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Morpho-anatomical Features of the Leaves of the Mediterranean Geophyte *Urginea maritima* (L.) Baker (Liliaceae)

Sharaf Al-Tardeh, Thomas Sawidis, Barbara-Evelin Diannelidis and Stylianos Delivopoulos*
Department of Botany, School of Biology, Aristotle University, Thessaloniki 541 24, Greece

The morpho-anatomy and histochemistry of the hysteranthous leaf of *Urginea maritima* (L.) Baker and its adaptive strategies to the Mediterranean climate were investigated. The leaf of *U. maritima* is 714 μm thick and possesses moderate specific leaf mass (8.564 mg cm^{-2}) and low tissue density (136.5 mg cm^{-3}). The epidermal cells are compactly arranged and covered with cuticle. The average density of stomata in lower epidermis is higher than that of the upper one. The mesophyll cells occupy 52.96% of the total volume of the leaf, while the mesophyll intercellular spaces and the air spaces occupy 30.41%. Idioblastic cells containing raphide bundles and different phenotypes of crystalloid inclusions, embedded in polysaccharides, occur in the lower side of the mesophyll. The presence of oil droplets and lipids is evident. Bundle sheath cells are hardly visible with no chloroplasts which are a pronounced C_3 plant character. Plastids containing protein crystalloid inclusions are abundant in the protophloem sieve elements. *U. maritima,* a deciduous plant, possesses leaves with mesophytic characters, in order to optimize its adaptation to the seasonal fluctuation of environmental conditions of the Mediterranean climate.

Keywords: geophytes, leaf anatomy, Mediterranean climate, morphology, morphometry, *Urginea maritima*

Urginea maritima (L.) Baker is a perennial bulbous geophyte, native to the Mediterranean basin and well adapted to its type of climate. It generally occurs in the slopes of hills, the sandy grounds near the Mediterranean Sea (Gentry *et al.*, 1987) and in certain regions of Northern Africa, (Bellakhdar, 1997), Middle East and Europe. *U. maritima* is a winter plant characterized by three phenological stages consisting of inflorescence, leaves and no above-ground biomass. Leaves first appear after the flowers have wilted in response to winter onset rains during November or December in Crete-Greece, and may remain green until late spring (May), depending on rainfall and temperature.

Some geophytes that flower without bearing leaves at the end of summer (September/October) are known as species with hysteranthous foliage. Most of the geophytes with hysteranthous leaves in the Mediterranean flora are from the Liliales: Liliaceae (*Urginea maritima* (L.) Baker), Amaryllidaceae and Iridaceae. Some other genera with similar phenological behavior are Primulaceae (*Cyclamen*) and Araceae (*Birarum*). The flower stimulation of the hysteranthous geophytes depends on temperature increase and the water supply stored in the bulb, while leaf appearance is connected with an external water supply (Dafni et al. 1981).

The Mediterranean-type climate is characterized by hot and dry summers alternating with cold and wet winters (Daget, 1977; Nahal, 1981). The seasonal fluctuations in soil moisture are considered as one of the limiting factors for growth and productivity of Mediterranean perennial species (Mitrakos, 1980; Specht, 1987). Restriction of water loss to a minimum is of decisive importance under conditions of severe drought. This is mainly achieved by the coverage of leaf surfaces with the cuticle (Schönherr, 1982).

Leaves are the plant organs mostly exposed to aerial conditions and the changes in their characteristics have been interpreted as an adaptation to specific environments (Fahn and Cutler, 1992). Many traits from leaf structure can be explained as adaptations to enhance CO_2 diffusion within the leaf for photosynthesis (Parkhurst, 1986). One morphological trait of the leaf that correlates with CO_2 assimilation is the specific leaf mass (SLM) (Mooney et al., 1978; Field and Mooney, 1983; Ellsworth and Reich, 1992).

Deciduous species in the dry regions possess leaves that undergo senescence and desiccation during the period of dryness (Orshan, 1963). Amphistomaty and leaf compartmentation have been repeatedly evaluated concerning leaf xeromorphy. Amphistomaty, which is more common in xeric habitats (Parkhurst, 1978; Fahn and Cutler, 1992), shortens the distance of CO_2 diffusion to mesophyll cells (Parkhurst et al., 1988; Terashima et al., 2005). Small but abundant stomata are also believed to lower the CO_2 diffusion resistance toward the photosynthesizing tissue; thus, non-succulent species show increased stomatal density (Sundberg, 1986). Mesophyll compartmentalization is supposed to protect the leaf against water stress (Terashima, 1992), but, unavoidably, increases the CO_2 diffusion resistance of the tissue (Miyazawa and Terashima, 2001). Abundant palisade tissues are also believed to increase the CO_2 absorbing surface of the mesophyll (Rhizopoulou and Psaras, 2003; Terashima et al., 2005). Thus, the leaf fine structure of a species, which provides the anatomical basis to explain physiological traits, always needs to be elucidated.

