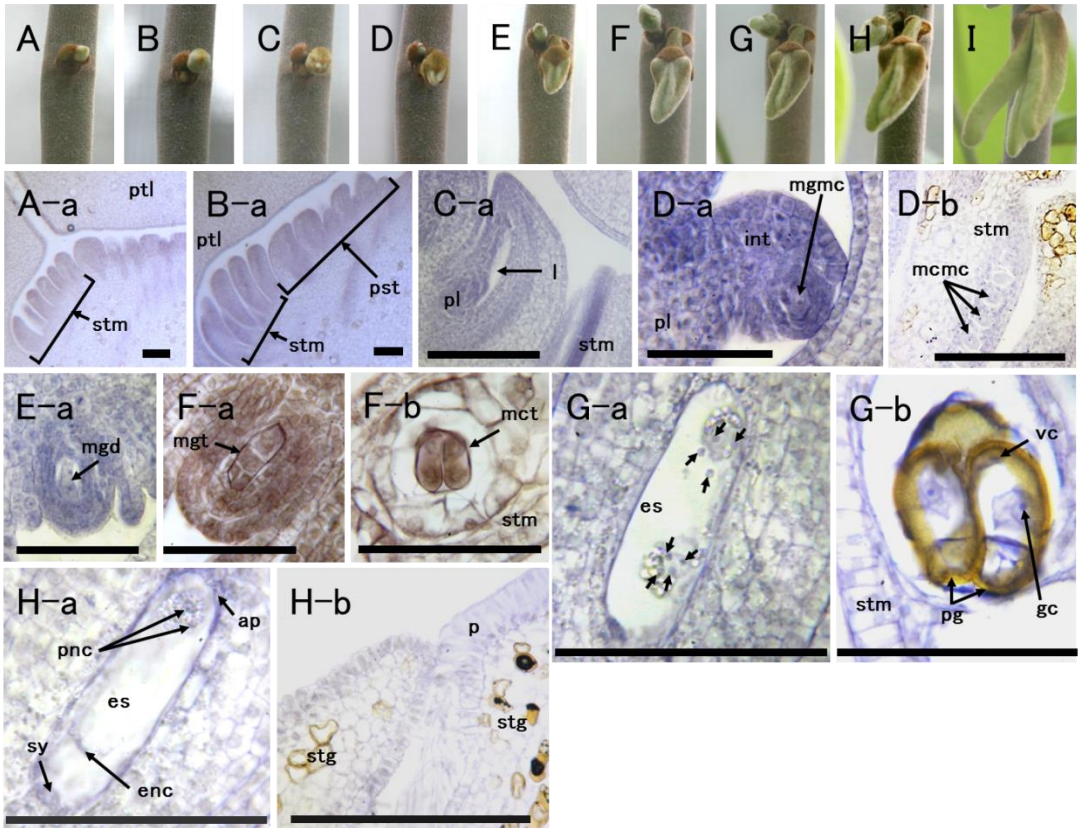

Plate 2.1 Floral morphogenesis process of cherimoya. A–I: external appearance of flower buds. From A-a to I-a: optical microscopic observations of flower buds every week from 8 weeks before anthesis to the day before anthesis. A-a and B-a: petal (ptl), stamen (stm), and pistil (pst) forming stages at 8 and 7 weeks before anthesis, respectively; C-a: locule (l) and placenta (pl) forming in pistil (pst) next to stamen (stm) at 6 weeks before anthesis; D-a: ovule primordia, composed of the integument (int) and one megaspore mother cell (mgmc) emerging from the placenta (pl), and D-b: microspore mother cells (mcmc) in the stamen (stm), at 5 weeks before anthesis; E-a: megasporic dyad (mgd) formation stage at 4 weeks before anthesis; F-a: megasporic tetrad (mgt) formation, and F-b: microsporic tetrad (mct) formation in the stamen (stm), at 3 weeks before anthesis; G-a: female gamete, which has the embryo sac (es) with octad nuclei (arrows), and G-b: pollen tetrad in the stamen formation stage, at 2 weeks before anthesis. Generative cells (gc) and vegetative cells (vc) in pollen grain (pg); H-a: embryo sac with two polar nuclei (pnc), two small synergids (sy), and one small egg cell nucleus (enc) at the micropylar end, and antipodal cells (ap) at the chalazal end at 1 week before anthesis; H-b: papillae (p) with some secretion on the surface of stigma (stg). Scale bars indicate 50 μm.

Chapter 3

Effects of Pre-pollination Temperatures on Pollen Vigor

Section 1

Observations of Pollen Starch Accumulation and Pollen Germinability as Affected by Pre-anthesis Night Temperature

Introduction

Lora *et al*. (2012) noted that not only pollen germinability but also decomposition of pollen starch was affected by the pre-anthesis temperatures: pollen starch was rapidly lost before anthesis when flowers were stored for 2 days at 25ºC, but not 15ºC. Pollen also showed lower germination at 15ºC. The accumulation and catabolism of starch may be affected by temperatures. Thus, the effects of temperature on pollen starch reserves and pollen morphogenic development need to be elucidated.

Metabolism of starch reserves was reported to be closely related to pollen germination. Inhibition of pollen starch accumulation caused male sterility in sorghum (Brooking, 1976), while a starch excess mutation in tomato resulted in

undecomposed residual starch even after anthesis, which was associated with low germination (Nashilevitz *et al.*, 2009). Also in cherimoya, pollen was reported to germinate little when starch was hardly decomposed after anthesis (Saavedra, 1977). Nevertheless, the correlation between temperature and starch accumulation is unknown and the germinability change under different developmental temperatures is not clarified.

Pre-anthesis night temperatures can alter pollen vigor by affecting the metabolism of pollen starch reserves, because pollen is generally regarded to germinate autotrophically (e.g. Iwanami, 1959; Baker and Baker, 1979). To examine thermal response of reproductive organs, secondary effects caused by the growth response of mother trees should be removed. This has been difficult, because accurate portable devices are not available to regulate the confined space temperature surrounding a target flower *in situ*. Dr. Ogata (Japan International Research Center for Agricultural Sciences) provides a compact hand-made temperature control device applying a thermoelectric cooling/heating element using the Peltier effect (Fig. 3.1), which facilitate the precise regulation of a specific target. This device has clarified the thermal response of reproductive organs in a tropical fruit tree; Kozai *et al*. (2014) reported that durian fertilization was inhibited by night temperatures below 15ºC.

In this section, the pre-anthesis night temperatures surrounding flowers were regulated at 4–35ºC, and the germination of collected pollen was tested to determine the critical night temperature ranges for depressing pollen germination. Changes in starch grains were anatomically examined at various developmental stages to evaluate the correlation between temperature and pollen starch accumulation.

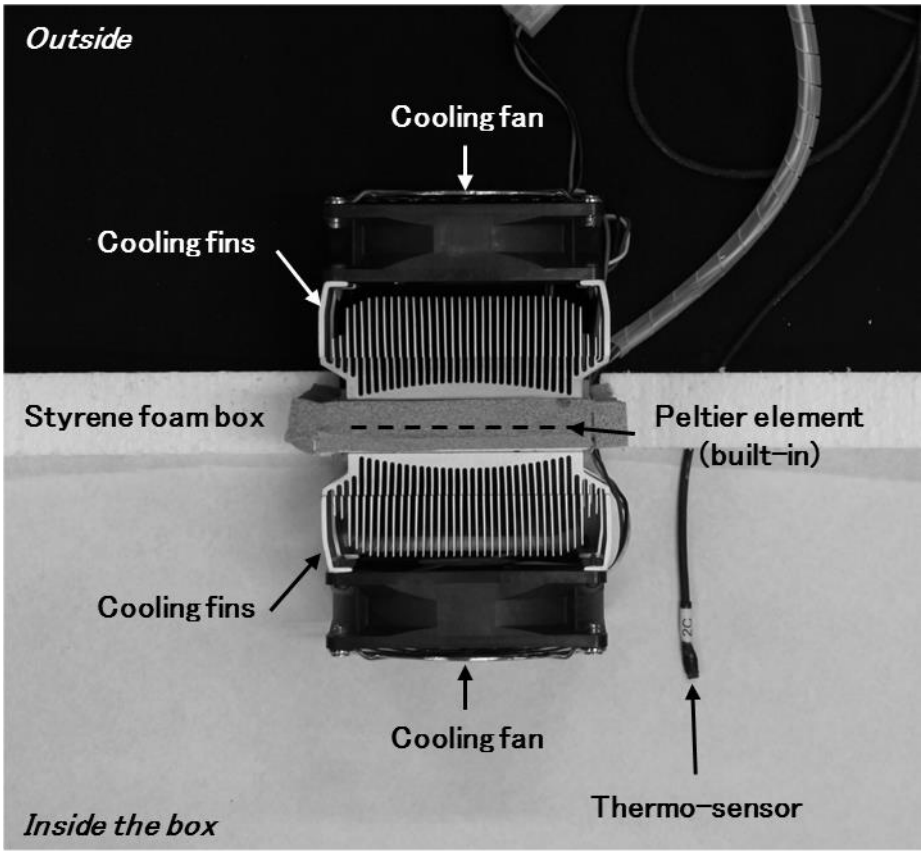

Fig. 3.1 A temperature control device applying thermoelectric cooling/heating element using Peltier effect. The Peltier element is sandwiched by two cooling-fin holders mounted with fans. The device is attached on a styrene foam box and can regulate the temperatures inside the box precisely without following outside-temperature fluctuation: device is governed by the Proportional-Integral-Derivative (PID) algorithm to keep a target temperature ±0.2ºC.

Materials and Methods

Eleven 17-year-old ‘Big Sister’ cherimoya trees grown in 40-L pots and nineteen 7-year-old trees grown in 15-L pots in a greenhouse (winter minimum temperatures >10ºC) at Kyoto University (35.0° N, 135.8° E) were used for the experiments. The experiments were conducted from May–June, 2014. Air temperature conditions during the experiments are shown in Fig. 3.2.

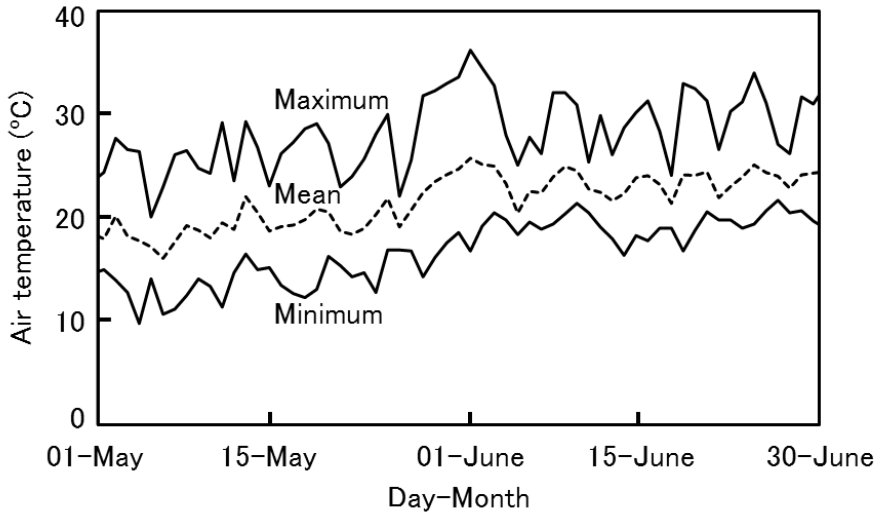

Fig. 3.2 Daily maximum, mean, and minimum air temperatures in the greenhouse during the experimental period from May–June, 2014.

Experiment 1: Temperature effect on pollen germination

Temperature regulation

An ≈8-L styrene foam box equipped with temperature-regulating units (Fig. 3.1) was installed on the tree to enwrap a flower (Fig. 3.3) during late afternoon on the previous day to the expected anthesis. Temperatures surrounding the flower were then regulated locally from 1800 h to 0900 h the next morning on the anthesis day.

Fig. 3.3 A styrene foam box equipped with the temperature control device (Fig. 3.1), being installed on the tree to enwrap a cherimoya flower.

Higher temperature regime

The sectional temperatures surrounding flowers were regulated at 12, 15, 18, 20, 22, 25, 27, 30, 32, or 35ºC during nighttime (1800–0900 h) previous to the anthesis, and some flowers were tagged and left untreated as a control(Cont-A). Immediately after recording anthesis time of each flower, when the petals opened together with pollen shedding, the pollen was collected using a small paintbrush. The collected pollen was placed on ≈1 mL of 2% agar medium containing 15% w/v sucrose, 100 ppm w/v boric acid, 300 ppm w/v $Ca(NO_3)_2 \cdot 4H_2O$, 200 ppm w/v $MgSO_4 \cdot 7H_2O$, and 100 ppm w/v KNO_3 (Brewbaker and Kwack, 1963; Yonemoto *et al.*, 1999), which was prepared on a glass microscopic slide. After placing the pollen on the agar medium, the medium on the glass slide was maintained in another small airtight plastic container together with wet Kimwipes™ under the slide to keep the agar medium from drying. The pollen and agar medium on the slides were then incubated at 25ºC for 24 h and the number of germinated pollen grains from the 1000 grains and the lengths of 10 pollen tubes on each slide were recorded as described by Matsuda and Higuchi (2013). Average germination percentages and tube lengths were calculated for each treatment. The higher temperature regime test was conducted in mid to late May, when the atmospheric daily maximum/minimum temperatures in the greenhouse ranged 23–29/13–17ºC (Fig. 3.2).

Lower temperature regime

To examine the effects of lower night temperatures, the temperatures surrounding flowers were regulated at 4, 6, 8, 10, 12, or 14ºC during nighttime and some flowers were left untreated as a control (Cont-B), as described above. Anthesis time was recorded. Pollen germination percentage and pollen-tube growth were also determined as described above. If the treated flower did not attain anthesis by 1800 h on the expected anthesis day, the temperature regulation was continued for another night, because the low temperature can postpone anthesis for other tropical species

(Kozai *et al.*, 2014).. The lower temperature regime test was conducted in mid to late June, when the daily maximum/minimum temperatures in the greenhouse ranged 25–33/16–21ºC (Fig. 3.2).

Statistical analysis

The temperature regulation experiment on pollen germination was conducted with a randomized block design in which each temperature treatment was tested on the same date and the test was repeated three times. The data were analyzed by analysis of variance followed by Tukey's multiple range test ($P < 0.01$).

Experiment 2: Changes in pollen starch

Developmental changes under orchard condition

To observe chronological changes in pollen morphology and starch grain density anatomically, flower buds from various developmental stages (Fig. 3.4) were sampled in May 2014, as cherimoya pollen grains were found to be formed about 2 weeks before anthesis in Chapter 2. Ten flower buds each were detached from trees at 1 and 2 weeks before anthesis. Another 10 flowers each were sampled at 1800 h on 3 days before anthesis, at 0900 h and 1800 h on 2 and 1 day before anthesis, at 0900 h on the anthesis day, and immediately before anthesis[1)] (1500–1600 h). Immediately after sampling, these samples were fixed in an FAA solution as described in Chapter 2.

The fixed flowers were then rinsed with distilled water and dehydrated in an alcohol series (30, 50, 70, 80, 85, 90, and 100% v/v ethanol and 100% v/v butyl alcohol), followed by embedding in paraffin wax (melting point: 52–54ºC) and sectioning longitudinally at 12 μm using a microtome. Serial sections of anthers were double stained as follows: first, starch was stained by periodic acid-Schiff (PAS) reaction procedure (McManus and Cason, 1950); then the nuclei were stained with Mayer's acid haemalum solution (Avwioro, 2011). The stained sections were

mounted to create permanent preparations using NEW M·X medium (Matsunami Glass Ind., Ltd., Japan), and then subjected to microscopic observation of pollen morphology and starch.

Temperature effect on pollen starch

In the same manner as described in Experiment 1, nighttime temperatures surrounding flowers were regulated at 4, 18, or 32ºC, or not regulated (control treatment). Three flowers per treatment were then sampled immediately after cessation of temperature regulations (0900 h on the anthesis day, but before anthesis). Developing pollen in these flowers was stained in serial sections on glass slides for starch observation as described above. The temperature regulation was conducted in late June, when the daily maximum/minimum temperatures in the greenhouse ranged 26–34/19–22ºC (Fig. 3.2).

Starch contents of freshly-shed pollen were also observed using the pollen obtained from Experiment 1. Pollen from each treatment was placed on agar medium of the same composition as in Experiment 1. After waiting for moisture absorption for 20–30 minutes, the pollen was stained with an I_2:KI = 0.05:0.2 mol·L^{-1} solution and covered with a cover glass. The starch was then observed.

