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# Establishment of growth medium and quantification of pollen grains of olive cultivars in Brazil's subtropical areas

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**ABSTRACT:** Pollen grain germination *in vitro* indicates viability and consequently provides information related to fruit set. It also assists in the development of hybrids. Along with a suitable species, a standard culture medium is essential for evaluating pollen viability. It should contain a gelling agent consisting of carbohydrates and enhancer elements as well as have the correct pH, temperature, and incubation time. The objective of this study was to optimise the culture medium, determine the pollen germination capacity, and quantify the number of pollen grains per flower of certain olive tree cultivars. A basic sequential culture medium for pollen grain germination was determined, always utilizing the best result from the previous experiment to continue the sequence. The factorial treatment

arrangement was: 1) agar *versus* boric acid; 2) pH *versus* sucrose; 3) calcium nitrate *versus* magnesium sulfate. After determining the culture medium components, two experiments were conducted evaluating temperature and incubation time. Another experiment evaluated both the germination percentage and the number of flower pollen grains of 28 cultivars. The culture medium should be composed of 4 g·L<sup>-1</sup> of agar, 90 g·L<sup>-1</sup> of sucrose, and 400 mg·L<sup>-1</sup> of boric acid with a pH adjusted to 5.79 and an incubation time of 60 h at 28 °C. The Manzanilla 215 cultivar had the highest germination rate while Ascolano 315 presented the highest number of pollen grains per flower.

**Key words:** *Olea europaea* L., germination rate, fruit set.

## INTRODUCTION

Despite the high quality of olive oil produced in Brazilian subtropical conditions, olive cultivation is still not a viable economic activity due to difficulties in adapting cultivars to the country's climate (Silva et al. 2012a). Low productivity that is associated with reduced fecundation due to self and inter-incompatibility is very often caused by insufficient pollen grain viability and germinability (Giordani et al. 2014).

Self and inter-incompatibility are determined by the presence of an allelic series in the haploid genome of the pollen. This allelic series controls the development of the pollen tube in the pistil (Rapaport 1999). The lack of an efficient pollinator cultivar may explain losses caused by low fruit set. Although this position is still controversial,

there is recognition that cross pollination might increase the fruit set rate (Cuevas et al. 2009; Shemer et al. 2014).

Knowledge of the characteristics of the flowers in available olive tree cultivars is very important in selecting the genitors used in hybridization. Therefore, field evaluations are an indispensable prerequisite in successfully carrying out the crossbreeding (Fabbri et al. 2009; Giordani et al. 2014).

Some cultivars have adapted well to subtropical conditions (Oliveira et al. 2012; Silva et al. 2012b). Nonetheless, there is a need to enhance breeding efforts to select higher-yielding individuals in these regions with the objective of producing fruits with a high agroindustrial output.

Fruit set percentage, pollen germination, and pollen viability are related. The culture medium must possess a gelling agent composed of carbohydrates and stimulating elements such as boric acid and calcium nitrate. The pH →

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of the culture medium also influences the viability and germination of pollen grains (Pio et al. 2012; Zambon et al. 2014). Factors such as temperature and incubation time are also important in determining pollen grain germination and pollen tube development (Chagas et al. 2010; Zambon et al. 2014).

There is a potential for orchards to remain unproductive or less productive when established with a single cultivar (Besnard et al. 2001). Therefore, determining which cultivars possess large amounts of pollen grains and a high germination rate is of fundamental importance in the selection of pollinating individuals as well as in the intercropping of two or more cultivars.

Verifying the germination capacity of cultivars introduced in Brazil is very important in order to find genotypes that can be used as pollinators and that are compatible in the crosses, resulting in an increased fruit set rate. Therefore, the development of a protocol using an ideal culture medium is essential in the evaluation of the germination capacity of pollen grains in different olive tree cultivars.

The objective of the present study was to determine the ideal culture medium composition, identify the time and temperature of incubation, and evaluate both the germination capacity and the number of pollen grains per flower of different olive cultivars in Brazil's subtropical areas.

## MATERIALS AND METHODS

The experiment was conducted in a Brazilian subtropical region (22°18'51"S, 45°23'24"W, with an average altitude of 1,276 m). The region's climate, according to the Koppen classification, is mesothermal with dry winters (Cwb), an average temperature of 17 °C and rainfalls of, approximately, 1,739 mm per year (Souza et al. 2013). Arbequina cultivar, characterized by its early flowering, was used to determine the ideal culture medium composition by maximising the germination of its pollen grains. Anthers of 10 flower buds were removed at full development at stage 65 (BBCH scale) (Sanz-Cortés et al. 2002) in the late afternoon and with the use of forceps. Anthers were stored in uncapped Petri dishes at a controlled room temperature (27 °C) in a drying oven for 12 h in the absence of light to allow for anthesis, complete dehiscence, and pollen grain release (Ramos et al. 2008).

