WAX CHEMICAL COMPOSITION AND MORPHOLOGY IN FOUR *PISTACIA* SPECIES FROM ALGERIA

DOGHBAGE Abdelghafour[1,2]*, BELHADJ Safia[3], GAUQUELIN Thierry[4], GREFF Stephane[4], TONETTO Alain[5], DERRIDJ Arezki[1] and MEVY Jean Philippe[4]
[(1)]Faculté des Sciences biologiques et agronomiques, Université Mouloud Mammeri de Tizi-Ouzou, Algérie
[(2)]Centre de Recherche en Agropastoralisme (CRA-past), 17000 Djelfa, Algérie
[(3)]Département Agro-vétérinaire, Faculté des Sciences de la Nature et de la Vie, Université Ziane Achour de Djelfa, 17000 Djelfa, Algérie
[(4)]Aix Marseille Université, Avignon Université, CNRS, IRD, IMBE, Marseille, France
[(5)] ix Marseille Université, CNRS, Centrale Marseille, FSCM, PRATIM, Marseille, France
E-mail: doghbage_abdelghafour@yahoo.com

(Received 11 May 2021 – Accepted 23 December 2021)

Abstract.- *Leaf samples of* Pistacia atlantica, P. lentiscus, P. terebinthus *and* P. vera *from different sites, in Algeria, were characterized according to their epicuticular wax chemical composition and morphology. The cuticular waxes were observed under scanning electron microscope and analyzed by gas chromatography coupled to mass spectrometry after cyclohexane extraction. Wax morphology revealed a glossy aspect with a smooth wax layer in* P. lentiscus *leaves while a thin structure is observed in* P. atlantica. *The qualitative composition of the waxes, were different for the four species. The principal components were aliphatics, phenols and terpenes. There were, as well, quantitative differences between the taxa, concerning phenol and aliphatic compounds. P. vera show the highest phenol content, while* P. atlantica *and* P. terebinthus *have the highest aliphatic contents. Regarding the infraspecific variation, P. atlantica from semi-arid-fresh site exhibit more aliphatic and terpene. For* P. lentiscus, *sub-humid-mild site exhibited more aliphatic compounds while the semi-arid-cold site had the highest terpenes amount.*

Key words: *Pistacia, leaves, waxes, morphology, chemical composition.*

COMPOSITION CHIMIQUE ET LA MORPHOLOGIE DE LA CIRE CHEZ QUATRE ESPECES DU GENRE *PISTACIA* EN ALGERIE

Résumé.- *Des échantillons de feuilles de Pistacia atlantica, P. lentiscus, P. terebinthus and P. vera, récoltés de différentes stations sur le territoire algérien, ont été chimiquement et morphologiquement caractérisés. Les cires cuticulaires ont été observées sous microscope électronique à balayage (MEB) et analysées par chromatographie à phase gazeuse couplée au spectrophotomètre de masse (CGSM) après leur extraction avec du cyclohexane. L'observation au MEB des surfaces foliaires a révélé un aspect brillant avec une couche lisse des cires chez les échantillons de feuilles de P. lentiscus et une structure plus fine pour P. atlantica. La composition qualitative des cires, diffère selon les quatre espèces. Les composés principaux sont les aliphatiques, les phénols et les terpènes. Des différences quantitatives ont également été détectées entre les taxa, concernant les composés phénoliques et les composés aliphatiques. P. vera, particulièrement, a enregistré la valeur la plus élevée pour les composés phénoliques, alors que P. atlantica et P. terebinthus enregistrent les valeurs les plus importantes pour les composés aliphatiques. Concernant la variation infraspecifique, P. atlantica de l'étage bioclimatique semi-aride frais montre plus de composés aliphatiques et terpéniques. Pour P. lentiscus, du subhumide doux, plus de composés aliphatiques ont été enregistrés alors que la station du semi-aride froid montre la valeur la plus élevée pour les terpènes.*

Mots-clés: *Pistacia, feuilles, cires, morphologie, composition chimique.*

Introduction

Plant surfaces represent the relationship between plants and their environment. Cuticles, that cover the primary above ground organs of plants, are very efficient transport barriers. Their main physiological functions are the protection against uncontrolled water loss by transpiration and the reduction of leaching of essential solutes from inside the cells [1,2]. The cuticle is mainly composed of a three-dimentional network of cutin, which is a polymeric polyester, and integrated superimposed soluble lipids called cuticular waxes.

Plant waxes are composite materials of aliphatic hydrocarbons and their derivatives with carbon chain lengths between 20 to 60 atoms [3,2]. Several properties of the cuticles are mainly based on the waxes. The ones that are deposited on the cuticular surface are referred as epicuticular waxes [4]. They strongly influence the wettability, self-cleaning behaviour and the light reflection at the cuticle interface; whereas, intracuticular waxes mainly function as water transpiration barrier. For most plants, the abaxial and adaxial leaf surfaces appear different under the electron microscope. Most of the epicuticular waxes form two-and three-dimensional structures, varying from hundreds of nanometers to micrometers, with great variations of their morphologies. They are composed mainly of very long aliphatic chain and exist as an amorphous film or as crystalline structures. The proportion of the different components in the wax differs among plant species and influence the fine structure of the deposits on the leaf surfaces [5,2]. The shape and density of waxes may be of some taxonomic value, but a plant may have more than one form of wax surface [6].

The chemical composition and the morphology of cuticular waxes have been studied in several plant species, using various extraction and imaging methods. To our knowledge, no work has been done for *Pistacia* L. species (Anacardiaceae) from Algeria or elsewhere. The members of this genus may be either evergreen or deciduous, characterized by alternate, pinnate leaves with a single layer of thin walled epidermal cells in, both, leaflet surfaces. The epidermal cells are covered with a relatively thick layer of cutin in *P. lentiscus*, *P. Mexicana* and *P. weinmannifolia*, but little or no cutin is observed in other species [7,8]. Although, the micromorphology of the leaf of various species of this genus have been studied by electron microscopy (SEM) [7-18], very little is known about the morphology and the chemical composition of their leaf waxes. The aim of this study is to investigate how leaf waxes morphology and composition vary among the *Pistacia* taxa. The study is based on SEM and gas chromatography coupled to mass spectrometry (GC-MS). This study provides more data that can be used in combination with morphological data to refine the taxonomic relationships among the different *Pistacia* species.

1.- Material and methods

1.1.- Plant material

Mature leaves of the studied species were collected, in summer time, from different locations (3 sites D_3, MS_1 and MS_2 for *P. vera* , 2 sites D_1 and MS_1 for *P. atlantica*, 3 sites B, D_2 and E for *P. lentiscus* and 1 site D_2 for *P. terebinthus*) (tab. I). In each area, six fully expanded leaflets were randomly sampled from different leaves, from three healthy trees (a total of 54 leaflets). The samples were allowed to dry under laboratory environmental conditions until use.

Table I.- Main Climatic Features Of The Experimental Selected Sites

Sites (locations)	Elevation (m)	M. (ºC)	m. (ºC)	An. Rain (mm)	Q_3	Climate type
Boumerdes (B)	495	32.8	5.9	650	82.9	Sub Humid Mild
Medea (E)	950	31	-1.3	490	52.1	Sub Humid Cold
Djelfa-Zaina (D1)	1092	34.6	1.4	320.9	34.7	Semi Arid Fresh
Djelfa-Senelba (D2)	1270	33	-1	320.9	34.4	Semi Arid cold
Djelfa-Rosslayoun (D3)	1153	34	1.4	320.9	33.5	Semi Arid Fresh
M'sila-Elmergueb (MS1)	630	36.6	2.7	308.6	31.2	Semi Arid Fresh
M'sila-Oued Soyeb (MS2)	469	38.2	3.7	214.3	21.3	Arid Mild

Source of climatic data: National Meteorology Office of Algeria (O.N.M.); Q_3: Pluviometric quotient of Emberger, M: Mean of the maxima temperatures of the warmest month, m: Mean of the minima temperatures of the coldest month.