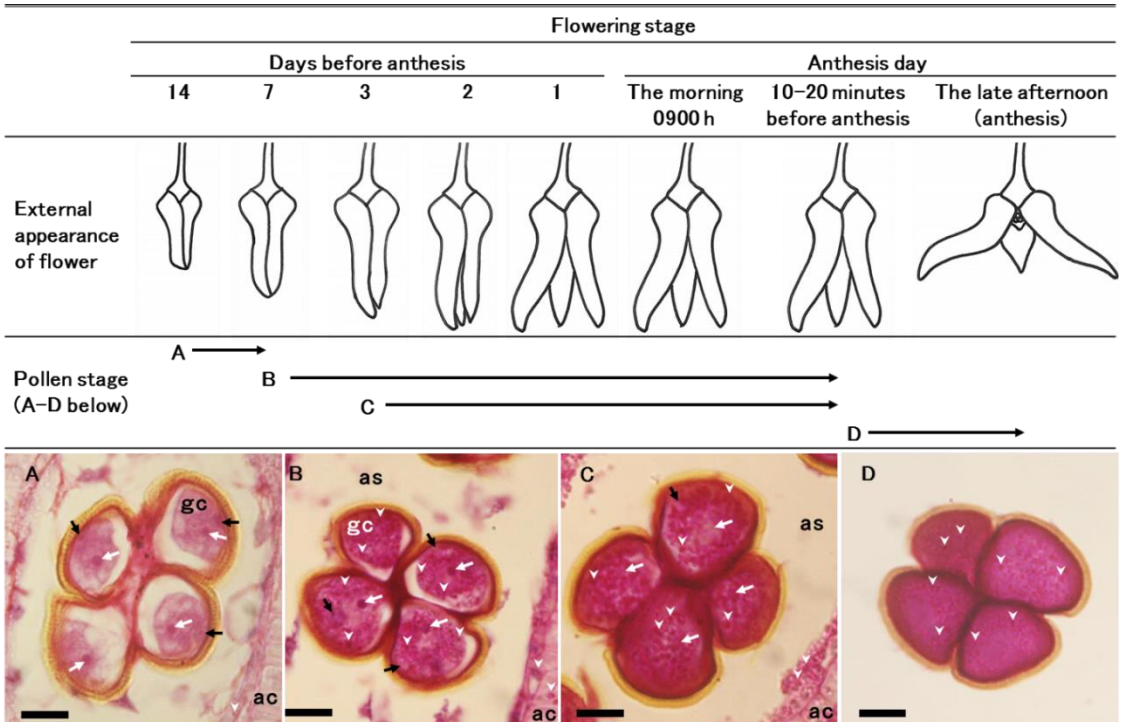

Fig. 3.4 Developmental changes in the external appearance of a ‘Big Sister’ cherimoya flower correlated with anatomical observations of the developing pollen tetrad and its accumulation of starch reserve from 2 weeks (A) to 10–20 minutes (D) before anthesis. A: Tetrad 2 weeks before anthesis. A generative cell (gc) including a generative nucleus (white arrow) was observed adjacent to the vegetative nucleus (black arrow) in each pollen grain. No starch grain was observed in pollen, but anther cells (ac) contained a few starch grains (arrowheads). B: Major tetrad from 1 week before anthesis to 0900 h the anthesis day. Many small starch grains (arrowheads) were observed around the generative nucleus (white arrow) in individual generative cell (gc). A vegetative nucleus (black arrow) was identified adjacent to the generative cell (gc). Many large starch grains were present in the anther cell (ac) 1 week before anthesis. C: Some of tetrad from 1800 h 3 days before anthesis to 0900 h the anthesis day. Many large starch grains (arrowheads) were observed in almost all portions of individual pollen grain and the edge of each generative cell became unclear. A generative nucleus (white arrow) was identified but a vegetative nucleus was not. Large starch grains were present in the anther sac (as). D: Major tetrad within 10–20 minutes before anthesis. Individual pollen grain was filled with starch grains (arrowheads) and the generative and vegetative nuclei were not identified. Scale bars indicate 10 μm.

Results

Experiment 1: Temperature effect on pollen germination

Higher temperature regime

Anthesis times of the flowers of which the surrounding temperatures were regulated overnight are shown in Fig. 3.5.A. Among the flowers regulated at 12–35ºC, those with pre-anthesis nighttime temperatures at 18–22ºC attained anthesis earliest (from 1500–1600 h). Anthesis tended to become later both in lower and higher temperatures, and was more than 2 h later at 35ºC. Pollen germination percentages immediately after anthesis were highest (≈70%) at 20–22ºC. The percentages at 20–22ºC were higher than that at Cont-A (Fig. 3.6.A). Germination percentages below 12ºC or over 27ºC nighttime temperatures were lower than the percentage at Cont-A i.e., less than 50%. At 35ºC, about 10% of the pollen germinated. Pollen-tube elongation tended to be high at 18–22ºC; temperatures higher and lower than this range negatively affected tube elongation.

Lower temperature regime

Among the flowers regulated at 4–14ºC, the Cont-B and 14ºC flowers attained anthesis earliest, from 1500–1600 h (Fig. 3.5.B). At lower temperatures than 14ºC, anthesis tended to be delayed. At 12ºC, flowers attained anthesis later than 1600 h. Below 8ºC, anthesis was often delayed to the following day. Pollen germination percentages immediately after anthesis exceeded 60% at Cont-B (Fig. 3.6.B), which was similar to Cont-A. The percentages at all temperatures below 14ºC were lower than the percentage at Cont-B. Pollen germinated little at 4ºC.

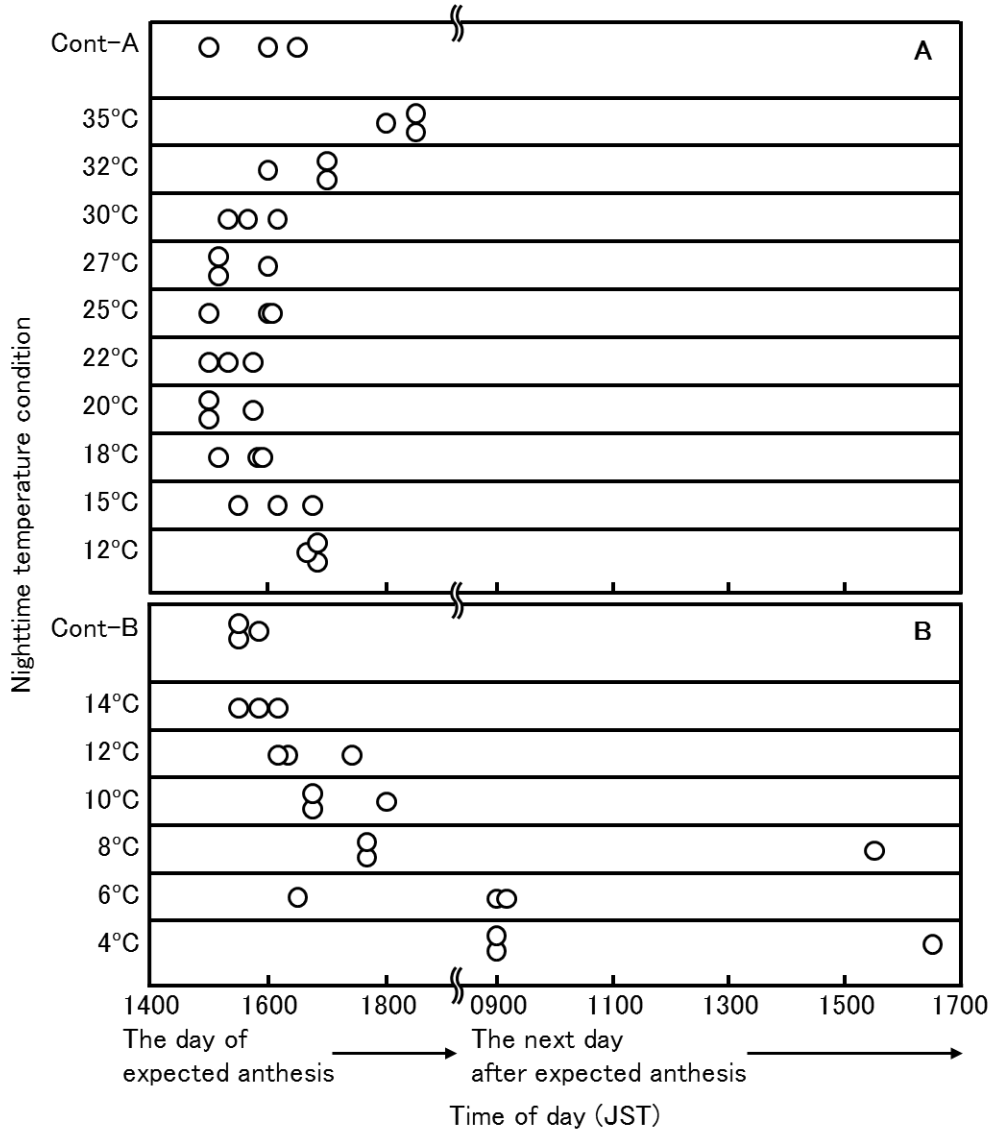

Fig. 3.5 Effects of the pre-anthesis nighttime (1800–0900 h) temperatures at 12–35ºC (A) and 4–14ºC (B) on anthesis time of 'Big Sister' cherimoya. The experimental periods differed between A and B. Thus, the results are arranged from low to high temperatures. Controls (Cont-A and -B) represent the cultivation condition inside the greenhouse, in which nighttime minimum temperatures ranged from 13–17ºC (A) and 16–21ºC (B).

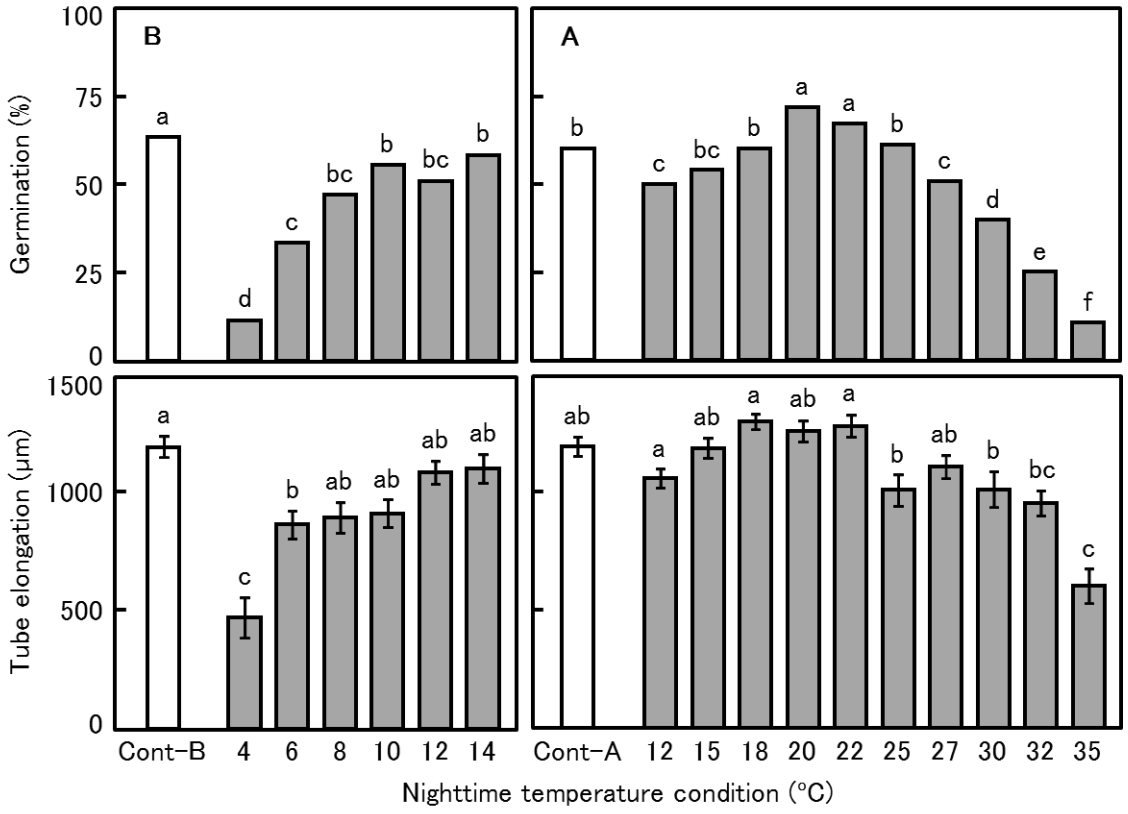

Fig. 3.6 Effects of the pre-anthesis nighttime (1800–0900 h) temperatures at 12–35ºC (A) and 4–14ºC (B) on *in vitro* germination percentages of pollen collected immediately after anthesis and tube elongation in 'Big Sister' cherimoya. The experimental periods differed between A and B. Thus, the results are arranged from low to high temperatures. Controls (Cont-A and -B) represent the cultivation condition inside the greenhouse, in which nighttime minimum temperatures ranged from 13–17ºC (A) and 16–21ºC (B). The different letters in each graph indicate statistical differences based on Tukey's multiple range tests at $P < 0.01$.

Experiment 2: Changes in pollen starch

Developmental changes under orchard condition

Developmental changes in the external appearance of flower buds and anatomical pollen morphology are shown in Fig. 3.4. Starch grains were scarcely observed in pollen at 2 weeks before anthesis and a generative cell including a generative nucleus was clearly observed adjacent to the vegetative nucleus. Starch grains emerged in anther cells. One week before anthesis, starch accumulated around the nucleus in the generative cell in many pollen grains, while no starch grains were present in the cytoplasm between the generative cell and the pollen cell wall. Starch grains in anther cells were larger than those 2 weeks previously.

In the late afternoon 3 days before anthesis, some pollen grains accumulated starch grains in the whole portions (Fig. 3.4) and the starch grains were larger than 1 week before. Although starch grains in anther cells did not change from 1 week before, many grains were present in the anther sac. Thereafter, the appearance and density of starch grains did not change until the morning on the anthesis day. Whereas 10–20 minutes before anthesis, almost all pollen grains were filled with starch grains and nuclei were not identified. Some parts of the anther wall already started to dehisce.

Temperature effect on pollen starch

Pre-anthesis nighttime temperature (4, 18, 32ºC, or not regulated) did not affect starch accumulation in the developing pollen inside the floral bud in the morning of the anthesis day. The starch density was found to be similar to that under cultivation condition (Fig. 3.4).

Freshly-shed pollen starch was also unaffected by nighttime temperature (Fig. 3.7): pollen tetrads[2)] collected from each treatment were filled with starch evenly in four grains or almost empty of starch in four grains, and an intermixture of those two types was observed at a similar ratio. At 4ºC, shriveled pollen grains were often

observed.

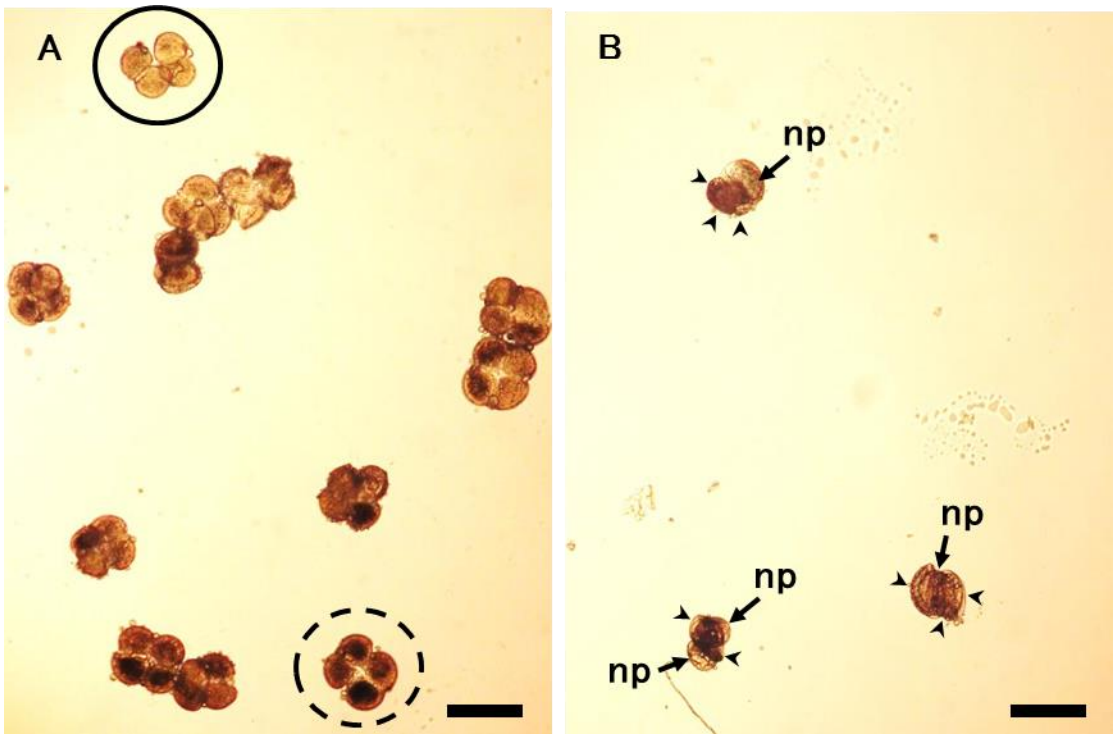

Fig. 3.7 Appearance of freshly-shed ‘Big Sister’ cherimoya pollen tetrads collected from 6–35ºC and controls (A) and 4ºC (B) nighttime (1800–0900 h) temperature treatments. Pollen was stained with I_2KI solution on agar medium. A: Three types of tetrad were observed at a similar ratio: tetrads in which (1) all pollen grains contained almost no starch (circle), (2) all grains contained much starch (dashed circle), and (3) grains containing much starch and almost no starch were mixed. B: Pollen tetrads often included some shriveled grains (arrowheads) together with some normal pollen grains (np). Scale bars indicate 50 µm.

Discussion

Pre-anthesis night temperatures at 20–22ºC increased pollen germination (Fig. 3.6), while night temperatures below 14ºC and over 27ºC, even for one night right before anthesis day, decreased germination.

Night temperatures below 8ºC often resulted in irregular anthesis times (Fig. 3.5), and germination decreased remarkably to ≈30% below 6ºC (Fig. 3.6), despite that pollen available for hand-pollination is empirically desired to hold higher germination percentage than 30%. With a night temperature at 4ºC, pollen germinated little and pollen grains were often observed to be shriveled (Fig. 3.7.B), seemed to be caused by water absorption failure. Germination also decreased to a level unsuitable for hand-pollination at temperatures above 30ºC (Fig. 3.6).

Lora *et al*. (2012) stored cut cherimoya flowers at 15, 25, 30, or 35ºC from the morning 1 day before anthesis to the time of anthesis and found that pollen germination decreased at 35ºC. The present results showed that temperatures even in a shorter time clearly affected pollen germination percentages in cherimoya: germination decreased below 14ºC and over 27ºC. Only the sectional temperatures surrounding flowers were regulated in the present experiment, and temperature effects were analyzed at 2–3ºC intervals. Thus the thermal response of reproductive organs was clearly detected.

In the present observation, about half of the pollen immediately after anthesis on agar medium lost its starch grains (Fig. 3.7.A) though the other half still contained many starch grains. Pollen in which starch was decomposed after anthesis was reported to germinate well by Saavedra (1977). Lora *et al*. (2012) also reported that the starch reserve was completely decomposed in pollen showing high germination after anthesis, although pollen starch catabolism occurred immediately before anthesis, unlike the present result in which pollen starch became most accumulated immediately before anthesis (Fig. 3.4.D). Saavedra (1977) and Lora *et*

al. (2012) commonly suggested that high germination is accompanied by promoted starch decomposition. Thus, pollen germination seems to need not only a sufficient pre-anthesis starch reserve but also smooth and rapid catabolism of starch. Pre-anthesis temperatures apparently affected pollen germination, suggesting that the pre-anthesis night temperature affects both starch accumulation and its subsequent catabolism. However, microscopic observation of pollen starch may not predict pollen germinability in practice, because potential metabolic activity is difficult to observe visually.

