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In vitro viability of pollen grains from genipap accessions

Viabilidade *in vitro* de grãos de pólen de acessos de jenipapeiro

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The aim of this study was to evaluate the *in vitro* viability of pollen from genipap accessions at ambient temperature in different culture media. Pollen grains were collected at the Genipap Active Germplasm Bank (BAG Jenipapo) of Embrapa Tabuleiros Costeiros at the Jorge Sobral Experimental Field in the municipality of Nossa Senhora das Dores, Sergipe, Brazil. Three culture media were evaluated in combination with 11 evaluation times and 4 accessions. *In vitro* viability through germination of the pollen tube using carmine acetic was evaluated in accessions AR2, AR4, CA, and CR3 cultivated in: Lora, Sousa, and modified Sousa medium. A significant effect ($p \leq 0.05$) was observed among the interactions of the time after inoculation, culture media, and accession factors regarding pollen germination *in vitro*. The highest *in vitro* viability was obtained by accession CR3 in Lora culture medium at 96 h of incubation (86.56%). Regarding pollen viability using carmine acetic 1%, there was no significant difference in viability time for accessions AR2 and CR3. However, a significant difference was observed among the accessions after 168 h, with a decrease in viability for the AR4 accession, to 64.74%. The Lora culture medium is superior for *in vitro* viability studies of pollen grains by germination of the pollen tube in genipap accessions, and there is a genetic-dependent response for viability of pollen grains from genipap accessions.
Keywords: *Genipa americana* L., germination, pollen tube.

Este trabalho teve como objetivo avaliar a viabilidade polínica in vitro de acessos de jenipapeiro em temperatura ambiente em diferentes meios de cultura. A coleta dos grãos de pólen foi realizada no Banco Ativo de Germoplasma de Jenipapo (BAG Jenipapo) da Embrapa Tabuleiros Costeiros, localizado no Campo Experimental Jorge Sobral, no Município de Nossa Senhora das Dores, Sergipe. Foram avaliados três meios de cultura combinados com 11 tempos de avaliação e 4 acessos. A viabilidade *in vitro* por meio da germinação do tubo polínico e com o uso do corante carmim acético foi avaliada nos acessos AR2, AR4, CA e CR3 em três meios de cultura: Lora, Sousa e Sousa modificado. Foi observado efeito significativo ($p \leq 0,05$) entre as interações dos fatores tempo após inoculação, meios de cultura e acessos quanto à germinação de pólen *in vitro*. A maior viabilidade *in vitro* foi obtida pelo acesso CR3 em meio de cultura Lora por 96 h de incubação (86,56%). Quanto à viabilidade polínica por corante carmim acético 1%, não houve diferença significativa do tempo de viabilidade para os acessos AR2 e CR3. No entanto, após 168 horas foi observada diferença significativa entre os acessos, com decréscimo da viabilidade para o acesso AR4 com 64,74%. Pode-se concluir que o meio de cultura de Lora é superior para estudos de viabilidade *in vitro* de grãos de pólen por germinação do tubo polínico em acessos de jenipapeiro e que existe resposta genético-dependente para a viabilidade de grãos de pólen de acessos de jenipapeiro.
Palavras-chave: *Genipa americana* L., germinação, tubo polínico.

1. INTRODUCTION

Genipa americana L. is a native species not endemic to Brazil of the Rubiaceae family, originating from the north of South America and widely distributed in South and Central America [1]. It is present in nearly all of Brazilian territory and in all the biomes, except for the Pampas [2].

The genipap tree is of significant economic importance; however, expansion of the agricultural frontier and other factors have led to loss of individuals, which can generate genetic erosion, changes in distribution patterns, and even extinction, making it necessary to conserve the species *ex situ* and *in situ* [3, 4].

