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# Use of microstructural characteristics of pollen and pistil to identify olive cultivars in Southern Brazil

**Andrés Chacón-Ortiz[1,2*], Carlos Busanello[1], Antonio Costa de Oliveira[1], Luciano Carlos Da Maia[1], Juliana Aparecida Fernando[3] and Camila Pegoraro[1]**

[1]Departamento de Agronomia, Centro de Genômica e Melhoramento Vegetal, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Campus Capão do Leão, Pelotas, Rio Grande do Sul, Brazil. [2]Department of Animal Production, Táchira National Experimental University, Sector 5001, Universidad Avenue, San Cristóbal, Táchira, Venezuela. [3]Departamento de Botânica, Instituto de Biologia, Universidade Federal de Pelotas, Campus Capão do Leão, Pelotas, Rio Grande do Sul, Brazil. *Author for correspondence. E-mail: aecortiz@gmail.com

**ABSTRACT.** The olive crop has a large diversity in cultivars around the world, whose identification have been based on leaf, stem or fruit traits. However, these traits may be influenced by the environment, which has led to the misidentification of cultivars. The variability of the pistil and pollen structure under electron scanning and light microscopy were studied using the cultivars 'Arbequina', 'Arbosana', 'Frantoio', 'Koroneiki', 'Manzanilla de Sevilla' and 'Picual', cultivated in Southern Brazil. The aim was to find unique patterns for their identification. This type of study has not been carried out in the conditions of Southern Brazil, where the identification of commercial cultivars sometimes is complex and confusing. There were significant differences in the variables, showing uniformity within each cultivar in different locations. The principal component analysis indicated a separation of cultivars according to similarities or micromorphological differences in the pollen wall, area of the lumen and the structural elements of the tectum. For the analysis of the pistil, the separation was even clearer, each cultivar individually forming a cluster, using as characters the length of the style in contrast to the length, width and area of the stigma. This evidenced little variation among individuals within the same cultivar. The information provided can contribute to the individual identification of very close olive tree cultivars in the conditions of Southern Brazil.

**Keywords:** Flower morphology; *Olea europaea*; Oleaceae.

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## Introduction

Historically, most of the olive tree cultivars (*Olea europaea* L.) have been identified based on the taxonomic and agronomic traits adopted by the International Olive Council, estimating more than 1275 cultivars recognized in at least 56 countries (Barranco & Rallo, 1984; Bartolini, Prevost, Messeri, & Carigani, 1998; Barrranco, Fernández-Escobar, & Rallo, 2008).

The olive cultivation has spread throughout the world by clonal replicas of a diverse group of cultivars, whose main origin is the Mediterranean Sea Basin (Mora, Tapia, Ibacache, Nunes-Martins, & Alberto-Scapim, 2008). However, there are in the olive tree classification, more than 3000 synonyms and homonyms still to be reclassified (Özkaya, Ergülen, Ülger, & Özilbey, 2004; Rallo et al., 2005; Pereira, Oliveira, Kanashiro, & Costa, 2015; Adakalic & Lazovic, 2018), generating confusion and uncertainty about their correct identity (Özkaya et al., 2004; Alba, Montemurro, Sabetta, Pasqualone, & Blanco, 2009). This identity problem is caused because the characters used to classify them are usually influenced by local environmental fluctuations (Levin & Lavee, 2005; Mora et al., 2008; Besnard, Rubio de Casas, Christin, &Vargas, 2009; Matías, Toro, Montalván, & Molina, 2010; Lubna et al., 2019; Ali et al., 2021). In Brazil, the ecological and cultivation conditions for the olive tree are different from the countries of origin, a situation that makes it difficult to use some of the descriptors developed by Barranco and Rallo (1984), adapted for Europe. The use of scanning electron microscopy has revolutionized bioscience, allowing the exploration of traits to determine plant taxa, obtain information on the anatomical, palynological and ecological importance of different plant groups (Gul et al., 2021; Ishtiaq et al., 2020; Majeed et al., 2020; Ali et al., 2020; Ali et al., 2021; Umber et al., 2022), identification in olive cultivars (Serrano, Suárez, Olmedilla, Rapoport, & Rodriguez-García, 2008; Castro et al., 2010), in addition of use in conservation or to select species for controlled crosses in breeding programs (Souza et al., 2021; Umber et al., 2022).

Pollen morphological traits have been essential to distinguish species that have similar physical appearance (Zhang et al., 2017). Ultrastructural characters of pollen in olive trees, particularly those of sporoderms, have been used repeatedly for the classification and discrimination of cultivars (Arzani & Javady, 2002). These traits present high polymorphic levels (Ganino, Bartolini, & Fabbri, 2006) and have low influence from the environment and different phenological stages (De La Rosa, James, & Tobutt, 2002).

On the other hand, ultrastructural studies in gynoecium in some olive cultivars have revealed differences that depend on the type of cultivar and plant development stages (Koubouris, Metzidakis, & Vasilakakis, 2012). However, few studies have focused on describing the differences that exist in the structure and composition of the pistil, differentiating each cultivar during the time of anthesis (Rapoport, 2008; Serrano et al., 2008). Floral morphology is a taxonomic trait that is barely evaluated in the olive tree (Serrano et al., 2008). Studies to characterize the reproductive system among cultivars of this species have never been developed in Brazil.

For this reason, the objective of this work was to establish a cultivar recognition system under optical and scanning microscopy based on the combination of pollen and pistil characters, analyzing six cultivars used in the Rio Grande do Sul state, Brazil.

## Material and methods

Three thousand perfect olive flowers (*Olea europaea* L.) of each cultivar were randomly collected in paper bags, showing a phenology between F and F1 (Fernández-Escobar & Rallo, 1981), showing turgid anthers, of the cultivars 'Arbequina', 'Arbosana', 'Frantoio', 'Koroneiki', 'Manzanilla de Sevilla' and 'Picual', from the companies 'Verde Louro' (UTM 31.474477S; 52.9473309W), city of Canguçu ; 'Olivas do Sul-Pomar' (UTM 30.009034S; 52.866627W), Cachoeira do Sul and 'Azeites Batalha' (UTM 31.5607715S; 53.511726W), Pinheiro Machado; all located in the Rio Grande do Sul state, Brazil. The collection was carried out of September to December of 2019 of trees of 5 to 10 years old. The local climate was characterized during flower harvest year, based on the data from the Meteorological Stations: Canguçu, WMO: A811, Bagé, WMO: A827 and Encruzilhada do Sul WMO: A893, Region S, belonging to the National Institute of Meteorology (INMET), of the *Ministério da Agricultura, Pecuária e Abastecimento*, Brazil. The climatic conditions characterized by presenting scattered precipitations, with high RH and solar radiation.

