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Scientific Article

Viability and conservation of genipap tree pollen grains

Viabilidade e conservação de grãos de pólen de jenipapeiro

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ABSTRACT - Conservation of plant genetic resources is vital for maintaining diversity for future use. The conservation of pollen grains is one of the tools complementary to conventional conservation strategies and plays an important role in breeding programs. Considering the importance of genipap trees and the lack of studies related to their floral biology and pollen conservation, the aim of this study was to evaluate the storage potential of pollen grains under different storage conditions. This study was conducted at the Plant Tissue Culture Laboratory of Embrapa Tabuleiros Costeiros, Aracaju, SE, Brazil. Flowers were collected in the pre-anthesis phase, and the pollen grains were placed in cryotubes and kept at different temperatures (i.e., 4°C, -20°C, -80°C, and -196°C) after extraction. Evaluations were performed after 30, 60, 90, 120, 150, and 180 days of storage. After 180 days, the germination percentage of the pollen tube was greater than 60.00% for all storage temperatures. The lowest percentage (60.21%) was observed under the storage condition of 4°C. Therefore, the other three temperatures are more highly recommended for ex situ conservation of pollen grains.

Keywords: Liquid nitrogen. *Genipa americana* L. Germination. Pollen tube.

RESUMO - A conservação de recursos genéticos vegetais é vital para manter a diversidade para uso futuro. A conservação de grãos de pólen é uma das ferramentas complementares às estratégias convencionais de conservação além de desempenhar um papel importante em programas de melhoramento. Considerando a importância do jenipapeiro e à escassez de trabalhos relacionados à biologia floral e conservação de pólen, este trabalho teve como objetivo avaliar o potencial de armazenamento de grãos de pólen em diferentes condições de armazenamento. O estudo foi conduzido no Laboratório de Cultura de Tecidos de Plantas da Embrapa Tabuleiros Costeiros, Aracaju, SE. A coleta de flores foi realizada na fase de pré -antese e após a extração, os grãos de pólen foram acondicionados em criotubos e mantidos nas seguintes temperaturas: 4°C, -20°C, -80°C e -196°C. As avaliações foram realizadas aos 30, 60, 90, 120, 150 e 180 dias de armazenamento. Após 180 dias, as porcentagens de germinação do tubo polínico foram superiores a 60,00% em todas as temperaturas. A menor porcentagem foi observada em condição de armazenamento de 4°C com 60,21%. Sendo as demais temperaturas as mais indicadas para conservação ex situ de grãos de pólen.

Palavras-chave: Nitrogênio líquido. *Genipa americana* L. Germinação. Tubo polínico.

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INTRODUCTION

Genipa americana L., popularly known as *jenipapeiro* or the genipap tree in Brazil, is widely distributed from Mexico and the Antilles to northern Argentina. It has different popular names depending on the region of occurrence. In Brazil, its presence has been confirmed in all states of the Federation, except for Rio Grande do Sul, and in all biomes, except Pampa (southern grasslands) (GOMES, 2023). All parts of the tree are used. The fruit is used as food for humans *in natura* and for making sweets, jams, juices, beverages, wine, and spirits. The liqueur is an important element in the June Festivals in the Northeast region of Brazil (SILVA; LEDO; SILVA JÚNIOR, 2020).

The species were listed among the ten native fruit-bearing plants with greatest potential for immediate use in Brazil by the "Plants of the Future" program, developed in a partnership between the Conselho Nacional de Desenvolvimento Científico e Tecnológico, World Bank, Global Environment Facility, Ministry of the Environment, and Project for Conservation and Sustainable Use of Brazilian Biological Diversity (SILVA; LEDO; MELO, 2018).

Diverse biotechnological techniques have been applied in the conservation of genetic resources. Most prominent among them are plant tissue culture-based in vitro conservation in the short and medium term and cryopreservation in the long term. Conservation or preservation of the genetic diversity of a plant is vital for maintaining its diversity for future use and plays an important role in breeding programs (WELEWANNI; JAYASEKERA; BANDUPRIYA, 2017).

