



Leaf surface characteristics and wetting in *Ceratonia siliqua* L.

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ABSTRACT

Compound leaves of *Ceratonia siliqua* L. (carob tree) exhibit a long life span and are exposed to environmental stimuli for approximately twenty months. The micromorphology of the adaxial and the abaxial leaflet surfaces was studied, in comparison with treated waxless epidermises (after the removal of cuticle and epicuticular waxes) and corresponding replicas, respectively. The microstructural surface features are evaluated as possible consistent parameters related to the wetness of leaves. The abaxial leaflet surface is more hydrophobic than the adaxial leaflet surface in *C. siliqua*, which may be particularly important for the ecophysiological status of its hypostomatic leaves.

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Introduction

The surfaces of leaves are usually covered by a continuous cuticular layer which contains substances nearly impermeable to water and forms an interface between the atmosphere and the tissues (Riederer, 2006). The cuticle is the first structure in contact with abiotic environmental conditions, dust and invading organisms, and concomitantly an effective barrier to water movement from the plant to the atmosphere (Koch et al., 2009; Taylor, 2011).

Ceratonia siliqua L. (carob tree) is a slowly growing, woody evergreen, sclerophyll species and widespread occurring as a native plant in the Mediterranean Basin. It is also distributed in Arabia and Oman, while it was introduced in California, Mexico and Southern Australia (Ramon-Laca and Mabberley, 2004). *C. siliqua* is considered a phylogenetically primitive species of tropical origin that has been cultivated in the Mediterranean area since historic times (Bures et al., 2004; Jahns, 2003; Zohary, 2002). It is an economically important plant (Biner et al., 2007; Makris and Kefalas, 2004; Özcan et al., 2007), which has been used for afforestation in semi-arid regions (Catarino, 1993; Makhzoumi, 1997).

In the Eastern Mediterranean region, *C. siliqua* is subjected to a prolonged drought period, which immediately follows the main period of growth of the evergreen sclerophyll shrubs (Rhizopoulou and Mitrakos, 1990; Rhizopoulou et al., 1991). The developing compound leaves of *C. siliqua* expand within a 3-months period; then they ceased growing, and are exposed to the environmental conditions for approximately 20 months (Diamantoglou and Mitrakos,

1981; Rhizopoulou and Davies, 1991). The sclerophyll leaves of *C. siliqua* are characterized by a very thick, unilayered adaxial epidermis, while stomata are present only on the abaxial surface (Christodoulakis, 1992; Nunes and Linskens, 1980). The root apices of the deep tap roots of *C. siliqua* sustain water flux to the leaves also for prolonged periods of topsoil drying (Pratikakis et al., 1998; Rhizopoulou, 2004; Rhizopoulou and Davies, 1991).

The purpose of this work was to study micromorphological features and wettability of the adaxial and the abaxial surface of fully expanded leaflets of *C. siliqua*. Comprehensive information about them could contribute to understand leaf functionality of this species, because relationships between surface hydrophobicity and leaf micromorphology have been reported to be of great ecological importance (Brewer and Nuñez, 2007; Holloway, 1969; Pandey and Nagar, 2003; Wiśniewska et al., 2003). To the best of our knowledge water repellent properties of the long-lived leaves of *C. siliqua* have not been hitherto reported. In addition, the micromorphology of leaf surfaces of *C. siliqua* is studied in the following, using treated surfaces, after the removal of the cuticle and epicuticular waxes from the surfaces, and using replicas of the original leaf surfaces. The replication technique is historically a very early method for studying surfaces of specimens that has recently being used in biomimetics (Miguens, 2008; Schulte et al., 2009).

Materials and methods

Plant material

Fully expanded and turgid leaves of *Ceratonia siliqua* L. (Leguminosae), which all sprouted at the same time in spring, were harvested early in the morning in autumn after the first rains,

