Characterization of epicuticular wax structures on leaves of urban plant species and its association with leaf wettability

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***Samira Muhammad [a], Karen Wuyts [a], Gert Nuyts [b], Karolien De Wael [b], Roeland Samson [a]**

[a] Laboratory of Environmental and Urban Ecology, Department of Bioscience Engineering, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp Belgium
[b] AXES Research Group, Department of Chemistry, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp Belgium
samira.muhammad@uantwerpen.be, karen.wuyts@uantwerpen.be, karolien.dewael@uantwerpen.be, roeland.samson@uantwerpen.be
*Corresponding author: samira.muhammad@uantwerpen.be, samira.muhammad@gmail.com
Tel: + 32 265 3569

ABSTRACT

Epicuticular wax (EW) protects the plant's integrity and acts as a barrier against biotic and abiotic stresses. The micro-structured three-dimensional EW's and presence of leaf trichomes influence the wettability of a leaf surface. In this study, leaves of 96 perennial urban plant species were examined to determine an association between epicuticular wax structure (EWS) types and leaf wettability and investigate their seasonal variation. The EWS types were identified using Scanning Electron Microscopy (SEM), while leaf wettability was analyzed by measuring the drop contact angle (DCA) on both the abaxial and the adaxial sides of leaves collected from a common garden in June and September 2016. Four distinct EWS types namely thin film, platelets, crusts, and tubules were observed on leaves of investigated plant species in both June and September. The EWS types varied significantly between functional plant types and plant families in both June and September. In June, the abaxial DCA ranged from 56° to 147°, and the adaxial DCA ranged from 56° to 136°. In September, the abaxial DCA ranged from 54° to 130°, and the adaxial DCA ranged from 51° to 125°. The effect of time, leaf side, and EWS type on leaf wettability were significant. Plant species which showed a change in EWS type or clustering from June to September did not show a more pronounced reduction in DCA compared to those species which exhibited a constant EWS type. Findings from our study illustrate that DCA is not a good indicator in determining the different EWS types due to overlapping DCA intervals between the identified EWS types. However, the identified EWS types remained fairly stable throughout the in-leaf season and do not require repeated measurements for characterization.

KEYWORDS: Leaf wettability, Epicuticular wax structures, Urban plant species, Drop contact angle, Leaf traits

1. Introduction

The leaf surface possesses a cuticle layer which creates a seemingly smooth layer known as the epicuticular wax (EW). The EW layer protects the leaf surface from ultraviolet (UV) radiation, and dehydration (Reicosky et al.1978). It also has a tendency to avert insects (Müller 2006) and pathogens (Carver et al. 2006). The structural appearance and chemical composition of EW has been extensively studied (e.g., Baker 1974; Barthlott et al. 1998; Buschhaus et al. 2007). The EW layer may vary in chemical

composition, thickness, height of wax crystals (Barthlott et al. 1998) and density per unit leaf area depending on the environment (Baker 1974; Reed and Tukey 1982) abiotic stress factors, i.e., drought, heat, cold, frost (Shepherd and Griffiths 2006) and air pollution (Cape 1983). Removal of the EW layer due to abrasion may pose a threat to the long-term sustainability of the plant itself, i.e., due to increased transpiration rate (Jenks and Ashworth 1999). Leaves of plants can regenerate EW which may be sufficient for a slight loss. However, in the case of an advanced loss of the EW layer, the regeneration may be trivial (Baker et al. 1986). Changes in the EW layer can be observed directly using Scanning Electron Microscopy (SEM: Hall and Jones 1961; Reicosky and Hanover 1976; Hutten and Laine 1981) or through indirect measures such as leaf surface wettability (Leyton and Juniper 1963). The advantage of SEM micrographs is that the morphology of the epicuticular wax structures (EWS) can be observed (Cape, 1983) whereas, the leaf wettability can elaborate on the extent of erosion of the EW layer between successive drop contact angle measurements (Fogg 1947; Percy and Riding 1978; Paoletti et al. 1998). The EW layer is responsible for maintaining the wettability of a leaf surface (Neinhuis and Barthlott 1997). Wettability of a surface can be characterized by the static contact angle between the water droplet and the surface also called the drop contact angle (DCA: Holloway 1969; Bhushan and Jung 2008; Koch and Barthlott 2009). A spherical shape droplet has a large DCA while, a droplet which spreads results in a small DCA. The classification of leaf surfaces being super-hydrophilic to super-hydrophobic as used in many previous studies (e.g., Bhushan and Jung 2008; Roach et al. 2008; Zhang et al. 2008) is as follows: super-hydrophilic DCA < 10°, hydrophilic 10° < DCA < 90°, hydrophobic 90° < DCA < 150°, super-hydrophobic DCA > 150°. The hydrophobicity of a leaf surface is an essential physiological aspect during the lifespan of a plant (Fogg 1947). Low leaf wettability, (i.e., large DCA) prevents a reduction in photosynthesis after rainfall events or when leaves are covered with dew. A thin film of water on the leaf surfaces may impede the gaseous exchange by blocking of the stomata (Smith and McClean 1989; Bradley et al. 2003; Dietz et al. 2007). Carbon dioxide (CO_2) diffuses 10^4 times slower in water compared to air (Neinhuis and Barthlott 1997; Hanba et al. 2004; Brewer et al. 2007; Wang et al. 2015). In addition, plant species with low leaf wettability may increase quantities of through fall, stemflow, and fog precipitation at a site by shedding water from the canopy (Holder 2007). On the contrary, when leaf wettability is high (i.e., small DCA), e.g., when the cuticle of plants is damaged, the leaf encounters a higher exchange and foliar uptake of dissolved nitrogen (Adriaenssens et al. 2011, Wuyts et al. 2015), increased dry deposition of water-soluble gases such as sulphur dioxide (Zhang et al. 2003), enhanced particle accumulation (Muhammad et al. 2019) and stimulated growth of phyllosphere microbial communities (Martin and Juniper 1970; Knoll and Schreiber 1998; Marcell and Beattie 2002).

Kardel et al. (2012) revealed that leaf wettability was univocally affected by habitat type when comparing industrial to semi-natural areas. Effects of particle accumulation on leaf surfaces causing high leaf wettability (Cape et al. 1989; Neinhuis and Barthlott 1997, 1998) and wax degradation (Crossley and Fowler 1986; Turunen and Huttunen 1990) have been examined under simulated conditions, i.e., acid rain (Percy et al. 1994) acid fog (Percy and Baker 1990) and gaseous and particle pollutants (Percy and Riding 1978; Schreuder et al. 2001; Burkhardt and Pariyar 2014). Most studies (Cape. 1983; Neinhuis and Barthlott. 1998; Schreuder et al. 2001; Knoll and Schreiber. 1998; Marcell and Beattie. 2002; Shepherd and Griffiths. 2006; Kardel et al. 2012) have demonstrated the variation in leaf wettability using either evergreen needle-like species or a limited number of plant species. Similarly, studies in which the EWS of a broad range of plant species is characterized are very few (but see Neinhuis and Barthlott 1997). Moreover, the dynamics of EWS types throughout time and their relationship with wettability are not well known. Our research aims to determine an association between EWS and leaf wettability and their dynamics. The specific objectives of the present study were to (i) characterize the EWS types on leaves of 96 perennial urban plant species belonging to different functional plant types (n = 5) using SEM, (ii) assess leaf wettability of these plant species by employing drop contact angle measurements on the abaxial and the adaxial leaf sides, (iii) analyze the relationship between EWS types and leaf wettability, (iv) investigate the seasonal variation in EWS and leaf wettability from early to late in-leaf season and (v) link this seasonal variation in DCA (Δ DCA) with seasonal variation in EWS.

The inclusion of an extensive number of selected perennial urban plant species belonging to distinct functional plant types (i.e., deciduous and evergreen) will enable us in the testing of the following null hypotheses (H_0)

(H_{01}) The epicuticular wax structure type is independent of functional plant type.

(H_{02}) Leaf wettability is independent of the epicuticular wax structure type.

(H_{03}) The effect of time on leaf wettability is independent of the epicuticular wax structure type.

