Cryopreservation of Peach and Nectarine Pollen Grains

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Abstract

The objective of this research was to investigate the conservation of pollen grains of peach and nectarine under different storage environments. Pollen of 'Aurora 1', 'Douradão', 'Centenária' and 'Colombina' were stored in cryotubes and conserved under four environments: ambient temperature (25°C), refrigerator (5°C), freezer (-12°C) and liquid nitrogen (-196°C, cryoconservation) for a 300 day period. After that a second field experiment was set up to test the viability of pollen grains. This was done through controlled crosses using the pollen grains from the previous conservation evaluation. Only pollen from refrigerator (5°C), freezer (-12°C) and liquid nitrogen (-196°C) storage were used because these were the only methods that conserved pollen viability. The liquid nitrogen (-196°C) was the best method to conserve the pollen grains of all the tested cultivars. Higher viability of pollen and on field fruit set percentage was obtained with the pollen conserved in liquid nitrogen (-196°C) for all cultivars. The highest percentage of field fruit set was obtained with 'Aurora 1' pollen conserved in liquid nitrogen (-196°C).

INTRODUCTION

Pollination and fertilization are essential for fruit set in peach. In its absence, fruit abscise since peaches do not naturally have the phenomena of parthenocarpy and apomixis (Barbosa et al., 1991).

In general, genetic improvement of peach and nectarine is made through conventional breeding methods. Thus, the development of new cultivars is obtained through controlled crosses that are made in the field. Controlled pollinations of peach and nectarine require reasonable available quantities of viable pollen. There is also a need to collect pollen grains at different times and store them for use at another time. However, this is not always possible due to rapid loss of pollen viability caused by improper storage

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(Barbosa et al., 1991). Hanna (1994) mentioned also that the storage of pollen grains for a period exceeding one year is important for the germplasm preservation, development of research on pollen, germplasm exchange and for the efficiency of genetic improvement programs.

The success of preservation of pollen independent of storage time, mainly depends on factors such as temperature and the storage environment, among others (Linsken, 1964; Dean, 1965; Khan et al., 1791; Gomes et al., 2003). The use of low temperatures usually is linked to reduction of pollen metabolism, which provides greater longevity. This can be achieved by reducing storage temperature using refrigerators and freezers which are easy to access but other more sophisticated methods are also used such as cryopreservation. This method is defined as the conservation of biological material in liquid nitrogen at -196°C, or in its vapor phase at -150°C (Kartha, 1985). The decision as to which method is used depends on the storage purposes and the characteristic of the species being preserved (Pio, 2003).

Several studies have been conducted to establish and/or standardize the conditions of storage or preservation of pollen grains. Oliveira Junior et al. (1995) stored peach pollen of 'Aurora 1' at room temperature for 15 and 30 days and had unsatisfactory results with 1.81% germination after 30 days of storage. Eenik (1983) stored lettuce pollen at room temperature (20°C) and in a refrigerator (4°C) and observed loss of viability after 2 days at room temperature. However, at cooler temperatures this loss occurred after 4 days, indicating that low temperatures are responsible for maintaining the viability of pollen for a prolonged period of time. Barbosa et al. (1991) working with conservation of pollen from low-chill cultivars, observed high rates of germination (70%) until the third day when stored at room temperature (25°C). After that there was significant loss of viability for the cultivars of peaches and nectarines studied. The same authors found that 60 days of storage in a desiccator at 1°C maintained adequate viability of pollen grains. Griggs et al. (1953) obtained success in the conservation of temperate tree pollen grains up to 3 years at -18°C. Recent studies have suggested that pollen from different fruit species can be stored for indefinite periods at -196°C (Lee et al., 1985; Gomes et al., 2003).

In this context, we tested different storage environments for the preservation of pollen grains of peach and nectarine.

MATERIALS AND METHODS

Pollen Grain Collection

Pollen grains were used from anthers from flower buds of peach cultivars 'Douradão' and 'Aurora I' and the 'Colombina' and 'Centenária' cultivars of nectarine trees collected in the "balloon" stage. These anthers were stored in petri dishes and incubated in a growth room at 27±1°C, with a photoperiod of 16 hours and irradiance of 32 $\mu mol.m^{-2}.s^{-1}$ for approximately 24 hours to occur anthesis.

