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Research Area: Ecophysiology and Sustainability

Wettability, polarity and water absorption of *Quercus ilex* leaves: effect of leaf side and age

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Summary

The highly pubescent abaxial side of *Q. ilex* leaves is unwettable and water-repellent. The adaxial side is however wettable and can take up water, which may be an adaptation to growing under Mediterranean conditions

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ABSTRACT

Plant trichomes play important protective functions and may have a major influence on leaf surface wettability. With the aim of gaining insight into trichome structure, composition and function in relation to water-plant surface interactions, we analyzed the adaxial and abaxial leaf surface of *Quercus ilex* L. (holm oak) as model. By measuring the leaf water potential 24 h after the deposition of water drops on to abaxial and adaxial surfaces, evidence for water penetration through the upper leaf side was gained in young and mature leaves. The structure and chemical composition of the abaxial (always present) and adaxial (occurring only in young leaves) trichomes were analyzed by various microscopic and analytical procedures. The adaxial surfaces were wettable and had a high degree of water drop adhesion in contrast to the highly unwettable and water repellent abaxial holm oak leaf sides. The surface free energy, polarity and solubility parameter decreased with leaf age, with generally higher values determined for the abaxial sides. All holm oak leaf trichomes were covered with a cuticle. The abaxial trichomes were composed of 8% soluble waxes, 49% cutin, and 43% polysaccharides. For the adaxial side, it is concluded that trichomes and the scars after trichome shedding contribute to water uptake, while the abaxial leaf side is highly hydrophobic due to its high degree of pubescence and different trichome structure, composition and density. Results are interpreted in terms of water-plant surface interactions, plant surface physical-chemistry, and plant ecophysiology.

INTRODUCTION

Plant surfaces have an important protecting function against multiple biotic and abiotic stress factors (Riederer, 2006). They may for example limit the attack of insects (Eigenbrode and Jetter, 2002), or pathogenic fungi (Gniwotta et al., 2005; Łaźniewska et al., 2012), avoid damage caused by high intensities of UV and visible radiation (Reicosky and Hanover, 1978; Karabourniotis and Bormann, 1999), help to regulate leaf temperature (Ehleringer and Björkman, 1978; Ripley et al., 1999), and chiefly prevent plant organs from dehydration (Riederer and Schreiber, 2001).

The epidermis of plants has been found to have major degree of physical and chemical variability, and may often contain specialised cells such as trichomes or stomata (Roth-Nebelsick et al., 2009; Javelle et al., 2011). Most aerial organs are covered with an extra-cellular, generally lipid-rich layer named cuticle, which is typically composed of waxes embedded into (intra-cuticular) or deposited on to (epi-cuticular waxes) a biopolymer matrix of cutin (forming a network of cross-esterified, hydroxy C_{16} and/or C_{18} fatty-acids) and/or cutan, with variable amounts of polysaccharides and phenolics (Domínguez et al., 2011; Yeats and Rose, 2013). Different nano- and/or micro-scale levels of plant surface sculpturing have been observed by scanning electron microscopy (SEM), generally in relation to the topography of epicuticular waxes, cuticular folds and epidermal cells (Koch and Barthlott, 2009). Such surface features together with their chemical composition (Khayet and Fernández, 2012) may lead to a high degree of roughness and hydrophobicity (Koch and Barthlott, 2009; Konrad et al., 2012). The interactions of plant surfaces with water have been addressed in some investigations (Brewer et al., 1991; Brewer and Schmidt, 1997; Pandey and Nagar, 2003; Hanba et al., 2004; Dietz et al., 2007; Holder, 2007a, b; Fernández et al., 2011, 2014; Roth-Nebelsick et al., 2012; Wen et al., 2012; Urrego-Pereira et al., 2013), and is a topic of growing interest for plant ecophysiology (Helliker and Griffiths, 2007; Aryal and Neuner, 2010; Limm and Dawson, 2010; Kim and Lee, 2011; Berry and Smith, 2012; Rosado and Holder, 2013; Berry et al., 2013; Helliker, 2014). On the other hand, the mechanisms of foliar uptake of water and solutes by plant surfaces are still not fully understood (Fernández and Eichert, 2009; Burkhardt and Hünsche, 2013), but they may play an important ecophysiological role (Limm et al., 2009; Johnstone and Dawson, 2010; Adamec, 2013; Berry et al., 2014).

The importance of trichomes and pubescent layers on water drop-plant surface interactions and on the subsequent potential water uptake into the organs has been analysed in some investigations (Fahn, 1986; Brewer et al., 1991; Grammatikopoulos and Manetas, 1994; Brewer and Smith, 1997; Pierce et al., 2001; Kenzo et al., 2008; Fernández et al., 2011, 2014; Burrows et al., 2013). Trichomes are unicellular or multi-cellular, glandular or non-glandular appendages, which originate from epidermal cells only, and develop outwards on the surface of plant organs (Werker, 2000). Non-glandular trichomes are categorised according to their morphology and exhibit a major variability in size, morphology, and function. On the other hand, glandular trichomes are classified by the secretory materials they excrete, accumulate or absorb (Johnson, 1975; Werker, 2000; Wagner et al., 2004). Trichomes can be often found in xeromorphic leaves and in young organs (Fahn, 1986; Karabourniotis et al., 1995). The occurrence of protecting leaf trichomes has been also reported for Mediterranean species such as *Quercus ilex* L. (Karabourniotis et al., 1995, 1998; Morales et al., 2002; Karioti et al., 2011; Camarero et al., 2012). There is limited information about the nature of the surface of trichomes, but they are also covered with a cuticle similarly to other epidermal cell types (Fernández et al., 2011, 2014).

In this study and using *Q. ilex* as model, we assessed for the first time the leaf surface-water relations of the abaxial (always pubescent) versus the adaxial (only pubescent in developing leaves and for a few months) surface, including their capacity to absorb surface-deposited water drops. Based on membrane science methodologies (Fernández et al., 2011; Khayet and Fernández 2012) and following a new integrative approach, the chemical, physical and anatomical properties of holm oak leaf surfaces and trichomes were analyzed, with the aim of addressing the following questions: (i) are young and mature adaxial and abaxial leaf surfaces capable of absorbing water deposited as drops on to the surfaces?, (ii) are young and mature abaxial and adaxial leaf surfaces similar in relation to their wettability, hydrophobicity, polarity, work-of-adhesion for water, solubility parameter and surface free energy?, and (iii) what is the physical and chemical nature of the adaxial versus the abaxial trichomes, chiefly in relation to young leaves?

RESULTS

Leaf surface water absorption

The water absorption results for both young and mature holm oak shoots are shown in Figure 1. The initial shoot water potential (Ψ_o) measured for young shoots were -2.47 ± 0.10 MPa. Twenty-four h after wetting the adaxial side of the leaves, the final water potential (Ψ_f) became -1.51 ± 0.15 MPa. In contrast, after wetting the abaxial side, Ψ_f reached -3.00 ± 0.09 MPa. As expected, the untreated (non-wetted) shoots, reached a lower Ψ_f of -3.19 ± 0.07 MPa (Fig. 1A). All Ψ_f values were statistically different from Ψ_o ($\alpha = 0.05$). A similar trend was found for mature shoots (Fig. 1B). In addition, the relative water content (RWC) varied according to the water potential variations. When the adaxial holm oak leaf side was wetted, RWC increased from 88.79 ± 0.54 to 93.45 ± 0.67%. When drops were applied on to the abaxial side, RWC decreased from 87.52 ± 0.63 to 84.22 ± 0.65%. Finally, the RWC of untreated leaves (non-wetted) decreased from 88.05 ± 0.99 to 82.48 ± 0.89% (Fig. 1C). Similar variations were recorded for mature leaves (Fig. 1D).

The adaxial and abaxial surface cuticular conductances of young holm-oak leaves were 3.8 ± 0.2 and 3.4 ± 0.1 m s^{-1} 10^{-5}, respectively. The cuticular conductance values measured for mature leaves were 3.1 ± 0.2 (adaxial) and 3.3 ± 0.3 (abaxial) m s^{-1} 10^{-5}. Regardless of leaf age, no significant cuticular conductance differences were found between upper and lower leaf sides. Statistically significant conductance differences were however recorded for the adaxial cuticle of young versus mature leaves ($P<0.05$).