Footnotes

1) Cherimoya anthesis occurs simultaneously regardless of the individual tree. From 10–20 minutes before anthesis, if flowers are stimulated by gently forcing open the petals with fingers, the petals thereafter visibly open completely by themselves simultaneously with anther dehiscence. Unless flowers are ready for anthesis, anther dehiscence does not occur, even if the petals are forced to open. Within a few days before anthesis, the day of anthesis can be predicted accurately by the appearance of the petal aperture.
2) In cherimoya, pollen was shed remaining as tetrads.

Section 2

Effects of Temperature and Humidity on Anthesis and Germinability

Introduction

In the previous section, cherimoya pollen germinability was found to decrease by pre-anthesis temperatures below 14ºC or over 27ºC, even for overnight previous to anthesis. The effect of anthesis daytime temperatures, however, is unknown. In addition, for more than 100 species, pollen germinability is reportedly strongly influenced by not only temperatures but also by humidity after anthesis (e.g., Pfundt, 1910; Holman and Brubaker, 1926; Wakisaka, 1963). Thus, cherimoya pollen germinability may be strongly influenced by temperature and/or humidity.

Cherimoya anthesis begins in the late afternoon; pollen collection and hand-pollination must be completed before sunset, because of the brittle pollen vigor described in Chapter 1. Despite such limited time for hand-pollination, anthesis time varies daily over several hours. Thus, these practices cannot be completed when anthesis is delayed. If the anthesis time could be estimated daily according to climate conditions, efficient orchard practices could be arranged. Various analyses of environmental factors that influence cherimoya anthesis time have been conducted using orchard climate data. However, no clear relationships have been reported to date. In cherimoya, the anthesis time is likely influenced by

micrometeorological factors. Such factors may be difficult to detect precisely under field conditions because only macroclimate data are usually recorded in orchards. Nevertheless, the anthesis hours of some fruit tree species are reportedly influenced by temperature and humidity (Yates and Sparks, 1993; Gradziel and Weinbaum, 1999). Similarly, cherimoya anthesis may be influenced by temperature and/or humidity.

To complete effective hand-pollination quickly during limited evening hours, growers must induce anthesis earlier and maintain high pollen germinability by controlling the growth environment. Therefore, the environmental conditions necessary to encourage early anthesis and maintain pollen vigor for a few hours must be clarified. In this section, to clarify the effects of temperature and humidity on cherimoya anthesis time and subsequent pollen germinability, flowers were detached in the morning, then transferred to culture liquid in the laboratory and incubated under various temperature/humidity (15–30ºC/40–90% RH) conditions. Subsequently, anthesis time was recorded, and pollen was collected hourly for 4 h after anthesis and placed on agar medium to test germination.

Materials and Methods

Cherimoya flowers from 18-year-old ‘Big sister’ trees were detached the morning of the expected anthesis day. The trees were grown in 40-L pots under greenhouse conditions (minimum temperature >10ºC) at Kyoto University (35.0° N, 135.8° E). Then, the flowers were incubated in liquid culture medium under various regulated temperature and humidity conditions in the laboratory. After anthesis, germination of the shed pollen was tested on agar medium and the germination percentages were recorded.

Saturated solutions of $MgCl_2 \cdot 6H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, or NaCl, were used to

regulate humidity conditions as a facile method in the laboratory. Saturated salt solutions are widely used for humidity regulation (e.g., Yates and Sparks, 1993; Buitink *et al.*, 2000). In particular, $MgCl_2 \cdot 6H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, and NaCl are inexpensive and allow relatively stable humidity irrespective of temperature: humidity at 15–30ºC is 34–33% RH, 56–47% RH, and 76–75% RH for $MgCl_2 \cdot 6H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, and NaCl (Winston and Bates, 1960).

First, the temperature and humidity conditions surrounding the pre-anthesis flowers were regulated. From May–June, 2014, a flower was detached from the tree at 0900 h on its expected anthesis day and the peduncle was immediately inserted into a glass vial that contained a second small vial, and both were sealed with Parafilm (Fig. 3.8.A). The small inner vial was filled with liquid culture medium comprising 2% sucrose, 1% plant nutrition additive (Menedael; Menedael Co., Ltd., Osaka, Japan), and 0.2% chemical fertilizer (Garden Meister; nitrogen: phosphorus: potassium [N:P:K] = 7:8:6; HYPONeX Japan Co., Ltd., Osaka, Japan) to protect the flower from wilting. Immediately following insertion, the glass vial containing the flower was housed in a small, airtight plastic container, which was closed tightly and sealed with Parafilm. To regulate humidity inside the container, ≈100-g $MgCl_2 \cdot 6H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$ or NaCl was placed in the bottom of the container (Fig. 3.8.B). Then, the container was left to stand for 20 to 30 min to achieve vapor pressure equilibrium between each saturated solution and the air inside the container, and humidity conditions at ≈40%, ≈60%, or ≈80% were obtained. In addition, a ≈90% humidity condition was obtained by placing wet Kimwipes™ in the container instead of salts. Humidity inside the containers was monitored by a small button-sized data logger (Hygrochron; KN Laboratories, Inc., Osaka, Japan) attached to the inside wall of the container at the same height as the flower. These containers were then incubated at 15, 20, 25, or 30ºC. Then, 16 treatment combinations were prepared: humidity (four levels) and temperature (four levels). The time of anthesis was recorded for each flower. Completely open petals were

considered to indicate anthesis, as cherimoya petal opening coincides with anther dehiscence and pollen shedding. After recording the time, the flowers remained inside the incubator until that night. The incubator was placed beside the window to receive gentle light without direct solar radiation, and the light was not artificially regulated. The humidity inside the container is shown in Fig. 3.9.

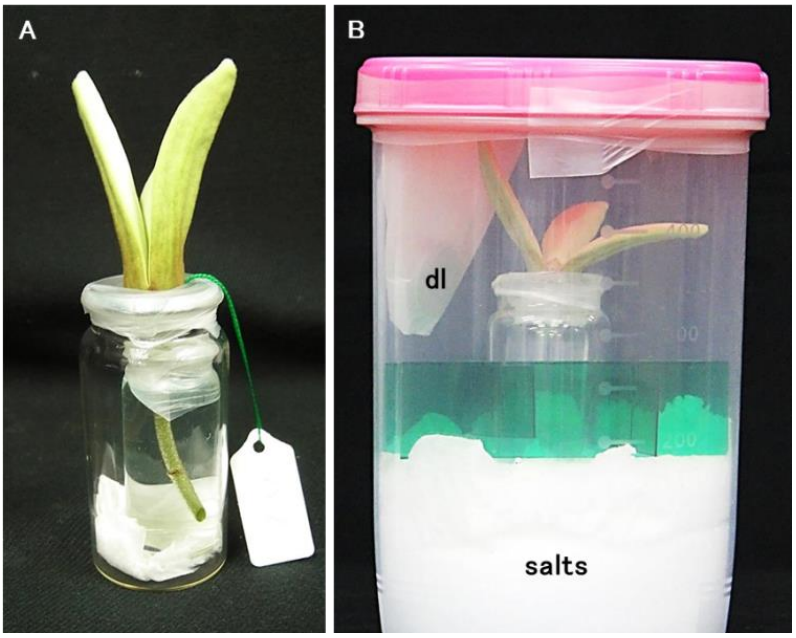

Fig. 3.8 A clipped ‘Big Sister’ cherimoya pre-anthesis flower (A), and a small airtight plastic container for humidity regulation enwrapping a flower at anthesis (B). A: The flower peduncle was inserted into in a small glass vial containing liquid culture medium, which was sealed with Parafilm. B: $MgCl_2 \cdot 6H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, or NaCl was placed in the plastic container together with the cut flower to regulate the relative humidity, and the humidity inside was recorded by a small button-sized data logger (dl).

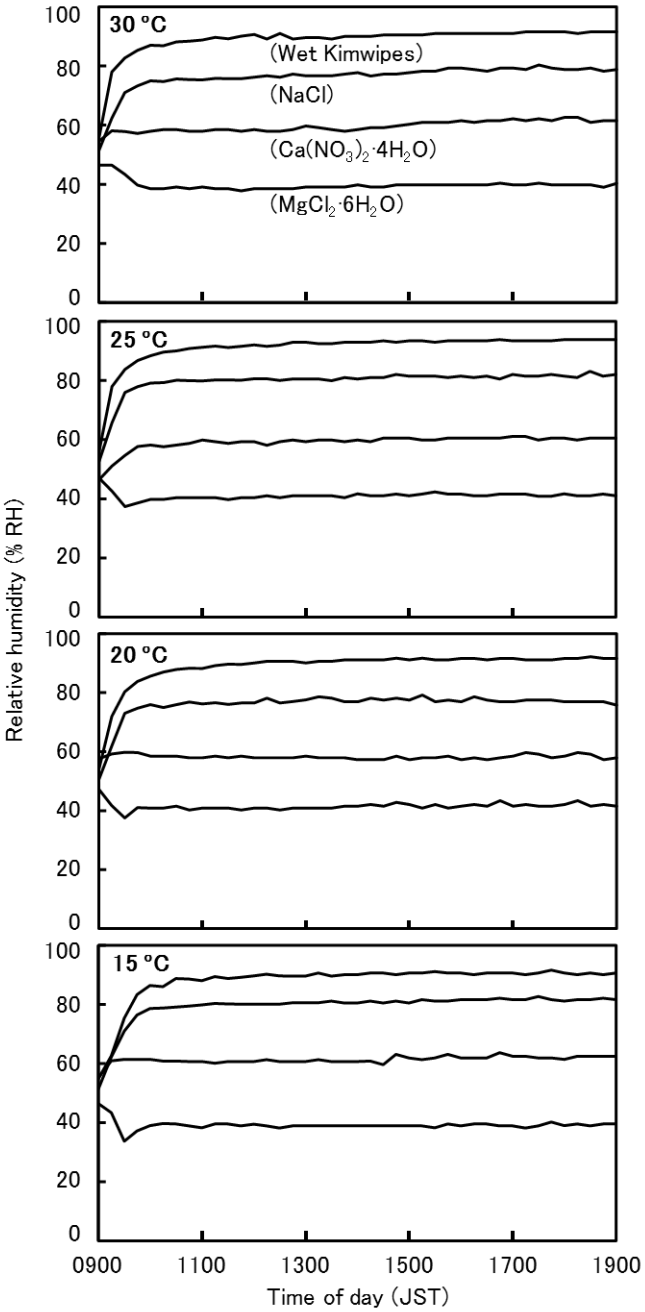

Fig. 3.9 Relative humidity conditions inside the airtight plastic container containing a flower detached from a 'Big Sister' cherimoya tree. The humidity was regulated at 15–30ºC. $MgCl_2 \cdot 6H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, NaCl, or Wet Kimwipes™ maintained a relative humidity (RH) of approximately 40, 60, 80, or 90%, respectively.

Second, pollen germination was tested. After recording the anthesis time, the plastic container from each treatment was removed from the incubator and opened to allow for collection of the shed pollen using a small paintbrush. Then, immediately after collection, the container was closed again, sealed with Parafilm, and placed back into the incubator. The collected pollen was placed on ≈1 mL of 2% agar medium containing 15% w/v sucrose, 100 ppm w/v boric acid, 300 ppm w/v $Ca(NO_3)_2 \cdot 4H_2O$, 200 ppm w/v $MgSO_4 \cdot 7H_2O$, and 100 ppm w/v KNO_3 (Brewbaker and Kwack, 1963; Yonemoto *et al*., 1999), which was prepared on a glass microscope slide. Pollen remaining on the flower was collected hourly and was placed on different agar medium until 4 h after anthesis, as described above. The pollen and agar medium was then incubated at 25°C for 24 h, as described in Section 1. Then, the number of germinated pollen grains from the 400 grains and the lengths of 10 pollen tubes on each glass slide were recorded as described by Matsuda and Higuchi (2013). The above incubation of the flowers and germination test were repeated four times for each treatment to calculate average germination percentages and tube lengths.

Results and Discussion

Cherimoya anthesis time was strongly influenced by temperature conditions (Fig. 3.10). Flowers attained anthesis earliest at 20 and 25ºC. Most anthesis occurred from 1400–1500 h at these temperatures; anthesis occurred slightly earlier at 20ºC, and was concentrated in the first 30 min. Although anthesis was delayed by 1 h at 15ºC compared to 20ºC and 25ºC, most of the flowers at this temperature attained anthesis within 1 h. However, anthesis at 30ºC varied widely from 1400–1700 h. Anthesis time during the mid-summer in warm temperate zones is often unstable; protracted anthesis from early to late hours is known to occur. Such a

phenomenon was duplicated under a controlled environment at 30ºC in the present study. Humidity conditions had little effect on anthesis.

The collected pollen germination percentage was influenced by pre-anthesis temperature conditions; the germination percentage just after anthesis was > 80% at 15–25ºC (Fig. 3.11), and slightly lower (≈70%) following the 30ºC treatment. The tendency was towards fewer germinative pollen grains following exposure to high temperature (30ºC) at high humidity (90% RH) and low temperature (15ºC) at low humidity (40% RH). On the contrary, pollen tube elongation just after anthesis was not influenced by temperature or humidity treatments.

Following anthesis, pollen germination tended to decrease more rapidly under higher temperature and lower humidity conditions: pollen germination percentage rapidly decreased at 30ºC and 40% RH and soon lost germinability. Whereas following higher humidity (> 80% RH) treatments, the germination percentages were > 70% within 4 h after anthesis at 20ºC and below. At higher humidity, pollen germination percentages at 25ºC decreased to less than 50% within 4 h; at 30ºC the germination percentage decreased earlier, to less than 10%. In addition to germination percentage, tube elongation tended to decrease more rapidly following lower humidity treatments. Tube elongation of pollen collected 3 h after anthesis was greatest following 20ºC treatment. To abrogate the germinability reduction following anthesis, temperature and humidity conditions of 20ºC and > 80% RH were favorable.

In the present study, the germination percentage of pollen collected just after anthesis was low at 30ºC (Fig. 3.11) and then continued to decrease to less than half of the initial value within 2 h, except under the high humidity (90% RH) condition. Similarly, Rosell *et al*. (2006) reported that the germination percentage of cherimoya pollen collected 2 h after mid-summer anthesis was about one third of that just after anthesis. This indicates that high-temperature conditions of 30ºC for 2 h result in deterioration of pollen sufficient to preclude hand-pollination.

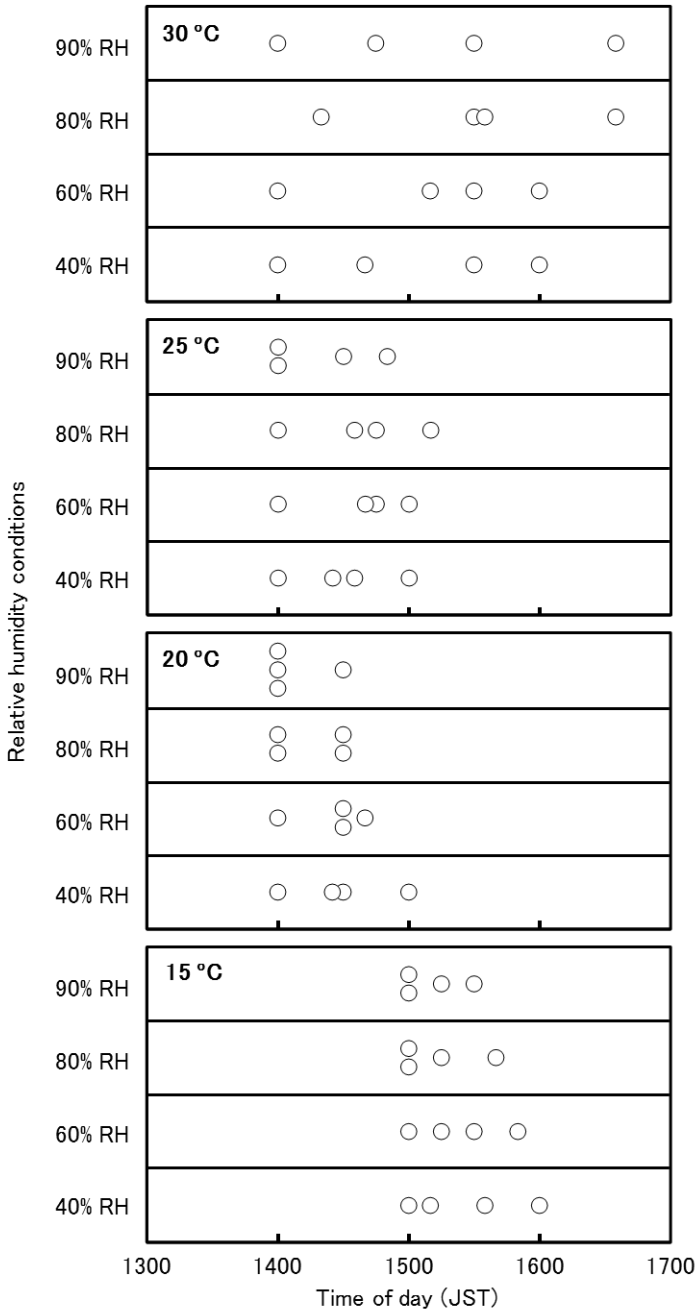

Fig. 3.10 The anthesis time of 'Big Sister' cherimoya flowers incubated under regulated temperature and humidity conditions (15–30ºC, 40–90% RH) from morning to evening on the day of anthesis. Each open circle indicates the anthesis time of an individual flower.

