

Short communication

Low temperature storage and in vitro germination of cherimoya (*Annona cherimola* Mill.) pollen

J. Lora, M.A. Pérez de Oteyza, P. Fuentetaja, J.I. Hormaza*

E.E. la Mayora-CSIC, Algarrobo-Costa, Málaga 29750, Spain

Received 25 October 2004; received in revised form 11 November 2005; accepted 9 December 2005

Abstract

Due to the protogynous dichogamy of cherimoya and to the absence of proper pollinating vectors, hand-pollination with fresh pollen is a common practice for cherimoya commercial production. In order to optimize the process of hand-pollination, in this work we have studied the conservation of cherimoya pollen at -20 , -80 and -196 °C for up to 3 months. In vitro pollen germination of fresh pollen was 57.1% and it was progressively reduced with conservation time at the three temperatures studied reaching a minimum after 3 months of storage of 10.4%, 14.2% and 13.6% at -20 , -80 and -196 °C, respectively. Differences in germination among temperatures were only significant during the first 2 weeks of storage. Field pollinations with pollen stored for up to 3 months at the three temperatures show no yield differences compared to pollinations performed with fresh pollen. The results indicate that pollen collected and stored at sub-zero temperatures at the beginning of the cherimoya blooming season can be used along the whole blooming season avoiding the need of collecting fresh pollen daily.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Annonaceae; Cryopreservation; In vitro pollen germination; Pollen viability

1. Introduction

Cherimoya (*Annona cherimola* Mill.) belongs to the Annonaceae, one of the largest tropical and subtropical families of trees, shrubs and lianas that include about 130 genera and 2300 species with a worldwide distribution, 900 of which are found in the Neotropics (Chatrou et al., 2004). Three genera (*Annona*, *Rollinia* and *Asimina*) contain species with edible fruits, such as cherimoya (*A. cherimola*), sugar apple (*A. squamosa*), atemoya (a hybrid between *A. cherimola* and *A. squamosa*), soursop (*A. muricata*), custard apple (*A. reticulata*), ilama (*A. macrophyllata*), soncoya (*A. purpurea*), rollinia (*Rollinia mucosa*) or pawpaw (*Asimina triloba*). Cherimoya and atemoya are the most important worldwide both commercially and for local consumption. Cherimoya seems to be originated in the interandean valleys of southern Ecuador and northern Peru (Van Damme et al., 2000) although Spain is currently the major world producer of cherimoyas with about 3000 ha followed by Chile with about 1000 ha. Cherimoyas are also produced at a limited commercial scale in other Latin

American countries where most of the cherimoya fruits sold or consumed are collected from the wild or from backyard trees (Van Damme et al., 2000).

Cherimoya has hermaphroditic flowers with a gynoecium and an androecium of pyramidal form surrounded by two whorls of three petals, the inner small and scale-like and the outer green-like and fleshy. The gynoecium is located in the centre of the conic receptacle and is composed of up to 300 carpels, each carpel containing a single ovule that will form a syncarp after fertilization. The androecium is found below the gynoecium forming a helicoidal structure with up to 200 stamens. Cherimoya presents protogynous dichogamy, with hermaphroditic flowers where female and male structures do not mature simultaneously generally preventing self-fertilization in the same flower (Schroeder, 1971). Moreover, flowers of the same genotype are synchronized and, consequently, transfer of pollen between different flowers of the same genotype is also prevented. At anthesis, usually at midday under our environmental conditions, the flowers are in female stage with receptive stigmas. The following day the flower switches to the male stage around 4–5 p.m., depending on environmental conditions, when the anthers start to dehisce and the stigmas are no longer receptive (Farré et al., 1976). Due to the lack of overlap between male and female stages in cherimoya and the

* Corresponding author. Tel.: +34 952552656; fax: +34 952552677.

E-mail address: ihormaza@eelm.csic.es (J.I. Hormaza).

lack of proper pollinating agents outside its native range, hand pollination with pollen and stamens together is a common practice for commercial production (Schroeder, 1971; Soria et al., 1990).

