Research Article

Pollen viability and germination in *Elaeis oleifera*, *Elaeis guineensis* and their interspecific hybrid[1]

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ABSTRACT

Elaeis oleifera chromosomes are similar to those of *E. guineensis*, with close gene pools for the production of interspecific O x G hybrids. The pollen viability and germination of *E. oleifera* 'Coarí' and *E. guineensis* 'La Mé' were compared to their interspecific hybrid O x G ('Coarí' x 'La Mé'). The pollen viability was determined by the acetocarmine staining method (0.5 %) and the pollen germination by *in vitro* incubation on agar-sucrose medium (1.2-11.0 g in 100 mL of distilled water). The pollen viability and germination of the 'Coarí' x 'La Mé' hybrid were significantly lower than those of their parents. The percentage of pollen viability by acetocarmine staining was higher than that of *in vitro* germination, indicating that not all pollen grains classified as viable germinated on the agar-sucrose medium. The pollen germination test is a more reliable indicator than the staining viability test, because the latter only reveals that the pollen contains the enzymes necessary to initiate germination, while the germination test determines the emission and development of the pollen tube.

KEYWORDS: Plant physiology, acetocarmine, pollination.

RESUMO

Viabilidade e germinação de pólen em *Elaeis oleifera*, *Elaeis guineensis* e seu híbrido interespecífico

Os cromossomos de *Elaeis oleifera* são semelhantes aos de *E. guineensis*, com pools genéticos próximos para a produção de híbridos interespecíficos O x G. A viabilidade e germinação do pólen de *E. oleifera* 'Coarí' e *E. guineensis* 'La Mé' foram comparadas com as de seu híbrido interespecífico O x G ('Coarí' x 'La Mé'). A viabilidade do pólen foi determinada pelo método de coloração com acetocarmina (0,5 %) e a germinação por incubação *in vitro*, em meio ágar-sacarose (1,2-11,0 g em 100 mL de água destilada). A viabilidade e germinação de pólen do híbrido 'Coarí' x 'La Mé' foram significativamente menores do que as de seus progenitores. A porcentagem de viabilidade do pólen pela coloração com acetocarmina foi superior à germinação *in vitro*, indicando que nem todos os grãos de pólen classificados como viáveis germinaram no meio ágar-sacarose. O teste de germinação de pólen é um indicador mais confiável que o de viabilidade por coloração, pois este apenas revela que o pólen contém as enzimas necessárias para iniciar a germinação, enquanto o teste de germinação determina a emissão e desenvolvimento do tubo polínico.

PALAVRAS-CHAVE: Fisiologia vegetal, acetocarmina, polinização.

INTRODUCTION

Interspecific oil palm O x G hybrids are obtained from the cross between the American oil palm (*Elaeis oleifera* HBK Cortes) and the African oil palm (*Elaeis guineensis* Jacq.). They are a valuable alternative for oil production, due to characteristics such as tolerance to pests and diseases, acceptable fresh fruit bunch production (20-25 t ha^{-1}), high oil quality (oleic 55 %; linoleic 11 %) and higher contents of vitamin E (Mozzon et al. 2013) and carotenes (Rey et al. 2004, Peláez et al. 2010, Rivera et al. 2013, Choo & Nesaretnam 2014).

The cultivated area for the O x G hybrid in Colombia is approximately 35,700 ha, representing 6.4 % of the total area cultivated with oil palm in the country (Sispa 2015). O x G hybrids produce few male inflorescences and have low pollen viability, what indicates that a certain genetic imbalance occurs (Alvarado et al. 2000). Peduncular bracts or spathes cover female inflorescences, hindering the pollen entry. These characteristics affect natural pollination and, therefore, bunch production and oil yield per hectare.

Hybrid plants may present some type of pollen sterility. Male sterility in plants implies an inability

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to produce or release functional pollen and is the result of a failure in the formation or development of functional stamens, microspores or gametes. Rodriguez-Rojas et al. (2015) pointed out that gametes of mutants and interspecific hybrids are mostly sterile.

