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In vitro germination and viability of pollen grain of coconut accessions[1]

Germinação *in vitro* e viabilidade de grãos de pólen de acessos de coqueiro

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ABSTRACT - Storage as a means of maintaining the pollen viability is important for the preservation of the genetic variability, facilitates the exchange of germplasm and greatly contributes to the generation of variability obtained from artificial crosses, increasing the efficiency of breeding programs. The objective of this study was to select different culture media for the *in vitro* germination of pollen grain of dwarf and tall coconut accessions, as well as to determine the viability of pollen grain at room temperature conditions. For this purpose, Brazil Green Dwarf (BGD) and Brazilian Tall (BRA) pollen grains derived from the Coconut Active Germplasm Bank of Embrapa Coastal Tablelands Sergipe were used. To evaluate the effect of different culture media on the *in vitro* germination of pollen grains of anão verde do Brasil de Jiqui (AVeBrJ) and gigante do Brasil Praia do Forte (GBrPF) accessions, they were inoculated on to Petri dishes containing 2 ml of culture media. The pollen viability was assessed by staining with 1% acetic carmine and *in vitro* germination at 0, 24, 48 and 72 hours. The culture medium of Lora is suitable to assess the *in vitro* germination of pollen grain of the AVeBrJ and GBrPF accessions. The pollen grain of the AVeBrJ accession showed intermediate viability (66.87%) at room temperature up to 23.14 hours by *in vitro* germination. The pollen grain of the GBrPF accession showed high viability, above 70%, at room temperature up to 120 hours by *in vitro* germination.

Key words: *Cocos nucifera*. *In vitro* culture. Culture media.

RESUMO - A conservação de recursos genéticos é importante para a preservação da variabilidade genética, facilita o intercâmbio de germoplasma e contribui na geração de variabilidade obtida por meio de cruzamentos artificiais aumentando a eficiência dos programas de melhoramento genético. O objetivo desse estudo foi selecionar meio de cultura para germinação *in vitro* de grãos de pólen de acessos de coqueiro anão e gigante, bem como determinar a viabilidade de grãos de pólen em condições de temperatura ambiente. Para tanto foram utilizados grãos de pólen dos acessos Anão verde do Brasil de Jiqui (AVeBrJ) e Gigante do Brasil Praia do Forte (GBrPF) provenientes do Banco Ativo de Germoplasma de Coco da Embrapa Tabuleiros Costeiros. Para avaliar o efeito de diferentes meios de cultura na germinação *in vitro*, grãos de pólen dos acessos BGD e BRA foram inoculados em placas de Petri contendo 2 ml de meio de cultura. A viabilidade do pólen foi avaliada por coloração com 1% carmim acético e por germinação *in vitro* a 0, 24, 48 e 72 horas. O meio Lora promove o maior percentual de germinação *in vitro* de grãos de pólen dos dois acessos. Grãos de pólen do acesso AVeBrJ apresentam viabilidade intermediária (66,87%) no ponto máximo da curva de 23,14 horas por germinação *in vitro*. Os grãos de pólen do acesso GBrPF apresentaram alta viabilidade acima de 70% até 120 horas em temperatura ambiente por germinação *in vitro*.

Palavras-chave: *Cocos nucifera*. Cultura *in vitro*. Meio de cultura.

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INTRODUCTION

Storage of pollen grains is important for the preservation of germplasm and to aid in research using stored biological materials to promote exchange and enhance breeding programs (HANNA, 1994). Furthermore, it is considered one of the main alternatives for the conservation of genotypes, which would allow carrying out crossings without the need for alternate cultivation of these pollinating strains in the field (GANESHAN *et al*., 1986).

In palms, the study of pollen grain storage has received special attention due to the production of hybrids being held in pollen grains, fresh and preserved at low temperatures (KARUN *et al*., 2006; KARUN; SAJINI, 2010; SOUSA; SCHEMBERG; AGUIAR, 2010; TOWILL; WATERS, 2000). The evaluation of the germination capacity (viability) during the storage of pollen grains from the male parent is crucial in the process of artificial hybridization (FRANÇA *et al*., 2009).

