

Full Length Research Paper

Intraspecific variation in pollen viability, germination and ultrastructure of *Olea europaea* L.

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Variability of pollen morphology and ultrastructure of *Olea europaea* L. (*Oleaceae*) cultivars ‘Koroneiki’, ‘Mastoidis’ and ‘Kalamata’ was studied with scanning electron microscopy to identify genotype-distinguishing characters that could be employed for morphological cultivar discrimination. Pollen viability and germination was also measured for the three cultivars. Significant variation was observed in minimum diameter, maximum/minimum diameter (L/W) ratio and size index of pollen grain of ‘Mastoidis’ and the other two cultivars. Regarding the exine pattern, muri width of ‘Koroneiki’ and ‘Mastoidis’ pollen was similar and significantly lower than that of ‘Kalamata’. The highest values of maximum diameter were observed in ‘Kalamata’ and differed significantly with ‘Mastoidis’ but not with ‘Koroneiki’. No marked variation was noted in minimum diameter and L/W ratio. ‘Kalamata’ was characterized by higher size index than the other two cultivars, though statistical difference was observed only when compared with ‘Mastoidis’. Higher pollen viability and *in vitro* germination was recorded for ‘Kalamata’ and ‘Mastoidis’ compared to ‘Koroneiki’. Acquired information could contribute to the establishment of a database for future olive germplasm classification studies.

Key words: Floral biology, olive, pollen ultrastructure, scanning electron microscopy.

INTRODUCTION

Olive (*Olea europaea* L.) is among the earliest domesticated tree crops (Zohary and Hopf, 1994) and has had major economic, social and cultural importance in the Mediterranean Basin (Loumou and Giourga, 2003). Determination of characters with stable expression in different contexts is critical for classification of the existing varietal heritage as well as for description and isolation of genotypes with valuable characteristics (Ganino et al., 2006). Morphological and biochemical markers have been widely employed for olive germplasm classification while ultrastructural analyses have had less application. Previously implemented morphological and cytochemical studies of olive flower gynoeceum (Ciampolini et al., 1983; Serrano et al., 2008) revealed differences possibly attributed to different cultivars used

and different developmental stages examined (Serrano et al., 2008).

Roselli (1979) (Ganino et al., 2006) was the first to use pollen ultrastructural analysis for olive cultivar identification. Electron microscopy has also been used for characterization of pollen exine pattern in taxonomic studies in olive (D'hallewin et al., 1990; Bartolini et al., 1992; Lanza et al., 1996) as well as in other plant species (Fogle, 1977; Maas, 1977; Ahmedullah, 1983; Marcucci et al., 1984) since it is considered genetically stable character in different genotypes (Thakur and Thakur, 1970). Though in certain cases, insufficient information was derived for the identification of individual cultivars (Bartolini and Petruccioli, 1994). The objective of the present study was to collect information regarding morphological and ultrastructural pollen characteristics of three widely cultivated olive cultivars in order for a database to be gradually formed including a large number of cultivars and applied in germplasm characterization and

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Table 1. Pollen grain parameters of 'Koroneiki', 'Kalamata' and 'Mastoidis'.

Parameter	Cultivar		
	Koroneiki	Kalamata	Mastoidis
Maximum diameter (L) (µm)	22.68 ^a	22.54 ^a	22.49 ^a
Minimum diameter (W) (µm)	15.48 ^a	15.22 ^a	14.62 ^b
L/W ratio	1.47 ^a	1.48 ^a	1.54 ^b
Size index	3.51 ^a	3.43 ^a	3.29 ^b

Different letters within a row indicate significant differences at $P = 0.05$, least significant difference (LSD) test.

identification.

MATERIALS AND METHODS

Plant materials and growth conditions

Observations and experiments were carried out in the Institute of Olive Trees and Subtropical Plants, N.AG.RE.F, Chania, Greece. 30 to 40 years old irrigated trees belonging to cultivars 'Koroneiki', 'Kalamata' and 'Mastoidis' were selected. Mean air temperature in this area is 18°C; relative humidity (RH) is 64% and annual rainfall is 600 to 800 mm (N.AG.RE.F. Meteorological Station, Chania).