Cryopreservation includes the conservation of plant material at an ultralow temperature provided by liquid nitrogen at -196°C, or by its vapor phase at -150° C (LEDO et al., 2020). The cryopreservation of pollen grains is often neglected. It is a safe and practical alternative for germplasm curators or plant breeders, conserving important alleles and overcoming reproductive difficulties, such as

asynchrony of flowering and insufficient pollen production (SAHA et al., 2023). It is important to emphasize that cryopreservation of pollen grains is an important tool (DINATO et al., 2020). It has been successfully applied to diverse fruit-bearing species, such as coconut (*Cocos nucifera* L.) Ledo and Vendrame (2021), pineapple (*Ananas comosus* L.) Souza, Souza and Silva (2018), mango (*Mangifera indica* L.) Veena et al. (2019), sugar-apple (*Annona squamosa* L.) Chander, Rajasekharan and Kurian (2019), olives (*Olea europaea* L.) Petruccelli et al. (2021), date palm (*Phoenix dactylifera* L.) Oliveira et al. (2023), and *Pisidium* sp. (VISHWAKARMA et al., 2021).

Applying plant tissue culture techniques is important for species such as genipap trees because they overcome the difficulties imposed by traditional propagation systems, allowing large-scale multiplication and conservation of this species with good prospects for the future (SOUZA et al., 2016). Ex-situ conservation of the genetic resources of the species was carried out through germplasm banks in the field.

Given the importance of the genipap tree and the lack of studies related to floral biology and pollen conservation, the aim of this study was to evaluate pollen viability determined by in vitro germination and pollen grain staining of *G. americana* under different storage conditions and to perform histological analysis of pollen grains.

MATERIAL AND METHODS

Collecting and obtaining pollen grains

This study was conducted at the Plant Tissue Culture Laboratory (Laboratório de Cultura de Tecidos de Plantas, LCTP) of Embrapa Tabuleiros Costeiros in the municipality of Aracaju, Sergipe, Brazil. The collection was made from an adult mother plant of the genipap tree in the municipality of Siriri, Sergipe, Brazil (10°60'30" S, 37°11"28" W, and altitude of 230 m), an area with a mean annual rainfall of 1,479 mm and mean temperature of 24.8°C. Functionally masculine flowers in the pre-anthesis phase (24 hours before opening) were selected to obtain the pollen grains. With the aid of scissors sterilized in ethyl alcohol, the flowers were cut at their pedicel, placed in a closed paper bag, and then placed in expanded polystyrene boxes (Isopor). Pollen grains were extracted from anthers opened over aluminum foil using fine-point tweezers in the laboratory. After the extraction, the pollen grains were inserted in 2.0-mL cryotubes and kept under the following storage conditions: T1, refrigerator (4°C); T2, freezer (-20°C); T3, ultrafreezer (-80°C); and T4, a drum with liquid nitrogen (-196°C).

Viability determined using acetocarmine staining

The samples were removed from the storage condition treatments, rehydrated for 2 hours, and placed on slides to evaluate the viability of pollen grains by staining under each storage condition. A drop of 1% acetocarmine was then added, followed by homogenization. The slide was then placed in a Petri dish (80 mm, Labomax Inc.) and incubated in a biological incubator at 30 ± 1°C for 25-30 minutes. The slides (26 × 76 mm, Global Glass) were analyzed for the number of viable and non-viable pollen grains per quadrant using a microscope (model DMSL, Leica, Bernsheim, Germany) at 10x magnification and a digital camera (Moticam C2300 model, Motic Instruments, Hong Kong, China). The pollen grains with intact walls stained red by the reaction in the presence of enzyme activity were considered viable, while unstained or red-stained grains with ruptured walls were considered unviable.

Viability determined using *in vitro* germination

Samples (0.0005 g) were inoculated in Petri dishes containing 2 mL of the culture medium described by Lora et al. (2006) to determine viability by in vitro germination of the pollen tube as recommended by Freire et al. (2022). The Petri dishes (80 mm, Labomax Inc.) were kept in an incubator for 24 hours at a temperature of 30 ± 1°C. Petri dishes were analyzed for the number of pollen grains that germinated using a microscope (model DMSL, Leica, Bernsheim, Germany) at 10x magnification with a digital camera (model Moticam C2300, Motic Instruments, Hong Kong, China). Pollen grains were considered to have germinated when they had a pollen tube length two times greater than the diameter.