For the intermediate and long-term storage success, one should master collecting, drying and storage techniques and pollen viability tests. For this, methodologies for these techniques must be developed, since each species behaves differently against these procedures, which can compromise pollen viability (SOUSA; SCHEMBERG; AGUIAR, 2010).

The *in vitro* germination of pollen grains is the most applied technique for pollen viability studies (SOARES *et al*., 2008). Various culture media are used for *in vitro* pollen germination and the Brewbaker and Kwack medium (BREWBAKER; KWACK, 1963) is the most widely applied. Changes in the boron and sucrose concentrations are necessary for optimal germination (TOWILL; WALTERS, 2000).

In palm, the specific conditions for pollen germination are wide and acceptable results are obtained with various sugars in liquid semi-solid culture media, with or without the addition of boric acid. In studies with areca palm (*Areca catechu* L.), tucumã palm (*Astrocaryum vulgare* Mart.) and queen palm (*Syagrus romanzoffiana* (S.) Cham) Liu, Huang and Li (2013), Oliveira, Coutunier and Beserra (2003) and Sousa, Schemberg and Aguiar (2010), respectively, observed high pollen viability. Ranasinghe *et al*. (2010) quantified the response of *in vitro* pollen germination in Asian coconut varieties at different temperatures. There are not reports on the pollen grain viability of Brazilian coconut varieties.

The aim of this study was to select culture medium for the *in vitro* germination of pollen grain of the anão verde do Brasil de Jiqui (AVeBrJ) and gigante do Brasil Praia do Forte (GBrPF) accessions and determine the viability of pollen grain at room temperature.

MATERIAL AND METHODS

Representative plants of anão verde do Brasil de Jiqui - AVeBrJ (Brazil Green Dwarf) and gigante do Brasil Praia do Forte - GBrPF (Brazilian Tall) accessions were select from the Coconut Active Germplasm Bank of Embrapa Coastal Tablelands Sergipe located at the experimental fields of Itaporanga and Betume, Sergipe, Brazil. For each plant, a spathe close to maturation was marked before opening, removed from the plants and maintained at room temperature in the laboratory (28 ± 1 °C) until anthesis. The pollen grains were collected and placed in cryotubes under room temperature.

To evaluate the effect of different culture media on the *in vitro* germination of pollen grains of the AVeBrJ and GBrPF accessions, they were inoculated on to Petri dishes containing 2 ml of culture media (Table 1).

The Petri dishes were kept in biological incubator at 24 ± 1 °C for a 24-hour period. In DMLS Leica microscope, 10X magnification and were analyzed at 0, 24, 48 and 72 hours after inoculation for the number of germinated pollen grains. The pollen grains were considered germinated when showing pollen tube length greater than their diameter. To calculate of *in vitro* pollen germination, the following formula was used:

Table 1 - Composition of the culture media evaluated for *in vitro* germination of coconut accessions.

Culture media	Composition
Brewbaker and Kwack (1963) modified by Sousa, Schemberg and Aguiar (2010)	100 mg L^{-1} H_3BO_3; 300 mg L^{-1} $Ca(NO_3)O_2.4H_2O$; 200 mg L^{-1} $MgSO_4.7H_2O$; 100 mg L^{-1} KNO_3
Lora *et al*. (2006)	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} of sucrose
Sousa, Schemberg and Aguiar (2010)	3 g L^{-1} of agar; 100 g L^{-1} of sucrose
Sousa, Schemberg and Aguiar (2010) modified by the author	80 g L^{-1} of sucrose; 1 g L^{-1} of agar; 100 mg L^{-1} H_3BO_3

In vitro pollen germination (%) = Number of germinated pollen grains / Number of counted pollen grains x 100.

For the determination of the *in vitro* pollen grains viability, pollen grains released from male flowers of the AVeBrJ and GBrPF accessions as previously described were placed in cryotubes and kept under room temperature until the completion of each experimental trial.

