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## Leaf surface wettability and fatty acid composition of *Arbutus unedo* and *Arbutus andrachne* grown under ambient conditions in a natural macchia

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**Abstract:** Features of the adaxial and abaxial surface microstructure of *Arbutus unedo* L. and *Arbutus andrachne* L. are evaluated as possible consistent parameters contributing to the wetness of leaves. The abaxial leaf surface of *A. andrachne* and *A. unedo* was determined to be more hydrophobic than the adaxial leaf surface. Hydrophobicity may be of particular importance for the ecophysiological status of the hypostomatic leaves of both *Arbutus* species, which exhibit a long lifespan and are exposed to various environmental stimuli. Water repellence may also be correlated to the increased presence of surface wax. Lipid analysis of the leaves of both plant species revealed an abundance of  $\alpha$ -linolenic acid, with palmitic acid as the second major contributor, followed by linoleic and oleic acid. Oleic and linoleic acid were present in slightly larger percentages in *A. andrachne* compared with *A. unedo*, whereas myristic, palmitic and  $\alpha$ -linolenic acids were found in elevated percentages in *A. unedo*. The fatty acid composition analysis of the leaf wax of *A. andrachne* and *A. unedo* ranged from C16 to C26, with fatty acids of an even longer chain length detected in the case of *A. andrachne*. Despite the similar fatty acid composition of total lipids, the composition of the wax fraction showed differences between the two *Arbutus* species, which may partly contribute to the foliar surface properties of the two species.

**Keywords:** adhesion; *Arbutus*; contact angle; fatty acids; leaf; Mediterranean; stomata; surface; wettability

### Introduction

Plant surfaces have evolved over millions of years, resulting in complex multifunctional interfaces that fulfil a multitude of functions in terrestrial plants, such as mechanical containment, limitation of water loss and protection against biotic and abiotic stresses (Neinhuis and Barthlott 1998; Müller and Riederer 2005; Holder 2007a; Koch, Bhushan, and Barthlott 2009; Nairn, Forster, and van Leeuwen 2011; Wang, Guo, and Liu 2014).

The objective of this work was to study and compare leaf surface morphology and wettability of *Arbutus unedo* L. and *Arbutus andrachne* L., as well as the fatty acid composition of leaf lipids, as part of a larger study of leaf wettability of Mediterranean plants (Kolyva et al. 2012; Fernández et al. 2014; Razeq et al. 2014). *Arbutus unedo* L. and *A. andrachne* L. belong to the Ericaceae family, and are relatively widespread in the Mediterranean region (Hileman, Vasey, and Parker 2001; Baslar, Dogan, and Mert 2002; Blondel et al. 2010). The strawberry tree, *A. unedo*, is a perennial, drought-tolerant evergreen, which is capable of regenerating after forest fires (Savé et al. 1993). The appearance of both fruits and flowers in late autumn and during the winter give the strawberry tree ornamental importance (Celikel, Demirsoy, and Demirsoy 2008; Oliveira et al. 2011); also, the edible fruits of *A. unedo* contain phenolic and antioxidant substances, and can be used to produce jams, jellies and alcoholic beverages (Ayaz, Kucukislamoglu,

and Reunanen 2000; Bouzid et al. 2014; Cavuşoğlu, Sulusoglu, and Erkal 2015). The oriental strawberry tree, *A. andrachne*, is a perennial evergreen that differs from *A. unedo* in lamina and panicle positions (Serçe et al. 2010); its fruits are smaller than those of *A. unedo*, but are of similar shape and colour. *Arbutus andrachne* is not cultivated, unlike *A. unedo*.

*Arbutus unedo* and *A. andrachne* may be a vicariant pair, geographically isolated from other *Arbutus* species (Blondel et al. 2010); *A. × andrachnoides* Link, also found in the Mediterranean region, is believed to be a hybrid between *A. unedo* and *A. andrachne* (Bertsouklis and Papafotiou 2013), whereas *A. canariensis* Veil is endemic to the Canary Islands (Hileman, Vasey, and Parker 2001).

A comparative study involving the two Mediterranean *Arbutus* species, *A. unedo* and *A. andrachne*, will hopefully aid in the understanding of their relationship and the possible causes of the speciation that led to their existence (Mesléard and Lepart 1989; Blondel et al. 1991; Ribeiro et al. 2014). Also, there is growing interest in botany, agricultural research and industry to obtain a more comprehensive understanding of the properties of foliar tissues expanded under ambient conditions (Stratakis et al. 2009; Kouvaris et al. 2012). To the best of our knowledge a study of the surface microsculpture and waxes of the two species has not hitherto been reported.