Viability was evaluated by germination and acetocarmine staining after 30, 60, 90, 120, 150, and 180 days of storage.

Experimental design and statistical analyses

A completely randomized design was adopted in a 4 × 6 factorial arrangement (four storage conditions combined with six storage periods) with four replicates. Each replicate consisted of a Petri dish containing four counting fields. Analysis of variance (ANOVA) was used to analyze the pollen grain viability data using the F-test at 5% significance. For qualitative factors (storage conditions), the mean values were compared using Tukey's test at 5% probability. The regression equations were fitted to the quantitative factor (storage time) using SISVAR.

In addition, viability was evaluated by germination 365 days after conservation, and the data from this evaluation were compared only with the data obtained at 180 days. Analysis of variance (ANOVA) alone was used for the storage conditions and storage time factors using the F-test at 5% significance. For this, a completely randomized design was adopted in a 2 × 4 factorial arrangement (two storage periods combined with four storage conditions) with four replicates. Each replicate was composed of a Petri dish with four count fields.

Histological analysis

The pollen grains used for histological analysis were stored for 60 days under the following storage conditions: T1, refrigerator (4°C); T2, freezer (-20°C); T3, ultra freezer (-80° C); and T4, drum with liquid nitrogen (-196°C). The pollen grains were fixed in 50% FAA for 72 hours, transferred to 70% ethanol, and dehydrated in a series of ethanol solutions of increasing concentrations (90% and 100%) at 2-hour intervals. They were infiltrated with an infiltration solution (Historesin kit, Leica Microsystems, Heidelberg, Germany) and polymerized in histomolds. Microtomes were prepared using the semiautomatic microtome (model YD335) with

sections of 6-µm thickness that were then stained with toluidine blue at pH 4.8 and observed under an optical microscope (Nikon Eclipse E100 connected to an Infinity 1 camera) where photomicrographs were taken.

RESULTS AND DISCUSSION

Viability of genipap pollen grains determined by in vitro germination

A significant difference was observed for the interaction between storage conditions and storage time on genipap pollen grain viability, as determined by in vitro germination of the pollen tube, using the F-test ($p < 0.05$).

After 180 days of storage under different conditions, the percentages of in vitro germination of pollen grains were higher than 60.00%, and the lowest percentage of germination of the pollen tube (60.21%) was observed at 4°C, with a severe decline in viability over time (Figure 1). However, no significant difference among the four storage conditions was observed when viability was analyzed after 120 days of storage. The in vitro germination test showed that the pollen grains were viable and formed pollen tubes (Figure 4).