2. Materials and methods

2.1. *Experimental setup and plant material*

The study was conducted as a common garden experiment located at 51º 10'46.0"N, 4º 25' 0.02"E on the premises of the University of Antwerp (Antwerp, Belgium). The set-up of the experiment has been fully described by Muhammad et al. (2019). In brief, 96 perennial urban plant species were selected of which 45 plant species were deciduous broadleaf/needle-like trees, 32 deciduous broadleaf shrubs, 12 evergreen, needle/scale-like, 5 evergreen broadleaves and 2 climber species. For each plant species, five replicates were bought from one nursery (Houtmeyers in Eindhout – Laakdal, Belgium). Each plant replicate was planted in a 15L pot with organic potting soil and controlled release fertilizer and placed randomly in a 1.5 m x 1.5 m arrangement at the experiment site by 24th March 2016. All plants were generously watered and left to grow in a spatially uniform environment and exposed to similar atmospheric and climatic conditions. Two sampling campaigns were organized during the growing season; first in June 2016 and the second in September 2016. In the June sampling campaign, leaves of deciduous broadleaf/needle-like tree and shrub species were developed and harvested from the current growing season whereas leaves of evergreen needle/scale-like, evergreen broadleaf, and climber plant species were about one year old. In September sampling campaign, leaves of evergreen plant species which emerged in June were harvested in September and therefore were 3-months old. All leaves sampled were fully developed. In both sampling campaigns (i.e., June and September), leaf samples from each investigated plant species and their respective replicates were collected from the south-east oriented side of the plant, to eliminate within canopy orientation bias. During the growing season (1st April – 30th September 2016) the mean total PM_{10} (i.e., particles with an aerodynamic diameter smaller than 10 µm) and $PM_{2.5}$ (i.e., particles with an aerodynamic diameter smaller than 2.5 µm) concentrations from the nearest air quality monitoring station (42R817, Antwerp Groenenborgerlaan, at 250 m from the experiment site operated by Flanders Environment Agency, VMM) were 21.8 and 11.2 µg/m^3 respectively. The meteorological data were obtained from the station Antwerpen Luchtbal (station 42M802, Havanastraat, Antwerp) operated by VMM. From April – September 2016 a mean total average rainfall, air temperature, wind speed, and relative air humidity of 74.3 mm, 15.4 °C, 3.1 m/s, and 72 % respectively were recorded.

2.2. *Drop contact angle (DCA) measurements*

Leaves of the investigated plant species (n = 96) were harvested in batches on a span of 10 days (13th - 24th June and 12th – 23rd September) for DCA measurements. Only healthy, undamaged leaves were used. Soon after harvesting, DCA measurements were conducted on the right side of the fresh leaf sample. At room temperature (21 ºC) a droplet of distilled water was placed on leaf samples from available replicates (~n = 3 - 5) of each plant species (n = 96). According to the method described by Kardel et al. (2012), leaf surfaces were fastened to a flat horizontal surface using double-sided tape with either the abaxial or the adaxial leaf side facing up. A 7.5 µL droplet of distilled water (for broadleaves) and 4 µL droplet (for needles) was placed on the sample avoiding the midrib and the leaf margin using a micropipette. Next, using a Canon EOS 550D camera attached to a macro lens (MP-E 65mm 1:2.8), digital images of the droplets were

acquired with 3x magnification for each leaf side (abaxial/adaxial). All measurements were completed within an hour after leaf harvesting in a temperature and light controlled room. Finally, the left and the right inside contact angles between the droplet outline intersecting the solid surface (i.e., leaf surface) within a droplet were measured on the image using ImageJ software (https://imagej.nih.gov/ij/) and the drop snake analysis plugin (Stalder et al. 2006). For this analysis, a polynomial fit was created around the droplet based on 10 – 12 manually placed points. The DCA for a single replicate was calculated as an average of the left and the right angle. The leaf wettability data for June and September 2016 have been previously reported in a study by Muhammad et al. (2019).

2.3. *Scanning electron microscopy (SEM)*

To characterize the EWS types, leaf discs (approximately 12 mm diameter) were punched out from the left side of the leaf using a leaf perforator. Same leaves were used as those analyzed for DCA for the majority (n = 75) of investigated plant species except for plant species with small leaves (n = 7) such as *Rosa*, *Salix* and species with needle/scale-like leaves (n = 14). To examine the EWS on each leaf side (abaxial/adaxial), two leaf discs avoiding the central vein were collected from each plant species (n = 96). The leaf discs were placed on an aluminum stub (Ted Pella Inc.) affixed in place using conductive double-sided tape (PELCO Tabs 12 mm, 16084-1) and left to dry at room conditions. Next, three days before imaging for EWS, the leaf discs were coated with a 20 nm layer of carbon (Leica EM ACE600) in a vacuum environment to avert charge build-up effects. Lastly, the leaf discs were inspected and imaged using a Quanta 250 Field Emission Gun Environmental Scanning Electron Microscope (FEG-ESEM) which requires a high vacuum setting of 10^{-3} Pa. A spot size of 2.5 was selected, the distance between the electron emitter and the sample stage was set to 10 mm. A magnification of 10,000 – 50,000x was used depending on the subtleness of the feature, and an accelerating voltage of 20 kV was set before imaging of the leaf samples. For highly pubescent leaf surfaces, several attempts of thorough inspection were required to eventually secure an area without any obstruction by trichomes for the purpose of characterization of EWS.

2.4 *Data analysis*

Based on SEM images, the EWS were classified according to the classification rules proposed by Barthlott et al. (1998). The change in clustering of the EW crystals (Marcell and Beattie 2002) was qualitatively assessed by comparing the digital SEM images of leaf samples collected in June against those collected in September. A linear mixed effects model (LMER: Bates et al. 2015) was applied to examine the effect of time (two levels: June and September), EWS type (four levels: thin film, platelets, crusts, and tubules), leaf sides (two levels abaxial and adaxial), and their interaction effects (fixed effects) on the DCA taking into account plant id as a random effect. The response variable DCA was transformed using the natural log (ln). Normality of residuals was checked by Shapiro-Wilk test and Normal Q-Q plot. To distinguish EWS types between functional plant types (n = 5, i.e., deciduous broadleaf trees, deciduous broadleaf shrubs, evergreen needle/scale-like, evergreen broadleaf, climber species and between families (n = 29), a Monte-Carlo Pearson's Chi-square Test of Independence (χ^2) was performed. The seasonal change in EWS from June to September was evaluated based on EWS type and the clustering of the EW crystals and was classified either as (i) a change in type of EWS, (ii) a decrease in clustering of wax crystals within the same type of EWS or (iii) no change in type or clustering of EWS. One-way analysis of variance (ANOVA) was performed on the change in EWS from June to September with the absolute difference in DCA from June to September (Δ DCA). The change in EWS (type or clustering) from June to September was tested for functional plant types (n = 5) and plant families (n = 29) using Monte-Carlo Pearson's Chi-square Test of Independence (χ^2). All analyses were performed using the software R, version 3.4.2 (R Development Core Team 2017), and the add-on package *lmerTest* (Kuznetsova et al. 2017). The box plots were generated using the *lattice* package (Deepayan, 2008).

3. Results

3.1 *Characterization of epicuticular wax structure (EWS) types*

Four distinct EWS types were identified on the leaves of 96 investigated urban plant species (Table 1) i.e., thin film (n = 32, n = 20), platelets (n = 30, n = 39), crusts (n = 18, n = 18), and tubules (n = 16, n = 19) in June and September respectively. The EWS type did not differ between the abaxial and the adaxial leaf sides of the investigated plant species. The frequency of EWS type thin film was reduced whereas the frequency of platelets was increased from June to September. The EWS type *thin film* shows a smooth surface and no fissures after drying (Fig.1 a, b). The EWS type *platelets* were attached to the surface at varying angles and height (Fig.1 c, d). The *crusts* showed coverings with visible engravings of more than one µm in thickness (Fig.1 e, f). The *tubules* were cylindrical hollow structures with openings on the upper side. The dimensions of tubules were considerably homogenous, approximately 0.5 – 5 µm in length and 0.2 - 0.3 µm in diameter (Fig.1 g, h).