Pollen Grain Conservation

After anther dehiscence the pollen was packaged in cryotubes and stored in four environments: laboratory at 25°C; in a refrigerator (5°C); freezer (-12°C); and in liquid nitrogen (-196°C). Using a small brush the pollen grains were placed on Petri dishes containing 20 ml of culture medium, consisting of 60 g L^{-1} of sucrose, 10 g L^{-1} of agar, 400 mg L^{-1} of boric acid and pH was adjusted to 6.5. Then the Petri dishes of all treatments were kept in a growth room at 25°C, constant photoperiod and irradiance of 32 $\mu mol.m^{-1}.s^{-1}$. After 12 hours the percentage of germinated pollen grains was evaluated with the aid of binocular microscope with objectives of 10 X. Seven evaluations were performed at 0, 10, 20, 30, 90, 180 and 300 days after storage.

Viability Test of Pollen Grains in the Field

This test was achieved by using controlled crosses with the pollen grains from the

storage test, in other words, the pollen after 300 days of storage in the refrigerator (5°C), freezer (-12°C) and liquid nitrogen (-196°C) environments.

Statistical Analysis

The experiments were conducted in a factorial arrangement and the experimental design was completely randomized with four replications of 100 pollen grains for conservation, and 200 for crosses in the field viability study of the pollen grains.

The data were submitted to analysis of variance by using the test F. Qualitative data were subjected to the comparison of means by Tukey test, calculating the least significant difference at 5% level of probability.

RESULTS AND DISCUSSION

Pollen Grain Conservation

The 'Douradão' cultivar had the highest percentage of pollen grain germination followed by the cultivar 'Colombina', which did not differ statistically from the 'Centenária'. The lowest percentages of germination were observed in the cultivars 'Centenária' and 'Aurora 1' (Fig. 1).

Table 1 presents a summary of the results from each cultivar according to the storage period and environment. With the 'Aurora 1', 'Colombina' and 'Douradão' cultivars, it was possible to observe the efficiency of the conservation methods after 10 days. During this period the storage in refrigerator (5°C), freezer (-12°C) and liquid nitrogen (-196°C) were higher than the room temperature (25°C). For the Centenária cultivar statistical differences were only observed after 20 days (Table 1). Barbosa et al. (1991), working with different environments for the peach pollen storage, also found cold storage (0±1°C) statistically superior compared to storage at room temperature (25°C).

After 90 days of storage there is a significant decrease in the percentage of germination of the pollen grains preserved in the refrigerator and freezer when compared with those maintained in liquid nitrogen. However, when comparing the methods of conservation in refrigerator and freezer, there was virtually no significant difference in the conservation period except to the 'Douradão' and 'Colombina' cultivars, which showed significant differences at the end of the reporting period, in other words, at 300 days (Table 1).

In the refrigerator environment there was a significant decrease in viability after 10 and 20 days of the storage for all cultivars. This decrease in pollen grain viability was 68, 77, 72 and 80% for pollen from 'Aurora 1', 'Centenária', 'Colombina' and 'Douradão' cultivars respectively (Table 1). Barbosa et al. (1991) also found marked loss of viability in pollen grains of peach 'Rei da Conserva' after being held for 60 days at 1°C. Similar results were obtained by Daniel et al. (2002) who observed significant loss in viability of pollen grains of the *Dioscorea rotundata* stored at 10 and 5°C after 100 days of storage.

Results in the freezer environment were similar to those in the refrigerator. With the exception of the 'Aurora 1' and 'Colombina' cultivars which showed loss of viability after 20 days, the others had losses after 10 days of storage. This germination loss was possible since, even under low temperatures, pollen grains do not completely stop their metabolic activities. It is unlikely that this occurs in extremely low temperatures, as observed in liquid nitrogen conditions.