The pollen grain viability by *in vitro* germination as previously described and by staining with 1% acetic carmine and *in vitro* germination at the following times after pollen extraction: 0, 24, 48, 72, 96 and 120 hours for BGD and at 0, 24, 48, 96, 120, 168 and 216 hours for the BGD in daily inoculations in the culture medium of Lora *et al.* (2006).

To assess the viability by staining, a pollen sample with approximately 0.02 grams was placed on a slide by adding a drop of 1% acetic carmine followed by homogenization. Then, the slide was placed in a Petri dish and kept in biological incubator at 37 ± 1 °C for 25-30 minutes. In microscope model DMSL (Leica, Bernsheim, Germany) at 10x magnification, with a digital camera model Moticam C2300 (Motic Instruments, Hong Kong, Chine) the slides were analyzed for number of viable and nonviable pollen grains per quadrant.

Pollen grains stained in red were considered viable (by the reaction of the presence of enzymatic activity) and with intact walls and nonviable, those colorless or stained in red with rupture of walls. To calculate of pollen grain viability, the following formula was used:

Pollen viability (%) = Number of stained pollen grains/Number of counted pollen grains x 100

The experimental design of culture medium selection was completely randomized in a 4 x 4 factorial scheme (four culture media combined with four evaluation times) and three replications. For *in vitro* viability test under room temperature, a completely randomized design with six treatments and three replicates was considered for the AVeBrJ accession and a completely randomized design with seven treatments and three replicates for the GBrPF accession.

The viability of pollen grain means were subject to an analysis of variance by F test at a 5% probability and were estimated the regression curves with SISVAR software (FERREIRA, 2011).

RESULTS AND DISCUSSION

There was a significant interaction between culture media and time for the *in vitro* germination of the pollen grains of the AVeBrJ and GBrPF accessions (Table 2).

The culture medium of Lora (LORA *et al.*, 2006) provided higher germination of the pollen tube for both

Table 2 - Summary of the analyses of variance of the pollen grains viability of the AVeBrJ and GBrPF coconut accessions under different culture media (CM) and times (T)

AVeBrJ accession				
Culture media	Time (hours)			
	0	24	48	72
Brewbaker and Kwack (1963) mod.	5.51 Bb	5.27 Bb	12.96 Bb	31.35 Ba
Lora et al. (2006)	58.80 Ab	66.86 Aab	74.98 Aa	65.01 Ab
Sousa, Schemberg and Aguiar (2010)	5.71 Bab	2.01 Bc	10.46 BCab	11.40 Ca
Sousa, Schemberg and Aguiar (2010) mod.	1.31 Ba	0.90 Ba	3.18 Ca	6.78 Ca
VC (%)	17.84			
GBrPF accession				
Culture media	Time (hours)			
	0	24	48	72
Brewbaker and Kwack (1963) mod.	27.48 Ba	31.27 Bb	9.05 Bc	13.49 Bab
Lora et al. (2006)	89.95 Aa	79.54 Aab	74.62 Aab	56.48 Ac
Sousa, Schemberg and Aguiar (2010)	9.28 Ca	10.71 Ca	8.31 Ba	6.50 Ba
Sousa, Schemberg and Aguiar (2010) mod.	16.62 BCa	1.55 Dc	5.10 Bab	5.56 Bab
VC (%)	17.37			

** Significant at 5% significance by the F test.; SV- Source of Variation; SF- Degrees of Freedom; SM- Mean Square

accessions (Table 3 and Figura 1), being notably the best culture medium for studies on the viability of pollen grains by *in vitro* germination of the pollen tube for coconut accessions.

Probably, the high viability shown by pollen grains in the culture medium of Lora *et al.* (2006) is due to the combination of boron, calcium, sucrose and its liquid physical condition. The basic culture medium used in most germination tests of pollen grains is composed of sucrose, and the combination of other nutrients may vary. Boron and calcium are essential for the onset of the intine prolongation and *in vitro* pollen tube formation (BREWBAKER; KWACK, 1963).