The paper bags containing the flowers were immediately taken to the Plant Genomics and Breeding Center of the *Universidade Federal de Pelotas* where the flowers were kept in ultra-freezer at -80°C. The analysis under light microscope were carried out at the 'Eliseu Maciel School of Agronomy', *Universidade Federal de Pelotas* (UFPel) and the observations under scanning electron microscope in the Southern Electron Microscopy Center (CEME-SUL), *Universidade Federal do Rio Grande* (FURG).

### Pollen grain count

Pollen grain counting and analysis were performed under a Leica DM750 model light microscope. One thousand five hundred flowers per cultivar were randomly selected. From each flower, an anther was chosen and preserved in 300 µL of ethanol (70 %) and methylene blue (0.5 %). The anthers were macerated and homogenized. Three aliquots of 20 µL were taken for the counting in a neubauer chamber under 100X objective. The average number of pollen grains was multiplied by 0.3. The number of pollen grains produced per flower was obtained, multiplying the average value by two, since the flower has two anthers.

### Pollen grain analysis

Observations and measurements were made on 4,000 pollen grains per cultivar under a light microscope with a 100X objective. Photographs were taken with a Nikon eclipse E200 photomicroscope equipped with a BEL software, version 7.1.1.7. The subsequent measures were performed with help of Motic software. The measured parameters included the maximum diameter (PL) and the minimum diameter (PW). Quotient between the maximum and the minimum diameter of the particle (L-W ratio), and the size index (SI), representing a simple calculation of the area of the grain (Lanza, Marsilio, & Martinelli, 1996; Koubouris et al., 2012).

For ultrastructure observations, 25 pollen grains of each cultivar taken separately at random from a flower stock, stored in Eppendorf tubes with silica gel, inside a deep freezer at -80°C were measured. These grains were fixed in 50 % ethanol, 3 % glutaraldehyde and 1 % formaldehyde solution for 48 hours. Subsequently, the samples were dehydrated in a series of 50-100 % ethanol plus 1 % formaldehyde, then they were dried at

28°C and stored at -20°C. The samples were sprinkled with powdered gold in a Desk V TSC. Pollen was examined under high vacuum scanning electron microscope, Jeol, JSM-6610LV, with EDS microprobe. Observations were made using magnifications from 4,000X to 25,000X. The ultrastructural traits analyzed were the tectum height (HT) and width (WT), the lumen area (LA) and the distances between the sculptural elements in the exine (DSE).

### Anatomical pistils analysis

Twenty-five flowers of each cultivar were randomly picked. They were prepared under a Meiji Techno EMZ-13 stereoscopic microscope, removing the petals and sepals, leaving the stigma, style and ovary free. Each pistil was individually fixed in 300 µL of 4°C Karnovsky solution. The fixed samples were dehydrated in ethanol (50-100%), embedded in plastic resin (Leica Historesin) and were sectioned (7 µm thick) using a Ancap rotary microtome. The longitudinal sections were stained with 0.05% toluidine blue in citrate–phosphate buffer, pH 4.5 and mounted in Entellan™ synthetic resin.

### Ultrastructural analysis of pistils

The samples were dehydrated in ethanol (50-100%), adding 1 % formaldehyde. They were dried at 28°C for 24 hours. Subsequently, gold was pulverized in a Denton metallizer vacuum desk and observed using magnification from 100X to 2,500X. The measurements taken were, ovary ratio (OR), which reflects the ratio between the length and width of the ovary, style length (SL), stigma length (StigL), width of the stigma (StigW) and the stigma area (StigA).

### Statistical analysis

The normality of the data was verified with the Shapiro-Wilks test. Since all the variables showed a normal distribution, no transformation was applied. The means, standard deviations were presented for the counting, morphometric measurements of the size of the pollen grains and the pistil. To compare the values of the means in all the variables analyzed, a Scheffé test was performed ($p \leqslant 0.05$). Although all variables were measured in the same unit (µm), the values were normalized by expressing them in standard deviation units.

The ANOVA, Scheffé test and principal component analysis (PCA) were performed with the help of the statistical package RStudio and PAST.

## Results

An analysis of variance was applied to all the variables measured in pollen and pistils, for the different cultivars. In all cases, at least one cultivar was significantly different (data not shown). Likewise, the comparison of plants from the same cultivar between different locations did not show statistical differences.

### Pollen grain count

'Koroneiki' presented the highest average value of pollen grains per flower (113,889 ± 11,928), while 'Manzanilla de Sevilla' did show the lowest average (53,811 ± 10,857), this value being close to half the amount of pollen grains of 'Koroneiki' (Table 1).

### Pollen grain analyses

The scanning electron microscopy analysis of the pollen grain revealed that there are differences in the exine pattern of each of the cultivars, which can include the variation in the size of the pores (lumen) and the shape of the structural elements of the mesh (Figure 1A-F). The observations made at 25,000X showed differences in the width and height of the tectum design, lumen area, the shape and distance between each sculptural elements of the wall surface in the exine. It was observed that 'Picual' and 'Manzanilla de Sevilla' have sculptural structures with notable sharp surfaces, while in 'Koroneiki' presented less noticeable angulations and in 'Arbequina', 'Arbosana' and 'Frantoio' tend to be rounded.

The measurements have been carried out under the same hydrated conditions. The pollen grains from 'Manzanilla de Sevilla' and 'Frantoio' presented the highest and the lowest SI values, respectively. Values of L/W > 1 indicated prolate shapes while close to unity values indicated sub-prolate shapes (Valdés, Díez, and Fernández, 1987; Koubouris et al., 2012). The cultivar that presented pollen grains with the most prolate shape was 'Arbequina', while 'Koroneiki' tends to present sub-prolate pollen grains (Table 2).

