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Pollen Ultrastructural Image Analysis among Ancient Native Olive Genotypes in the Central Eastern Tunisia

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ABSTRACT

The olive tree (*Olea europaea* L.) is considered as one of the oldest and the most important fruit crops of the Mediterranean basin, which is characterized by the existence of a considerable number of different olive cultivars. Therefore, the olive cultivar identification is crucial to safeguard the genetic patrimony of this species. Different morphological and molecular markers were used to discriminate among cultivars. The aim of the present work was to describe different pollen morphological and ultrastructural parameters (shape, size and exine pattern) as an additional tool for the identification of olive cultivars. Observations were carried on seventy centennial olive accessions grown in the Central Eastern part of Tunisia using Scanning Electron Microscopy (SEM) and Image analysis (ImageJ). Pollen were three-zonocolpate and elliptical-prolate or subprolate. Pollen morphological qualitative traits revealed specific differences among the studied genotypes including variation in whole grain shape and also exine pattern ornamentation as meshes profile and regularity and muri thickness. The quantitatively measured traits were significantly different among pollen from diverse genotypes. Polar and equatorial diameters varied from 21.80 to 29.88 µm and from 14.47 to 21.14 µm, respectively, while the pollen area ranged between 274.58 and 466.35 µm^2. Frequency distributions of most measured pollen parameters depicted a normal distribution. The three principal components of the Principal Component Analysis (PCA) accounted for more than 97% of the total variation. The first Principal Component (PC1) was correlated to pollen size. The second (PC2) and the third (PC3) were correlated to exine texture and to pollen shape, respectively. Both morphometric features and exine pattern observations were potentially relevant tools to discriminate among the studied genotypes. Further combination between pollen ultrastructural analysis, morphological and molecular markers is fully desirable, in subsequent work, to improve both reliability and discriminative ability for cultivars classification.

Keywords: Exine pattern, *Olea europaea* L., Pollen micromorphology, Scanning electron. Microscopy.

INTRODUCTION

The Tunisian olive (*Olea europaea* L.) germplasm is rich and is one of the oldest Mediterranean genetic centers of olive tree. Olives were cultivated 3,000 years ago in Tunisia (Loussert and Brousse, 1987) and were introduced in North Africa by Phoenicians. Other Mediterranean civilizations, such as Greeks and Romans, have spread this culture throughout the Mediterranean Basin. The distribution of *Olea*

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europaea L. varieties in the Mediterranean basin is very complex and it is marked by the existence of a considerable number of different olive cultivars (Bartolini *et al*., 2005).

The olive cultivars identification is crucial to safeguard the genetic patrimony of this species. Up to date, different morphological, agronomic, and chemical parameters have been used to discriminate among cultivars (Bartolini *et al*., 2005; Trentacoste and Puertas, 2011). However, these markers are still lacking where environmental or agronomic factors have influenced phenotype modification and are not used reliably for plant identification (Hannachi *et al*., 2007; Padula *et al*., 2008). During the last decade, molecular markers (RAPD, AFLP, ISSR, SSR, SNP) have been used in order to improve both reliability and discriminative ability (Gomes *et al*., 2012; Muzzalupo *et al*., 2014; Trujillo *et al*., 2014). The best taxonomic results were achieved by combining morphological and molecular markers (Lanza *et al*., 1996).

With the aim to supply useful identification tools in modern taxonomy and cultivar classification, Scanning Electron Microscopy (SEM) studies on pollen have been carried out (Pacini *et al*., 1980). The pollen structure, morphology, morphometry and the exine pattern are genetically stable, providing an important taxonomic descriptor (Thakur and Thakur, 1970; Stanley and Linskens, 1974). As these pollen descriptors are derived from genetic action, it is unlikely that they would be conditioned by environment and agronomic techniques (Heslop-Harrison, 1971; Mulas *et al*., 1987). In this sense, pollen morphology studies offer possibilities for distinguishing species and could be used also to fingerprint cultivars within species.

Pollen ultrastructure has been used to determine interspecific relationships (Nazeri Joneghani, 2008; Wrońska-Pilarek, 2011; Li *et al*., 2014; Al Watban *et al*., 2015). Pollen ultrastructure has been also studied to determine intraspecific relationships in apple (Currie *et al*., 1997), pear (Li *et al.,* 2002), apricot (Dezhong *et al*., 1995; Arzani *et al*., 2005), almond (Talaie and Imani, 1998; Sorkheh *et al*., 2008), citrus (Beris *et al*., 1993), pomegranate (Varasteh and Arzani, 2009), grape (Inceoglu *et al*., 2000) and sweet cherry (Radičević *et al*., 2013).

