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Storage of pollen and properties of olive stigma for breeding purposes[1]

Armazenamento de pólen e propriedades do estigma de oliveira para fins de melhoramento genético

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ABSTRACT - In order to ensure success in controlled hybridizations in olive tree cultivation, the information on pollen viability and stigma receptivity is essential. The aim was to establish methodologies that increase the preservation of pollen viability and to establish the time to perform crossbreeds in hybridization studies with olive trees. Three experiments were performed with plants from the cultivar Arbequina, in Maria da Fé, MG, Brazil. In the first experiment, the description of the flower events was performed. In the second, anthers were desiccated in eppendorfs, being stored at three different conditions for pollen viability test: room temperature (27 °C), refrigerator (8 °C) and freezer (-10 °C). In order to evaluate the *in vitro* germination, culture medium for olive pollen grains was used. In this respect, pollen grains were transferred in Petri dish containing culture medium and placed in a BOD (biochemical oxygen demand) chamber at 28 °C for 60 h, being counted. The first evaluation was performed prior to the assembly of the experiment, testing the initial viability, whereas the second occurred 24 h after storage. Subsequently, seven evaluations were performed fortnightly. In the third experiment, the stigma receptivity was verified by the 3% hydrogen peroxide method, with flowers in pre-anthesis, anthesis and post-anthesis, evaluated hourly in the period from 7 a.m. to 6 p.m. for three days. In the description of the flower events, it was verified that the olive tree shows diurnal anthesis, with flower opening between 10 a.m. and 11 a.m. The anthers stored in a freezer preserved the viability for 60 days and the stigmas were receptive since the pre-anthesis.

Key words: *Olea europaea* L.. Pollen viability. Pollen grains. Hybridization.

RESUMO - Para assegurar o sucesso em hibridizações controladas na cultura da oliveira, é fundamental informações sobre a viabilidade polínica, assim como a receptividade do estigma. Objetivou-se estabelecer metodologias que aumentem a preservação da viabilidade polínica e estabelecer o momento para se realizar cruzamentos em trabalhos de hibridização com oliveiras. Foram realizados três experimentos com plantas da cultivar Arbequina, em Maria da Fé, MG. No primeiro experimento realizou-se a descrição dos eventos florais. No segundo, anteras foram dessecadas em eppenddorfs, sendo armazenados em três condições diferentes para teste de viabilidade polínica : temperatura ambiente (27 °C), geladeira (8 °C) e freezer (-10 °C). Para avaliação da germinação *in vitro*, utilizou-se meio de cultura para grãos de pólen de oliveira. Para isso, grãos de pólen foram transferidos em placa de Petri, contendo meio de cultura e colocadas em estufa do tipo BOD (Demanda Biológica de Oxigenio) a 28 °C por 60 h, sendo contabilizados. A primeira avaliação foi realizada antes da montagem do experimento, testando a viabilidade inicial: a segunda 24 horas após armazenamento. Posteriormente, foram realizadas sete avaliações quinzenais. No terceiro experimento verificou-se a receptividade estigmática pelo método de peróxido de hidrogênio 3%, com flores em pré-antese, antese e pós-antese, avaliados a cada hora, no período de 7 h as 18 h por três dias. Na descrição dos eventos florais, verificou-se que a oliveira apresenta antese diurna, com abertura floral entre 10 e 11 horas. As anteras armazenadas em freezer preservam a viabilidade por 60 dias e os estigmas apresentaram-se receptivos desde a pré-antese.

Palavras-chave: *Olea europaea* L.. Viabilidade polínica. Grãos de pólen. Hibridação.

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INTRODUCTION

The olive tree (*Olea europaea* L.) is characterized as an allogamous plant with self-incompatibility of pollen-pistil or gamete, being observed an abundant flowering and a low fertilization (lower than 20% of the flowers are differentiated into fruits) (KOUBOURIS; METZIDAKIS; VASILAKAKIS, 2009). Its inflorescences are constituted by actinomorphic flowers with regular symmetry, composed of four sepals, four petals, two stamens and one central pistil (OLIVEIRA *et al*., 2012).