**Table 1.** Counting of pollen grains. Number of pollen grains per flower of six olive cultivars 'Arbequina' (aq), 'Arbosana' (as), 'Frantoio' (f), 'Koroneiki' (k), 'Manzanilla de Sevilla' (m) and 'Picual' (p), in each of the studied localities.

| Cultivars | | Verde Louro n=1,500 | Azeites Batalha n=1,500 | Olivas do Sul n=1,500 | Total n=4,500 |
|---|---|---|---|---|---|
| | $\bar{x}$ | 109,249 [a] | 110,119 [b] | 115,056[b] | 111,474[a] |
| aq | $s^2$ | 245,853x10$^3$ | 35,0796x10$^3$ | 198,552 x10$^3$ | 265,067 x10$^3$ |
| | $s$ | 15,679 | 18,729 | 14,090 | 16,280 |
| | $\bar{x}$ | 109,110 [a] | ---- | ---- | 109,110[a] |
| as | $s^2$ | 428,495x10$^2$ | ---- | ---- | 428,495x10$^2$ |
| | $s$ | 6,565 | ---- | ---- | 6,565 |
| | $\bar{x}$ | 101,816 [b] | 98,979 [d] | ---- | 100,397[b] |
| f | $s^2$ | 112,548 x10$^3$ | 212,693 x10$^3$ | ---- | 218,894 x10$^3$ |
| | $s$ | 10,608 | 14,584 | ---- | 14,795 |
| | $\bar{x}$ | 108,471 [a] | 115,213 [a] | 117,985[a] | 113,889[a] |
| k | $s^2$ | 932,450 x10$^2$ | 182,634 x10$^3$ | 151,001x10$^3$ | 142,293 x10$^3$ |
| | $s$ | 9,656 | 13,514 | 12,288 | 11,928 |
| | $\bar{x}$ | 56,415 [c] | 51,208 [f] | ---- | 53,811[c] |
| m | $s^2$ | 115020 x10$^3$ | 120,770 x10$^3$ | ---- | 117,895 x10$^3$ |
| | $s$ | 10724 | 10,990 | ---- | 10,857 |
| | $\bar{x}$ | 58153 [c] | 57,979 [e] | ---- | 58,066[c] |
| p | $s^2$ | 218547 x10$^3$ | 358,529 x10$^3$ | ---- | 288,538 x10$^3$ |
| | $s$ | 14783 | 18,935 | ---- | 16,986 |

a) n = total pollen measured and means ($\bar{x}$), variance ($s^2$) and standard deviation (s). b) Different letters within a column indicate significant differences by Scheffé test ($p \leq 0.05$). Letters are not presented between different columns, since there were no differences between different locations. ($p \leq 0.05$). c) --- It translates into the absence of data for the cultivar.

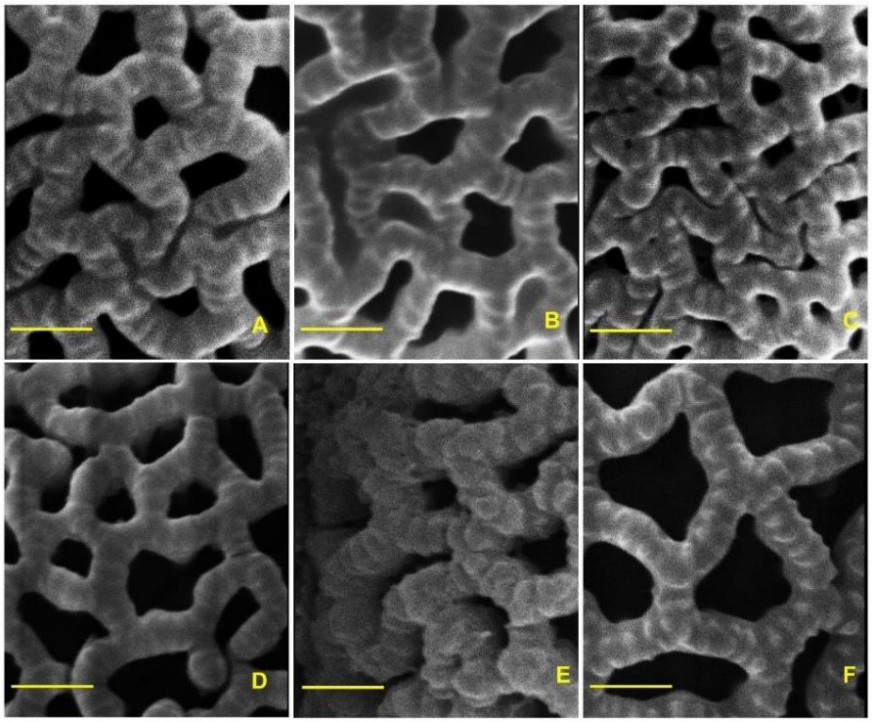


**Figure 1.** Scanning electron micrographs of olive (*Olea europaea*) pollen grain morphology observed under 25,000X. The samples correspond to the cultivars: A. Arbequina, B. Arbosana, C. Frantoio, D. Koroneiki, E. Manzanilla de Sevilla and F. Picual. Line indicates a scale of 1 µm

Tectum measurements indicated a significant variation of HT between the highest and lowest values, belonging to 'Manzanilla de Sevilla' and 'Frantoio', respectively. However, 'Arbosana' and 'Arbequina' didn't show significant differences between them. For WT, a wider range of values was observed, according to the Scheffé test, all values presenting differences between pairs of means. In turn, the mean value of LA for 'Picual' is almost twice the value calculated for 'Koroneiki', which follows it on second place. 'Arbosana' and 'Frantoio' did not show differences in their LA values.

The DSE of 'Picual' and 'Manzanilla de Sevilla' presented the highest values among sculptural elements, not differing significantly. In turn, 'Arbequina' and 'Frantoio' showed similarities in their averages, differing significantly from 'Picual' and 'Manzanilla de Sevilla'.