In pollination, the pollen grain is transferred and reaches the stigma of flowers and, then, by germinating and emitting the pollen tube, may cause a double fertilization of the ovary (Dafni & Firmage 2000). There are fundamental differences between the terms pollen germination and pollen viability. Viability refers to the fact that the pollen grain has all the necessary enzymes that enable it to germinate (Dafni & Firmage 2000, Shivanna & Tandon 2014), while germination refers to the fact that the pollen grain emits and develops a pollen tube when it reaches the stigma of flowers (Gaaliche et al. 2013, Shivanna & Tandon 2014). Therefore, viability tests do not necessarily indicate whether the pollen is capable of reaching the ovary to fertilize the ovules, whereas germination evaluates the pollen's ability to produce a pollen tube as a measure of its fertility (Godefroid et al. 2010).

One of the techniques used to evaluate the pollen viability is acetocarmine staining, which has been widely described by some authors (Pio et al. 2007, Sánchez & Romero 2013). Acetocarmine binds to the core chromatin of viable pollen grains, which is dyed in shades ranging from pink to deep red, while sterile pollen grains are often constricted, do not stain, and remain translucent (Marutani et al. 1993). The determination of pollen viability and germination are fundamental to breeding programs, in order to guarantee the formation of improved varieties and new hybrids; however, there are few studies dedicated specifically to comparing the germination performance of hybrid pollen with that of its progenitor species.

This study performed a comparative analysis of pollen viability and germination of the American oil palm 'Coarí' (*Elaeis oleifera* HBK Cortes) and the African oil palm 'La Mé' (*Elaeis guineensis* Jacq.) with those of their interspecific hybrid 'Coarí' x 'La Mé'.

MATERIAL AND METHODS

The study was carried out at the Indupalma plantation, in San Alberto, Cesar, Colombia (10º20'N, 73º11'W and altitude of 125 m), with maximum temperature of 34 ºC, minimum temperature of 22 ºC, relative humidity of 72.3 %, annual rainfall of 2,497 mm, annual evaporation of 1,208 mm, 2,130 hours of sunshine/year, and agroecological conditions corresponding to a tropical humid forest life zone - bh-T.

Pollen from the American oil palm 'Coarí' (*Elaeis oleifera*), African oil palm 'La Mé' (*Elaeis guineensis*) and their interspecific hybrid 'Coarí' x 'La Mé' were used. The pollen samples were collected from an experimental plot known as 'Jardín Granero' (Indupalma Company) during the 2017 crop season. The female and male parents for the interspecific hybrid of the breeding population of restricted origin were 'Coarí' and 'La Mé', respectively. For the pollen collection, on all genotypes, the male inflorescences were identified in pre-anthesis II (stage 602), corresponding to a 30 % tearing of the peduncular bracts (Hormaza et al. 2012). The male inflorescences were covered with isolation bags fitted with an elastic band, to avoid contamination from surrounding natural pollen.

The male inflorescences were cut at anthesis (stage 607), according to a scale proposed by Hormaza et al. (2012) (Figure 1), where more than 70 % of the male flowers were open and almost ready to release pollen. Afterwards, they were taken to the laboratory and the finger-like spikelets were separated on Kraft paper and dried in a controlled convection oven, for 12 hours, at 39 ºC, to humidity of 6 %. Then, they were shaken carefully to release pollen grains from the anthers, which were sieved with nº. 100 and 200 sieves to remove impurities, vacuum-packed and stored at -4 ºC (Rodríguez et al. 2011).

To determine the viability, the staining method was used with a 0.5 % acetocarmine solution (Pio et al. 2007). Each pollen sample was spread on slides with a swab, and two drops of acetocarmine were added and covered with coverslips for a better dispersion. After 10 minutes, the pollen grains were observed under a fluorescence microscope with 40× magnification. Four experimental units were established for each material, and each material was observed under four fields of view. Each field observation was made for 50 pollen grains. To photograph the viable grains, an Olympus CX21 microscope coupled to a 10-megapixel digital camera was used, and the counts were done with the ImageJ™ software (Rasband 2018). The pollen grains that stained red or pink were considered viable, signifying the presence of

Figure 1. Development stages of the male inflorescence for pollen collection. A) Stage 602; B) bagging; C) stage 607.

nuclear chromatin, and grains pale or translucent were considered non-viable.

The viability percentage was calculated using the following formula: Viability (%) = [(number of viable grains)/(number of total grains)] × 100, where the number of total grains = viable grains + non-viable grains.