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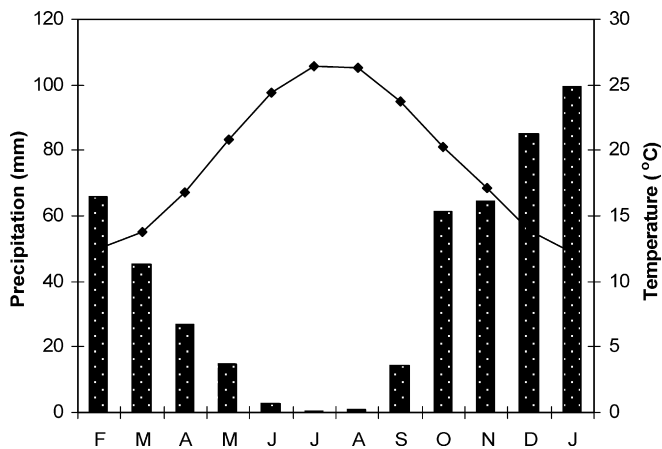


Fig. 1. Climatic data of the closest to the sampling site meteorological station; the order of months is from February up to January. Continuous line represents mean monthly temperature and bars represent total, monthly precipitation.

between 2009 and 2011; the collection was made from a stand of 4 m tall trees grown under ambient conditions in Crete (85 m above sea level, 35°18'21"N, 25°04'24"E). Autumn is physiologically important for *C. siliqua*, when resources are needed for fruit enlargement, flower induction and leaf growth. Climatic data recorded by the closest to the sampling site meteorological station give average values of five years (Fig. 1) and were kindly provided by the Hellenic National Meteorological Service. During the study period, the average monthly precipitation ranged from 61 mm to 85 mm, and the average monthly temperature from 17°C to 12°C (Fig. 1).

Microscopy

The study was carried out on the adaxial and the abaxial surfaces of the lamina of expanded leaflets of *C. siliqua* leaves which are subdivided into three pairs of leaflets; the middle pair of these leaflets has been used. Samples from leaflets were carefully cut in square pieces (4 mm²) and fixed in 3% glutaraldehyde in Naphosphate buffer at pH 7, at room temperature, for 2 h. Plant material was washed three times by immersion in buffer for 30 min each time; then, it was post fixed in 1% OsO₄ in the same buffer at 4°C and dehydrated in acetone solutions. Dehydrated tissues were embedded in Spurr resin (Serva Chemicals, D). Semi-thin sections of resin-embedded tissue (LKB Ultratome III microtome, Bromma, S) were stained in Toluidine Blue 'O', in 1% borax solution, photographed and digitally recorded using a Zeiss Axioplan light microscope (Carl Zeiss, Thornwood, N.Y.) equipped with a digital camera (Zeiss AxioCam MRc5). Dehydrated samples were dried at the critical point in a Bal-tec CPD-030 dryer (Balzers, LI), mounted with double adhesive tape on stubs, sputter coated with 20 nm gold in a Bal-tec SCP-050. The adaxial and abaxial leaf surfaces were viewed using the scanning electron microscope JEOL JSM 840 (SEM, JEOL Ltd., Tokyo, Japan) that has been proven to be very effective for screening surface structures (Koch et al., 2009).

Replication process

Negative and positive replicas of the expanded and fully turgid leaflets of *C. siliqua* were created. The negative replicas were initially transferred to elastomeric polydimethylsiloxane PDMS (SYLGARD 184, Dow Corning). Briefly, the leaf was covered with a viscous liquid prepolymer of PDMS, which consisted of a "base" and a "curing agent" that are typically mixed in a ratio 10:1 (w:w). The polymer coated structure was placed into a chamber at room

temperature for three days; vacuum and/or heating treatment for removal of cuticular relief was not applied in order to avoid formation of air bubbles within the PDMS. Then, a mold which holds the negative replica of the original pattern was peeled off the leaf. Using such a PDMS mold, that represents the negative leaf morphology, replicas of the initial morphology (positive replicas) could be made out (Koufaki et al., 2011). The resulting successful reproduction of the initial leaf morphology was investigated on ORMOCER (ORGanically MODified CERamic), a UV photocurable organic–inorganic hybrid material, with particularly interesting properties, which is used in optical and biological applications. For the preparation of positive ORMOCER replicas a droplet of the liquid prepolymer containing the radical photoinitiator (Ormocomp Acryl Polymer microresist technology GmbH, D) was poured onto the PDMS mold; then, ORMOCER was polymerized by illumination with UV light for 2 h. Once solidification of ORMOCER had occurred, the PDMS mold was separated from the positive ORMOCER replica.