Holm oak leaf surface structure

Fresh, young and mature holm oak leaves were examined by SEM. The young leaves were covered with trichomes, the adaxial surfaces being much less pubescent than the abaxial surfaces, where dense, imbricated trichome layers were found (Fig. 2, A and B). An abaxial dense indumentum was also present in mature leaves, but their upper surfaces had almost no trichomes (Fig. 2, C and D).

Concerning the adaxial leaf epidermis, a rather smooth epicuticular wax layer could be observed in mature leaves, which was only partially visible as patches in the young leaves (Fig. 2, A and C). The adaxial side of young leaves had a trichome density of approximately 87 per mm^2 leaf surface. The cuticle surface covered with trichomes was

194 estimated to be about 43% (Fig. 2A). Some protruding trichome-scars were especially
195 visible in the mature leaf adaxial surface (Fig. 2C).

196 Trichomes were observed to be non-glandular, multicellular, stellate, with the arms
197 fusing into a short, erect stipe and then diverging horizontally (Hardin, 1976). Most of
198 the adaxial trichomes of the young leaves had eight arms, while single long hairs, and
199 from four to seven-arm-trichomes were also eventually found. Their length and
200 diameter were measured on eight-arm-trichomes from the fusing point and at 10 µm
201 above, respectively. The length of the arms was approximately 115 ± 24 µm, and the
202 diameter was 6.2 ± 0.6 µm. The overlapping of trichome arms and the existence of
203 various trichome layers on the lower leaf sides prevented the accurate estimation of the
204 number of arms, arm length and diameter.

205 When analyzing the epidermal structure of holm oak leaves we noticed that the base
206 of abaxial and adaxial trichomes was cutinized, since it was stained with Sudan IV (Fig.
207 3A). The structure of the base of adaxial versus abaxial trichomes is different (Fig. 3, B
208 to E). Abaxial trichomes are anchored deeper into the epidermis by a group of cells
209 (Fig. 3, C and E), while those present in the upper side of young leaves are bound to the
210 underlying tissue in a more superficial manner, the union largely formed by cuticle and
211 cell wall material appearing more susceptible to shedding (Fig. 3, B and D). The scars
212 remaining in the upper leaf sides after trichome abscission seemed to have a
213 heterogeneous topography in both young (Fig. 3F) and mature leaves (Fig. 3G).
214 Trichomes and trichome scars were often seen directly or in the vicinity of bundle
215 sheath extensions (Fig. 3, A and G).

216 **Chemical composition of trichomes**

217 The proportion of chemical constituents of isolated holm oak trichomes was assessed
218 by the weight loss after successive chemical treatments, coupled with Fourier transform
219 infrared spectroscopy (FTIR) measurements. Isolated trichome SEM observations,
220 however, led us to discard the data concerning the adaxial side, since epidermal pieces
221 of considerable size were removed together with the trichomes due to their scarcity.
222 Hence, we are only showing the chemical composition of trichomes isolated from the
223 lower leaf side to avoid misinterpretations.

224 The fractions corresponding to depolymerized material (chiefly cutin) represented
225 the greatest percentage of the trichomes (49.0%). After cutin depolymerization a residue

(mainly of polysaccharides) of 42.9% was determined. A proportion of soluble waxes of 7.9% of the trichome mass was recorded after chloroform extraction.

The FTIR spectra of isolated intact and chemically treated abaxial holm oak trichomes are shown in Figure 4. Cuticular material assignment was based on previous data (Ramírez et al., 1992; Villena et al., 2000). For intact hairs, bands associated with different chemical functional groups, and consequently to cuticle components, were identified (Fig. 4A): long-chain aliphatic waxes and cutin (intense and thin asymmetric and symmetric CH_2 stretching absorptions at 2919 and 2849 cm^{-1} and the CH_2 bending one at 1463 cm^{-1}), the polyester cutin (C-O stretching vibration in ester environments band at 1729 cm^{-1} and the asymmetrical and symmetrical C-O-C stretching absorptions at 1166 and 1107 cm^{-1}, respectively), polysaccharides (mainly for the band associated with the glycosidic bond at 1054 cm^{-1}), and some unsaturated/aromatic compounds (stretching of C=C double bonds, aromatic rings, and aromatic rings conjugated with double bonds at 1653, 1610 and 1515 cm^{-1}, respectively). The broad and very strong band at 3350 cm^{-1}, assigned to the O-H stretching vibration, could be related to tissue hydration. When waxes were extracted in chloroform, a remarkable reduction of the aliphatic bands was observed (CH_2 stretching absorptions decreased and broadened; Fig. 4B). Finally, the saponification step led to the depolymerization of cutin and hence decreasing its characteristic absorption peaks. The remaining material can be considered a polysaccharide fraction with a small contribution of unsaturated/aromatic compounds (Fig. 4C).

When examining the isolated trichomes of young holm oak leaves by SEM, a generally smoother surface with some shallow cracks was noticed after wax removal as compared to the intact trichomes (Fig. 5, A to D). This phenomenon could be observed more clearly in the abaxial trichomes, since they initially had a rougher epicuticular wax layer as compared to adaxial ones (Fig. 5, B and D). After cutin depolymerization, trichomes appeared to be flatter, with twisted arms, and showing the cell wall structure (Fig. 5, E and F). Adaxial, intact (isolated and attached to the leaf surface) and chloroform-extracted trichomes seemed to be covered with only a partially-developed cuticle (Figs. 5, A and C and 6A).

The effect of immersing holm oak leaves in cellulase and pectinase solutions for 4 or 7 days is shown in Figure 6. The adaxial trichomes of young leaves were hydrolyzed when immersed in 2% cellulase (Fig. 6C). The process of trichome degradation seemed to take place from the fusing point of each arm at the side which is exposed to the

environment (Fig. 6B). After seven days of cellulase hydrolysis, almost only the cuticle covering the base of the trichomes remained attached to the epidermis underneath (Fig. 6C), which finally led to trichome shedding. The opening of the cuticle fusing line (observed as a central furrow in the arms) located near the base in some arms of intact trichomes (Fig. 6A) may somehow facilitate cellulase accessibility and cell wall degradation. These cuticular fractures in the adaxial trichomes were more common in the fusing point and towards the tip of the arms (see Fig. 6A as an example). Leaf immersion in cellulase did not alter the structure of abaxial leaf trichomes which were similar to those present in intact holm oak surfaces (Fig. 6D). Leaf pectinase digestion did not affect the structure of either the adaxial or the abaxial trichomes (data not shown).

Contact angle measurements

The mean θ values measured after depositing drops of three liquids with different polar and apolar surface tension components on to the adaxial and abaxial surfaces of young and mature holm oak leaves are summarized in Table I. The presence of trichomes in young leaf adaxial surfaces led to initially high θ values for water and especially glycerol, which sharply decreased over time before reaching a steady value when the drop became static. This phenomenon was however not observed for the fully apolar diiodomethane drops which remained static when deposited on to the adaxial young leaf surfaces. This indicates that water and glycerol drops (having a significant polar (acid-base) surface tension component) interacted with the trichomes present in the adaxial surface of young leaves which led to a sharp θ decrease shortly after drop deposition (Fig. 2A; Table I). On the contrary, the glabrous upper leaf surface of mature leaves and the highly pubescent abaxial young and mature leaf surfaces enabled the measurement of static contact angles immediately after drop deposition which implies a lower degree of chemical interactions between such liquids and the plant surfaces analyzed (Fig. 2, B to D).

With regard to leaf age, higher water and glycerol θ values were measured on the upper side of mature versus young leaves, but similar results were recorded for all abaxial leaf surfaces. In contrast, diiodomethane θ measurements were similar for the adaxial surface of young and mature leaves and slightly higher for the abaxial side of young leaves.