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## Materials and methods

### Plant material and study area

The collection of plant material was made from a stand of *Arbutus unedo* L. (strawberry tree) and *Arbutus andrachne* L. (oriental strawberry tree) trees with average heights of 2 m and 3 m, respectively. The trees had grown under ambient conditions in a 40-year-old natural macchia, near the campus of the University of Athens (37°57'42" N, 23°47'55" E; altitude 370 m above sea level). Investigations were made on fully expanded leaves, harvested in the morning in late autumn and early winter. The climate of the study area is Mediterranean; values of precipitation and air temperature were obtained from a meteorological enclosure about 2.5 km from the research site, over 10 years. During the study period the mean monthly air temperature and monthly rainfall were 15°C and 139 mm in November, and 9.5°C and 154 mm in December. Leaf longevity in *A. andrachne* ranges between 8 and 18 months, and in *A. unedo* between 10 and 18 months (Diamantoglou and Mitrakos 1981). Therefore, expanded leaves of the selected shrubs for each species were exposed to the natural variations of precipitation from September to May, and to the prolonged dry period from June to August. Leaf area was measured in 35 mature leaves collected from at least five individual plants, according to Rhizopoulou and Psaras (2003).

### Microscopy

The adaxial and abaxial surfaces of fully expanded leaves of *A. unedo* and *A. andrachne* were treated to be viewed with scanning electron microscopy. Selected leaves were washed with a 2% TWIN 20 solution overnight at 4°C, followed by immersion in sodium phosphate buffer at pH 7 at room temperature for 30 min. Small pieces of leaves were carefully cut and immersed in buffer for 30 min, then fixed in 3% glutaraldehyde in sodium phosphate buffer at pH 7, at room temperature for 2 h. The samples were then washed three times with buffer for 30 min each time, and post-fixed in 1% OsO<sub>4</sub> in the same buffer at 4°C. Dehydration of the material was accomplished with acetone solutions of increasing concentrations. Dehydrated samples were critical-point-dried in a Leica Microsystems CPD-030 dryer (Leica Microsystems, Balzers, Liechtenstein), mounted with double adhesive tape on stubs, and sputter coated with 20 nm gold in a Leica Microsystems SCP-050. Samples of the adaxial and abaxial leaf surfaces were then viewed using a JEOL JSM-6390LV scanning electron microscope (JEOL Ltd, Tokyo, Japan).

### Total lipid extraction and fatty acid methylation from whole leaves

Fully expanded leaves of *A. andrachne* and *A. unedo* plants were selected, dried at 60°C for 3 days and then ground up. Extraction of lipids was accomplished using

a chloroform–methanol (2 : 1, volume/volume) solution. Briefly, 25 ml of the chloroform–methanol solution was added to 300 mg of powdered leaf material and agitated for 1 h. The solution was then filtered into a separating funnel along with a further 20 ml of the chloroform–methanol solution and 10 ml 0.9% NaCl, carefully agitated and left for 20 h. The lower phase of lipids dissolved in chloroform was then transferred to a flask, heated gently and evaporated under a constant stream of nitrogen gas (Bligh and Dyer 1959). Fatty acid methyl esters were then produced by saponification of the lipids (Stoffel, Chu, and Ahrens 1959); 10 ml methanol and 0.5 ml 98% sulphuric acid were added to the lipids and the flask was placed in a water bath at 80°C, while connected to a condenser supplied with cool water for 2 h. After being transferred to a separating funnel, 10 ml petroleum ether and 10 ml distilled water were added. The upper phase of methyl ester-containing petroleum ether was collected, heated gently and evaporated under a constant stream of nitrogen gas. The methyl esters were then re-dissolved in hexane for gas chromatographic analysis.

### Leaf wettability

Static contact angle (CA) measurements were performed by an automated tensiometer, using the sessile drop method, on each side of the same expanded leaves (Boyce and Berlyn 1988; Nairn, Forster, and van Leeuwen 2011). A droplet (2 µl) of distilled and deionized water was gently positioned, using a microsyringe, on the adaxial and abaxial leaf surfaces mounted with double adhesive tape on stubs, and images were captured to measure the angle formed at the liquid–solid interface, i.e. the CA, according to Zorba et al. (2008). The ImageJ image processing and analysis software with the Brugnara plug-in was used for the measurements (Williams et al. 2010). The mean value was calculated from nine different randomly selected leaves from three different plants for each species.