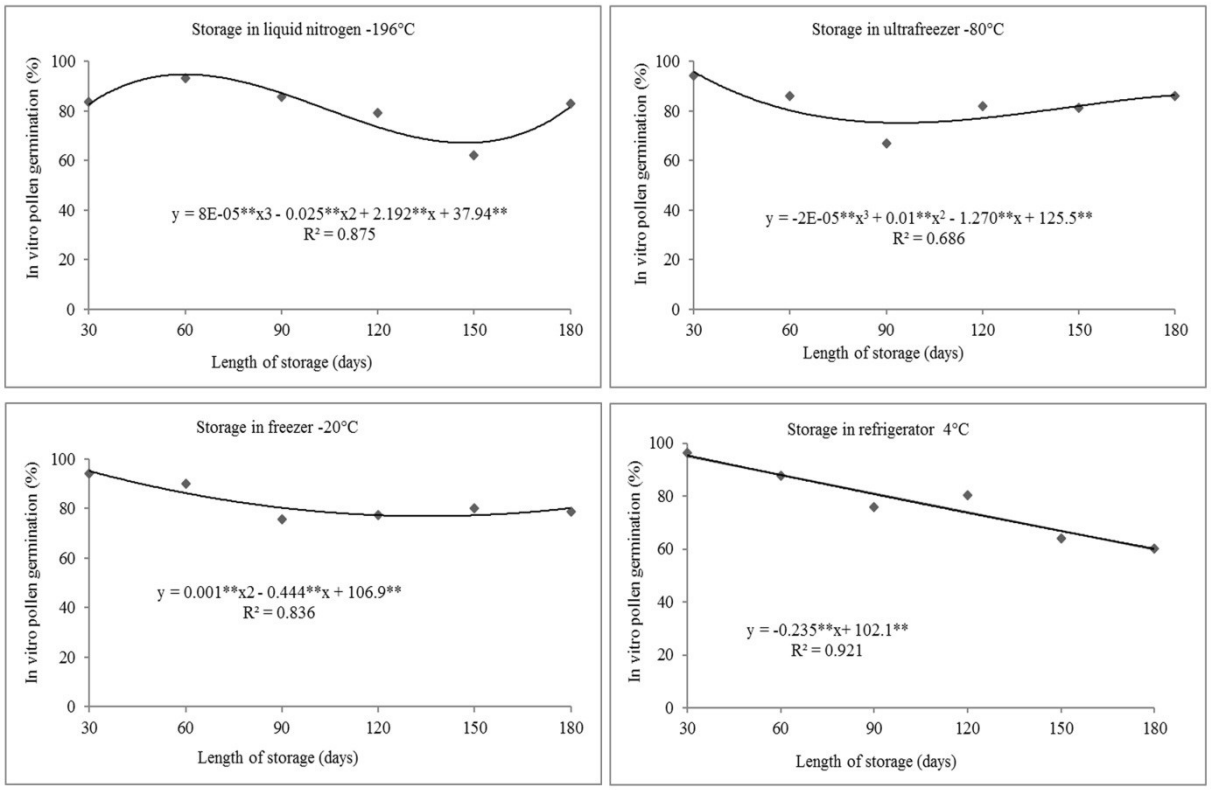

Figure 1. Viability response of genipap pollen grains at different storage conditions and length of storage (days) determined using in vitro germination.

According to Souza, Pereira and Martins (2002), values above 70% are high pollen viability, 31-69% are intermediate pollen viability, and up to 30% are low pollen viability. As shown in Table 1, the results indicated viability values ranging from intermediate to high up to 180 days under different storage conditions. Although in vitro germination of pollen grains enabled the experimental conditions to be controlled, it does not completely reproduce the in vivo growth of the pollen tube, where interactions with the stigma surface are more complex (NOVARA et al., 2017).

The success of pollen grain storage for long periods can contribute to an increase in in vitro pollination techniques and hybridization, facilitating transport and allowing storage so that plants of different ages or flowering times can be pollinated or hybridized (GIOVANNINI et al., 2017).

The pollen tubes showed low developed and were fragile after 90 days of storage at -20°C and -80°C. Germination was difficult to read because the genipap pollen grains had a translucid appearance. Good development was observed under the storage conditions and conservation times. The fragility of the pollen tube may be related to its handling. According to Giovannini et al. (2017), pollen degradation during storage may be due to dehydration, which results in the loss of colloidal properties and may interfere with the development of the pollen tube.

Table 1. Mean values of viability of pollen grains (%) of the Siriri accession under different storage conditions determined using in vitro germination.

Time (days)	-196°C	-80°C	-20°C	4°C
30	83.52 B	94.30 A	94.15 A	96.20 A
60	93.32 A	86.07 B	90.05 AB	87.79 AB
90	85.66 A	67.11 C	75.62 B	75.97 B
120	79.39 A	81.94 A	77.43 A	80.27 A
150	62.06 B	81.36 A	80.03 A	64.07 B
180	83.02 AB	86.21 A	78.87 B	60.21 C
CV 1 (%):	3.83			
CV 2 (%):	3.70			

Mean values followed by the same uppercase letters in the same row do not differ according to Tukey's test at 5% significance.

A comparison of the mean values of viability determined by germination at 180 and 365 days of conservation under different storage conditions showed that the storage conditions of -80°C and -196°C were more suitable (Figure 2). Once cryopreserved at the temperature of -196 °C, the pollen would theoretically have an infinite period of longevity. Thus, it could overcome the environmental specificities of the species, such as the problems of asynchronous flowering and early or late flowering of male plants (RAJASEKHARAN; GANESHAN, 2019; VISHWAKARMA et al., 2021). Dinato et al. (2020) reported an improvement in pollen viability after cryopreservation and emphasized the importance of this technique in breeding programs to prolong the length of the storage period in various seasons.

