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Viability, production and morphology of pollen grains for different species in the genus *Manihot* (Euphorbiaceae)

Lívia de Jesus Vieira[1,6], Taliane Leila Soares[1], Mônica Lanzoni Rossi[2], Alfredo Augusto Cunha Alves[3], Francisco de Assis Ribeiro dos Santos[4] and Fernanda Vidigal Duarte Souza[5]

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RESUMO

(Viabilidade, produção e morfologia de grãos de pólen de diferentes espécies do gênero *Manihot*, Euphorbiaceae). O objetivo deste trabalho foi caracterizar a viabilidade, produção e morfologia de grãos de pólen de diferentes espécies do gênero *Manihot*. Botões florais de acessos do gênero *Manihot* foram coletados dos bancos de germoplasma da *Embrapa Mandioca e Fruticultura*. A viabilidade do pólen foi avaliada por testes *in vitro*, *in vivo* e testes colorimétricos. A estimativa da produção de pólen foi realizada por meio da contagem do número de grãos de pólen produzidos por botão floral. O diâmetro do pólen foi determinado medindo-se o comprimento transversal do grão. O delineamento experimental utilizado foi o inteiramente casualizado. Estudos da ultraestrutura polínica foram realizados por meio de microscopia eletrônica de varredura. A viabilidade dos grãos de pólen é elevada nos testes colorimétricos, intermediária nos testes *in vivo* e não houve germinação *in vitro*. A produção média observada entre todos os acessos foi de 1.253 grãos de pólen. Verificou-se que nos acessos silvestres, o tamanho variou de 132 a 163 µm, e de 129 a 146 µm nos acessos cultivados. Os grãos de pólen de todos os acessos analisados são muito grandes, apolares, esféricos e inaperturados, com exina ornamentada com pilos organizados no chamado padrão-*Croton*. Os acessos silvestres, de maneira geral, produzem mais pólen e apresentam maior tamanho, quando comparados com os cultivados.

Palavras-chave: *Manihot esculenta* Crantz, pré-melhoramento, recursos genéticos

ABSTRACT

(Viability, production and morphology of pollen grains for different species in the genus *Manihot* (Euphorbiaceae)). The objective of this work was to characterize the viability, production and morphology of pollen for different species in the genus *Manihot*. Floral buds from *Manihot* accessions were collected from two germplasm banks at Embrapa Cassava & Fruits. The viability of the pollen was assessed via colorimetric, *in vitro* and *in vivo* assays. The diameter of the pollen grains was determined by measuring the transversal length of the grain. The experimental design was entirely randomized. Studies on pollen ultrastructure were performed via scanning electron microscopy. Pollen viability was high in the colorimetric tests and intermediate *in vivo* tests; there was no germination in the *in vitro* tests. The average production for all accessions was 1,253 pollen grains per floral bud. The size of the pollen grains varied from 132 to 163 µm in the wild accessions, and 129 to 146 µm in the cultivated accessions. The pollen grains for all accessions were very large, apolar, spherical as well as inaperturate, with an exine ornamented with pila organized in a *Croton* pattern. The wild accessions, in general, produced more and larger pollen grains compared with the cultivated accessions.

Key words: *Manihot esculenta* Crantz, pre-breeding, genetic resources

[1] Universidade Federal do Recôncavo da Bahia, Cruz das Almas, BA, Brazil
[2] Universidade de São Paulo, Centro de Energia Nuclear na Agricultura, Piracicaba, SP, Brazil
[3] Embrapa LABEX, USDA/ARS National Center for Genetic Resources Preservation, Fort Collins, CO, USA
[4] Universidade Estadual de Feira de Santana, Feira de Santana, BA, Brazil
[5] Embrapa Mandioca e Fruticultura, Cruz das Almas, BA, Brazil
[6] Autor para correspondência: liviabiol@gmail.com

Introduction

Cassava (*Manihot esculenta* Crantz), a species originally from the American continent, is a halophyte and a perennial shrub that belongs to the Euphorbiaceae. It is drought tolerant and can adapt to a wide range of soil and climate conditions. Its tuberous roots, which are rich in starch, are used as food for humans and animals as well as raw material for different industries. The wild cassava species are essential for a breeding program because they have a high level of variability, can adapt to a broad spectrum of conditions, and therefore they offer many traits for developing more productive plants, such as resistance to pests and disease, as well as a higher tolerance to abiotic stresses (Horsfall & Abia 2003; Nassar *et al.* 2007 a,b).