Estimating the wettability

Static contact angle measurements (θ) were performed by an automated tensionmeter, using a droplet (2 μ l) of distilled and deionized water that was gently positioned on the surfaces of lamina and images were captured to measure the angle formed at the liquid–solid interface, i.e. the contact angle (θ), according to Zorba et al. (2008). Measurements were made on each side of the same leaflets. Contact angle measurement is a very sensitive measure of the repellence of a surface and provides information on the characteristics of the outermost layer of a cuticle interfacing the liquid water and the atmosphere (Pandey and Nagar, 2003). A smaller contact angle indicates better wettability, a bigger one a greater hydrophobicity. Estimates were made on twenty randomly selected healthy leaflets (untreated specimens) from ten different trees and by five individual measurements per leaflet.

Removal of cuticular layer

Cuticular layer and epicuticular waxes of leaflets were removed by dipping the tissues in chloroform for 14 min (Cameron et al., 2002). Microscopic examination of leaf surfaces was used to confirm removal of the cuticle and epicuticular waxes (treated specimens), and lack of dehydration.

Data analysis

One-way ANOVA was performed to compare measurements on surfaces and their replicas. The statistical analysis was realized with the SPSS statistical program.

Results and discussion

Expanded leaflets of *C. siliqua*, grown under ambient conditions in open habitats, possess a rough cuticle on the adaxial (Fig. 2A) and the abaxial surface (Fig. 2C), consistent of wax rods on the adaxial (Fig. 2B) and star-formed wax platelets on the abaxial surface (Fig. 2C). Scanning electronic micrographs reveal stomata on the abaxial surface of leaflets of *C. siliqua* (Figs. 2D–3B) and wax platelets surrounding the stomatal pores (Fig. 2D). Epicuticular waxes of leaves form a barrier that enables the plant to control the exchange of water, solutes, gases and vapours, adhesion and penetration of dust and invading microorganisms (Riederer, 2006; Wiśniewska et al., 2003).

The results of the contact angle measurements for both the adaxial and the abaxial untreated waxy (θ_x), and treated waxless (θ_s) surfaces of the leaflets of *C. siliqua* are presented in Fig. 3 and Table 1. The highest θ_x was detected on the abaxial surface (Table 1).