After the release of the pollen grains, three factorial experiments were conducted to determine the ideal

components of the culture medium: 1) agar (4, 6, 8 and 10 g·L<sup>-1</sup>) *versus* boric acid (0, 400, 800 and 1,200 mg·L<sup>-1</sup>); 2) pH (3.5, 4.5, 5.5 and 6.5) *versus* sucrose (0, 30, 60 and 90 g·L<sup>-1</sup>); and 3) calcium nitrate (0, 200, 400 and 800 mg·L<sup>-1</sup>) *versus* magnesium sulfate (0, 0.5, 1.0 and 1.5 mg·L<sup>-1</sup>). The pollen tube emission time was also tested in isolation (0, 12, 24, 36, 48, 60 and 72 h after inoculation) as was the temperature of pollen grain incubation (24, 26, 28, 30 and 32 °C). Tests were performed in a BOD-type germination chamber, and the best results from the previous experiment were used for the subsequent experiment.

For each step, the pollen was inoculated on the surface of a Petri plate containing 20 mL of culture medium with the aid of a brush to obtain a uniform distribution over the surface of the medium. Subsequently, the Petri dishes were capped and kept in the absence of light for 72 h. The numbers of germinated and non-germinated pollen grains were counted with the aid of a microscope with a 10x objective lens. Grains were considered germinated if the size of the pollen tube was greater than twice the diameter of the grain (Chagas et al. 2010). Experiments were conducted in a completely randomised design with four replications, where each replication corresponded to one Petri dish and consisted of five fields of view.

After determining the components of the culture medium (4 g·L<sup>-1</sup> of agar amended with 90 g·L<sup>-1</sup> of sucrose and 400 mg of boric acid·L<sup>-1</sup>; pH adjusted to 5.79), as well as the time and temperature of pollen tube emission (72 hours and 28 °C), a new *in vitro* germination experiment was conducted with the pollen grains of 28 olive cultivars: Alto D'Ouro, Arbequina, Arbosana, Ascolano 315 (MGS ASC315), Ascolano USA, Cerignola, Clone 113 (MGS NEBLINA), Clone 0025, Cornicabra, Galega, Grappolo 541 (MGS GRAP541), Grappolo 553, Grappolo 561 (MGS GRAP561), Grappolo 575, JB1, Koroneiki, Manzanilla 215, Manzanilla 234, Maria da Fé (MGS MARIENSE), Mission, Negroa, Penafiel SP, Pindolino, Salomé 488, Santa Catalina, Tafahi 390, Tahafi 391 and Zalmate 002. The experimental design was completely randomised with four replications, where each replication corresponded to one Petri dish and consisted of five fields of view.

Five flower buds of each cultivar (stage 65 - BBCH Scale) were randomly collected to count the number of pollen grains per flower. Thereafter, each pair of anthers was removed and stored separately in uncapped Eppendorf tubes at a controlled temperature (27 °C) for 24 h in the absence

of light to allow for dehiscence and pollen grain release, according to the recommendations of Ramos et al. (2008) and Zambon et al. (2014).

After 24 h a solution of 1,000  $\mu\text{L}$  of lactic acid was added to the Eppendorf tubes and after 48 h a sample of 10  $\mu\text{L}$  from each tube was placed onto a counting chamber (Neubauer) to determine the number of pollen grains with the aid of an optical microscope with 100x objective lens (Zambon et al. 2014).

The experimental design used in this study was completely randomised with 28 treatments (cultivars), where each treatment included 12 replicates and each plot consisted of average four Neubauer chamber readings.

The total number of pollen grains per flower was calculated by multiplying the average number of pollen grains from each sample by the volume of the lactic acid solution (1,000  $\mu\text{L}$ ) and then dividing this value by the product of the volume of the lactic acid sample (10  $\mu\text{L}$ ) and the number of anthers from each tube (five). This value was then multiplied by two (the number of anthers that exist in each olive flower).

The results obtained were subjected to an analysis of variance; a regression analysis was used for quantitative data, and Scott and Knott was used for qualitative data. The analyses were performed using the System for Analysis of Variance (SISVAR) software (Ferreira 2011).