However, primary problems using wild *Manihot* species in improvement programs include a lack of synchrony during the flowering periods, as well as difficulty with the propagation and maintenance of these species in the field (Ceballos *et al.* 2002). There is little information regarding pollen grain fertility for wild cassava species, despite their potential use in crosses for hybrid production.

The viability of pollen grains can be estimated using a variety of colorimetric methods (Munhoz *et al.* 2008). In addition to these methods, *in vivo* and *in vitro* germination can be used as tools to test viability. The *in vitro* method is the most used in these studies because it is an efficient method, fast and easy to observe (Marcellán & Camacho 1996; Geetha *et al.* 2004; Chiai *et al.* 2009). However, results from these viability assessments in cassava have not been encouraging, thus demanding additional efforts in this regard (Orrego & Hershey 1984; Mbahe *et al.* 1994).

Studies addressing pollen viability, production and its morphology are, therefore, necessary and important for cassava breeding programs and for supporting future crosses between cultivated and wild *Manihot* species.

Materials and methods

This study was performed from March 2009 to January 2010 using wild and cultivated cassava accessions from the germplasm banks at Embrapa Cassava & Fruits in Cruz das Almas, Bahia. The average annual rainfall for the region is 1,224 mm, the average annual temperature is 23.8 ºC, and the relative air humidity is 80%.

Pollen viability

Floral buds, at the pre-anthesis stage, were collected from eight different available *Manihot* accessions; six wild species and two varieties from the cultivated species, *Manihot esculenta* Crantz (Table 1). For the *in vitro* germination test, pollen grains were obtained from flowers during pre-anthesis between 12:00h and 14:00h. Pollen grains from approximately ten flowers were obtained and immediately transferred to Petri dishes, without a disinfection procedure, in three culture medias: (1) 0.03% $Ca(NO_3)4H_2O$, 0.02% $Mg(SO_4)7H_2O$, 0.01% KNO_3, 0.01% H_3BO_3, 15% saccharose, solidified with 0.8% agar, the pH adjusted to 7.0; (2) 0.08% $Ca(NO_3)4H_2O$, 0.02% H_3BO_3, 10% saccharose, solidified with 1.0% agar, the pH adjusted to 6.5; and (3) 0.015% H_3BO_3, 0.045% $Ca_3(PO_4)_2$, 25% saccharose, solidified with 0.6% agar, the pH adjusted to 6.5. After inoculating the pollen grains, the Petri dishes were maintained in the dark, at a controlled temperature of 27±1 ºC for 24 hours, before counting the germinated pollen grains via a microscope.

For *in vivo* germination, all accessions were crossed with the same female parent plant, the 'Rosa' variety (BGM 260), due to the great number of female flowers available. Ten female flowers were covered with fabric bags and, while the flowers were open, manual crosses were performed during the hottest time of the day, from 12:00h to 14:00h, when the stigmas were most receptive (Mbahe *et al.* 1994). The pollen grains were collected from the different *Manihot* species at pre-anthesis (Table 1). Pollen grains were considered viable if they formed fruits. The viability was estimated as a percentage.

To analyze pollen viability with stains, the same accessions examined in the *in vitro* tests were used. The pollen was scattered on a glass slide and treated with three types of stain, acetic carmine (Kearns & Inouye 1993), Sudan IV (Baker & Baker 1979) and Alexander's (Alexander 1980), to investigate the most efficient stain for estimating pollen viability.

After preparing the slides, the pollen grains were observed for ten minutes after being stained. Using a microscope to observe a sample population of the stained pollen grains, we counted 100 grains/slide/accession, performing three replications for each treatment, for a total of 300 pollen grains for each stain tested.