1.2.- Scanning electron microscopy

Standard procedures were followed for Scanning Electron Microscope (SEM) to study the epidermal leaf surfaces. Three specimens from each location were examined. A sample section of $5mm^2$ of the dry leaf surface (both abaxial and adaxial faces) was fixed on a labelled stub. The samples were coated with gold and scanned in a Philips XL 30ESEM (USA). Micromophological observations included wax distribution, occurrence and morphology. SEM pictures were digitally recorded in different magnifications. A mean of 3 micrographs were recorded for each sample (162, in total).

2.3.- Cuticular compounds extraction

The extraction method used consisted of suspending leaf dry matter ($0.785cm^2$) in cyclohexane (analysis grade, Carlo Erba ®) for 2 mn under sonication (Ultrasonics 88169, Bioblock Scientific ®). Three specimens from each location were analyzed.

2.4.- Analysis of compounds

Leaf matter was manually removed, and extracts were let to dry until complete evaporation of solvent at room temperature for 24 hours. The residue was then suspended in 200 µl of cyclohexane and analyzed with a gas chromatograph Agilent® GC 6890 coupled to a mass selective detector 5973 Network. The system was fitted with an HP-5MS capillary column 30m-0.25mm, 0.25 µm. Two (2) µl of extracts were injected through an automatic injector ALS 7683 in splitless mode. Purge was set at 50 mn ml^{-1} after 1 mn. Injection temperature was maintained at 250°C. Helium was used as carrier gas. A constant flow rate of 1 ml mn^{-1} was set throughout the run. The oven temperature initially set at 160°C for 2 mn was increased to 240°C at a rate of 10°C mn^{-1}, then increased to 320°C at a rate of 4°C mn^{-1} and remained constant for 5 mn. The MSD transfer line heater was maintained at 330°C. Mass detector parameters are : ion source, 230°C; quadrupole, 150°C; EI, 70eV; EMV, 2500V; acquisition in scan mode from 40 à 800 uma. Cuticular compounds were identified by comparison of their retention index and mass spectra obtained from literature and instrument database "NIST/EPA/NIH Mass Spectral Library (NIST 08)". Quantitation is expressed in percentage relative to Total Ion Chromatogram.

1.5.- Statistical analysis

The data were subjected to one-way ANOVA with post-hoc *Tukey* test (or the *t*-test) to determine whether the differences were significant or not, using Statistix analytical software (version 1.0, 1996).

2.- Results

2.1.- Wax occurrence and morphology

The main morphological wax features of each species are reported with electron microscope images (fig. 1-3). Both surfaces are completely covered with differently shaped wax structures found on the leaves surfaces. Thin layers or flakes and some crystal deposits are obviously apparent on all the studied leaves. However, in the abaxial face, particular flakes type deposits were observed particularly in *P. terebinthus* (fig. 1A) and *P. vera* (fig. 3) while a glossy aspect with a smooth and homogenous layer of waxes is particularly observed in all *P. lentiscus* leaves (fig. 2). In *P. atlantica*, a thin structure with flakes deposits is observed (granular film with dusty-like structure) (fig. 1C and E). Wax crystalloids are overall distributed in the entire leaf surface in *P. atlantica* and *P. vera* (fig. 1E, 3C). Obvious wax of irregularly distributed crystalloids layers cover completely the adaxial epidermis in *P. terebinthus, P. atlantica*, and *P. vera* (fig. 1B-1D, 3B-3D-3E). Some wax crusts are seen in the leaf adaxial face from Zaina (D1) site for *P. atlantica* (fig. 1F) and some cracks in the wax crust are also seen in some leaves in Senalba site for *P. vera* adaxial leaf surface (fig. 3D). Both surfaces of *P. lentiscus* leaves, show an interrupted wax layer with several lacks and cracks (fig. 2A, 2C, 2D, 2F). Many areas show large amounts of wax entirely coating the trichomes (fig. 2F) and the stomata (fig. 1A, 3A).

2.2.- Chemical composition

Forty six compounds were identified, corresponding to 91% of the total (seventy five detected compounds). The wax extracts of *Pistacia* species revealed the presence of various typical plant wax constituents, including phenols, aliphatics and terpenes. Considerable amounts of aliphatics (52%) and terpenes (24.4%), followed by phenols (14.1%) some unknown compounds (9%) and others (0.5%), were identified (tab. II). Concerning the phenolics, the components were alkyl phenols (8.7%), with the phenol, 3-tridecyl- as major component (6.7%), and alkenyl phenols (5.4%) with the phenol, 3-(x-heptadecenyl)- as major component (3%). The aliphatic components (52%) in the waxes of *Pistacia* leaves were alcanes (41%), aldehydes (7%) and esters (3.7%). About twenty seven molecules were detected for this group. The major components for the three classes were, heptacosane (5.6%), nonacosane (9.5%) and hentriacontane (16%) for the alcanes; hexacosanal (1.3%), octacosanal (1.7%) and triacontanal (2.6%) for the aldehydes. For the third group (esters), the amounts detected are smaller as compared to two first groups (alcanes and aldehydes). Only one component (hexacosanoic acid, methyl ester) was detected above 1%. The terpenic components ranged in two different classes: diterpenes (1.%) and triterpenes (23.2%). Four different molecules were detected among the diterpenes, but in small amounts (less than 0.5%) while five molecules were detected for the triterpenes in, relatively, high amounts (more than 2%). The heptadecan-4-olide,4,8,12,16 tetramethyl (0.5%) was the major components for the diterpenes while the α-amyrin ester (5.24%) and α-tocopherol,acetate (9.9%) were the major components for the triterpenes. Two other molecules, classified as others, as the tridecane, 2-phenyl-, and

the 1-oxaspiro[4.5]deca-6,9-diene-2,8-dione, 7,9-bis(1,1-dimethylethyl)- were also detected in very small amounts (0.4%). Twenty nine unknown compounds were also detected in relatively small amounts (9%). The chemical composition of the waxes varied quantitatively and significantly among the four *Pistacia* species, for the phenols and the aliphatics while there were no significant differences among the studied species for the terpenes and the others group (tab. II).