## RESULTS AND DISCUSSION

According to the analysis of variance, there was a significant interaction between the factors agar and boric acid. The concentrations of 4  $\text{g}\cdot\text{L}^{-1}$  of agar and 400  $\text{mg}\cdot\text{L}^{-1}$  of boric acid promoted a greater germination of the pollen grains (28.94%) (Figure 1).

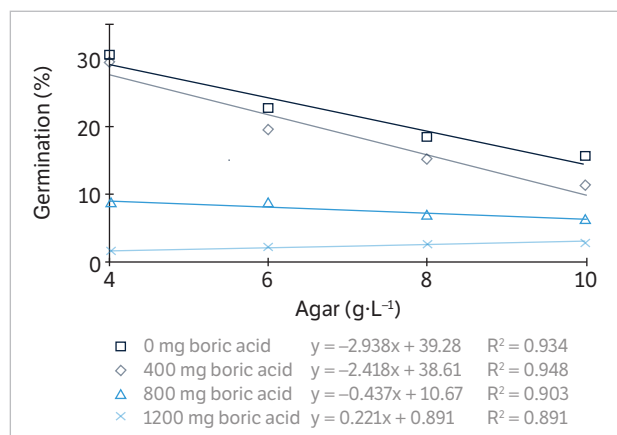
One possible explanation for the increased germination percentage observed at the lowest agar concentration (4  $\text{g}\cdot\text{L}^{-1}$ ) is that the smaller amount of solidifying agent led to relatively poor culture medium reliability, favouring the absorption of water and nutrients from the medium by the pollen grains.

However, boric acid plays a more specific role in the formation and development of the pollen tube and may show different responses depending on the species. As boron plays a role in pollen grain germination and pollen tube growth, both processes are compromised when boron levels are absent or insufficient. In addition, the absence of

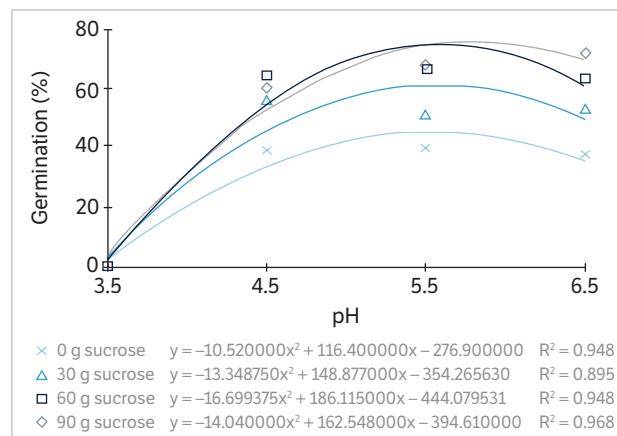
boron results in a low sucrose absorption efficiency and a decrease in the growth rate of the pollen tube (Lewis 1980).

In this study, the addition of 400  $\text{mg}\cdot\text{L}^{-1}$  of boric acid to the culture medium supported better the germination of pollen grains compared to when the boric acid was absent (1.41% increase). However, when the concentration of boric acid exceeded 400  $\text{mg}\cdot\text{L}^{-1}$ , the germination percentage decreased. This was most likely related to a gradual increase in the solute concentration of the medium that disrupted the integrity of the pollen grain cell structure (Dantas et al. 2005). The application of boric acid in the field raised the pollen grain germination of loquat (Nogueira et al. 2014), which proves the need for boric acid in pollen grain germination.

There was a significant interaction between sucrose and pH. The best results were observed when 90  $\text{g}\cdot\text{L}^{-1}$  of sucrose was added to the medium and the pH was adjusted to 5.79 (75.88% germination) (Figure 2).



**Figure 1.** Percentage of *in vitro* germination of pollen grains of Arbequina olive tree under different concentrations of agar and boric acid.



**Figure 2.** Percentage of *in vitro* germination of Arbequina olive tree pollen grains under different pH levels and sucrose concentrations.

The addition of sucrose as a carbohydrate source is used to meet the metabolic demands involved in growth. It can act both as a source of energy generation and as a source of carbon skeletons for biosynthetic cell, differentiation processes (Chagas et al. 2009). Thus, the high percentage of germination under increased sucrose concentrations may be explained by the increased energy supplied in the form of carbohydrates, favouring the growth of the pollen tube. A study of pollen germination of blackberry and loquat concluded that the highest germination rates were achieved at the highest concentrations of sucrose added to the medium (Figueiredo et al. 2013; Nogueira et al. 2014).