**Table 2.** Variables measured in pollen grains: diameters, maximum (PL) and minimum (PW), L/W ratio, size index (SI); height (HT) and width of the tectum (WT), lumen area (LA) and distance between sculptural elements (DSE), made in the olive cultivars 'Arbequina' (aq), 'Arbosana' (as), 'Frantoio' (f), 'Koroneiki' (k), 'Manzanilla de Sevilla' (m) and 'Picual' (p).

| Cultivar | | PL | PW | L/W | SI | HT | WT | LA | DSE |
|---|---|---|---|---|---|---|---|---|---|
| | | n=4,000 | | | | n=25 | | | |
| aq | $\bar{x}$ | 17.158 [b] | 13.565 [e] | 1.264 [a] | 2.327 [d] | 0.150 [d] | 0.475 [b] | 0.380 [c] | 0.200 [b] |
| | *s* | 1.130 | 1.346 | 0.140 | 0.310 | 0.018 | 0.071 | 0.048 | 0.020 |
| as | $\bar{x}$ | 16.929 [d] | 15.146 [c] | 1.117 [c] | 2.564 [c] | 0.151 [d] | 0.249 [f] | 0.208 [d] | 0.151 [d] |
| | *s* | 1.981 | 2.063 | 0.109 | 0.666 | 0.008 | 0.031 | 0.081 | 0.012 |
| f | $\bar{x}$ | 13.455 [f] | 11.589 [f] | 1.161 [d] | 1.559 [e] | 0.114 [e] | 0.431 [c] | 0.217 [d] | 0.210 [b] |
| | *s* | 1.521 | 1.327 | 0.056 | 0.354 | 0.010 | 0.033 | 0.031 | 0.008 |
| k | $\bar{x}$ | 16.238 [e] | 14.599 [d] | 1.112 [c] | 2.370 [d] | 0.255 [b] | 0.363 [d] | 0.527 [b] | 0.183 [c] |
| | *s* | 1.044 | 1.104 | 0.068 | 0.300 | 0.035 | 0.033 | 0.102 | 0.007 |
| m | $\bar{x}$ | 19.752 [a] | 16.982 [a] | 1.163 [b] | 3.354 [a] | 0.391 [a] | 0.529 [a] | 0.154 [e] | 0.224 [a] |
| | *s* | 0.915 | 1.307 | 0.098 | 0.320 | 0.045 | 0.025 | 0.052 | 0.020 |
| p | $\bar{x}$ | 16.998 [c] | 15.011 [b] | 1.132 [e] | 2.551 [b] | 0.210 [c] | 0.324 [e] | 1.069 [a] | 0.226 [a] |
| | *s* | 0.939 | 1.366 | 0.074 | 0.375 | 0.022 | 0.058 | 0.122 | 0.023 |

The measures (PL, PW, HT, WT and DSE) are expressed in μm; (LA and SI) are expressed in $\mu m^2$, and L/W is unitless. The data is presented as means ($\bar{x}$) and standard deviation (s). Different letters within a column indicate significant differences by Scheffé test ($p \leq 0.05$).

## Anatomical pistils analysis

Although the six olive cultivars analyzed belong to the same species, they show different anatomical patterns into the female reproductive system (Figures 2 A-R) that can be used as identification tools. In the case of 'Arbequina' (Figures 2 A-C), it presented 2-3 layers of slightly round glandular cells in the stigma, with a sharply conical transmission tissue and rows of small elongated cells, with thick walls. The base of the stigma is formed by a continuation of the parenchymal tissue of the style. The style has several layers of cells. The cortex showed irregularly shaped cells of different size and the ovary has a single layer of epidermis, covered by a dense cuticle. Ovules protected by several layers of the ovary, whose cells showed thick walls.

In 'Arbosana' (Figures 2 D-F), as in 'Arbequina', 2 to 3 layers of slightly elongated glandular cells in the stigma and a slightly conical transmission tissue were observed. The base of the external stigma is a continuation of the parenchymal tissue of the style. The style has several layers of cells. The cortex had regular and vacuolated cells with thicker walls. The ovary showed a layer of epidermis and small cells, covered by a dense cuticle. Ovules protected by several layers of the ovary, whose cells showed thick walls.

In 'Frantoio' (Figures 2 G-I), 1 to 2 layers with phenolic compounds are observed in the stigma. The transmission tissue is conical with rows of elongated cells with thick walls. The base of the stigma shows a clear separation from the parenchymal tissue of the style. The style has several layers of cells. The cortex is characterized by quadratic cells with large nuclei. The ovary has an epidermis with cells of reduced size and irregular shapes. Ovules protected by several layers of the ovary, whose cells show thick walls.

'Koroneiki' (Figures 2 J-L) shows 1 to 2 layers of slightly elongated glandular cells on the stigma. The transmission tissue has a conical shape with two small projections directed to the lobes. The base of the stigma presented external cells as a continuation of the parenchymal tissue of the style. The style has several layers of cells. The cortex has irregular and vacuolated cells with thick walls. The ovary shows a single-layered epidermis, covered by a dense cuticle. Ovules protected by several layers of the ovary, whose cells show thick walls.

In 'Manzanilla de Sevilla' (Figures 2 M-O), the stigma presents 3 layers of epidermal papillae with an elongated vacuolar structure. The transmission tissue has slightly conical to straight shape with no apparent projections. The base of the stigma shows a clear separation between the cells of the stigma and the parenchymal tissue of the style. The style has several layers of cells. Irregular, elongated and vacuolated cells with thin walls are observed in the cortex. The ovary has a single-layered epidermis, covered by a dense cuticle. Ovules protected by several layers of the ovary, whose cells show thick walls.

In the case of 'Picual' (Figures 2 P-R), the stigma has 3 layers of elongated glandular cells. The transmission tissue has a well defined conical structure, with two large, sharp projections to the lobes. The base of the stigma does not show clear insertion of the cells of the style parenchyma tissue. The style has several layers of cells. The cortex is composed of rows of elongated, vacuolated cells. The ovary has a single epidermal layer, covered by a fine cuticle. Ovules are protected by several layers of the ovary, whose cells show thick walls.