The best conservation results were obtained when the pollen grains were preserved in liquid nitrogen (-196°C). Except for 'Colombina' cultivar, the others largely maintained their initial viability during the conservation period. Lee et al. (1985) reported that pollen grains can be kept for indefinite periods in the process of cryopreservation (liquid nitrogen at -196°C). Similar results have been obtained by several authors testing the preservation of pollen grains in ultralow temperatures like -80°C and in liquid nitrogen (-196°C) (Bomber et al., 1999; Parton et al., 2002; Daniel et al., 2002; Sparks and Yates, 2002; Metz et al., 2000).

For the ‘Colombina’ cultivar the effect was synergistic. It was observed that conservation in liquid nitrogen increased the viability of pollen grains that initially had a germination of 50.2% and the end of 300 days it was obtained 78.2% (Table 1). This phenomenon has not been well understood, but researchers like Kartha (1985) also found higher viability of frozen pollen, compared to those recently harvested. According to this author, the process of freezing may release of some necessary nutrients for pollen germination.

Viability Test of Pollen Grains in the Field

Higher percentage of fruit set in the field was obtained when pollen grains from the liquid nitrogen method of storage were used in the controlled crosses. This result was significantly higher than the percentage of fruit set when using pollen grains from other methods, such as refrigerator and freezer. The second best result was obtained using pollen stored in the freezer. Moreover, the worst result was found when the pollination was made with pollen from the refrigerator environment (Table 2). This result confirmed the results obtained in Table 1.

The pollen grains obtained from cultivars ‘Centenária’, ‘Colombina’, ‘Aurora 1’ and ‘Douradão’ stored in the refrigerator and freezer environments did not differ in their ability to stimulate fruit set. Pollen grains from liquid nitrogen caused higher percentages of fruit set with the ‘Aurora 1’ cultivar, followed by ‘Douradão’, ‘Colombina’ and ‘Centenária’ cultivars. Taking into account the data presented in Figure 1 and Table 1 it was expected that the best results would be obtained with the ‘Douradão’ cultivar. However, this did not happen possibly because of the largest rustic stadium of the ‘Aurora 1’ cultivar, as shown previously in field pollination trials for obtaining new cultivars. Also the crosses were performed in periods of higher temperatures and it is expected that the genetic characteristics of low-chill cultivars, such as the ‘Aurora 1’, contribute to greater success in the crosses made.

The results obtained in this work are important, because according to Barbosa et al. (1991), research on conservation and germination of pollen on fruit set are indispensable for the genetic improvement of peach. In works with controlled pollination of peaches and nectarines there is usually a high amount of pollen needed for immediate use. But this is not always possible due to its rapid deterioration caused by improper storage. In this study, we found that the method of preservation in liquid nitrogen was very satisfactory.

CONCLUSIONS

- Storage in liquid nitrogen (-196°C) was the best method for pollen grain conservation for the ‘Aurora 1’, ‘Centenária’, ‘Colombina’ and ‘Douradão’ cultivars, keeping them viable until 300 days after storage.
- Higher viability of pollen and percentage of fruit set in the field was obtained with pollen grains stored in liquid nitrogen (-196°C) for the ‘Aurora 1’, ‘Centenária’, ‘Colombina’ and ‘Douradão’ cultivars.

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Tables

Table 1. Percentage of in vitro germinated pollen grains of 'Centenária' and 'Colombina' nectarines and 'Aurora 1' and 'Douradão' peach submitted for different times and environments of conservation. Lavras, UFLA, 2009.