For germination, the medium described by Turner & Gilbanks (1974) was used, consisting of 11 g of sucrose and 1.2 g of agar dissolved in 100 mL of distilled water and boiled for 5 minutes; then, the medium was placed in Petri dishes and allowed to cool to room temperature. The pollen was sprinkled on Petri dishes and incubated for two hours at 35 ºC (Chia et al. 2008). The readings were taken at 40× magnification on an Olympus CX21 microscope, and the pictures were taken with a 10-megapixel digital camera coupled to the microscope. Only those that developed a pollen tube greater than or equal to the diameter of the pollen grain were counted as germinated grains (Kakani et al. 2002), using the ImageJ™ software.

The germination percentage was calculated using the following formula: Pollen germination (%) = [(number of germinated pollen grains)/(total number of pollen grains on a slide)] × 100, where the number of total grains = germinated grains + non-germinated grains.

A completely randomized design was used to evaluate the pollen viability and germination of each genotype. The genotypes were 'Coarí', 'La Mé' and the interspecific hybrid 'Coarí' x 'La Mé'; and the experimental units were four per treatment, with four Petri dishes per experimental unit. The generated data were subjected to analysis of variance and correlation analysis, and the mean comparison carried out with the Tukey test ($p \leq 0.05$), using the SAS™ 9.0 software.

RESULTS AND DISCUSSION

The acetocarmine staining made it possible to easily differentiate the viable pollen grains, which were stained in shades from pink to deep red, and non-viable pollen grains, which did not stain but remained translucent or stained very faint pink (Figure 2). The analysis of variance showed highly significant differences between the genotypes for the viability methods with acetocarmine staining and *in vitro* germination (Table 1). There were significant differences between the 'Coarí' and 'La Mé' genotypes and the O x G hybrid (Figure 3). There was a great variation in the 'La Mé' genotype, with viability of 85 % and germination of 62 %. The general average pollen viability for all genotypes was 62.95 %, while the germination was 51.70 % (Table 1). Regarding the

Table 1. Analysis of variance for pollen germination and viability from the American oil palm 'Coarí' (*Elaeis oleifera* HBK Cortes), African palm 'La Mé' (*Elaeis guineensis* Jacq.) and their interspecific hybrid.

Source of variation	Degrees of freedom	Mean square	
		Viability	Germination
Genotype	2	6,567.59**	5,350.44**
Error	9	4.24	6.20
CV (%)		3.23	4.18
Average		62.96	51.70

** Highly significant ($p < 0.01$).

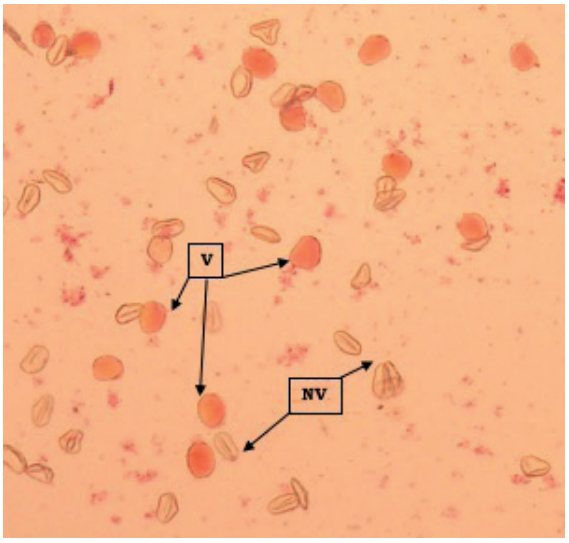

Figure 2. Microscope observation of pollen viability with acetocarmine staining. The viable pollen grains (V) are stained red and the non-viable pollen grains (NV) are unstained and translucent.

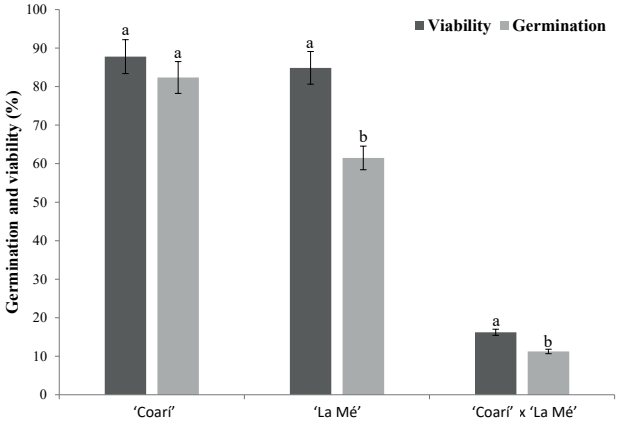

Figure 3. Germination and viability of pollen from American oil palm 'Coarí' (*Elaeis oleifera* HBK Cortes) and African palm 'La Mé' (*Elaeis guineensis* Jacq.), compared to their interspecific hybrid 'Coarí' x 'La Mé'. Bars with different letters for each variable (viability or germination) are statistically different according to the Tukey test ($p \leq 0.05$).