This study focuses on the morpho-anatomical features of the leaf of the Mediterranean geophyte *U. maritima*, to provide the background knowledge for understanding further experimental work implicating physiological parameters. The ultimate goals are to identify the structure and the function of the leaf, and to give some points on the abundance of the plant in the Mediterranean region. More specifically, the aim of this paper is to elucidate the plant's adaptive strategy to semiarid environments, and to find its defense

*Corresponding author; fax +30-2310-99-83-89
e-mail delivopo@bio.auth.gr

mechanisms against herbivores.

MATERIALS AND METHODS

Plant Material

Individuals of *Urginea maritima* (L.) Baker, growing wild in a hill about 10 km NW of Chania, on the island of Crete, southern Greece, were used in this study. Leaves of the same age (3rd leaf from the rosette basis) and size (~20 cm length) were used in our investigations. Experiments and measurements were performed during the growing period (November-May) of 2004 and 2005. In all cases, leaf main drip was excluded.

Microscopy (LM, TEM and SEM)

Leaf segments were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodylate buffer for 2h. After post-fixation in 1% osmium tetroxide and dehydration in an ethanol series, the tissue was embedded in Spurr's epoxy resin (Spurr, 1969). Semi-thin sections (1 μm thick) from resin embedded tissue were obtained with a Reichert OM U_2 ultra microtome; they were stained with 0.5% toluidine blue O in 5% borax (Pichett-Heaps and Northcote, 1969) and photographed using an inverted photomicroscope ECLIPSE TE2000-S (Nikon). Ultrathin sections (0.08 μm thick) were stained with uranyl acetate and lead citrate (Reynolds 1963) and examined using a Zeiss 9 S-2 transmission electron microscope (TEM) (Sawidis et al., 2005; Al-Tardeh et al. 2006). For scanning electron microscopy (SEM), the specimens, after fixation and dehydration, were critical-point dried in a Balzers CPD 030 device and then coated with carbon in a JEE-4X vacuum evaporator. Observations were made with a JSM 840-A scanning electron microscope (Kofidis et al., 2003).

Leaf Histochemistry

To stain lipophilic substances, semi-thin sections of fixed material and hand-cut sections of fresh leaves were stained with 1% Sudan Black B (Bronner, 1975; Sawidis et al., 2005). A freshly saturated solution of Sudan Black B in 70% ethanol was prepared and kept in a closed container for 12 h at 37°C, then filtered. Glass slides with semi-thin sections were immersed in 70% ethanol for 1-2 min, and then transferred into the freshly filtered saturated solution of Sudan Black B at 60°C in an oven for 35 min. The slides were rinsed in 70% ethanol for one minute, and then washed with water. For polysaccharide staining, semi-thin sections of fixed material and hand-cut sections of fresh leaves were treated with periodic acid-Schiff's reagent (PAS) according to Nevalainen et al. (1972) and examined with a light microscope (LM) (Sawidis, 1991;1998; Sawidis et al. 2000).

Leaf Density and Thickness

Leaf samples from 30 individuals of almost the same age and size, were investigated. The leaf surface area (LSA) was measured using the MK_2 (Image Analysis System) area meter (Delta_T Devices Ltd, Cambridge, UK) connected to a TC7000 Series Camera (Burle Industries Inc. Lancaster, PA, USA). Leaf thickness was measured with an electronic digital caliper. Leaf dry mass (LDM, mg) was determined by drying at 80°C to constant weight. Leaf water content was measured (LWC, fresh leaf mass – dry leaf mass / fresh leaf mass x 100%) according to Cappelletti (1954). Specific leaf mass (SLM, mg cm^{-2}) was calculated as the ratio of leaf dry weight to unifacial leaf area. Specific leaf area (cm^2 g^{-1}) was determined as leaf area per unit leaf mass (Reich et al., 1992). Leaf tissue density (LTD, mg cm^{-3}) was calculated as the ratio of LDM to leaf volume (V, leaf area x leaf thickness cm^{-3}) (Witkowski and Lamont, 1991), in order to express leaf compactness (Chrstodoulakis and Mitrakos, 1987).

The total leaf thickness and the thickness of the histological components, viz. of the adaxial and abaxial cuticles, the adaxial and abaxial epidermises and the mesophyll were measured from 15 light micrographs of leaf cross-sections (x 50).

Morphometry

For the morphometric evaluation of the relative volume of the histological components of the leaf, a transparent sheet bearing a square lattice of points arrays, 10 mm apart, was laid over light micrographs of leaf cross-sections (x 50). The point-counting technique analysis was then applied (Steer, 1981). To determine the density of stomata, stereoscopic photographs of the adaxial and abaxial leaf surfaces (x 64) were used.