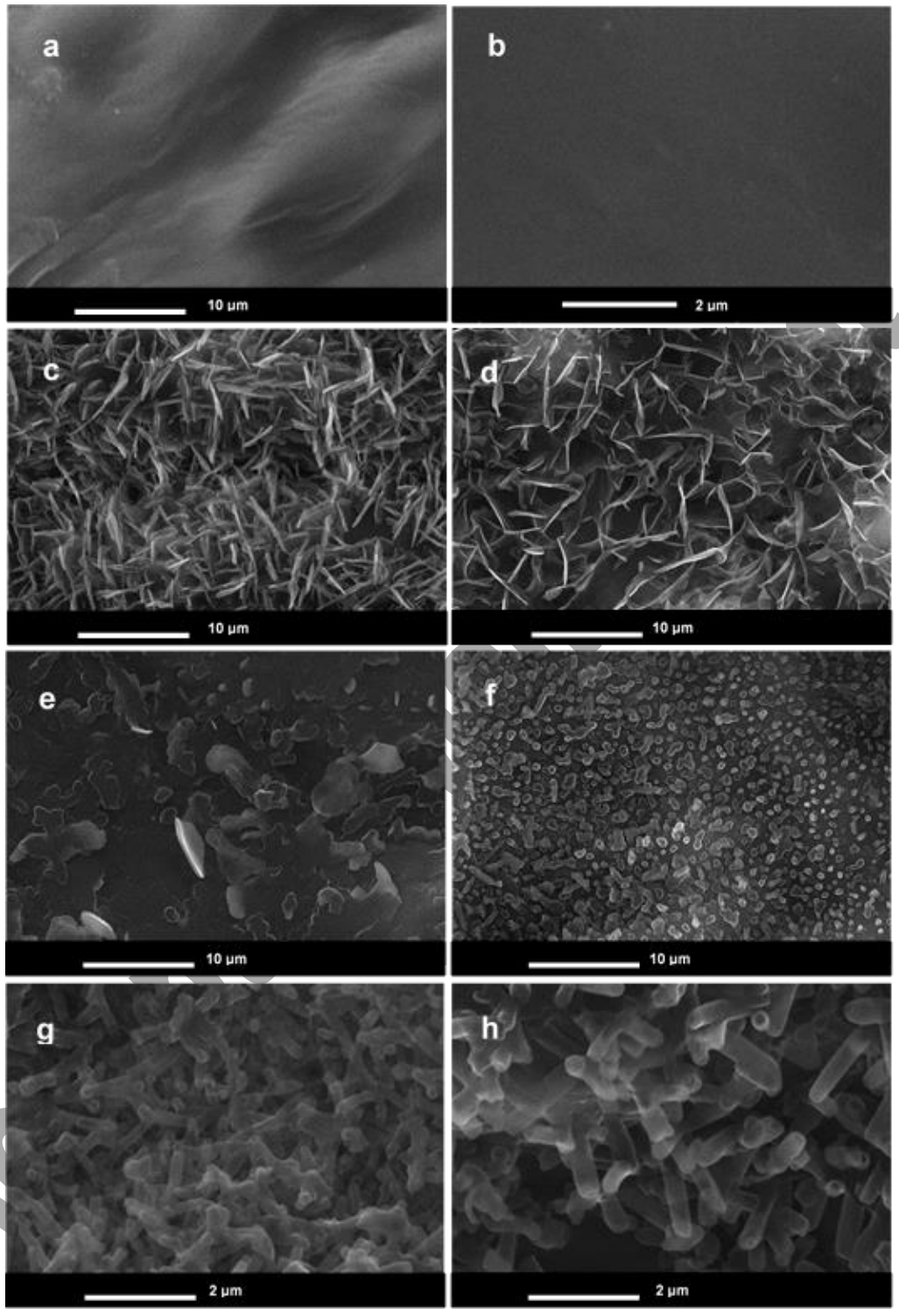

Fig. 1. Epicuticular wax structures on the abaxial leaf side for **(a)** *Aesculus hippocastanum* (**b)** *Cornus alba* (**c)** *Quercus petraea* (**d)** *Prunus padus* (**e)** *Crataegus monogyna* (**f)** *Liriodendron tulipifera* (**g)** *Juniperus communis* and (**h)** *Lonicera tartarica,* showing the types - thin film (a-b), platelets (c-d), crusts (e-f), and tubules (g-h). Scale bar of a, c, d, e, and f = 10 µm and for b, g, and h = 2 µm.

Table 1

Mean (± standard error) drop contact angle (DCA in °) on the abaxial (AB) and the adaxial (AD) leaf sides calculated from 3 – 5 available replicates per plant species and the epicuticular wax structure type in June and September for leaves of 96 perennial urban plant species. The change in epicuticular wax structure types from June to September is "↓" a decrease in clustering of wax crystals within the EWS type, "Δ" a change in EWS type or "↔" no change in EWS type or clustering of the epicuticular wax structure. Selected plant species belonged to 29 families and five functional plant types (EN = evergreen needle/scale-like, E.B = evergreen broadleaf, DT = deciduous broadleaf and needle-like trees, DS = deciduous broadleaf shrubs, CL = climber). Of the plant species with names in bold text, sampled leaves were about one-year-old in June 2016 and three months old in September 2016. DCA in bold text indicates a decrease in DCA by ≥ 20 ° from June to September.

		Drop contact angle								Epicuticular wax structure type		
		June				September						
Family	**Plant Species**	**AB**		**AD**		**AB**		**AD**		**June**	**September**	**June - September**
Adoxaceae	*Sambucus nigra* (DS)	56	±1.4	64	±1.8	54	±2.3	64	±4.6	Thin film	Thin film	↔
	Viburnum lantana (DS)	**79**	±4.3	76	±5.5	**58**	±5.5	71	±2.8	Platelets	Platelets	↔
	Viburnum opulus (DS)	95	±2.0	74	±2.8	77	±5.3	71	±2.4	Thin film	Platelets	Δ
Altingiaceae	*Liquidambar styraciflua* (DT)	98	±1.2	**98**	±0.6	83	±3.8	**67**	±3.8	Platelets	Thin film	Δ
Apiaceae	***Hedera helix* (CL)**	74	±1.3	82	±5.6	72	±1.1	74	±1.8	Platelets	Platelets	↓
Aquifoliaceae	***Ilex aquifolium* (E.B)**	93	±0.6	89	±0.5	80	±3.8	83	±1.5	Thin film	Platelets	Δ
Berberidaceae	***Mahonia aquifolium* (E.B)**	**132**	±2.3	86	±0.9	**89**	±3.0	69	±1.7	Thin film	Platelets	Δ
Betulaceae	*Alnus glutinosa* (DT)	65	±2.7	65	±5.3	59	±2.3	58	±3.8	Thin film	Platelets	Δ
	Alnus incana (DT)	115	±5.6	75	±5.9	98	±11	69	±3.0	Thin film	Crusts	Δ
	Betula pendula (DT)	73	±4.0	75	±3.4	76	±3.8	74	±3.8	Platelets	Platelets	↔
	Carpinus betulus (DT)	**89**	±2.0	76	±3.4	**67**	±4.6	74	±2.8	Platelets	Crusts	Δ
	Corylus avellana (DS)	77	±4.4	76	±6.5	63	±0.7	69	±2.8	Platelets	Platelets	↓
	Corylus colurna (DT)	62	±2.4	56	±2.7	57	±3.7	63	±6.6	Thin film	Thin film	↔
Bignoniaceae	*Catalpa bignonioides* (DT)	94	±6.8	79	±1.7	80	±3.4	62	±1.8	Thin film	Thin film	↔
Caprifoliaceae	***Lonicera periclymenum* (CL)**	**134**	±0.9	**123**	±1.7	**105**	±6.1	**93**	±6.0	Tubules	Tubules	↔
	Lonicera tatarica (DS)	**137**	±1.1	**136**	±1.4	**112**	±4.0	**58**	±3.6	Tubules	Tubules	↔
	Lonicera xylosteum (DS)	**140**	±1.9	**134**	±1.5	**112**	±1.7	**69**	±2.3	Tubules	Tubules	↓
	Symphoricarpos × chenaultii (DS)	140	±1.7	**135**	±0.2	126	±2.8	**92**	±6.4	Tubules	Tubules	↔
Celastraceae	*Euonymus europaeus* (DS)	**88**	±1.2	88	±4.0	**63**	±1.8	74	±6.5	Platelets	Platelets	↓
Cornaceae	*Cornus alba* (DS)	120	±5.6	88	±2.5	111	±4.0	73	±3.8	Thin film	Platelets	Δ
	Cornus mas (DT)	78	±3.9	83	±1.7	62	±2.9	74	±1.9	Platelets	Platelets	↓
	Cornus sanguinea (DS)	81	±5.1	74	±2.4	63	±0.8	74	±3.2	Thin film	Platelets	Δ
Cupressaceae	***Chamaecyparis lawsoniana* (EN)**	111	±0.8	117	±6.2	108	±1.0	104	±5.7	Tubules	Tubules	↓
	***Juniperus communis* (EN)**	99	±6.0	89	±7.5	81	±2.4	72	±3.3	Tubules	Tubules	↓
	***Thuja plicata* (EN)**	104	±2.2	83	±1.9	93	±1.1	64	±2.8	Tubules	Tubules	↓