The experimental design was entirely randomized, for each 9 x 3 (accessions x stains) factorial treatment, with three replications. Each replication consisted of three slides. Before the analysis of variance, the data, expressed as percentages, was arcsine-transformed to meet the ANOVA assumptions. Means were compared by Tukey's grouping test, with a 5% probability, and by using SAS (2000) computer software.

Production and pollen grain size

Floral buds in pre-anthesis were extracted from 15 different available accessions of *Manihot*; five of them were from cultivated *M. esculenta* varieties, and 10 accessions were from wild species (Table 2).

The estimation of pollen production was determined by counting the number of pollen grains produced per floral bud. For each genotype, three flowers were collected and placed in 3.5 x 2 cm plastic jars. The pollen grains were washed in 1.0 mL of 70% ethanol and microcentrifuged for one minute at 2000 rpm. After this procedure, the supernatant was carefully discarded to avoid losing the precipitated

material. Finally, the pollen grains were suspended in 1.0 mL of 50% glycerol (Vidal *et al*., 2008).

Five slides containing 30.0 µL of the pollen grain-glycerol suspension were prepared from each flower, totaling fifteen slides per accession. The pollen grains that remained adhered to the flower stamens were placed on a glass slide, immediately covered with a cover slip and then counted to include these grains in the total pollen count. The pollen diameter was determined by a random measurement of 45 pollen grains from each accession evaluated.

To estimate pollen production in each accession, three replications were evaluated, with each replication consisting of five slides; five pollen grains were sampled for the pollen size estimate. The data were subjected to an analysis of variance, and the means were compared via the Scott-Knott test with a 5% probability. These statistical analyses were performed using SAS (2000) software.

Exine ultrastructure

For this study, we collected male flowers from each accession. Pollen grains were retrieved and treated by placing them in Eppendorf tubes with a modified Karnovsky fixing solution, consisting of 2% glutaraldehyde, 2% paraformaldehyde, 5 mM calcium chloride buffered with sodium cacodylate (0.05 M pH 7.2), for 48 hours. Samples were dehydrated using a series of increasing ethanol concentrations (35%, 50%, 60%, 75%, 85%, 95% and 100%). Samples were critical point dried using liquid CO_2. Pollen grains were then immediately mounted on metallic platforms, coated with gold and observed using a Zeiss LEO 745 VP scanning electron microscope (SEM).

Results

All of the accessions had a high level of pollen viability when evaluated using the colorimetric techniques (Table 1). The *in vivo* germination tests showed high viability for the pollen grain from accessions FLA 005-05 and PER-002-02, with 71% and 60%, respectively. There was intermediate viability for the accessions 'Rosa', Aipim bravo, CEC-A019-13 and VIO-A 001-14, with 40%, and low viability (25%) in

Table 1. Viability (%) of cassava pollen grains evaluated using three different stains and *in vivo* experiments (fruit formation). Means followed by the same lowercase letter on the same line and means followed by the same uppercase on the same column are not significantly different using Tukey's test, 5% probability.

Accessions	Acetic carmine	Sudan IV	Alexander's	*In vivo*
CEC A019-13 (M. *cecropiaefolia* Pohl)	95.7 abA	97.3 aA	95.0 abA	40.0 B
DIC 602-06 (*M. dichotoma* Ule)	95.00 aA	72.0 bD	91.7 aB	0.0 D
Aipim Bravo (*M. esculenta* Crantz)	83.0 aC	80.0 aC	90.7 aB	40.0 B
Rosa (*M. esculenta* Crantz)	75.0 aC	62.7 abD	76.0 aC	40.0 B
FLA 005-05 (*M. flabellifolia* Pohl)	90.7 aB	87.3 aB	88.6 aB	71.0 A
PER 002-02 (*M. peruviana* Müll. Arg.)	98.0 aA	98.0 aA	88.0 bB	60.0 A
TOM 001-06 (*M. tomentosa* Pohl)	84.7 bC	91.0 abB	95.0 aA	25.0 C
VIO A 001-14 (M. *violacea* Pohl)	92.3 abB	91.3 abB	95.3 aA	40.0 B

Table 2. Pollen grain production per flower and pollen grain size for the wild and cultivated varieties of cassava. Measurements recorded with the same letter belong to the same group determined by the Scott-Knott test, 5% probability.

