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Leaf structural dynamics associated with adaptation of two *Ebenus cretica* ecotypes

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Abstract

Morphological and anatomical features of *Ebenus cretica* leaflet, such as lanceolate shape, reduced size, dense cover with non-glandular hairs, epidermis of small cells, compact mesophyll, amphipleurous presence of palisade parenchyma, thick cuticle, development of numerous mesophyll phenol-storing cells and the amphistomatic type, disclose the xeromorphic character of the plant. In the island of Crete two ecotypes of *E. cretica,* ecotype A and ecotype C, are greatly extended. In ecotype A leaflets, the above features are more prominent than in ecotype C. This fact accomplished by physiological data favours the suggestion that plants of ecotype A are better adapted to the xerothermic environment of the island of Crete. This may be the reason that ecotype A occupies the major portion of the island and is predominant in the western and central regions. The distinction of ecotypes A and C, by evaluating the strategies these plants used in order to better adapt and the characteristics of their inflorescences may be used as a criterion for the selection of the most appropriate ecotype for application in floriculture and ornamental horticulture.

Additional key words: cuticle, epidermis, *Fabaceae,* floriculture, leaf anatomy, mesophyll, xeromorphic environment.

Introduction

The introduction of wild flowering plants in floriculture is an event of particular economic importance. Such plants are robust, not water demanding and adapt easily to unfavorable cultivating conditions.

The genus *Ebenus* belongs to the family of Fabaceae and includes 18 species distributed from Eastern Mediterranean to Central Asia. *Ebenus cretica* is an endemic of the island of Crete (Greece). The plant has been known in Western Europe since 1737 and it has been listed in the greenhouses of many European botanical gardens. *E. cretica* is an herbaceous perennial evergreen subshrub, up to 60 cm in height. Leaves are palmately compound and consist of 3 - 7 leaflets, 1 - 3 cm long and 4 - 6 mm wide. Flowers are vivid pink or purple and they form thick racemes, 5 - 15 cm in length. *E. cretica* plants bloom from early April to late June, depending on altitude and latitude (Vlahos 1996).

A wide environmental variation often occurs within the natural range of a plant species. Adaptation of a species to this variation may reflect different morphological and physiological characteristics, resulting in the development of ecotypes (Kubiske and Abrams 1992, Zheng *et al.* 2000). Four ecotypes (A, B, C and D) have been distinguished in *E. cretica* (Syros *et al.* 2003). In Western and Central Crete, ecotype A is predominant. Ecotype B has been localized repeatedly in Western Crete and sporadically in Central Crete. Ecotype C has been locally found in Western Crete and quite often in Central Crete. Ecotype D has been exclusively met in Central Crete. According to dendrogramic data based on the analysis of the total seed storage proteins, ecotypes A and B belong to the same genetic group, as ecotypes C and D do. Both groups have a significant genetic distance (Syros *et al.* 2003).

The present work aims at the distinction of the *Ebenus cretica* ecotypes A and C (dominant of each genetic group) by evaluating the morphological and physiological strategies these plants use in order to better adapt to the specific environment they grow. The results may be used for the selection of the most appropriate ecotype for application in floriculture and ornamental horticulture.

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Materials and methods

Ebenus cretica L. plants were raised from seeds harvested from ecotypes A and C grown wild on the island of Crete. Plants from each ecotype (4-month-old), grown in pots, were maintained in a computer-controlled greenhouse at the Aristotle University of Thessaloniki, under controlled temperature (22 ± 2 °C) and relative humidity (85 ± 10 %).

A portable photosynthesis system (*LCi*, *ADC BioScientific*, Hoddesdon, UK), equipped with a square (6.25 cm^2) chamber, was used for leaf net photosynthetic rate (P_N) measurements. Data collections were performed on mature leaflets (third node from the basis of annual stems) at 2 h intervals from early morning (08:00, local time) to late afternoon (20:00). Irradiance was in the range of 40 - 360 μmol m^{-2} s^{-1} and natural CO_2 concentration.

Chlorophyll content of leaflets was determined using the method of Wintermans and De Mots (1965).