The higher pH levels caused an increase in the pollen grain germination percentage. However, the germination rate decreased with a pH of 5.79 or higher. Relate this decrease may to a greater or poorer availability of components (nutrients) and/or an osmotic imbalance of the culture medium.

An optimal pH for the physiological processes involving pollen grains is associated with higher pollen germination capacity, thus ensuring greater chances of fertilisation, larger and better fruit sets, and a better production rate in the field (Salles et al. 2006).

In the experiment with varied levels of calcium nitrate and magnesium sulfate, a significant interaction was found among these factors. However, the highest rate of pollen grain germination was obtained in the absence of both components (72.05%) (Figure 3). Similar results were observed when using calcium nitrate in the *in vitro* germination of pollen grains of peach and pear trees (Chagas et al. 2009). Similarly, according to Nogueira et al. (2014), the culture medium absent of calcium nitrate promoted more germination of loquat pollen grains.

Among the evaluated temperatures, it was observed that the highest rate of pollen grain germination occurred at 28.21 °C (72.43% germination) (Figure 4).

The temperature to which the pollen is exposed during the germination phase is directly related to the development of the pollen tube. Very low temperatures lead to decreased metabolic activity, thereby preventing germination, whereas very high temperatures cause the degradation of proteins and enzymes fundamental to the development of the pollen tube (Wahid et al. 2007).

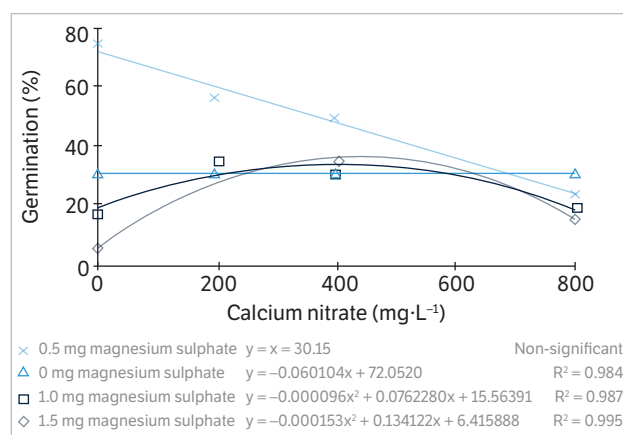
These results agree with a study of the development of pollen tubes of pear rootstocks, which found that temperatures of up to 28 °C favoured germination while temperatures higher than this resulted in a decreased percentage of germination (Chagas et al. 2010).

Under field conditions, the correct environmental temperature is fundamental for the favourable germination of pollen grains as well as pollen tube development and flower and fruit set. A comparison of two temperatures (15.6 and 32.2 °C) in the germination and development of olive tree pollen grains under glasshouse conditions found that pollen tube growth was faster at higher temperatures (Bradley et al. 1961).

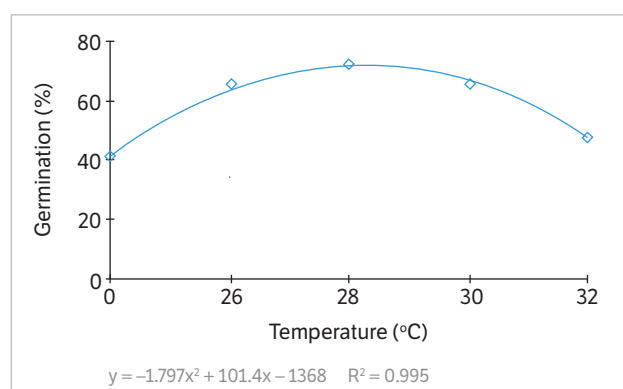
The present study found that a pollen tube emission time of 72 h resulted in a germination rate of 73.89% (Figure 5).

Regarding pollen grain germination, a large variability in the germination capacity was observed for the different olive cultivars examined in this study (Table 1).