The total surface free energy (γ) of the holm oak leaf surfaces evaluated was determined from contact angle measurements (Fernández et al., 2011, 2014) using the so-called Lifshitz-van der Waals, van Oss, Good, and Chaudhury method (van Oss et al., 1987, 1988). The γ of young leaf adaxial surfaces was higher than that of mature surfaces (Table II). In contrast, the abaxial surface of mature leaves had a higher γ value as compared to the upper side. On the other hand, the total γ results of young and mature adaxial leaf surfaces were higher than the values determined for the corresponding abaxial surfaces. The γ values of the upper surfaces can be mainly ascribed to their relatively higher dispersive component (γ^{LW}). For the adaxial surface, the non-dispersive component (γ^{AB}) was higher for young leaves and decreased with maturity (Table II).

The highest degree of surface polarity was generally determined for the abaxial leaf side. For the upper side, the surface polarity values of young leaves were however higher than those determined for mature leaves (Table II).

The work-of-adhesion (W_a; Kwok and Neumann, 1999) for the three liquids and the solubility parameter (δ) were calculated as described by Fernández et al. (2011, 2014) and Khayet and Fernández (2012) (Table III). The adaxial surface of young and mature holm oak leaves exhibited a higher adhesion for water drops, followed by glycerol and diiodomethane, compared to the water drop repellence of the lower leaf side. Of the three liquids, the highest W_a values were recorded for water drops deposited on to the upper leaf side. Regardless of leaf age, the abaxial surfaces were found to have similar W_a values for water and diiodomethane, which were higher than those for glycerol. The solubility parameter (δ) values of the adaxial surface of young and mature leaves were considerably higher than those of the lower leaf side. Regarding the effect of leaf age, younger leaves had higher δ values as compared to mature leaves.

DISCUSSION

In this study, we analyzed the physico-chemical properties of the adaxial and abaxial surface of holm oak (*Q. ilex L.*) leaves as model for a typically Mediterranean species, and also in relation to leaf maturity. We selected this species since it constitutes an interesting system for assessing the nature and functionality of adaxial and abaxial leaf surfaces, and also for evaluating leaf water absorption and solid-liquid interactions. As a preliminary trial, we assessed the water absorption capacity of holm oak leaves by measuring the leaf water potential 24 h after depositing water drops either on to the adaxial or abaxial leaf side. After gaining evidence for leaf hydration via the upper leaf

side (independently of leaf age), we analyzed in detail the physico-chemical and structural properties of abaxial and adaxial holm oak leaf surfaces, focussing on trichomes. The structure and morphology of the adaxial and abaxial trichomes were similar to those described in previous holm oak leaf studies (e.g., Karabourniotis et al., 1998; Karioti et al., 2011; Camarero et al., 2012).

Trichome chemical composition and structure

The presence of trichomes in developing organs has been reported in previous studies (e.g., Karabourniotis et al., 1995). The adaxial and abaxial holm oak leaf side performed differently in terms of wettability (Table I), as previously found for other plant species (e.g., Brewer and Smith, 1997; Fernández et al., 2014). We showed that the hydrophobic character of the abaxial leaf side is clearly associated with the roughness provided by the high density, chemical composition and structure of trichomes. According to the classification suggested by Brewer et al. (1991) and Brewer and Smith (1997) the abaxial oak leaf trichomes may belong to the "lifting strategy" group, while further trials will required for characterizing the performance of liquid water with the adaxial oak leaf side at a macroscopic level.

Leaf surface hydrophobicity has been often interpreted as either an adaptive trait to dry climates (Holder, 2007a) or to wet environments (Holder, 2007b). The occurrence of trichome layers on the abaxial leaf surface may be considered as a xeromorphic character which provides adaptive advantages mainly under biotic and abiotic stress conditions (Karabourniotis et al., 1992; Karabourniotis and Bornman, 1999; Liakopoulos et al., 2006). In spite of this, the relationship between leaf surface hydrophobicity and the ecological conditions where a plant species is native to remains controversial.

By measuring the contact angles of three liquids with different polarity, we gained evidence for the wettable character of holm oak adaxial leaf surfaces. The increased wettability and work-of-adhesion for water of the adaxial young leaf surfaces is associated with the occurrence of hydrophilic trichomes, which interacted with polar liquids (i.e., water and glycerol) immediately after drop deposition. The degradation of adaxial trichomes after leaf immersion in cellulase showed that they were permeable to water in contrast to the abaxial ones which were not altered by the enzymatic treatment. The cuticular irregularities observed in the surface and base of the adaxial trichomes are

likely to be the main uptake pathway for the cellulase solution, which finally led to trichome degradation.

The gradual extraction of cuticular components of the abaxial leaf surface trichomes coupled to SEM observations, facilitated the quantification of cuticular constituents which amounted to 8% soluble waxes, 49% depolymerized material (likely cutin) and 43% residue (chiefly polysaccharides). The adaxial holm oak leaf trichomes were largely found to have a higher proportion of cutin and a lower content of polysaccharides as compared to the peach fruit trichomes analyzed by Fernández et al. (2011; having 15% waxes, 19% cutin and 66% polysaccharides). Scanning and transmission electron microscopy observations also in relation to the chemical removal of cuticular components showed that both the adaxial and abaxial trichomes are covered with a cuticle, which seemed to be homogeneously distributed along the surface of the more long-lasting, abaxial trichomes but appeared to develop heterogeneously over the cell wall of the adaxial trichomes of young holm oak leaves. Apart from being hydrophilic, such trichomes fractured more easily. Moreover, trichome shedding left a scar on the adaxial leaf surface, and the base of the abscised trichomes may represent a site for water entry since the cuticle in this area was observed to be thinner and more heterogeneous (Fig. 3, F and G). In addition, adaxial trichomes were often located in the vicinity of bundle sheath extensions (Fig. 3, F and G). Bundle sheath extensions may facilitate the transport of water in such heterobaric leaves (Wylie, 1943), and may be related to water economy (Nikolopoulos et al., 2002).

Leaf surface wettability, polarity, solubility parameter and work-of-adhesion

Measurement of water contact angles provides information about the wettability and retention of water drops by different leaf surfaces, and may serve as tool to classify species growing in contrasting environmental conditions (e.g., Brewer et al., 1991; Brewer and Nuñez, 2007; Holder, 2007a, b). Additional determinations of contact angles of liquids with different polarity (Fernández et al., 2011; Khayet and Fernández, 2012), facilitate the quantitative characterisation of plant surfaces and their solid-liquid interactions, in relation to the combined effect of surface chemistry and roughness.

The most abundant epicuticular waxes found on *Q. ilex* young leaves are *n*-alkyl esters, representing up to 56% of the total chloroform soluble waxes, followed by *n*-primary alcohols (Martins et al., 1999). While an increased surface roughness due to pubescence (Fernández et al., 2011) seems again to have a major effect on leaf surface

wettability, the contribution of surface chemistry should also be considered, and is the major factor affecting water-solid interactions in the adaxial surface of mature leaves (rather flat and glabrous). The predominant role of surface chemistry in relation to the adaxial, mature leaf side was supported by the solubility parameter value obtained experimentally (approximately 16 $MJ^{1/2}$ $m^{-3/2}$), which is within the range calculated by Khayet and Fernández (2012) for model epicuticular wax molecular structures (between 16 to 17 $MJ^{1/2}$ $m^{-3/2}$). In contrast, the dense indumentum of the lower leaf sides led to extremely low solubility parameter values, which were even below those determined for the almost super-hydrophobic juvenile *Eucalyptus globulus* leaf surface (Khayet and Fernández, 2012). This may be due to the high level of surface roughness and to the occurrence of air pockets within the trichome layers covering the abaxial leaf side (Fernández et al., 2011), which may be even denser when leaves are young.