Brazil is the eighth largest importer of olive oil and table olive in the world (COI, 2017). Research on olive groves has been performed in some regions of the states of Rio Grande do Sul and Minas Gerais (SILVA *et al.*, 2012a).

The carpometric trait of fruits and olive pits is decisive to define the exploitation potential of the cultivar, either highlighting its purpose of usage, for olive oil extraction or canning. In this respect; some cultivars are highlighted, such as Negroa, MGS ASC322, MSG MISS293, MGS JB1, MGS GRAP556, MGS GRAP541 and Arbequina (SILVA *et al.*, 2012b).

Arbequina cultivar is the most commonly used by the producers and hence is the most representative cultivar in the Brazilian olive groves among the cultivars with productive potential evaluated in the South and Southern regions (SILVA *et al*., 2016). It is a cultivar originating from Spain used to produce olive oil, which was very well adapted to the environmental conditions of the South and Southeast of Brazil (SILVA *et al.*, 2012a).

Currently, the productive performance of different cultivars introduced in the recent years in the country is already known (SILVA *et al*., 2012b). Brazil also has varieties developed from Brazilian breeding programs, whose study was performed based on natural hybridizations, with subsequent evaluation of the performance of genotypes from these crossbreeds (OLIVEIRA *et al*., 2012). However, the new premise for breeding studies would be the use of controlled hybridizations, in which information would be obtained from both parents, increasing the possibility of finding a cultivar with the desired characteristics within a selection study.

To ensure success in hybridizations, the pollen should essentially show high rate of viability and germination. Theoretically, the pollen collection in the proper and correctly prepared flower stage does not require a viability test (BRITO *et al.*, 2010).

However, there is often no possibility of immediate use, such as in cases where there is a lack of flower synchronicity among the cultivars, or when the pollen grains are collected in a region far from that of hybridization. In these situations, it is necessary to evaluate the viability before using the material, as well as to test and develop techniques that extend its viability (MOURA; MACHADO; LÈDO, 2015).

The reproductive structure denominated stigma is another fundamental factor, since the aptitude of flowers in the fertilization process is directly related to its receptivity to the pollen grain. Such receptivity can last only a few hours, such as in some species of the family Anacardiaceae, or several days as in several species of the family Solanaceae (SCHIFINO-WITTMANN; DALL'AGNOL, 2002). In some plants, the ovary receptivity is indicated by the moisture of the estimate, which allows adhering the pollen grain. (SCHIFINO-WITTMANN; DALL'AGNOL, 2002).

Therefore, the aim was to establish methodologies that preserve pollen viability over time and to determine the moment of stigmatic receptivity of olive flowers to perform crossbreeds in hybridization studies.

MATERIAL AND METHODS

The present study was performed with the cultivar Arbequina, aged eight years, in Maria da Fé, MG, Brazil (22°18'51" S, 45°23'24" W and 1,276 m altitude) between August and September 2015 (SILVA *et al.*, 2016).

1st experiment: description and flower events

For the determination of anthesis, daily quantitative observations of 50 flower buds from 10 randomly selected individuals were performed and noted from 7 a.m. to 6 p.m. for 20 days (BRITO *et al.*, 2010). In this phase, the main flower opening time, flower synchronicity (within an inflorescence and among different inflorescences), onset of anthesis and flower longevity were observed. Results were expressed as a percentage.

2nd experiment: conservation of pollen grains

In this second phase, 15 plants were selected, being collected randomly 560 flower buds at "balloon" stage, in pre-anthesis in the morning.