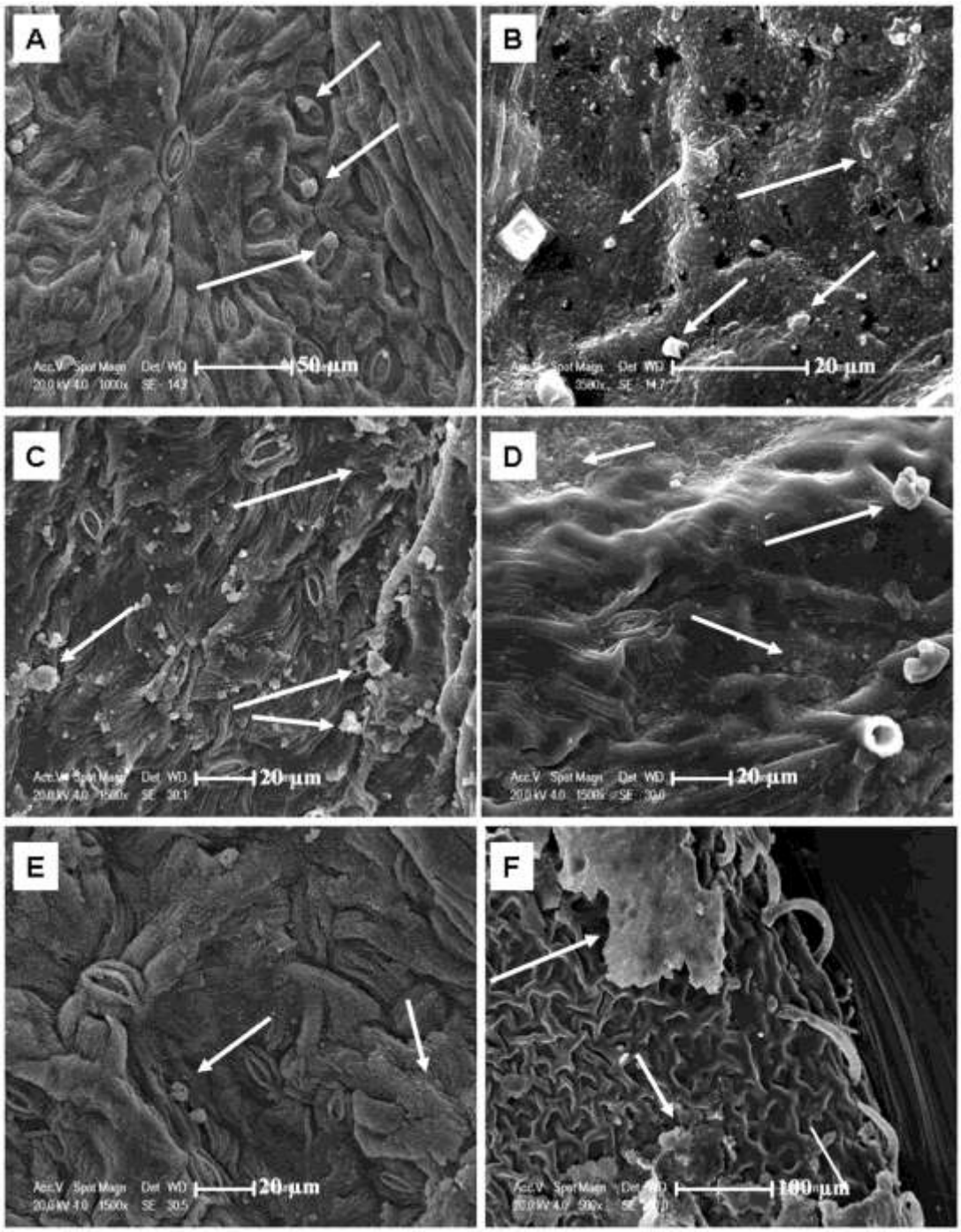

Figure 1.- SEM micrographs of abaxial and adaxial leaf surfaces of *Pistacia* species from different populations, showing waxes' features. (A-B) *P. terebinthus*, (A) abaxial face, wax flakes deposits nearby the stomata (arrows) (B) crystalloid deposits in the adaxial face. (C-E) *P. atlantica*, (C and E) abaxial face from El Mergueb site, (granular film with dusty like structure), (D) wax crust in the

leaf adaxial face from Zaina site.

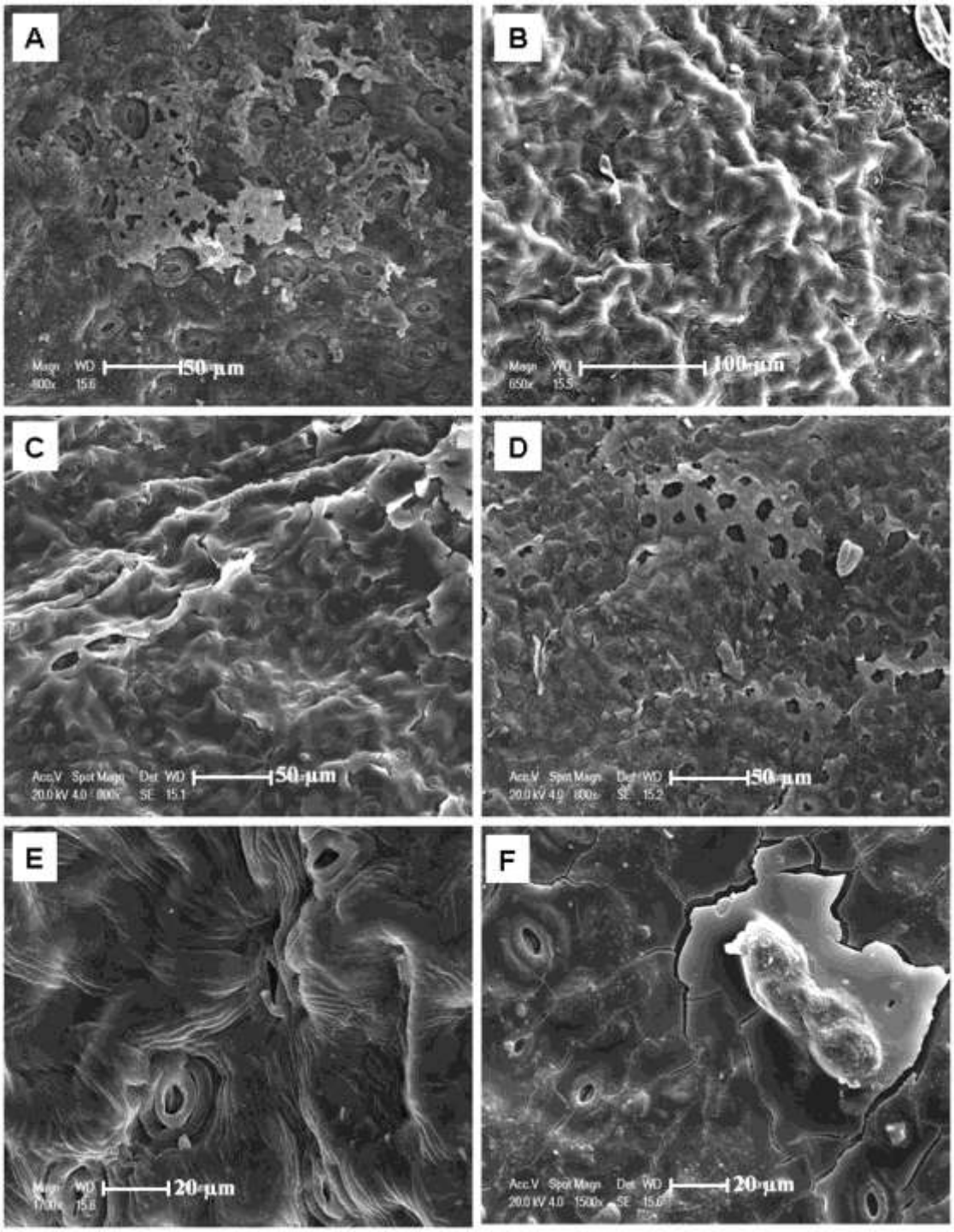

Figure 2.- SEM micrographs of abaxial and adaxial leaf surfaces of *P. lentiscus* from different populations showing waxes' features. (A-B) granulate and anomorphous deposits in thin layers of waxes over the epidermis surface in Boumerdes site (A and E) abaxial face, (B) adaxial face. Glossy aspect of the wax layer on the abaxial face in Senalba site (C), granulate deposits on the adaxial face in Hamdania site (D), wax crust on the adaxial leaf surface (F).