The content of total phenolics in the leaflets was determined using the Folin-Ciocalteu reagent and the results were expressed as gallic acid equivalents (GAE) (Singleton *et al.* 1999). Standard concentrations of gallic acid between 0 and 80 μg cm^{-3} were used to prepare calibration curves.

Small pieces of leaflets were initially fixed for 3 h with 5 % glutaraldehyde in 0.05 M phosphate buffer (25 °C, pH 7.2). After washing in buffer, the specimens were postfixed for 2 h with 2 % osmium tetroxide, similarly buffered. Samples were dehydrated in an alcohol series followed by propylene oxide. The samples were then embedded in Spurr's (1969) resin. Semi-thin sections for light microscopy were obtained in a *Reichert* (Vienna, Austria) *OM* U_2 ultramicrotome, stained with toluidine blue O and photographed in a *Zeiss III* (Jena, Germany) photomicroscope.

For the morphometrical assessment of the relative volume of the leaflet histological components, a transparent sheet bearing a square lattice of point arrays, 10 mm apart, was laid over light micrographs of leaf cross-sections (×800). The point-counting analysis technique was then applied (Steer 1981). Similar sections were used to estimate the thickness of leaflet lamina. The densities of stomata and non-glandular hairs were determined by using paradermal sections. Calculation of epidermal and mesophyll cell densities was provided on the same sections.

All morphological, anatomical, morphometrical and physiological data were analyzed using one-way *ANOVA*. Duncan's multiple comparison tests were used to separate significant differences in means.

Results

Ebenus plants of A and C ecotypes remarkably differ in their external morphology. Plants of ecotype A form long branches with lanceolate leaflets being acute at their tips. Plants of ecotype C are somehow shorter with larger leaflets, also lanceolate, rounded at their apices.

Cross-sections of leaflets of both ecotypes showed an amphistomatic structure (Fig. 1). In the mesophyll, the palisade parenchyma extends on either side of the spongy parenchyma, which occupies a small portion at the middle region of the leaflet. The whole leaflet structure is fairly compact, since intercellular spaces occupy only a small volume. Large phenolic-containing cells, most of which are in contact with the epidermal cells, are ordinarily found in the palisade tissue (Fig. 1). Those facing the upper side of the leaflet are bigger, thicker and more oblong, extending up to the spongy parenchyma. Both leaflet surfaces are densely covered with non-glandular hairs. The upper surface, however, is more pubescent than the lower one.

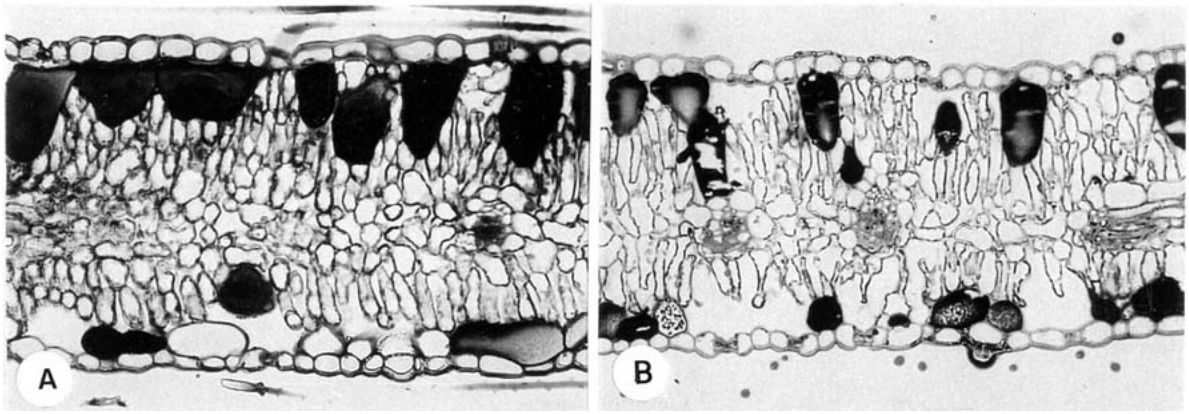

Fig. 1. Leaflet cross-sections of ecotype A (*A*) and ecotype C (*B*) plants (×180).