The main bioactive compound of the genipap fruit, called jenipapo, is genipin, a powerful natural blue dye with polar characteristics that, in addition to being a colorant, has antioxidant, anticarcinogenic, and neuroprotective activity; and it acts against liver diseases [5]. The fruit is also a source of non-polar compounds, such as fatty acids, though extraction of these compounds has not yet been examined [6]. Genipap has been used more expressively in making jellies, jams, preserves, liqueurs, and wines; the fruit has been collected in an extractive manner from natural populations and has contributed to the family income of rural and riverside populations [7].

Advances in propagation and promotion of the species are important for conservation and plant breeding programs. Plant tissue culture techniques have been successfully applied to the propagation and conservation of the genipap tree [4, 8-11]. Pollen viability tests are also performed to identify a suitable culture medium for pollen germination of each species. A study was carried out on pollen viability of genipap by Freire et al. (2022) [12]. The results obtained previously were used to conduct the present study. However, few studies have been conducted on the *in vitro* viability and conservation of pollen grains. Cryopreservation of pollen grains is an important strategy for conservation and directed crosses [13].

Cryopreservation consists of conservation at an ultralow temperature provided by liquid nitrogen at -196 °C, or by its vapor phase at -150 °C. It is considered a safe and practical alternative for germplasm curators or plant breeders, conserving important alleles and overcoming reproductive difficulties, such as asynchronous flowering and insufficient pollen production [14]. *In vitro* pollen conservation is a strategy that complements germplasm conservation in the field or seed bank, and it has been successfully applied to diverse fruit-bearing species, such as coconut (*Cocos nucifera* L.) [15]; pineapple (*Ananas comosus* L.) [16, 17]; mango (*Mangifera indica* L.) [18]; sugar apple (*Annona squamosa* L.) [19]; olive (*Olea europaea* L.) [20]; date palm (*Phoenix dactylifera* L.) [21]; and *Psidium* sp. [22].

Considering the *in vitro* conservation of pollen grains, some factors must be kept in mind to obtain efficient protocols, considering the high specificity of the germination response to the culture media. It is essential to define culture media for each species and for genotypes within the same species [23]. The culture medium should be composed of organic and inorganic elements that most nearly reproduce the conditions offered by the female flower structure upon receiving the pollen grain, and these conditions may differ from may differ between species [17]. The genotype factor has been considered for *in vitro* responses.

Successful pollen storage for genetic resources conservation depends on the many factors, and it is essential that the chosen procedure maintain high genetic integrity and diversity, vigor, and germination percentages [13]. Thus, the aim of the present study was to evaluate pollen viability by *in vitro* pollen tube germination and by staining of different genipap accessions at ambient temperature over time in different culture media and in vitro culturing time.

2. MATERIALS AND METHODS

The study was conducted in the Plant Tissue Culture Laboratory of Embrapa Tabuleiros Costeiros at Aracaju, Sergipe, Brazil. Functionally male flowers were collected in pre-anthesis (24 h before opening) between 9:00 and 10:00 a.m. in four accessions of the Genipap Active Germplasm Bank (BAG Jenipapo) of Embrapa Tabuleiros Costeiros. Plants were chosen from the following accessions: Lagarto/Silo (CR3), Caueira (CA), Arauá 2 (AR2), and Arauá 4 (AR4).

2.1 Viability of pollen grains from genipap accessions by *in vitro* germination of the pollen tube

For these analyses, functionally male flowers were cut at their pedicel using a scissors sterilized in ethanol; they were placed and sealed in paper bags and stored in expanded polystyrene (Isopor®) boxes. In the laboratory, fine point tweezers were used to extract pollen grains from the anthers opened over aluminum foil. After that procedure, the pollen grains were kept in a biological incubator at a temperature of 30±1 ºC.

To evaluate the effect of different culture media on *in vitro* germination of the pollen tube, approximately 0,0005 g of pollen grains were inoculated in 35-mm-diameter sterile disposable Petri dishes containing 2 mL of the culture media shown in Table 1.