		Drop contact angle								Epicuticular wax structure type		
		June				September						
Family	**Plant Species**	**AB**		**AD**		**AB**		**AD**		**June**	**September**	**June - September**
	Elaeagnus angustifolia (DT)	**147**	±5.8	85	±4.5	**124**	±2.8	79	±4.2	Crusts	Crusts	↔
Elaeagnaceae	*Hippophae rhamnoides* (DS)	117	±2.2	86	±2.8	101	±1.3	84	±3.0	Thin film	Thin film	↔
Ericaceae	***Rhododendron* (E.B)**	58	±3.2	76	±1.6	55	±3.3	59	±1.6	Platelets	Platelets	↓
	Laburnum anagyroides (DT)	133	±2.7	**113**	±3.6	115	±3.6	**76**	±1.1	Platelets	Platelets	↓
Fabaceae	*Robinia pseudoacacia* (DT)	141	±1.2	132	±1.7	125	±2.5	123	±2.7	Platelets	Platelets	↔
	Castanea sativa (DT)	68	±4.5	73	±5.8	64	±7.7	70	±3.0	Crusts	Crusts	↔
	Fagus sylvatica (DT)	**92**	±4.0	90	±1.2	**69**	±2.3	75	±1.0	Crusts	Crusts	↓
	***Quercus ilex* (E.B)**	**130**	±0.9	71	±3.2	**100**	±6.5	66	±3.3	Platelets	Platelets	↓
Fagaceae	*Quercus palustris* (DT)	**99**	±4.6	**87**	±2.4	**57**	±3.5	**65**	±4.6	Crusts	Crusts	↔
	Quercus petraea (DT)	**133**	±2.2	93	±0.9	**110**	±2.5	75	±3.7	Platelets	Platelets	↓
	Quercus robur (DT)	**131**	±1.6	**119**	±1.3	**94**	±4.3	**80**	±2.7	Platelets	Platelets	↓
	Quercus rubra (DT)	**122**	±1.9	**104**	±3.1	**76**	±5.5	**75**	±1.9	Platelets	Platelets	↓
Ginkgoaceae	*Ginkgo biloba* (DT)	131	±3.0	**127**	±2.2	117	±2.1	**70**	±2.8	Tubules	Tubules	↔
Juglandaceae	*Juglans regia* (DT)	76	±3.5	71	±3.8	60	±2.1	69	±2.0	Crusts	Crusts	↓
	Liriodendron tulipifera (DT)	135	±0.7	**133**	±2.9	125	±2.2	**93**	±1.9	Crusts	Crusts	↓
Magnoliaceae	*Magnolia kobus* (DT)	**101**	±4.7	**104**	±5.9	**77**	±2.0	**64**	±2.7	Platelets	Platelets	↓
	Hibiscus syriacus (DS)	77	±3.9	73	±6.0	60	±3.2	62	±2.0	Thin film	Platelets	Δ
Malvaceae	*Tilia cordata* (DT)	74	±4.3	66	±3.0	70	±4.3	76	±2.5	Platelets	Platelets	↓
	Tilia platyphyllos (DT)	**84**	±2.5	59	±3.8	**61**	±3.2	59	±0.5	Thin film	Platelets	Δ
	Fraxinus excelsior (DT)	71	±3.0	80	±3.8	55	±3.1	64	±1.9	Thin film	Thin film	↔
	Fraxinus ornus (DT)	80	±6.7	67	±6.1	67	±1.1	67	±1.7	Thin film	Thin film	↔
Oleaceae	*Ligustrum ovalifolium* (DS)	**85**	±1.2	79	±2.2	**60**	±2.6	71	±1.5	Thin film	Thin film	↔
	Ligustrum vulgare (DS)	95	±4.2	**98**	±0.4	85	±4.8	**74**	±0.9	Thin film	Thin film	↔
	Syringa vulgaris (DS)	56	±0.6	79	±3.4	56	±0.6	63	±2.5	Thin film	Thin film	↔
	***Abies fraseri* (EN)**	73	±2.0	72	±3.0	90	±2.9	56	±3.1	Crusts	Crusts	↓
	***Abies koreana* (EN)**	115	±4.2	**89**	±3.3	111	±1.7	**66**	±2.6	Crusts	Tubules	Δ
	***Abies nordmanniana* (EN)**	72	±5.7	68	±2.1	64	±6.9	64	±1.2	Crusts	Crusts	↔
	***Cedrus deodara* (EN)**	**96**	±2.1	**101**	±1.9	**71**	±2.3	**79**	±1.7	Tubules	Tubules	↓
	Larix decidua (DT)	**114**	±3.0	**105**	±3.5	**84**	±3.6	**76**	±7.7	Tubules	Tubules	↓
Pinaceae	*Larix kaempferi* (DT)	111	±3.8	**112**	±3.0	101	±3.0	**87**	±4.1	Tubules	Tubules	↓
	***Picea abies* (EN)**	**100**	±3.9	**104**	±1.0	**66**	±1.0	**82**	±2.5	Tubules	Tubules	↓
	***Picea pungens Glauca* (EN)**	80	±1.2	82	±1.4	88	±2.9	93	±1.9	Tubules	Tubules	↓
	***Pinus nigra* (EN)**	76	±4.0	86	±2.2	75	±3.5	77	±4.2	Crusts	Tubules	Δ
	***Pseudotsuga menziesii* (EN)**	90	±4.4	84	±1.2	91	±3.6	76	±0.7	Tubules	Tubules	↔
Platanaceae	*Platanus × acerifolia* (DT)	**99**	±4.1	83	±0.7	**55**	±2.8	80	±1.0	Platelets	Thin film	Δ
Rhamnaceae	*Rhamnus cathartica* (DS)	84	±0.9	68	±2.2	76	±1.9	68	±4.3	Platelets	Platelets	↓
	Rhamnus frangula (DS)	**91**	±13	83	±2.7	**62**	±1.7	71	±2.7	Thin film	Crusts	Δ
	Amelanchier lamarckii (DS)	**113**	±4.3	85	±4.8	**77**	±2.6	85	±5.5	Tubules	Tubules	↓
Rosaceae	*Crataegus monogyna* (DT)	**98**	±3.9	78	±2.2	**72**	±4.5	65	±2.2	Crusts	Thin film	Δ
	Malus sylvestris (DT)	**93**	±1.9	81	±5.6	**87**	±6.5	76	±1.0	Thin film	Platelets	Δ
	Mespilus germanica (DT)	**92**	±3.4	85	±1.1	**71**	±1.8	74	±3.2	Thin film	Thin film	↔
	Prunus avium (DT)	87	±3.3	86	±4.0	74	±3.9	64	±4.5	Platelets	Platelets	↓

		Drop contact angle								Epicuticular wax structure		
		June				September						
Family	Plant Species	AB		AD		AB		AD		June	September	June - September
Rosaceae	***Prunus laurocerasus* (E.B)**	85	±1.2	85	±1.2	81	±0.8	78	±3.1	Thin film	Platelets	Δ
	Prunus padus (DS)	**126**	±1.9	**92**	±2.1	**96**	±6.2	**69**	±3.3	Platelets	Platelets	↓
	Prunus spinosa (DS)	**100**	±2.1	**86**	±0.7	**82**	±3.1	**66**	±0.6	Thin film	Platelets	Δ
	Rosa canina (DS)	97	±2.7	**123**	±1.3	89	±4.2	**103**	±4.1	Crusts	Crusts	↔
	Rosa glauca (DS)	131	±0.8	129	±1.5	126	±4.5	124	±1.7	Crusts	Crusts	↔
	Rosa pimpinellifolia (DS)	**128**	±1.5	**128**	±0.6	**90**	±6.4	**80**	±4.9	Platelets	Platelets	↔
	Rosa rubiginosa (DS)	69	±1.8	**89**	±1.8	59	±2.2	**66**	±5.4	Thin film	Thin film	↔
	Rosa rugosa (DS)	**124**	±1.6	**81**	±1.0	**100**	±3.5	**58**	±3.5	Crusts	Platelets	Δ
	Sorbus aria (DT)	139	±0.9	**82**	±6.1	130	±1.6	**61**	±2.5	Thin film	Thin film	↔
	Sorbus aucuparia (DT)	**131**	±1.7	78	±1.6	**86**	±6.3	75	±3.2	Platelets	Tubules	Δ
	Sorbus intermedia (DT)	**135**	±1.6	79	±2.2	**110**	±7.0	63	±3.3	Thin film	Thin film	↔
	Sorbus torminalis (DT)	**84**	±3.3	77	±1.3	**61**	±4.7	59	±2.5	Platelets	Platelets	↔
Salicaceae	*Populus alba* (DT)	**93**	±2.0	85	±1.5	**75**	±4.9	76	±4.0	Thin film	Thin film	↔
	Salix alba (DT)	125	±3.6	74	±3.5	110	±4.9	67	±1.3	Crusts	Crusts	↓
	Salix aurita (DS)	134	±2.1	**120**	±4.6	126	±1.3	**68**	±3.1	Platelets	Platelets	↔
	Salix caprea (DT)	133	±2.4	71	±3.9	125	±2.6	64	±3.1	Crusts	Crusts	↓
	Salix cinerea (DS)	130	±1.1	85	±3.4	124	±2.9	83	±5.2	Crusts	Crusts	↔
	Salix purpurea (DS)	130	±2.4	**132**	±2.9	121	±1.4	**112**	±2.8	Platelets	Platelets	↔
	Salix repens (DS)	129	±1.2	69	±3.4	123	±2.5	81	±2.0	Crusts	Crusts	↔
	Salix rosmarinifolia (DS)	137	±0.9	69	±2.3	128	±2.1	78	±1.4	Platelets	Platelets	↔
	Salix viminalis (DS)	130	±0.8	85	±1.1	128	±1.8	84	±5.5	Thin film	Platelets	Δ
Sapindaceae	*Acer campestre* (DT)	69	±4.1	83	±3.2	67	±6.3	78	±5.1	Thin film	Thin film	↔
	Acer ginnala (DT)	**88**	±1.4	81	±4.4	**61**	±2.2	73	±5.6	Thin film	Thin film	↔
	Acer platanoides (DT)	86	±1.4	**96**	±3.0	76	±3.6	**67**	±1.1	Platelets	Platelets	↔
	Acer pseudoplatanus (DT)	**133**	±3.3	76	±3.8	**106**	±0.8	63	±2.3	Platelets	Platelets	↔
	Aesculus hippocastanum (DT)	97	±3.1	84	±6.0	88	±5.0	62	±2.1	Thin film	Crusts	Δ
Scrophulariaceae	*Buddleja davidii* (DS)	133	±3.4	76	±2.6	124	±1.3	63	±3.5	Thin film	Thin film	↔
Taxaceae	***Taxus baccata* (EN)**	94	±2.4	75	±2.1	86	±4.0	66	±3.1	Tubules	Tubules	↔
Ulmaceae	*Ulmus glabra* (DT)	85	±5.4	**85**	±2.5	67	±7.1	**55**	±2.0	Platelets	Platelets	↔