Regarding the olive pollen studies, numerous researches have been carried out for cultivars characterization (Pacini and Vosa, 1979; D'hallewin *et al*., 1990; Bartolini *et al*., 1992; Lanza *et al*., 1996; Lanza and Marsilio, 1999; Javady and Arzani, 2001; Koubouris *et al*., 2012; Ribeiro *et al*., 2012). However, studies about olive pollen grain genetic diversity remain limited and restricted to some olive clones and defined germplasm cultivars. No palynological study has been reported on the Tunisian olive germplasm. The aim of the present study was to examine olive pollen (shape, size and exine pattern) of seventy centennial olive accessions in the Central Eastern Tunisia, using a combination of scanning electron microscope, image processing software and statistical analysis to provide knowledge about olive pollen morphology and to individualize discriminate features of identification value on *Olea europaea* cultivars.

MATERIALS AND METHODS

Pollen

Samples were collected from archeological sites located in Central Eastern Tunisia (Figure 1). The study was carried out on 70 autochthonous olive accessions (*Olea europaea* L.). They were picked respectively from Msaken (35° 43' 60" N; 10° 34' 60" E), Bouficha (36° 18' 6" N; 10° 27' 17" E) and Sidi Bou Ali (35° 57' 24" N; 10° 28' 23"). All olive groves under study were in rain-fed conditions and subject to the traditional cultural practices in the area. Before anthesis, three branches from various parts of each tree were isolated with paper bags to avoid contamination from other pollen sources. The anthers were kept dry to allow pollen release. Pollen was collected in small vials and stored at 4ºC until used.

Scanning Electron Microscopy and Image Analysis

Small quantities of pollen grains were placed by fine brush on conductive carbon tabs mounted on the aluminium stub surface. Grains were observed using Fei Quanta 200 Environment Scanning Electron Microscope (ESEM), Fei Corporation, Eindhoven, The Netherlands, operating in low-vacuum mode (the chamber pressure was kept at 1 Torr) at 15 kV, without pre-treatment of the samples.

Damaged or poorly positioned pollen grains were excluded. Pollen grains were photographed at different magnification (2,400X for whole grain, 10,000X for exine pattern). Experience indicated that no new diagnostic morphological characters appeared at magnifications higher than 10,000X (Lanza *et al*., 1996). Thirty pollen grains of each olive accession were viewed before selecting as a representative pollen grain. The best were stored as images in files. The measurements were based on ten mature and fully developed pollen grains in each genotype. The morphometric parameters measured on pollen were extracted from micrographs using Imaging software analysis (ImageJ/FIJI 1.46, 2012). The original images were reduced to an array of picture elements (pixels). Using successive processing steps, the images binarized were used for the detection of features.

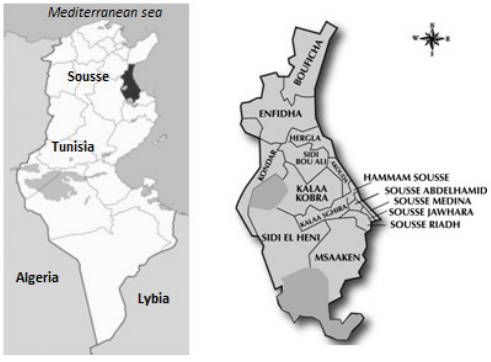


Figure 1. Geographic position of the different archaeological olive groves studied.

Parameters Measured and Statistical Analysis

Thirteen quantitative features, describing pollen's size and shape and exine pattern texture, were determined after morphometric analysis of the captured images using the ESEM.

Size Descriptors: Area (area of selection in square pixels or in calibrated square units, μm^2), perimeter (the length of the outside boundary of the selection, µm), width and height (related to the smallest rectangle enclosing the selection), major and minor axis (the primary and secondary axes of the best fitting ellipse), Feret and MinFeret (related to the maximum and minimum caliper diameters) and Size Index [SI = (Minor axis×Major axis)/100].

Exine Pattern Descriptors: Mode (most frequently occurring gray value within the selection corresponding to the highest peak in the histogram) and mean (average gray value within the selection).

Shape Descriptors: Circularity [Circ.= $4\pi\times(\text{Area})\ (\text{Perimeter})^2$], Aspect Ratio [AR= (Major Axis) (Minor Axis)] and Roudness [Round= $4\times(\text{Area})\ \pi\times(\text{Major axis})$].

Statistical Analysis

These morphological parameters were statistically analyzed by descriptive analysis (minimum, maximum, mean) and by running ANOVA. The mean values were compared by using Duncan's multiple range test. Correlations between features were determined by Pearson's test. Frequency distribution and Principal Component Analysis (PCA) were done. All statistical analyses were done by using the SPSS software (version 20.0).