Table 1: Composition of the culture media tested for in vitro germination of pollen grains from different accessions.

Culture media	Composition
A - Lora et al. (2006) [24]	200 mg L^{-1} $MgSO_4.7H_2O$; 300 mg L^{-1} Ca (NO_3) $O_2.4H_2O$; 100 mg L^{-1} KNO_3; 100 mg L^{-1} H_3BO_3; 40 g L^{-1} sucrose
B - Sousa et al. (2010) [25]	100 g L^{-1} sucrose; 3 g L^{-1} agar
C - Sousa et al. (2010) [25] modified by Machado et al. (2014) [26]	80 g L^{-1} sucrose; 1 g L^{-1} agar; 100 mg L^{-1} H_3BO_3

The number of germinated pollen grains was analyzed under a microscope 10X objective lens at 24-h intervals, up to 264 h after beginning incubation. Pollen grains were considered germinated when the pollen tube length was twice the diameter, according to the methodology suggested by Cook and Stanley (1960) [27]. For better visualization of the pollen tube and assistance in counting and photomicrography, the cultures were stained with Evans blue dye. The *in vitro* germination percentage was obtained by applying the following formula: *in vitro* germination of pollen grains (%) = (number of germinated pollen grains / total number of pollen grains) × 100.

2.2 Viability of pollen grains from genipap accessions by staining

For determination of viability by staining at ambient temperature, the pollen grain samples were collected as already described and placed in 2.0-mL cryotubes and kept at ambient temperature at 30±1 °C up to the end of the experimental trial. The viability of the pollen grains was evaluated by carmine acetic 1%. The number of viable and inviable pollen grains per quadrant was analyzed under a microscope 20X objective lens at 24-h intervals up to 264 h, or until reaching values below 50-60%.

Samples of approximately 0.0005 g of pollen grains were arranged on glass slides (26 × 76 mm, Global Glass), and then a drop of carmine acetic 1% was added and homogenized. After that, the slides were placed in Petri dishes (80 mm, Labomax Inc.) and kept in a biological incubator for 25 to 30 min at a temperature of 30±1 ºC.

Pollen grains stained red (through reaction of the occurrence of enzyme activity) and with intact walls were considered viable, and those not stained red or stained red with ruptured walls were considered inviable [26]. The following formula was considered for calculation of the percentage: viability of the pollen grains by staining (%) = (number of stained pollen grains / total number of pollen grains) × 100.

2.3 Experimental designs and statistical analyses

The experimental design for *in vitro* viability by germination of the pollen tube was completely randomized in a 3 × 4 × 11 triple factorial arrangement (three culture media × four accessions × eleven evaluation times) and four replications. Each replication consisted of a Petri dish with four counting fields.

The experimental design for viability by staining was completely randomized in a 4 × 10 double factorial arrangement (four accessions × ten evaluation times) and four replications. Each replication consisted of a slide with 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 (accession and culture medium), the mean values were compared using Tukey's test at 5% probability. The regression equations were fitted to the quantitative factor (in vitro culturing time), using the SISVAR statistical program [28].

3. RESULTS AND DISCUSSION

3.1 Viability of pollen grains from genipap accessions by *in vitro* germination of the pollen tube

A significant effect was observed for the interactions of the accession and culture medium factors, the accession and *in vitro* culturing time factors, and the *in vitro* culturing time and culture medium factors for the pollen grain viability.

In the accession as a function of the *in vitro* culturing time interaction, the accession CR3 obtained better performance than the others up to 192 h of inoculation, with a value of 43%, the AR2 e CA accessions did not show a sudden reduction in their viability over time according to the adjusted regression equations (Figure 1). According to Souza et al. (2002) [29], values higher than 70% are considered high pollen viability; from 31% to 69%, intermediate viability; and up to 30%, low pollen viability. Accordingly, the accessions AR2, AR4, and CA exhibited intermediate and low viability, and the accession CR3, high to intermediate viability (Table 2). It can be considered that there were variations between the percentages of pollen viability in the genotypes evaluated, in which it was observed that the CR3 accession presented values higher than 39% of viability up to 264 hours after collection, the other genotypes presented lower values, providing evidence that the genotypes differ from each other (Table 2).