In the laboratory, the anthers were separated with the aid of a forceps, being placed four anthers by eppenddorfs, totaling 280 eppenddorfs. Afterwards, the tubes were desiccated with silica gel and maintained at 28 °C for 24 h, so that the anthesis occurred and the pollen grains had their moisture content reduced (CUCHIARA; SILVA; BOBROWSHI, 2012). Then, the test was performed to determine the initial viability of

pollen grains, using 10 eppendorfs, being one per Petri dish, using culture medium specific for olive pollen germination (SILVA *et al.*, 2016). Subsequently, the remainder of eppendorfs were distributed among the treatments (90 epperddorfs per treatment).

The used treatments consisted of keeping the pollen grains stored under three different conditions for later viability test: room temperature (27 ºC), refrigerator (8 °C) and freezer (-10 °C). After 24 h, a new evaluation was performed, followed by seven evaluations fortnightly. A total of 10 eppendorfs per treatment (replicates) were tested in each evaluation period.

The experimental design was completely randomized in a 9 x 3 factorial design (storage time x environments) and 10 replicates, each replication consisting of one Petri dish, with the content of 1 eppendorf, where germinated and non-germinated pollen were counted in five randomly selected fields of view.

In order to determine the viability of pollen grains, the *in vitro* germination method was used through culture medium specific for germination of olive pollen grains. The culture medium consisted of 4 g L^{-1} agar plus 90 g L^{-1} sucrose, 400 mg L^{-1} of boric acid and pH adjusted to 5.79, in the absence of calcium nitrate and magnesium sulfate, kept for 60 h at 28 °C (SILVA *et al.*, 2016).

For each stage, the pollen grains were transferred with the aid of a brush to a Petri dish surface containing 20 mL of culture medium. Subsequently, the Petri dishes were kept in germination chamber (BOD) in the absence of light at 28 °C for 60 h, being counted germinated and ungerminated pollen grains using a microscope with 10x objective lens (SILVA *et al.*, 2016).

The pollen grain, whose length of the pollen tube exceeded twice the diameter was considered as germinated (FIGUEIREDO *et al.*, 2013). This experiment was performed in a completely randomized design with three replicates, being each replicate consisting of one eppendorfs and five fields of view per Petri dish.

3rd experiment: receptivity of stigma

Stigma receptivity was tested using the 3% hydrogen peroxide (H_2O_2) method. This method consists of the deposition of 3% hydrogen peroxide on the stigma of flowers, in which the stigmas that showed bubble formation were considered as receptive (KEARNS; INOUYE, 1993).

For this experiment, a completely randomized design was used, and the evaluations were performed from 7 a.m. to 6 p.m. for three days, with 10 replicates. Stigma receptivity was evaluated in three flower development stages: pre-anthesis, anthesis and post-anthesis.

For the characterization experiment of flower events, a description of flower habits was performed and compared with those available in the literature. All the results were recorded through photography and expressed as percentage.

For the experiments of pollen viability and stigma receptivity, the data were submitted to an analysis of variance, being the pollen viability experiment subjected to linear or quadratic regression at 5% probability level. The stigma receptivity experiment was subjected to Scott-Knott test at 5% error probability. All of the analyses were performed on the Sisvar® statistical software (FERREIRA, 2011).