RESULTS AND DISCUSSION

Olive Pollen Grain Observation

Pollen grains of the examined olive accessions were trizonocolpate with

subdivision of the surface area into three equal parts (Figures 2 and 3). This result agrees with a large number of

studies in olive pollen (Moore and Webb, 1983; Lanza *et al*., 1996). Moreover, the aperture system can be also trizonocolporate like indicated in others studies (Pacini and Vosa, 1979; Ribeiro *et al*., 2012).

The pollen grains investigated revealed important variability in terms of size [Figure 4 (A, B)] and shape [Figure 4 (C, D, E, F)]. Regarding the grain outlines in equatorial view, the olive pollen were mostly elliptic, rarely slightly elongated or triangular, and more rarely, slightly circular. The maximum equatorial diameter presented two principal positions [Figure 4 (G, H)]. It was in the medium for some olive accessions (80%) and in the bottom of pollen grain for other accessions (20%). The three germinal furrows extended differently the length of grain [Figure 4 (I, J, K)]. These furrows can be more or less parallel for most studied pollen (60%). Moreover, the distance between these furrows can be also maximal in the medium (30%) or in the bottom (10%) of pollen grain.

The microstructural analysis of the exine patterns showed interesting characteristics. This exine is formed by bacula carrying reticulated muri which has verrucosities on its surface. The thickness of the muri delimits the meshes which have various forms and dimensions in the different cultivars (nomenclature suggested by Erdtman, 1966). Thickness of muri was not constant [Figures 4 (L, M)]. It was mostly medium (50%) and sometimes thin (23%) or thick (27%). Differences in exine structure can be also noted by the presence of incomplete fusion of the muri [Figure 4 (N, P)]. This last cited characteristic was found particularly more or less evident (19 and 30%, respectively) in the studied genotypes. The major part of pollen grains noted a complete muri (Figure 4-O).

In discussing the exine pattern characteristics, the muri's incomplete closure can be considered a characteristic of normally developed pollen grains and is therefore cultivar specific when this feature is present in all the pollen grains (Pacini and Vosa, 1979), like noted in the present study.

The verrucosities of the muri vary from numerous and prominent at exine surface [Figure 4 (Q, R)] on some genotypes (22%)

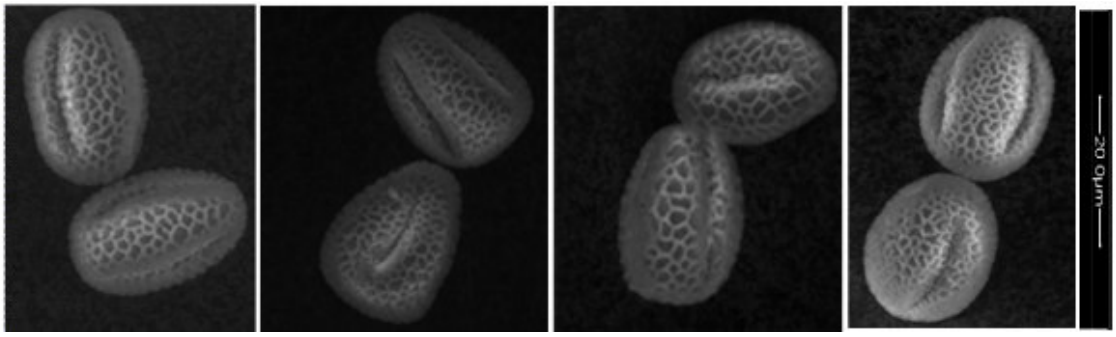


Figure 2. Olive pollen grain in equatorial view; exine and ectocolpi are visible.

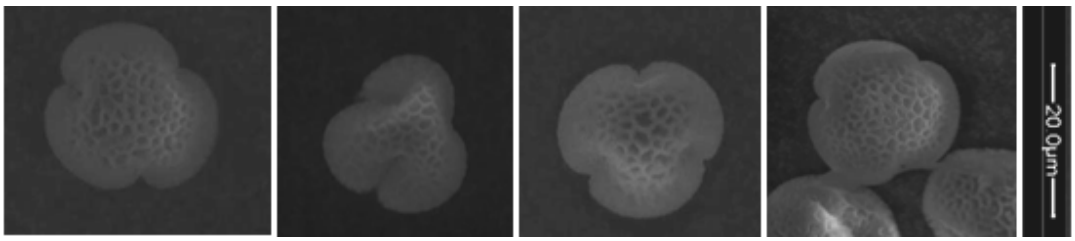


Figure 3. Olive pollen grain in polar view; exine and trizonoectocolpi are visible.