RESULTS AND DISCUSSION

Leaf anatomy

The leaves are 714.2±33.6 μm thick (Table 1 and Fig. 1A). The upper epidermis is slightly thicker (periclinal) (51.97±1.08 μm) than the lower epidermis (48.91±0.76 μm) (Table 1). Both the upper and lower epidermal cells are compactly arranged and covered with a relatively thick cuticle (~10 μm) (Table 1, Fig. 1B). The epidermal cells are very similar, polygonal and possess rounded margins. In cross section, however, the epidermal cells are isodiametric and periclinally oriented (Figs 1A&B). The epidermal cells, especially those that are close to the guard cells, possess myelin-like structures (Fig. 1C).

In the leaf of *U. maritima*, the stomata are arranged in

Table 1. Average thickness (μm) of leaf tissues in cross-sections [n=15, ± standard deviation (SD)] and stomatal density (No. mm^{-2} ± SD) in longitudinal sections (n=15).

Leaf tissue	Average leaf tissue thickness (μm)	Density of stomata (No. mm^{-2}±SD)
Adaxial cuticle	10.25±1.16	
Adaixial epidermis	51.97±1.08	736.83±105.35
Mesophyll	594.7±33.3	
Abaxial epidermis	48.91±0.76	867.06±66.09
Abaxial cuticle	8.41±0.58	
Total leaf thickness	714.2±33.6	