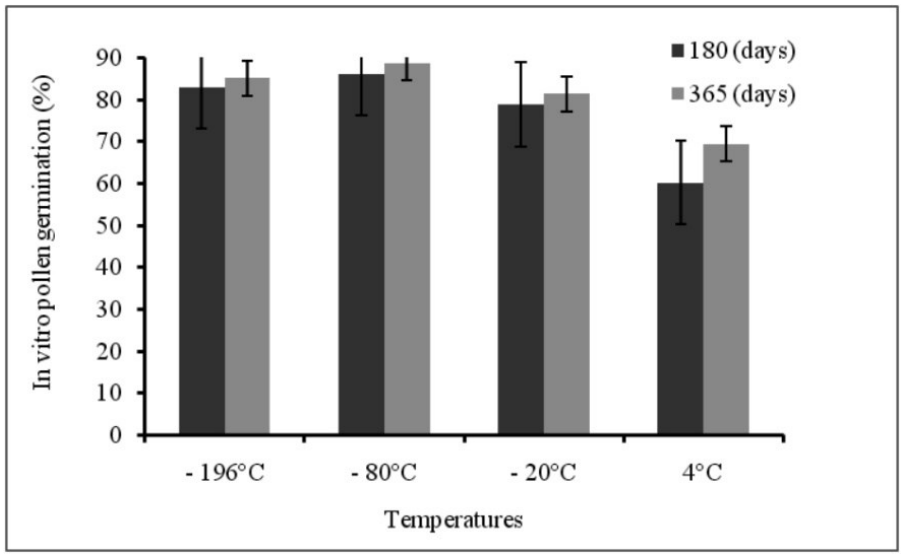

Figure 2. Viability of genipap (%) pollen grains after 180 and 365 days at different storage conditions determined using in vitro germination.

In relation to storage time, the highest percentages of in vitro germination of pollen grains for all the temperatures was detected at 365 days, and the storage condition with the lowest germination percentage was that of 4°C. Similar results were observed by Anushma et al. (2018) with date palm pollen grains, with a reduction in the germination percentage as the storage temperature increased.

The reduction in pollen grain viability is likely caused by the methods of handling the pollen before storage, moisture in the pollen, temperature stability during the storage period, and starch concentration, which results in a reduction in the soluble sugar concentration (MEHAREB et al., 2017).

Viability of genipap pollen grains determined using acetocarmine staining

There was a significant difference in the F test ($p < 0.05$) for the interaction between storage conditions and time on the viability of genipap pollen grains, as determined using acetocarmine staining.

The estimation of pollen viability by staining with 1% acetocarmine acid showed that all storage conditions studied led to viability greater than 88.00% after 180 days of storage (Figure 3).