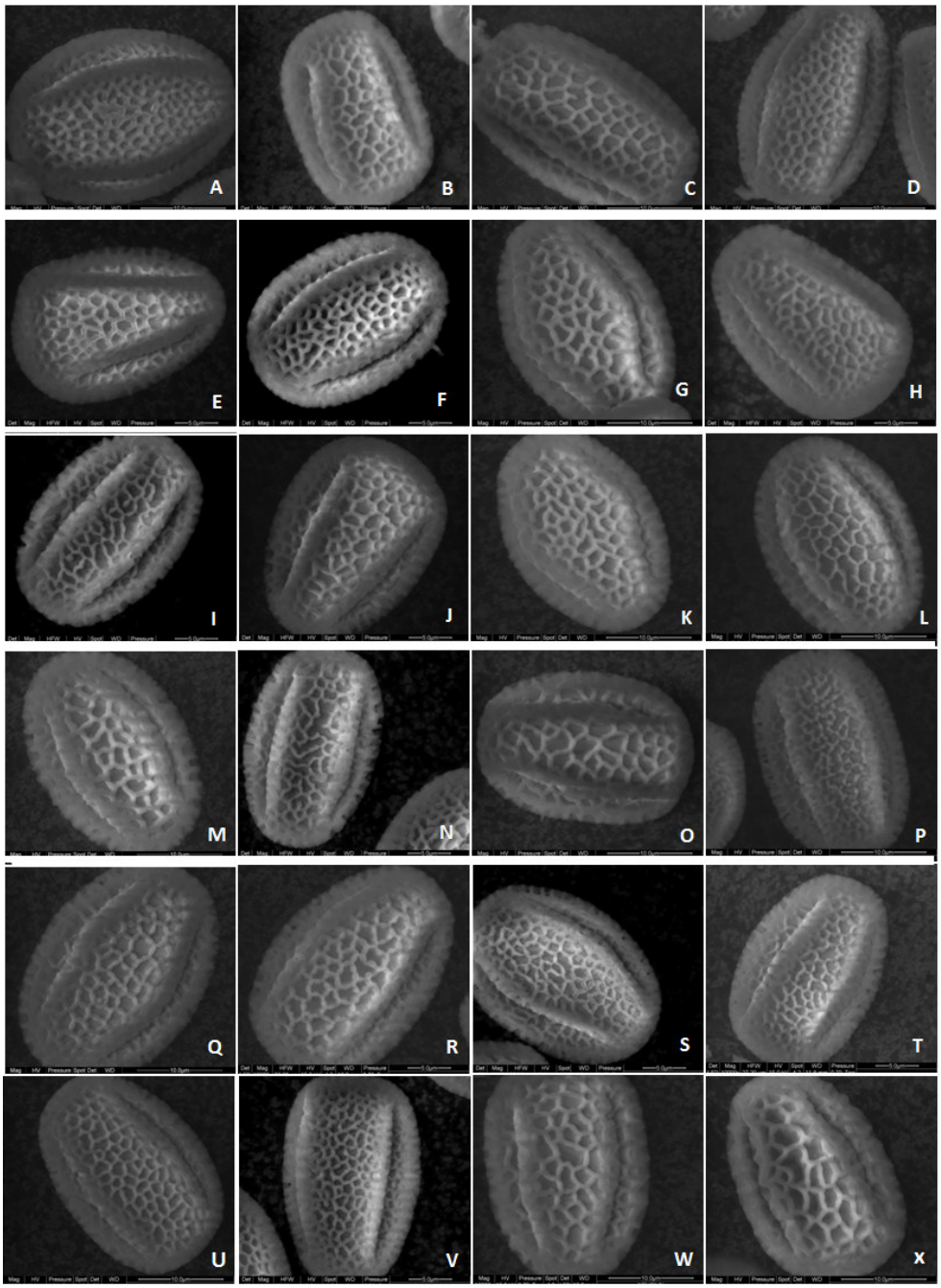


Figure 4. Photomicrographs showing typical size, shape, and exine patterns taken with the PGs orientation.

to less evident and altogether smaller (Plate) on other ones (35%). However, these verrucosities remained slightly visible for most studied pollen (43%). The bacula's diameter was not constant. It seemed greater than that of the muri [Figure 4 (S, T)] on some genotypes (18%) and less evident (38%) or practically not visible on other ones (44%).