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3.2 *Epicuticular wax structures types: differences between functional plant types, and families*

No EWS type was exclusively linked to one functional plant type, but associations between EWS type and functional plant type were clear from the contingency table of the different EWS types over the five functional plant types (Table 2). The EWS types were significantly associated with functional plant types in June [χ^2 (df = 12, n = 96) = 48.98, p < 0.001] and in September [χ^2, (df = 12, n = 96) = 56.92, p < 0.001] (Table 2). Leaves of *Lonicera* species, and evergreen and deciduous needle/scale-like species (n = 14) predominantly possessed tubules in June and September as EWS type, but some had crusts. Leaves of the investigated evergreen broadleaf species (n = 5) had either platelets or thin film in June and September as EWS type. Leaves of both deciduous broadleaf tree (n = 43) and shrub species (n = 32) showed all four EWS types (Table 2). Leaves of deciduous broadleaf trees predominantly had platelets in June and September. However, leaves of deciduous broadleaf shrubs (such as *V. opulus, C. alba*, *C. sanguinea*, *H. syriacus*, *P. spinosa*) widely had an EWS type of thin film in June, but more platelets were observed in September. Leaves of climber species (n = 2) had an EWS type of tubules and platelets (Table 2) in both June and September.

The Monte-Carlo Chi-square test of independence indicated differences in EWS type between families [χ^2 (df = 84, n = 96) = 136.97, p < 0.001] in June and in September [χ^2 (df = 84, n = 96) = 145.25, p < 0.001]. The EWS type of thin film was observed on leaves of plant species within the Oleaceae family (n = 5) in both June and September (Table 1). In September, plant species within the family Cornaceae (n = 3), Fabaceae (n = 2), and Malvaceae (n = 3) undoubtedly had an EWS type of platelets. Plant species within the family Rosaceae (n = 17) showed all four types of EWS in both June and September, but the EWS type of thin film (n = 7) was predominantly observed in June whereas, platelets (n = 7) were predominantly observed in September. Plant species attributed to the family Salicaceae (n = 9), Betulaceae (n = 6), and Fagaceae (n = 7) incorporated crusts and platelets as EWS type in both June and September. The families mentioned above were few examples of inter/intra family variation and similarities in EWS types (Table 1).

Table 2

Observed contingency table of epicuticular wax structures types (n = 4) and functional plant types (n = 5): deciduous broadleaf tree, deciduous broadleaf shrub, evergreen and deciduous needle / scale-like, evergreen broadleaf, and climber species in June and September 2016.

	June						September					
	Deciduous broadleaf tree	Deciduous broadleaf shrub	Evergreen & deciduous needle/scale-like	Evergreen broadleaf	Climber	**ROW TOTAL**	Deciduous broadleaf tree	Deciduous broadleaf shrub	Evergreen & deciduous needle/scale-like	Evergreen broadleaf	Climber	**ROW TOTAL**
Crusts	9	5	4	0	0	**18**	11	5	2	0	0	**18**
Tubules	1	4	10	0	1	**16**	2	4	12	0	1	**19**
Platelets	18	9	0	2	1	**30**	17	16	0	5	1	**39**
Thin film	15	14	0	3	0	**32**	13	7	0	0	0	**20**
TOTAL	**43**	**32**	**14**	**5**	**2**	**96**	**43**	**32**	**14**	**5**	**2**	**96**

3.3 *Drop contact angle: effect of time, epicuticular wax structures, and leaf sides*

The results of linear mixed effects model (Table 3) indicated a significant effect of time on DCA. Leaf wettability increased from (mean ± SE) June (92 ± 2.5°) to September (77 ± 2.1°), i.e., smaller DCA's were observed in September compared to June. A significant effect of leaf side was indicated with higher leaf wettability on the adaxial leaf side (81 ± 2.9°) compared to the abaxial leaf side (94 ± 3.1°). The effect of EWS on DCA was significant (Table 3) in the following order, thin film < platelets = crusts < tubules (Fig.

2). The interaction effect between Time x EWS was significant (Table 3), indicating that the change in DCA from June to September differed between EWS types. For tubules the difference between June and September (22 ± 2.5°) was more pronounced than for the other EWS types, the smallest difference between June and September was observed in crusts with a mean decrease in DCA of (13 ± 0.1°). The interaction effect of leaf side x EWS was not significant (p = 0.124).

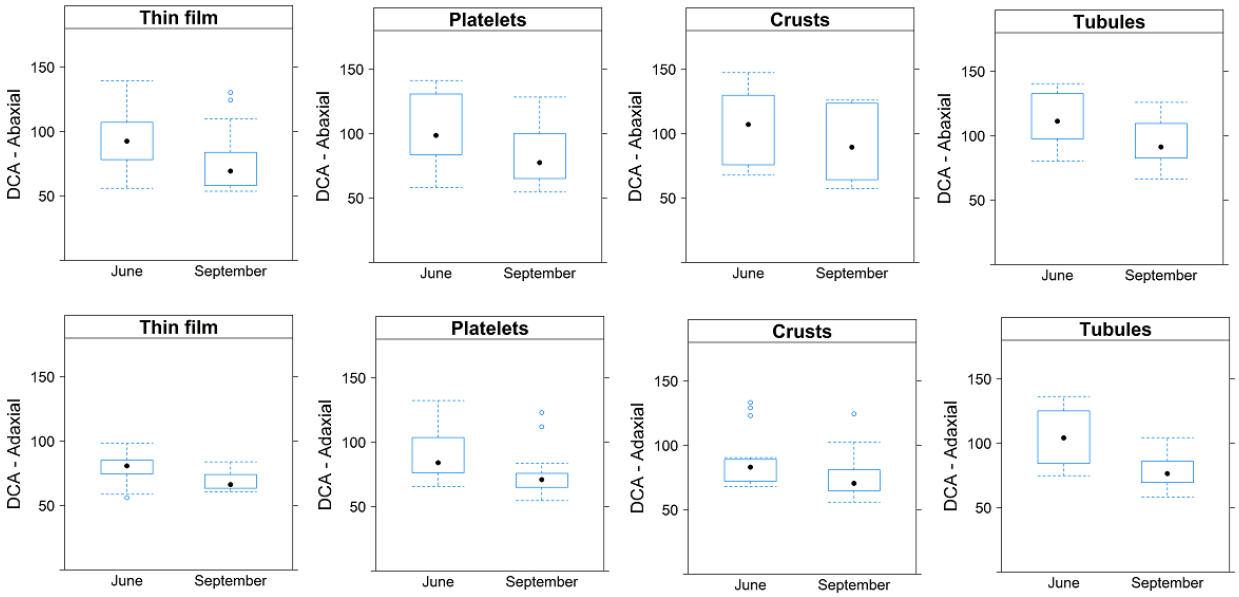


Fig. 2. Box plots of drop contact angles (DCA) on the abaxial (top) and the adaxial (bottom) leaf side for the four observed epicuticular wax structure types in June and September. The bars extending vertically from both sides of the box are the upper and lower whisker indicating the minimum, and maximum, the bottom of the box indicates the first quartile while the top indicates the third quartile. The black filled dots indicate median DCA. The hollow circles above the upper whisker indicate outliers.

Table 3

ANOVA of fixed effects in the linear mixed effect model with drop contact angles - ln(DCA) as the response variable and plant id as a random effect. The fixed effects with second-order interaction were time (June, September), leaf side (abaxial, adaxial) epicuticular wax structure - EWS (thin film, platelets, crusts, and tubules). DF = degrees of freedom, significant effects (p ≤ 0.05) are shown in bold)

Fixed effects	DF	F-value	p-value
Time	1	289.602	**< 0.001**
Leaf side	1	178.192	**< 0.001**
EWS	3	5.335	**0.001**
Time x Leaf side	1	0.012	0.911
Time x EWS	3	3.255	**0.020**
Leaf side x EWS	3	1.922	0.124

3.4 *Changes in epicuticular wax structure and DCA with time*

The leaf surfaces of about one-third of the investigated plant species (n = 31) mainly with platelets and tubules as EWS type showed a reduction in clustering of the wax crystals from June to September, for example, *L. anagyroides M. kobus*, *P. pungens Glauca,* and *Q. robur* (Table 1). The placement of wax crystals was far apart from each other with a smooth, rippleless layer appearing between the wax crystals.

The EWS type changed from June to September for a small number of plant species (n = 23) of which leaves of 12 plant species showed a change in EWS type of thin film to platelets such as *M. sylvestris, C. alba*, *H. syriacus*, *V. opulus***,** *P. spinosa* (Table 1). A majority of the investigated plant species (n = 42) showed no change in either the clustering of the wax crystals or the type of EWS from June to September, for example, *B. davidii*, *T. baccata*, and *U. glabra*. The results of the one-way ANOVA on ΔDCA did not indicate a significant difference in DCA change in time between the levels of EWS change for both the abaxial (p = 0.30) and the adaxial (p= 0.15) leaf sides (Fig. 3). However, the SEM micrographs seemingly exhibit a loss of wax crystals in September compared to June (Fig.4).