### Wax extraction

Cuticular layer and epicuticular waxes of fully expanded leaves of *A. andrachne* and *A. unedo* were removed by dipping the tissues in chloroform for 15–20 min (Buschhaus, Herz, and Jetter 2007a; Kolyva et al. 2012), at room temperature while being gently agitated. Microscopic examination of leaf surfaces was used to confirm removal of the wax.

### Fatty acid composition of extracted leaf epicuticular wax

The chloroform solution containing the extracted waxes was evaporated by heating gently under a constant stream of nitrogen gas. The remaining waxes were re-dissolved

in 5 ml methanol and transmethylated with 5 ml 2M sodium solution in methanol, followed by 2 ml 37% HCl drop-wise, while gently agitating for 20 min. The contents were transferred into a separating funnel, and 30 ml distilled water and 10 ml petroleum ether were added. The upper phase of petroleum ether containing the methyl esters of the fatty acids was collected in a flask, and the petroleum ether was evaporated by heating gently under a constant stream of nitrogen gas. The flask was washed three times with 5 ml petroleum ether, and the

contents were transferred into a test tube that was then heated gently under a constant stream of nitrogen gas for the petroleum ether to evaporate. The test tube was then washed three times with 0.5 ml chloroform and the contents were transferred into a vial and evaporated as above. The methyl esters were re-dissolved in hexane for gas chromatographic analysis.

#### Gas chromatography

Aliquots of fatty acid methyl esters in hexane were analysed with a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector and a 30-m HP-INNOWAX glass capillary column coated with polyethylene glycol (0.5- $\mu\text{m}$  film thickness) with nitrogen as a carrier gas (1 ml  $\text{min}^{-1}$ ). After an initial oven temperature of 150°C, temperature was increased by 15°C  $\text{min}^{-1}$  to 200°C, followed by a further increase of 2°C  $\text{min}^{-1}$  to 240°C, which was then maintained for 20 min. Injector and detector temperatures were 220°C and 275°C, respectively. Identification of fatty acid methyl esters was performed by comparison of retention times with authentic standards (AccuStandard, FAMQ-005). Data processing was accomplished with a computer equipped with the HP ChemStation software. The amounts of fatty acids are given as percentages of the total fatty acids (Emre et al. 2010).

#### Statistical analysis

One-way analysis of variance was performed to compare the measurements, and analysis was carried out using the



Fig. 1. *Arbutus andrachne* (left) and *Arbutus unedo* (right) from engravings of the monumental edition *Flora Graeca* (Sibthorp and Smith 1823), based on watercolours by Ferdinand Bauer (1760–1826), reproduced with permission of the National Library of Greece.