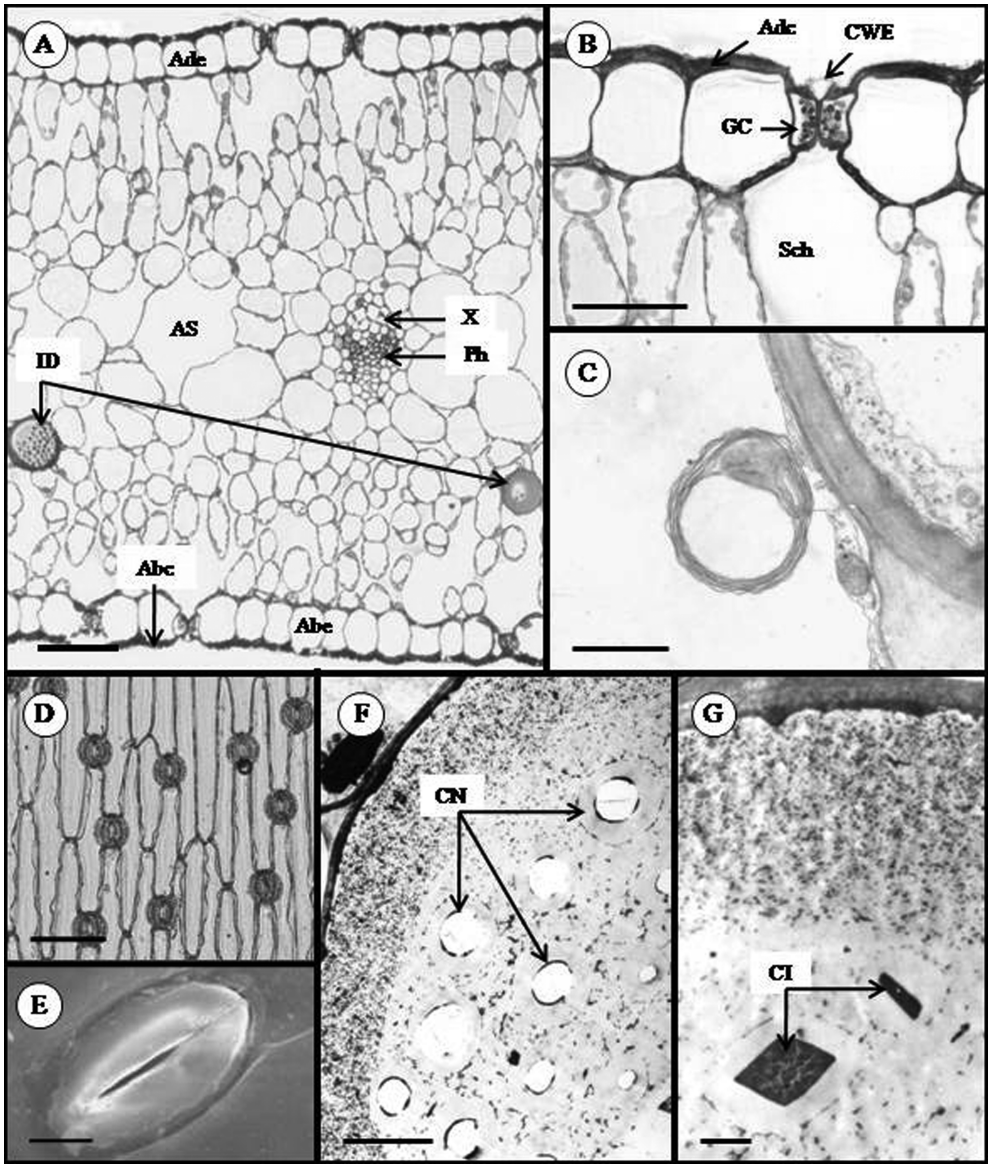


Figure 1. Leaf morpho-anatomy. (**A**) LM micrograph (overview) of a transverse section of an *Urginea maritima* leaf revealing idioblastic cells (Id) with bundles of raphides. (Abc: abaxial cuticle, Abe: abaxial epidermis, Ade: adaxial epidermis, AS: air space, X: xylem, Ph: phloem). Scale bar = 100 μm. (**B**) LM micrograph of a transverse section of an *U. maritima* leaf showing stomatal aperture in adaxial epidermis. (Adc: adaxial cuticle, CWE: cell wall extension, Gc: guard cell, Sch: stomatal chamber). Scale bar = 50 μm. (**C**) TEM micrograph of a transverse section of an *U. maritima* leaf showing an epidermal cell, beside the guard cell, with myelin-like structure. Scale bar = 1 μm. (**D**) LM micrograph of a hand-cut paradermal section of an *U. maritima* leaf showing stomata in rows parallel to the leaf main drip. Scale bar = 500 μm. (**E**) SEM micrograph of an *U. maritima* leaf revealing anomocytic stomata. Scale bar = 10 μm. (**F**) TEM micrograph of a transverse section of an *U. maritima* leaf revealing crystal needles (CN) embedded in polysaccharidic content. Scale bar = 5 μm. (**G**) TEM micrograph of a transverse section of an *U. maritima* leaf showing crystalloid inclusion (CI) of different shapes present in the idioblastic cell. Scale bar = 1 μm.

rows parallel to the long axis of the leaf (Fig. 1D). Their development begins at the tips of the leaves and progresses downwards. The leaves possess sunken stomata on both sides of the leaf (amphistomaty). The density of stomata in upper epidermis (736.83±105.35 mm^{-2}) is, somewhat, lower than that of the lower epidermis (867.06±66.09 mm^{-2}) (Table 1). The guard cell pairs have an average length of 45.4±2.3 μm and 22.8±2.1 μm width, as determined from SEM micrographs (Fig. 1E), and possess cell wall extensions (Fig. 1B). The stomatal apparatuses are anomocytic, i.e., real subsidiary cells are absent (Figs. 1D&E). The stomatal chambers are almost 2-fold bigger in size than the adjacent mesophyll cells (Figs. 1A&B).

The leaves of *U. maritima* are equifacial, i.e., in terms of cuticle, epidermis and spongy cells, which are found on both leaf sides. The mesophyll is 594.7±33.3 μm thick. The spongy cells on the upper side of the leaves are elongated, while those of the lower side are oval-rounded (Fig. 1A). However, the lower side of the leaf possesses idioblastic cells containing bondles of raphides which reflect the light under light microscope (Fig. 1A). Raphides occur within the central vacuole. Under the electron microscope, the vacu-