RESULTS AND DISCUSSION

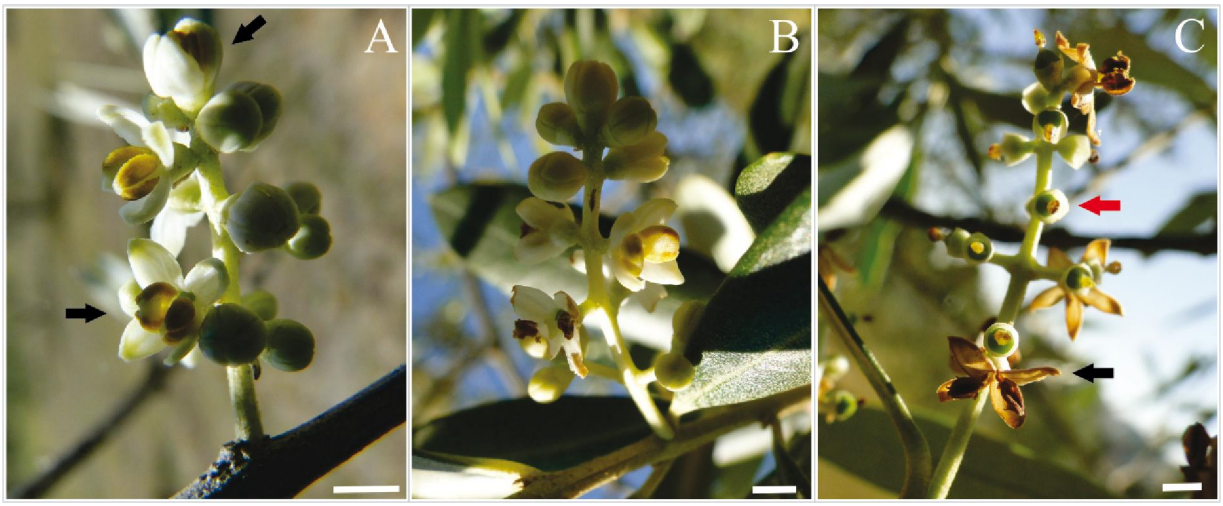
During the evaluation of flower events, it was verified that all the flower buds of the inflorescences did not show a synchronous opening (lower than 6%), thus observing flowers at different stages in the same inflorescence (Figures 1A, B and C).

This information goes against the strategy of several fruit species in temperate regions, where the plants come into dormancy in autumn and winter, showing a fast and simultaneous flowering during the spring, guaranteeing quick pollination and fertilization, which causes homogeneity in the fruit development (GUO *et al.*, 2014).

However, such non-uniformity in olive flowering is intrinsic to the species, similarly as other phenological records found in the literature (OLIVEIRA *et al.*, 2012; SADOK *et al.*, 2013; SANZ-CORTÉS *et al.*, 2002). This fact is probably due to the olive tree diminishing the metabolic activity, but without coming into complete dormancy in the colder seasons, which affects the synchrony during the differentiation of flower buds in the spring. Furthermore, thermal oscillations during the winter period may lead to the physiological decompensation of these buds, specifically related to the chill accumulation, also causing uniformity and reduction in flowering (GUO *et al.*, 2014).

Also during the evaluation, it was observed that in the pre-anthesis (closed bud), there was no release of pollen grains on the stigma; all the flowers showed indehiscent anthers at the time of flower opening (Figure 1A), refuting the possibility of occurrence of cleistogamy in flowers.

Figure 1 - Inflorescences of olive tree (*Olea europaea* L.), Arbequina cultivar. (A) Onset of flowering, showing pre-anthesis flowers with different sizes and flowers at the beginning of the anthesis - detail of non-dehiscent anthers (arrows). (B) Inflorescence containing flowers in pre-anthesis, anthesis and post-anthesis in the same inflorescence. (C) Post-anthesis of all flowers of an inflorescence - detail for the senescence of the corolla (black arrow), flowers without corolla, with stigma showing an intense yellow coloration (red arrow). Bar: 5 mm



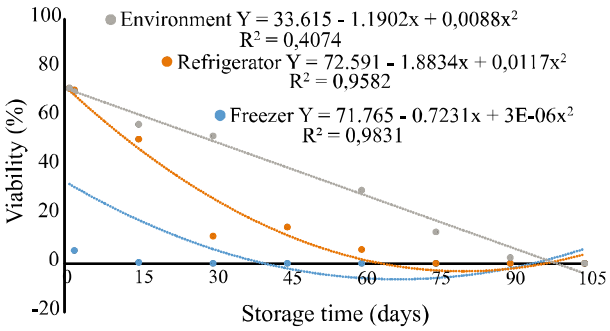
Flower opening occurs more frequently between 10 a.m. and 11 p.m. However, the anthers are not dehiscent in 82% of the evaluated flowers; its desiccation and release of pollen grain occur gradually throughout two or more days in 80% of the flowers (Figure 1A). This characteristic of gradual release of pollen grains throughout the days is sharper in plants with anemophily, in which the temperature and relative humidity directly influence the anther desiccation and release of the pollen grain (MARTIN; CHAMECKI; BRUSH, 2010).