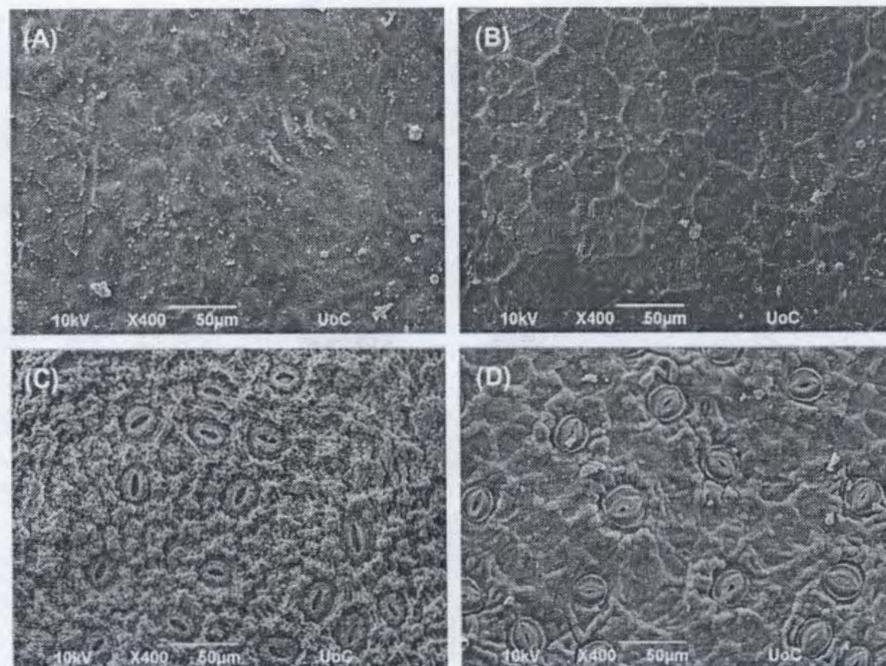


Fig. 2. Scanning electron micrographs of the adaxial leaf surface of (A) *Arbutus andrachne* and (B) *Arbutus unedo*, and the abaxial leaf surface of (C) *A. andrachne* and (D) *A. unedo* (magnification 400 $\times$ ).

SPSS statistical program (SPSS Inc., Chicago, IL, USA). The reported values are means  $\pm$  standard deviation.

**Results**

Leaves of *A. andrachne* (Figure 1, left) are larger than those of *A. unedo* (Figure 1, right); the average area of the alternate and simple leaves gathered was found to be  $28.2 \pm 4.2 \text{ cm}^2$  for *A. andrachne* and  $13.7 \pm 3.4 \text{ cm}^2$  for *A. unedo*.

The presence of wax was observed on the adaxial and abaxial surfaces of *A. andrachne* and *A. unedo* (Figures 2 and 3). The adaxial surface of *A. andrachne* (Figure 2A) appeared to be covered by a continuous wax

film, with occasional small wax clusters and crystal patches. The adaxial surface of *A. unedo* (Figure 2B) was similar, but with far fewer wax clusters and almost no crystal patches observed. The abaxial surface of *A. andrachne* (Figures 2C and 3A) revealed more complex waxy microsculptural formations than that of *A. unedo* (Figures 2D and 3B). Also, waxy and waxless adaxial and abaxial leaf surfaces of *A. andrachne* and *A. unedo* show different micromorphology on both surfaces. Comparison of scanning electron micrographs of the abaxial leaf surfaces of *A. andrachne* and *A. unedo* before (Figure 4A and C, respectively) and after removal of epicuticular wax (Figure 4B and D, respectively) revealed the influence of the underlying

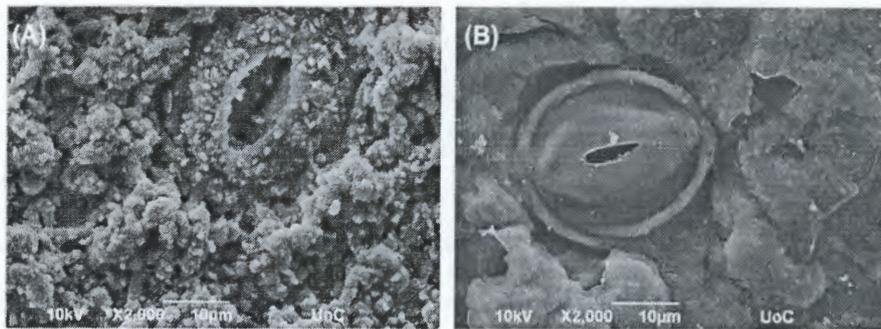


Fig. 3. Scanning electron micrographs of abaxial leaf surface of (A) *Arbutus andrachne* and (B) *Arbutus unedo* showing in detail the topography and microsculpturing of epicuticular waxes (magnification 2000  $\times$ ).