The change in EWS from June to September was tested between functional plant type and plant families. The results of Monte-Carlo Pearson's Chi-square Test of Independence did not indicate a significant difference between functional plant types [χ^2 (df = 8, n = 96) = 14.35, p = 0.06], however, significant differences between plant families [χ^2 (df = 56, n = 96) = 74.71, p = 0.01] were indicated. Plant species, for example, within the Elaeagnaceae and Oleaceae family did not show a change in either the type or clustering of wax crystals from June to September. Most of the plant species, for example, within the Cupressaceae, Fagaceae, Magnoliaceae, and Pinaceae family showed a decrease in clustering of the wax crystals. In addition, plant members within the Fagaceae and Pinaceae family showed an increase in leaf wettability (i.e., smaller DCA) from June to September by at least 20 ° on both leaf sides, together with a decrease in clustering of wax crystals (Table 1). Plant species within the Betulaceae, Cornaceae, and Malvaceae family were observed to be more prone to a change in EWS type (Table 1).

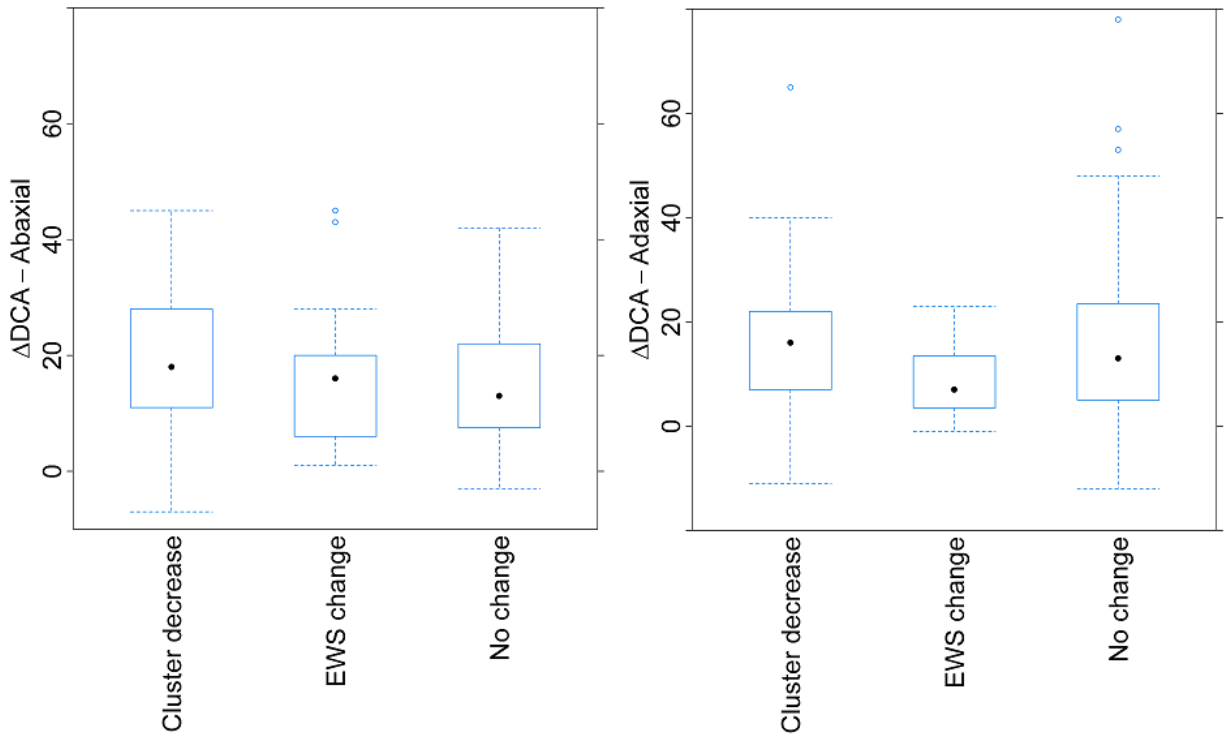
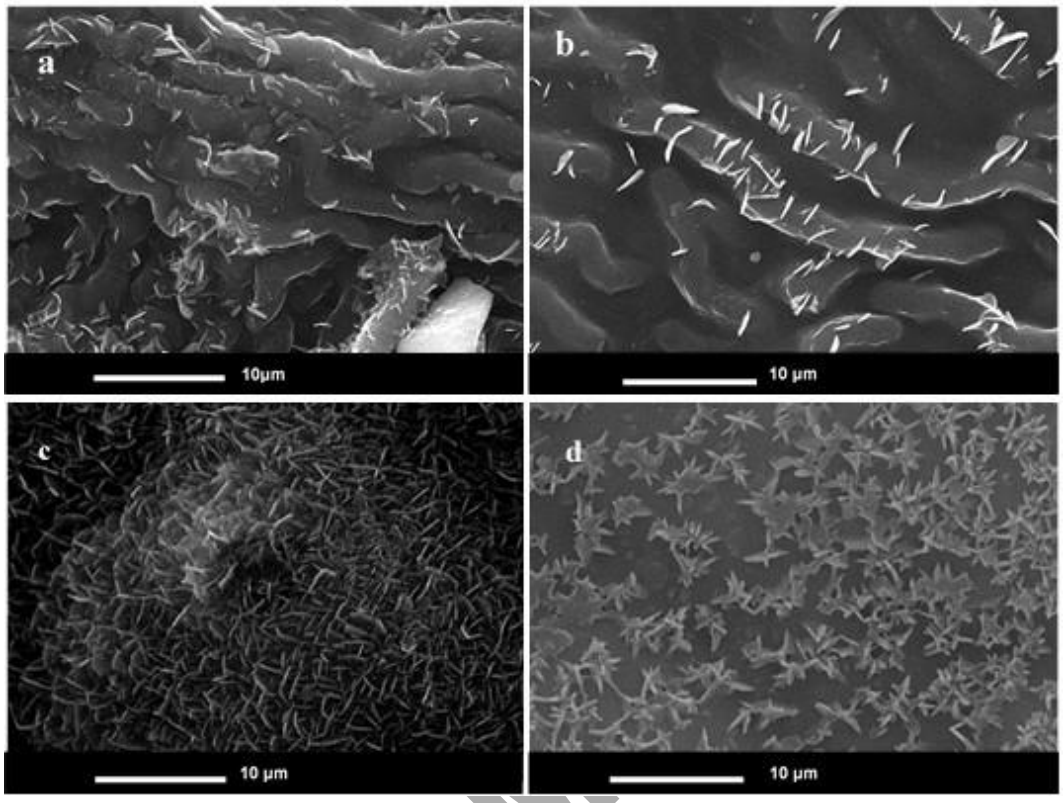


Fig. 3. Box plots of absolute difference in the abaxial (left) and the adaxial (right) DCA (Δ DCA) from June to September of the investigated plant species (n = 96) grouped by changes in clustering or types of EWS. "EWS change" = a change in EWS type (n = 23), "Cluster decrease" = an increase in gaps or reduced clustering of wax crystals (n = 31). "No change" = no change in either EWS type or clustering of wax crystals (n = 42) (see Table 1). Shown are the median ΔDCA, the upper and lower whiskers indicating the minimum and maximum Δ DCA within 1.5 times the inter-quartile range of the lower and upper quartile, the first and the third-quartile indicated by the lower and top end of the box and the outliers of Δ DCA.

312 **1)**



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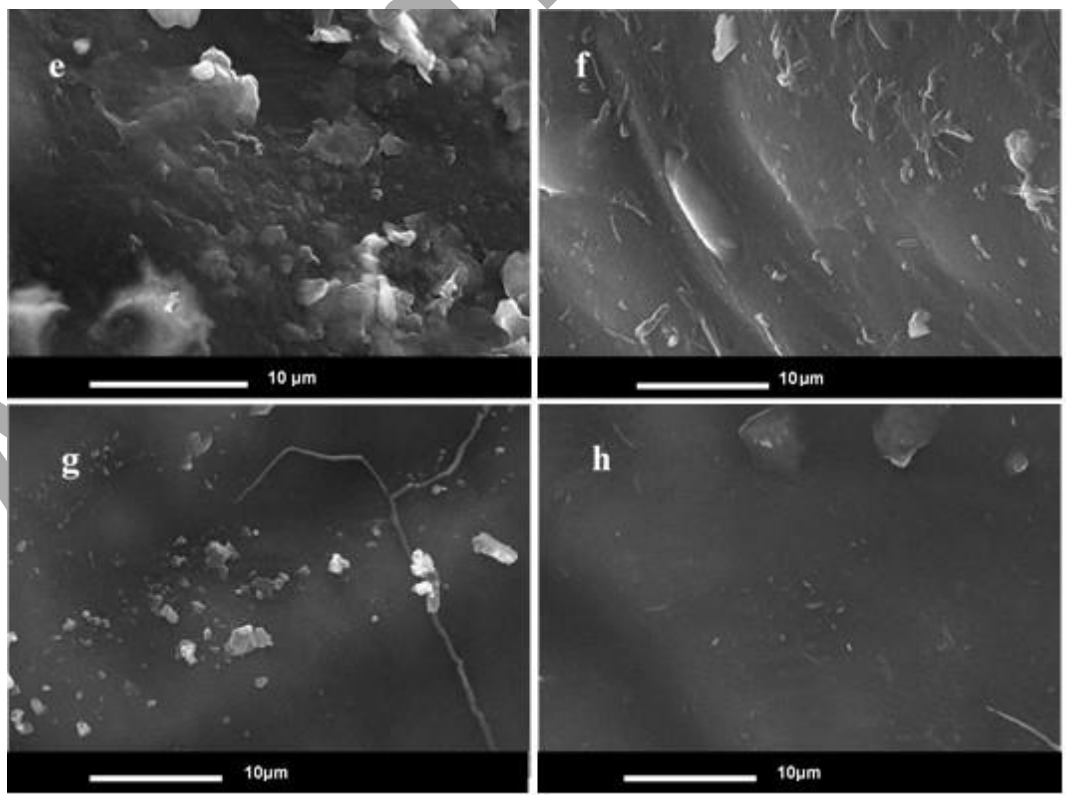
314 **2)**