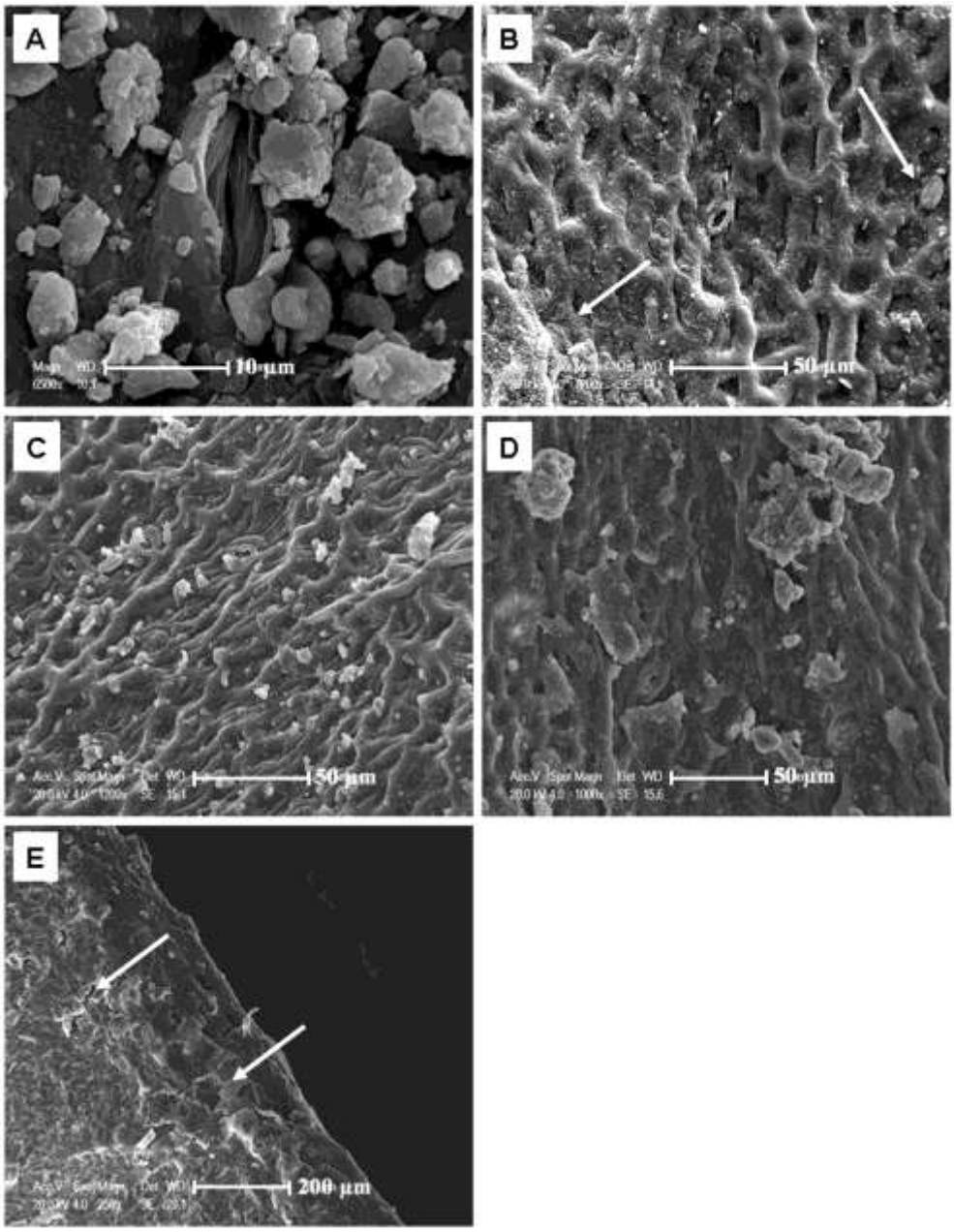

Figure 3.- SEM micrographs of abaxial and adaxial leaf surfaces of *P. vera* from different populations showing waxes' features. Crystalloids deposits around stomata on the abaxial face in Elmergueb site (A), granulate deposits on the adaxial face (B), granulate deposits on a thin wax layer in Senalba site, on the abaxial face (C) and adaxial face (D), cracks in the wax crust in Senalba site (Arrows).

For the phenols, the variations were highly significant with the highest amount (39.3%) recorded for *P. vera.* The variations were highly significant for the alkyl phenols and very significant for the alkenyl phenols, with the highest amounts found in *P. vera* (23.3% and 16%, respectively). For the aliphatics, the variations were highly significant for the three compound classes (alcanes, aldehydes and esters). The highest aliphatic amounts were recorded for *P. atlantica* (70.4%) and *P. lentiscus* (70%) followed by *P. terebinthus* (64.3%). *P. lentiscus* (66.1%) and *P. atlantica* (51.1%) showed more alcanes, while *P. terebinthus* showed more aldehydes (41%) and *P. atlantica* the highest amount in esters (13.6%). The Chemical composition varied also qualitatively as no phenols were detected

in *P. lentiscus*, no esters in *P. vera*, no alkenyl phenols in *P. atlantica* or other compounds (Tridecane,2-phenyl- and 1-oxaspiro [4.5] deca-6,9-diene-2,8-dione,7,9-bis (1,1-dimethylethyl)-) in *P. terebinthus.* However these qualitative variations were significant only among *P. vera*, *P. atlantica* and *P. lentiscus*, for the following compounds (esters, alkenyl phenols and phenols, respectively) (tab. II).

Concerning the infraspecific chemical composition of the waxes, the variations were different quantitatively and qualitatively (tab. III, IV and V). For *P. atlantica*, M'sila site recorded higher aliphatics (72,6%) and terpenes (20,9%), while, some phenols (0.5%) were detected solely in this site. In Zaina site (D_1), 23.2% of other and unknown compounds were detected against 5.9%, in MS1 site. These variations were highly significant for the aliphatics and significant for the terpenes while no significant differences were recorded for the phenols although 0.5% of alkyl phenols were detected in MS_1 site (tab. III).

Table III.- Epicuticular wax composition of *Pistacia atlantica* leaves from two sites

Compound	*P. atlantica* (MS_1) (%)	*P. atlantica* (D_1) (%)	*t*-test
Phenols	**0.50±0.50**	--	**NS**
-*Alkyl phenols*	0.50±0.50	--	NS
Phenol, 3-tridecyl-	0.50±0.50	--	NS
Aliphatics	**72.60±9.16**	**68.27±3.87**	***
-*Alcanes*	58.87±8.64	43.49±1.04	***
Tetracosane	--	1.33±0.68	NS
Pentacosane	2.60±1.42	3.50±1.78	*
Hexacosane	0.63±0.63	1.50±0.76	NS
Heptacosane	11.80±1.01	10.73±1.21	***
Octacosane	3.40±0.61	1.80±0.06	**
Nonacosane	33.27±4.46	17.90±3.22	**
Triacontane	0.90±0.90	0.33±0.33	NS
Hentriacontane	5.17±1.43	3.53±0.46	**
Dotriacontane	1.10±1.10	--	NS
Tritriacontane	--	0.30±0.30	NS
-*Aldehydes*	6.63±1.40	4.73±2.87	**
Tetracosanal	--	0.47±0.47	NS
Hexacosanal	1.17±0.66	1.40±0.70	*
Octacosanal	0.67±0.67	1.90±1.04	NS
Triacontanal	4.80±0.84	0.97±0.97	*
-*Esters*	7.13±2.41	20.17±4.42	*
Tetracosanoic acid, methyl ester	1.33±0.67	6.00±2.17	NS
Hexacosanoic acid, methyl ester	3.87±0.99	5.60±1.93	**
Acetic acid, hexacosanyl ester	0.87±0.87	4.77±0.49	*
Octacosanoic acid, methyl ester	--	0.13±0.23	NS
Acetic acid, octacosanyl ester	1.10±1.10	3.63±0.07	*
Terpenes	**20.93±9.53**	**8.73±3.20**	*
-*Diterpenes*	3.13±3.13	1.20±1.20	NS
Trans-phytol	2.37±2.37	--	NS
Phytone	--	1.20±1.20	NS
Pseudo phytol	0.47±0.47	--	NS
Heptadecan-4-olide,4,8,12,16tetramethyl	0.30±0.30	--	NS
-*Triterpenes*	17.80±7.38	7.53±3.63	*
Squalene	0.80±0.80	0.10±0.10	NS