Flower longevity (period between anthesis and corolla senescence) in cv. Arbequina was approximately one week in 84% of the analyzed flowers. This information also corroborates with Suàrez, Castro and Rapoport (2012), which worked with morphological and histochemical alterations of the pistil of olive trees and found during their phenological evaluation that it can take more than a week between the onset of anthesis and its senescence.

In the pollen grain conservation experiment, it is verified through linear regression (Figure 2) that the pollen viability was drastically decreased at room temperature after the second day of storage (close to 5%), being that the material no longer responded to the germination test in culture medium after 40 days.

For several studies with fruit trees, 30% germination of pollen grains is considered an acceptable rate to ensure good fertilization and fruiting (ALBUQUERQUE JÚNIOR, 2010; SILVA *et al.*, 2016). Considering the germination rate around 5% (Figure 2), it can be considered as very low for the use of this material in hybridization studies in olive trees.

Figure 2 - Pollen viability of olive grains (*Olea europaea* L.), Arbequina cultivar, stored for 105 days at different storage locations (room temperature at 27 °C, refrigerator at 8 °C and freezer at -10 °C



Regarding the other treatments, the storage of pollen grains at 8 °C showed a viability around 56%, being that there was a drastic decrease in the pollen germination after that period, becoming unviable after 63 days (Figure 2).

The freezer storage method at -10 °C showed higher results in relation to the other treatments, preserving the material viability for longer time (Figure 2). At 60 days of storage, the pollen grains still had a germination rate around 30%, i.e., an acceptable parameter for the use, according to the literature (ALBUQUERQUE JÚNIOR, 2010; HAUAGGE; BRUCKNER, 2002). Fifteen days after this period, the material still showed a low germination rate when subjected to the culture medium, becoming completely unviable only 99 days after the experiment.

With respect to the material viability, it is possible to observe that the pollen grains stored at 27 °C (ambient temperature) lost viability quickly. This fact may be related to the high temperature of the environment, which maintains or accelerates the material's metabolic activity, making it unviable quickly. The storage at ambient temperature also makes the material more susceptible to moisture absorption, which accelerates the metabolism, besides making pollen grains more susceptible to attack by fungi and microorganisms (KOUBOURIS; METZIDAKIS; VASILAKAKIS, 2009).

It was also possible to observe that treatments with lower temperatures showed greater durability. The use of low temperatures is related to the reduction of the metabolism of pollen grains, which favors greater longevity of the material (CUCHIARA; SILVA; BOBROWSHI, 2012).

Regarding the stigma receptivity evaluation, it was verified that there was no statistical difference among the treatments; the stigmas of all development stages and at all times show bubble formation in the stigma cavity when subjected to the 3% hydrogen peroxide test, indicating activity of the peroxidase enzyme (Figure 2).