The cherimoya blooming season in Spain starts in late May and lasts through July whereas the regular harvesting season extends from October to December. However, under appropriate cultural practices (mainly pruning before the blooming season), it is possible to induce flowering in August and early September; the fruits set by these flowers are harvested in March–April. Thus, in order to optimize the pollination for both fall and spring harvesting it could be interesting to conserve pollen collected during June for pollination in June, July and August. Cherimoya pollen is tricellular and is dispersed from the anthers in tetrads. The individual pollen grains are released after contact with a proper germination medium (Rosell et al., 1999). Similarly to other tricellular pollen (Brewbaker, 1967; Hanna and Towill, 1995; Yamaguchi and Ishikawa, 2000; Towill and Walters, 2000), cherimoya pollen has a short viability and a high sensitivity to desiccation and, consequently, conservation is problematic. In this sense, no previous reports are available on the conservation of cherimoya pollen at sub-zero temperatures. Thus, the main objective of this work is to develop a method for mid-term storage of cherimoya pollen (up to 3 months) comparing different sub-zero temperatures in order to optimize the current process of hand-pollination in this species.

2. Materials and methods

Pollen from the cherimoya cultivar Campas was used in this work. Flowers in female stage were collected from the field at 4:00 p.m., approximately half an hour before they switch to the male stage, and carried to the laboratory. Immediately after anther dehiscence, pollen was subjected to different treatments. A first experiment consisted in evaluating pollen longevity at 4 °C; for this, pollen was stored at 4 °C and in vitro pollen germination evaluated daily as described below. The other experiments involved desiccation of pollen prior to the storage.

In order to optimize desiccation time we first checked the effect of four different moisture contents (obtained by increasing the desiccation time) at a fixed storage temperature (−20 °C). Afterwards, dehisced anthers with pollen from 25 flowers were dehydrated on glass Petri dishes in a silica gel desiccator during 90 min since previous observations showed a decrease in pollen germination after longer desiccation times. The moisture content of the pollen was determined by drying the pollen in an oven at 85 °C for 2 days. After the desiccation step, pollen was placed in 1.5 mL cryovials and stored at −20,

−80 and −196 °C in liquid nitrogen. Pollen was thawed at 1, 7, 15, 30, 60 and 90 days after storage by keeping the samples at room temperature for 5 min. After thawing pollen was hydrated in a covered tray with wet filter paper for 200 min at room temperature.

For in vitro pollen germination, approximately 0.02 g of pollen with stigmas (Rosell et al., 1999) were placed on a 35 mm Petri dish with 1–2 mL of liquid germination medium at room temperature. A slightly modified pollen germination medium based on those described by Rosell et al. (1999) was used. This medium consisted of 8% sucrose, 200 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 250 mg/L $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 100 mg/L KNO_3 and 100 mg/L H_3BO_3 . Pollen germination was observed under a Leica DML microscope. Data were collected on four Petri dishes with at least 200 pollen grains in each one. Pollen was considered as germinated when the length of the tube was longer than the grain diameter. Data were subject to arcsine root square transformation and an ANOVA analysis was carried out. Duncan's multiple range tests were used for means separation ($p \leq 0.05$). Statistical analyses were performed with SPSS 12.0. statistical software (SPSS Inc. Chicago, USA).

Field pollinations with fresh pollen and pollen stored at sub-zero temperatures were performed by applying the pollen with the anthers with a paintbrush on the stigmas just before flower opening; the pollinated flowers were covered by a cotton plug to avoid additional pollination with unwanted pollen.

3. Results and discussion

Pollen germination of freshly collected pollen was 57.1% (Table 1) a similar value to tricellular pollen from other species such as *Zea mays* (Inagaki, 2000) or *Brassica campestris* (Mulcahy and Mulcahy, 1988). However, this is a higher value than that reported previously for cherimoya (Rosell et al., 1999) probably due to the fact that in the later work a storage step during 20 h at 7–8 °C is performed before germination. Our results indicate that fresh cherimoya pollen stored at 4 °C showed a short longevity and no in vitro pollen germination was obtained 6 days after the pollen is released from the anthers (Table 1).