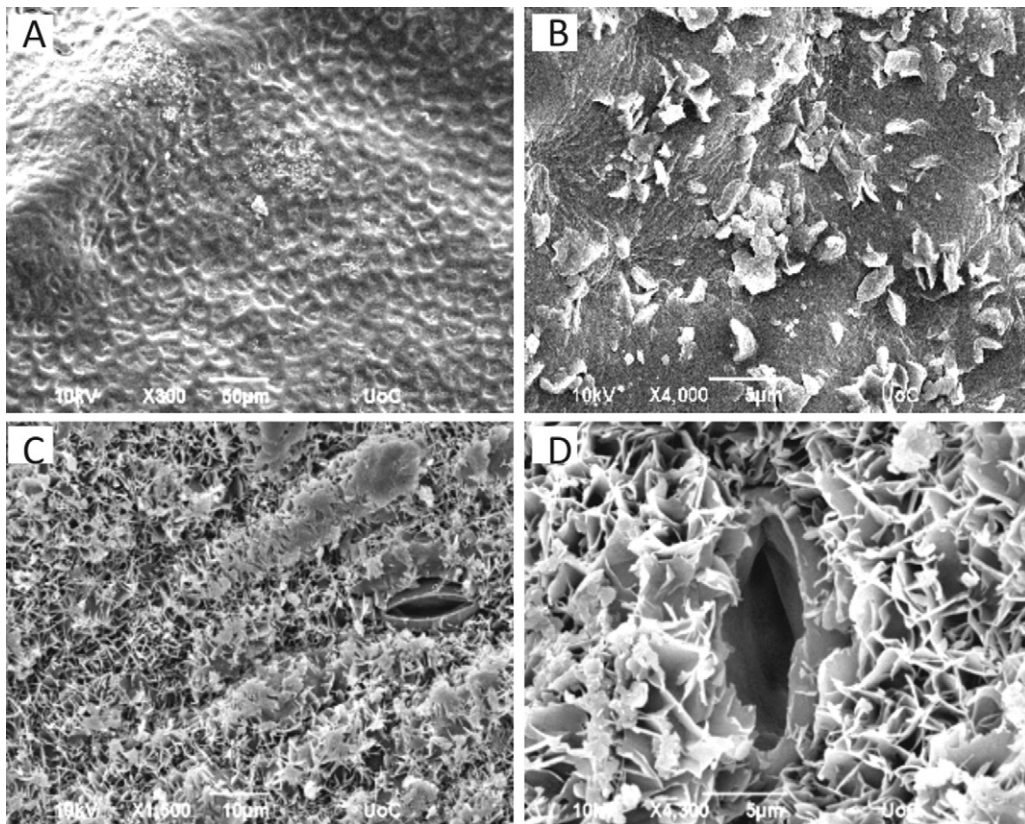


Fig. 2. Scanning electron micrographs of untreated adaxial (A, M: 300 \times) and abaxial (C, M: 1500 \times) leaflet surface of *Ceratonia siliqua* showing in detail the structure of epicuticular wax rods at the adaxial surface (B, M: 4000 \times) and wax platelets surrounding a stomatal pore at the abaxial surface (D, M: 4300 \times); M: magnification.

Upon wax removal the contact angle of the abaxial leaflet surface in *C. siliqua* is greatly reduced (θ_s , Table 1), which indicates that wetness will depend on synergy effects of roughness and chemistry (Stratakis et al., 2011). It seems likely that both the tissue microsculpture and the waxy coverage increase the roughness of the abaxial surface of the leaflets, which is more hydrophobic than the adaxial surface (Figs. 2A, C and 4A, B). Hydrophobic properties of leaf surfaces may play a significant role in resisting water loss from the cuticle, and higher reflectance of a wax-rich leaf surface can reduce also the temperature of leaves exposed to dry conditions (Jordan et al., 1984; Nizam Uddin and Marshall, 1988; Sánchez

et al., 2001). During the prolonged dry season, leaf rolling occurs in *C. siliqua* (personal observation) as a typical response to water deficit (Kadioglu et al., 2012). This change exposes more intensely the adaxial, astomatous leaf surfaces to the environmental stimuli, and protects the abaxial leaf surfaces from the ambient conditions.

Estimates of θ_s of treated abaxial leaflet surfaces of *C. siliqua*, i.e. after the removal of cuticle and epicuticular waxes, render similar values as the angles found for water droplets on a paraffin wax surface (Kamusewitz et al., 1999). Based on this, wettability of a cuticle-free leaf surface would principally be low. However, waxless surfaces of leaflets of *C. siliqua* possess a micromorphology

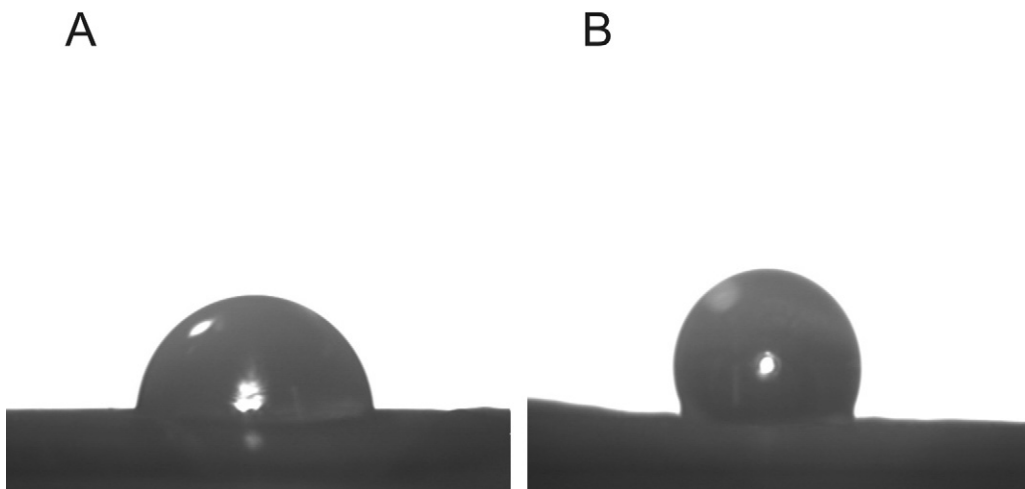


Fig. 3. Shape of a water droplet on the untreated adaxial (A) and abaxial (B) surface of leaflets of *Ceratonia siliqua*.