Pio *et al.* (2007) and Ramos *et al.* (2008) observed that the boron concentration of 200 mg L^{-1} stimulated the germination of pollen grains of citrus cultivars.

However, the *in vitro* germination of apple pollen grains (*Malus* sp.) had no positive effective in the presence of boric acid (DANTAS *et al.*, 2005). As previously mentioned, the addition of boron to the culture medium showed variable responses, depending on the species, and for the BRA and BGD accessions, the presence of boron was positive.

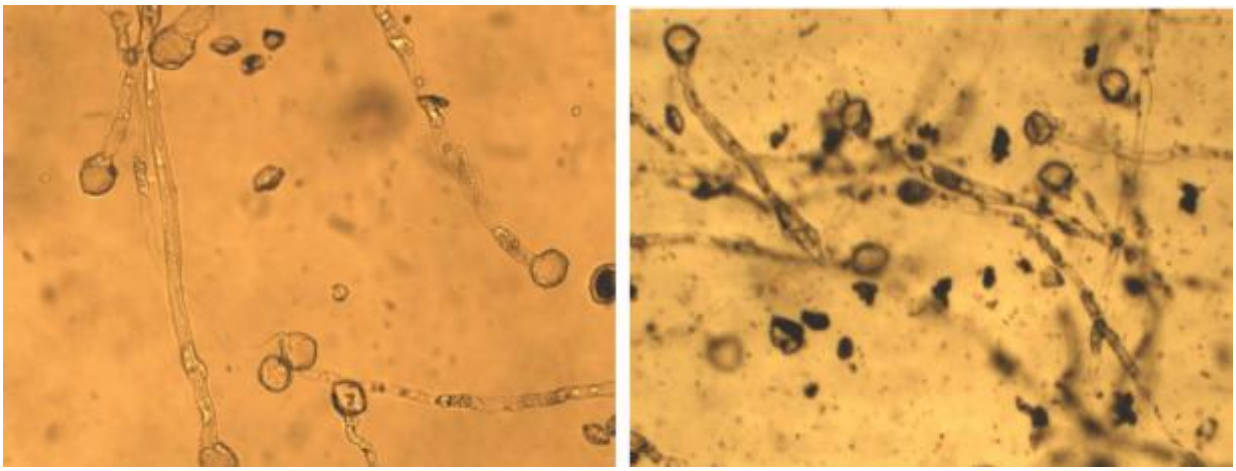
Some broken pollen tubes of the BRA accession in the presence of media of Sousa, Schemberg and Aguiar (2010) and Sousa, Schemberg and Aguiar (2010) modified were observed. According to Salles et al. (2006), pollen tubes may break due to high humidity and changes in the environment caused by increased osmotic pressure and low resistance of the cell wall. Boron stimulates pollen tube growth and decreases the likelihood of breaking up (FRANZON; RASEIRA, 2006). The rupture observed in

Table 3 - Pollen grain viability of the AVeBrJ and GBrPF coconut accessions under different culture media and times by *in vitro* germination

SV	DF	AVeBrJ (GER)		GBrPF (ACA)	
		SM	F	SM	F
Time	5	2279.70	55.74**	29.25	1.725^{ns}
Error	12	40.90		16.75	
VC (%)		14.51		4.44	
SV	DF	BRA (GER)		BRA (ACA)	
		SM	F	SM	F
Time	6	490.46	8.345 **	79.15	6.81**
Error	14	58.77		11.63	
VC (%)		10.36		3.80	

Means followed by same capital letter in the column or small letter in the line do not differ by the Tukey test at 5% significance level. VC- Variation Coefficient; mod- modified.

Figure 1 - General view of the *in vitro* pollen tube germination in the culture medium of Lora (10x): A- AVeBrJ accession; B- GBrPF accession



this study can be attributed to the absence of boron in the composition of the media of Sousa, Schemberg and Aguiar (2010) and Sousa, Schemberg and Aguiar (2010) modified.