The moisture content of pollen after desiccation for 90 min was 49.7% compared to 85.7% of fresh pollen just after anther dehiscence. Although this can be considered a high moisture content for pollen storage compared to the results in other species (Hanna and Towill, 1995), tricellular pollen shows a high metabolic activity and moisture content compared to bicellular pollen (Barnabás and Kovács, 1997) and, consequently, a common feature of tricellular pollen is that desiccation below some critical level can induce injury.

Table 1
Cherimoya pollen germination (mean \pm S.D.) after storage at 4 °C during six consecutive days

	Time (days)						
	0	1	2	3	4	5	6
% Pollen germination	57.1 \pm 11.5 a	42.7 \pm 12.4 b	39.4 \pm 10.9 b	20.1 \pm 13.9 c	11.7 \pm 6.3 cd	4.8 \pm 2.8 d	0 e

Means followed by the same letter in a row are not significantly different ($p \leq 0.05$) by Duncan's multiple range test.

Table 2

Cherimoya pollen germination (mean \pm S.D.) of different moisture contents stored during 7, 15, 30 and 60 days at -20°C

Moisture content (%)	Time (days)			
	7	15	30	60
49.7	37.3 \pm 3.4 a	31.1 \pm 8.4 a	23.2 \pm 7.6 a	20.1 \pm 7.1 a
29.1	17.6 \pm 3.4 b	23.2 \pm 3.1 a	19.5 \pm 4.9 ab	11.6 \pm 5.9 ab
15.4	8.7 \pm 0.8 c	6.8 \pm 1.1 b	11.4 \pm 2.4 b	15.7 \pm 3.1 ab
11.9	13.4 \pm 3.4 bc	12.1 \pm 0.2 b	7.7 \pm 6.8 b	9.2 \pm 3.2 b

Means followed by the same letter in a column are not significantly different ($p \leq 0.05$) by Duncan's multiple range test.

Table 3

Cherimoya pollen germination (mean \pm S.D.) after storage up to 3 months at -20 , -80 and -196°C

Temperature ($^{\circ}\text{C}$)	Days of storage					
	1	7	15	30	60	90
-196	51.5 \pm 3.4 a	40.3 \pm 6.6 a	35.5 \pm 0.9 a	24.1 \pm 8.0 a	25.2 \pm 4.5 a	13.6 \pm 6.0 a
-80	30.8 \pm 2.6 b	23.5 \pm 6.4 b	26.7 \pm 9.7 a	23.1 \pm 3.1 a	15.7 \pm 5.6 a	14.2 \pm 3.2 a
-20	28.6 \pm 4.3 b	37.3 \pm 3.4 a	31.1 \pm 8.4 a	23.2 \pm 7.6 a	20.1 \pm 7.1 a	10.4 \pm 0.5 a

Means followed by the same letter in a column are not significantly different ($p \leq 0.05$) by Duncan's multiple range test.

We have observed this effect in cherimoya where desiccation below 50% resulted in lower germination percentages after storage (Table 2). Thus, a compromise has to be reached since exposure of pollen with high water content to low temperatures can also lead to intracellular ice formation, cell death and loss of germination (Hanna and Towill, 1995).

Results of pollen germination after 1 day of storage at -20 , -80 and -196°C showed a significant higher germination of pollen stored at -196°C with 51.5% germination; no significant differences were observed between pollen stored at -20 and -80°C with 28.6% and 30.8% germination respectively (Table 3). Differences in germination are reduced with storage time and, thus, although significant differences are maintained after 7 days of storage, no significant differences in germination percentages were recorded among the three different treatments at 15, 30, 60 and 90 days. This is somehow unexpected since usually the decline in pollen germination is much faster at -20°C than at -80 or -196°C (Hanna and Towill, 1995). Consequently, although pollen conservation at -196°C can be interesting for long-term storage such as conservation of genetic resources, for hand-pollination both for commercial fruit production and breeding, pollen can be efficiently stored at -20°C .