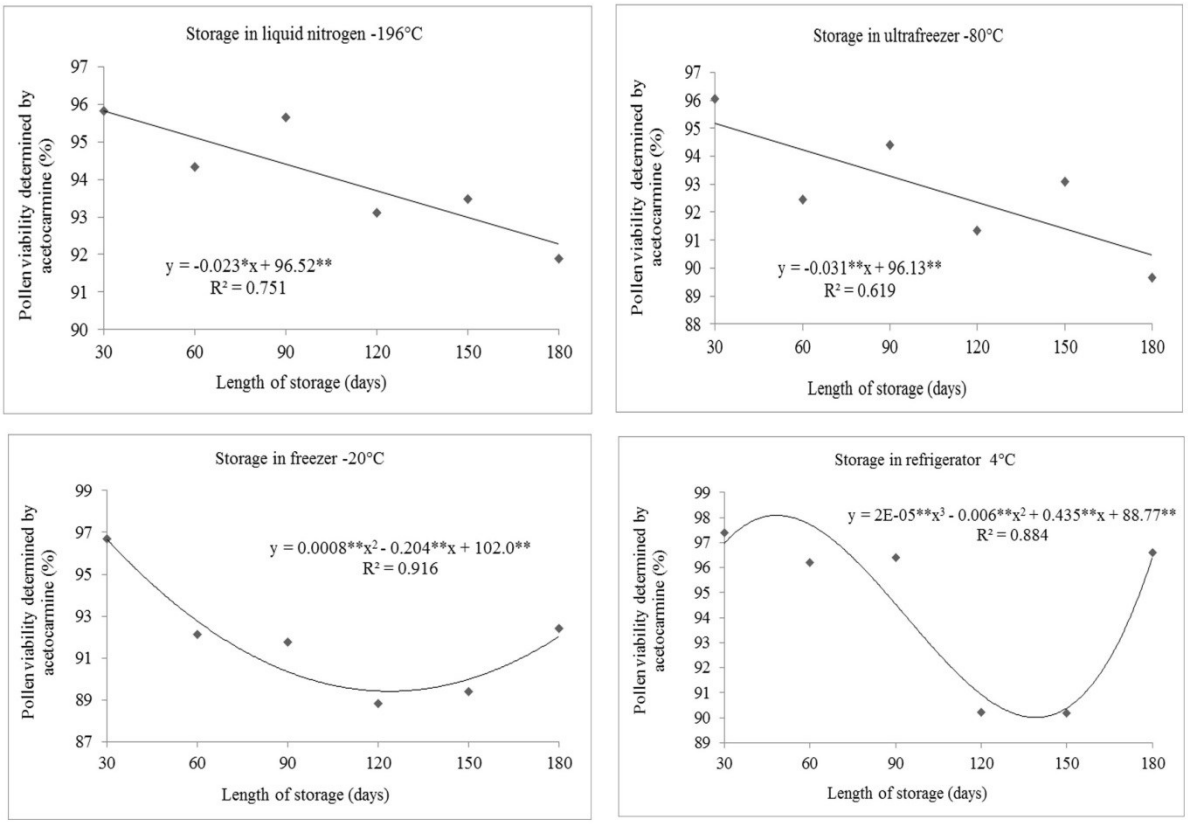

Figure 3. Viability response (%) determined by acetocarmine staining of genipap pollen grains under different storage conditions and lengths of storage (days).

Pollen viability is widely tested using staining because of the simplicity and speed of the procedure (Figure 4). However, this may over-or underestimate the results. Thus, it is fundamental to adopt different methods for determining pollen viability to obtain more accurate results. The viability test for in vitro germination is a reliable and widely used method (ALEXANDER, 2019).

The acetocarmine stain used in the present study indicated the presence of structural elements and integrity of the pollen grains. Therefore, it is used as an indicator of pollen viability by indicating chromosomal integrity, which is used for chromatin integrity observation and reacts with cell components present in mature pollen grains (RUZZA et al., 2023)

In a study conducted by Ruzza et al. (2023), the highest mean percentage of pollen viability in *G. americana* was obtained using acetocarmine staining, with 97.96% of the pollen grains showing chromatin integrity.