Pollen morphology

Fresh flowers from 'Koroneiki', 'Kalamata' and 'Mastoidis' trees, were collected 1 day before anthesis and stored in formaldehyde: glacial acetic acid: ethyl alcohol (FAA) solution and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, at pH 7.2 overnight at 4°C for SEM. After fixation, specimens were rinsed in buffer, post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 h, then rinsed in distilled water, dehydrated in an ethanol series and critical point dried in a BAL – TEC CPD 030 Critical Point Dryer (BAL – TEC, JAPAN). Specimens were mounted on aluminium stubs, sputter-coated with gold in a BAL – TEC SCD 050 sputter coater (BAL – TEC, JAPAN), and viewed in a JEOL JSM 840 SEM (JEOL, JAPAN) at an accelerating voltage of 10 kV. Images were recorded on ILFORD FP4 PLUS film (ILFORD, Athens, GREECE).

Images were analysed with MCID Analysis (InterFocus Imaging Ltd, Linton, UK). The parameters measured were described in detail by Lanza et al. (1996) which include the following: (1) maximum diameter (L): the maximum length is the longest free distance between any pair of boundary points. The maximum length itself is a distance and therefore independent of the orientation of the particle; (2) minimum diameter (W): the minimum length is the shortest free distance between any pair of boundary points; (3) L/W ratio: the ratio of the maximum diameter and the minimum diameter of the particle; (4) size index (SI) = $L * W / 100$. SI is the result of the multiplication between maximum diameter and minimum diameter; (5) muri width: the muri width is the thickness of exine walls measured between boundaries of two adjacent particles.

At least 30 measurements for every cultivar and parameter were implemented.

Pollen viability and germination

In each of the three years, ten inflorescences from each of four trees of the three olive varieties were collected and pollen of each cultivar was mixed and used for viability and germination

experiments. The fluorescein diacetate (FDA, Fluka BioChemika, Athens, Greece) test was employed according to Heslop-Harrison and Heslop-Harrison (1970) in order to assess pollen viability on five fields containing over 100 pollen grains each, for each cultivar. Stained pollen was observed using epifluorescence microscopy (Nikon ECLIPSE E800). In order to assess genotype dependence of pollen germination characteristics, pollen samples were *in vitro* cultured at 25°C in a growth chamber (Kottermann 2770, D3162, Hanigsen, Germany) on solid medium consisting of 0.8% (w/v) agar (Fluka BioChemika, Athens, Greece), 15% (w/v) sucrose (Fluka BioChemika, Athens, Greece), 100 ppm boric acid (Riedel-de Haen, Athens, Greece) and 60 ppm tetracycline hydrochloride (Sigma, Athens, Greece) according to Pinney and Polito (1990) with a slight modification. Indeed, a micropipette with liquid medium of the same composition excluding agar was used, in order to spread pollen uniformly on the solid medium. Pollen germination was evaluated in a light microscope (Olympus BH-2, JAPAN) after 24 h incubation in the dark, on three fields containing over 50 pollen grains each, in each of four different Petri dishes for each cultivar and experiment was repeated twice.

Statistical analysis

Data were analyzed using SPSS (SPSS Inc., Chicago, USA) and were subjected to one-way analysis of variance (ANOVA). The least significant difference tests at $P = 0.05$ were used to distinguish treatment differences.

RESULTS AND DISCUSSION

Pollen morphology

Maximum diameters of 'Koroneiki', 'Mastoidis' and 'Kalamata' pollen grains were 22.68, 22.49 and 22.54 µm, respectively and did not differ from each other significantly ($P = 0.506$, $P = 0.638$, $P = 0.839$) (Table 1, Figure 1). In contrast, significant variation was observed in minimum pollen grain diameter of 'Mastoidis' and the other two cultivars ($P = 0.000$, $P = 0.001$). As expected, 'Mastoidis' also had higher L/W ratio than 'Koroneiki' ($P = 0.003$) and 'Kalamata' ($P = 0.012$) which had similar values ($P = 0.797$). Furthermore, size index of 'Mastoidis' pollen grain was significantly lower than the other two cultivars ($P = 0.003$, $P = 0.036$) which once again did not differ from each other ($P=0.283$). Details of exine different layers are visible in Figure 2.