Species	Accessions	Average production	Diameter of pollen grains (µm)
M. anomala Pohl	ANO 049V-05	1.165.0 d	142.0 b
M. dichotoma Ule	DIC 001	1.963.0 b	163.0 a
M. dichotoma Ule	DIC 587-05	3.637.6 a	160.6 a
M. esculenta Crantz	BGM 001	1.193.3 d	129.0 c
M. esculenta Crantz	BGM 116	932.0 d	132.0 c
M. esculenta Crantz	BGM 549	613.3 d	142.0 b
M. esculenta Crantz	COL 2215	863.7 d	146.0 b
M. esculenta Crantz	'Rosa'	965.5 d	145.3 b
M. flabellifolia Pohl	FLA 005-01	730.0 d	147.0 b
M. flabellifolia Pohl	FLA 005-05	697.7 d	136.0 c
M. flabellifolia Pohl	FLA 029-01	579.3 d	153.0 a
M. peruviana Müll. Arg.	PER 005-01	634.3 d	138.0 c
M. tomentosa Pohl	TOM 001-18	1.351.7 c	140.0 b
M. tomentosa Pohl	TOM 001-24	1.156.0 d	135.0 c
M. violacea Pohl	VIO A001-14	1.823.0 b	143.0 b

accession TOM-001-06. One exception to these results was observed in accession DIC 602-06 (*M. dichotoma* Ule), which did not produce fruit.

From the eight accessions studied, five were significantly different for the three stains used. The highest values, above 90% for all stains, were observed for accessions CEC A019-13 (*M. cecropiaefolia* Pohl) and VIO A001-14 (*M. violacea* Pohl), which, in turn, formed fruits in 40% of the *in vivo* viability tests (Table 1).

The integrity of the chromatin was confirmed using acetic carmine, and the mean percentage of pollen grains that stained with an intense red was 88% (Table 1 and Figure 1). The Alexander dye assay suggested that 90% of the pollen grains had intact protoplasm and cell walls, as indicated by the pink color of the protoplasm and the fine green contour of the cell wall (Table 1 and Figure 2).

With Sudan IV, the pollen grains from all of the accessions had a positive reaction, indicating the presence of lipids in the pollen grains both internally and externally. In the latter, reactivity may have been due to the pollenkitt.

In the estimation of pollen viability, no *in vitro* germination was observed for all evaluated species in any of the three media proposed. Further, we did not observe a correlation between the colorimetric assays and the *in vivo* viability experiments.