cultivars, there were significant differences between them for pollen viability using both methods. The average viability was higher for 'Coarí' (85.08 %), followed by 'La Mé' (73.18 %) and the O x G hybrid (13.71 %) (Figure 3).

Figure 3 shows that the percentage of pollen viability by acetocarmine staining was higher than for *in vitro* germination, indicating that not all pollen grains classified as viable germinated in the agar-sucrose medium. In many species, *in vitro* pollen germination is dependent on the addition of key substrates, such as calcium nitrate, boron and magnesium, to the germination media. Indole-3-acetic acid promotes pollen tube growth in the pistil and stimulates pollen tubes to grow long and straight (Abdelgadir et al. 2012). However, staining tests with nuclear dyes, such as acetocarmine, are very accurate for determining pollen fertility, because they indicate the integrity of the nucleus when dyeing the chromosomes (Sunilkumar et al. 2013). In general, it is expected that staining methods overestimate the pollen viability (Sulusoglu & Cavusoglu 2014). Instead, an *in vitro* germination test can yield results that better approximate the actual pollen viability, as the sucrose-agar medium emulates the stigma exudate where pollen is deposited (La Porta & Roselli 1991). The low viability observed in the pollen of 'Coarí' x 'La Mé' was a consequence of its origin, hybridization between genetically close parents, as evidenced in a previous study (Rodríguez-Rojas et al. 2015). While some hybrids can break postzygotic barriers, it is expected that they present a lower fertility, relatively to the parental species (Marques et al. 2011). The grains of pollen of the hybrid present low percentages of viability and germination due to the fact that they vary in size and form, with regard to *E. oleifera* and *E. guineensis*, and, during their formation, they undergo frequent divisions of abnormal cells (Sánchez & Romero 2013).

The germination of pollen grains was evident 2 hours after incubation in the sucrose-agar medium (Figure 4). However, the percentage of pollen germination in the hybrid 'Coarí' x 'La Mé' was significantly lower than that in the female 'Coarí' or male 'La Mé' (Figure 3). The pollen fertility of the interspecific hybrid was very low, as a consequence of its hybridization between the American 'Coarí' and African 'La Mé' palms, with a common genetic origin. This agrees with the findings of Alvarado et al. (2000), who found 6.2 % of pollen germination for the hybrid 'Amazon' (O x G), as compared to 66.9 % in the mother *E. oleifera* and 61.8 % in the father *E. guineensis*. Related species develop reproductive barriers to avoid hybrid formation and maintain species integrity (Xie et al. 2017). Reproductive barriers to gene flow include prezygotic barriers that act before fertilization, preventing interspecific gene flow, and postzygotic barriers that reduce the fertility of hybrids (Widmer et al. 2009).