The second part of the ultrastructural study of pollen

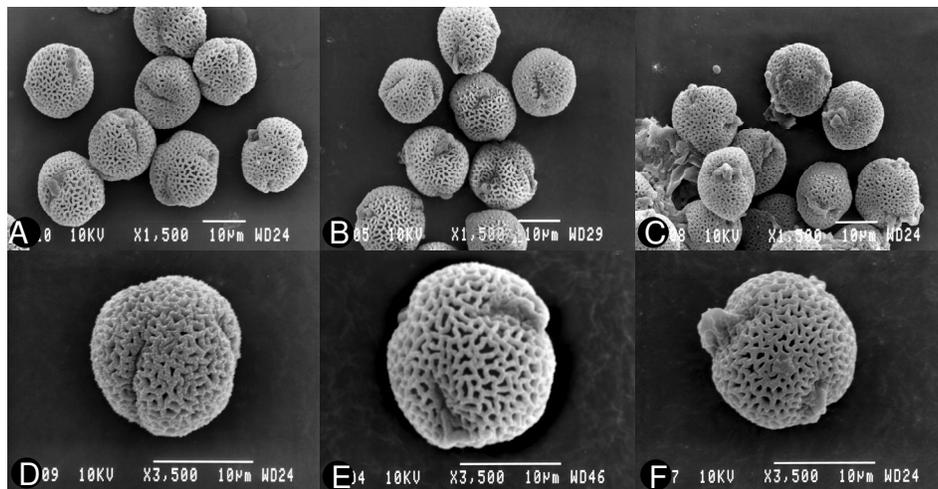


Figure 1. Scanning electron microscopy images of pollen grains of 'Koroneiki', 'Kalamata' and 'Mastoidis'. Scale bar = 10 µm. A to C, overview of pollen grains. (magnification X 1,500); D to F, representative pollen grain (magnification X 3,500).

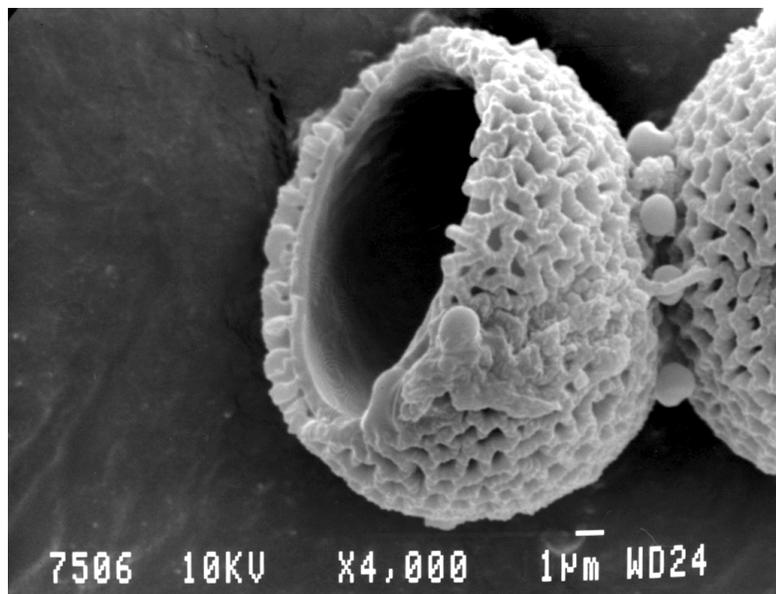


Figure 2. Scanning electron micrograph (SEM) of olive pollen grain section where details of exine different layers are visible.

grains consisted of observations and measurements regarding the exine pattern. Muri width of 'Koroneiki' and 'Mastoidis' was similar (Table 2) and significantly lower than 'Kalamata' ($P = 0.000$, $P = 0.000$). The highest values of maximum diameter were observed in 'Kalamata' and differed significantly from 'Mastoidis' ($P = 0.003$) but not from 'Koroneiki' ($P = 0.275$). No marked variation was noticed in minimum diameter and LW ratio (Table 2). 'Kalamata' was characterized by higher size index than the other two cultivars, though statistical

difference was observed only when compared with 'Mastoidis' ($P = 0.037$). Pollen ultrastructure has been employed to study genetic variation between cultivars, to comprehend developmental processes as well as to determine response of reproductive systems in environmental triggers (Pacini and Juniper, 1979a, b; Pacini and Vosa, 1979; Fernandez and Rodriguez-Garcia, 1988; Rodriguez-Garcia and Fernandez, 1990; Wunnachit et al., 1992; Lanza et al., 1996) implying wealth of information that could be revealed.

Table 2. Exine pattern parameters of 'Koroneiki', 'Kalamata' and 'Mastoidis'.

Parameter	Cultivar		
	Koroneiki	Kalamata	Mastoidis
Muri width (μm)	0.54 ^a	0.70 ^b	0.53 ^a
Maximum diameter (L) (μm)	1.17 ^{a,b}	1.26 ^b	1.02 ^a
Minimum diameter (W) (μm)	0.44 ^a	0.5 ^a	0.44 ^a
L/W ratio	3.02 ^a	3.01 ^a	2.39 ^a
Size index	0.005 ^{a,b}	0.006 ^b	0.005 ^a

Different letters within a row indicate significant differences at $P = 0.05$, least significant difference (LSD) test.