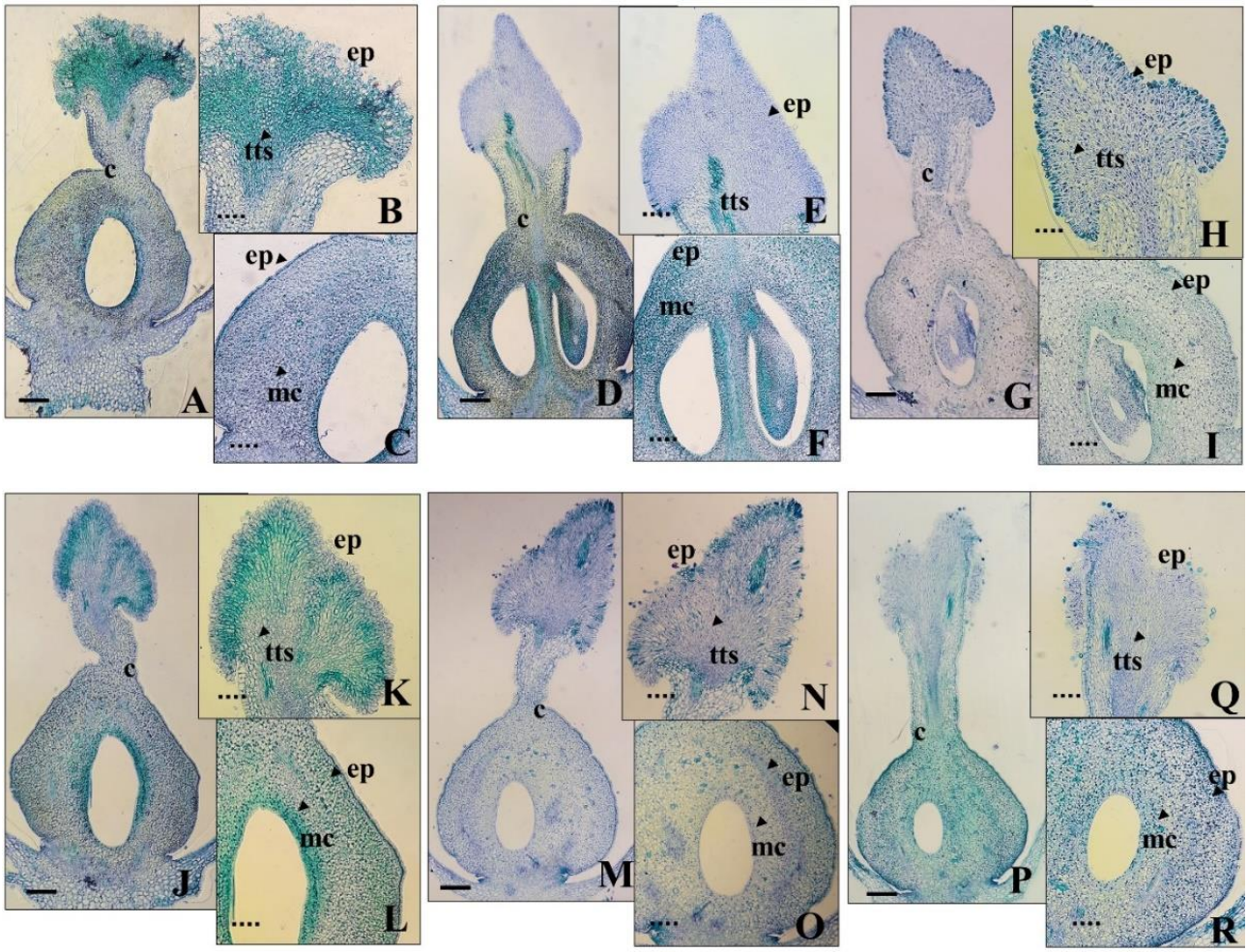


**Figure 2.** Micrography of histologic cuts of the pistils in olive. The samples correspond to the cultivars: A-C. Arbequina, D-F. Arbosana, G-I. Frantoio, J-L. Koroneiki, M-O. Manzanilla de Sevilla and P-R. Picual. Longitudinal section of a complete pistil (4X). A bilobed stigma, a style showing a core of transmitting tissue (tts) and the cortex (c), and an ovary. Details of a stigma longitudinal section showing externally oriented papillae and inner stigmatic cells with intercellular spaces and ovary longitudinal section showing an outer cuticle covering the epidermis (ep), mesocarp (mc) and the innermost cells of the endocarp (10X). The solid line in images A, D, G, J, M and P indicates a scale of 200 µm. The dashed line in images B, C, E, F, H, I, K, L, N, O, Q and R indicate a scale of 500 µm.

## Ultrastructural pistils analysis

In the microscopic observations, the pistil of the olive tree generally consists of a superior ovary, relatively short and simple style, and a papillary stigma with two asymmetric lobes (Figures 3 A-F). However, presented notable differences in terms of size and shape between the six cultivars, allowing individual recognition.

In 'Frantoio', the stigma presents fused and indistinguishable lobes, while in the remaining five cultivars the two lobes present cleft with different degrees of separation; being 'Arbequina' the one that presents total separation of the lobes from the base of the stigma. Likewise, in 'Arbosana' and 'Koroneiki', the separation of half the length of the stigma lobes is not clearly distinguished and in 'Picual' and 'Manzanilla' de Sevilla a separation of the lobes in the distal area is seen.

The OR could be classified into two types: spherical and ovoidal, depending if the value is equal to 1 or if it deviates from 1, respectively. The first group includes the cultivars 'Frantoio', 'Manzanilla de Sevilla' and 'Koroneiki', while 'Arbequina', 'Arbosana' and 'Picual' are ovoid, although 'Arbequina' and 'Picual' have a transverse axis greater than the longitudinal axis (Table 3).

Morphometric data suggest that 'Koroneiki' has the longest StigL, while 'Arbequina' the shortest. However, 'Frantoio' presented the StigW, which differs significantly from the rest. 'Arbequina', 'Arbosana', 'Koroneiki' and 'Manzanilla de Sevilla', did not show significant differences between them. 'Picual', on the other hand, had the lowest value of StigW, being significantly different from all the other cultivars.

Taking into account the StigA; 'Koroneiki'' presented the highest value with contact potential for pollen, while 'Picual' was the cultivar that showed the smallest area. The longest style (SL) corresponded to 'Picual', doubling the length of the 'Manzanilla de Sevilla' cultivar.