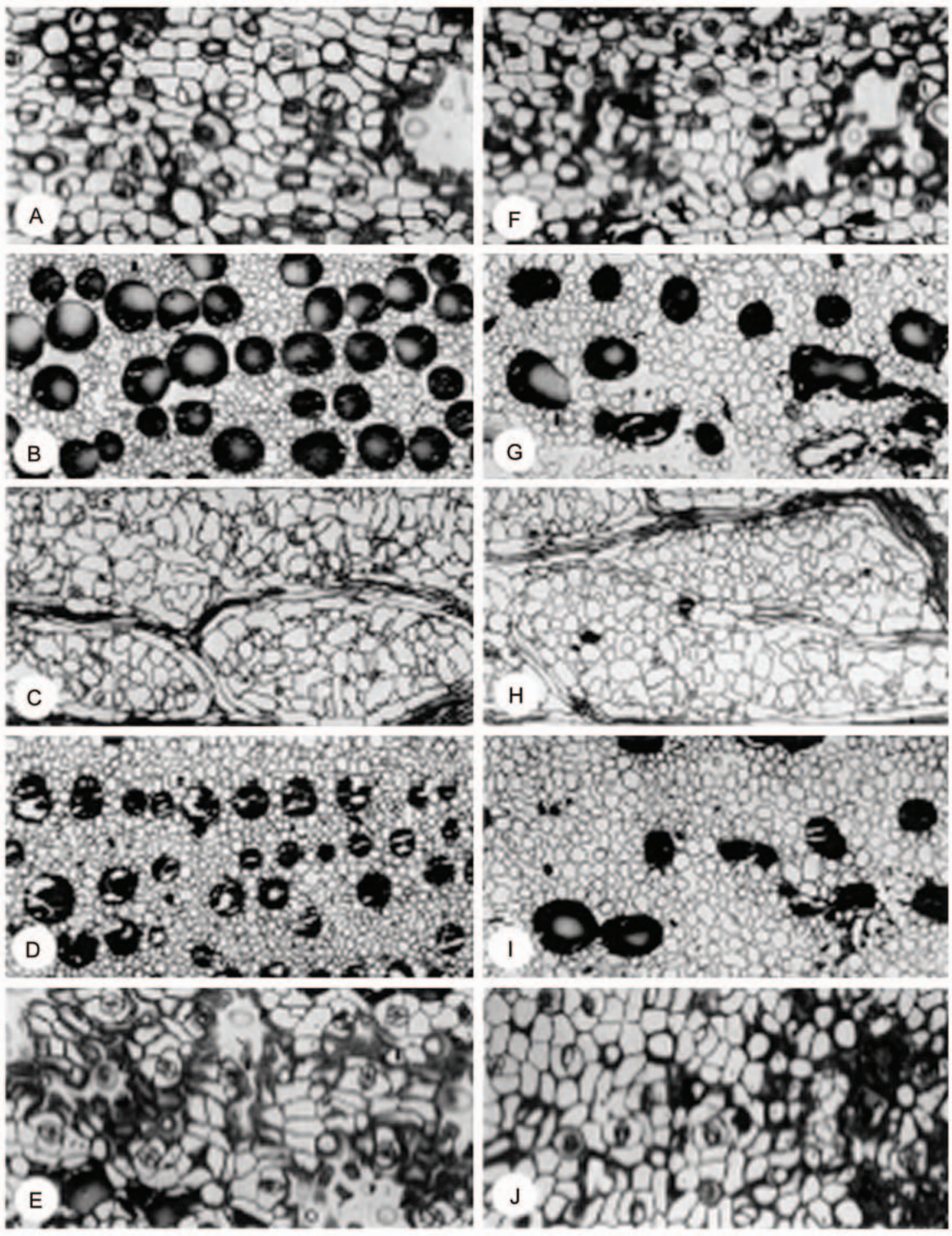

Fig. 2. Leaflet anatomy of ecotype A (*A* - *E*) and ecotype C (*F* - *J*) plants in paradermal sections serially cut from the upper to the lower epidermis: *A*, *F* - upper epidermis; *B*, *G* - upper palisade parenchyma; *C*, *H* - spongy parenchyma; *D*, *I* - lower palisade parenchyma; *E*, *J* - lower epidermis (×180).