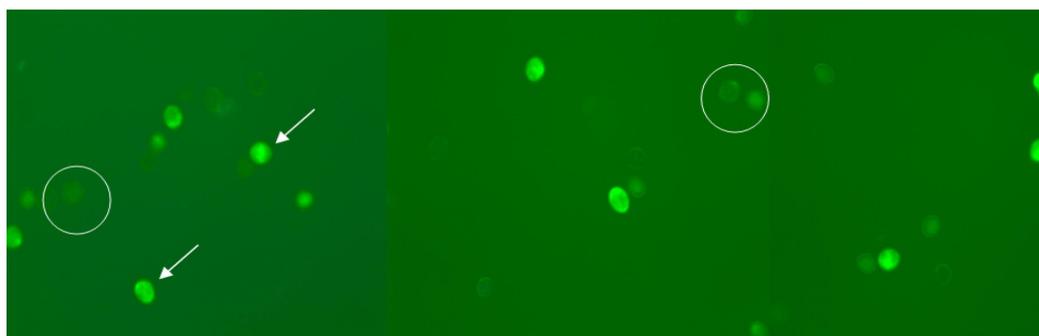


Figure 3. Olive pollen prepared for viability measurements under ultraviolet light. Viable pollen grains are marked with arrows while non-viable grains are circled.

According to our results, minimum diameter, L/W ratio and size index of pollen grains could be exploited for cultivar characterization and discrimination. In a previous paper, Lanza et al. (1996) reported significant differences only in pollen grain L/W ratio but not in maximum diameter, minimum diameter or size index of Italian olive cultivars 'Grossa di Cassano', 'Picholine', 'Leccino' and 'Coratina'. Those results indicate that only one of these four indicators was efficient to discriminate pollen of the four Italian cultivars. However, exine pattern parameters were more suitable for cultivar discrimination compared to pollen morphology. In fact, according to our results, muri width, maximum diameter and size index of pollen exine could be exploited for cultivar characterization and discrimination. In the case of Lanza et al. (1996), 13 exine pattern parameters were efficient to discriminate Italian olive cultivars 'Grossa di Cassano', 'Picholine', 'Leccino' and 'Coratina'. Similar reports on different olive cultivars could contribute to the establishment of a database with pollen ultrastructure data that would be a useful tool for future olive germplasm classification studies.

Pollen viability and germination

'Kalamata' and 'Mastoidis' without statistical differences (Table 3). However, 'Koroneiki' pollen showed high

values of pollen viability were observed in cultivars significantly lower viability. Overall, pollen viability was over 70% in all measurements indicating sufficient pollen quality of the studied cultivars (Figure 3). Pollen germination was similar for 'Kalamata' and 'Mastoidis' and significantly lower for 'Koroneiki' (Table 3). Pollen germination measurements were always lower than those regarding viability in agreement with Ferri et al. (2008). Marked variation in pollen viability of different cultivars ranging between 14 to 79% (Wu et al., 2002) and 68 to 86% (Reale et al., 2006) was also reported in previous works revealing the role of genetic factors.

Differences in germination between various cultivars' pollen grains on the same stigma could be partially attributed to differences in their pollen viability. In fact, correlation between viability and *in vivo* germination was observed in cultivars 'Kalamata', 'Frantoio', 'Manzanillo', 'Pendolino' and 'Picual' (Wu et al., 2002). However, the role of pollen - stigma interaction on pollen germination should not be underestimated (Pfahler et al., 1997) since cross-incompatibility between olive cultivars is commonly observed (Griggs et al., 1975; Cuevas and Polito, 1997; Cuevas et al., 2001; Wu et al., 2002; Diaz et al., 2007).

Conclusion

Significant genetic variability between olive cultivars

Table 3. Pollen viability and germination of olive cultivars 'Koroneiki', 'Kalamata' and 'Mastoidis'.

Cultivar	Pollen viability (%)	Pollen germination (%)
Koroneiki	74.9 ^a	52.2 ^a
Kalamata	80.7 ^b	61.9 ^b
Mastoidis	78.4 ^b	63.4 ^b

Different letters indicate significant differences at $P = 0.05$, least significant difference (LSD) test.

'Koroneiki', 'Mastoidis' and 'Kalamata' was revealed through pollen grain ultrastructure study. Ultrastructure measurements in pollen of a large number of cultivars would contribute in selecting suitable indicators for cultivar discrimination. Acquired information could be exploited as a first step in order to establish a database for future olive germplasm classification studies when relevant measurements for a large number of cultivars will be available.

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