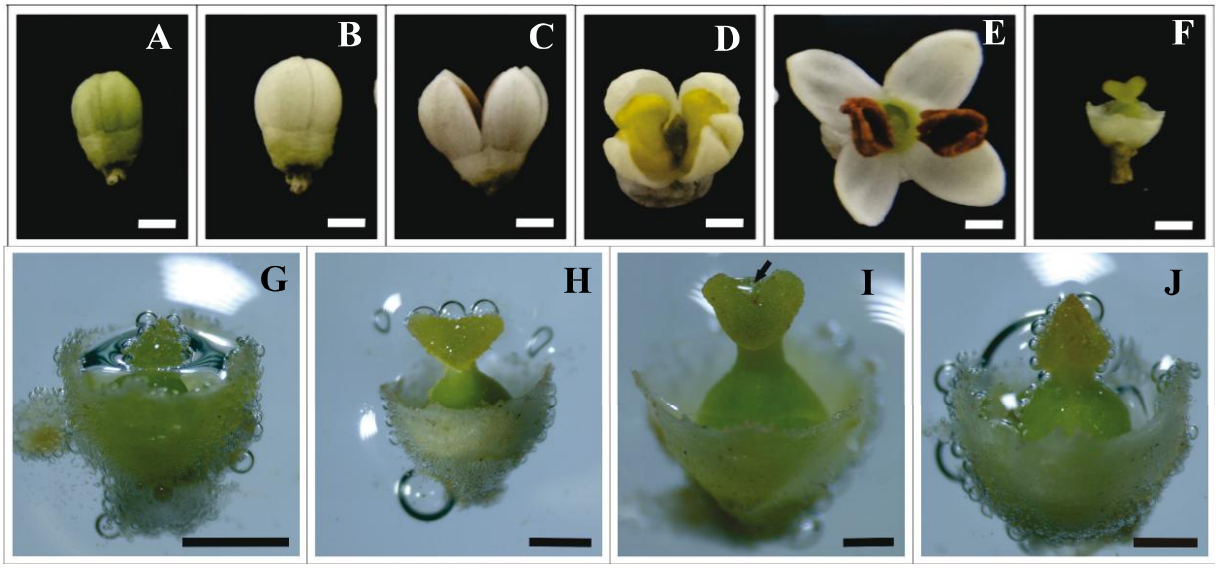
These results are highly relevant for breeding experiments. Once the pre-anthesis flowers are receptive, controlled hybridization can be performed without contamination of pollen grains other than the selected material (Figures 3A, 3B, 3G and 3H).

However, it is recommended to the breeder the choice of developed flower buds and as close as possible to the anthesis (Figures 3B and 3H), since although the stigma of olive tree is receptive, it is only close to the flower anthesis that the style transmission will begin to break the starch and hence provide nutrients for the development of the pollen tube (SUÀREZ; CASTRO; RAPOPORT, 2012).

Regarding the time of an open flower, the duration of gynoecium receptivity will be directly related to the supply of pollen grain in the environment (LANKINEN; MADJIDIAN, 2011). In other words, the higher the pollen grain supply, the lower the time of stigma reception, the lower the pollen supply, the longer the time of its receptivity.

Additionally, several studies with different species have shown that flower longevity increases according

Figure 3 - Flower development of olive tree (*Olea europaea* L.), Arbequina cultivar, and stigma receptivity at different development stages, using the 3% hydrogen peroxide (H_2O_2) method. (A and B) Pre-anthesis flowers. (C and D) Anthesis flowers. (E and F) Post-anthesis flowers. (G) Extremely green flower bud, pre-anthesis, showing receptive stigma. (H) Flower bud stigma, fully developed, pre-anthesis and receptive. (I) Anthesis flower, detail for receptive stigma cavity (arrow). (J) Post-anthesis flower with receptive stigma. Bar: 1mm



to the altitude. This basically occurs due to the lower pollination rates, and/or due to the lower temperatures that lead to decreased metabolic activity, delaying the anthesis (MU *et al.*, 2011; PACHECO *et al*., 2016; PÉLABON *et al*., 2013; STEINACHER; WAGNER, 2010), which is also a possible explanation for the receptivity period of this experiment.

CONCLUSIONS

1. The olive pollen grains, Arbequina cultivar, are preserved as viable for use in hybridizations for up to 60 days when stored in the freezer at -10 °C;
2. The stigmas are receptive since pre-anthesis, with receptivity frequency prevailing throughout the day, allowing performing hybridizations at different flower development stages and at different daytimes.

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