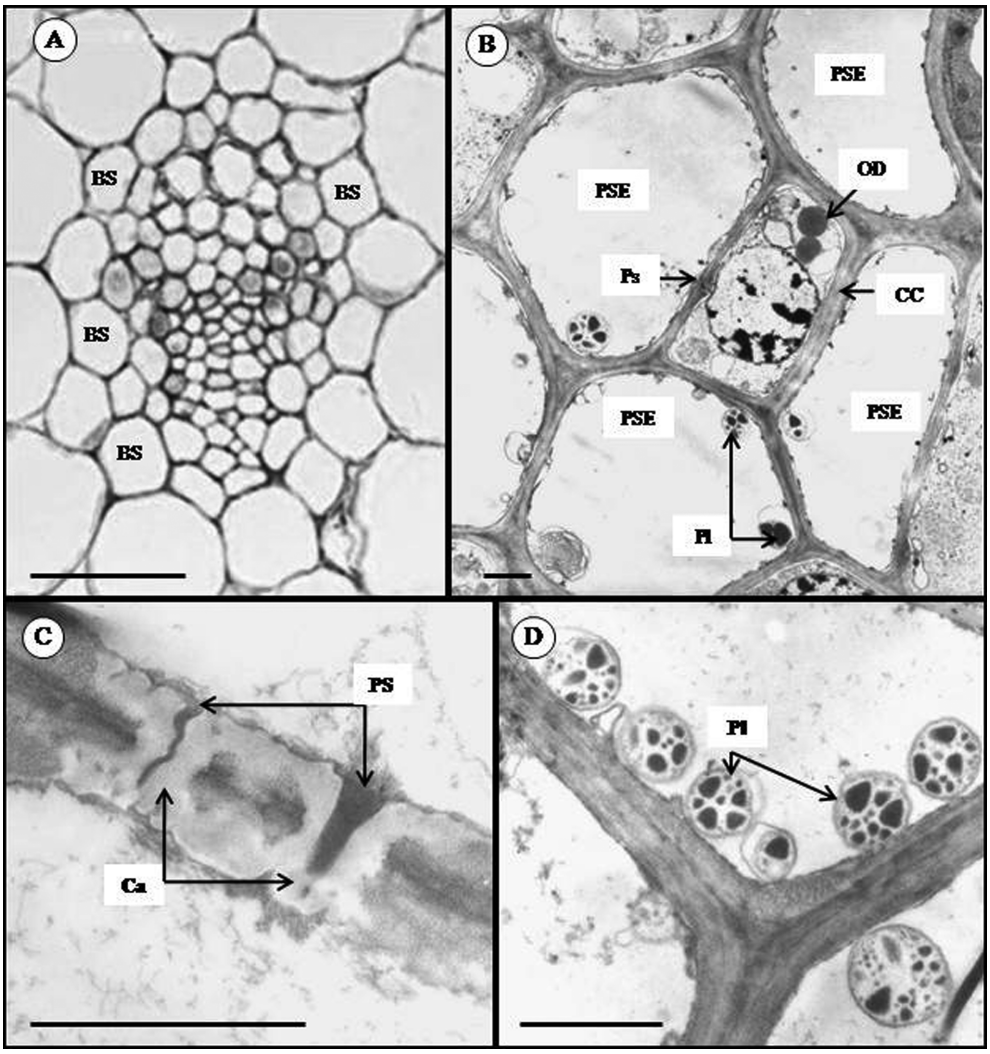


Figure 2. The vascular system. (**A**) LM micrograph of a transverse section of an *Urginea maritima* leaf revealing the bundle sheath (BS) and the vascular system. Scale bar = 50 μm. (**B**) TEM micrograph of a transverse section of an *U. maritima* leaf showing the protophloem sieve element (PSE) and the companion cell (CC). (OD: oil droplets, Pl: plastid, Ps: plasmodesmata). Scale bar = 1 μm. (**C**) TEM micrograph of a transverse section of an *U. maritima* leaf showing plasmodesmata (Ps) between PSE and CC provided by callose (Ca). Scale bar = 1 μm. (**D**) TEM micrograph of a transverse section of an *U. maritima* leaf revealing plastids (Pl) inside PSE. Scale bar = 1 μm.

olar content appears foamy and each crystal needle is embedded in a translucent homogeneous substance (Fig. 1F). The idioblastic cells contain different phenotypes of crystalloid inclusions (Fig. 1G) and myelin-like structures. In addition, the cell wall of the idioblastic cells contains oil droplets and starch granules.

The bundle sheath of the leaf of *U. maritima* is hardly seen and does not possess either chloroplasts or bundle sheath extensions (Fig. 2A). The vascular bundles (the main veins) occur on rows parallel to each other and are crossed by small veinlets. The xylem consists of vessel members. Phloem cells lose most of their organelles during maturation. The phloem sieve elements are supported by the companion cells in order to maintain the osmotic pressure of the cell during transportation (Fig. 2B). The presence of plasmodemata between the phloem sieve elements and the companion cells is evident. Plasmodesmata are provided with callose to control the movements of the substances (Fig. 2C). Plastids of the protophloem sieve elements (PSE) are characterized by peculiar protein crystalloid inclusions (Fig. 2D).

The phenology of the chloroplast is correlated with the cell that belongs to, i.e., the choloroplasts of the upper spongy mesophyll cells are elongated (Fig. 2A) and those of the lower ones are ovulated (Fig. 3B), while some other chloroplasts are rounded and/or irregularly shaped (Fig. 3C). The chloroplasts exhibit abundant oil droplets, phenolic compounds (Fig. 3D) and putative starch granules (Fig. 3E). Myelin-like structures, oil droplets and mitochondria usually occur close to the chloroplasts (Fig. 3D).

Plants in the Mediterranean ecosystems, semiarid and arid regions are typically limited by water and/or nutrients rather than carbon (Pate and Dixon, 1982; Dixon et al., 1983; Pate and Dell, 1984; Bloom et al., 1985; Stock et al., 1987; Gutterman and Boeken, 1988; Boeken, 1990; Chapin et al., 1990; Witkowski, 1990; Boeken and Gutterman, 1991). In the Mediterranean environment, perennial species are sclerophyllous, summer (drought) deciduous, or seasonally