Lup-20(29)-en-3-one	1.03±1.03	--	NS
α.-Tocopherol, acetate	13.13±7.02	--	NS
β -Amyrin ester	2.83±2.83	7.40±3.70	NS
Others	--	**2.67**±1.45	**NS**
Tridecane, 2-phenyl-	--	0.13±0.13	NS
1-oxaspiro[4.5]deca-6,9-diene-2,8-dione, 7,9-bis(1,1-dimethylethyl)-	--	2.53±1.44	NS
Unknown	**5.97±3.09**	**20.40±5.15**	**

SE, standard error; tr, traces (<0.1%); --, not detected. Each percentage is the mean of three replicates±SE; Mean separation within lines, by *t*-test (*P<0.05, ** P<0.01, *** P<0.001, NS: not significant).

No significant differences were recorded among *P. vera* sites (Table 4). It should be noted that the three major compounds were recorded in the studied sites in different amounts; 59.8% in MS2, against 32.2% in MS1 and 25.8% in D3, for the phenolic compounds. For the aliphatics, 30.9% in MS1, 13.5% in D3 and 8.7% in MS2 and finally, 56.1% in D3, 36.1% in MS1 and 25.5% in MS2 for the terpenes. For the second group, the aliphatics, no aldehydes were detected in D3 and MS2 sites.

Table IV.- Epicuticula wax composition of *Pistacia vera* leaves from three different sites

Compound	***P. vera* (D_3) (%)**	***P. vera* (MS_2) (%)**	***P. vera* (MS_1) (%)**	***Tukey test***
Phenols	**25.80±8.44**	**59.83±17.0**	**32.20±16.63**	**NS**
-Alkyl phenols	17.57±9.08	29.93±7.03	22.43±13.05	NS
Phenol, 3-undecyl-	0.30±0.30	0.13±0.07	--	NS
Phenol, 3-tridecyl-	15.87±8.59	23.47±6.21	19.60±11.40	NS
Phenol, 3-pentadecyl-	0.87±0.87	4.93±0.71	2.83±1.64	NS
Phenol, 3-heptadecyl-	0.57±0.57	1.37±0.37	--	NS
-Alkenyl phenols	8.23±2.14	29.90±10.12	9.80±5.52	NS
Phenol, 3-(x-tridecenyl)-	3.77±1.16	11.93±5.31	2.37±2.37	NS
Phenol, 3-(x-pentadecenyl)-	0.83±0.42	0.73±0.26	0.93±0.93	NS
Phenol, 3-(x-heptadecenyl)-	3.60±0.91	17.26±6.27	6.50±3.26	NS
Aliphatics	**13.50±7.11**	**8.73±5.11**	**30.93±16.89**	**NS**
-Alcanes	13.50±7.11	8.73±5.11	29.33±17.88	NS
Docosane	--	0.23±0.23	--	NS
Tetracosane	0.27±0.27	0.43±0.34	--	NS
Pentacosane	5.23±5.23	--	0.40±0.40	NS
Hexacosane	0.23±0.23	0.67±0.52	0.57±0.57	NS
Heptacosane	4.77±1.09	3.50±1.35	5.60±5.60	NS
Octacosane	0.53±0.27	0.97±0.67	0.67±0.67	NS
Nonacosane	2.23±0.63	2.40±1.56	1.53±1.53	NS
Triacontane	--	0.13±0.13	--	NS
Hentriacontane	0.20±0.20	0.40±0.30	--	NS
-Aldehydes	--	--	1.60±1.02	NS
Tetracosanal	--	--	0.43±0.43	NS
Heptacosanal	--	--	1.17±1.17	NS
Terpenes	**56.13±16.36**	**25.47±6.71**	**36.13±11.45**	**NS**
-Diterpenes	2.37±1.76	2.33±1.39	0.83±0.52	NS
Trans-phytol	0.27±0.27	0.20±0.12	--	NS
Phytone	0.67±0.67	0.33±0.33	--	NS
Heptadecan-4-olide,4,8,12,16tetramethyl	1.40±1.16	1.77±1.02	0.83±0.52	NS

-Triterpenes	53.77±18.11	23.10±5.35	35.33±11.78	NS
Squalene	6.90±3.48	1.23±0.82	9.57±7.70	NS
Lup-20(29)-en-3-one	0.53±0.27	0.63±0.54	--	NS
α.-Tocopherol, acetate	20.07±10.25	7.83±3.73	17.50±10.51	NS
β.-Amyrin ester	5.47±1.26	2.23±1.76	1.20±1.20	NS
α.-Amyrin ester	20.83±4.58	11.20±7.36	7.07±7.07	NS
Others	**0.23±0.23**	**0.47±0.47**	--	**NS**
1-oxaspiro[4.5]deca-6,9-diene-2,8-dione, 7,9-bis(1,1-dimethylethyl)-	0.23±0.23	0.47±0.47	--	NS
Unknown	**4.33±1.41**	**5.57±4.74**	**0.77±0.50**	**NS**

SE, standard error; tr, traces (<0.1%); --, not detected. Each percentage is the mean of three replicates ±SE; Mean separation within lines, by *Tukey* test (NS: not significant).

As well, for *P. lentiscus*, we recorded significant differences among the sites. In Boumerdes (B) site, 93.3% of aliphatics were recorded against 77.6% in Medea (E) site and 39.1% in Senalba (D_2) site. D_2 site, recorded more terpenes (40.7%) than the two remaining sites. In the aliphatics group, alcanes are the major components with hentriacontane as the major component in each case (63.1% for B site, 53% for E site and 19.9% for D_2 site). Similarly, in the terpenes group, the triterpenes are more important in each case, with the Lup-20(29)-en-3one as major component (20.7% in D_2 site, 3.4% in E site and finally, 0.3% in B site. No diterpenes compounds were detected in D_2 and B sites (tab. V).

Table V.- Epicuticular wax composition of *P. lentiscus* leaves from three different sites

Compound	*P. lentiscus* (D2) (%)	*P. lentiscus* (B) (%)	*P. lentiscus* (E) (%)	*Tukey test*
Aliphatics	**39.13b±5.66**	**93.30a±1.35**	**77.57ab±4.41**	*******
-Alcanes	28.70b±2.37	92.30a±1.33	77.33a±4.45	***
Docosane	--	--	0.17±0.17	NS
Tetracosane	--	--	0.30±0.30	NS
Pentacosane	--	1.57±0.38	--	**
Hexacosane	--	0.23±0.23	0.67±0.67	NS
Heptacosane	0.93ab±0.93	3.83a±0.71	0.60ab±0.60	*
Octacosane	--	0.73±0.73	1.20±0.67	NS
Nonacosane	7.87±1.62	9.43±1.23	7.07±0.37	NS
Triacontane	--	5.60a±0.97	6.07a±0.33	***
Hentriacontane	19.90b±2.46	63.07a±0.73	53.03a±2.05	***
Dotriacontane	--	3.97a±0.61	4.77a±0.38	***
Tritriacontane	--	3.90a±0.75	3.47ab±0.69	**
-Aldehydes	10.43±5.23	0.23±0.23	0.23±0.23	NS
Triacontanal	10.43±5.23	0.23±0.23	0.23±0.23	NS
-Esters	--	0.80±0.49	--	NS
Hexacosanoic acid, methyl ester	--	0.80±0.49	--	NS
Terpenes	**40.73a±9.81**	**2.50b±0.87**	**3.67b±0.28**	******
-Diterpenes	--	--	0.23±0.23	NS
Heptadecan-4-olide,4,8,12,16tetramethyl	--	--	0.23±0.23	NS
-Triterpenes	40.73a±9.81	2.50b±0.87	3.43b±0.52	**
Squalene	4.10±4.10	--	--	NS
Lup-20(29)-en-3-one	20.67a±7.54	0.33b±0.33	3.43ab±0.52	*
α.-Tocopherol, acetate	10.07±10.07	--	--	NS
α.-Amyrin ester	5.87±3.29	2.17±1.17	--	NS

