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

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

4 genético

5

6 **ABSTRACT -** In order to ensure success in controlled hybridizations in olive tree cultivation, the

7 information on pollen viability and stigma receptivity is essential. The aim was to establish

8 methodologies that increase the preservation of pollen viability and to establish the time to

9 perform crossbreeds in hybridization studies with olive trees. Three experiments were performed

10 with plants from the cultivar Arbequina, in Maria da Fé, MG, Brazil. In the first experiment, the

11 description of the flower events was performed. In the second, anthers were desiccated in

12 eppendorfs, being stored at three different conditions for pollen viability test: room temperature

13 (27 °C), refrigerator (8 °C) and freezer (-10 °C). In order to evaluate the *in vitro* germination,

14 culture medium for olive pollen grains was used. In this respect, pollen grains were transferred in

15 Petri dish containing culture medium and placed in a BOD (biochemical oxygen demand)

16 chamber at 28 °C for 60 h, being counted. The first evaluation was performed prior to the

17 assembly of the experiment, testing the initial viability, whereas the second occurred 24 h after

18 storage. Subsequently, seven evaluations were performed fortnightly. In the third experiment, the

19 stigma receptivity was verified by the 3% hydrogen peroxide method, with flowers in pre-

20 anthesis, anthesis and post-anthesis, evaluated hourly in the period from 7 a.m. to 6 p.m. for three

21 days. In the description of the flower events, it was verified that the olive tree shows diurnal

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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.

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, M., *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, A., *et al*., 2012). However, the new premise for breeding studies would be the use of controlled

70 hybridizations, in which information would be obtained from both parents, increasing the
71 possibility of finding a cultivar with the desired characteristics within a selection study.

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

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

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

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

90 **MATERIAL AND METHODS**

91 The present study was performed with the cultivar Arbequina, aged eight years, in Maria da
92 Fé, MG, Brazil (22°18'51" S, 45°23'24" W and 1,276 m altitude) between August and September
93 2015 (SILVA *et al.*, 2016).

94 **1st experiment: description and flower events**

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

100 **2nd experiment: conservation of pollen grains**

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

103 In the laboratory, the anthers were separated with the aid of a forceps, being placed four
104 anthers by eppenddorfs, totaling 280 eppenddorfs. Afterwards, the tubes were desiccated with
105 silica gel and maintained at 28 °C for 24 h, so that the anthesis occurred and the pollen grains had
106 their moisture content reduced (CUCHIARA; SILVA; BOBROWSHI, 2012). Then, the test was
107 performed to determine the initial viability of pollen grains, using 10 eppendorfs, being one per
108 Petri dish, using culture medium specific for olive pollen germination (SILVA *et al.*, 2016).
109 Subsequently, the remainder of eppendorfs were distributed among the treatments (90
110 epperddorfs per treatment).

111 The used treatments consisted of keeping the pollen grains stored under three different
112 conditions for later viability test: room temperature (27 °C), refrigerator (8 °C) and freezer (-10
113 °C). After 24 h, a new evaluation was performed, followed by seven evaluations fortnightly. A
114 total of 10 eppendorfs per treatment (replicates) were tested in each evaluation period.

115 The experimental design was completely randomized in a 9 x 3 factorial design (storage
116 time x environments) and 10 replicates, each replication consisting of one Petri dish, with the

117 content of 1 eppendorf, where germinated and non-germinated pollen were counted in five
118 randomly selected fields of view

119 In order to determine the viability of pollen grains, the *in vitro* germination method was
120 used through culture medium specific for germination of olive pollen grains. The culture medium
121 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,
122 in the absence of calcium nitrate and magnesium sulfate, kept for 60 h at 28 °C (SILVA *et al.*,
123 2016).

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

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

132 **3rd experiment: receptivity of stigma**

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

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

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

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

147 RESULTS AND DISCUSSION

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

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

157

158 This information goes against the strategy of several fruit species in temperate regions,
159 where the plants come into dormancy in autumn and winter, showing a fast and simultaneous

160 flowering during the spring, guaranteeing quick pollination and fertilization, which causes
161 homogeneity in the fruit development (GUO *et al.*, 2014).

162 However, such non-uniformity in olive flowering is intrinsic to the species, similarly as
163 other phenological records found in the literature (OLIVEIRA, M., *et al.*, 2012; SADOK *et al.*,
164 2013; SANZ-CORTÉS *et al.*, 2002). This fact is probably due to the olive tree diminishing the
165 metabolic activity, but without coming into complete dormancy in the colder seasons, which
166 affects the synchrony during the differentiation of flower buds in the spring. Furthermore,
167 thermal oscillations during the winter period may lead to the physiological decompensation of
168 these buds, specifically related to the chill accumulation, also causing uniformity and reduction in
169 flowering (GUO *et al.*, 2014).