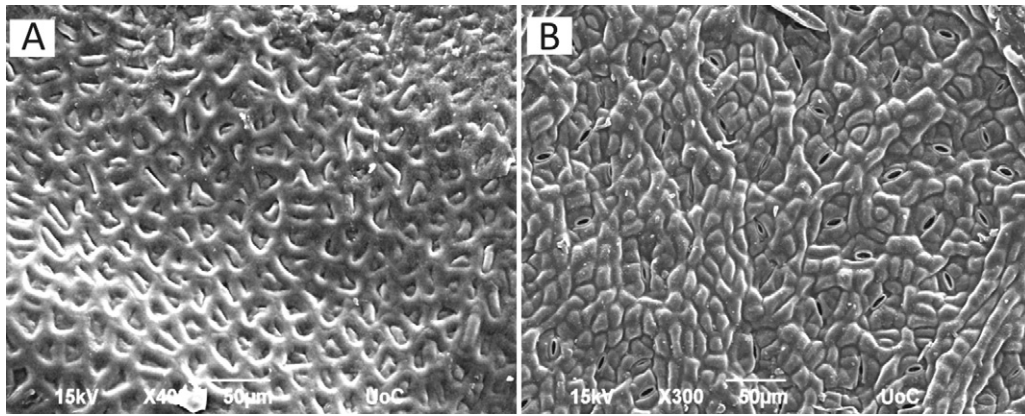


Fig. 4. Scanning electron micrographs of treated, waxless adaxial (A, M: 400 \times) and abaxial (B, M: 300 \times) leaflet surfaces of *Ceratonia siliqua*; M: magnification.

(Fig. 4) that would permit water droplets to flow easily into depressions of the surface, wetting the lamina and making leaf tissues vulnerable to long-lasting water adhesion (Wagner et al., 2003). Cuticle and epicuticular waxes are therefore essential keeping the leaves healthy.

Leaf wetting can cause instantaneous suppression of photosynthesis, since water droplets or a thin water layer can clog stomata and impede CO₂ transfer into the leaf (Ishibashi and Terashima, 1995; Smith and McClean, 1989). Also, hygroscopic particles may work as “desiccants,” reducing the drought tolerance of plants (Burkhardt, 2010). On one hand, it is therefore important that water does not accumulate between the rolled margins and the abaxial, stomatous leaf surface in *C. siliqua* (Nunes et al., 1992), i.e. the high

water repellency of the abaxial surface has high functional importance. On the other hand, water repellence of the exposed parts of long-lived leaves of *C. siliqua* can canalise water off the adaxial surface, by this way washing away dust, microbial pathogens and pollutants (Meletiου-Christou et al., 2011).

The stomata-free hydrophobic adaxial surfaces of leaflets of *C. siliqua* (Figs. 2A and 4A) seem to be an effective barrier to water loss from the cuticle to the atmosphere during the dry season (Burton and Bhushan, 2006; Koch and Ensikat, 2008; Taylor, 2011). The micromorphology does not seem to enhance the overall roughness of the adaxial surface, as concluded from the values of θ_x and the corresponding θ_s of untreated and replicated surfaces, respectively (Table 1).