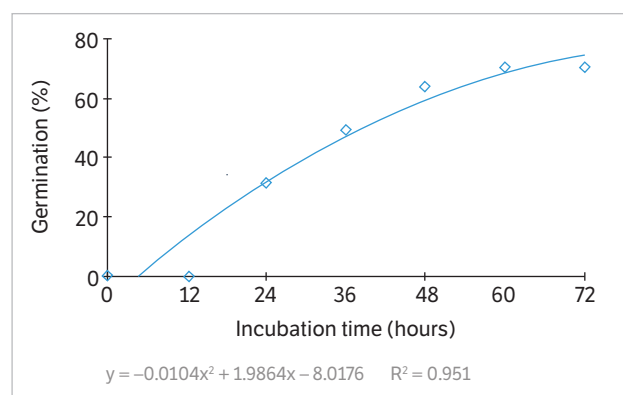
The best result was obtained with the cultivar Manzanilla 215 (81.56%), and poorer results were found with cultivars Penafiel SP, Alto D'Ouro, and Mission (5.50, 8.92, and 9.64%, respectively). These results agree with Giordani et al. (2014), which found a difference in the germination →



**Figure 3.** Percentage of *in vitro* germination of pollen grains of Arbequina olive tree under different concentrations of calcium nitrate and magnesium sulfate. NS = não significativo.



**Figure 4.** Percentage of *in vitro* germination of pollen grains of Arbequina olive tree in different temperatures.



**Figure 5.** Percentage of *in vitro* germination of pollen grains of Arbequina olive tree under different incubation times.

capacity of pollen grains between different olive tree cultivars.

There was also a large variation in the number of pollen grains per flower of each cultivar (Table 1). Cultivar Ascolano 315 (MGS ASC315) presented higher amounts of pollen (14,545.83), and cultivars Maria da Fé (MGS MARIENSE) (5,667.67), Koroneiki (6,200.00), Grappolo 575 (6,304.17), Arbosana (6,777.08), Cerignola (6,862.50), Salomé 488 (6,881.25), and Tafahi 391 (6,912.50) produced smaller amounts.

Certain olive cultivars are self-incompatible and produce little or no fruit in mono-varietal orchards. In this case, cross-pollination favours larger and more regular yields. Although self-incompatibility is an important feature to the production of olives, the mode of inheritance of this self-incompatibility remains unknown for this species. This issue is a major concern for growers and economists and has hindered possible market forecasts (Breton and Bervillé 2012).

## CONCLUSION

The culture medium used for the germination of olive pollen grains should be composed of 4 g·L<sup>-1</sup> of agar amended with 90 g·L<sup>-1</sup> of sucrose and 400 mg·L<sup>-1</sup> of boric acid. The pH should be adjusted to 5.79, and calcium nitrate and magnesium sulfate should be omitted. Pollen should be incubated for 60 h at 28 °C.

Cultivar Manzanilla 215 showed the highest germination rate (81.56%), and cultivar Ascolano 315 (MGS ASC315) had the highest number of pollen grains per flower (14,545.83).

**Table 1.** Average germination and number of pollen grains per flower of different olive tree cultivars.

Cultivar	Germination (%)	Number of pollen grains per flower
Alto D'Ouro	8.92 i	9,581.25 c
Arbequina	73.65 b	9,204.17 c
Arbosana	48.47 e	6,777.08 e
Ascolano 315 (MGS ASC315)	58.59 d	14,545.83 a
Ascolano USA	62.35 c	8,000.00 d
Cerignola	50.65 e	6,862.50 e
Clone 113 (MGS NEBLINA)	49.14 e	7,464.58 d
Clone 0025	29.95 g	10,529.17 c
Cornicabra	13.21 h	10,862.50 b
Galega	25.02 g	9,493.75 c
Grappolo 541 (MGS GRAP541)	57.35 d	10,418.75 c
Grappolo 553	64.64 c	8,206.25 d
Grappolo 561 (MGS GRAP 561)	27.02 g	9,641.67 c
Grappolo 575	52.05 e	6,304.17 e
JB1	32.41 f	11,389.58 b
Koroneiki	15.97 h	6,200.00 e
Manzanilla 215	81.56 a	8,427.08 d
Manzanilla 234	68.17 b	11,612.50 b
Maria da Fé (MGS MARIENSE)	16.16 h	5,667.67 e
Mission	9.64 i	10,018.75 c
Negroa	18.72 h	10,018.75 c
Penafiel SP	5.50 i	7,508.33 d
Pindolino	38.00 f	7,343.75 d
Salomé 488	36.31 f	6,881.25 e
Santa Catalina	68.17 b	8,089.58 d
Tafahi 390	70.50 b	11,262.50 b
Tafahi 391	62.61 c	6,912.50 e
Zalmate 002	24.76 g	11,697.92 b
<b>CV (%)</b>	<b>12.35</b>	<b>20.36</b>

Means followed by the same letter do not differ in the columns by the Scott-Knott test ( $p \leq 0.05$ ). CV = Coefficient of variation.

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