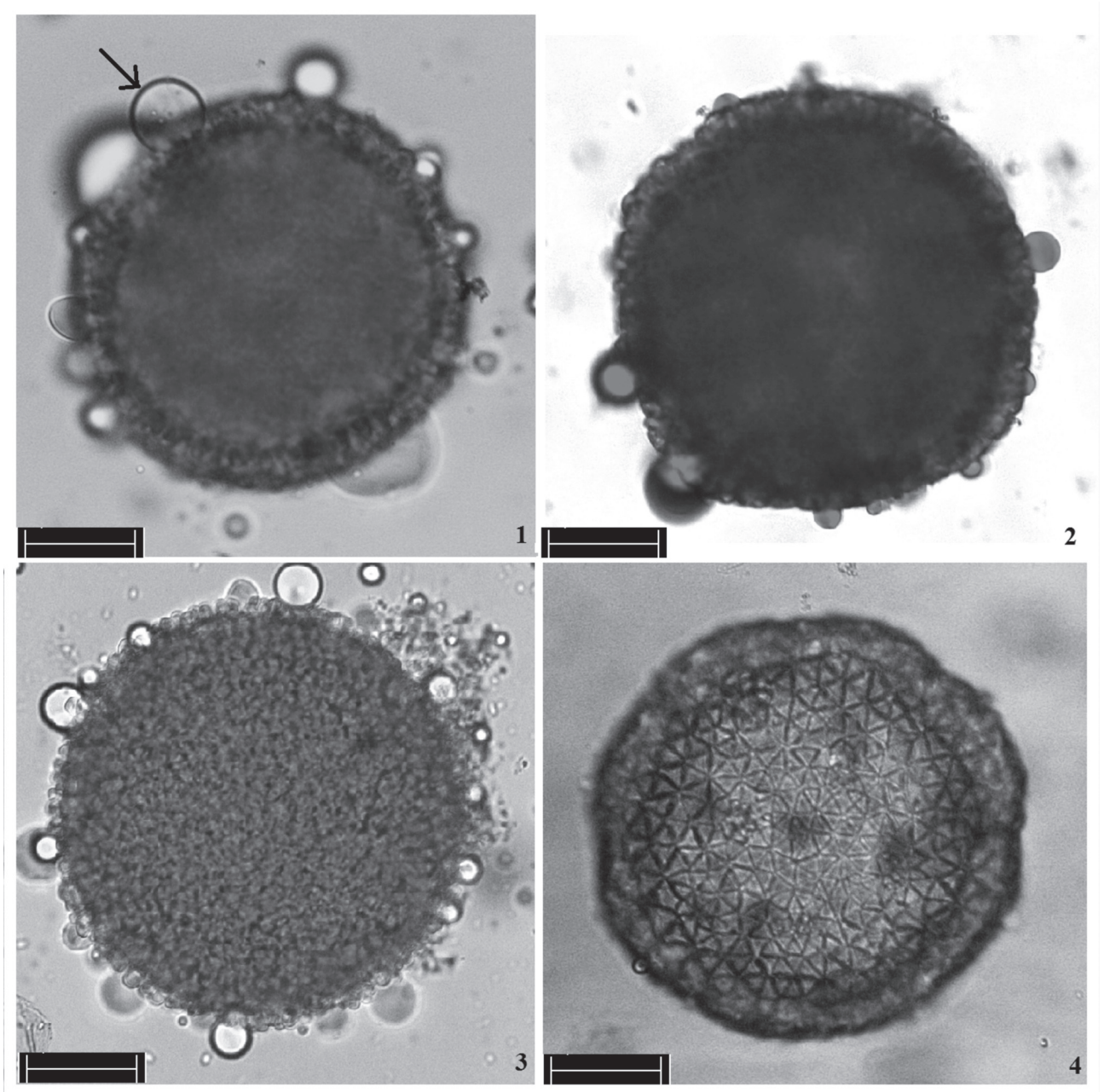

Figure 1-4. Viability of cassava pollen grains. 1. Stained with acetic carmine, detail for pollenkitt (arrow); 2. Stained with Alexander solution; 3. Stained with Sudan IV; 4. Detail of stained pollen (unviable - arrow) with Alexander solution. Scales = 50 µm.