This is the first report on storage of cherimoya pollen at low temperatures. Although a decrease in pollen germination was recorded along with conservation time, field pollinations with pollen stored for up to 3 months at the three temperatures showed no yield differences compared to pollinations performed with fresh pollen (around 60% of the flowers produced fruits in both cases). This is not unexpected since pollination is made by hand and, consequently, a high pollen/ovule ratio is applied in the pollinated carpels. Moreover, the cherimoya flower can have about 300 carpels and the fertilization of less than 1/4 of those is sufficient for the development of fruits of commercial value (the average seed index is around 10% both with fresh pollen and pollen stored at

low temperatures). In Spain, the cherimoya blooming season lasts from early June to the end of July and with appropriate cultural practices blooming can also be induced during August and early September. Consequently, pollen collected and stored at sub-zero temperatures in June can be used for field pollinations along the whole cherimoya blooming season avoiding the need of collecting pollen daily to perform hand-pollinations the following day.

Acknowledgements

We gratefully acknowledge J.M. Farré, J.M. Hermoso, and E. Guirado for valuable information on cherimoya production. Financial support for this work was provided by MCYT (Project Grant AGL2003-05318-C02-01).

References

- Barnabás, B., Kovács, G., 1997. Storage of pollen. In: Shivanna, K.R., Sawhney, V.K. (Eds.), *Pollen Biotechnology for Crop Production and Improvement*. Cambridge University Press.
- Brewbaker, J.L., 1967. The distribution and phylogenetic significance of binucleate and trinucleate pollen grains in the angiosperms. *Am. J. Bot.* 54, 1069–1083.
- Chatrou, L.W., Rainer, H., Maas, P.J.M., 2004. Annonaceae. In: Smith, N., Mori, S.A., Henderson, A., Stevenson, D.W., Heald, S.V. (Eds.), *Flowering Plants of the Neotropics*. Princeton, New Jersey, USA.
- Farré, J.M., Hermoso, J.M., González, M.A., 1976. Ensayos sobre polinización, cuajado y crecimiento del fruto en chirimoya. *Anales INIA/Serie Producción Vegetal* 6, 63–92.
- Hanna, W.W., Towill, L.E., 1995. Long-term pollen storage. *Plant Breed. Rev.* 13, 179–207.
- Inagaki, M., 2000. Use of stored pollen for wide crosses in wheat haploid production. In: Engelmann, F., Tagako, H. (Eds.), *Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Applications*. International Plant Genetic Resources Institute, Rome, Italy, pp. 130–135.
- Mulcahy, G.B., Mulcahy, D.L., 1988. The effect of supplemented media on the growth in vitro of bi- and trinucleate pollen. *Plant Sci.* 55, 213–216.

- Rosell, P., Herrero, M., Galan-Sauco, V., 1999. Pollen germination of cherimoya (*Annona cherimola* Mill.) In vivo characterization and optimization of in vitro germination. *Sci. Hort.* 81, 251–265.
- Schroeder, C.A., 1971. Pollination of cherimoya. *California Avocado Society Yearbook*, vol. 44. pp. 119–122.
- Soria, J.T., Hermoso, J.M., Farré, J.M., 1990. Polinización artificial del chirimoyo. *Fruticultura Profesional* 35, 15–22.
- Towill, L.E., Walters, C., 2000. Cryopreservation of pollen. In: Engelmann, F., Tagako, H. (Eds.), *Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Applications*. International Plant Genetic Resources Institute, Rome, Italy, pp. 115–129.
- Van Damme, P., Van Damme, V., Scheldeman, X., 2000. Ecology and cropping of cherimoya (*Annona cherimola* Mill.) in Latin America. New data from Ecuador. *Fruits* 55, 195–206.
- Yamaguchi, E., Ishikawa, M., 2000. Cryopreservation of maize (*Zea mays* L.) pollen. In: Engelmann, F., Tagako, H. (Eds.), *Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Applications*. International Plant Genetic Resources Institute, Rome, Italy, pp. 369–370.