Irrespective of leaf age, the upper leaf side had a higher surface free energy than the lower one, while polarity followed an inverse trend. The work-of-adhesion for water provides a quantification of the degree of water drop adhesion or repellence to a certain plant surface (Fernández et al., 2014). The work-of-adhesion for water was extremely high for the adaxial holm oak leaf surface as compared to the values previously obtained for other pubescent or glabrous plant materials (Khayet and Fernández, 2012; Fernández et al., 2014). The presence of trichomes in the adaxial side of the young holm oak leaves additionally increased the adhesion of water drops to such surfaces. However, the lower leaf sides had a degree of water drop repulsion similar to the one of the peach fruit (Fernández et al., 2011), but their work-of-adhesion for water was above the values estimated for the even more water-repellent and unwettable, hairy, adaxial wheat leaf surface (Fernández et al., 2014).

Leaf water uptake and ecophysiological implications

The ability to capture water via the leaf has been indirectly reported in species occurring in deserts (Martin and von Willert, 2000), tropical climates (Yates and Hutley, 1995), cloud-immersed mountain habitats (Berry and Smith, 2012; Berry et al., 2013, 2014) and in coastal mountain regions where fog is a significant climatic contributor (Burgess and Dawson, 2004). However, this is the first time when foliar water uptake has been analysed in strict physico-chemical terms, while recording significant water potential changes in response to leaf hydration.

Regardless of leaf age, we observed a markedly different performance of the adaxial (wettable and retaining water drops) versus the abaxial (unwettable and water-repellent) holm oak leaf surfaces when in contact with water drops. We have demonstrated that the major wettability of the adaxial side of holm oak leaves allows for leaf rehydration both in terms of RWC and leaf water potential (Fig. 1). Drop adherence to the plant surface is a pre-requisite for foliar uptake to occur (Fernández and Brown, 2013; Fernández et al., 2014). Thereby, in the case of holm oak leaves, foliar absorption of pure water (i.e., in the absence of surfactants) may only take place through the upper leaf side to which water drops strongly adhere (having a high work-of-adhesion for water; Table III). In contrast, the repulsion of water drops by the abaxial leaf side will impede the penetration of liquid water. The hydrophobic nature of the abaxial leaf trichomes will ensure the occurrence of an air layer above stomatal pores, and hence help to preserve an adequate gas exchange rate even under wet conditions.

The permeability of plant surfaces to water and solutes has been a matter of scientific interest since the last century, but the mechanisms involved are still not fully characterised (Fernández and Eichert, 2009). Water deposited on to a leaf surface may penetrate via stomata, the cuticle, cuticular cracks and irregularities, or through specialised epidermal cells such as trichomes (Fernández and Brown, 2013). In the case of holm oak adaxial leaf surfaces, trichomes and the remaining scars after trichome shedding may play a key role in water absorption. The high surface tension, polarity and H-bonding capacity of water theoretically pose restrictions for the penetration of this liquid through the cuticle (Guzmán et al., 2014 a,b) and also via stomata (Schönherr and Bukovac, 1972; Burkhardt et al., 2012). The actual contribution of leaf trichomes to the absorption of water and solutes remains unclear (Fernández et al., 2014). While some studies suggest that trichomes may actively participate in the uptake of foliar-applied nutrient solutions (e.g., Benzing et al., 1976; Schlegel and Schönherr, 2002), and water in Bromeliads (e.g., Pierce et al., 2001; Reyes-García et al., 2011), the contribution of *Phlomis fruticosa* leaf trichomes to the absorption of water could not be clarified (Grammatikopoulos and Manetas, 1994). A traditional problem when investigating on the mechanisms of foliar penetration is the occurrence of technical constraints associated with optical and fluorescence microscopy and attempting to observe the uptake of specific dyes and solutes by leaf surface micro- and nano-structures (Fernández and Eichert, 2009). Fahn (1986) and more recently Burrows et al. (2013) reported that leaf trichomes having a cutinised base would fail to take up the surface-

applied dye solutions and to transport them into the leaf interior. While the base of the adaxial and abaxial holm oak leaf trichomes that we analysed was cutinised, when applying water drops on to the adaxial surface of young *Q. ilex* leaves we gained evidence for hydration as derived from the resulting leaf water potential increase . Our results suggest that there may be a major degree of variability among trichome structures and functions that may sometimes impede or facilitate the uptake of water.

Foliar water uptake due to natural phenomena such as fog or dew may be an important mechanism of hydration in some areas of the world subjected to temporary drought (e.g., Burgess and Dawson, 2004; Oliveira et al., 2005; Breshears et al., 2008; Limm et al., 2009; Simonin et al., 2009; Limm and Dawson, 2010; Berry et al., 2013, 2014; Gotsch et al., 2014).

Holm oak is a species native to Mediterranean-type climates where summer drought is imposed by a combination of high temperatures and low precipitation. In spite of the lower precipitation level during this season, summer rainfall constitutes 24% of the whole year precipitation (referring to the Iberian Peninsula; www.aemet.es). In this season, short-term storms are the most common precipitation form, being up to 100% of the fallen rain during July and August (Mosmann et al., 2004). While most of the water falling after high precipitation summer storms is not available for plant roots due to storm water run-off, low precipitation storms (below 1 mm) are considered negligible in terms of soil water balance (Allen et al., 2000), the latter accounting for 54% of the total summer rainfall (www.aemet.es). Therefore, direct water uptake by the foliage may positively contribute to water economy of holm oak during the summer. Since the beginning of a storm (high or low precipitation), the environmental conditions (air temperature and humidity, and vapour pressure deficit (VPD) sharply change as shown in Figure 7 (data gathered with a Hobo Pro temp/RH (Onset Computer Bourne, MA, USA) and a Rain Collector II (Davis Instruments, CA, USA)). The extreme VPD reduction (close to 0 kPa, as simulated in our rehydration trials) provides optimal conditions for foliar water absorption, since the low VPD retards the evaporation of water drops hence increasing the chance for water uptake by the foliage.

An additional important source for leaf water absorption for evergreen holm oak is the formation of dew due to condensation, which may occur all year round but may be especially relevant during the summer. This mechanism occurs on approximately 20% of summer days in the holm oak growing areas of the Iberian Peninsula (www.aemet.es). Of the surface condensation mechanisms described (Nourbakhsh,

1989) two can be expected to occur under field conditions: (i) film condensation on the wettable adaxial leaf side, and (ii) dropwise condensation on the unwettable abaxial surface. While film condensation may lead to direct foliar water uptake, dropwise condensation will favour drop dripping either to the soil or to the foliage below, which may again turn available for foliar absorption. Finally, a further water deposition phenomenon of limited relevance under Mediterranean conditions is the occurrence of fog, in which the expected leaf wetting and water absorption mechanisms will be similar to those described above for dew deposition.

It could be reckoned that adaxial leaf trichomes and scars may also contribute to water loss and leaf dehydration. However, the mechanisms of uptake of liquid water and loss probably as water vapour (Rockwell et al., 2014), and processes of water adsorption and desorption in the cuticle (Reina et al., 2001) may be different and will require further investigation. The functional advantage of water absorption by the upper leaf side is not linked to a higher cuticular conductance, since no statistical differences between adaxial and abaxial cuticular conductances were found.

In summary, in this study we analysed the water-leaf surface interactions of the adaxial and abaxial leaf side of holm oak, a typical evergreen Mediterranean species. While the adaxial trichomes present in young leaves were hydrophilic, the dense indumentum of the lower leaf side led to a high degree of hydrophobicity. The upper holm oak leaf side of young and mature leaves were wettable and absorbed water, and the uptake mechanisms have been related to the presence of trichomes and trichomes scars. It is concluded that trichome structure, chemical composition and function may vary even for the same organ, but in relation to their occurrence in the abaxial versus the adaxial leaf side. The water absorption capacity of holm oak leaves may be ecologically advantageous for competition under Mediterranean conditions and in relation to different means of water precipitation (e.g., condensation, rain or fog) all year round, especially during summer and when growing in soils with low water storage capacity.