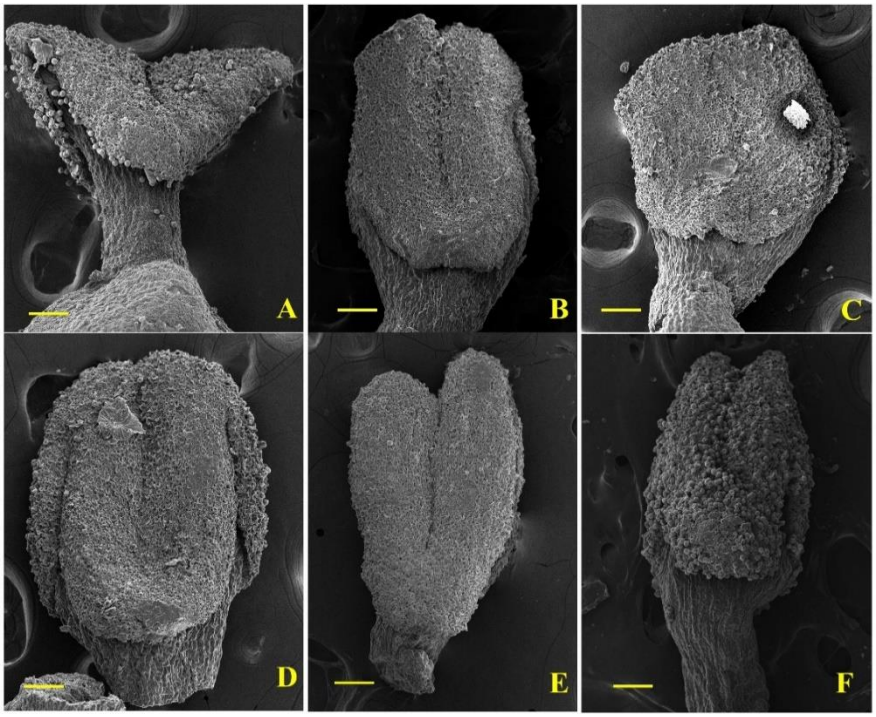


**Figure 3.** Scanning electron micrographs of olive flower stigma morphology observed under 100X. The samples correspond to the cultivars: A. Arbequina, B. Arbosana, C. Frantoio, D. Koroneiki, E. Manzanilla de Sevilla and F. Picual. Line indicates a scale of 100 µm.

**Table 3.** Variables measured in the pistil: Ovary Ratio (OR), Style Length (SL), Style Width, (SW), Stigma Length (StigL), Stigma Width (StigW), Stigma Area (StigA), made in the olive cultivars 'Arbequina' (aq), 'Arbosana' (as), 'Frantoio' (f), 'Koroneiki' (k), 'Manzanilla de Sevilla' (m) and 'Picual' (p).

| Cultivar | | OR | SL | StigL | StigW | StigA |
|---|---|---|---|---|---|---|
| | | | | n=25 | | |
| Aq | $\bar{x}$ | 0.833 $^{c}$ | 295.571 $^{c}$ | 502.162 $^{f}$ | 518.316 $^{b}$ | 259,748.33 $^{b}$ |
| | *s* | 0.012 | 7.773 | 18.749 | 18.698 | 13,356.31 |
| As | $\bar{x}$ | 1.357 $^{a}$ | 343.021 $^{b}$ | 682.705 $^{c}$ | 505.190 $^{b}$ | 344,870.54 $^{a}$ |
| | *s* | 0.096 | 18.618 | 8.042 | 751.729 | 18,864.17 |
| F | $\bar{x}$ | 1.034 $^{b}$ | 332.051 $^{b}$ | 561.410 $^{e}$ | 632.029 $^{a}$ | 357,536.82 $^{a}$ |
| | *s* | 0.054 | 11.707 | 10.254 | 62.451 | 36,906.52 |
| K | $\bar{x}$ | 1.064 $^{b}$ | 314.007 $^{b\,c}$ | 810.619 $^{a}$ | 473.471 $^{b}$ | 383,996.14 $^{a}$ |
| | *s* | 0.032 | 17.253 | 19.036 | 17.490 | 37,647.34 |
| M | $\bar{x}$ | 1.014 $^{b}$ | 237.260 $^{d}$ | 750.767 $^{b}$ | 474.862 $^{b}$ | 356,844.07 $^{a}$ |
| | *s* | 0.006 | 23.020 | 10.504 | 21.264 | 19,742.73 |
| P | $\bar{x}$ | 0.785 $^{c}$ | 478.790 $^{a}$ | 628.684 $^{d}$ | 345.677 $^{c}$ | 217,471.59 $^{b}$ |
| | *s* | 0.074 | 12.226 | 11.302 | 7.473 | 3,080.53 |

The measures (SL, StigL and StigW) are expressed in µm; (StigA) is expressed in µm$^2$, and (OR) is unitless. The data is presented as means ($\bar{x}$) and standard deviation (*s*). Different letters within a column indicate significant differences using Scheffé test ($p \leq 0.05$).

## Multivariate analyzes

A principal component analysis (PCA) was performed based on the ultrastructural measurements of the pollen grains (Figure 4 A).

The first two axes explain 76 % of the total variation. The first axis (CP1) represents the morphometric variation gradient of the variables (LA and a fraction of DSE), while the second axis (CP2) represents the trend of the data based on the variables WT, HT and part of DSE). On the axis CP1, LA clearly separates ‘Picual’, ‘Arbosana’ and ‘Manzanilla de Sevilla’. In the upper zone of the axis, ‘Picual’ represents pollen grains with a larger LA. Towards the opposite end of this axis, ‘Manzanilla de Sevilla’ and ‘Arbosana’ can be observed, that represent cultivars with pollen grains of LA smaller.

Towards the left end over the axis CP2; DSE, HT and WT separate the group of observations related to ‘Arbosana’ which is associated with low values, while ‘Manzanilla de Sevilla’ and a fraction of ‘Arbequina’ show high values of the variables, separating from the rest of the cultivars. ‘Frantoio’, ‘Koroneiki’ and part of ‘Arbequina’ make up a group that are located in the center of data dispersion and that can not be easily separated by the selected axes.

A PCA was performed based on the measurements of the pistils (Figure 4 B), whose ordering shows the first axis (CP1), defined by StigL and StigW. The second axis (CP2), clearly characterized by SL and StigA. The total variance absorbed in the first two axes is 85 %. The axis CP1 showed a descending gradient in StigL values that separates 'Koroneiki', 'Manzanilla de Sevilla', 'Arbosana', 'Frantoio' and 'Arbequina'. However, StigW has a component on the same axis but in the opposite direction. The axis CP2, clearly separates 'Picual', which is characterized by having greater SL and less potential area for the contact of the stigma lobes.