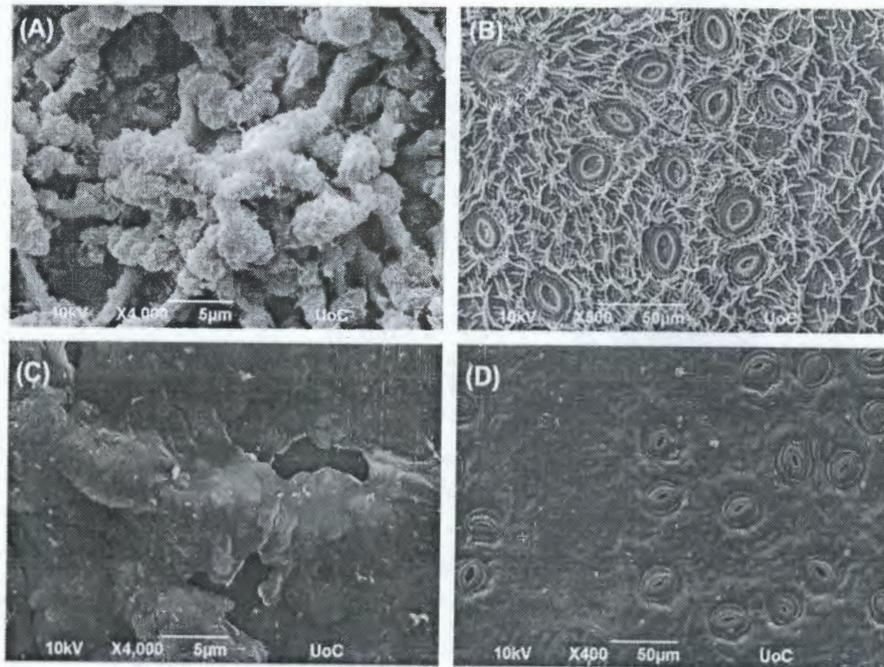


Fig. 4. Scanning electron micrographs of waxy abaxial leaf surface of (A) *Arbutus andrachne* (magnification 4000  $\times$ ) and (C) *Arbutus unedo* (magnification 4000  $\times$ ), and waxless abaxial leaf surface of (B) *A. andrachne* (magnification 500  $\times$ ) and (D) *A. unedo* (magnification 400  $\times$ ).

cell expansion and morphology of the cuticle on that of the wax film of both species. Scanning electron micrographs of waxless, abaxial leaf surfaces of *A. andrachne* (Figure 4B) were interesting; the cuticle appeared to possess distinctive wrinkles, on which the wax accumulates. In contrast, the abaxial surface of *A. unedo* (Figure 4D) displayed no such wrinkles, whereas the adaxial leaf surface (Figure 2B) was similar to the adaxial leaf surface of *A. andrachne* (Figure 2A). Stomata were observed only on the abaxial surfaces of *A. andrachne* (Figures 2C and 3A) and *A. unedo* (Figures 2D and 3B); stomata of *A. andrachne* appeared to be extensively covered by wax clusters (Figure 3A), but those of *A. unedo* (Figure 3B), with raised cuticular flanges, seemed to be covered by a wax film.

Total lipid extraction and purification from whole leaves and waxy leaf surfaces of the two species was performed. The results of fatty acids determination for expanded leaves of both plant species (Table 1) indicated an abundance of  $\alpha$ -linolenic acid (18:3<sup>A9,12,15</sup>), with palmitic acid (16:0) being the second major contributor, followed by linoleic (18:2<sup>A9,12</sup>) and oleic (18:1<sup>A9</sup>) acids. Oleic and linoleic acids were present in larger percentages in *A. andrachne* compared with that of *A. unedo*, whereas myristic, palmitic and  $\alpha$ -linolenic acids were found in elevated percentages in *A. unedo* (Table 1). The percentages of palmitoleic (16:1), stearic (18:0) and arachidic (20:0) acids were small (Table 1). Overall the fatty acid compositions of total lipids of the expanded leaves of the two plant species were similar (Table 1).

The fatty acid composition analysis of the leaf wax of *A. andrachne* and *A. unedo* (Table 2) ranged from C16 to C26, with fatty acids of an even longer chain length detected in the case of *A. andrachne* (14.3%), which could not be conclusively identified (Table 2). In the case of *A. andrachne* C22 fatty acids were dominant (Table 2). In contrast, in the case of *A. unedo* C26 fatty acids were the largest contributors; of the remaining fatty acids, the C16 and C20 fatty acids were found in similar

percentages in both species, whereas higher percentages of C18 and C24 fatty acids were observed in *A. unedo* and *A. andrachne*, respectively.