519 ## MATERIALS AND METHODS

520 ### Plant material

521 *Quercus ilex* L. seeds were planted in 500 ml containers filled with a mixture of 80%
522 substratum and 20% perlite and were kept in a greenhouse. Seedlings were transplanted
523 to 25 L containers after the first growing season, and they were subsequently
524 transplanted to an experimental field plot (CITA, Zaragoza, Spain). Plants always grew
525 under typically Mediterranean environmental conditions and were irrigated when
526 necessary, avoiding also the incidence of pests and diseases. Ten-year-old trees were
527 finally selected for the development of this study. Shoots with undamaged, fully
528 developed, 2-month old leaves with pubescent adaxial and abaxial sides (hereinafter
529 referred to as young leaves) were collected at early summer, whereas shoots having 8-
530 month old leaves (hereinafter referred to as mature leaves) with a glabrous adaxial and a
531 pubescent abaxial surface were collected at late autumn.

532 ### Leaf surface water absorption

533 By mid-June, two holm oak twigs per tree from five different trees of *Q. ilex* were
534 cut and enclosed in plastic bags until measurement. The initial water potential (Ψ_o) and
535 initial fresh weight (FW_o) were measured in three young shoots of each of the ten twigs
536 selected. Then, 2 mL of distilled water were carefully deposited on to: (i) the adaxial
537 side of the leaves of one of the shoots, and (ii) the abaxial side of the leaves of another
538 shoot. For comparison, the third shoot was used as an untreated control shoot.
539 Thereafter, young shoots were enclosed in single plastic bags and stored in the dark at
540 room temperature (at approximately 20º C). After 24 h, the weight and water potential
541 were measured again in all shoots to obtain the final water potential (Ψ_f) and final fresh
542 weight (FW_f). Then, shoots were dried in an oven (65ºC, 24h) and they were weighed to
543 obtain the dry weight (DW). With the remaining young shoots of each twig not used in
544 this process, we obtained a relationship between shoot full saturation weight (turgid
545 weight, TW) and dry weight (DW). Afterwards, weight measurements were used to
546 calculate the relative water content (RWC) for each shoot as RWC = (FW-DW)/(TW-
547 DW). A t-test paired sample comparison was used to compare leaf water potential and
548 relative water content before (Ψ_o, RWC_o) and after (Ψ_f, RWC_f) surface wetting. This
549 leaf surface water absorption process was repeated again at late autumn on mature
550 leaves.

551 **Cuticular conductance**

552 Cuticular water losses were measured gravimetrically on fully rehydrated young and
553 mature leaves (Anfodillo et al., 2002). To differentially assess the transpiration loss of
554 adaxial and abaxial surfaces, silicon grease was applied on to the opposite surface to
555 seal it against water loss. After initially weighing the leaves, they were placed for 72 h
556 in a dark chamber with an electric fan ensuring air circulation, under constant humidity
557 and temperature conditions (22.4 ± 0.1 ºC and 76.4 ± 0.1 %). Cuticular conductance
558 (ms^{-1}) was calculated from the transpiration data and according to Anfodillo et al.
559 (2002).

560 **Quantitative and qualitative estimation of chemical components of holm oak**
561 **trichomes**

562 Adaxial and abaxial trichomes of young holm oak leaves were mechanically isolated
563 by gently scraping the leaf surfaces with a scalpel. Trichomes were subjected to the
564 successive removal of soluble cuticular lipids and cutin, while performing simultaneous
565 Fourier transformed infrared spectra (FTIR) analyses using a Nexus 670-870 NICOLET
566 FTIR spectrometer (Thermo Fisher Scientific, Waltham, USA; transmission mode, 3850
567 to 850 cm^{-1} with 4 cm^{-1} resolution, and accumulating 64 scans). For FTIR, samples were
568 ground with 1% potassium bromide, and thin tablets were subsequently formed and set
569 into the apparatus. A blank corresponding to the spectrum of potassium bromide was
570 also recorded and substracted from sample spectra. For chemical removal, hairs were
571 first immersed in chloroform for 4 h, and then cutin was depolymerized by
572 saponification in 1M sodium hydroxide (KOH) for 24 h under reflux conditions.
573 Percentages of each chemical fraction (soluble waxes, cutin and remaining residue)
574 were calculated according to the corresponding weight loss.

575 Additionally, young holm oak leaves were enzymatically digested for four and seven
576 days in either 2% cellulase or 2% pectinase (both from Novozymes, Bagsvared,
577 Denmark) plus 2 mM sodium azide, adjusting the pH to 5.0 by adding sodium citrate.

578 **Microscopy**

579 Gold-sputtered intact and enzymatically digested adaxial and abaxial holm oak leaf
580 surfaces were examined with a variable pressure scanning electron microscope (SEM;
581 Hitachi S-3400 N, Tokyo, Japan; acceleration potential, 20 kV; working distance, 15-17

mm). Changes in adaxial and abaxial isolated trichome structure were analysed in intact hairs, and after soluble lipid extraction and alkaline hydrolysis.

For transmission electron microscopy (TEM) Samples were fixed in 2.5% glutaraldehyde-4% paraformaldehyde (both from Electron Microscopy Sciences (EMS), Hatfield, USA) for 6 h at 4ºC, rinsed in ice-cold phosphate buffer, pH 7.2, four times within a period of 6 h and left overnight. Tissues were then post-fixed in a 1:1 2% aqueous osmium tetroxide (TAAB Laboratories, Berkshire, UK) and 3% aqueous potassium ferrocyanide (Sigma-Aldrich) solution for 1.5 h. Samples were then washed with distilled water (x3), dehydrated in a graded series of 30, 50, 70, 80, 90, 95 and 100% acetone (x2, 15 min each concentration) and embedded in acetone-Spurr's resin (TAAB Laboratories) solutions (3:1, 2h; 1:1; 2h; 1:3; 3h (v:v)) and in pure resin overnight at room temperature. Final embedding was done in blocks which were incubated at 70°C for 3 days for complete polymerization. Prior to TEM observation, sections were post-stained with Reynolds lead citrate (EMS) for 5 min.

Thin, cryo-sectioned leaf tissues (30 µ thick) were observed with a Olympus BX40 fluorescence microscope. Transversal sections were examined by either visible light transmission or under UV excitation (emission of blue fluorescence by simple phenols and lignin) after immersion in 10% (w:v) KOH for 2 min, followed by a thorough distilled water rinse. A U-MWU filter combination (exciter filter 330-385 nm, barrier filter 420 nm) was used. Leaf sections were also stained with Sudan IV. Microphotographs were taken using a Olympus DP71 digital camera (Olympus Corporation, Tokyo, Japan).

The average number of trichome arms, arm length, arm diameter, trichome densities, and leaf area covered with hairs were assessed by image analysis of adaxial and abaxial SEM micrographs (ImageJ 1.45s, W.R., National Institutes of Health, Bethesda, Maryland, USA).

Contact angle determinations and leaf surface properties

Advancing contact angles (θ) of drops of double-distilled water, glycerol and diiodomethane (both 99% purity; Sigma-Aldrich) were measured at room temperature (25ºC) using a CAM 200 contact angle meter (KSV Instruments, Helsinki, Finland) equipped with a CCD camera, frame grabber and image analysis software. Contact angles were determined on adaxial and abaxial surfaces of intact, young and mature leaves. After removing the midrib and margins, leaf sections of approximately 2 x 0.5

615 cm^2 were cut with a scalpel and mounted on a microscope slide with double-sided
616 adhesive tape. Two μL drops of each liquid were deposited onto the adaxial or abaxial
617 holm leaf surfaces with a manual dosing system holding a 1 mL syringe with 0.5 mm
618 diameter needle (30 repetitions). Side view images of the drops were captured at a rate
619 of 6 frames s^{-1}. Contact angles were automatically calculated by fitting the captured
620 drop shape to the one calculated from the Young-Laplace equation. For mature leaves
621 and all abaxial leaf surfaces, θ for all the liquids were measured shortly after drop
622 deposition. On young adaxial surfaces, however, the θ of water and chiefly glycerol
623 were initially high and decreased over time. Hence, θ values were recorded immediately
624 after drop deposition and also when they were static (taking approximately 15 sec for
625 water and 10 min for glycerol).