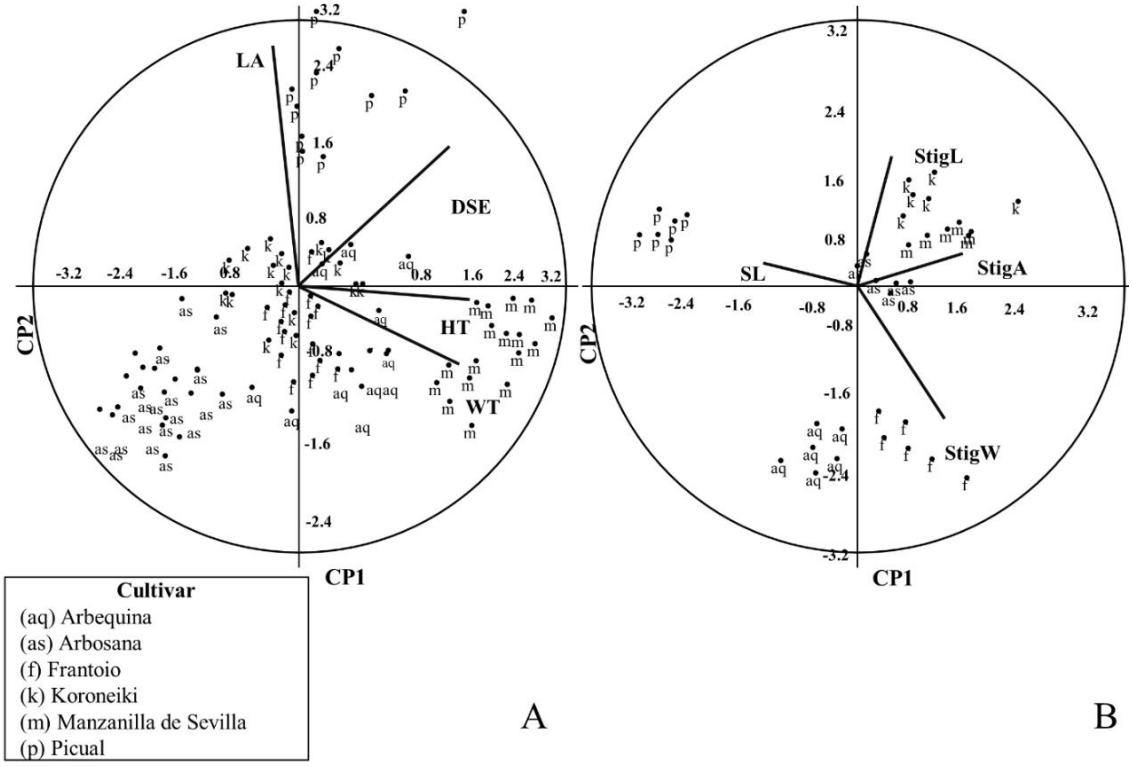


**Figure 4.** Principal component analysis. A. Parameters of the pollen of 'Arbequina', 'Arbosana', 'Frantoio', 'Koroneiki', 'Manzanilla de Sevilla' and 'Picual' cultivars, based on the vectors of the variables of lumen area (LA), distance sculptural elements (DSE), height (HT) and width tectum (WT). B. Parameters of the pistil, based on the vectors of the variables style length (SL), stigma length (StigL), stigma width (StigW) and stigma area (StigA).

## Discussion

Despite the worldwide spread of olive cultivation, the information available on the description of their reproductive structures is scarce and fragmented. It is generally presented as a small flower, actinomorphic with a size between 4 and 5 mm, with four white or cream petals and the same amount of cup-shaped green sepals (Özkaya et al., 2004; Serrano et al., 2008). The pistil is composed of a superior bilocular ovary with a short style and a relatively long and highly variable bifid stigma (Serrano et al., 2008). Meanwhile, the androecium is formed by two large yellow anthers, longitudinally dehiscent at maturity and with a variable number of pollen grains that seem to depend on the cultivar (Rojo, Salido, & Pérez-Badia, 2015). To date, the use of pollen morphological traits as discriminating elements has been very limited.

The characterization of pollen morphological traits have been essential in distinguishing species that have a similar physical appearance (Zhang et al., 2017; Du, Zhao, & Liu, 2018). The ultrastructural characters of olive pollen, particularly those of the sporoderms, have been repeatedly used for cultivar classification and discrimination (Arzani & Javady, 2002). These characters apparently have low dependence on both the environment and different phenological conditions (De La Rosa et al., 2002), in addition to presenting high polymorphic levels (Ganino et al., 2006).

On the other hand, ultrastructural studies of the gynoecium of some olive cultivars have revealed differences that depend on the type of cultivar and the stages of plant development (Koubouris et al., 2012). However, few studies have focused on partially describing the structure and composition of the pistil, differentiating each cultivar during the anthesis season (Serrano et al., 2008). More complete descriptions of the architecture, organization and composition of the stigma, style and ovary of the different olive cultivars

are still lacking. As well as combining the different morphological characters to establish statistical patterns that allow a reliable classification and discrimination of cultivars.

Given this little detailed information and its differences between cultivars, it was decided to carry out a morphological characterization of pollen and pistil, using them as tools to the identification of cultivars, under different environments in the Southern region of Brazil.

## Pollen

Studies carried out on the production, viability, development and size of pollen grains produced depend on environmental factors, such as photoperiod, irradiation, temperature, relative humidity (Ejsmond et al., 2011; García, Rivero, & Droppelmann, 2015). As the temperature rises, plants produce larger grains. However, a larger size does not fully compensate for the increase in desiccation rates, the grains have less chance of successful pollination. Nevertheless, temperature does not appear to significantly affect the shape or structure of pollen (Lenoir, Gégout, Marquet, Ruffray, & Brisse, 2008; Ejsmond et al., 2011). Therefore, the environmental conditions of Southern Brazil should not influence changes in the morphology, and can be used to discriminate between cultivars.

The studies showed that 'Koroneiki' and 'Arbequina' presented the highest values of the pollen grain count, while the lowest values corresponded to 'Picual' and 'Manzanilla de Sevilla'. The cultivars with pollen grains of larger size produced little amounts, while those with small pollen grains produced large amounts, the latter strategy being the best adapted to anemophily pollination (Faegri & Van der Pijl, 1979; Yamamoto, Kinoshita, & Martins, 2007; Cariñanos & Marinangeli, 2021). The number of pollen grains per flower for 'Arbequina' (111,474) and 'Arbosana' (109,110) were similar to those reported by Ferrara, Camposeo, Palasciano, and Godini (2007), for the same cultivars in Italy (111,258 and 90,180 respectively). Likewise, the value obtained in 'Picual' (58,066) in this study, was similar to the reported by Aguilera and Valenzuela (2012) in Spanish localities.