Contact angles of both leaf surfaces of *A. andrachne* and *A. unedo*, i.e. of the adaxial (Figure 5A and B, respectively) and abaxial (Figure 5C and D, respectively) surfaces, indicated that the surfaces of both sides of *A. andrachne* leaves were more hydrophobic compared with those of *A. unedo*. The abaxial surfaces of *A. andrachne* (Figure 5C) and *A. unedo* (Figure 5D) were more hydrophobic than the respective adaxial surfaces (Figure 5A and 5B, respectively), showing greater CA (Figure 5). Statistical analysis conducted to compare CA measured on all leaf surfaces, indicated a significant difference found at the  $p < 0.05$  level [ $F_{(3,16)} = 17.73$ ]. Post-hoc comparisons were conducted to identify significant differences between particular surfaces; the Tukey Honest Significant Difference test

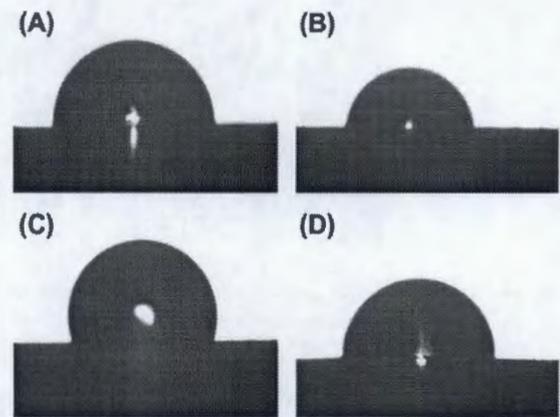


Fig. 5. Images used to capture measurements of contact angle (CA)  $\pm$  standard deviation (SD) of water droplets positioned on the adaxial surface of (A) *Arbutus andrachne* (CA  $97.0 \pm 3.7^\circ$ ) and (B) *Arbutus unedo* (CA  $84.2 \pm 1.7^\circ$ ), and the abaxial surface of (C) *A. andrachne* (CA  $111.1 \pm 3.7^\circ$ ) and (D) *A. unedo* (CA  $88.2 \pm 0.8^\circ$ ).

Table 1. Fatty acid composition of lipids of fully expanded leaves of *Arbutus andrachne* and *Arbutus unedo*.

Fatty acid (%) of total fatty acids $\pm$ SD									
Source	C14:0**	C16:0**	C16:1	C18:0	C18:1**	C18:2**	C18:3*	C20:0	Unknown
<i>A. andrachne</i>	3.0 $\pm$ 0.2	22.6 $\pm$ 0.3	0.7 $\pm$ 0.03	2.3 $\pm$ 0.2	9.8 $\pm$ 0.1	13.0 $\pm$ 0.2	42.4 $\pm$ 0.6	1.5 $\pm$ 0.1	4.6 $\pm$ 0.2
<i>A. unedo</i>	5.3 $\pm$ 0.3	25.5 $\pm$ 1.2	0.8 $\pm$ 0.02	2.8 $\pm$ 0.1	6.8 $\pm$ 0.3	7.9 $\pm$ 0.1	44.2 $\pm$ 0.6	1.3 $\pm$ 0.02	5.4 $\pm$ 0.5

Asterisks indicate significant difference in mean values at \* $p < 0.05$  and \*\* $p < 0.01$ .

Table 2. Fatty acid composition of epicuticular leaf wax of *Arbutus andrachne* and *Arbutus unedo*.

Fatty acid (%) of total fatty acids $\pm$ SD							
Source	C16	C18**	C20	C22**	C24**	C26**	Unknown**
<i>A. andrachne</i>	2.4 $\pm$ 0.2	4.9 $\pm$ 0.1	9.3 $\pm$ 0.4	44.0 $\pm$ 2.3	10.5 $\pm$ 1.1	11.1 $\pm$ 2.4	17.5 $\pm$ 2.5
<i>A. unedo</i>	5.6 $\pm$ 0.9	14.0 $\pm$ 0.4	7.6 $\pm$ 0.1	27.8 $\pm$ 1.1	5.6 $\pm$ 0.2	35.9 $\pm$ 1.2	3.6 $\pm$ 0.5

Asterisks indicate significant difference in mean values at \*\* $p < 0.01$  level.

Table 3. Contact angle (CA) of adaxial and abaxial leaf surfaces of *Arbutus andrachne* and *Arbutus unedo*.

Source of variation	Mean difference significance
CA of adaxial leaf surfaces of <i>A. andrachne</i> and <i>A. unedo</i>	**
CA of abaxial leaf surfaces of <i>A. andrachne</i> and <i>A. unedo</i>	**
CA of adaxial and abaxial leaf surfaces of <i>A. andrachne</i>	**
CA of adaxial and abaxial leaf surfaces of <i>A. unedo</i>	*

Asterisks indicate significant difference in mean values at \* $p < 0.05$  and \*\* $p < 0.01$ .

indicated that CA of the abaxial surface of *A. andrachne* was significantly different from that of the adaxial surface (Table 3), as well as of both surfaces of *A. unedo* ( $p < 0.01$ ).