Times of conservation (Days)	Environment of Conservation [z]			
	Room temperature 25°C	Refrigerator 5°C	Freezer -12°C	Liquid nitrogen -196°C
		Aurora 1		
0	55.5 aA	57.5 aA	57.7 aA	57.5 aA
10	21.0 cB	34.5 abB	45.0 abAB	54.0 aAB
20	15.5 cBC	32.7 bBC	41.0 bBC	52.7 aAB
30	7.2 cCD	23.0 bCD	42.2 aBCD	44.0 aB
90	0.0 cD	20.2 bCD	29.7 bCDE	46.2 aAB
180	0.0 cD	13.0 cD	28.2 bDE	55.7 aAB
300	0.0 cD	18.2 bD	19.0 bE	53.5 aAB
		Centenária		
0	58.5 aA	61.5 aA	61.5 aA	62.5 aAB
10	30.2 cB	28.7 cB	44.2 bB	66.0 aAB
20	5.2 dCD	21.5 cB	36.0 bBC	64.5 aAB
30	8.7 cCD	20.2 bB	31.2 bBCD	63.2 aAB
90	14.2 cC	16.2 bcBC	25.7 bCDE	70.0 aA
180	12.2 bCD	17.5 bBC	17.7 bE	62.0 aAB
300	0.0 cD	14.2 bC	19.0 bDE	63.0 aAB
		Colombina		
0	49.7 aA	47.2 aA	50.0 aA	50.2 aD
10	22.5 bB	42.5 aAB	49.2 abA	59.2 aCD
20	7.2 cC	26.2 bCD	32.7 bBC	51.2 aD
30	2.7 cC	29.5 bC	28.7 bAB	86.5 aA
90	1.2 dC	20.5 cCDE	45.0 bAB	83.7 aA
180	0.5 dC	14.5 cDE	41.2 bAB	69.0 aBC
300	0.0 dC	13.0 cE	24.7 bC	78.2 aAB
		Douradão		
0	64.0 aA	65.7 aA	65.7 aA	67.2 aA
10	15.0 dB	30.0 cB	52.5 bB	71.0 aA
20	9.2 dBC	27.0 cB	49.5 bB	69.5 aA
30	6.0 cBC	28.0 cB	47.2 bBC	62.7 aA
90	2.7 cBC	30.5 bB	36.0 bCD	61.5 aA
180	0.0 cC	23.7 bBC	34.2 bCD	68.7 aA
300	0.0 cC	13.0 cC	23.7 bD	65.0 aA

[z]Averages separation at 5% level (DMRT). Averages followed by the same letter in the same column, does not differ significantly between them by Tukey's test.

Table 2. Percentage of fruit set in the field using pollen grains of 'Centenária' and 'Colombina' nectarine and 'Aurora 1' and 'Douradão' peach stored for 300 days in different environments. Lavras, UFLA, 2009.

Times of conservation days (°C)	Percentagem of fruit fixated in field[z]			
	Centenária	Colombina	Aurora 1	Douradão
5	3.0 cA	5.5 bA	3.5 cA	8.5 cA
-12	14.7 bA	12.7 bA	16.7 bA	18.7 bA
-196	41.2 aC	57.5 aB	69.5 aA	53.5 aB

[z]Averages separation at 5% level (DMRT). Averages followed by the same letter in the same column and the same line, do not differ significantly as determined by Tukey's test.

Figures

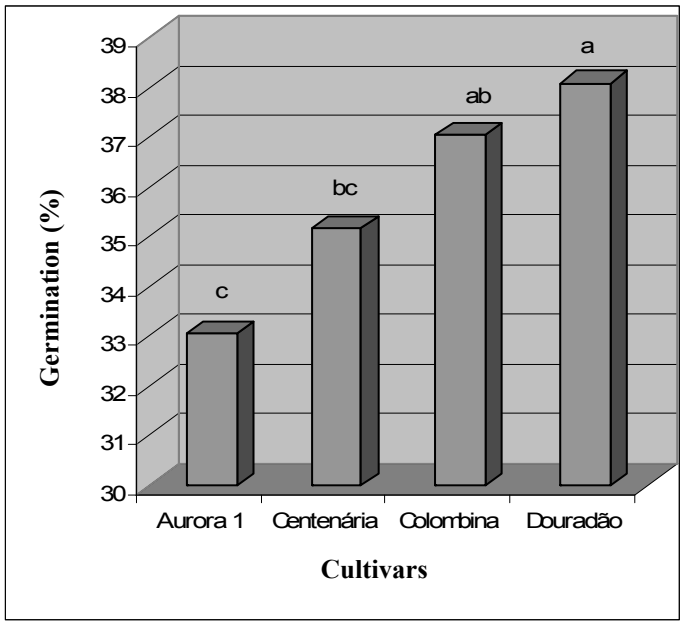

Fig. 1. Percentage of in vitro germinated pollen grains of 'Centenária' and 'Colombina' nectarines and 'Aurora 1' and 'Douradão' peach submitted a different times and environments of conservation. Lavras, UFLA, 2009.