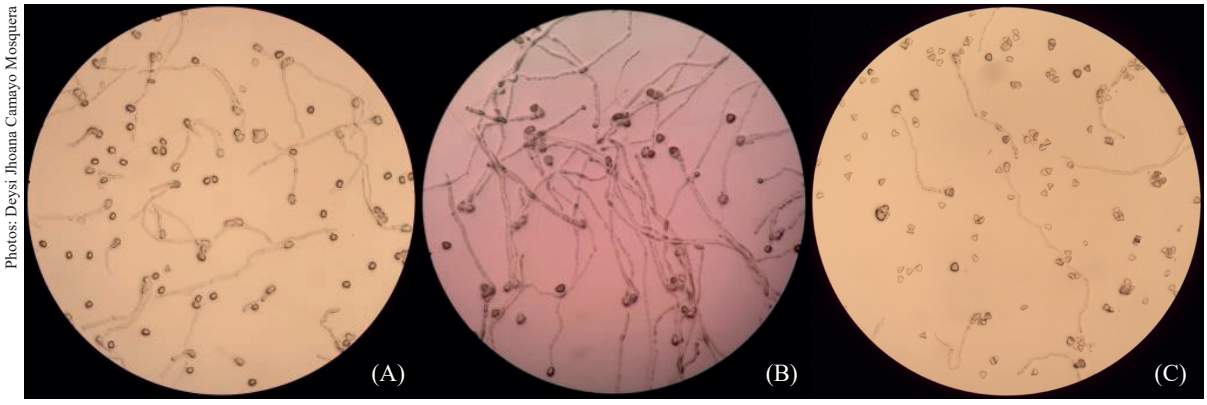

Figure 4. *In vitro* pollen germination under 10× microscopic magnification for the genotypes 'Coarí' (A) and 'La Mé' (B) and their interspecific hybrid 'Coarí' x 'La Mé' (C). Germinated pollen grains develop a pollen tube.

When the pollen grain reaches the receptive stigma, it imbibes water and germinates, giving rise to the pollen tube, which grows very quickly. This rapid growth of the pollen tube indicates the ability of the pollen grain to complete the next stage of ovary fertilization (Godefroid et al. 2010, Shivanna & Tandon 2014) and, therefore, is a rapid determinant of its fertility. Rejón et al. (2010) reported that the conditions for germination in an *in vitro* medium were different from those of the stigma of the flower, and, therefore, the germination percentage obtained in a laboratory test will always be different from that achieved under natural conditions. These differences have been demonstrated in comparative studies between *in vitro* pollen germination and germination in the presence of stigmatic tissues of flowers or pistils extracts (Abdelgadir et al. 2012).

Notably, the pollen germination does not express its viability alone, but it is an estimator. The germination loss does not indicate that the pollen has died, but that the conditions are not optimal for germination. Sunilkumar et al. (2011), in a study carried out to determine the optimal culture medium for oil palm pollen germination, confirmed that, despite the basic requirement of an artificial medium for pollen tube growth, the optimal composition may vary depending on genotype requirements. The use of non-optimal culture media could underestimate the pollen quality. This suggests that, in nature, the stigma of female flowers provides the optimal conditions for pollen to germinate normally. In the case of O x G hybrids, the composition and concentration of the stigmatic fluid are still unknown, and further studies are needed.

Sunilkumar et al. (2011) asserted that the germination rate of pollen in culture media is a more reliable indicator than the estimated viability of staining, what leads to the presumption that pollen that does not germinate or that shows little development of a pollen tube in the usual laboratory tests is very likely not to be effective at fertilizing the ovary. In fact, the viability test only reveals whether the pollen contains the enzymes required to initiate germination, while the germination test assesses the emission and development of a pollen tube. Therefore, viability does not necessarily indicate whether the pollen is capable of reaching the ovary to fertilize the ovules, while germination does (Godefroid et al. 2010). Thus, a high value on the viability test (> 90 %) does not guarantee that the pollen will normally germinate in the stigma of the flowers, nor low pollen germination (< 70 %) indicates that the pollen is not suitable to guarantee a successful pollination of inflorescences. Additionally, some extrinsic factors, including temperature, may affect the pollen germination (Lin et al. 2017).

In the present study, a highly significant ($p < 0.01$) and positive correlation coefficient of 0.9663 was observed for the association between viability and *in vitro* germination percentage, that is, a low viability implied a low pollen germination percentage and vice-versa, while a high viability generated high germination values for the pollen grains.

The production of bunches and oil depend on abiotic and biotic factors among the pollen supply; therefore, assisted pollination is a mandatory agronomic practice in O x G interspecific hybrids that have low pollen viability and germination,

with respect to their progenitors 'Coarí' and 'La Mé'. Since the success of assisted pollination in O x G hybrid crops fundamentally depends on pollen viability and germination, it is necessary to perform laboratory tests before applying pollen to commercial lots, because the results may vary with the prevailing weather conditions.

CONCLUSIONS

1. The low pollen viability and germination observed for 'Coarí' x 'La Mé' confirmed the male sterility that characterizes the hybridization between genetically similar parents;
2. The pollen germination test is a more reliable indicator than the staining viability test; therefore, it is recommended to carry out both tests before applying commercial pollen.

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