Leaflets of ecotype C plants are significantly thinner (256.6 ± 9.3 μm) than those of ecotype A plants (271.2 ± 13.1 μm) (Fig. 1). The assessments of the relative volume percentage of the leaflet histological components (cross-section) revealed no important differences between the two *E. cretica* ecotypes (Table 1). The relative volume

percentage of the upper epidermis in ecotype A is significantly smaller than that in ecotype C, whereas that of the upper palisade parenchyma is larger in the leaflets of ecotype A. No significant differences between the two ecotypes are observed as concerns the spongy parenchyma, the lower palisade parenchyma and the lower epidermis (Table 1).

The density of epidermal and mesophyll cells in leaflet paradermal sections was found to differ significantly between the two ecotypes (Table 1, Fig. 2). The density of cells is significantly higher in ecotype A, as concerns the upper palisade parenchyma, the spongy parenchyma and the lower palisade parenchyma. Phenol-storing cells in the upper and lower palisade parenchymas are also more densely arranged in leaflets of ecotype A. No significant differences between the two ecotypes are noticed concerning the upper and lower epidermal cell density.

Stomata and non-glandular hairs are present on both leaflet surfaces, but they are generally more numerous on the lower leaflet surface of both ecotypes (Table 2, Fig. 2). The density of stomata on the upper leaflet surface of ecotype A is lower than that of ecotype C, while no significant differences in stomatal density are noticed on the lower surface (Table 2). On the other hand, the density of non-glandular hairs is higher in the leaflets of ecotype A, compared to ecotype C, for both leaflet surfaces (Table 2).

The total chlorophyll content is significantly higher in ecotype C [9.1 ± 0.4 mg g^{-1}(f.m.)] than in ecotype A [7.9 ± 0.4 mg g^{-1}(f.m.)]. The total phenolics in leaflets of ecotype A are by 72 % more abundant [25.5 ± 0.5 mg(gallic acid) g^{-1}(f.m.)] as compared to those of ecotype C [14.8 ± 0.3 mg(gallic acid) g^{-1}(f.m.)].

Leaflets of ecotype A are superior to those of ecotype C in net photosynthetic rate (P_N) during the day. In ecotype C, P_N is extremely low during the whole day (up to 1 µmol m^{-2} s^{-1}), whereas in ecotype A, P_N reaches a peak at about 10:00 (up to 3.5 µmol m^{-2} s^{-1}) and then sharply declines at 14:00, reaching the values of ecotype C. Thereafter, P_N goes to another peak at 16:00 (up to 8 µmol m^{-2} s^{-1}).

Table 1. Relative volume percentage of the leaflet histological components (cross-sections) and density of epidermal and mesophyll cells in leaflet paradermal sections of two *Ebenus cretica* ecotypes (means ± SD, n = 12, * - significantly different at $P < 0.05$; phen. cells - phenol-storing cells).

Ecotype	Relative volume [%] A	C	Density [mm^{-2}] A	C
Upper epidermis	5.2 ± 0.3	5.9 ± 0.4*	1538.4 ± 51.3	1524.7 ± 38.8
Upper palisade parenchyma	44.0 ± 1.1	42.6 ± 1.2*	2082.4 ± 120.9	1830.3 ± 67.6*
Upper palisade parenchyma (phen. cells)			390.9 ± 32.3	277.7 ± 33.0*
Spongy parenchyma	18.4 ± 0.4	18.6 ± 0.2	2089.7 ± 54.9	1833.9 ± 51.3*
Lower palisade parenchyma (phen. cells)			238.2 ± 31.0	178.8 ± 19.6*
Lower palisade parenchyma	26.6 ± 1.0	26.8 ± 1.6	2571.9 ± 40.8	2119.4 ± 78.4*
Lower epidermis	5.8 ± 0.3	5.9 ± 0.2	1515.3 ± 33.2	1500.8 ± 41.0

Table 2. Density of stomata and non-glandular hairs in leaflets of two *Ebenus cretica* ecotypes (means ± SD, n = 12, * - significantly different at $P < 0.05$).