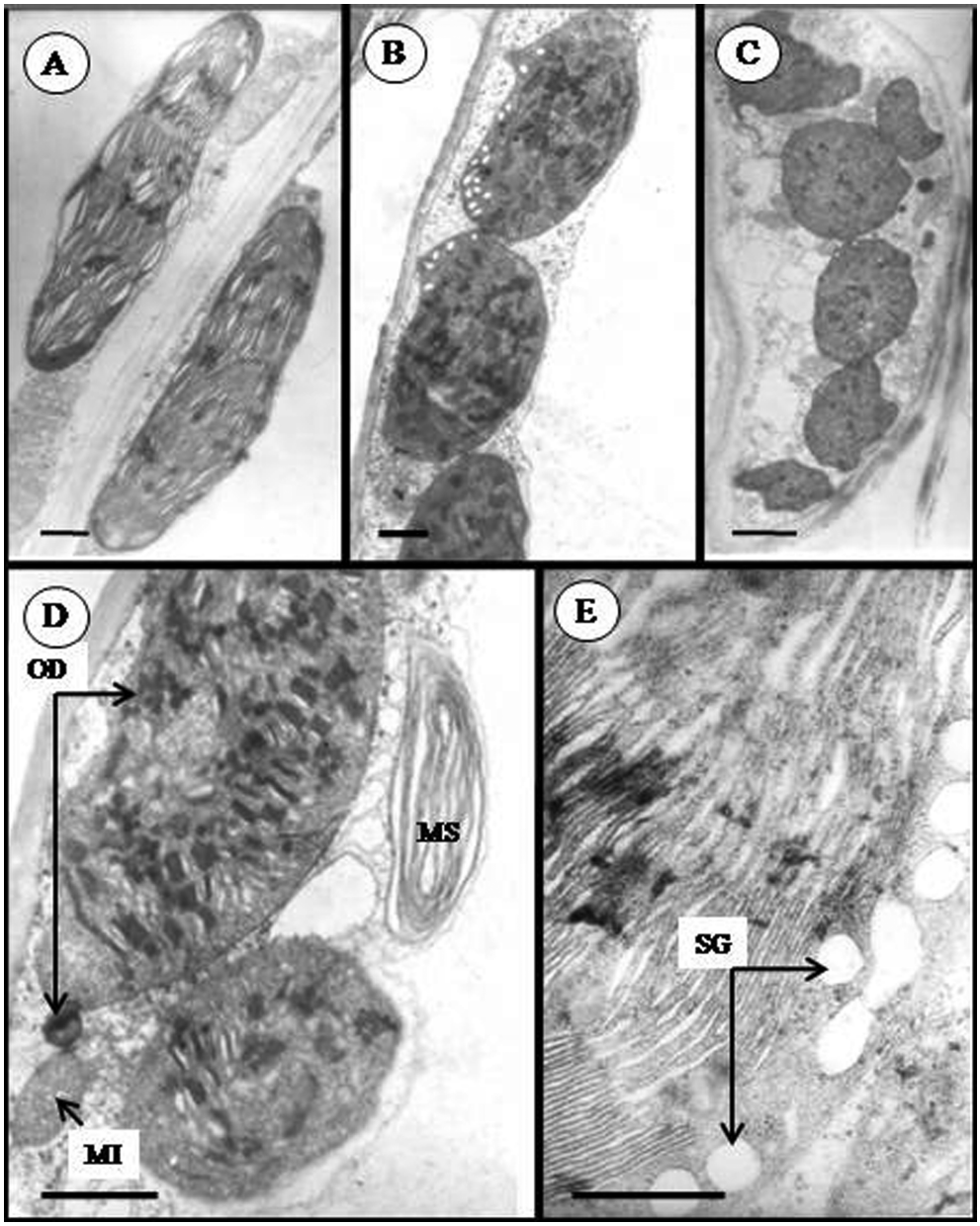


Figure 3. TEM micrographs of a transverse section of an *Urginea maritima* leaf showing chloroplasts with different shapes. Scale bar = 1 μm except for Fig. 3D, Scale bar = 0.5 μm. (**A**) In upper spongy mesophyll. (**B**) In meddle spongy mesophyll. (**C**) In lower spongy mesophyll. (**D**) Organelles occur close to chloroplast that contains oil droplets (OD). (Mi: mitochondria, MS: myelin-like structure). (**E**) Chloroplast with putative starch granules (SG).

dimorphic (Margaris, 1981). The flat structure of the lamina is characteristic of mesomorphic leaves (Strasburger et al., 1982). C_3 species abundance higher than that of C_4 was consistent with moist climates (850 mm rainfall). Therefore, C_3 species tend to be distributed in moist and low temperature conditions (Wang, 2005). In the case of *U. maritima*, the absence or the presence of very few chloroplasts inside the bundle sheath cells and the frequent occurrence of chloroplasts inside mesophyll cells are a typical characteristic of C_3 plant.

Abundant stomata on both leaf surfaces and the absence of any kind of compartmentalization, as well as the fine structure of spongy parenchyma, i.e. cell arrangement and chloroplasts distribution along cell walls facing intercellular spaces, may decrease CO_2 diffusion resistance (Parkhurst et al., 1988; Psaras et al., 1996; Evans and Loretto, 2000). Moreover, the wide mesophyll intercellular spaces and air spaces are reserved for gas exchange, which in turn increases the photosynthetic rate. Before leaf senescence, the nutrients are translocated to the storage organs of the plant (Al-Tardeh et al., 2006).