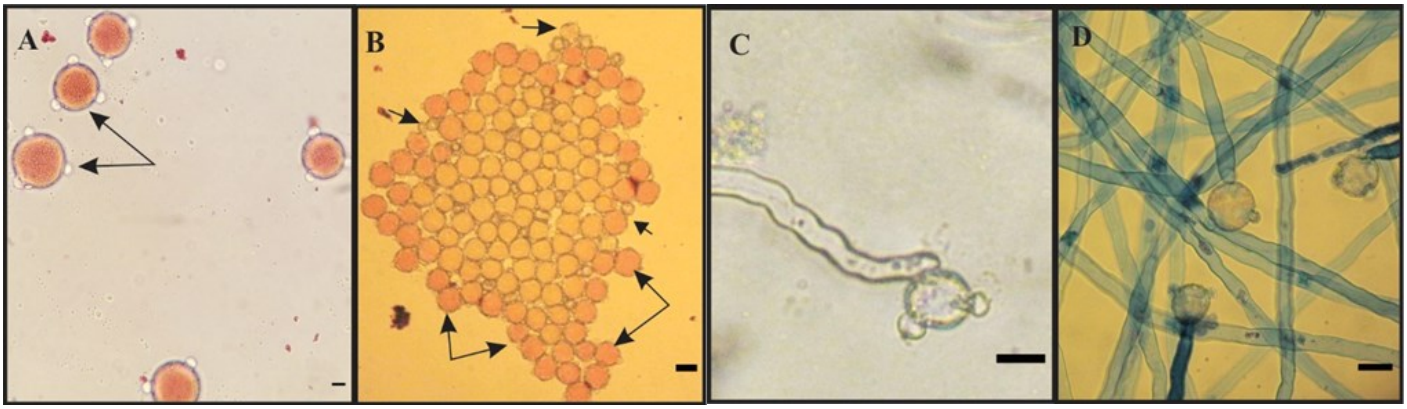

Figure 4. Viability test of pollen grains of *G. americana*, where A and B exhibit viability determined using 1% acetocarmine staining, and C and D exhibit viability determined using germination of the pollen tube. Symbols: A single arrow represents non-stained pollen, and a double arrow represents stained pollen.

Histological analysis

Some pollen grains showed a dark blue nucleus, generally located in the central region, and a pollenkitt joined to the exine surface (Figure 5). Genipap pollen grains are medium-sized, tricolporate, and spherical, with a reticulate exine, and their shape is oblate and circular scope (MATOS et al., 2014). All species release their pollen grains in bicellular form, and the generative cell is spindle-shaped (CORTEZ et al., 2022).

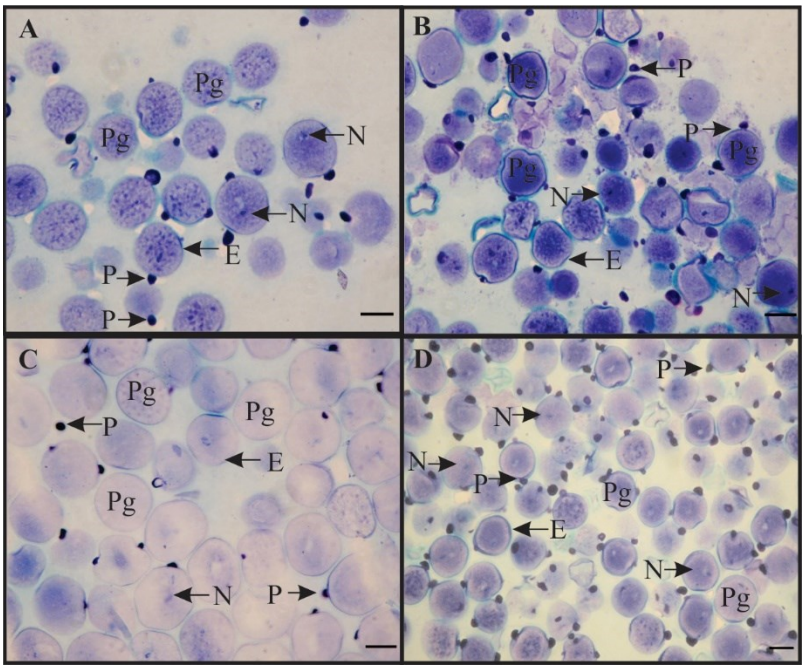

Figure 5. Histological analysis of the pollen grains of *G. americana* under different storage conditions. A in a refrigerator (4°C), B in a freezer (-20°C), C in an ultra freezer (-80°C), and D in a drum with liquid nitrogen (-196°C). N nucleus, E exine, Pg pollen grains, and P pollenkitt.

The morphology and structural characteristics of the pollen grains were similar under all four storage conditions. This may indicate that modifications in pollen structure did not occur even after conservation. Regarding the presence of pollenkitt in the pollen grain of *G. americana*, it is a structure present in most angiosperms; in most entomophilous species, it is always present with parietal or amoeboid tapetum, and it has important functions during dispersal of pollen grains, participating in adherence to the body of pollinators (DETTKE; SANTOS, 2011).

The results obtained indicate that the conservation of genipap pollen grains at different temperatures is promising for breeding, pollination, and hybridization studies. Pollen grains maintained excellent viability profiles after long-term storage at low temperatures for long periods.

CONCLUSION

These results indicate that conserving genipap pollen grains at lower temperatures is promising. The storage temperature of 4°C reduced pollen viability, determined using in vitro germination, over the storage period.

Based on the histological analysis, it can be concluded that the morphology and structural characteristics of the pollen grains were very similar under all four storage conditions.

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