626 For all the surfaces evaluated, the total surface free energy or surface tension (γ) and
627 its components, i.e. the Lifshitz-van der Waals (γ^{LW}) and acid-base (γ^{AB}; γ^+ and γ^-)
628 components, as well as the work-of-adhesion for the three liquids and the solubility
629 parameter were calculated, considering the surface tension components of water (γ^{LW} =
630 21.8 mJ m^{-2}, $\gamma^+ = \gamma^-$ = 25.5 mJ m^{-2}), glycerol (γ^{LW} = 34.0 mJ m^{-2}, γ^+ = 3.92 mJ m^{-2}, γ^- =
631 57.4 mJ m^{-2}) and diiodomethane (γ^{LW} = 50.8 mJ m^{-2}, $\gamma^+ = \gamma^{--}$ = 0 mJ m^{-2}) (Fernández et
632 al., 2011; Khayet and Fernández, 2012).

633

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TABLES

Table I. *Contact angles of water (θ_w), glycerol (θ_g) and diiodomethane (θ_d) with the adaxial and abaxial surface of young and mature holm oak leaves*

Leaf age	θ_w (º)		θ_g (º)		θ_d (º)	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
Young	40.7±3.8	130.1±6.5	50.2±5.1	141.4±4.4	56.6±4.9	128.4±6.5
	91.1±6.0[i]		143.5±6.0[i]			
Mature	55.5±5.1	134.3±10.1	68.6±5.9	142.5±3.9	59.1±4.1	119.3±10.2

[i]Initial θ of H_2O and glycerol when deposited on to the adaxial young leaf surfaces. The remaining θ values were recorded when the drops were stable and did not vary when deposited on to the surfaces.

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Table II. *Surface free energy per unit of area. Lifshitz van der Waals component (γ^{LW}), Acid-base component (γ^{AB}), total surface free energy (γ) and surface polarity ($\gamma^{AB}\ \gamma^{-1}$) of the adaxial and abaxial surface of young and mature holm oak leaves*

Leaf side	γ^{LW} (mJ m^{-2})		γ^{AB} (mJ m^{-2})		γ (mJ m^{-2})		$\gamma^{AB}\gamma^{-1}$ (%)	
	Young	Mature	Young	Mature	Young	Mature	Young	Mature
Adaxial	30.55	29.11	12.50	0.55	43.05	29.66	29.04	1.85
Abaxial	1.82	3.31	2.42	2.45	4.24	5.76	57.02	42.53

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Table III. *Solubility parameter (δ) and work-of-adhesion for water ($W_{a,w}$), glycerol ($W_{a,g}$) and diiodomethane ($W_{a,d}$) and solubility parameter (δ) of intact, adaxial and abaxial surfaces of young and mature holm oak leaves*

Leaf age	$W_{a,w}$ (mJ m^{-2})		$W_{a,g}$ (mJ m^{-2})		$W_{a,d}$ (mJ m^{-2})		δ ($MJ^{1/2}$ $m^{-3/2}$)	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
Young	127.99	25.93	104.98	14.00	78.79	19.25	20.85	3.67
Mature	114.04	21.99	87.40	13.25	76.91	25.94	15.77	4.62

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FIGURES LEGENDS

Figure 1. **Leaf water potential (ψ) and relative water content (RWC) for young (A, C) and mature (B, D) shoots of *Q. ilex* before (grey bars) and after (black bars) surface wetting**. AdW = leaf adaxial wetting; AbW = leaf abaxial wetting; NW = no wetting. Significant difference before and after surface wetting were found in all cases.

Figure 2. **SEM micrographs of intact adaxial and abaxial holm oak leaf surfaces.** (A, B) Adaxial and abaxial sides of young leaves, (C, D) adaxial and abaxial sides of mature leaves.

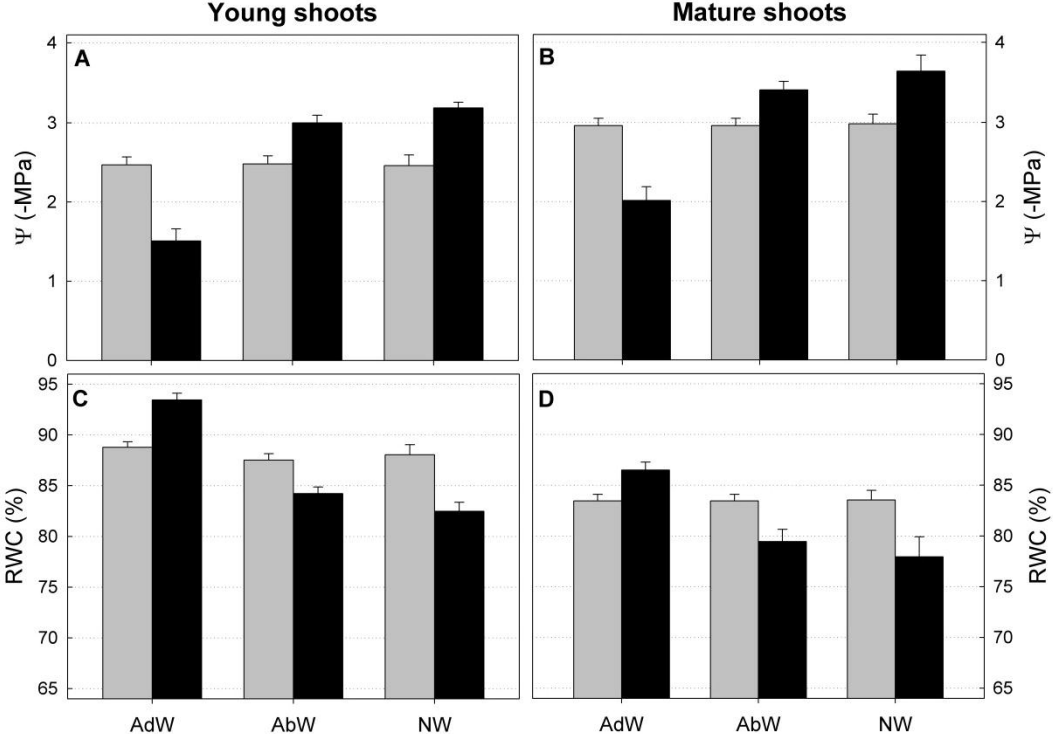
Figure 3. **Optical and transmission electron microscopy micrographs of intact adaxial and abaxial surfaces of holm oak young (A to F) and mature (G) leaves**. (A) transversal section of a young leaf stained with Sudan IV, (B) adaxial leaf trichome, (C) abaxial leaf trichome, (D) base of an adaxial leaf trichome observed by TEM, (E) base of an abaxial leaf trichome observed by TEM, (F) detail of scar on a young, adaxial leaf surface after trichome shedding, and (G) detail of two scars on a mature, adaxial leaf surface after trichome shedding. Note that the bases are near the bundle sheath extension (dark blue).

Figure 4. FTIR spectra of isolated (A), dewaxed (B), and cutin depolymerized (C) abaxial holm oak leaf trichomes.

Figure 5. **Isolated trichomes of adaxial and abaxial holm oak leaf surfaces after gradual extraction of chemical components**. (A, B) intact trichomes, (C, D) trichomes after chloroform extraction, and (E, F) trichomes after cutin depolymerization.

Figure 6. Effect of cellulose hydrolysis on holm oak adaxial (A, B, C) and abaxial (D) leaf surfaces. (A) intact, adaxial leaf surface, (B) adaxial leaf surface after 4 days in cellulase solution, (C) adaxial leaf surface after 7 days in cellulase solution, and (D) abaxial leaf surface after 7 days in cellulase solution.

910 **Figure 7. Hourly evolution of Temperature (T), Relative Humidity (RH) and**
911 **Vapor Pressure Deficit (VPD) during five consecutive summer days in a typically**
912 **Mediterranean holm oak area**. The gray area indicates a precipitation event lower
913 than 1 mm.