### Discussion

The adaxial surface of the smaller leaves of *A. unedo* exhibited fewer wax clusters and almost no crystal patches, in comparison to the larger leaves of *A. andrachne*, which is in accordance with earlier observations (Mitrakos and Christodoulakis 1981; Christodoulakis and Mitrakos 1987; Bačić, Lawrence, and Cutler 1992; Gratani and Ghia 2002).

Plant lipids are a diverse group of molecules with varied functions, such as storage compounds, membrane structural components, signalling, protein modification, waterproofing and surface protection (Buchanan, Grissem, and Jones 2000; Müller and Riederer 2005; Buschhaus, Herz, and Jetter 2007a). In both *Arbutus* species,  $\alpha$ -linolenic and palmitic acids accounted for over two-thirds of the total fatty acid content (70% in *A. unedo* and 65% in *A. andrachne*). The dominance of  $\alpha$ -linolenic acid was not surprising considering its frequent abundance in photosynthetic plant tissues (Diamantoglou and Meletiou-Christou 1981; Meletiou-Christou, Rhizopoulou, and Diamantoglou 1992, 1994). Despite the similar fatty acid composition of total lipids, the composition of the wax fraction revealed significant differences between the two *Arbutus* species. This different composition may partly contribute to the respective surface properties of the leaves of the two species (Buschhaus, Herz, and Jetter 2007b; Samuels, Kunst, and Jetter 2008; Domínguez et al. 2010; Declercq et al. 2014). It is worth noting that a CA  $> 90^\circ$  indicates a hydrophobic surface, whereas a CA  $< 90^\circ$  indicates a rather hydrophilic surface (Boyce and Berlyn 1988; Bhushan and Jung 2006; Kolyva et al. 2012). Hence, the elevated CA measured on the abaxial surface of *A. andrachne* may relate to the increased presence of surface wax microsculpturing on that surface compared with the rest of the examined leaf surfaces (Abas and Simoneit 1998).

*Arbutus andrachne* appears to be better protected than *A. unedo* against water loss. The increased presence of surface wax on the abaxial surface of *A. andrachne*, which has a different fatty acid composition compared with that of *A. unedo*, as well as its hydrophobic

properties revealed by CA measurements, both indicate a plant adapted to the warm and dry summers of the region it inhabits. The differences observed between the two species might be better explained by examination of the particular habitats they are usually found in. *Arbutus unedo* has long been a cultivated species because of its fruits, as well as its use folk medicine (Soufleros, Mygdalia, and Natskoulis 2005; Pallauf et al. 2008; Antolín and Jacomet 2014; Miguel et al. 2014). In contrast, *A. andrachne* is more often found wild, with far less access to a water regimen. Another possible explanation may be found by examining the size of the leaves of the two species. The larger size of the *A. andrachne* leaves compared with those of *A. unedo* could be the driving force behind the need for a compensating mechanism to avoid water loss.

The abaxial leaf surfaces of *A. andrachne* and *A. unedo* were found to be more hydrophobic than their adaxial surfaces, which is at first glance surprising, because usually adaxial surfaces are more hydrophobic (Wang et al. 2014) and exposed to greater variations of environmental stimuli than the abaxial surfaces. However, a high hydrophobicity at the underside of leaves may play a significant role in sustaining photosynthesis during the rainy season, which coincides with the flowering and fruiting period of these species, keeping the gas exchange of the hypostomatic leaves unimpeded (Lange, Tenhunen, and Beyschlag 1985; Harley, Tenhunen, and Lange 1986; Raschke and Resemann 1986; Vitale and Manes 2005; Holder 2007b; Grassi et al. 2009); also, a hydrophobic adaxial leaf surface minimizes cuticular transpiration during the dry season.

It is likely that surface microsculpturing generally affects the wettability of leaves of strawberry trees to a degree that they can remain healthy irrespective of their long lifespan. Also, such structures are able to regulate the water status of the plants and microhabitats, both by influencing the diffusion boundary layer of the leaf surface and by regulating, through water-drop shedding, soil water availability (Rotondi et al. 2003; Holder 2012; Konrad et al. 2012).

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