Sousa, Schemberg and Aguiar (2010) observed higher *in vitro* germination of queen palm pollen (*Syagrus romanzoffiana* (S.) Cham) in culture medium with 3 g L^{-1} agar and 100 g L^{-1} sucrose.

According to Stanley and Linskens (1974), sucrose promotes osmotic balance between the pollen and the germination medium and provides energy to the development of the pollen tube. Reis *et al.* (2011) worked with banana pollen (*Musa* sp.) and observed that the standard culture medium for the germination of pollen grains supplemented with 15% sucrose provided higher germination percentage when compared to medium without sucrose and supplemented with 6.7% of the carbohydrate.

There was a significant effect of time on the viability by *in vitro* germination of pollen grains of the AVeBrJ accession submitted at room temperature. There was no effect of time on the viability by staining (Table 4).

The viability by *in vitro* germination of pollen grains of the AVeBrJ accession presented a quadratic variation with significant decrease after 23,14 hours. The viability by staining with 1% carmine acetic acid presented a cubic variation (Figure 2).

The highest viability by *in vitro* germination of the pollen tube obtained by the maximum point of the regression curve was the 66.87% at 23.14 hours after inoculation.

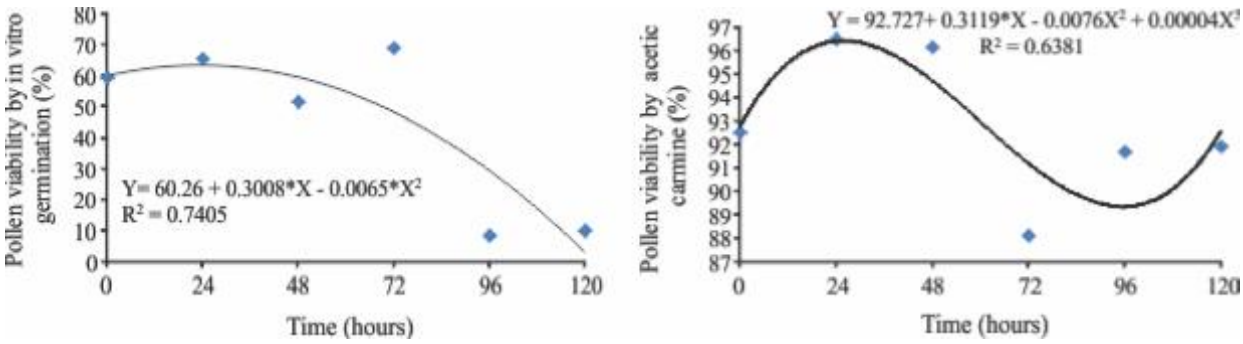
The AVeBrJ accession reached the highest viability compared with results obtained by Armendariz *et al.* (2006) for Malayan green dwarf (MGD) coconut. This reinforces that viability depends on the genotype and other factors, as reported by Ganeshan *et al*. (2008). In *Passiflora suberosa*, Cruz *et al.* (2008) the viability obtained by *in vitro* germination showed smaller value (21.82%).

Table 4 - Summary of the analyses of variance of the pollen grain viability of the AVeBrJ and GBrPF coconut accessions in different times under room temperature by *in vitro* germination (GER) and staining with 1% acetic carmine (ACA)

SV	DF	AVeBrJ (GER)		GBrPF (ACA)	
		SM	F	SM	F
Time	5	2279.70	55.74**	29.25	1.725^{ns}
Error	12	40.90		16.75	
VC (%)		14.51		4.44	
SV	DF	BRA (GER)		BRA (ACA)	
		SM	F	SM	F
Time	6	490.46	8.345 **	79.15	6.81**
Error	14	58.77		11.63	
VC (%)		10.36		3.80	

** Significant at 5% significance by the F test.; SV- Source of Variation; DF- Degrees of Freedom; SM- Mean Square; VC – Variation Coefficient.