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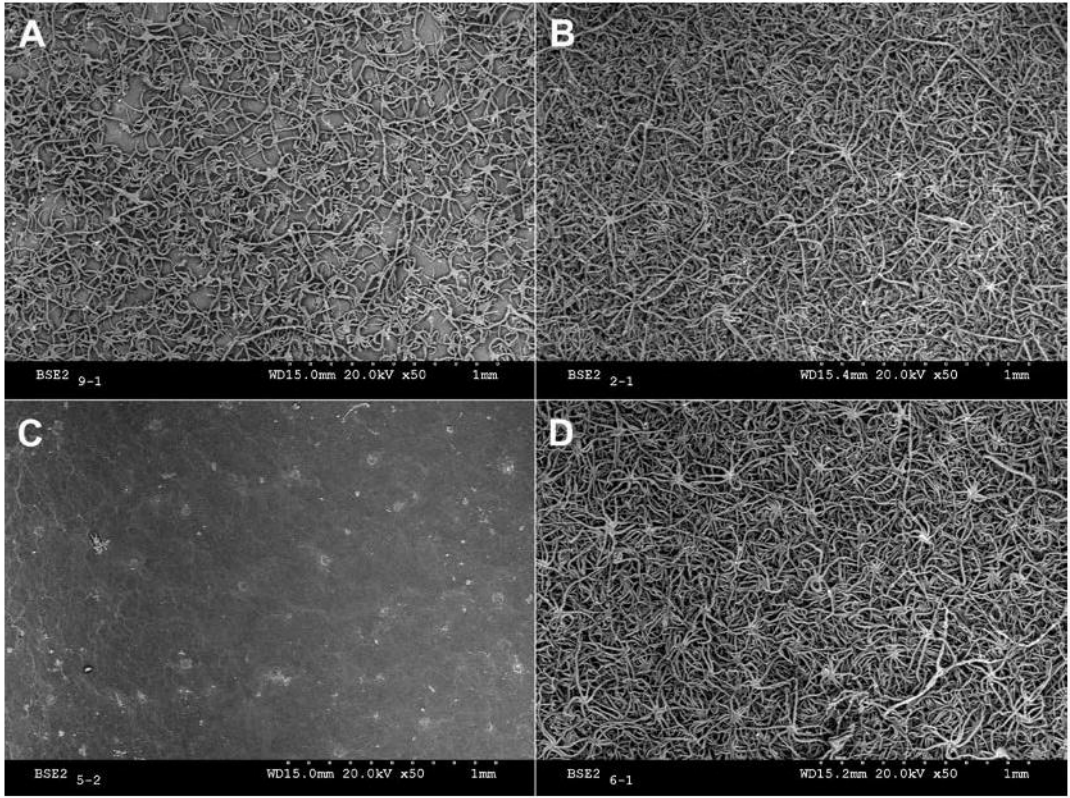

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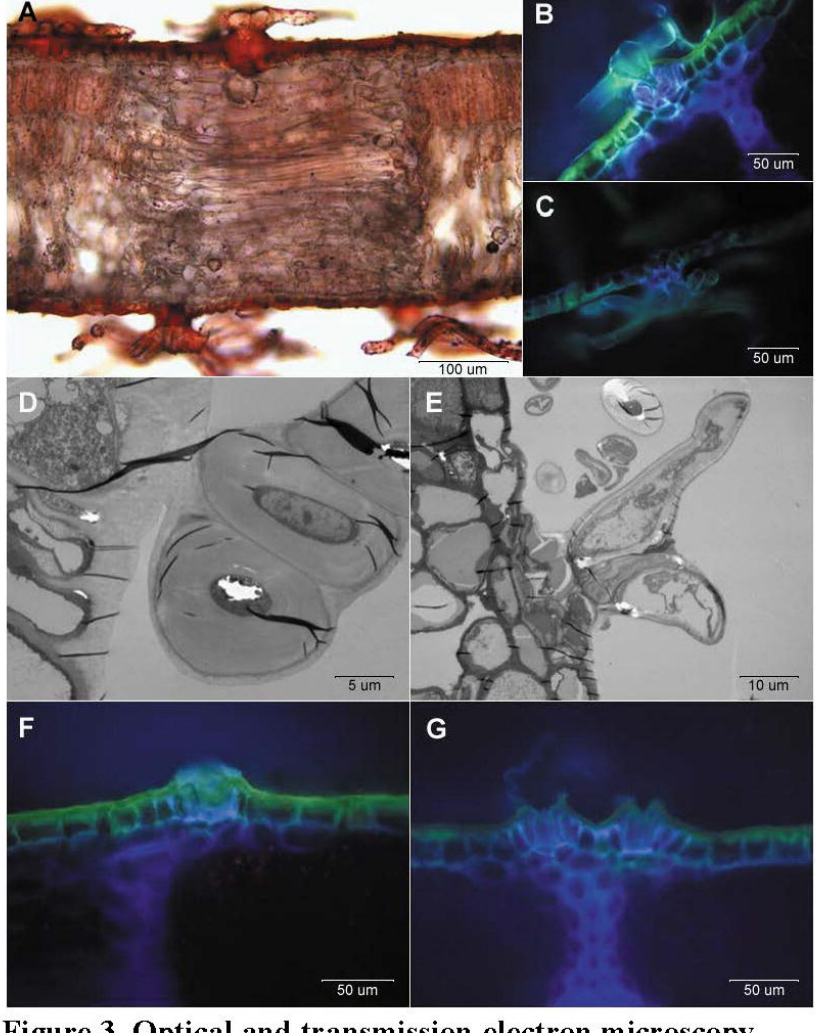

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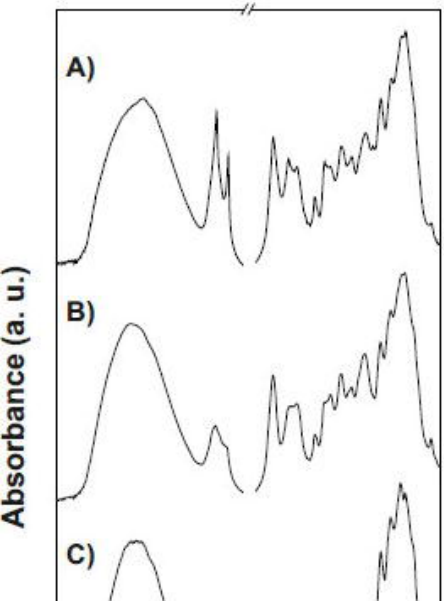

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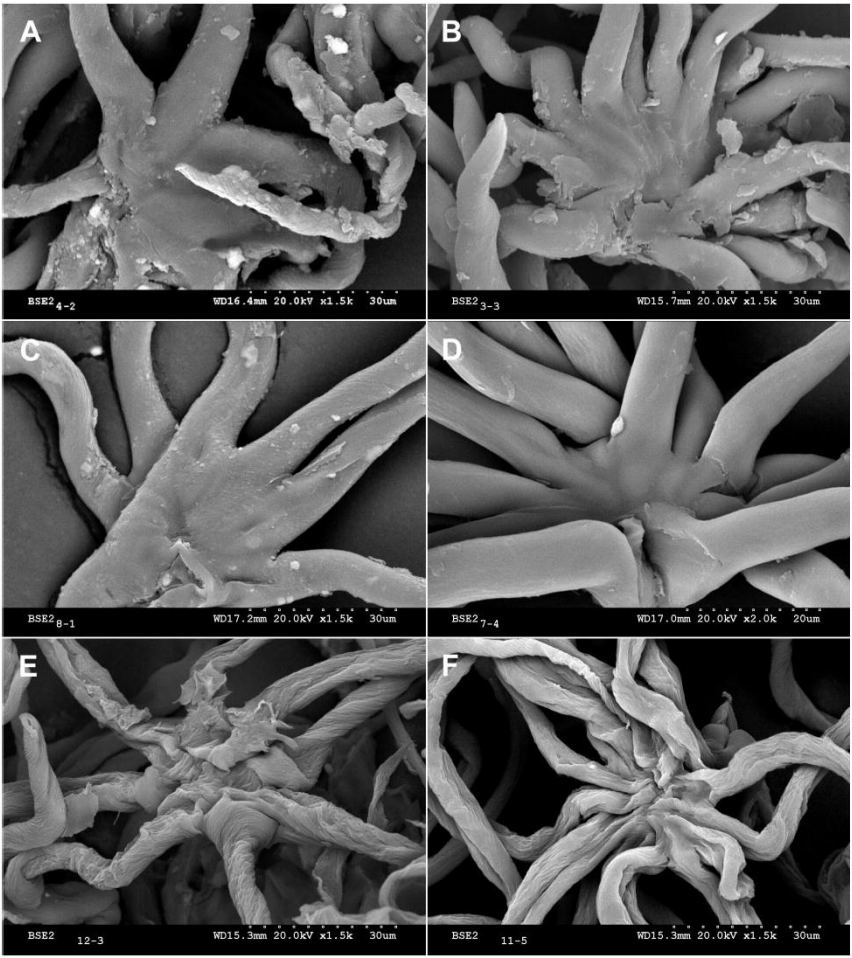