Leaf Morphology and Morphometry

The average leaf (3rd leaf from the rosette basis) traits of the studied species are shown in Table 2. The leaves of *U. maritima* are 2-5 cm wide, 20-30 cm long, lanceolated in

Table 2. Morphological leaf (3rd leaf from the rosette basis) traits of *U. maritima* in semiarid Meditearranean climate (N=30±SD).

Leaf parameter	Ave ± SD
Range of leaf length (cm)	20-30
Range of leaf width (cm)	2-5
Leaf surface area (LSA, cm^2)	87.26±13.93
Leaf volume (V, cm^{-3})	6.232±0.995
Leaf dry mass (LDM, mg)	843.5±111.0
Specific leaf area ($cm^2\ g^{-1}$)	103.5±11.19
Leaf tissue density (LTD, mg cm^{-3})	136.5±14.41
Specific leaf mass (SLM, mg cm^{-2})	8.564±3.139
Leaf water content %	84.59±1.424

Table 3. Relative volumes (%) of leaf adaxial cuticle, adaxial epidermis, spongy mesophyll, abaxial epidermis and abaxial cuticle (n=15, ± SD).

Leaf tissue	Relative volume %
Adaxial cuticle	2.41±0.20
Adaxial epidermis	6.01±0.97
Spongy mesophyll cells	52.9±1.42
Spongy air spaces and intercellular spaces	30.4±1.13
Abaxial epidermis	6.00±0.21
Abaxial cuticle	2.21±0.15

shape, somewhat undulated at the margin, shiny and dark-green in texture. The leaf surface area is almost 87.26± 13.93 cm^2. The SLM and LTD of the leaf are 8.564±3.139 mg cm^{-2} and 136.5±14.41 mg cm^{-3}, respectively.

A morphometric analysis, listed in Table 3, was carried out in order to explain the relation between the leaf components and their contributions to the total leaf volume. The relative volume of the spongy mesophyll cells is the highest (52.96±1.42%), and that of the abaxial cuticle is the lowest (2.21±0.15%), while the corresponding one of the spongy air spaces and intercellular spaces is intermediate (30.41± 1.13%).

Reduced leaf size, increased thickness, thick external walls of the epidermal cells, high stomatal density and palisade parenchyma developed at the expense of spongy parenchyma are common features of plants grown in xeric environments (Fahn and Cutler, 1992; Shields, 1950). The leaf of *U. maritima* is thicker compared to that of evergreen sclerophylls (714.2±33.6 μm vs. 250-550 μm, Christodoulakis and Mitrakos, 1987; Rotondi et al., 2003) and that of semi-deciduous species (714.2±33.6 μm vs. 100-150 μm). Thus, relatively thick leaves of *U. maritima* possess certain structural characteristics of xeromorphic leaves, considered to facilitate CO_2 uptake by the mesophyll (Terashima et al., 2005). However, *U. maritima* leaf could account for mesomorphic leaves, since they possess flat lamina (Strasburger et al., 1982).

LTD of the leaf of *U. maritima* is lower than that of evergreen sclerophylls (416-669 mg cm^{-3}) (Gratani and Bombelli, 2001), semi-deciduous species (610-690 mg cm^{-3}) (Gratani and Bombelli, 2000) and of that of evergreen shrub species (504-756 mg cm^{-3}) (Gratani and Varone, 2004). The lower LTD allows a better CO_2 movement through the air spaces between the cells (Parkhurst, 1986), resulting in a higher photosynthetic rate during the favourable period. On the contrary, leaves with a higher amount of biomass per unit area may be more efficient in water use during drought. The leaf of *U. maritima* is adapted to such conditions by possessing low LTD and moderate SLM for a better CO_2 movement (Gratani and Varone, 2004) and thick cuticle, which increase leaf reflectance, thus reducing solar inception, heat