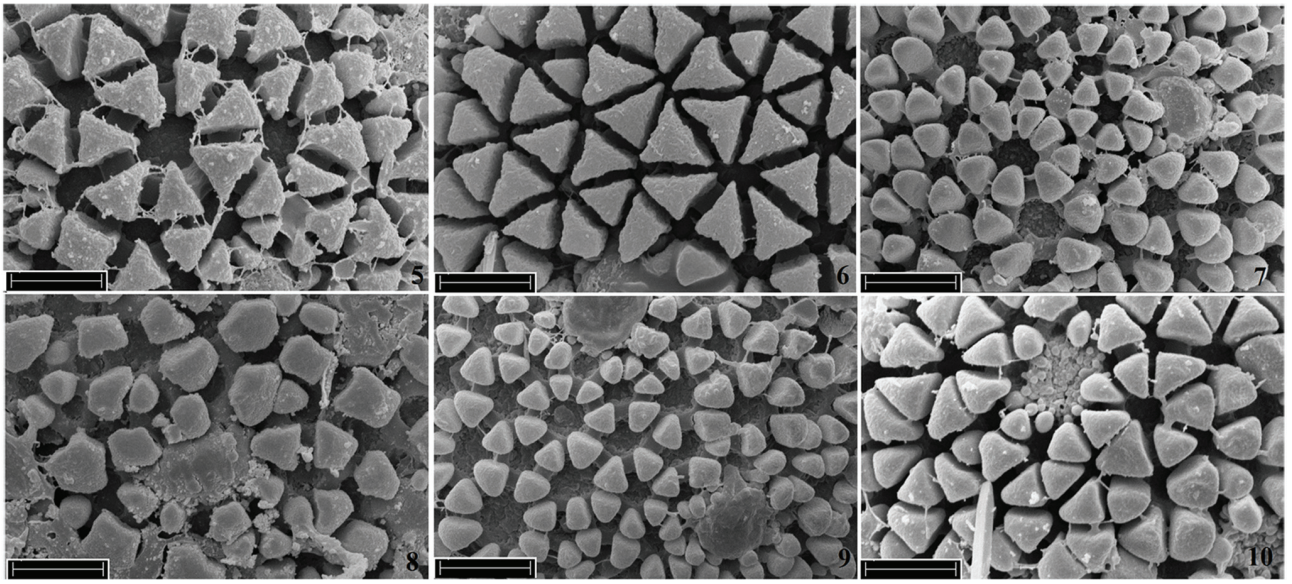

Figure 5-10. Electron micrographs of *Manihot* spp. pollen grains. 5. *M. flabellifolia* (FLA 005-07); 6. *M. dichotoma* (DIC 602-06); 7. *M. tomentosa* (TOM 001-18); 8. *M. violacea* (VIO A001-14); 9. *M. cecropiaefolia* (CEC A019-13); 10. *M. esculenta* Crantz ('Rosa'). Scales = 10 μm.

A highly significant variation in the production of pollen grains was observed among the *Manihot* accessions (Table 2). In general, pollen production was higher for wild accessions than in the cultivated cassava varieties. Among the wild species, the accession with the highest pollen grain production was DIC 587-05, while the lowest values were obtained from FLA 029-01. It was verified that, for the cultivated cassava varieties, the largest number of pollen grains obtained was significantly smaller compared with the wild accessions DIC-001, DIC-587-05, TOM 001-18 and VIO A001-14 (Table 2).

The pollen grains from all of the accessions were evaluated with an SEM (Fig. 5-10). The pollen grains are very large (>100 μm), apolar, spherical, inaperturate, with an exine ornamented with pila organized in the so-called *Croton* pattern. The ornamentation predominantly has 6-7 subunits positioned over a circular area and with sub-triangular, psilate clava, as well as small, rounded projections on surface; no granules were observed in the lumen of these subunits. Ornamentation of the exine was most similar between *M. flabellifolia* Pohl (FLA 005-07), *M. dichotoma* Pohl (DIC 602-06) and *M. esculenta* Crantz of the 'Rosa' variety (Figures 5, 6 and 10, respectively).

For accessions of *M. tomentosa* Pohl (TOM 001-18) and *M. violacea* Pohl (VIO A001-14) (Figures 7 and 8, respectively), the head of the pila was more globular in the first and more irregular in the latter; however, the *M. violacea* Pohl pollen grains had the same arrangement as those in *M. tomentosa* Pohl. In contrast, in *M. cecropiaefolia* Pohl (CEC A019-13), the *Croton* pattern was more defined, and the pila had a head with a transversal triangular section.

Significant differences were observed in the diameter of the pollen both among different species and among accessions within the same species. All of the accessions presented pollen grains with diameters larger than 100 μm. Thus, it was possible to establish three size classes: pollen grains <140 μm, 140 to 149 μm and ≥150 μm (Table 2).

The largest pollen grains were observed in *M. dichotoma* Pohl (in both accessions) and *M. flabellifolia* Pohl (accession FLA 029-01). *Manihot flabellifolia* Pohl presented the largest variation in pollen size among the accessions, and it has accessions with pollen grains placed in all of the three size classes established in this study. Pollen grains of the accessions from *M. esculenta* Crantz varied between two size classes, while, in *M. peruviana* Müll. Arg., the accession evaluated contained pollen grains set to the smaller size class.

Discussion

The results presented here corroborate those discussed in the literature about cassava, where the pollen viability rate (assessed with stains) in species of *Manihot* was above 60% (Silva *et al.* 2001; Vidal *et al.* 2008).