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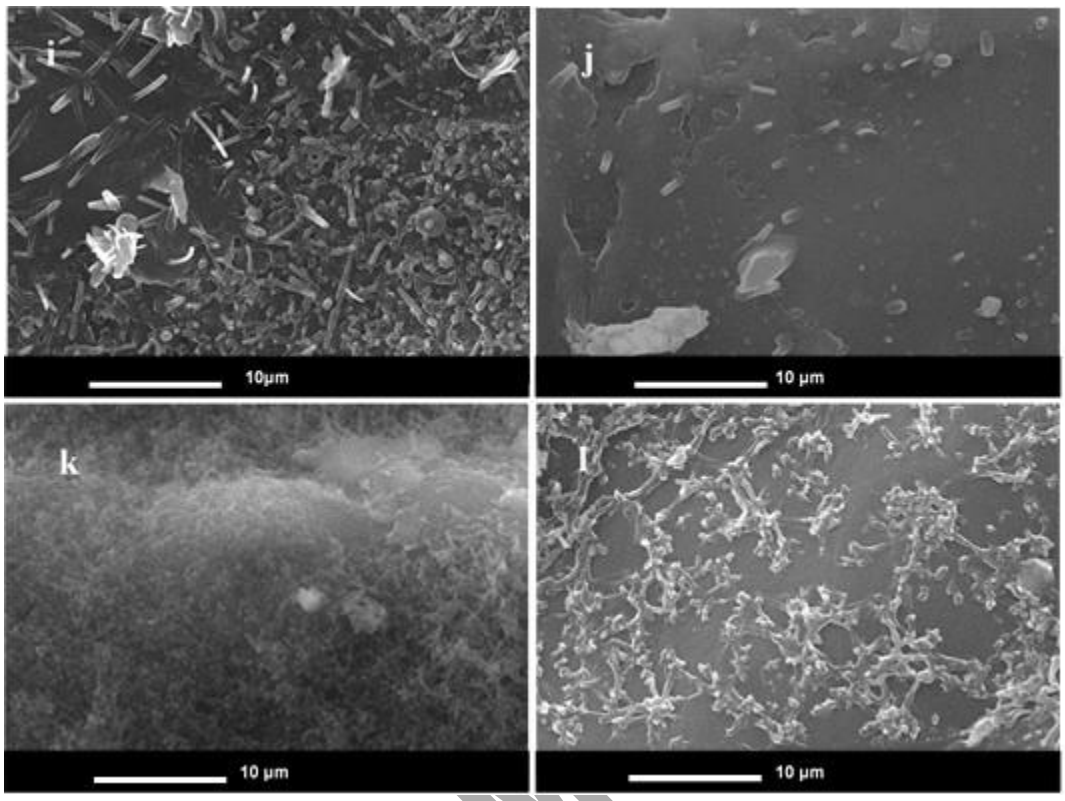
316 **3)**



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318 **Fig. 4.** A qualitative analysis of scanning electron micrographs of epicuticular wax structures on the adaxial
319 leaf surfaces illustrating a reduction in wax crystals from June (a, c, e, g, i, k) to September (b, d, f, h, j,l).
320 **Panel 1** showing platelets – (a, b) *Prunus avium* (c, d) *Laburnum anagyroides.* **Panel 2** showing crusts –
321 (e, f) *Abies fraseri*, (g, h) *Salix alba*. **Panel 3** showing tubules – (i, j) - *Amelanchier lamarckii*, (k, l) - *Picea*
322 *pungens Glauca*. Scale bar = 10µm.

323
324
325 **4. Discussion**

326 4.1 *Epicuticular wax structure types: differences between plant species and functional plant types*

327 Four distinct types of EWS were identified on leaves of perennial urban plant species investigated in this
328 study (n = 96) in both June and September (Table 1). The observed EWS types consisted of thin film,
329 platelets, crusts, and tubules, following the characterization and terminology proposed by Barthlott et al.
330 (1998). Of the six main EWS types recently described by Jeffree (2006) 'thin film', 'granules', 'tubules',
331 'platelets', 'rodlets' and 'filaments' three types were observed in our study. However, the EWS types
332 identified on leaves of investigated plant species in our study were in agreement with those included in the
333 review of Jeffree (2006). The three EWS types not observed (i.e., granules, rodlets, filaments) in our study
334 were identified by Jeffree (2006) on leaves of plant species that were not included in our study (e.g.,
335 herbaceous/tropical species). The identified EWS types in our study were also in agreement with those of
336 other authors for the corresponding investigated plant species (Hanover and Reicosky 1971; Jeffree 1976;
337 Crossley and Fowler 1986; Neinhuis and Barthlott 1997; Neinhuis et al. 2001; Wagner et al. 2003;
338 Buschhaus et al. 2007; Kardel et al. 2012). For some plant species such as *G. biloba* (tubules) and *B.*
339 *pendula* (platelets), the EWS types identified in our study (Table 1) were not in agreement with Neinhuis
340 and Barthlott (1997) and Kardel et al. (2012), respectively. Neinhuis and Barthlott (1997) observed platelets
341 on leaves of *G. biloba,* and Kardel et al. (2012) observed thin film on leaves of *B. pendula*. A majority of

leaf samples in the study of Neinhuis and Barthlott (1997) were collected from a botanical-garden, but some leaf samples were collected from different regions of the world (i.e., Europe, Mediterranean, Central Africa, and South America). For *G. biloba,* the reported dissimilarity can be ascribed to an alteration in the morphology due to differences in climatic conditions, i.e., irradiation, temperature, humidity (Baker 1974; Koch et al. 2006a; Shepherd and Griffiths 2006) in the region where *G. biloba* leaves in the study of Neinhuis and Barthlott's (1997) were collected. Koch and Ensikat (2008) indicated that disparity in EWS type of a plant species could be either due to the aging or erosion of waxes leading to undetermined shapes of wax crystals. For *B. pendula*, the discrepancy in the identification of EWS type between our study and that of Kardel et al. (2012) can be attributed to mechanical stress. The EW layer in the study of Kardel et al. (2012) may have likely been eroded during episodes of rain, dust or leaf-to-leaf contact (Shepherd and Griffiths 2006). Hence, the absence of undamaged or intact EW layer may have likely resulted in an imprecise classification.

The EWS types were significantly associated within a functional plant type, henceforth, rejecting the H_{01} that EWS types were independent of functional plant type. We observed that leaves of evergreen needle/scale-like species mainly had tubules as EWS type while leaves of deciduous broadleaf tree and shrub species possessed all four EWS types but chiefly platelets and thin film. In addition to similarities in EWS types between functional plant types, significant similarities in EWS types within families were also observed (Table 1). For example plant species within the Caprifoliaceae, Cupressaceae, Fabaceae, Oleaceae showed a distinct EWS type (Table1). Koch and Ensikat (2008) suggest that different wax morphologies arise from self-assembly of wax molecules. On the one hand, it has been established that morphological differences between EWS arise due to differences in their chemical composition (Holloway et al. 1976; Baker. 1982; Jeffree 1986; Jetter and Riederer 1995; Kunst and Samuels. 2003; Koch et al. 2006b). On the other hand, studies of Jetter and Schäffer (2001), Shepherd and Griffiths (2006), Buschhaus et al. (2007) and Koch and Ensikat (2008) have established that chemical composition of EWS has a formative but not exclusive influence on the morphology of EWS. A possible explanation for similarities in EWS types between functional plant and plant families could be the phylogeny of plant species. Givnish (1987) explained that leaf traits such as size, shape, thickness, stomatal density, epidermal cells are all influenced by ecological patterns. A modest overview of leaf traits within a plant family for example Oleaceae, showed that all plant members had small oval/elliptic-shaped leaves with high wettability, no trichomes on their leaf surfaces (Muhammad et al. 2019) and an EWS type of thin film as identified in this study (Table 1). Similarly, plant members within the Fabaceae family were observed to have low wettability, a trichome density of 11 – 32 mm^{-2} (Muhammad et al. 2019) and platelets as EWS type. Hence, it can be concluded that the distinct appearance of EWS types within a functional plant type or families can possibly be attributed to the phylogeny of the respective plant members.