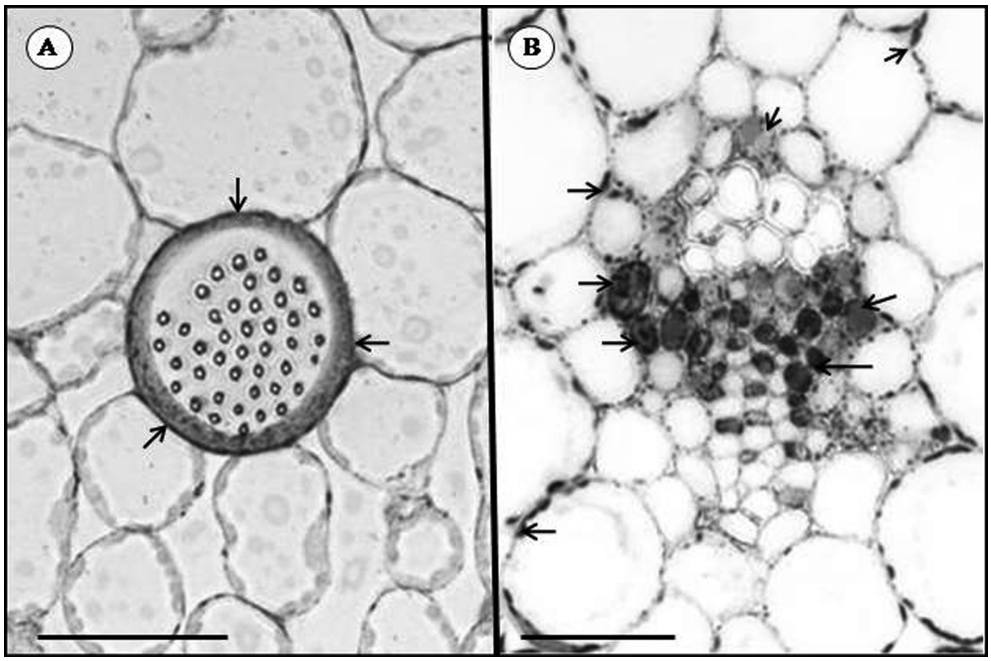


Figure 4. Leaf histochemistry. (**A**) LM micrograph of a transverse section of an *Urginea maritima* leaf showing idioblast cell with bundle of raphides embedded in polysaccharidic content stained red with Schiff's reagent (arrows). Scale bar = 50 μm. (**B**) LM micrograph of a transverse section of an *U. maritima* leaf showing numerous cells with lipids stained brown to black with Sudan Black B (arrows). Scale bar = 50 μm.

load and therefore water deficit (Gausman and Quisenberry, 1990). However, the latter may disturb the CO_2 movement through the stomata; this undesired condition may be compensated by possessing high density of stomata on both sides of the leaf which reduces the diffused distances of CO_2.

SLM is considered as a useful index of xeromorphism (Witkowski and Lamont, 1991). SLM of the leaf of *U. maritima* is lower than that of evergreen sclerophylls (14-20 mg cm^{-2}) (Gratani and Bombelli, 2001) and of drought semideciduous species (8.5-14.7 mg cm^{-2}) (Gratani and Bombelli, 2000), while, it is somewhat, higher than that of the non woody perennial species (6-9 mg cm^{-2}) (Yadav et al., 2004) and of the geophyte *Pulmonaria officinalis* (4.4 mg cm^{-2}) (Gaberščik et al., 2001). Therefore, SLM of the leaf of *U. maritima* could account for considering the plant to be adapted as a drought deciduous and mesophytic species.

Like other bulbous plants (Evenari and Gutterman, 1985; Gutterman and Boeken, 1988; Boeken, 1989; Kamenetsky, 1994), *U. maritima* lacks specific xerophytic adaptations such as sclerophyllous or succulent leaves or special physiological mechanisms to cope with drought.

Histochemistry

The presence of cells with polysaccharidic content in the leaf is evident after employing the Schiff's reagent. The idioblastic cells containing bundles of rapides are positively reacted (Fig. 4A) (arrows). When semi-thin or hand-cut sections are treated with Sudan Black B, numerous cells appear intensely stained brown to black (Fig. 4B) (arrows). Oil droplets occurring in the nucleus, chloroplasts (Fig. 3D) and vacuoles are positively reacted with this stain.

Presence of the ultraviolet-B-absorbing components (Grammatikopoulos et al., 1999) is evident as phenolic compounds and oil droplets. These essential oils may be efficient in reducing both transpiration and overheating (Todoroviæ and Stevanoviæ, 1994). Polysaccharides of the idioblastic cells could be fructans and fructo-oligosaccharides (Sims et al., 2001). The defense mechanisms of *U. maritima* could be viewed not only by raphide crystals (Al-Tardeh et al., 2006), but also by other stored compounds acting against microbial agents, herbivores, rodents, fungi and insects (March et al., 1991; Sathiyamoorthy et al., 1999; Fitzpatrick, 1952; Miyakado et al., 1975). Moreover, the cuticle is considered as the outermost defensive barrier of the plant leaves against the pathogens and the environmental hazards (Stenglein et al., 2005).

In conclusion, the equifacial leaves of *U. maritima* seem to play an important role in protecting the plant functions from environmental hazards. Structurally, these functions are based on the thick cuticle and the presence of the crystalloid inclusions. Furthermore, leaves of *U. maritima* constitute a means of synchronization of the plant with the seasonality of the Mediterranean climate, by the high production of nutrients in a short period (rain season). The high rate of photosynthesis, in reference to CO_2 availability, could be achieved by the high density of the sunken stomata on both sides, the fine structure of the mesophyll cells, the distribution of the chloroplasts parallel to the cell wall facing the intercellular spaces, the large air spaces in the mesophyll area and the low LTD and SLM. Finally, understanding the leaf structure of *U. maritima* under natural conditions will be useful for further studies dealing with the physiology of this species.

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