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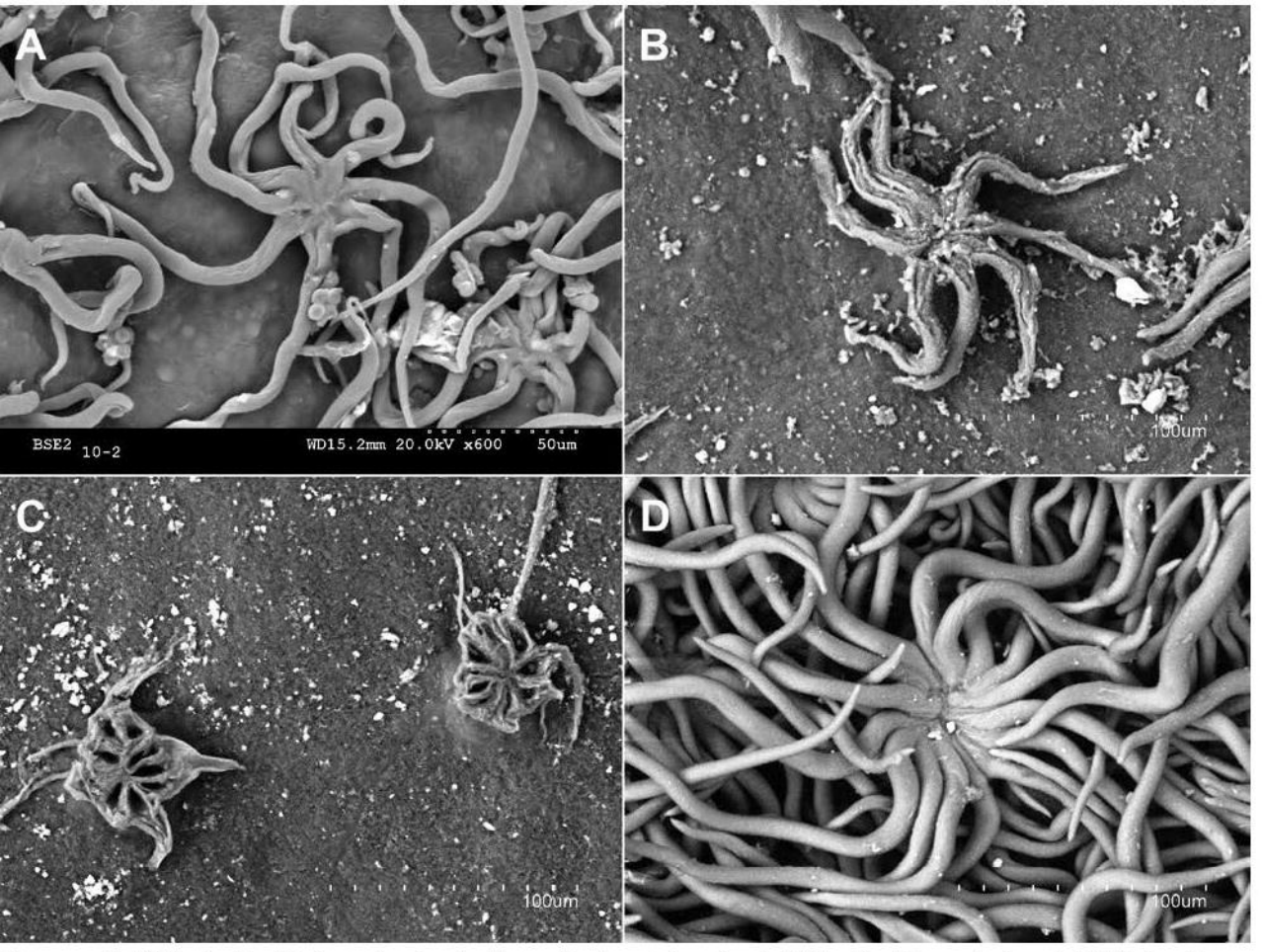

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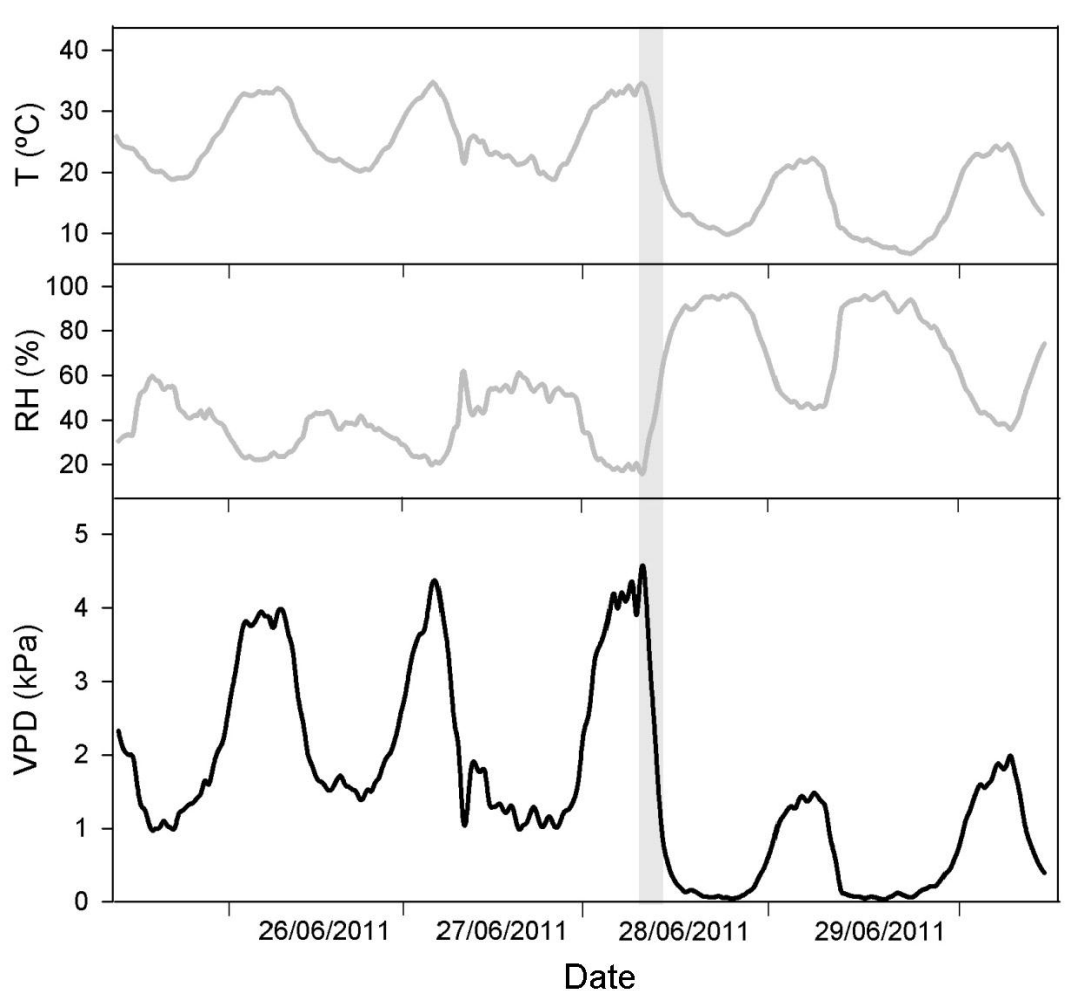

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