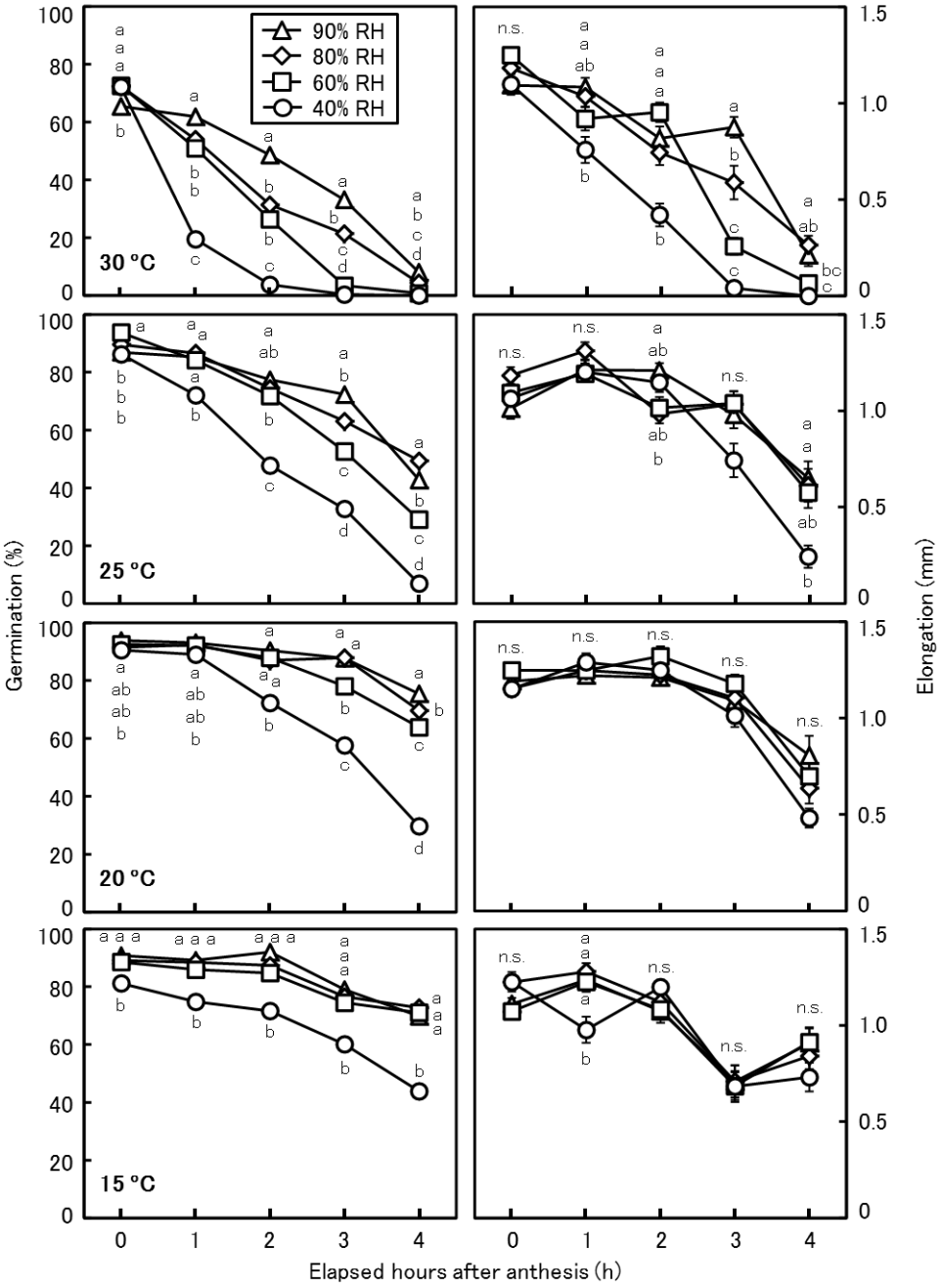

Fig. 3.11 Effects of temperature and humidity during anthesis on 'Big Sister' cherimoya pollen germination *in vitro* (left) and tube elongation (right) within 4 h after anthesis under regulated temperature and humidity conditions (15–30ºC, and 40–90% RH). Symbols with different letters in the same hours indicate statistical significance at $P < 0.01$, as determined by Tukey's test.

In addition, the germination percentage of pollen collected just after anthesis was also influenced by humidity, albeit to a lesser extent than temperature; indeed, an interaction between these factors was also evident: germination percentages at high temperature (30ºC) decreased at high humidity (90% RH), while those at low temperature (15ºC) decreased at low humidity (40% RH). However, the cause was not identified.

Cherimoya anthesis time was not affected by humidity in the present experiment. On the contrary, anthesis time is reportedly affected by humidity in many plant species: the times of anthesis in pecan (Yates and Sparks, 1993) and *Prunus* spp. (i.e., apricot, peach, and almond; Gradziel and Weinbaum, 1999) were reportedly advanced under lower humidity conditions. These species differ from cherimoya, in that cherimoya anthers are enveloped in large succulent petals and are thus not exposed to the ambient atmosphere. Therefore, humidity might not affect anthesis in a clear manner.

Cherimoya petals open for a short time at anthesis, which coincides with anther dehiscence and pollen shedding. Koura *et al*. (2001) observed a change in cherimoya petal water content just before petal opening and indicated that the difference in petal cell turgor pressure may drive petal opening. Under the high-temperature (30ºC) condition in the present experiment, petals were slightly drooping, showed slightly loose tension, and opened unusually slowly at anthesis. Anthesis time connected with petal opening varied widely at this temperature (Fig. 3.10). This flowering vacillation may be due to insufficient cell moisture caused by an increased vapor pressure deficit at high temperature, which results in cell turgor pressure being unable to control petal movement. In contrast, the delayed anthesis at low temperature (15ºC) may be caused by metabolic reduction.

Germination percentages of the lab-collected pollen just after anthesis exceeded 90% under the 20ºC condition at anthesis (Fig. 3.11). These percentages are higher than those of field-collected pollen, which exhibited 60–70% germination

in previous reports (Higuchi *et al*., 1998; Yonemoto *et al*, 1999; Rosell *et al*., 2006; Lora *et al*., 2011). In the present study, anthesis was induced under controlled environments. Such artificially stable conditions might have enabled more precise elucidation of the environmental effects than in previous studies.

Chapter 4

Effect of Post-pollination Temperature on Pollen-tube Growth

Introduction

Pollen germinability was found to decrease by pre-anthesis low night temperatures below 14ºC, and was deteriorated remarkably below 6ºC in Section 1 of Chapter 3. As well as such germinability decrease, immature pollen (Saavedra, 1977) was considered to partly be the cause of low fruit set in early spring described in Chapter 1. However, in greenhouse cultivation in Japan, even when the weather becomes warmer and pollen germination increases, fruit set did not increase during the earlier period of flowering (Yonemoto, 2002). Not only the pollen germinability but also the lower night temperatures after pollination were thought to have been related to this phenomenon.

Cherimoya pollination is generally conducted immediately after anthesis as described in Chapter 1. After pollination, 5–9 h are reportedly required for the pollen tube to reach the ovule (Ikeda *et al.*, 1994), suggesting that fertilization occurs from midnight to early dawn, when the temperature is generally decreasing. In typical Japanese plastic house cultivation, the minimum temperatures in late April

(corresponding to the early flowering period) is often around 10°C. The effect of such a cool temperature on pollen-tube growth in the pistil has not been investigated, although the effect of temperature on pollen viability has been studied extensively both *in vitro* (Yonemoto *et al.*, 1999; Koura *et al.*, 2001) and *in vivo* (Rosell *et al.*, 1999).

In this chapter, flowers immediately after hand-pollination were incubated at different temperatures for sufficient time to complete fertilization, and observed ovary cross sections by fluorescence microscopy to determine the abortive temperature range for pollen tubes before reaching the ovule. Then, pollen-tube growth was observed hourly within the attainable temperature range to estimate the time requirement for pollen tubes to reach the embryo sac after pollination.

Materials and Methods

Twenty-four trees of potted 'Big Sister' cherimoya grown in a greenhouse at Kyoto University (35.0° N, 135.8° E) were used for microscopic observations. The study was carried out in 2009 and 2010. In both years, the flowering periods were from April to July. The maximum and minimum temperatures during the periods were monitored.

Experiment 1: Specification of the pollen-tube abortive temperature range

Fresh pollen was collected from flowers at anthesis at 1600–1700 h in May and June 2009. Immediately after pollen collection, the flowers at one day before anthesis were hand-pollinated using a small paintbrush. Just after the pollination, the flower with the bearing stem was detached from each branch and placed into bottle filled with a culture liquid containing 2% sucrose, 1% plant nutrition additive (Menedael; Menedael Co., Ltd., Osaka, Japan), and 0.2% chemical fertilizer (N:P:K

= 7:8:6, Garden Meister; HYPONeX Japan Co., Ltd., Osaka, Japan) as described in Section 2 of Chapter 3.

The pollinated flowers were incubated in a styrene foam box of 50 × 30 × 20 cm^3 equipped with a handmade temperature control unit using a Peltier device (*cf*. Fig. 3.1), and the temperature was kept at 12, 15, 18, 20, 22, 25, 27, 30, 32, or 35°C, including the optimal pollen germination temperature range for cherimoya of 20–30°C, as reported for agar medium by Yonemoto *et al*. (1999). The flowers were incubated for 18 h, which is more than sufficient time for the pollen tubes to penetrate the ovule, as Ikeda *et al*. (1994) noted that cherimoya fertilization occurs within 5–9 h after pollination. Following the incubation, the pistils were fixed in an FAA solution as described in Chapter 2.

After rinsing with distilled water overnight, the fixed pistils were dehydrated in an alcohol series (see Chapter 2), followed by embedding in paraffin wax and sectioning longitudinally at 16 μm. The sections were stained with a 0.1% aniline blue solution containing 0.1M K_3PO_4 for 12 h, and observed under a fluorescence microscope (CyScope; Partec GmbH, Saarbrüchen, Germany). Then, whether the pollen tube reached the ovule was recorded. As cherimoya has multiple flowers, many ovules were observed together, and some ovules were examined in each flower. Thus the position of the longest pollen-tube tip among the examined ovules was recorded as the flower's record. Pollen germination on the stigma was also noted.

Experiment 2: Time requirement for pollen tubes to reach the embryo sac at different temperatures

To clarify the effect of temperature on pollen-tube growth in the pistil, flowers hand-pollinated from April through July 2010 in the same manner as Experiment 1 were placed in a temperature-gradient incubator kept at 15, 18, 20, 24, 27, 30, or 32°C. These temperatures are in the range in which the pollen tube reached the embryo sac in Experiment 1. Two to seven flowers at each temperature

were brought out hourly from the incubator until 10 h after pollination, and their pistils were fixed in FAA.

Sections of the pistils stained with the aniline blue solution were prepared from the fixed samples in the same manner as in Experiment 1. The pollen-tube growth in the pistils was chronologically observed by fluorescence microscopy. Because cherimoya has anatropous ovules, to record the deepest pollen-tube position, the pathway of the pollen tubes was divided into seven portions: stigma; style; upper, middle, and lower part of ovary; micropyle (including nucellus); and embryo sac (Fig. 4.1) following the manner of Beppu and Kataoka (1999). The correlation between the deepest pollen-tube position and the elapsed time was examined, and the time requirement for the pollen tube to reach the embryo sac was estimated for every temperature.

The flowering period was divided in two: early (April 23–June 8) and later (June 9–July 4), with the latter based on a daily minimum temperature exceeding 15°C. This criterion was determined according to the results of Experiment 1. The difference in the pollen-tube growth rates between the periods was examined.

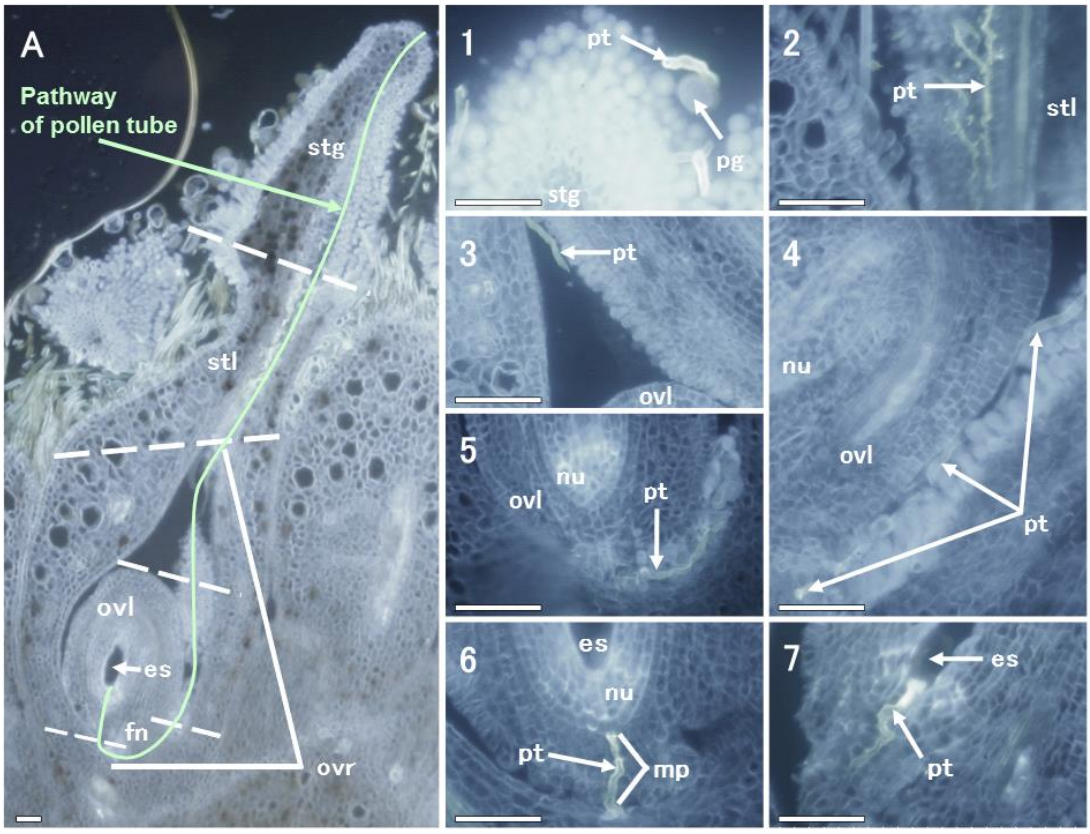

Fig. 4.1 Fluorescence microscopic observations of pollen-tube elongation into the cherimoya pistil. The pathway of the pollen tube (pt) is divided into seven portions (A): (1) pollen grains (pg) germinating on the stigma (stg); (2) pollen tube penetrating through the style (stl); (3) pollen tube arriving at the upper part of the ovary (ovr); (4) the middle part of the ovary; (5) pollen tube elongating along the funicle (fn) at the basal part of the ovary; (6) pollen tube penetrating into the micropyle (mp) or the nucellus (nu) in the ovule (ovl); and (7) pollen tube reaching the embryo sac (es). Scale bars indicate 50 μm.

Results

Experiment 1: Specification of the pollen-tube abortive temperature range

No pollen tube reached the embryo sac within 18 h after pollination at 12 or 35°C (Fig. 4.2.A). Even at 18 and 32°C, a few pollen tubes reached the embryo sac. In contrast, the pollen tubes in all of the flowers at 20–27°C reached the embryo sac. Pollen-tube tips were observed in the middle of the micropyle or the nucellus at 15 or 18°C (Fig. 4.2.B & 4.3). Pollen germination was observed on the stigmas of all of the flowers at 15–30°C, but fewer germinated at 35, 32, and 12°C (Fig. 4.2.C).

Experiment 2: Time requirement for the pollen tube to reach the embryo sac at different temperatures

Pollen germinated on the stigma, and pollen tubes elongated through the style canal to the ovarian locule (Fig. 4.1). The pollen tubes extended farther along the ovarian wall and then penetrated into the micropyle through the lower part of the ovary from the funicle side. After passing the micropyle, the pollen tubes penetrated into the embryo sac through the nucellus cells.

The relationship between the deepest position of the pollen tube and the hours after pollination is shown in Fig. 4.4. At 20–27°C, the growth rates of pollen tubes in the later flowering period (June 9–July 4) were higher than those in the earlier period (April 23–June 8). The times that the pollen tubes took to pass each portion of the pistil illustrated in Fig. 4.1 were almost constant. The linear approximations of the time requirement from pollination to the embryo sac were calculated to be 10 h at 18°C, 7–9 h at 20°C, 6–8 h at 24°C, and 5–6 h at 27°C according to the regression line drawn in Fig. 4.4.

Pollen-tube growth rate tended to be faster at higher temperatures within the attainable temperature range under 27°C. Pollen-tube growth was greater at 20–27°C than at 18°C, and was slowest at 15°C (Fig. 4.4). At 30°C, although the growth was

rapid at the beginning, the growth rate slowed after passing the ovary, and at 32°C, many of the pollen tubes stopped growth before reaching the middle part of the ovary.

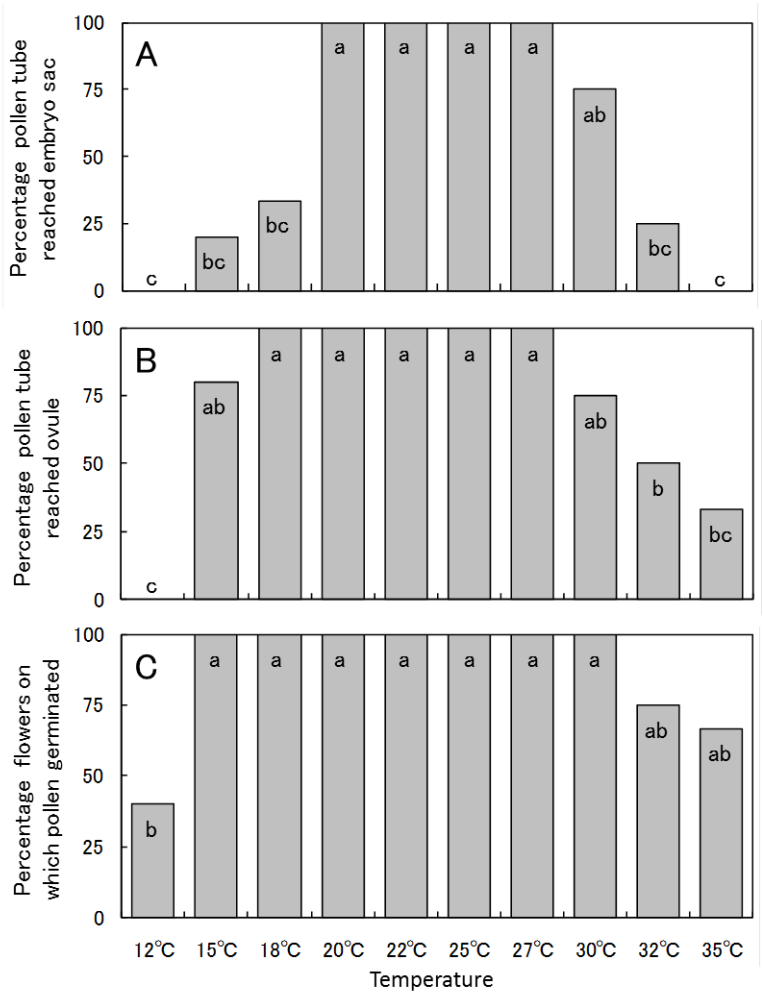

Fig. 4.2 Percentage of flowers in which the pollen tube reached the embryo sac (A) and the micropyle (B), and in which pollen germination was observed on the stigma (C), in flowers incubated at different temperatures for 18 h after pollination. Different letters indicate significant differences at $P < 0.05$ as determined by Fisher's exact test.