Others	--	--	**0.73±0.73**	**NS**
1-oxaspiro[4.5]deca-6,9-diene-2,8-dione,7,9-bis(1,1-dimethylethyl)-	--	--	0.73±0.73	NS
Unknown	**20.17a±4.16**	**4.17b±0.72**	**18.07ab±4.36**	*****

SE, standard error; tr, traces (<0.1%); --, not detected. Each percentage is the mean of three replicates±SE; the letters a,b,c, indicate Tukey test significant differences (*P<0.05, ** P<0.01, *** P<0.001, NS: not significant).

3.- Discussion

Plant cuticles play an important role in protecting the living tissue from external aggression and from uncontrolled water loss [3]. Cuticles may be covered with waxes which have considerable structural diversity and are of fundamental importance to plants in their interactions with the environment [19]. The extent to which waxes develop is dependent on species and environment [20-22]. Their functional significance has previously been related firstly to aridity and high irradiance. In many xeromorphic plants, wax particles cover stomata to prevent water loss due to high radiation [23]. Three-dimensional wax crystals appearing together with an underlying wax film has been reported by several authors [2]. The leaf waxy surface of higher plants exhibits a variety of structures ranging from smooth wax films to structured crystals. Again, these epicuticular crystals show a variety of forms and specific shapes which have been suggested to be correlated with high percentages of a single compound (class) [24]. According to [25], the shapes of wax crystals are strongly correlated with their chemistry, and are expression of the intrinsic wax properties. However, the morphology of certain plant waxes, when recrystallized *in vitro*, was influenced by the rate and method of recrystallisation. The same authors stated that further observations provided evidences that the crystals preceded the preparation procedures employed to image them.

Basically, the chemical composition of the plant waxes is well established, the main component classes are usually primary and secondary alcohols, ketones, fatty acids, and aldehydes. Alcanes are widely distributed but occur usually in low concentrations [2]. Hydrocarbons are one of the most ubiquitous waxes class being present in almost all plant surface waxes in percentages varying from traces to over 50% of the whole wax. Other compounds found in these waxes include acetates, different diterpenes and triterpenes including triterpene acetates, sterols and flavonoids [3,26]. Some of the aliphatic compounds are also found esterified, making up the ester fraction of the cuticular waxes. Esters can be a significant part of the cuticular waxes. The present results concerning the cuticular waxes of the *Pistacia* leaves are similar to those reported for other plant species, with respect to the main classes of compounds. The data concerning the presence/absence of waxes in *Pistacia* leaves are conflicting. ElOqlah [12] reported waxes in some *Pistacia* species, while other authors have indicated that *Pistacia* leaves are unwaxy in *P. eurycarpa*, *P. terebinthus* [27] and *P. atlantica* [28,27]. In our case, all the samples were waxy with various wax morphology. A common strategy to almost all the species populating the Mediterranean macchia is the increase of cuticular resistance to water loss with waxes, arranged in rods, platelets or crystalloids often covering both epidermises. This is considered as a xeromorphic feature of the leaves of some species [29], while in our case, some samples from less arid sites (B site sub humid mild and E site sub humid cold, both for *P. lentiscus*) were covered as well with wax particles.

SANTOS *et al.* (2007) reported that the amounts, morphology and chemical composition of cuticular waxes are extremely variable and their composition is influenced

by genetic (among or within the species), ontogenetic (different leaves of one organ) and environmental factors [3]. Although environmental factors may change the chemical composition, for the most of the plant, the variation is rather quantitative than quanlitative, for instance, increase in the quantity (wax amount) along with the drought or for higher light intensities [2]. However, our study shows clear qualitative and quantitative differences and the majority of our leaf waxes were characterized by the occurrence of aliphatics as major components in *P. atlantica*, *P. lentiscus* and *P. terebinthus* while the terpenes and the phenols were more important in *P. vera*. No phenols detected in *P. lentiscus* and no ester compounds detected in *P. vera*.

Wax preserves the water balance of the plant, minimizes mechanical damage to leaf cells and inhibits fungal and insect attack. It has been suggested that the physical arrangement, the morphology, of the epicuticular waxy covering is all important, but the chemical constituents must also determine its role to a very considerable extent [6]. The relationship between xerophytic character and quantity of wax has aroused some controversy in the literature. Egliton & Hamilton [6], reported no correlations between xeromorphic adaptation and the amount of surface waxes. The removal of *Trifolium repens* leaf surface increases the rate of transpiration, even when the stomata are closed. Populations of *Poa colensoi*, in more humid regions are green while those in semi arid sites are glaucous. In *Poa colensoi*, there were negative correlations between leaf surface wax and precipitation, and a slight positive correlation with the mean temperature. Also, the surface of the needles of *Pinus sylvestris* is covered by two morphologically different types of wax. Waxy projections cover the exposed surface, while the enclosed portion of the needle inside the sheath completely lacks these projections.

In the genus *Pistacia*, the glandular trichomes are reported to exude resin to coat the leaves with thin layer of varnish [30]. Moreover, the resin produced contains substantial amount of volatile oils [31,32], which are recognized as secondary metabolites involved in some Mediterranean plants adaptation to drought and high temperatures [33]. In a previous study, Baker and Procopiou (34] reported that the wax leaf surface of plants growing wild under arid conditions was greater than under naturally irrigated conditions. The effect was most apparent for the larger leaved *P. lentiscus*, greater deposits occur on the leaves of non-irrigated plants. The same study reveals that the pentacyclic triterpenoids were the principal components of the epicuticular waxes of pistachio species. The contradictions between some of our results and previous studies could rise from differences in the climatic conditions where the plants have grown. In response to the diversity in the climatic conditions, vegetation of the Mediterranean type ecosystems has developed an array of adaptations to water stress, resulting in a high diversity of life habits and growth forms. This diversity may derive in a variation of leaf ecophysiological traits [35].

Conclusion

Our study showed a variation in the wax morphology as well as in a quantitative and qualitative difference in the chemical composition of the different studied species. It seems that environmental factors (temperature, elevation and rainfall) play an important role in this variation, as epicuticular waxes constitute a barrier against water loss by transpiration. The genetic characteristics of the trees may also influence the leaf wax structure. These factors influence the plant's biosynthetic pathways and consequently the main characteristic components and their percentage. Although these results are consistent with the present taxonomic grouping, analyses of fresh plant material collected from

several individuals of each species at different developmental stages and from different origin will be needed to ascertain the stability and/or the variability of the chemical characters. Also, it would be interesting to perform further studies to understand how different ecophysiological traits combine to provide specific adaptations to adverse environments.

Acknowledgements

The authors are grateful to the Algerian Forest Conservation units for their technical assistance and cooperation during the sampling periods.