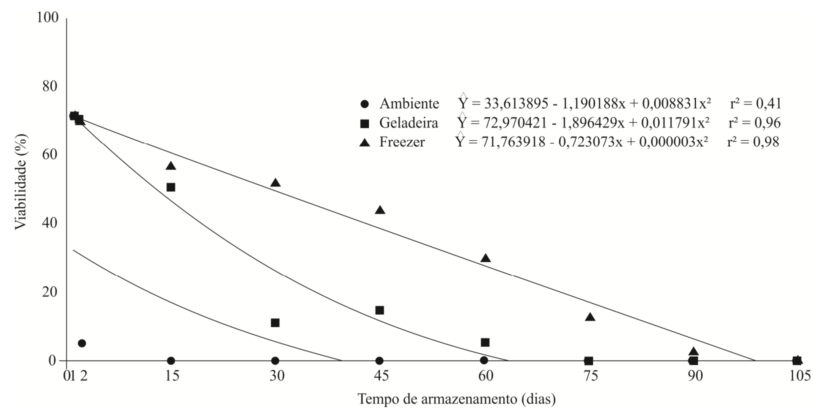
170 Also during the evaluation, it was observed that in the pre-anthesis (closed bud), there was
171 no release of pollen grains on the stigma; all the flowers showed indehiscent anthers at the time
172 of flower opening (Figure 1A), refuting the possibility of occurrence of cleistogamy in flowers.

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

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

184 In the pollen grain conservation experiment, it is verified through linear regression (Figure 185 2) that the pollen viability was drastically decreased at room temperature after the second day of 186 storage (close to 5%), being that the material no longer responded to the germination test in 187 culture medium after 40 days.

188 **Figure 2 -** Pollen viability of olive grains (*Olea europaea* L.), Arbequina cultivar, stored for 105 189 days at different storage locations (room temperature at 27 °C, refrigerator at 8 °C and freezer at - 190 10 °C

191 

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193 For several studies with fruit trees, 30% germination of pollen grains is considered an 194 acceptable rate to ensure good fertilization and fruiting (ALBUQUERQUE JÚNIOR, 2010; 195 SILVA *et al.*, 2016). Considering the germination rate around 5% (Figure 2), it can be considered 196 as very low for the use of this material in hybridization studies in olive trees.

197 Regarding the other treatments, the storage of pollen grains at 8 °C showed a viability 198 around 56%, being that there was a drastic decrease in the pollen germination after that period, 199 becoming unviable after 63 days (Figure 2).

200 The freezer storage method at -10 °C showed higher results in relation to the other 201 treatments, preserving the material viability for longer time (Figure 2). At 60 days of storage, the 202 pollen grains still had a germination rate around 30%, i.e., an acceptable parameter for the use, 203 according to the literature (ALBUQUERQUE JÚNIOR, 2010; HAUAGGE; BRUCKNER,

204 2002). Fifteen days after this period, the material still showed a low germination rate when
205 subjected to the culture medium, becoming completely unviable only 99 days after the
206 experiment.

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

214 It was also possible to observe that treatments with lower temperatures showed greater
215 durability. The use of low temperatures is related to the reduction of the metabolism of pollen
216 grains, which favors greater longevity of the material (CUCHIARA; SILVA; BOBROWSHI,
217 2012).

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

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

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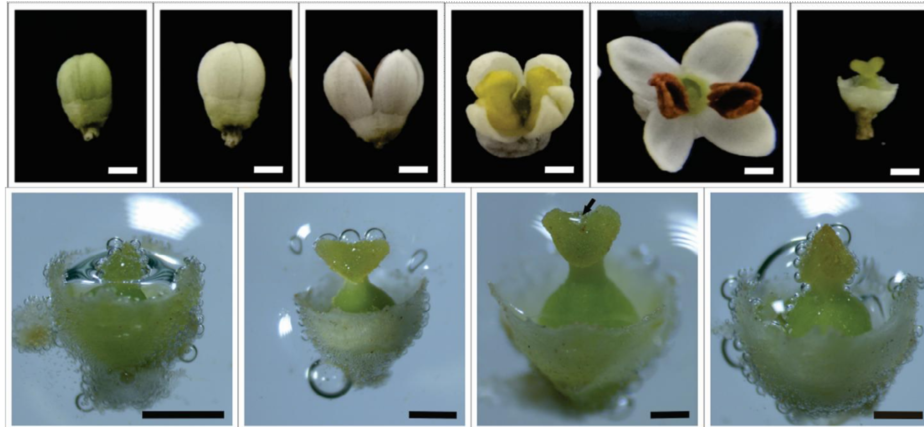

230 These results are highly relevant for breeding experiments. Once the pre-anthesis flowers
231 are receptive, controlled hybridization can be performed without contamination of pollen grains
232 other than the selected material (Figures 3A, 3B, 3G and 3H).

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

238 Regarding the time of an open flower, the duration of gynoecium receptivity will be
239 directly related to the supply of pollen grain in the environment (LANKINEN; MADJIDIAN,
240 2011). In other words, the higher the pollen grain supply, the lower the time of stigma reception,
241 the lower the pollen supply, the longer the time of its receptivity.

242 Additionally, several studies with different species have shown that flower longevity
243 increases according to the altitude. This basically occurs due to the lower pollination rates, and/or
244 due to the lower temperatures that lead to decreased metabolic activity, delaying the anthesis
245 (MU *et al*., 2011; PACHECO *et al*., 2016; PÉLABON *et al*., 2013; STEINACHER; WAGNER,
246 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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