According to the exine meshes characteristics, two main types exist: the first type characterized by small meshes (Figure 4-V) which was generally isodiametric, and the second type

characterized by larger meshes [Figure 4 (W, X)], sometimes elongated and often with gaps and incomplete muri (Figure 4-I). There are also intermediate forms which are characterized by the presence of both types of meshes (reduced meshes mixed with larger one) or by the presence of regular medium mesh (Figure 4-U). The major part of the studied pollen presented regular medium meshes (30%) or small and larger meshes mixed together (25%). Irregular mesh forms (18%), small (12%) and larger meshes (15%) were not frequently present.

The pollen grain's ultrastructure and exine ornamentation have been studied by various authors (Pacini and Vosa, 1979; Pacini *et al*., 1980; Lanza *et al*., 1996). Differences in exine pattern among some cultivars have been noted in apricot (Arzani *et al*., 2005), pomegranate (Varasteh and Arzani, 2009), almond (Sorkheh *et al*., 2008) and in apple clones (Currie *et al*., 1997). Lanza and Marsilio (1999) had studied pollen morphology of olive germplasm using SEM in order to distinguish germplasm differences and polymorphism. They reported that such pollen morphology examination using SEM revealed the relationships (differences and similarities) between little-related, closely related, and more related cultivars.

Frequency Distribution and Descriptive Analysis

Frequency distributions of different pollen parameters are shown in Figure 5. All histograms were superimposed by the Gaussian curve, which represents the normal distribution determined by the mean and the standard deviation of the studied olive pollen. The tallest bar gave the value that occurred more often and was called the mode of the distribution. The shape of the histogram gave information about the distribution of the data around the mode. The majority of the frequency distributions of pollen traits depicted a normal distribution which showed that most frequency values lie around the center of the distribution. The perimeter and circularity histograms showed a slight deviation from the normal distribution and presented an asymmetric distribution.

The pollens of the investigated olive

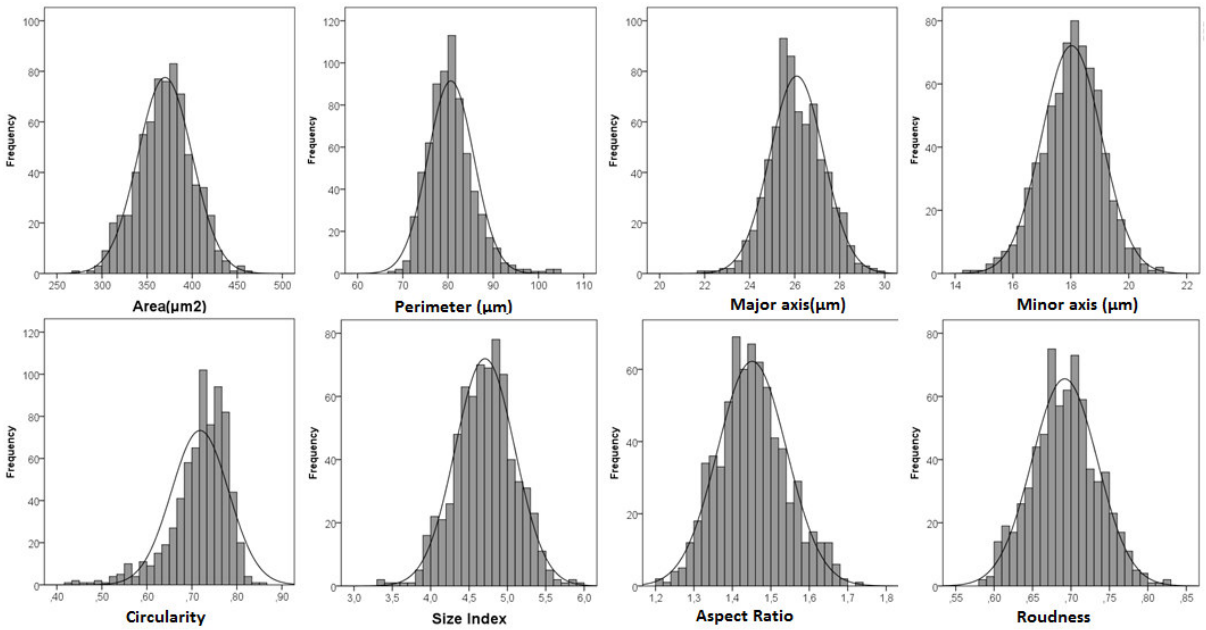


Figure 5. Frequency distribution histograms with superimposed curves of the normal probability density functions for morphometric descriptors on olive pollen grain.

genotypes showed variability in shape and size features (Table 1). With regard to polar (Major) and equatorial (Minor) diameters, the studied pollen samples were significantly different. The polar diameter's value ranged between 21.80 and 29.88 µm. The equatorial diameter's value was lower varying from 14.47 to 21.14 µm. Similar differences were noted on width, height, feret, and minferet which were correlated to major and minor axes. The area and the perimeter varied from 274.58 to 466.35 μm^2 and from 68.33 to 104.19 µm, respectively.