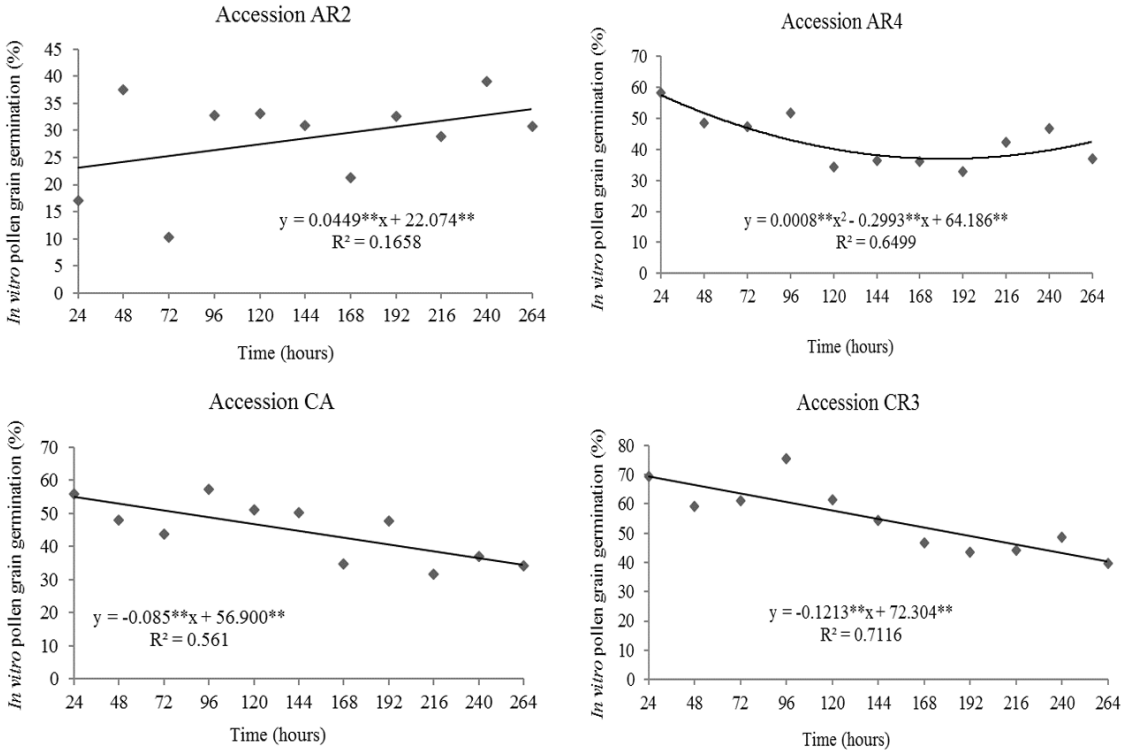

Figure 1: Viability response of genipap pollen grains at different accessions as a function of in vitro culturing time.

Table 2: In vitro viability of pollen grains by germination of the pollen tube (%) in different accessions of the Genipap BAG as a function of in vitro culturing time.

Time (h)	Accessions			
	AR2	**AR4**	**CA**	**CR3**
24	16.97 C	58.41 B	55.92 B	69.41 A
48	37.45 C	48.39 B	48.07 B	59.20 A
72	10.35 C	47.31 B	43.61 B	61.10 A
96	32.73 C	51.72 B	57.17 B	75.34 A
120	33.09 C	34.43 C	51.07 B	61.23 A
144	30.85 B	36.47 B	50.19 B	54.32 A
168	21.35 C	36.07 B	34.66 B	46.79 A
192	32.57 B	32.86 B	47.76 A	43.44 A
216	28.83 B	42.31 A	31.68 B	44.14 A
240	39.11 AB	46.71 AB	37.03 B	48.57 A
264	30.70 A	37.11 A	34.07 A	39.72 A

Mean values followed by the same uppercase letters in the same row do not differ according to Tukey's test at 5% significance ($p \geq 0.05$).