Ecotype	Stomata [mm^{-2}] upper leaf surface	lower leaf surface	Non-glandular hairs [mm^{-2}] upper leaf surface	lower leaf surface
A	155.3 ± 22.4	269.4 ± 20.6	177.8 ± 7.9	279.6 ± 9.1
C	223.8 ± 20.6*	278.6 ± 40.3	139.8 ± 6.5*	222.2 ± 7.9*

Discussion

Morphological and anatomical features of *Ebenus cretica* leaflet, such as a lanceolate shape, a reduced size, a dense cover with non-glandular hairs, an epidermis of small cells, a compact mesophyll, an amphipleurous presence of palisade parenchyma, a thick cuticle, a development of numerous phenol-storing cells, *etc*., indicate the xeromorphic character for the plant (Fahn and Cutler 1992). The density of non-glandular hairs was significantly greater in the leaflets of ecotype A than of ecotype C, for both surfaces. It has been postulated that a thick indumentum of non-glandular hairs may remarkably affect transpiration, leaf overheating, leaf reflectance, radiation

absorptance, insect attack, *etc*. (Johnson 1975, Ehleringer 1984, Karabourniotis *et al.* 1992). As to the presence of stomata on both leaflet surfaces, the amphistomatic type of *E. cretica* leaflet is not well-correlated to its xeromorphic character, since xeromorphic leaves have been generally observed to be hypostomatic (Christodoulakis 1989, Christodoulakis and Bazos 1990, Bosabalidis and Kofidis 2002).

Phenolic compounds are commonly found in plants and they have been reported to have multiple biological activities (Larson 1988). High accumulation of phenolics is a common characteristic for summer leaves of Mediterranean plants, such as *Thymus capitatus* (Christodoulakis and Bazos 1990), *Phlomis fruticosa* (Christodoulakis 1989) and *Origanum vulgare* (Kofidis *et al.* 2003). Phenolics are biosynthesized during a metabolic process responsible for the non-destructive dissipation of the excess of excitation energy in photosynthetic tissues exposed to high irradiance but unable to carry on photosynthesis (Christodoulakis 1989). High air temperature, high irradiance and water deficiency, which apparently inhibit the photosynthetic rate (Alexander *et al.* 1995, Higuchi *et al.* 1999), may be responsible for the appearance of phenolics. A principal role played by phenolics in green leaves is protection of the photosynthetic machinery and the nucleus from UV-B radiation (Karabourniotis *et al.* 1992).

The *E. cretica* ecotype A, compared to ecotype C, accumulates larger amounts of phenolic compounds, presumably owing to its higher photosynthetic capacity. Reduction of CO_2 assimilation rate in ecotype A, particularly during noonday (12:00 - 14:00), can be partly related to stomatal closure, a performance determined by environmental conditions (Dai *et al.* 1992, Epron 1997, Flexas and Medrano 2002). This may reflect a sacrifice of carbon fixation in order to reduce transpiration. Considering the significant differences in photosynthetic capacity between the ecotypes, the higher values observed in ecotype A, could be attributed to the larger photosynthetic tissue (thicker mesophyll) rather than to stomatal density.

The above data accomplished by the morphological and anatomical observations, that leaflets of ecotype A, compared to those of ecotype C, are smaller, thicker, more densely covered with non-glandular hairs (on both sides), have smaller and more densely arranged epidermal cells, thicker cuticle and more numerous and voluminous phenolic cells, favour the suggestion that plants of ecotype A are better adapted to the xerothermic environment of the island of Crete. This might be the reason that ecotype A occupies the major portion of *Ebenus cretica* ecotypes and is the predominant ecotype in Western and Central Crete (Syros *et al.* 2003).

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