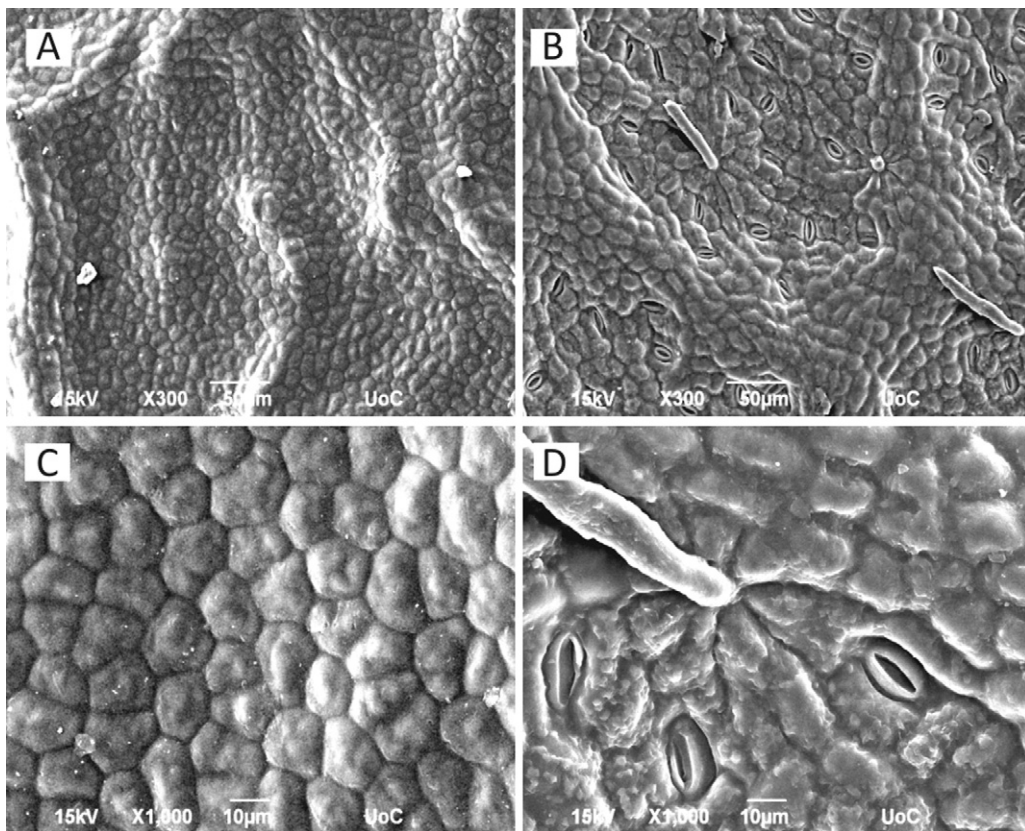


Fig. 5. Scanning electron micrographs of positive replicas of treated, waxless adaxial (A, M: 300 \times) and abaxial leaflet surfaces (B, M: 300 \times) of *Ceratonia siliqua*; high magnification reveals polygonal papillae cells on the adaxial epidermis (C, M: 1000 \times), and guard cells and open stomatal pores on the abaxial epidermis (D, M: 1000 \times); M: magnification.