The circularity (Antonym: Elongation) provides information about the elongation of the particle and showed differences between studied pollens. It varied from 0.43 to 0.85. The roundness (antonym: angularity) showed also differences between the studied pollens, it ranged between 0.58 and 0.83. The Major/Minor ratio (AR) of grains ranged from 1.21 to 1.72. According to Erdtman (1971) terminology, pollen grains were usually prolate –elliptical (ratio ranged from 1.33 to 2.00) and less frequently subprolate (ratio ranged from 1.14 to 1.33).

The differences noted in polar, equatorial and diametric ratio in the Tunisian autochthonous olive genotypes were similar or often wider than Iranian (Javady and Arzani, 2001), Portuguese (Ribeiro *et al*., 2012) and Italian clones and cultivars (Pacini and Vosa, 1979; Bartolini *et al*., 1992; Lanza *et al*., 1996).

In order to express grain size more satisfactorily, an index size obtained from the product of the diameters expressed in hundredths (Roselli, 1979) was used. It ranged between 3.30 and 5.94. Almost, similar size index was reported in other studies on olive pollen (Bartolini *et al*., 1992; Lanza *et al*., 1996). Size index has proved to be substantially in agreement with those related to the diameters (mainly polar diameters), but it furnishes more complete information on true pollen grain size (Bartolini *et al*., 1992).

Correlation and Principal Components Analysis

Correlation coefficient analysis was used to recognize correlation and relationship information between different traits of olive pollen(Figure 6). A strong relationship was found between MinFeret and Minor axis (r= 0.988), Feret and Major axis (r= 0.944) and area and size index (r= 1.000). The area, perimeter and size index were significantly correlated with all pollen size parameters related to the two diametric axes. The minor axis and the area were negatively correlated to the shape factor (r= -0.634 and r= -0.388). This result suggested that pollen with a big size had generally subprolate shape (low shape factor). However, height and width parameters showed weak correlation to other diametric axis measurement. This can be explained by the fact that an oval instance

Table 1. Descriptive statistical analysis of morphometric descriptors (minimum, maximum, mean and standard deviation) of pollen grain evaluated for 70 olive tree genotypes.

	Min	Max	Mean	*SD*
Area (μm^2)	274.58	466.35	369.92	30.00
Perimeter (µm)	68.33	104.19	80.62	5.09
Width (µm)	14.89	27.48	21.94	2.89
Height (µm)	16.10	24.07	22.32	1.82
Major (µm)	21.80	29.88	26.09	1.19
Minor (µm)	14.47	21.14	18.03	1.03
Size index	3.30	5.94	4.71	0.39
Circularity	0.43	0.85	0.72	0.06
Feret (µm)	21.60	30.10	26.22	1.23
MinFeret (µm)	14.28	21.34	17.93	1.08
Aspect ratio	1.21	1.72	1.45	0.09
Round	0.58	0.83	0.69	0.04

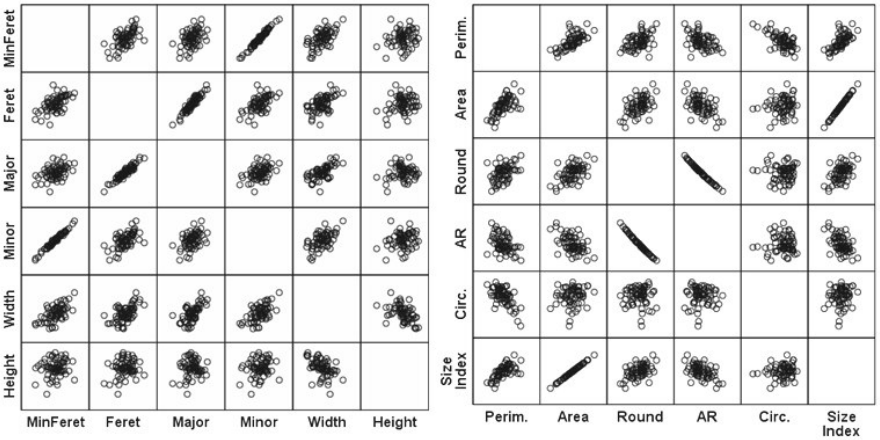


Figure 6. Correlation graphics among some pollen characteristics for the studied olive genotypes.

had distinct bounding rectangles depending on the coordinate space. Each bounding rectangle reflected the pollen orientation.