Considering the culture medium and accession interaction, the highest mean values of *in vitro* viability were found in culture medium A for the four accessions, with values greater than 64%, achieving high and medium viability (Table 3). The Lora culture medium [24] was likely superior through being more complete in relation to nutrients such as magnesium sulfate, calcium nitrate, potassium nitrate, and boric acid.

Table 3: Pollen viability (%) by in vitro germination of the pollen tube of genipap accessions as a function of the culture medium.

Culture medium	Accessions			
	AR2	**AR4**	**CA**	**CR3**
A	64.83 Da	74.17 Ca	80.40 Ba	91.70 Aa
B	6.26 Cb	11.71 Bc	15.31 Bc	23.68 Ac
C	9.99 Cb	34.87 Bb	32.11 Bb	42.46 Ab

Mean values followed by the same lowercase letter in the column and uppercase letter do not differ from each other by Tukey's test ($p \geq 0.05$). A - Lora et al. (2006) [24], B - Sousa et al. (2010) [25], and C - Sousa et al. (2010) [25] modified by Machado et al. (2014) [26].

In the B and C culture media, viability was intermediate and low in all the accessions. The B and C culture media are in gel form, a condition that may have created difficulties for germination of the pollen tube of the accessions. Selection of the culture medium depends on the physiological need of each species for development of the pollen tube. Use of the liquid medium brought about results superior to those of the gelled media. A similar result was observed by Zortéa et al. (2022) [30] in studies on *Vochysia divergens* Pohl (Vochysiaceae) pollen grains. The authors found that the culture media with the addition of agar and boric acid led to lower germination of the pollen tube. In contrast, Freire et al. (2022) [12], in their work on genipap pollen germination, observed that the best culture medium for pollen tube germination of Siriri accession was the medium of Sousa (2012) [25] modified by Machado et al. (2014) [26] with the addition of agar. This fact may be an indication that there are nutritional requirements for development not only among different species, but also among genotypes.

The CR3 accession stood out, showing greater viabilities of 91.70%, 23.68%, and 43.46% in the A, B, and C culture media, respectively (Table 3). Adhikari and Campbell (1998) [31] report that pollen grain viability is affected by genotypic differences and the physiological stage of the

plant and of the flower. Although *in vitro* germination of pollen grains makes for a controlled experimental system, it does not fully reproduce the growth of the pollen tube *in vivo*, where interactions can occur between the compositions of the culture media and different plant genotypes [32].

In vitro germination of the pollen tube of the genipap is easily observed at microscope and use of Evans blue dye (Figure 2).