Figure 2 - Response of the pollen grains viability of the AVeBrJ accession at different times under room temperature by *in vitro* germination and staining with 1% acetic carmine



* Parameters of the equation show for the model $p < 0.05$

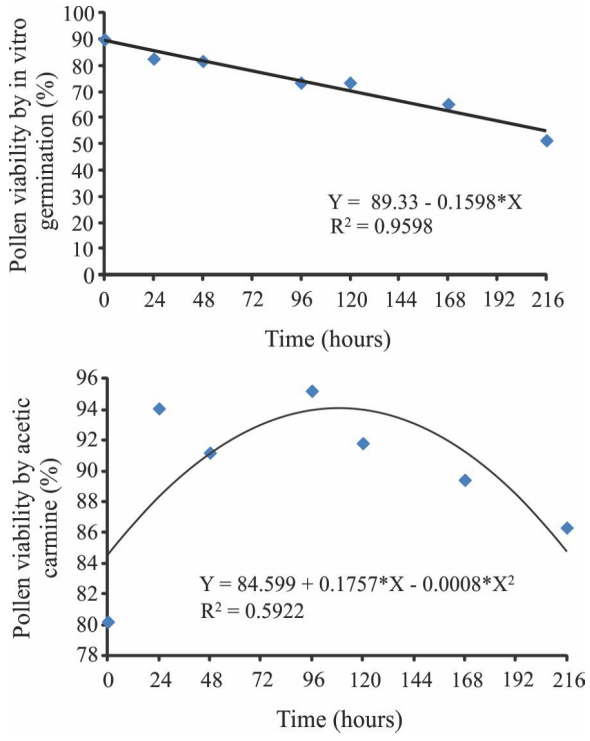
There was a significant effect of time on the viability of pollen grains of the GBrPF accession by germination of the pollen tube and by staining at room temperature (Table 4). The viability by in vitro germination presented a decrease linear with values above 70% up to 120 hours and the viability by staining with 1% carmine acetic acid presented a quadratic variation with significant decrease after 109.81 hours (Figure 3). The highest viability obtained by the maximum point of the regression curve was the 94.24% at 109.81 hours after inoculation.

According to Souza, Pereira and Martins (2002), values above 70% are considered as high pollen viability; from 31 to 69% as intermediate and up to 30% as low pollen viability. The results of this study indicate intermediate and high viability values up to nine days after the extraction of pollen grains of GBrPF accession. Viability times higher than those reported by Santos *et al*. (1996) indicate viability under field conditions for *Cocos nucifera* L. of only six days.

In the MGD coconut, Armendariz *et al*. (2006) found that the viability obtained by tetrazolium solution showed higher values (93.4 ± 8.9%) than those obtained by *in vitro* germination (40.2 ± 15.6%). The authors also reported the highest variation coefficient for results obtained for viability by *in vitro* germination compared to tetrazolium and suggest that the *in vitro* germination test should be used to assess the pollen grain viability of *Cocos nucifera* L. Probably the variation be explained by the fact that observed the methodologies involving dyes solution weren't efficient to estimate pollen viability evaluation, because of the deficient pollen staining, as reported by França *et al.* (2009). Einhardt, Correia and Raseira (2006) worked with peach pollen grain and observed that the method used for assessing the viability of pollen grain by staining overestimates viability while the *in vitro* test underestimates it. This may explain the antagonism found between results obtained using both methods.

However, the efficiency of the use of dyes has been variable depending on the species and positive results have been reported for various staining techniques on different plant species (ABDELGADIR; JOHNSON; VAN STADEN, 2012).

Figure 3 - Response of the pollen grains viability of the GBrPF accession at different times under room temperature by *in vitro* germination and staining with 1% acetic carmine



* Parameters of the equation show for the model $p < 0.05$

CONCLUSIONS

1. The culture medium of Lora (2006) is suitable to assess the *in vitro* germination of pollen grain of the AVeBrJ and GBrPF accessions;
2. The pollen grain of the AVeBrJ accession present intermediate viability (66.87%) at room temperature up to 23.14 hours by *in vitro* germination;
3. The pollen grain of the GBrPF accession present high viability (above 70%) at room temperature up to 120 hours by *in vitro* germination.

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