According to the classification system proposed by Valdés et al. (1987), the mean values of the maximum diameters obtained in this study, regardless of the three localities analyzed, ranged between 12 and 20 µm, and can be considered small in size. These results are lower than those reported in Italy, Spain or Portugal, reaching between 19 and 38 µm (Lanza et al., 1996; Castro et al., 2010; Koubouris et al., 2012; Ribeiro, Cunha, Calado, & Abreu, 2012). This smaller pollen size could be due to an eco-physiological response to adapt to the conditions of higher humidity in Southern Brazil, compared to the hydric stress and drought in the Mediterranean Sea Basin.

The high quantity of pollen grains compensates for the loss during their transport through the air, affected by excessive humidity during the development of the flower, limiting and further compromising the days of effective pollination (Cuevas, Lee, Hart, & Deaktor, 2005; Castro et al., 2010; Suárez, Castro, Rapoport, & Rodríguez-García, 2012).

In 'Koroneiki', PL, L/W and SI are lower than those reported by Koubouris et al. (2012), whose measurements were 22.68 µm; 1.47 and 3.51 $\mu m^2$, respectively. However, PW tended to be similar. 'Picual', showed values of PL and PW that barely reached half of the values reported by Castro et al. (2010). Furthermore, when comparing the L/W, the grains analyzed here tend to be sub-prolate while those of Castro et al. (2010) are markedly prolate (1.37) when dry and tend to be sub-prolate when hydrated (1.08). Although 'Arbequina' presented the highest L/W, its value is still lower than the minimum value of the three cultivars studied by Koubouris et al. (2012). The case of 'Koroneiki' is more evident, whose quotient is well below the value of 1.45 for the same cultivar. Even though the pollen grains of 'Manzanilla de Sevilla' were the largest, they reached the category of small grains, according to the range established by Valdés et al. (1987).

Details of the exine in each cultivar are visible and they can be used to morphologically separate cultivars, being this variation maintained in different environments. Specific differences were found in the exine pattern in Italy, Portugal and Spain using scanning microscopy (Pacini & Vosa, 1979; Fernández-Fernández & Rodriguez-García, 1985; Lanza et al., 1996; Ribeiro et al., 2012).

'Frantoio' and 'Manzanilla de Sevilla' showed differences, since they are characterized by a reduced lumen and a wide-walled structure, with sharp, conspicuous and widely spaced sculptural elements. On the other hand, 'Arbosana', 'Arbequina', 'Picual' and 'Koroneiki', can be easily separated by having a larger lumen area with slightly round and unremarkable structural wall elements. This combination of characters increases the possibility of a correct identification, since most of the publications usually use only grain size and exine size (Castro et al., 2010; Ribeiro et al., 2012).

By comparing the lumen area, it can be inferred that, the pollen of 'Frantoio' and 'Manzanilla de Sevilla', have small lumen areas and wide walls, and could be heavy to be transported by the wind, while 'Picual', 'Koroneiki', 'Arbequina' and 'Arbosana', whose lumen is larger and thin-walled, have greater surface/volume ratio and therefore could travel a greater distance, which makes them good candidates as pollen donor cultures.

### Pistil

Differences in the size and shape of the pistil have been found in the cultivars studied. Anatomical analyses of the pistils showed a stigma with exudates on the surface. According to some reports, these exudates allow the adherence, recognition and hydration of pollen grains (Serrano et al., 2008). Also, a style with compact cellular organization and a bilocular ovary, coincident with Ciampolini, Cresti, and Kapil (1983), were observed. In 'Picual', it is observed that it is composed of papillae arranged in a pseudostratified way, similar to the multicellular structure indicated by Serrano et al. (2008), for the same cultivar.

The differences among the cultivars suggest that the pistil could have a more stable and useful morphological character in the identification of olive trees. 'Picual' was similar with the description indicated by Serrano et al. (2008), who mentions that it has a wet bilobed papillary stigma. Also, the cultivar has a simple and solid style similar to 'Arbosana', 'Manzanilla' and 'Koroneiki', a feature previously reported by Rapoport (2008).

In the stigma of 'Picual' and 'Koroneiki', the epidermal papillae clearly show a larger contact area, although showing a smooth surface, in contrast to 'Manzanilla'. The conformation of the surface of the papillae present in 'Arbequina' is complex, presenting small fissures that further increase the pollen adhesion surface.

Despite the different tissue patterns observed in stigma and style, a common structural feature in all cultivars was the similarity in the conical shape of the stigma papillary receptive tissue and its continuity with the transmitter tissue in the style. This configuration could serve as a filter for the development of the pollen tube, since it has been observed that a reduced number of pollen grains penetrate beyond the first layers of stigma cells (Cuevas & Polito, 1997; Serrano et al., 2008; Sánchez-Estrada & Cuevas, 2019).

### Multivariate analyses

In this study the characters analysed grouped in a PCA, seem to be effective to separate cultivars. However, this idea does not support the propose by Xu and Kirchoff (2008), who mention that the measures analysed separately provide a better way to explore the differences between cultivars, when compared with complex multivariate analyses, in which the differences of the cultivars apparently are not clear.

## Conclusion

The number of pollen grains does not seem to vary when the same cultivar was evaluated in different locations, however, they showed differences between cultivars.

The 'Koroneiki' and 'Arbequina' cultivars presented higher values of small pollen grain count, which makes them good candidates as pollen donors, in addition to being better adapted to anemophily pollination.

Characters such as lumen area, width, height, shape and distance between structural elements in the wall of pollen grains observed under scanning electron microscopy, allow to separate the different olive cultivars. Likewise, the use of anatomical and ultrastructural characters of the pistils such as style length, length, width and area of the stigma, showed a remarkable uniformity within each examined cultivar, being different among cultivars.

The use of principal component analysis could be a statistical tool that allows to morphologically separate olive cultivars based on floral characters, independently of the local conditions of the state of Rio Grande do Sul, Brazil.

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