References

[1].- Burghardt M., and Riederer M.: 2006.- Cuticular transpiration. In Biology of the cuticle, Riederer, M. & Müller, C. (Eds.), Pp. 292-309. Blackwell Pub. Annu Plant Rev 23.

[2].- Koch K. and Ensikat H.J., 2008.- The hydrophobic coatings of plant surfaces: epicuticular wax crystals and their morphologies: crystallinity and molecular self-assembly. Micron 39(7): 759-72.

[3].- Santos S., Schreiber L. and Graça J., 2007.- Cuticular waxes from ivy leaves (Hedera helix L.): analysis of high-molecular-weight esters. Phytochem Anal, 18: 60–69.

[4].- Koch K., Barthlott W., Koch S., Hommes A., Wandelt K., Mahmoud W., De-Feyter S. and Broekmann P., 2006.- Structural analysis of wheat wax (*Triticum aestivum*, CV, *Naturastar* L.): From the molecular level to three dimentional crystals. Planta 223: 258-270.

[5].- Bargel H., Koch K., Cerman Z. and Neinhuis C., 2006.- Structure-function relationships of the plant cuticle and cuticular waxes-a smart material? Functional Plant Biology. Evans Rev. 3: 893-910.

[6].- Eglinton G. and Hamilton R. J., 1967.- Leaf Epicuticular Waxes. Science, 156 (3780): 1322-1335.

[7].- Lin T. S., Crane J. C., Ryugo K., Polito V.S. and Dejong T.M., 1984.- Comparative study of leaf morphology, photosynthesis, and leaf conductance in selected *Pistacia* species. J Amer Soc Hort Sci, 109 (3): 325-330.

[8].- Al-Saghir M.G. and Porter D.M., 2005.- Stomatal distribution in *Pistacia* species (Anacardiaceae). Int J Bot 1: 83-187.

[9].- Yaltirik F., 1967.- *Pistacia* L. In Flora of Turkey and the east Aegean Islands, Davis, P.H. (Eds.), Edinburgh University Press. 2. Pp. 542-548..

[10].- Alyafi J., 1978.- New characters differentiating Pistacia atlantica subspecies. Candollea, 33: 201–208.

[11].- Alyafi J., 1979.- Approches systématique et écologique du genre Pistacia L. dans la région Méditerranéenne. Dissertation. University of Aix – Marseille, pages ????.

[12].- El-Oqlah A.A., 1996.- Biosystematic research on the genus *Pistacia* in Jordan. In Taxonomy, distribution, conservation and uses of *Pistacia* genetic resources, Padulosi, S. Caruso, T. & Barone, E. (Eds.), Palermo, Italy, Pp 12-19.

[13].- Özeker E. and Misirli A., 2001.- Research on leaf properties and stomata distribution of some Pistacia spp. Cah Opt Med, 56: 237–241.

[14].- Çaglar S. and Tekin H., 1999.- The stomata density of Pistachio cultivars on different *Pistacia* rootstocks. Turk J Agric Forest, 23: 1029–1032.

[15].- Al-Saghir M.G., 2010.- Phylogenetic analysis of the genus *Pistacia* L. (Anacardiaceae) based on morphological data. Asian J Plant Sci, 9: 28-35.

[16].- Belhadj S., Derridj A., Aigouy T., Gers C., Gauquelin T. and Mevy J.P., 2007.- Comparative morphology of leaf epidermis in eight populations of Atlas pistachio (*Pistacia atlantica* Desf., Anacardiaceae). Microsc Res Tech, 70: 837–846.

[17].- Belhadj S., Derridj A., Auda Y., Gers C. and Gauquelin T., 2008.- Analyse de la variabilité morphologique chez huit populations spontanées de *Pistacia atlantica* en Algérie. Botany 86 (5): 520-532.

[18].- Belhadj S., Derridj A., Moriana A., Gijon M.D.C., Mevy J.P. and Gauquelin, T., 2011.- *Comparative analysis of stomatal characters in eight wild atlas pistachio populations (Pistacia atlantica* Desf.; Anacardiaceae*).* Int Res J Pantl Sci, 2 (3): 060-069.

[19].- Barthlott W., Neinuis C., Cutler Ditsch F., Meusel I., Theisen, I. and Wilhelmi, H., 1998.- Classification and terminology of plant epicuticular waxes. Bot J Linn Soc, 126: 237-260.

[20].- St-Laurent L., Baum B.R., Akpagana K. and Arnason J.T., 2000.- Leaf trichome morphology and density in West African Trema spp. (Ulmaceae: Celtidoideae). Can J Bot, 78: 34–39.

[21].- Holmes M.G. and Keiller D.R., 2002.- Effects of pubescence and waxes on the reflectance of leaves in the ultraviolet and photosynthetic wavebands: A comparison of a range of species. Plant Cell Env, 25: 85-93.

[22].- Carpenter R.J., Hill R.S. and Jordan G.J., 2005.- Leaf cuticular morphology links Platanaceae and Proteacea. Int J Plant Sci, 166: 843–855.

[23].- Weng J.H. and Hsu F.H., 2001.- Gas exchange and epidermal characteristics of *Miscanthus* populations in Taiwan varying with habitats and nitrogen application. Photosynthetica 39: 35–41.

[24].- Dragota S. and Riederer M., 2008.- Epicuticular Wax Crystals of *Wollemia nobilis* : Morphology and Chemical Composition. Ann Bot, 100: 225-231.

[25].- Jeffree C.E. and Sandford A.P., 1982.- Crystalline structure of plant epicuticular waxes demonstrated by cryostage scanning electron microscopy. New Phytol 91, 549-559.

[26].- Saber M., Kashmiri M.A., Mohy-ud-din A., Ahmed M. and Khanum R., 2010.- Epicuticular wax of *Tamarix aphylla* L. J Chem Soc Pak, 32(5): 662-667.

[27].- Kafkas S., Kafkas E. and Perl-Treves R., 2002.- Morphological diversity and a germaplasm survey of three wild *Pistacia* species in Turkey. Gen Res Cro Evol 49: 261-270.

[28].- Zohary M., 1952.- A monographical study of the genus *Pistacia*. Palestine J Bot, 5 (4): 187-228.

[29].- Rotondi A., Rossi F., Asunis C. and Cesaraccio C., 2003.- Leaf xeromorphic adaptations of some plants of a coastal Mediterranean macchia ecosystem. J Med Ecol, 4 (3-4): 25-35.

[30].- Metcalfe C.R. and Chalk L., 1950.- Anatomy of the dicotyledons: leaves, stem, and wood in relation to taxonomy. Oxford: Clarendon Press.

[31].- Duru M.E., Cakir A., Kordali S., Zengin H., Harmandar M., Izumid S. and Hirata T., 2003.- Chemical composition and antifungal properties of essential oils of three *Pistacia* species. Fitoterapia, 74: 170-176.

[32].- Klaas Jan V. D. B., Jerre V. D. H., Jaap J. B. and Olof O. S., 1998.- Cis-l,4-poly-b-myrcene; the structure of the polymeric fraction of mastic resin (Pistacia lentiscus L.) elucidated. Tetrahedron Lett, 39: 2645-2648.

[33].- Castola V., Bighelli A. and Casanova J., 2000.- Intraspecifc chemical variability of the essential oil of Pistacia lentiscus L. from Corsica. Biochem Syst Ecol, 28: 79–88.

[34].- Baker E.A. and Procopiou J., 2000.- The leaf and fruit cuticles of selected drought tolerant plants. International Symposium on growth and development of fruit crops. ISHS Acta Hort, 527: 85-93.

[35].- Galmés J. Flexas, J. and Savé R., 2007.- Water relations and stomatal characteristics of Mediterranean plants with different growth forms and leaf habits: responses to water stress and recovery. Plant Soil, 290: 139-155.