Taking into account the poor discriminant ability of some parameters to distinguish the studied olive genotypes and the high relationship existing among some parameters, it was considered suitable to eliminate, for the principal component analysis, all of the perimeter, size index, width, height, Feret, MinFeret, round and circularity.

For the entire data matrix, four whole pollen parameters (area, minor, major and AR) and two exine texture details (Mean and Mode), were subjected to principal analysis. The data were auto scaled before principal component computation in order to access the same weight for each variable. The three principal components expressed more than 97% of the total variation (Table 2). The PC scores, associated with each variable on the three principal components, distinguish the variable that mostly describes them (Table 3). The first principal component was correlated with pollen size (area, major and minor axis). The second principal component was correlated with exine texture (mode, mean), while the last one was correlated with pollen shape (AR). This result is in agreement with Lanza *et al.* (1996) who found that PC1 was defined mainly by perimeter, size index, and maximum diameter, PC2 by meshes shape, muri width and other exine pattern parameters, and, finally, PC3 by the diametric ration of the whole pollen grain and shape factor.

The projections of the loadings defined by the first two principal components (Figure 7) allowed to demonstrate the position of the studied olive accessions. Genotypes located on the left-hand side of the plane (PC1-PC2) were distinguished by a big pollen size while those on the right-hand side were distinguished by a small pollen size. Genotypes distributed and overlapped randomly on the center of the plane had

Table 2. Percentage variance contributions by Principal Components (PCs) for the characteristics of olive pollen grains.

Principal components	Eigenvalues		
	Total variance	Variance (%)	Cumulative variance (%)
1	2.783	46.378	46.378
2	1.839	30.642	77.020
3	1.223	20.380	97.400

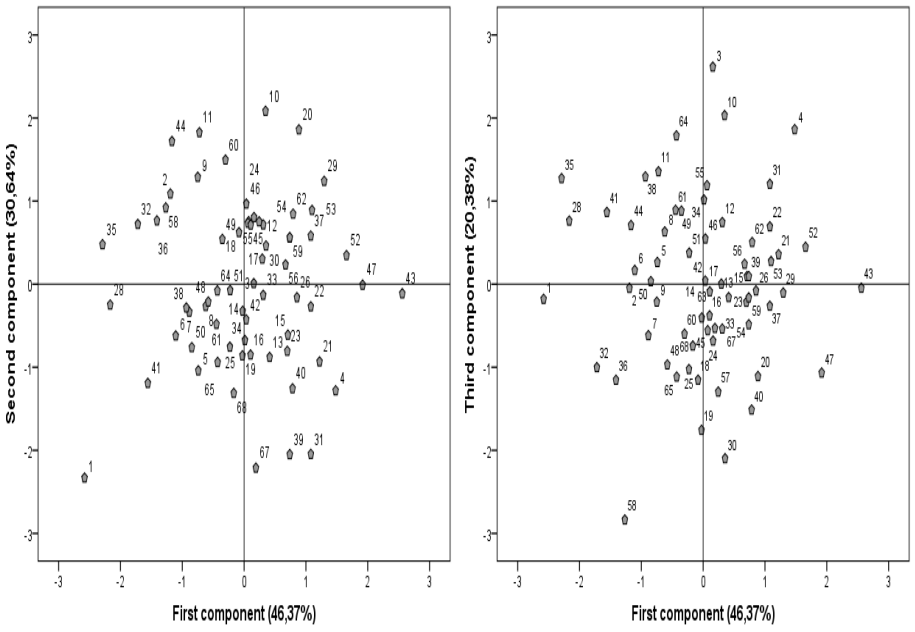
Table 3. Principal Component (PC) scores of variables on the three components for the characteristics of olive pollen grains.

Component Matrix			
	Component		
	1	2	3
Area	0.977	0.060	0.204
Minor	0.980	0.062	-0.189
Major	0.613	0.042	0.788
Mean	-0.059	0.959	0.008
Mode	-0.125	0.953	-0.008
Aspect ratio	-0.688	-0.045	0.724

generally medium pollen size. Genotypes located on the upper part of the plane (PC1-PC2) presented generally thicker and complete muri or numerous meshes. In opposite, the genotypes located on the bottom part of the plane (PC1-PC2) had generally thinner and incomplete muri or meshes with low density. The third component (PC3) distributed genotypes according to diametric ratio (AR). The genotypes located in the upper part of the plane (PC1-PC3) had elongated pollen while genotypes located in the lower part had more rounded ones. This result is consistent with the analytic key defined by Bartolini *et al.* (1992) who used the size index values and the mean mesh area to distinguish the clones of Leccino cultivar. This analytic key distinguished between clones with big size and smaller meshes and clones with opposite characteristics. Other olive clones showed features half-way between the two extremes.