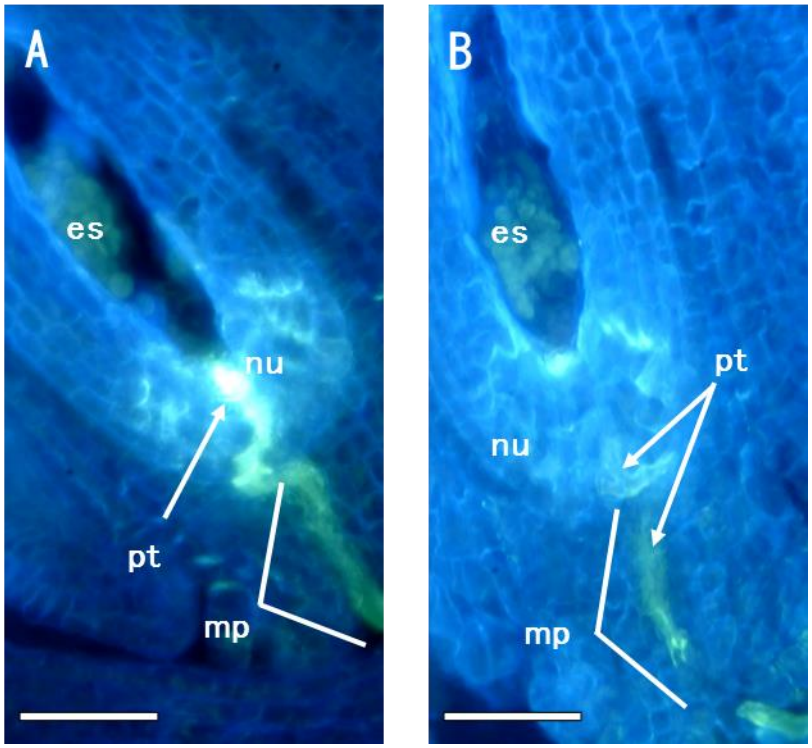

Fig. 4.3 Fluorescence microscopic observations of pistils. The pollen tube reached the ovule at 18 h after pollination. The pollen tube (pt) penetrated into the embryo sac (es) through the micropyle (mp) at 20°C (A), whereas it was suspended at the nucellus (nu) at 15°C (B). Scale bars indicate 50 μm.

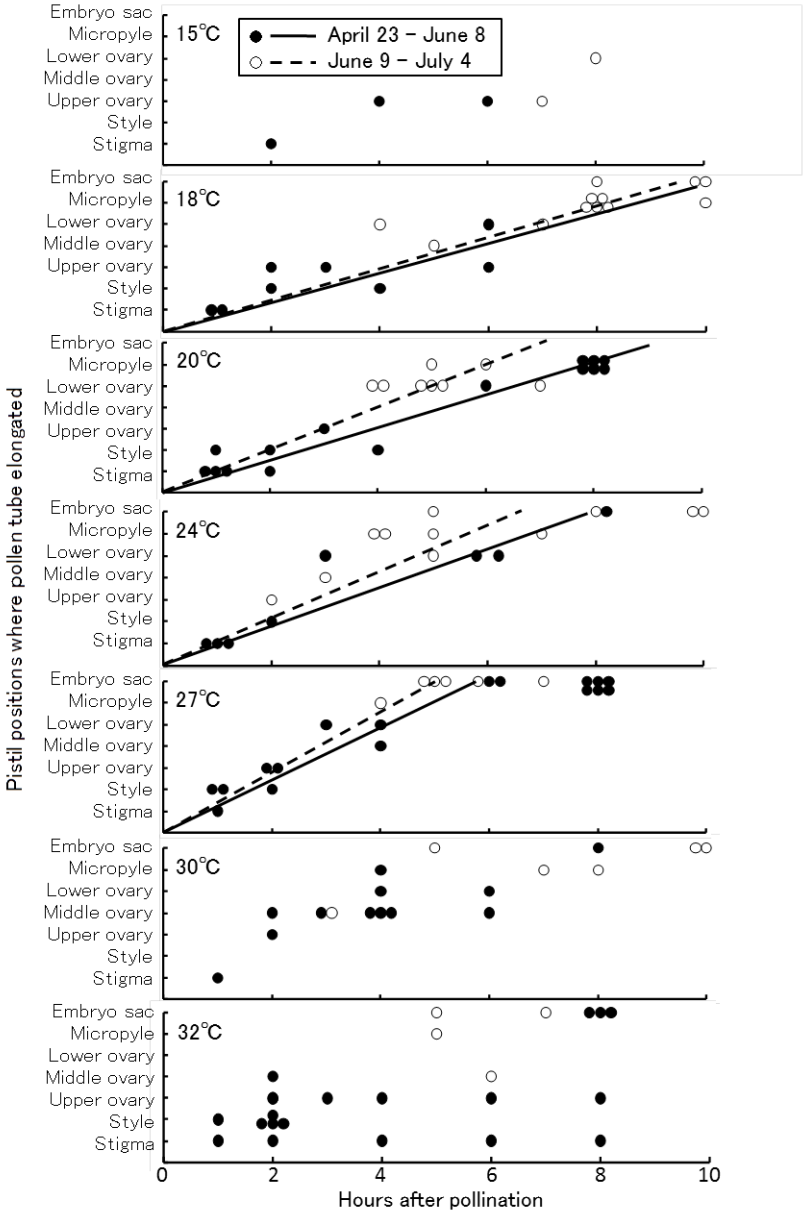

Fig. 4.4 Hourly elongation of pollen tubes at different temperatures in cherimoya pistils. The data are presented according to the deepest position of the pollen tube in the pistil, dividing the flowering season into two periods of April 23–June 8 (closed) and June 9–July 4 (open). The regression lines approximate the relationship between the germination and the arrival to the embryo sac.

Discussion

Yonemoto (2002) monitored daily changes in cherimoya fruit-set and pollen-germination percentages in Japanese greenhouse cultivation, and noted that fruit set was low in the early flowering period (April–May), although the pollen germinability increased with the temperature in mid-May. In the present experiment, pollen-tube growth in the pistil was inhibited at 15°C, which was around the minimum temperature in mid-May (Fig. 4.5). Pollen tubes might be sensitive to cool temperatures, and poor fruit set in the early flowering period is thought to be caused by the decreasing night temperatures following evening anthesis and pollination. Pollen tubes may possibly be refused to enter the embryo sac.

Later in the flowering period, the time required to reach the embryo sac became shorter (Fig. 4.4). Under greenhouse cultivation in Japan, cherimoya shoots flush in spring, and the leaf number and leaf area increase toward summer. Unless cherimoya trees are well leafed out, the fruit set remains low (Schroeder, 1943). This suggests that the *in vivo* growth of the pollen tube and subsequent fertilization depend not only on the pollen viability but also the pistil condition. The pistil receptivity of mango has also been reported to change within the flowering season (Dag *et al*., 2000).

Cherimoya pollen-tube growth has been found to be suitable at 22–25°C on artificial medium (Yonemoto *et al*., 1999) or 25°C *in vivo* (Rosell *et al*., 1999). In the present experiment, the growth in the pistil was better at 24–27°C (Fig. 4.4), a range a bit higher than that used in the artificial medium experiments. The pistil receptivity may be suitable at a slightly higher temperature than the optimal pollen germination temperatures on artificial medium.

The time required for cherimoya pollen tubes to reach the embryo sac after pollination was affected by the temperature. The attainment took a longer time at lower temperatures. The times varied from 5 to 10 h, shorter than the required 14 h

in cacao (Sato and Sakaguchi, 1968), 12–18 h in passion fruit (Ishihata *et al.*, 1987), and 24 h in avocado (Sedgley, 1979).

Karapanos *et al.* (2010) reported that pollen-tube growth was restricted by suppressed respiration at lower temperatures in tomato, whereas at higher temperatures, it became slow or stopped following decreased respiration after rapid exhaustion of the substrate. In the present experiment, it was the pistils, not like artificial medium, that supplied the nutrition for pollen-tube elongation. Thus, the slowdown in pollen-tube growth at lower temperatures such as the 15°C tested in the present study might be strongly related to suppressed respiration, and the rapid growth decline observed at higher temperatures such as 30 and 32°C might be related to exhaustion of the substrate due to the rapid respiration of both the female organs and the pollen. This appears to be a cause for high-temperature inhibition of cherimoya fruit set often observed over 30°C. Whereas at lower temperatures, pollen-tube growth was retarded, and in the predawn hours the temperature may decrease furthermore to fall sometimes below 15°C. In these temperatures, the pollen tubes cannot attain the embryo sac. The low fruit set in the early flowering season is likely to be attributed to this spiral.

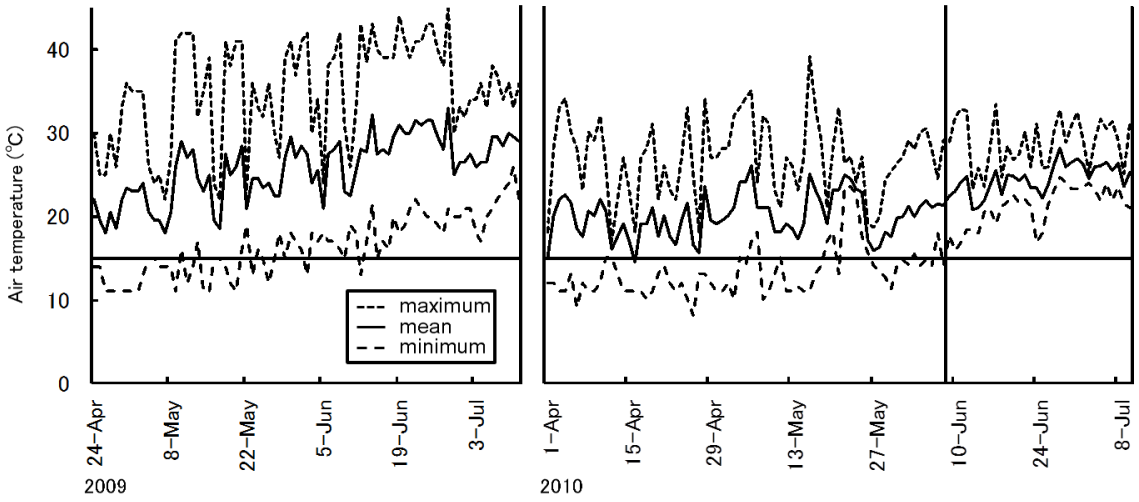

Fig. 4.5 Daily maximum, mean, and minimum air temperatures in the greenhouse during the flowering periods from April 24 to July 11, 2009, and from April 1 to July 10, 2010. Vertical and horizontal lines indicate June 8 and 15°C, respectively.

Chapter 5

Anatomical Study on Seasonal Changes in Pistil Receptivity

Introduction

The difficulty in fruit set during early spring is considered to be caused by not only low pollen vigor (Saavedra, 1977; Section 1 of Chapter 3) or retarded pollen-tube growth below 15ºC observed in Chapter 4 but also nutrient competition with vegetative flushing (Schroeder, 1943; Richardson and Anderson, 1996). The limited fruit set in mid-summer (Higuchi *et al*., 1998) is caused by high temperatures resulting in inhibited pollen-tube growth over 30ºC (Chapter 4) or fewer flowers (George and Nissen, 1987; Higuchi and Utsunomiya, 1999) and low relative humidity (George and Nissen, 1988). Although flowering occurs from spring to summer, it is empirically well documented that the beginning and ending periods of flowering, i.e., before mid April and after late July, are marked by remarkably unstable fruit set, which is a troubling problem for cherimoya growing in warm temperate regions with large seasonal differences in temperature.

The effect of postpollination temperature on pollen-tube growth in pistils of cherimoya was examined in the previous chapter and the pollen tube elongated well

at 20–27°C and penetrated into the embryo sac within 5–9 h. Moreover, even under the same incubation temperature, pollen tubes were found to elongate faster in the pistils during the warmer season, indicating that temperature before pollination can also affect pistil receptivity. In New Zealand, cherimoya pollinated at the beginning of flowering set small and asymmetrical fruit (Richardson and Anderson, 1996), whereas in southern Spain, fruit size increased with the number of seeds when flowering was extended into the warmer season by tip pruning and defoliation (Soler and Cuevas, 2008), suggesting that pistil receptivity may change seasonally.

In this chapter, pollen-tube growth in pistils incubated after pollination was observed chronologically, and the critical periods for changes in pistil receptivity were specified. In addition, leaf development, relative chlorophyll content, temperature, and pistil morphology were examined to explain the seasonal difficulty in fertilization and subsequent fruit set.

Materials and Methods

Eleven trees of potted ‘Big Sister’ cherimoya grown in a greenhouse at Kyoto University (35.0°N, 135.8°E) were used for observations of floral morphogenesis until anthesis and pollen-tube growth in pistils after pollination. Each plant was irrigated daily and fertilized weekly. The experiments were carried out from April to July, the flowering seasons in 2010 and 2011. Daily maximum and minimum air temperatures were monitored during April–July to assess the correlation between seasonal changes in pistil receptivity and temperature.

Experiment 1: Anatomical examination of floral morphogenesis

Pistils were observed to examine seasonal differences in morphology. On the day before anthesis, 3–5 unpollinated flowers were sampled and fixed in FAA (see

Chapter 2) at 1600–1700 h every 2 weeks during April–July 2011. After measuring the diameters and heights of the ovaries, the fixed flowers were sectioned, stained, and mounted to create permanent preparations in the same manner as described in Chapter 2, and pistil morphology was evaluated.

Experiment 2: Factors affecting pistil receptivity

Seasonal changes in pollen-tube growth in vivo

Pollen-tube growth in pistils was observed hourly to investigate seasonal differences in pollen-tube growth rates during April–July 2011. Fresh pollen was collected at 1600–1700 h. Flowers at 1 day before anthesis were pollinated using a paintbrush and immediately inserted into a bottled culture solution as described in Section 2 of Chapter 3, which was incubated in a temperature-gradient incubator maintained at 15°C, 20°C, 24°C, and 27°C. Flowers were removed from the incubator 4, 5, 6, 7, 8, 9, and 10 h after pollination for fixation in FAA. These procedures were repeated throughout the flowering season, except in April, when only the incubation temperature of 20°C was tested, due to the limited number of flowers during the early season. A total of 60–130 flowers were examined at each temperature.

After measuring the diameter and height of the fixed ovaries, samples were sectioned as well as Experiment 1, and stained for 12 h with aniline-blue solution as described in Chapter 4. Then, pollen-tube growth in pistils was observed under a fluorescence microscope (see Chapter 4). To record the deepest pollen-tube positions, the pathway of the pollen tube was divided into eight sections: stigma, style, upper ovary, middle ovary, lower ovary, micropyle, nucellus, and embryo sac. Because cherimoya has a multiple flower, about 10 pistils were observed in a single ovarian section, and the deepest pollen-tube position in each ovary was recorded in two ways: the position attained by the fastest pollen tube and the position of the average pollen tube.

Changes in fruit set, pollen germination, and flower number during the flowering season

To examine changes in fruit-set percentage during the flowering season, some of the pollinated flowers were left on the trees. The ovaries of these flowers enlarged 2 weeks after pollination, at which point they were considered to have set fruit, and fruit-set percentage was calculated. To monitor the changes in pollen germination percentage, fresh pollen used in the above experiment was placed on a semisolid medium consisting of 1% w/v agar, 15% w/v sucrose, 500 ppm H_3BO_4, 200 ppm $MgSO_4 \cdot 7H_2O$, 300 ppm $Ca(NO_3)_2 \cdot 4H_2O$, and 100 ppm KNO_3 (Brewbaker and Kwack, 1963; Yonemoto *et al*., 1999), and pollen germination was measured under an optical microscope after 12 h and 20°C incubation under moist conditions (see Section 1 of Chapter 3). In the same manner as described in Section 1 of Chapter 3, the number of germinated pollen grains from the 1000 grains each was recorded to calculate the daily germination percentage of pollen. Daily flower number was recorded for all trees.

Changes in assimilation capacity during the flowering season

Leaf number, leaf area, and SPAD value (relative chlorophyll content) were recorded every 2 weeks to estimate the assimilation capacity of the trees. Leaf length, width, and SPAD were measured on 10 randomly selected leaves from each tree.

Results

Experiment 1: Anatomical examination of the floral morphogenesis process

Ovular cells in flowers that opened in late May to mid-June were largest throughout the flowering season when well-developed large synergids were observed (Plate 5.1.A), whereas many ovules had small synergids in flowers before early May and after late June (Plate 5.1.B). Defective nucellus cells were peeled away from the integuments of many ovules in the flowers during April (Plate 5.1.C). Some ovules exhibited shrinkage of nucellus cells and embryo sacs. Synergids were occasionally not clearly recognized. Such peeling off and shrinkage were also observed during July. Stigmatic papillae were barely covered with secretions until mid-May (Plate 5.1.D), whereas secretion increased in late May to cover the papillae completely with ample secretion before mid-June (Plate 5.1.E). The secretion decreased after late June, and the tips of some papillae in large parts of the stigma were exposed (Plate 5.1.F) when pigmented cells were often observed in the stigma.

The ovarian dimensions measured in Experiments 1 and 2 are shown in Table 5.1. Both ovarian diameter and height were larger from May 20 to June 20 than during other periods.