Table II.- Wax composition in leaves of different species of the genus *Pistacia*.

Compound	M.W.	RI	*P. vera*	*P. atlantica*	*P. terebinthus*	*P. lentiscus*	Mean
Phenols			39.28a±8.96	0.25b±0.25	8.80ab±8.40	--	14.13±16.43***
-Alkyl phenols			23,31a±5.33	0,25ab±0.25	8,33ab ±7.94	--	8.75±10.27***
Phenol, 3-undecyl-	248.4	2098	0.14±0.09	--	--	--	0.05±0.17NS
Phenol, 3-tridecyl-	276.5	2294	19.64a±4.63	0.25b±0.25	1.20ab±0.83	--	6.71±8.2***
Phenol, 3-pentadecyl-	304.5	2502	2.88±0.82	--	7.13±7.13	--	1.75±3.9NS
Phenol, 3-heptadecyl-	332.6	2709	0.64±0.28	--	--	--	0.21±0.49*
-Alkenyl phenols			15,98a±4.87	--	0,47ab ±0.47	--	5.38±8.62**
Phenol, 3-(x-tridecenyl)-	274.4	2266	6.02±2.27	--	--	--	2.0±4.02*
Phenol, 3-(x-pentadecenyl)-	302.5	2479	0.83a±0.31	--	0.47ab±0.47	--	0.33±0.59*
Phenol, 3-(x-heptadecenyl)-	330.6	2687	9.12±2.92	--	--	--	3.04±5.17**
Aliphatics			17.71b±6.44	70.43a±4.55	64.27ab±6.47	70.00a±8.32	52.03±19.60***
-Alcanes			17,19b±6.54	51,13ab±5.21	18,03ab±3.38	66,11a±9.72	41.13±21.62***
Docosane	310.6	2201	Tr	1.20±0.85	--	Tr	0.31±0.99NS
Tricosane	321.6	2296	6.86±6.86	Tr	--	--	2.30±12.1NS
Tetracosane	338.7	2398	0.23±0.14	0.67±0.42	0,17±0.17	0.10±0.10	0.28±0.58NS
Pentacosane	352.7	2502	1.88±1.73	3.05±1.04	3,87±2.02	0.52±0.28	1.91±3.48NS
Hexacosane	366.7	2602	0.49±0.24	1.07±0.48	1,10±0.78	0.30±0.22	0.62±0.89NS
Heptacosane	380.7	2701	4.62ab±1.72	11.3a±0.75	8,27ab±1.05	1.79b±0.64	5.56±3.40***
Octacosane	394.8	2801	0.72b±0.29	2.60a±0.45	0,83ab±0.69	0.64b±0.34	1.13±1.01**
Nonacosane	408.8	2899	2.06b±0.67	25.58a±4.23	3,87b±1.37	8.12b±0.69	9.51±5.17***
Triacontane	436.6	3000	Tr	0.62b±0.45	--	3.89a±1.02	1.45±1.87*
Hentriacontane	436.6	3097	0.20b±0.12	4.35b±0.77	--	45.33a±6.59	16.14±11.69***
Dotriacontane	450.9	3201	--	0.55ab±0.55	--	2.91a±0.77	1.09±1.49**
Tritriacontane	464.9	3301	--	0.15b±0.15	--	2.45a±0.68	0.85±1.22*
-Aldehydes			0,53b±0.40	5,68b±1.49	41,03a±10.32	3,63b±2.28	7.21±6.88***
Docosanal	324.6	2429	--	--	0.43±0.43	--	0.05±0.22*
Tricosanal	338.6	2532	--	--	1.00±0.58	--	0.11±0.29***
Tetracosanal	352.6	2634	0.14b±0.14	0.23b±0.23	6.17a±3.08	--	0.78±1.62***
Pentacosanal	366.7	2736	--	--	1.57±0.92	--	0.17±0.47***
Hexacosanal	380.7	2839	--	1.28b±0.43	9.47a±2.94	--	1.34±1.58***
Heptacosanal	394.7	2940	0.39b±0.39	--	2.63a±0.59	--	0.42±0.75***

Octacosanal	408.7	3040	--	1.28b±0.62	12.67a±2.73	--	1.69±1.56***
Triacontanal	436.8	3247	--	2.88±1.03	7.13±0.27	3.63±2.28	2.64±4.19NS
-*Esters*			--	13,65a±3.68	5,20ab±2.63	0,27b±0.19	3.70±4.43***
Tetracosanoic acid, methyl ester	382.7	2733	--	3.67±1.46	--	--	0.81±1.66**
Hexacosanoic acid, methyl ester	410.7	2933	--	4.73a±1.05	--	0.27b±0.19	1.14±1.24***
Acetic acid, hexacosanyl ester	424.7	3012	--	2.82±0.98	--	--	0.63±1.12***
Octacosanoic acid, methyl ester	438.8	3129	--	Tr	--	--	0.01±0.08NS
Acetic acid, octacosanyl ester	452.8	3212	--	2.37±0.75	--	--	0.53±0.86***
Hexadecanoic acid, eicosyl ester	537.0	3762	--	--	1.40±0.87	--	0.15±0.44***
Hexadecanoic acid, docosyl ester	565.0	3988	--	--	3.80±1.90	--	0.42±0.97***
Terpenes			39.24±7.56	14.83±5.26	25.60±10.51	15.63±6.89	24.43±19.82NS
-*Diterpenes*			1,84±0.71	2,17±1.56	1,30±0.66	tr	1.27±2.21NS
Trans-phytol	296.5	2126	0.16±0.09	1.18±1.18	0.60±0.60	--	0.38±1.39NS
Phytone	268.5	1844	0.33±0.24	0.60±0.60	--	--	0.24±0.80NS
Pseudo phytol	306.5	2010	--	0.23±0.23	0.70±0.70	--	0.13±0.44NS
Heptadecan-4-olide,4,8,12,16tetramethyl	324.5	2363	1.33±0.49	0.15±0.15	--	tr	0.50±0.89NS
-*Triterpenes*			37,40±7.82	12,67±4.33	24,30±9.98	15,56±6.91	23.17±19.78NS
Squalene	410.7	2832	5.90±2.74	0.45±0.39	3.30±1.76	1.37±1.37	2.89±5.51NS
Lup-20(29)-en-3-one	424.7	3381	0.39±0.20	0.52±0.52	--	8.14±3.84	2.96±6.83NS
α.-Tocopherol, acetate	472.7	3150	15.13±4.75	6.57±4.29	20.97±8.21	3.36±3.36	9.95±12.15NS
β.-Amyrin ester		3357	2.97ab±0.96	5.12a±2.32	--	--	2.13±3.15*
α.-Amyrin ester		3403	13.03a±3.82	--	--	2.68b±1.32	5.24±7.15*
Others			0.23±0.16	1.33±0.88	--	0.24±0.24	0.45±1.13NS
Tridecane, 2-phenyl-	260.0	1916	--	Tr	--	--	0.01±0.07NS
1-oxaspiro[4.5]deca-6,9-diene-2,8-dione,7,9-bis(1,1-dimethylethyl)-	276.4	1932	0.16±0.16	1.30±0.76	--	0.24±0.24	0.41±1.11NS
Unknown	29 compounds	2113-3774	3.56b±1.61	13.18a±4.20	1.47bc±0.79	14.13a±3.06	8.99±7.78*

M.W., Mass weight; RI, retention indices; SE, standard error; tr, traces (<0.1%); --, not detected. Each percentage is the mean of three replicates ±SE; the letters a,b,c, indicate *Tukey* test significant differences (*P<0.05, ** P<0.01, *** P<0.001, NS: not significant).