The inefficiency of using these stains in assessing pollen viability has been previously suggested by other authors (Munhoz *et al.* 2008). However, other factors like barrenness, incompatibility between accessions and stigma receptibility can influence fruit production besides pollen viability. Even so, this type of stain-based experiment allows certain important inferences to be made regarding the integrity of the pollen grains, is a relatively safe method to estimate the amount of viable pollen grains and, therefore, should not be ignored.

Studies on pollen *in vitro* germination in species of Euphorbiaceae are rare. However, an adequate number of

studies have been performed on *in vitro* germination of pollen grains of castor bean (*Ricinus communis* L.), a plant belonging to the cassava family. Vargas (2006) obtained a low percentage of *in vitro* castor bean germination in all of the treatments evaluated, with a variation ranging from 0.4 to 0.82% for germinating pollen grains. One potential hypothesis is that there may be a specific substance found in the stigma that induces pollen tube formation. The results from that author did not differ from those obtained by Orrego & Hershey (1984) who, when studying *in vitro* germination of *Manihot esculenta* Crantz pollen, also did not observe the growth of the pollen tube in any of the culture media assessed. On the other hand, Mbahe *et al.* (1994) found a variation from 0.4 to 0.57% for pollen germination in cultivated cassava.

The lower pollen production in the cultivated cassava varieties and the wild species *M. flabellifolia* Pohl and *M. peruviana* Müll. Arg. could represent one of the consequences from the initial steps in the domestication process. The process primes the vegetative propagation of the species to the detriment of sexual propagation and, consequently, the use of pollen. For genetic improvement of cassava, particularly concerning the use of wild accessions, the production of pollen could be a relevant trait, especially if the need to store pollen arises from asynchrony during the flowering periods. The rate of adherence for pollen in cassava crosses is generally low, thus, crosses involving the same parents will likely depend directly on the quantity of available viable pollen.

The sizes of the pollen grains from the accessions are within the degree of variation that Vidal *et al.* (2008) found, in which the diameter of the pollen grains varied from 128 to 169 μm. There are few studies addressing *Manihot* pollen morphology, and size is a variable that ought to be considered, as it can potentially influence the compatibility between accessions.

From these results, it is possible to assert that the capacity for pollen production and the size of the pollen grains produced are important traits that may be factors in the reproductive behavior for the different varieties, directly affecting the genetic flow for each variety (Williams & Rouse 1990). DIC 602-06 (*M. dichotoma* Ule) was the accession that showed the most disparate traits in pollen grain size and pollen production compared with the cultivated species (*M. esculenta* Crantz). This difference seems to be related to the genetic incompatibility between DIC 602-06 and the cultivated Cassava 'Rosa' accession, because the wild accession showed higher pollen productivity and larger grains. These data are important when the objective is to select parent plants for interspecific hybridizations.

The study of pollen morphology is important, particularly for plant identification and taxonomic studies, allowing inferences to be made regarding the potential crosses between species. An important observation to note from this study is that lipids adhered to the cassava pollen grain cell wall, suggesting the presence of pollenkitt or tryphine, a substance secreted by the tapetum and related to protection against water loss and normal pollen grain development, which guarantees its viability (Pacini & Franci 1993; Shivanna *et al.* 1997).

Regarding the exine, Nowicke (1994), studying 13 species of *Croton* (Euphorbiaceae) with an SEM, verified that there is a variation in the size, distribution and ornamentation for subunits in the *Croton* pattern. The observations made by this author are very similar to those made for the *Manihot* species in this study.

The results from the production and the size of pollen grains in both groups, cultivated and wild, are similar, which suggests that there is a greater evolutionary proximity among them, and would explain the results from the *in vivo* experiment, where 70% of the crosses produced fruits. This is consistent with the hypothesis that *M. flabellifolia* Pohl descended from two *M. esculenta* Crantz ancestors (Allen 1999). The same argument can be made for *M. peruviana* Müll. Arg. (PER 002-02) that had 60% viable crosses and similarities in pollen production, pollen grain ultrastructure and size.

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