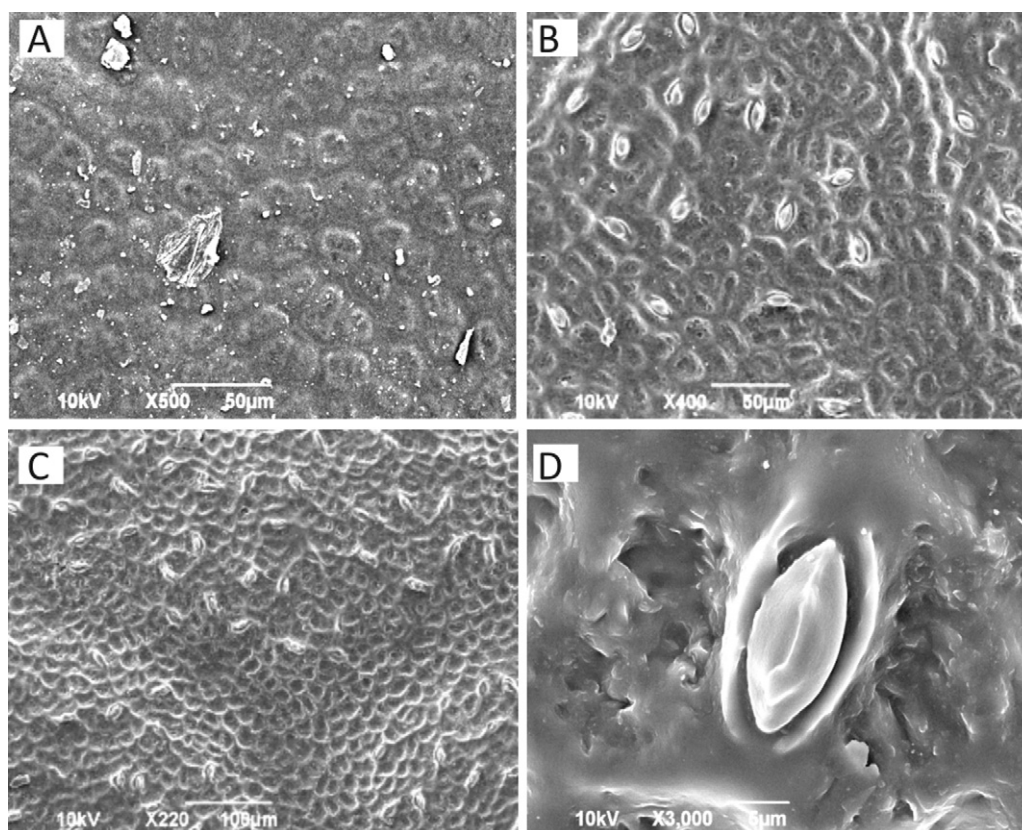


Fig. 6. Scanning electron micrographs of negative replicas of treated, waxless adaxial (A, M: 500 \times) and abaxial (B, M: 400 \times) leaflet surfaces of *Ceratonia siliqua*, with numerous stomata (C, M: 220 \times) and a closed stomatal pore, the guard cells and two subsidiary cells (D, M: 3000 \times); M: magnification.

Upon removal of the wax layers (treated samples) numerous stomata are well visible among rectangular epidermal cells (Figs. 4B and 5B) and occasionally well preserved unicellular hairs (Fig. 5D) at the abaxial surfaces of leaflets of *C. siliqua* (Fig. 4B) and at the surface of the corresponding replicas (Fig. 5B). Also, stomata appeared on negative replicas of the abaxial surfaces (Fig. 6B–D).

Plants in dry habitats are well adapted to minimizing the amount of surface area in contact with water and thus their leaves are mostly hydrophobic (Brewer and Nuñez, 2007; Wagner et al., 2003). Our results show that also leaf surfaces of carob tree are quite hydrophobic and this corresponds well to the origin and

the distribution of *C. siliqua* in Southern rather than in Northern Mediterranean regions (Mitrakos, 1980; Suc, 1984).

Conclusion

Both surfaces of *Ceratonia siliqua* leaflets are quite hydrophobic. However, their abaxial surface was found to be still more hydrophobic than the adaxial surface, which is at the first glance surprising, since usually the adaxial surface is more rain-exposed than the abaxial one. However, a high hydrophobicity at the underside of leaves may play a significant role in sustaining photosynthesis during the rainy season, keeping unimpeded the gas exchange of the hypostomatic leaves. High hydrophobicity of the adaxial leaf surface, on the other hand, minimizes cuticular transpiration during the dry season. Wetting properties of the surfaces after removal of the wax coverage indicate that water repellency of the tissues is remarkably affected by the micromorphology of their waxy relief. Adaxial and abaxial sides of leaflets of *C. siliqua* show differential micromorphologies of both the waxy leaf surfaces and the waxless cell wall surfaces. The waxy microstructures generally reduce the wettability of carob tree leaves to a degree that they can remain unaffected from longer lasting water films and remain healthy irrespective of their extremely long individual lifetime.

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Table 1

Contact angle of the adaxial and the abaxial untreated (θ_x) and treated (θ_s) leaflet surfaces (after the removal of their relief), from carob trees (*Ceratonia siliqua*) grown outdoors, as well as contact angle of the corresponding replicas (θ_{xr} and θ_{sr} , respectively). Asterisks indicate significant difference * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in mean values \pm S.D.

Untreated surface	Leaflet θ_x ($^\circ$)	Replica θ_{xr} ($^\circ$)
Adaxial	88 \pm 2	92 \pm 1
Abaxial	120 \pm 4	108 \pm 3
Treated surface	Leaflet θ_s ($^\circ$)	Replica θ_{sr} ($^\circ$)
Adaxial	85 \pm 3	92 \pm 2
Abaxial	102 \pm 5	107 \pm 2
Source of variation	p	
θ_x of adaxial and θ_x of abaxial surface	***	
θ_x and θ_s of adaxial surface	*	
θ_x and θ_s of abaxial surface	***	
θ_x and θ_{xr} of adaxial surface	*	
θ_x and θ_{xr} of abaxial surface	**	
θ_s and θ_{sr} of adaxial surface	**	
θ_s and θ_{sr} of abaxial surface	**	

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