The differences found in the olive pollen grain included differences in size, shape, and exine patterns which depended, itself, on the meshes' dimension and shape, the muri's thickness, and the muri and meshes regularity. These differences in pollen grain can be related to the differences in size and shape of the leaf, fruit, and endocarp as well as in general appearance of the tree (Pacini and Vosa, 1979; Petruccelli *et al*., 2014). Pacini and Vosa (1979) noted that every olive cultivar possesses its own morphological pollen characteristics which are constant and are found in successive years.

Moreover, olive has been cultivated since prehistoric times. The distribution area of



(a) (b)

Figure 7. Scores of olive genotypes on the first three principal components (a) PC1-PC2 (b) PC1-PC3.

wild and cultivated forms was very wide. This is why there are a large number of cultivars with high morphological diversity and different pollen and exine appearance.

CONCLUSIONS

The pollen grain ultrastructure morphology has proved a useful diagnostic tool to distinguish and identify the possible differences between olive genotypes. In addition, descriptive examination of pollen using SEM analysis can be an important contribution to improve knowledge about the phenotypic differences existing in the olive germplasm. It can also be used like a tool for the taxonomic characterization in breeding programs.

In conclusion, the application of image analysis to SEM micrographs allowed standing out variability in pollen's shape and size, as well as exine sculpture with various profiles of meshes, muri, bacula and verrucosities. The obtained information could be exploited as a first step in order to establish a guideline of olive pollen descriptors for future characterization studies.

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تفسیر تصویر فرا-ساختمانی گرده ژنوتیپ های بومی و کهن زیتون در مرکز بخش شرقی تونس

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چکیده

درخت زیتون (*Olea europaea* L.) به عنوان یکی از کهن ترین و مهم ترین درختان میوه در حوضه مدیترانه محسوب میشود که مشخصه آن وجود کولتیوارهای بسیار متعدد است. بنا بر این، شناسایی کولتیوارهای زیتون از اهمیت زیادی درحفظ میراث ژنتیکی این گونه گیاهی برخوردار است. در این پژوهش از نشانگرهای مختلف مورفولوژیکی و مولکولی برای متمایز کردن کولتیوارها از هم استفاده شد. هدف این پژوهش تشریح پارامتر های مختلف مورفولوژیکی و فرا-ساختمانی گرده (شکل، اندازه، و طرح exine) به عنوان ابزاری اضافی برای شناسایی کولتیوارهای زیتون بود. مشاهدات مربوطه با استفاده از میکروسکپ الکترونی (SEM)و تفسیر تصویر (ImageJ) روی ۷۰ نمونه (accession) صد ساله (centennial) زیتون که در مرکز بخش شرقی تونس کاشته می شود انجام شد. گرده ها دارای سه شیارقطری (three-zonocolpate) و بیضی شکل یا دوکی شکل بودند.صفات کیفی مورفولوژیکی گرده ها تفاوت های مشخصی در میان ژنوتیپ های مطالعه شده آشکار کرد که شامل تغییرات در شکل کلی گرده ها و طرح پیرایشی exine ونظم و مشخصات شبکه و ضخامت muri بود. در میان ژنوتیپ ها متنوع، صفاتی که به صورت کمّی اندازه گیری شد به گونه معناداری متفاوت بودند. قطرهای قطبی و کمربندی به ترتیب در محدوده ۲۹/۸۸ – ۲۱/۸ میکرومتر و ۱۴/۴۷-۲۱/۱۴ میکرومتر تغییر می کرد در حالیکه مساحت گرده ها در دامنه ۲۷۴/۵۸ و ۴۶۶/۳۵ میکرومتر مربع قرار داشت. توزیع بسآمد بیشتر پارامترهای اندازه گیری شده گرده ها یک توزیع نرمال را نشان می داد. سه جزء اصلی تجزیه به مولفه های اصلی (PCA) بیش از ۹۷٪ تغییرات کل را توجیه می کرد. اولین مولفه اصلی(PC1) با اندازه گرده همبسته بود. دومین مولفه اصلی(PC2) و سومین مولفه (PC3) به ترتیب با بافت exine و شکل گرده همبستگی داشتند. در این مطالعه، ویژگی های

مورفومتریک و مشاهدات الگوی exine هر دو ابزار مناسبی برای متمایز کردن ژنوتیپ های از یکدیگر بودند. به این قرار، برای بهبود وافزایش اعتماد به نتایج و توانایی تمایز کولتیوار ها برای طبقه بندی آن ها، ترکیب بیشتر تجزیه فرا-ساختمانی گرده ها و نشانگرهای مورفولوژیکی و مولکولی کاملا مفید است.