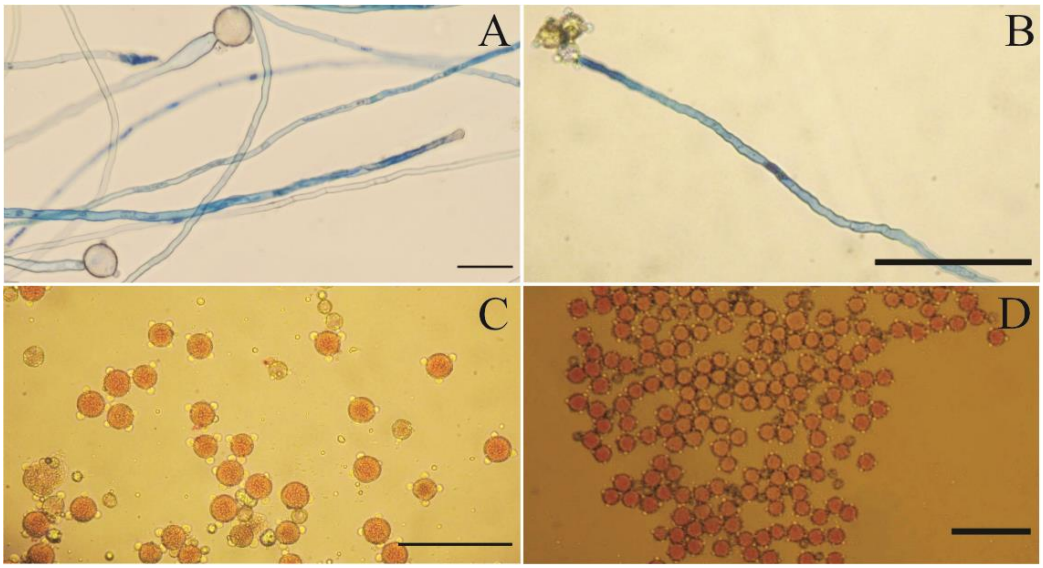

Figure 2: A and B: emergence of the pollen tube in the medium of Lora et al. (2006) [24] stained with Evans blue dye, and structures of the pollen tube and pollen grains; Figures C and D: pollen grains stained with carmine acetic 1%. Figures A, B, and C at 20X – bar = 50 μm; Figure D at 10X – bar = 200 μm.

There was significant interaction between culture media and *in vitro* culturing times (Table 4). The Lora medium (A) maintained high pollen viability throughout the evaluation time up to 264 h. This result may be related to the factors mentioned above, such as mineral and physical constitution of the medium.

Table 4: Pollen viability (%) by in vitro germination of the pollen tube of genipap accessions as a function of the culture medium and in vitro culturing time.

Time (h)	**Culture medium**		
	Medium A	**Medium B**	**Medium C**
24	65.63 A	23.76 C	57.39B
48	66.82 A	18.37 C	53.35 B
72	70.94 A	16.92 C	31.33 B
96	86.56 A	27.95 C	43.82 B
120	85.59 A	7.62 C	30.10 B
144	82.71 A	10.55 C	24.51 B
168	69.30 A	10.14 C	22.50 B
192	82.69 A	7.72 C	21.61 B
216	81.89 A	10.92 B	9.63 B
240	86.53 A	13.40 B	15.75 B
264	77.62 A	8.22 B	10.02 B

Mean values followed by the same uppercase letters in the same row do not differ according to Tukey's test at 5% significance ($p \geq 0.05$). Medium A - Lora et al. (2006) [24], Medium B - Sousa et al. (2010) [25], and Medium C - Sousa et al. (2010) [25] modified by Machado et al. (2014) [26].

The viability by in vitro germination of genipap pollen grains showed quadratic variation for the culture media with a significant decrease for media B and C; however, germination in medium A remained high throughout the evaluation period (Figure 3). The highest viability by in vitro germination of the pollen tube obtained by the maximum point of the regression curve was 86.56 % at 96 hours after inoculation.

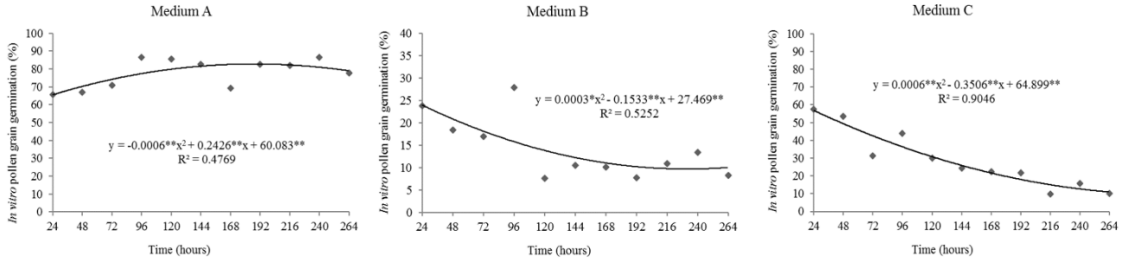

Figure 3: Response of viability by in vitro germination of genipap pollen grains as a function of the culture medium and in vitro cultivation time.