Table 5.1 Ovary size of cherimoya flowers in the four divided flowering periods, 2011

Flowering periods	Ovary diameter (mm)	Ovary height (mm)
I . Apr 14 − Apr 30	4.28 ±0.052 ab	4.10 ±0.041 bc
II . May 1 − May 19	4.10 ±0.034 b	4.06 ±0.029 c
III . May 20 − Jun 20	4.27 ±0.025 a	4.34 ±0.025 a
IV . Jun 21 − Jul 25	4.14 ±0.029 b	4.23 ±0.025 ab

Data are shown as mean ±S.E. Different letters within columns indicate statistical significance by Tukey's test at $P < 0.01$.

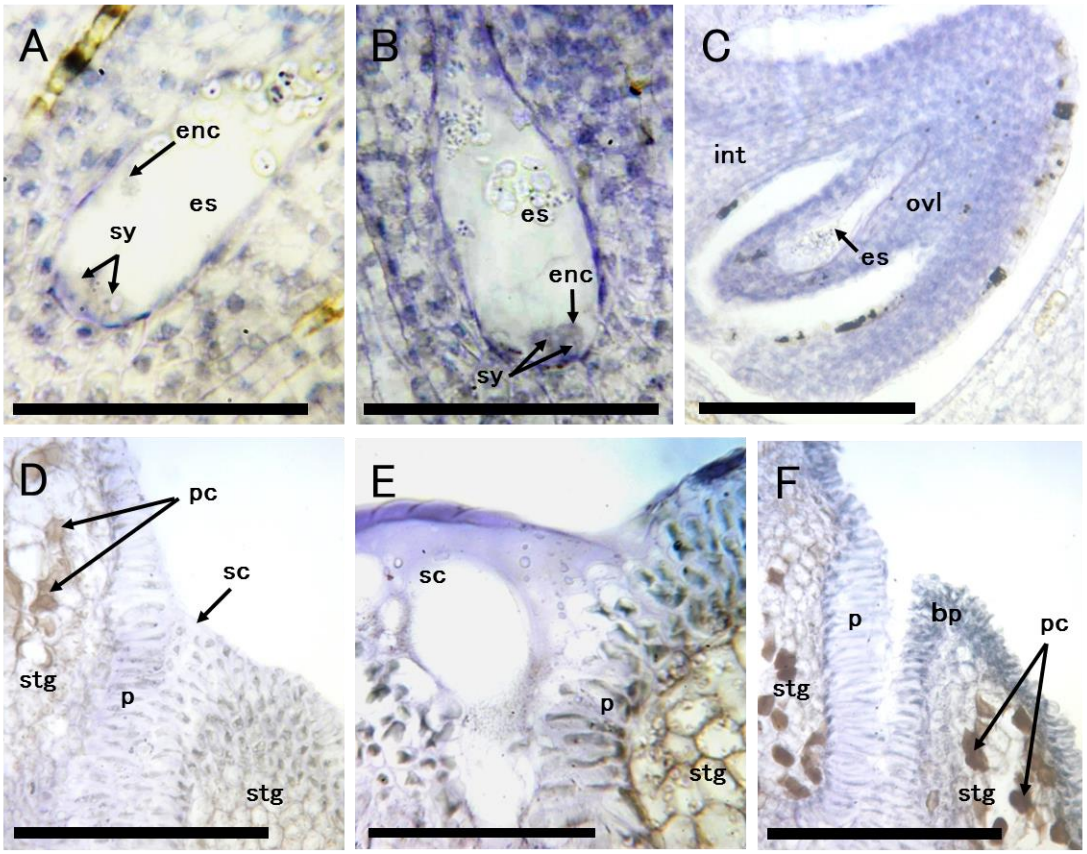

Plate 5.1 Optical microscopic observations of pistil morphology. A: well-developed ovule with a large embryo sac (es) possessing large synergids (sy) and the egg cell nucleus (enc) at the flowering period from May 20 to June 20; B: normal ovule with normal sized synergids (sy) and an egg cell nucleus (enc) at the flowering periods from May 1 to May 19 and in later June; C: abortive ovule during the flowering periods in April and July–August. The embryo sac (es) was shrunken, and the ovule (ovl) was partially peeled from the integument (int); D: papillae (p) covered by some secretion (sc) on the stigma (stg) during the flowering period from April to mid-May. Some pigmented cells (pc) were observed in the stigma (stg); E: covered by ample secretion (sc) during the flowering period from late May to mid-June. Few pigmented cells were observed in the stigma (stg); F: browning papillae (bp) had limited secretion and many pigmented cells (pc) were observed in the stigma (stg) from late June to August. Scale bars indicate 50 μm.

Experiment 2: Factors affecting pistil receptivity

Effect of air temperature on pistil receptivity during flower bud development

The air temperature during the flowering season of 2011 increased gradually from April to mid-May and then became stable at a high level (Fig. 5.1). The minimum temperatures were around 10°C in April, whereas it increased to exceed 15°C frequently in early to mid-May and was over 15°C constantly after late May. After late June, minimum temperatures were consistently over 20°C, and maximum temperatures were constantly over 30°C.

The correlation between pollen-tube position and hours after pollination during different flowering periods is shown in Figure 5.2. Pollen-tube growth in pistils was the slowest in April, with those in most pistils not reaching ovaries within 4 h after pollination. Although the pollen tubes continued to gradually elongate, they rarely reached the embryo sacs within 10 h.

From May 1 to 19, pollen-tube growth was faster than in April, and penetration into embryo sacs increased remarkably within 10 h. At incubation temperatures of 20°C, 24°C, and 27°C, more than half of the pistils in flowers had pollen tubes penetrating the embryo sacs within 10, 8, and 6 h, respectively. However, the number of those flowers did not increase subsequently. Pollen tubes scarcely reached embryo sacs within 10 h at 15°C.

From May 20 to June 20, the pollen-tube growth in this period was fastest throughout the flowering season. At 20°C and at 24°C or 27°C, more than half of the pistils had pollen tubes penetrating the embryo sacs within 7 and 5 h, respectively. The number of such flowers increased to 100% of the tested flowers within 10 h after pollination.

Pollen tubes stopped elongating at the locules in many pistils after June 21. In particular, growth stopped mostly at the style canals after mid-July.

Changes in fruit set, pollen germination, and flower number during the flowering

season

Fruit-set percentage was low before late April, maintained nearly 100% from May to mid-June, and then decreased in late June (Fig. 5.3.A). Fruit did not set after mid-July. The pollen germination percentage was 25–50% until mid-June, and decreased rapidly to about 10% in and after late June (Fig. 5.3.B). After June 26, most flowers did not reach anther dehiscence. The number of flowers increased rapidly in May, peaked in mid-June, and decreased rapidly in mid-July (Fig. 5.3.C).

Changes in assimilation capacity during the flowering season

Leaf number, area, and SPAD value increased rapidly from late April to mid-May (Fig. 5.4). Leaf number and area continued to increase until mid-June, stopped in late June to early July, and increased again in mid-July. SPAD values stabilized after early June.

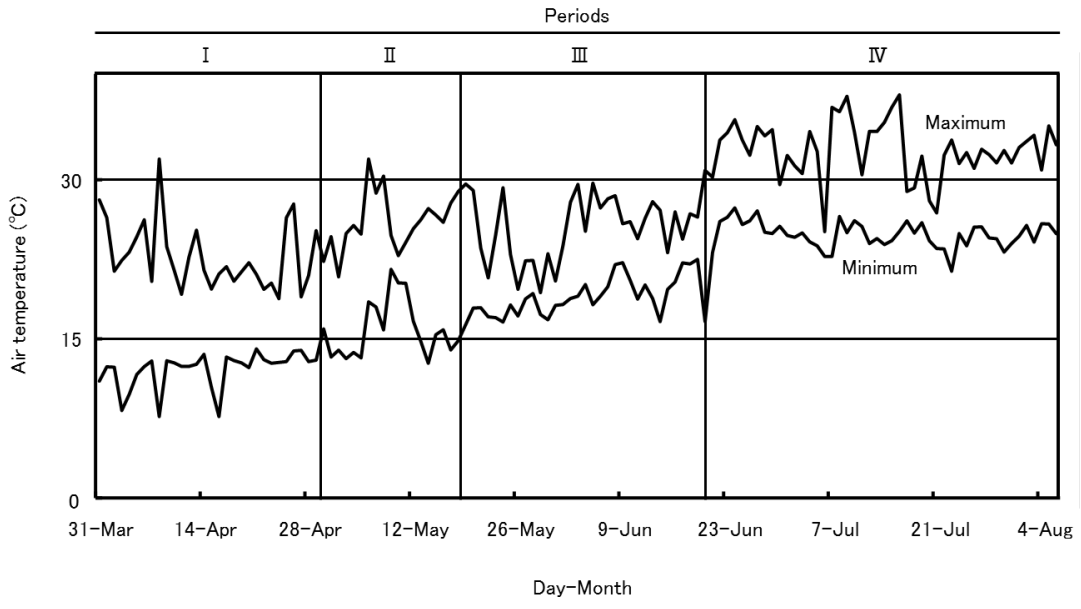

Fig. 5.1 Daily maximum and minimum air temperatures in the greenhouse during the flowering season from March 31 to August 7, 2011. Upper and lower horizontal lines indicate 30°C and 15°C, respectively, and left, middle, and right vertical lines indicate May 1 and 20 and June 21, respectively.

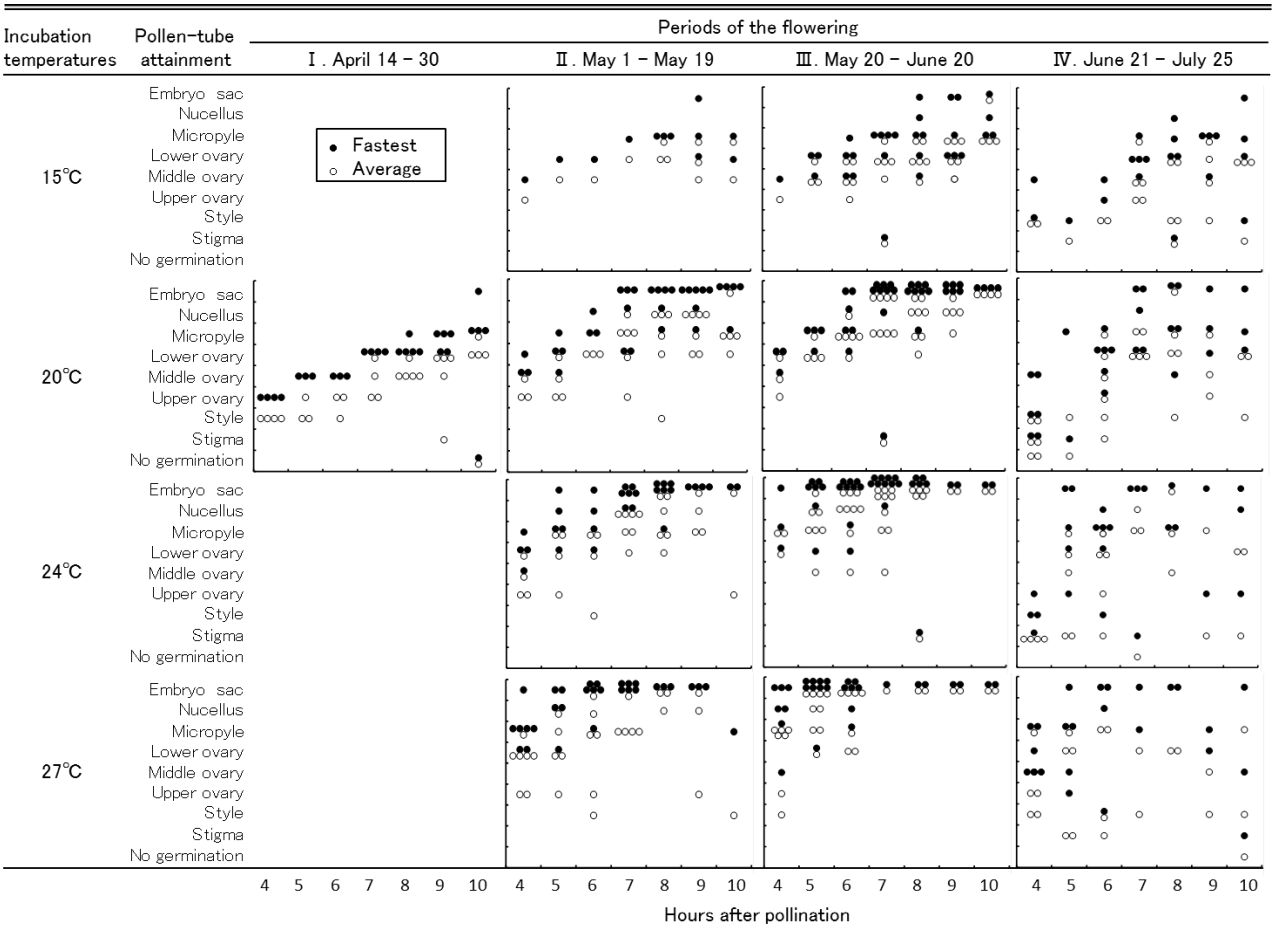

Fig. 5.2 Hourly elongation of pollen tubes in cherimoya pistils under four temperature conditions and divided flowering periods in 2011. Data are presented according to the two deepest positions of the pollen tube in pistils from each flower: a pistil in which pollen-tube elongation was the fastest (●), and the average (○).

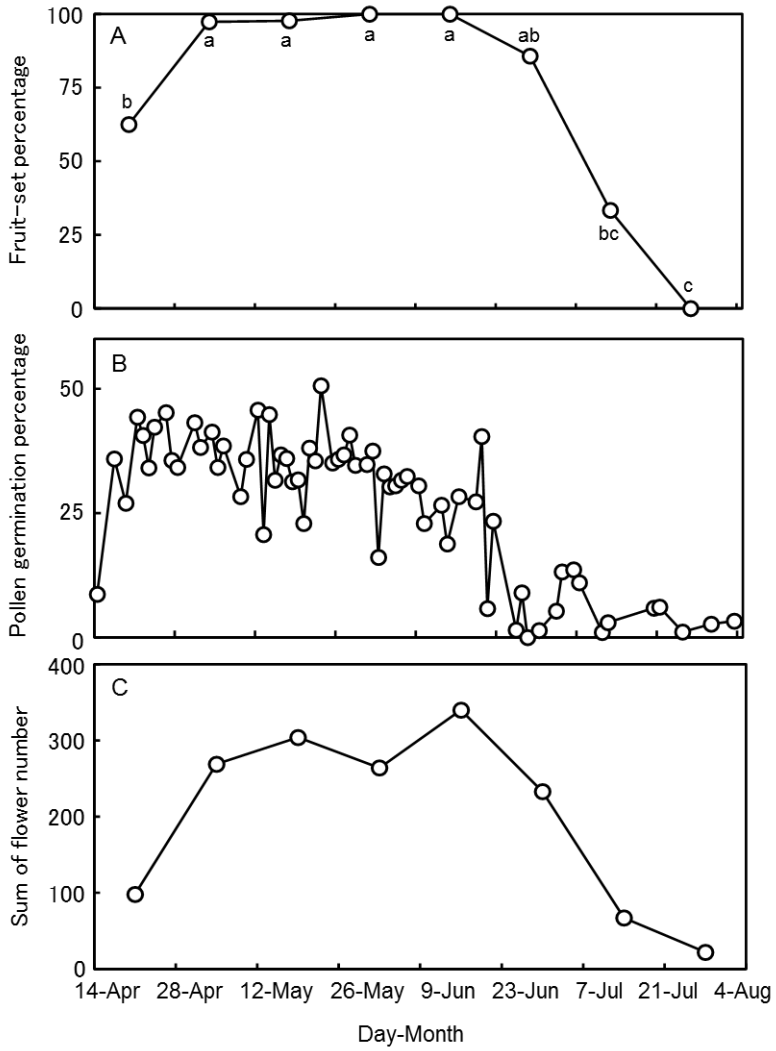

Fig. 5.3 Percentage of fruit set every 2 weeks (A), daily germination of pollen incubated on agar media (B), and the total number of flowers observed every 2 weeks (C) during the flowering season in 2011. Different letters indicate significant differences at $P < 0.05$ as determined by Fisher's exact test.

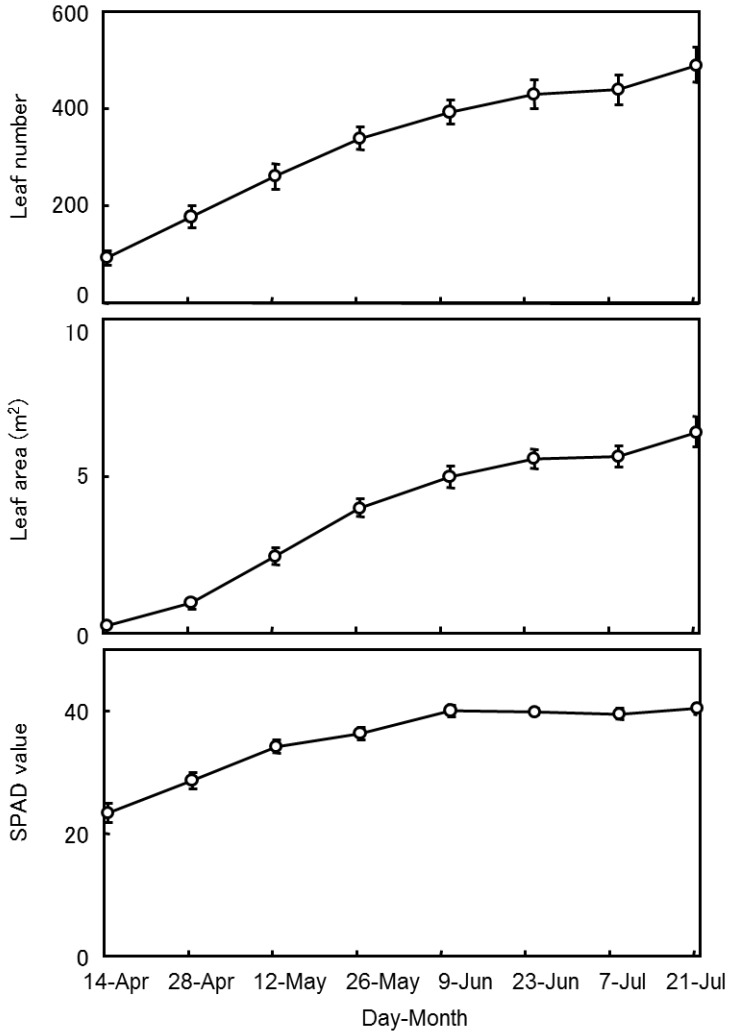

Fig. 5.4 Leaf number (top), leaf area (middle), and average SPAD value (bottom) every 2 weeks during the flowering season in 2011.

Discussion

Pollen-tube growth was suppressed and fruit set was still low in April (Figs. 5.2 and 5.3.A). Pistil receptivity was low until mid-May, and a few pollen tubes reached embryo sacs within 10 h. This suppression might reflect a shortage of photosynthate accumulation in flowers. Flushing and subsequent rapid growth of young shoots requires energy, but leaf area and SPAD were still low during these periods (Fig. 5.4). Thus, this shortage may not have been caused only by low assimilation capacity but also by competition with vigorous vegetative growth. Considering the heterotrophic elongation of pollen tubes in pistils (Cruzan, 1986; Herrero and Arbeloa, 1989), pollen-tube growth is supposed to be controlled by nutrient shortage in pistils. Stored nutrition functions little to support cherimoya flowering, not like most of deciduous temperate fruit trees.

Many impaired ovules were observed in April when minimum temperatures were approximately 10°C (Plate 5.1.C and Fig. 5.1). During this period, nutrient shortages in flowers and the low temperatures were considered to have caused the impaired ovule development, and this might be one reason for the low fruit set (Fig. 5.3.A). Fruit set in avocado decreased due to malformed pistils in early spring, when minimum temperatures were about 10°C (Inoue and Takahashi, 1990; 1991). A similar result has also been reported in mango (Dag *et al*., 2000).

Faster pollen-tube growth and less impaired ovules were observed after May 1, when the minimum temperature increased to more than 15°C. These warm conditions may have led to the decrease in defective ovule development. A large deviation in pistil receptivity, even in the same flower, was observed in early to mid-May (Fig. 5.2). This deviation might have been a result of nutrient competition between pistils. Despite the increase in minimum temperature and the reduction in impaired ovule development, the nutrient supply to flowers during that period may still have been insufficient.

Pistil receptivity was highest and differences among pistils decreased in late May to late June (Fig. 5.2) when temperatures were 15–30°C (Fig. 5.1). This high receptivity was attributed not only to warm temperatures but also to the completion of photosynthetic organ growth, which provided sufficient nutrients for the reproductive organs and resulted in well-developed reproductive organs (Plate 5.1.A and Table 5.1).

However, pistil receptivity decreased remarkably in mid-summer, i.e., after late June when nighttime temperatures often exceeded 25°C (Fig. 5.1), followed by the decrease in fruit set (Fig. 5.3.A). Pollen tubes did not elongate further than locules in most flowers (Fig. 5.2). This was not only because of the low pollen viability in this period (Fig. 5.3.B) but also because of pistil decline. High temperatures increase flower respiration and result in drastic exhaustion of substrate. Thus, pistil nutrient status may have become insufficient to support pollen-tube growth during this period.

Besides the inhibition of pollen-tube growth in pistils, other factors to decrease fruit set can occur under high temperature condition, such as the defect of stigma receptivity or the inhibition of floral development. Cherimoya stigma receptivity was shortened at 30°C (Lora *et al*., 2011) and was lost when secretions on the stigmatic surface disappeared (Koura *et al*., 2001). Lora *et al*. (2010) suggested that the secretion on the stigmatic surface assists pollen-tube growth. The secretion disappeared after late June (Plate 5.1.F). The stigma was considered to have dried out due to the high temperatures. The disappearance of the secretion also seemed to be a reason for the remarkable reduction in pistil receptivity.

High day/night temperatures inhibit pistil or flower bud development in passion fruit (Utsunomiya, 1992) and olive (Cuevas *et al*., 1994) as well as cherimoya (Higuchi and Utsunomiya, 1999). Malformed ovules and less flowering were observed in July (Plate 5.1.C and Fig. 5.3.C) when day/night temperatures often exceeded 30/25°C. Thus, the malformed ovules and less flowering were likely

to be attributed not only to heat during the daytime but also to high respiration caused by high nighttime temperatures during this period. This could result in the low fruit set in July (Fig. 5.3.A). Such effects of similarly high temperatures on inhibiting pistil and flower bud development were reported in avocado (Sedgley and Annells, 1981) and passion fruit (Menzel *et al*., 1987).

The present results, therefore, showed that fruit set decreased because of impaired pistil development at nighttime temperatures below 15°C or above 25°C.

The male organ, also, may have detrimental effects under high day/night temperatures. The pollen germination percentage of cherimoya *in vitro* decreased remarkably when flowers were exposed to temperatures over 35°C for several hours before anthesis (Koura *et al*., 2001). In the present study, pollen germination percentage was remarkably low in and after late June (Fig. 5.3.B) when day/night temperatures often exceeded 30/25°C (Fig. 5.1). Anther dehiscence failed frequently during this period, as the male organ was damaged by heat.

The pollen germination percentage was at a high level in April (Fig. 5.3.B), whereas pistil receptivity was lowest in April, as indicated by suppressed growth of pollen tubes into pistils (Fig. 5.2). The suppression of pollen germination occurred after late June (Fig. 5.3.B), which was earlier than the ovule malformation observed in July (Plate 5.1.C). These sexual differences in seasonal response suggest that the female organ is more sensitive to cool temperatures than is the male organ in cherimoya, although ovules were affected by heat stress later than male organs. A similar trend was reported by Higuchi *et al*. (1998) in which the female organ was less sensitive to heat stress than the male organ under 30/25°C day/night temperature conditions.

Chapter 6

Critical Post-pollination Night Temperatures to Fruit Set

Introduction

The fruit set decline in cherimoya can be ascribed to the direct effects of low night temperatures as well as secondary effects such as nutritional competition between reproductive and vegetative organs; impaired ovule development was observed frequently in spring when vigorous sprouts and subsequent rapid shoot growth coincided (Chapter 5). Low night temperatures like in the spring, i.e. below 14ºC, decreased pollen germinability (Section 1 of Chapter 3); A decline in pollen-tube growth in the pistils below 15ºC was observed in Chapter 4 that may have been also caused by the low night temperatures, although the detailed effects of post-pollination low temperatures on the fertilization and subsequent fruit set are unclear.

In the Section 1 of Chapter 3, the compact temperature control device applying a thermoelectric cooling/heating element using the Peltier effect (Fig. 3.1) enabled detecting the physiological response of male organs to low temperatures separately from the secondary effects on mother trees.

In this chapter, the effects of various night temperatures on fruit set, weight, symmetry, seed formation, and total soluble solid contents were examined together

with the anatomical observations of *in situ* pollen-tube growth and fertilized embryos to determine the critical temperature that negatively affects fertilization.

Materials and Methods

Experiment 1: Post-pollination night temperature effect on the fruit set

Hand-pollinated cherimoya flowers were exposed to various constant air temperatures regulated in a small box during nighttime (1800–0900 h). Eleven 15-year-old 'Big Sister' trees grown in 40-L pots and nineteen 5-year-old trees grown in 15-L pots in a greenhouse at Kyoto University (35.0° N, 135.8° E) were used. The minimum temperature in the greenhouse was maintained at 10ºC throughout the winter. During the summer, an electric fan was automatically operated to maintain air temperatures below 35ºC. The flowering season ranged from April to June in both 2011 and 2012. Hand-pollination was conducted daily. Pollen was collected from flowers at anthesis from 1600–1700 h, followed by pollinating flowers in the female stage (one day before anthesis) using a small paintbrush. Temperature treatment was conducted during the middle season of flowering, when vegetative shoot growth was sufficient and fruit-set percentage of hand-pollinated flowers was almost 100%, to minimize unexpected effects of other factors causing fruit-set decline, e.g. nutritional competition and impaired development of reproductive organs. Swollen ovaries were able to be observed 2 weeks after pollination and were regarded as to be set fruit. Then, fruit thinning was performed to maintain a leaf/fruit ratio > 50. Daily maximum/minimum air temperatures under greenhouse conditions during the experimental period were 19–32/10–22ºC in 2011 and 18–38/10–21ºC in 2012.

The remaining fruit was harvested within 140 days after pollination and weighed immediately. The symmetry of each fruit was evaluated and classified into

five grades: 1 (deformative) to 5 (symmetrical). The typical appearance of fruit from the grade 1 and 5 is shown in Fig. 6.1. After ripening to be softened under room temperature for several days, the total soluble solid content (°Brix) of each fruit was measured using a refractometer, and the number of seeds per fruit was counted.

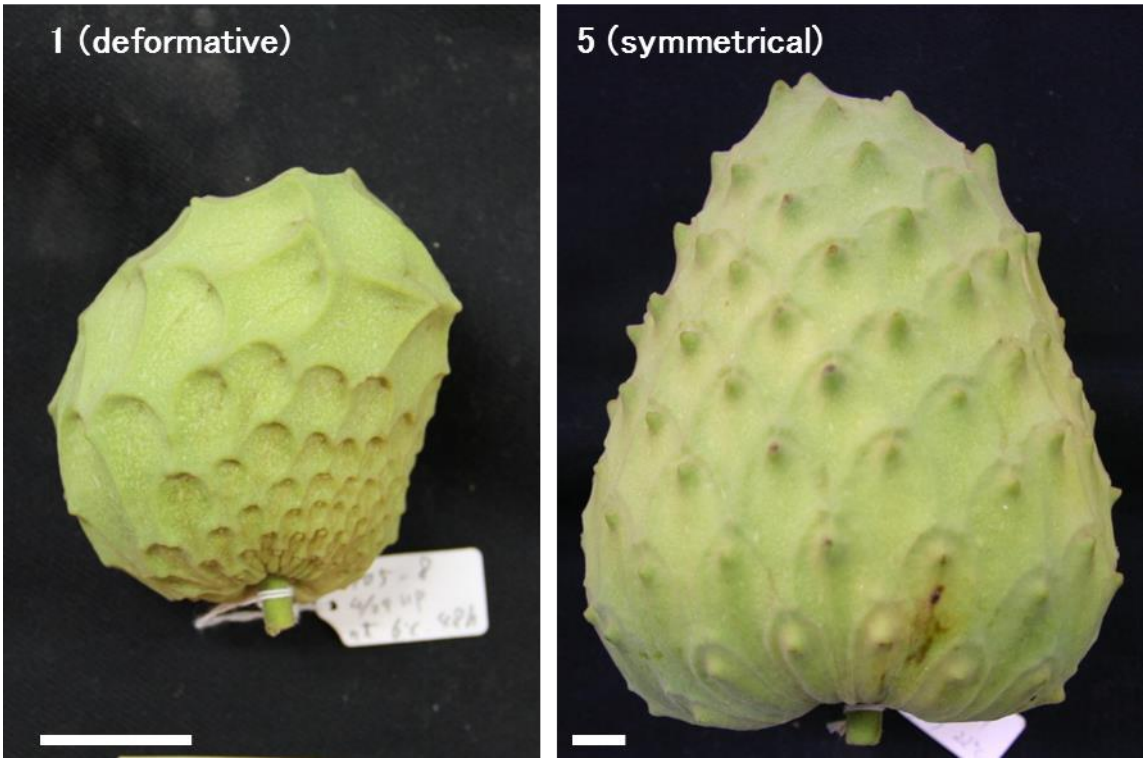

Fig. 6.1 Typical fruit appearance of ‘Big sister’ cherimoya from the symmetry grade 1 (deformative) and 5 (symmetrical). Scale bars indicate 1 cm.

Effects of night temperatures of 12–35ºC

The regulation of post-pollination floral temperatures was started in May–June 2011. Immediately after hand-pollination, a ≈20-L styrene foam box equipped with the temperature regulating units, using a Peltier thermoelectric element, was installed on the tree by strings to enwrap a pollinated flower together with the bearing branch inside the box (*cf*. Fig. 3.1 and 3.2). Air temperature inside the box was regulated at 12, 17, 22, 27, 32, or 35ºC from 1800–0900 h for 15 h, since cherimoya pollen tubes were found to reach the embryo sac within 5–10 h at 18–27ºC in Chapter 4. Fruit set percentage was calculated 2 weeks after pollination, after which some fruit was thinned as described above.

Effects of low night temperatures of 4–14ºC

To investigate the effect of temperatures below 14ºC, the nighttime air temperature surrounding post-pollinated flowers exclusive of the other parts of the tree was regulated in late-April to June 2012. The temperature was regulated constantly at 4, 6, 8, 10, 12, or 14ºC for two nights after pollination, as described above. At the end of the first night regulation (0900 h), the styrene foam box was opened to follow atmospheric temperature fluctuation through the daytime. Then the box was closed in the evening (1800 h) to start the second night regulation. A small data logger (Ondotori Jr. TR52-i, T & D Corp., Japan) monitored changes in temperature inside the box. Although anthesis of the cherimoya flower can be predicted within a few days, the date of anthesis was recorded because the low temperature can postpone anthesis for other tropical species (Kozai *et al*., 2014). The fruit-set percentage was calculated as described above. For comparison, many hand-pollinated flowers were left unregulated as a control.

Experiment 2: Anatomical observation of pistils exposed to post-pollination low night temperatures

To observe pollen-tube growth and embryo sac morphological development at low night temperatures after pollination, the air temperatures surrounding hand-pollinated flowers were regulated at 4, 6, 8, 10, 12, or 14ºC for two nights in the same manner as described in Experiment 1. These flowers were compared with the control flowers, which were exposed to atmospheric temperatures. Within 48 h after pollination, three to four flowers from each treatment were detached and fixed in an FAA solution (37% formaldehyde: acetic acid: 50% ethyl alcohol = 1: 1: 18). Control flowers were also fixed for comparison. These fixed ovaries were rinsed with distilled water and dehydrated in an alcohol series (30, 50, 70, 80, 85, 90, and 100% v/v ethanol and 100% v/v butyl alcohol), followed by embedding in paraffin wax (melting point: 52–54ºC) and sectioning longitudinally at 14 μm using a microtome.

Half of the preparation slides of ovarian serial sections from each flower were used for pollen-tube observation and were stained for 12 h with aniline-blue solution described in Chapter 4. After adding cover glass over these stained sections, pollen tubes in the pistil were observed using a fluorescence microscope (see Chapter 4). The number of embryo sacs in which the whole parts were visible was recorded for each syncarp. The number of embryo sacs that accepted pollen tubes was also recorded. Then, the percentage of embryo sacs into which the pollen tube penetrated was calculated for each treatment.

The other half of the preparation slides were stained with Mayer's acid haemalum solution (Avwioro, 2011) for morphological observation of the embryo sacs. The stained sections were mounted to create permanent preparations as well as in Chapter 2.

Results

Experiment 1: Effects of post-pollination night temperature on the fruit set

Effects of night temperatures of 12–35ºC

Fruit-set percentage at each treatment in the range 12–32ºC was 100% (Fig. 6.2), whereas at 35ºC, the fruit-set percentage was remarkably low. Fruit weight, number of seeds, and symmetry were best at 22ºC. The number of seed formations and symmetry decreased at 12ºC and 32ºC. Soluble solid content (°Brix) did not differ among treatments.

Effects of low night temperatures of 4–14 ºC

All flowers at 10–14ºC set fruit (Fig. 6.3), but some failed to set fruit at temperatures below this range. Flowers rarely set fruit at 4ºC. The number of seeds tended to decrease at lower temperatures of 4–14ºC. The symmetry and weight of the fruit also tended to decrease at lower temperatures. Flowers at 10–14ºC set fruit and formed seeds similarly to the control, although flowers at 12ºC developed smaller fruit than did the control. Similarly to Experiment 1, post-pollination temperatures did not affect °Brix. Flowers at 10–14ºC reached anthesis during 1600–1700 h on the next day after pollination, similarly to the control. However, anthesis of many flowers at 6 ºC and below that was delayed by 1 day and the anthesis occurred 2 days after pollination (Table 6.1). At 6ºC and 8ºC, anthesis of some flowers was delayed for several hours (data not shown).

Experiment 2: Anatomical observation of pistils exposed to post-pollination low night temperatures

Pollen tubes passed the micropyles and penetrated into the embryo sac within 48 h after pollination in all treatments ranging from 4–14ºC (Plate 6.1.A). At 8 ºC and below that, the percentage of embryo sacs that accepted pollen tubes was

significantly lower than that of the control (Table 6.2). A significant decrease in the percentage was observed at 6 ºC and below that, at these temperatures the pollen tubes scarcely reached the embryo sacs (Plate 6.1.B). At 10–14ºC, the percentages were as high as those of the control.

On the contrary, morphological development of the embryo sac was not affected by temperature; double-fertilized embryo sacs endowed with zygote nucleus in the micropylar side and endosperm nucleus in the center (Plate 6.1.C) were observed within 48 h after pollination under any condition at 4–14ºC, similarly to the control. A degenerated synergid was observed adjacent to the zygote nucleus in the micropylar side of the double-fertilized embryo sac, and pollen tubes were observed between the micropyle and nucellus. In ovules that had not been penetrated by a pollen tube, an egg cell nucleus and two polar nuclei were observed in the embryo sacs (Plate 6.1.D).

Table 6.1 Occurrence of one-day postponement of anthesis of 'Big Sister' cherimoya under low nighttime (1800–0900 h) temperatures

Nighttime temperature condition (ºC)	Percentage of flowers postponed anthesis day (%)		Number of flowers observed
Control (12–20)	0.0		82
14	0.0		12
12	0.0		14
10	0.0		18
8	5.6		18
6	37.5	**	16
4	100.0	**	7

Control was the open condition with no regulation of temperature.
**: statistical significance compared with the control based on Fisher's exact test at $P < 0.01$.