3.2 Viability of pollen grains from genipap accessions by staining

A significant effect was observed by the F test in the interactions of the accession and *in vitro* culturing time factors. There was no significant difference of *in vitro* culturing time for accessions AR2 and CR3. However, after 168 h, a significant difference was observed among the accessions, with a decline in viability for the accession AR4 (Table 5).

There was a significant effect of *in vitro* culturing time and accessions interaction by germination of the pollen tube and by staining in carmine acetic 1% at ambient temperature. Viability by *in vitro* germination showed a decline in comparison with viability by staining with carmine acetic 1% (Tables 2 and 5).

Analyses of pollen viability by staining should not be used in an isolated manner, because they indicate the presence of cell content, which does not necessarily imply formation of the pollen tube and later fertilization (Figure 1). *In vitro* tests of germination of the pollen tube are necessary to confirm this viability more reliably [33].

Table 5: Pollen viability (%) by carmine acetic 1% of genipap accessions as a function in vitro culturing time.

Time (h)	Accessions			
	AR2	**AR4**	**CA**	**CR3**
0	95.92 Aa	98.10 Aab	95.48 Aab	96.00 Aa
24	97.94 Aa	93.66 Aabc	96.52 Aab	95.30 Aa
48	97.36 Aa	95.46 Aabc	96.79 Aab	96.22 Aa
72	98.36 Aa	97.18 Aab	96.22 Aab	97.56 Aa
96	99.23 Aa	93.01Babc	100.00 Aa	97.32 ABa
120	95.37 Aa	98.26 Aa	94.17 Aab	95.73 Aa
144	98.50 Aa	96.12 Aab	99.60 Aa	97.79 Aa
168	96.52 ABa	91.35 Bbc	96.34 ABab	97.27 Aa
192	98.52 ABa	89.01 Cc	93.45 BCab	100.00 Aa
216	96.68 ABa	64.74 Cd	91.28 Bb	96.88 Aa

Mean values followed by the same lowercase letter in the column and uppercase letter in the row do not differ from each other by Tukey's test ($p \geq 0.05$).

Several authors report obtaining overestimates of pollen grain viability by staining in comparison with *in vitro* germination in various species [23, 25, 34-36].

Determination of pollen viability by staining and by germination of the pollen tube is fundamental for fertilization and assists in identification of the best male parents to maximize establishment of crosses and breeding programs [37]. Information on the viability and development of pollen grains in different accessions are fundamental for studies on reproductive biology and plant breeding, increasing the chances of success in controlled crosses, as well as assisting in identification of more promising crosses for use in hybridization programs [34, 38, 39, 23].

According to Ferreira et al. (2021) [23], germination of pollen in a culture medium is still the main technique used to determine viability, and it may provide results like those that will likely occur *in vivo*. However, as time proceeds, viability of the pollen grain declines and, consequently, its efficiency in fertilization.

4. CONCLUSIONS

The Lora culture medium is superior for *in vitro* pollen grain viability studies of genipap accessions as it presents nutritional requirements for maintaining the pollen tube germination up to 264 h. Furthermore, there is a genotype-dependent response to the viability of pollen grains from genipap accessions.

Through the viability analyses of genipap pollen grains in different accessions as a function of the in vitro cultivation time, it was observed that the pollen grains of genipap accessions lost viability 264 hours after collection and storage at room temperature.

It was possible to see through this study that the viability of pollen grains by staining method with carmine acetic 1% is overestimated.

5. ACKNOWLEDGMENTS

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