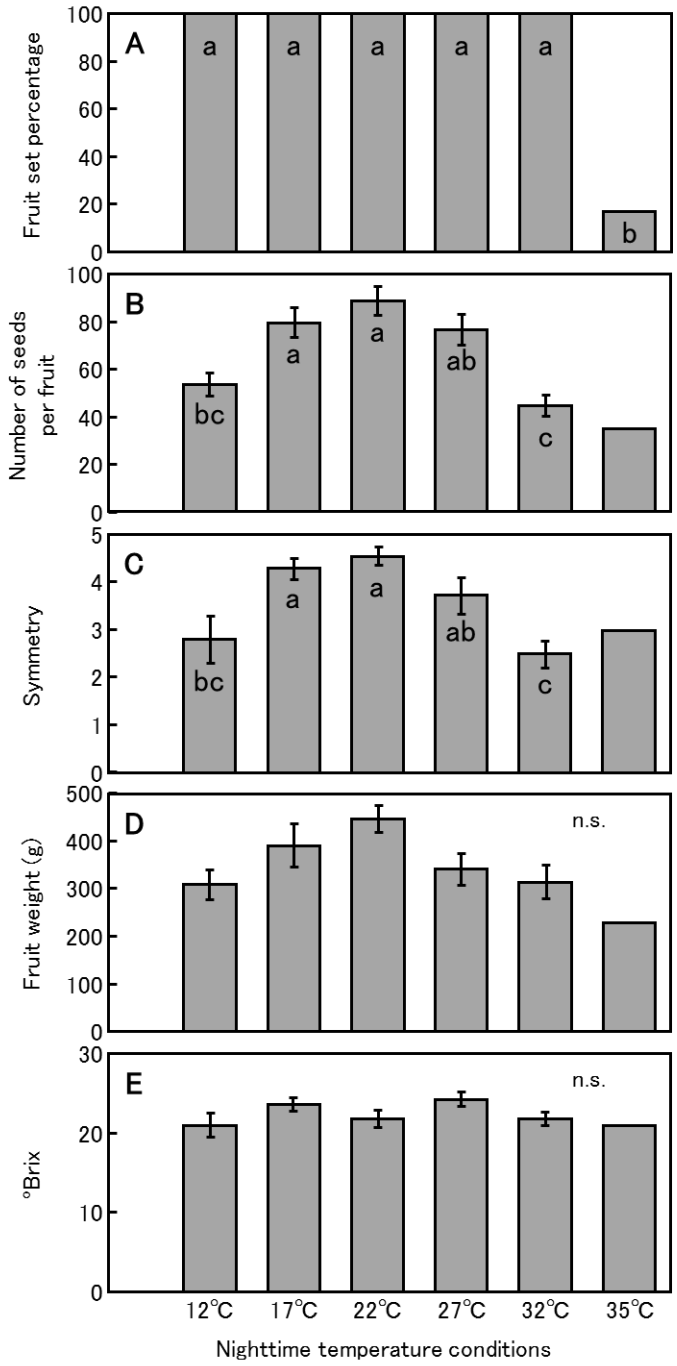

Fig. 6.2 Effect of post-pollination nighttime (1800–0900 h) temperatures ranging from 12 to 35ºC on the fruit-set percentage, number of seeds, symmetry, fruit weight, and soluble solid content (°Brix) of 'Big Sister' cherimoya. The different letters indicate statistical differences based on the Fisher's exact test (A) and Tukey's test (B–E) at $P < 0.05$. n.s.: not significant. Treatment comparisons at 35ºC could not be performed in B–E because the number of obtained fruit was one. Vertical bars indicate standard errors.

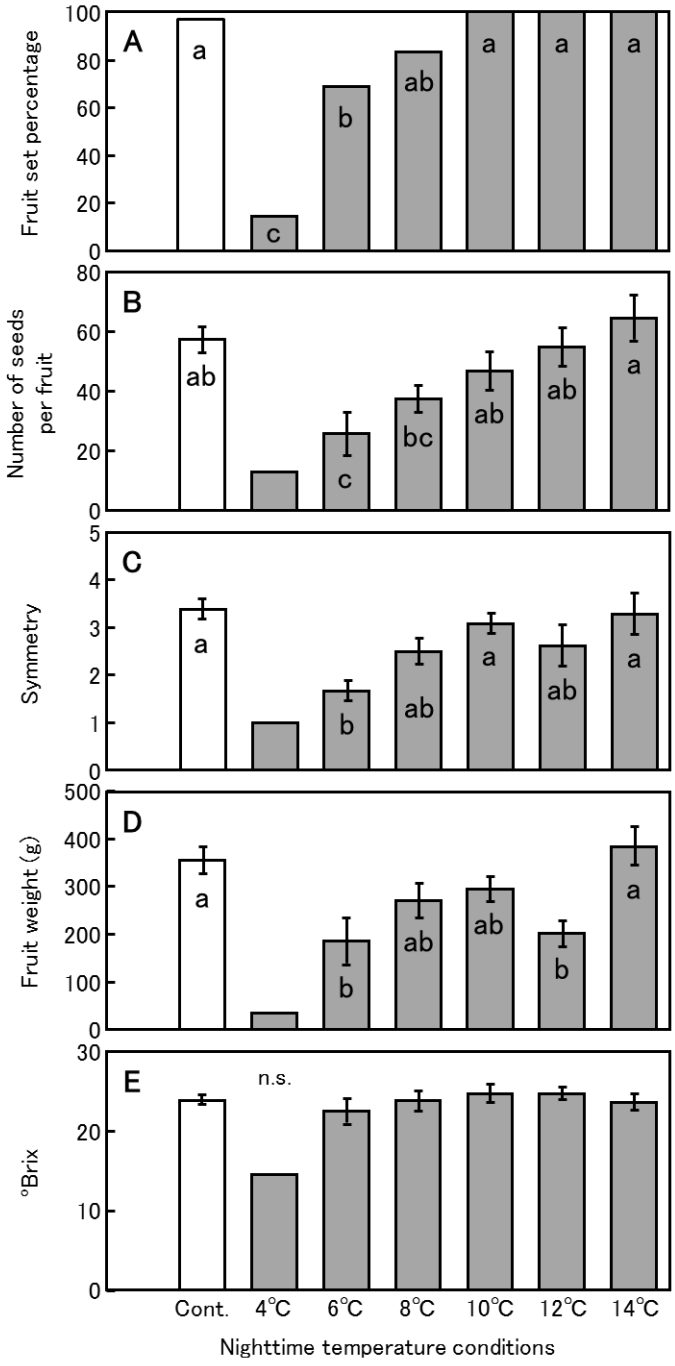

Fig. 6.3 Effect of post-pollination nighttime (1800–0900 h) temperatures below 14ºC on the fruit-set percentage, number of seeds, symmetry, fruit weight, and soluble solid content (°Brix) of 'Big Sister' cherimoya. Control (Cont.) represents the unregulated atmospheric temperature conditions inside the greenhouse, the minimum of which ranged from 12–20ºC. Different letters indicate statistical differences based on Fisher's exact test (A) and Tukey's test (B–E) at $P < 0.05$. n.s.: not significant. Treatment comparisons at 4ºC could not be performed in B–E because the number of obtained fruit was one. Vertical bars indicate standard errors.

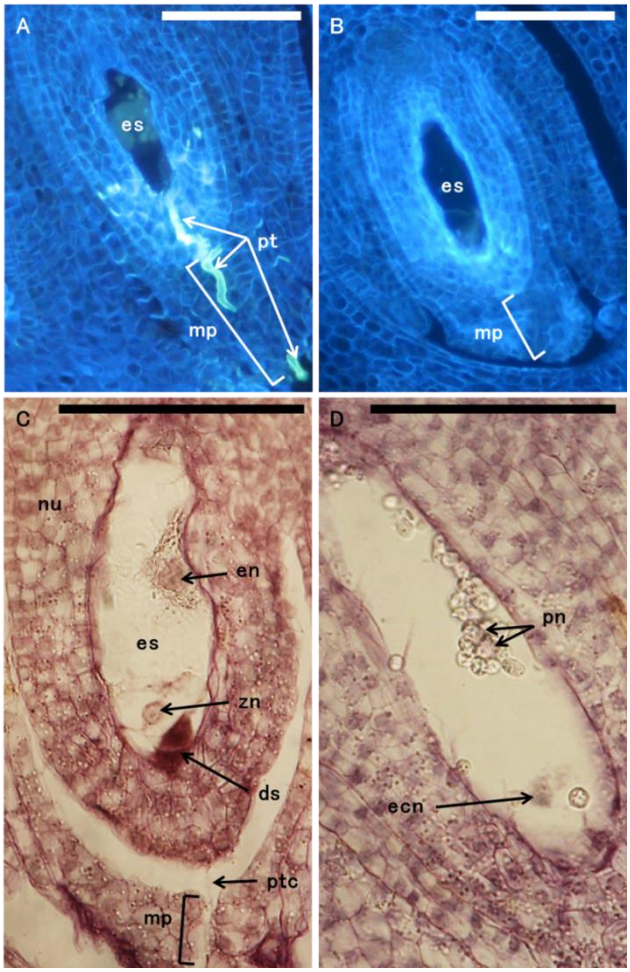

Plate 6.1 Fluorescence (A and B) and optical (C and D) microscopic observations of ‘Big Sister’ cherimoya ovules exposed to regulated post-pollination nighttime (1800–0900 h) temperatures ranging from 4–14ºC or left unregulated. A: the embryo sac (es) attained by pollen tubes (pt) 48 h after pollination, which was observed in flowers under each temperature condition. The pollen tube passed through the micropyle (mp). B: the embryo sac (es) that was not attained by the pollen tube (pt) 48 h after pollination, which was observed mainly in flowers under nighttime temperature conditions of 4–6ºC. C: the embryo sac (es) after completing double fertilization within 48 h after pollination under each condition. The endosperm nucleus (en) was observed in the center of the embryo sac (es), while the zygote nucleus (zn) and degenerated synergid (ds) were observed on the micropylar (mp) side. Pollen-tube cell wall (ptc) was observed between the micropyle (mp) and nucellus (nu). D: the embryo sac (es) in which the ovule was not penetrated by pollen tubes. Inside the embryo sac (es), two polar nuclei (pn) and one egg cell nucleus (ecn) were observed. Scale bars indicate 50 µm.

Table 6.2 Effect of low nighttime (1800–0900 h) temperatures on pollen-tube penetration into embryo sac within 48 h after pollination in 'Big Sister' cherimoya

Nighttime temperature condition (ºC)	Percentage of embryo sac that penetrated by pollen tube (%)		Number of embryo sacs (syncarp) observed	
Control (12–20)	67.5		83	(4)
14	67.4		86	(4)
12	59.0		83	(4)
10	63.6		88	(4)
8	42.5	**	87	(4)
6	10.4	**	77	(4)
4	15.8	**	76	(3)

Control was the open condition with no regulation of temperature.
**: statistical significance compared with the control based on Fisher's exact test at $P < 0.01$.

Discussion

Fruit set decreased at cool nighttime temperatures below 8ºC, while 100% of the fruit set was observed at temperatures above 10ºC (Fig. 6.3). Similarly in avocado, Gillespie (1956) observed a frequent drop of seedless fruit when night temperatures decreased below 8.3ºC.

Higher nighttime temperatures over 32ºC inhibited fruit setting (Fig. 6.2), and fruit rarely set at 35ºC. Under these temperatures, rapid respiration can cause exhaustion (Higuchi *et al.*, 1999), and that may inhibit pollen-tube growth or embryonic development after fertilization. Fertilization was inhibited by a temperature of 30ºC in the olive (Cuevas *et al.*, 1994) and by 28ºC in the avocado (Sedgley and Annells, 1981) and purple passion fruit (Utsunomiya, 1992).

The detrimental temperature range below 8ºC in the present results may seem to be lower than the growers' empirical knowledge from orchards, although the present results may also seem to indicate higher detrimental temperatures of over

32ºC than their knowledge. This can be ascribed to the sectional temperature control of target flower only: mother trees were excluded from the present regulation of temperatures. The present sectional temperature control enabled accurate resolution of the post-pollination night temperature effect over a wide range (4–35ºC) on particular physiological response of cherimoya reproductive organs, which might never be detected by analyzing field condition data only.

In this study, both the percentages of fruit set (Fig. 6.3) and of pollen-tube penetration into embryo sacs (Table 6.2) decreased as nighttime temperatures decreased from 14 to 4ºC. According to Chapter 4, cherimoya pollen-tube growth in the pistil was inhibited below 15ºC. Thus, pollen tubes were thought to elongate slowly or diapause at 4–14ºC during the night.

A cherimoya flower shows protogynous dichogamy (Schroeder, 1941) and can set fruit if it is pollinated several hours prior to anther dehiscence (Koura *et al.*, 2001), indicating that the pistils remain receptive and that pollen tubes in the pistil are still able to elongate until at least around noon on the next day after pollination. In the morning and under increasing temperatures, diapausing pollen tubes were thought to resume elongation and fertilize the embryo sacs.

The next day after pollination in the present experiment, pollen tubes were supposed to be attainable to the embryo sacs within the daytime, because pollen tubes reached the embryo sac within 5–10 h after pollination at 18–27ºC (Chapter 4).

At nighttime temperatures below 6ºC, anthesis was often postponed (Table 6.1) and pollen-tube growth was largely inhibited (Table 6.2), even though some embryo sacs were fertilized (Plate 6.1). The postponed anthesis and inhibited pollen-tube growth may be attributed to the decreased development of reproductive organs caused by low temperatures. In Chapter 5, the cherimoya fruit set was low during a season when daily minimum temperatures decreased below 10ºC. During such a cool period, impaired ovule development was common. The low fruit set was ascribed to the low receptivity of the pistil grown at low temperatures. Accordingly,

not only pollen tube growth but also embryonic development after fertilization are likely inhibited below 6ºC. In the avocado, a reduction of pistil function was reported below 7ºC (Inoue and Takahashi, 1990), and in mango, impaired embryonic development was induced by decreasing night temperatures down to 10 ºC 3 days after pollination (Sukhvibul *et al*., 2000). Cherimoya showed a similar thermal response in the avocado rather than mango.

Many large and symmetrical fruits were obtained at 17–27ºC nighttime temperatures (Fig. 6.2). This temperature range promoted pollen-tube growth and allowed ovule fertilization. These results support those in Chapter 4, in which cherimoya pollen-tube growth in the pistil was inhibited below 15ºC and over 30ºC.

Chapter 7

Conclusions

Recently, cherimoya has become relatively well known in Japan, with the domestic demand expected to increase further due to its high fruit quality, particularly its excellent flavor. Supply presently depends on importation from abroad; however, thanks to its delicate post-harvest ripening and storage characteristics, ripening disorder often occurs following long shipping, resulting in low-quality fruit. With the increase in domestic demand, high-quality fruits are therefore desired, and thus, the market is expected to demand an increase in domestic products to provide a steady supply.

In the 1990s, cherimoya remained a rare fruit and establishment of practical cultivation methods had mainly been examined in traditional cultivation areas such as California, Spain, and locations close to its origin. As a result of recent globalization, however, cherimoya has grown in popularity, with a further rise expected in the future. This demand is likely to continue worldwide, along with the introduction of cultivation methods for new crops. Cherimoya is very sensitive to both high and low temperatures, with the favorable zone in which cultivation

requires no artificial protection being highly restricted. This acts as a barrier to its introduction into temperate zones such as Japan. Temperature management is therefore essential, especially during the early flowering period in cool seasons. However, thermal responses of the reproductive organs have been examined only partly at higher temperatures. For suitable temperature management, the critical low temperature ranges resulting in deterioration of the reproductive organs, pollination, and fruit set must also be clarified as well as the optimal ranges.

In the present study, following observations of reproductive organ morphogenesis in Chapter 2, the thermal response of the reproductive organs was investigated by sectional temperature regulation and anatomical observations using 'Big Sister', a major cultivar from California. The critical temperature range resulting in a decline in pollen vigor, pollen-tube growth, fertilization, and fruit set were subsequently determined. The results obtained are considered similarly applicable to many other cultivars. The results presented in Chapters 3–6 are summarized and recommendations for a suitable temperature management protocol during the flowering season are as follows.

The present results indicate that minimum night temperatures lower than 10ºC may negatively affect the development of both male (Section 1 of Chapter 3) and female (Chapter 5) organs: Irregular anthesis occurred and ovule malformation was often observed. In Section 1 of Chapter 3, even 1 night prior to anthesis, temperatures below 14ºC were found to decrease pollen vigor. Daytime temperatures of 20ºC at >80% relative humidity were found to be favorable for anthesis and abrogated the subsequent reduction in germinability, as shown in Section 2 of Chapter 3. Pistil receptivity was highest when minimum temperatures ranged from 15–25ºC (Chapter 5). Pollen tubes were found to elongate well under temperature conditions from 20–27ºC, reaching the embryo sac within 5–10 h, while tube elongation was inhibited below 15ºC as shown in Chapter 4. Post-pollination night temperatures below 8ºC remarkably inhibited pollen-tube growth, resulting in a

decrease in fertilization and subsequent fruit set (Chapter 6).

The higher detrimental range of night temperatures was over 25ºC for both male and female development. Meanwhile, daytime temperatures over 30ºC negatively affected anthesis and resulted in a decrease in pollen vigor. Pollen-tube growth and fruit set also decreased over 30ºC, while temperatures higher than 32ºC appear to be lethal for the reproductive organs.

Recommended temperature management during the flowering season to prevent fruit-set failure is as follows. Daily minimum temperatures must be maintained at 10ºC by heating, with maximum temperatures kept below 30ºC. Humidification, fog cooling, mist irrigation and so on seem effective in controlling temperature, with the pollen preferring high humidity conditions. Especially during the night prior to pollination in cool seasons, minimum temperatures should be maintained at 15ºC by heating to mitigate any reductions in pollen vigor. On the day of pollination, to ensure collection and application of vigorous pollen, the daytime temperature and humidity should be maintained at around 20ºC with >80% RH. Daytime temperatures of 20ºC also induce more flowers to open simultaneously in the early hours, thus enabling hand-pollination of a greater number of flowers compared to late afternoon anthesis. This should help result in reliable fruit set and high productivity. If economically feasible, to promote pollen-tube growth and fertilization during cool seasons, the minimum night temperature just after pollination should be kept at 17ºC by heating and the following daytime temperature at over 20ºC. Pollination work should be ceased when minimum night temperatures exceed 25ºC or maximum day temperatures increase above 32ºC.

The above temperature management protocol could help avoid fruit-set failure during cool seasons in warm-temperate zones. Abrogation of fruit-set failure caused by low temperatures will remove this cultivation barrier, allowing the introduction of cherimoya into new areas.

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