4.2 *The relationship between epicuticular wax structure types and leaf wettability*

The linear mixed effects model indicated a significant effect of EWS type on leaf wettability (Table 3) thus rejecting H_{02} that leaf wettability is independent of EWS type. The EWS types differed in DCA in the following order, thin film < platelets = crusts < tubules. It was observed that leaves of plant species with tubules or crusts as EWS type (e.g., *L. tulipifera, L. periclymenum, R. pseudoacacia, R. glauca, S. purpurea,* and *S. chenaultii*) had low leaf wettability in June and September (Table 1, Fig. 2) while leaves of plant species with thin film as EWS type (such as *A. campestre*, *F. excelsior*, and *F. ornus*) had high leaf wettability in June and September (Fig. 2). Neinhuis & Barthlott (1997) examined leaves of 200 water repellent plant species and identified that plant species exhibiting water repellency on their leaf surfaces was due to multiple-length-scale roughness which is caused by trichomes, convex or papillose epidermal cells, and superimposed three-dimensional waxes (Neinhuis and Barthlott 1997). These findings were corroborated by Neinhuis and Barthlott (1998), Bhushan and Jung (2008) and Koch et al. (2009). Leaves of *A. lamarckii*, *G. biloba, P. abies, R. pseudoacacia,* with convex epidermal cells as observed by Neinhuis and Barthlott (1997), were found to have low leaf wettability in our study and that of Neinhuis and Barthlott (1997). Moreover, leaves of plant species mentioned above showed a high trichome density, i.e., 19 – 45 mm^{-2} (Muhammad et al. 2019) apart from having tubules and crusts as EWS type. Neinhuis and Barthlott (1997)

highlight that wettability of leaves with trichomes strongly depends on the presence or absence of wax crystals on the trichomes. It is possible that when a droplet of water is deposited on leaves with trichomes, it may bend the trichomes but the stiffness of the trichomes prevents contact from the epicuticular wax of ordinary epidermal cells (Otten and Herminghaus 2004). Thus in the case of high trichome density, the DCA does not reflect the wettability of the epicuticular wax, and as such DCA and EWS type are disconnected. For leaves with non-waxy trichomes, water-repellency may be short term because the water droplet usually penetrates between the trichomes after some minutes (Neinhuis and Barthlott 1997). We did not differentiate between waxy/non-waxy trichomes which warrants future research. In summary, although leaf wettability was significantly different on leaves with thin film and tubules, we were unable to procure large enough intervals in DCA to affirmatively identify each EWS type using DCA measurements. Therefore, it can be concluded that DCA measurements cannot be a good indicator or a complementary approach in identifying the different EWS types.

4.3 *Seasonal variation in epicuticular wax structures and leaf wettability*

It was observed that most of the investigated plant species (n = 42) did not show a change in EWS, neither in type nor in clustering of wax crystals. However, a change in EWS types from June to September occurred in few plant species (n = 23), of which leaves of 12 plant species showed a change in EWS type from thin film to platelets (for example *M. sylvestris, C. alba*, *H. syriacus*, *V. opulus,* and *P. spinosa* Table 1). Crossley and Fowler (1986) observed weathering of EWS with time on leaf surfaces of *Pinus sylvestris*. The authors identified the signs of a change in EWS as thickening of tubular waxes, the loss of wax plugs from the epistomatal chamber and the formation of plate-like structures. The latter change was also observed in our study. Karhu and Huttunen (1986) indicated that exposure to gaseous pollutants such as NO_2 and SO_2 either singularly or in combination causes a change in the morphology of EWS. The change in EWS type found in our study was observed in only 23 species and thus cannot be imputed on air pollution because we assume that with the common-garden approach, the effect of air pollution would be similar for all investigated plant species. Moreover, an alteration in EWS types due to air pollution or mechanical stress may result in erosion of wax crystals, and a display of amorphous thin film would likely be expected, whereas we observed a change in EWS type of thin film to platelets from June to September. Jenks and Ashworth (1999) indicated that the structure and chemistry of the EW layer does not remain static but changes during plant development and varies considerably among plant species. A plausible explanation for a change in EWS type from thin film to platelets can likely be attributed to the defense mechanism of plants against fungal pathogens (Nutman and Roberts 1960; Peries 1962; Jenks et al. 1994). Epicuticular wax crystals (i.e., platelets) may elevate the fungal spores above the leaf surface, thus limiting the spore's ability to receive physical or chemical signals from the plant, which are essential for spore development (Jenks and Ashworth 1999). An alteration in the topography of the leaf surface due to altered wax crystallization patterns may influence the successful penetration attempts by hyphae of fungi. However, the SEM micrographs analyzed in our study did not display colonization by phyllosphere bacteria or fungi; hence we believe that these changes in EWS type were likely a species-specific development shift in EWS as was observed by Crossley and Fowler (1986) and Jenks and Ashworth (1999).

Leaves of one-third of the plant species (n = 31) exhibited a decrease in clustering of the wax crystals from June to September (Table 1). A decrease in wax crystals was mainly for platelets and tubules (Table 1). Plant species such as *C. lawsoniana, T. plicata, L. decidua, L. kaempferi, Q. robur,* and *M. kobus* which were either evergreen needle/scale-like or deciduous tree species and more precisely plant members of the Cupressaceae, Pinaceae, Fagaceae and Magnoliaceae family (Table 1) showed a decrease in clustering of wax crystals. No significant association between EWS change neither for plant species, nor at functional plant type was indicated however, a significant association between EWS change and plant families was indicated. Shepherd and Griffiths (2006) emphasize that wax morphology can be influenced by temperature, light intensity, and humidity. Baker (1974) observed that with an increase in temperature from 15 to 35 °C, tubular waxes turned to dendrites, i.e., thinner and stretched apart, as was observed in our study from June to September (Fig. 4 k and l). Tubules are thermodynamically unstable due to their

high surface area/volume ratio (Shepherd and Griffiths 2006) indicating their sensitivity for cluster reduction. Generally, wax erosion advances with leaf age and air pollution, but to date, it has not been possible to link specific air pollutants to the erosion of EW layer (Cape and Fowler 1981; Crossley and Fowler 1986). One of the most documented symptoms of wax erosion is an increase in leaf wettability by both aging and interaction with atmospheric pollutants (Crossley and Fowler 1986; Turunen and Huttunen 1990).

A significant increase in leaf wettability from June to September (Table 3) was observed in this study. An estimated increase of 15° in leaf wettability on average for all investigated plant species was observed. The interaction effect of Time x EWS on the DCA was significant and illustrates that a change in DCA throughout time within a species depends on its EWS type (Table 3). This prompts us towards rejecting our H_{03} that the effect of time on leaf wettability is independent of EWS. For tubules the difference between June and September was (22 ± 2.5°) more pronounced than for the other EWS types, the smallest difference between June and September was observed in crusts with a mean decrease in DCA of (13 ± 0.1°). Neinhuis and Barthlott (1998) observed a considerable increase in leaf wettability of oak leaves which were observed to have platelets as EWS type. A possible explanation could be that platelets, in general, are more susceptible to alteration due to their shape and chemical composition compared to tubules (Jeffree 1986). Leaf wettability increased with time even in species of which the leaves did not show any morphological change in EWS type or clustering with time. Cape (1996) indicated that leaf wettability is influenced by the physicochemistry of the cuticular wax and to a lesser extent to leaf turgor. Neinhuis and Barthlott (1998) examined leaves of *G. biloba*, *Q. robur* and *F. sylvatica* for variation in particle load and leaf wettability throughout the growing season. It was observed that *G. biloba* maintained low leaf wettability throughout the growing season while *Q. robur* showed an increase in leaf wettability with leaf expansion and leaf aging. The authors also observed that leaves of *F. sylvatica* were highly wettable (i.e., 70° to 90°) and leaf wettability did not change significantly during the growing season thus no change in EWS type was either observed. However, Markstädter (1994) investigated the seasonal dynamics of epicuticular waxes on leaves of *F. sylvatica* and indicated that the chemical composition of EWS changes considerably with leaf aging. Neinhuis and Barthlott (1997) reported that it is difficult to ascertain if low wettability/water repellency depends on the very dense arrangement of wax crystals because theoretically, a leaf surface exhibits low wettability if air is enclosed between the surface structures (e.g., trichomes, epicuticular waxes) and water droplet (Holloway 1970). Based on our findings and those of both studies by Neinhuis and Barthlott (1998) and Markstädter (1994), it can be concluded that a change in epicuticular waxes with leaf aging may not be evident as a physical change in EWS (type or clustering) but rather a change in chemical composition which may likely alter the leaf wettability as it is dependent on the chemistry of the leaf surface (Neinhuis and Barthlott 1998). The effect of leaf side on leaf wettability was also observed to be significant (Table 3). The abaxial leaf sides had low wettability compared to the adaxial leaf sides as was observed in previous studies of (Neinhuis and Barthlott 1997; Holder 2007; Kardel et al. 2012). It is to be expected because the presence of stomata, trichomes, convex epidermal cells and epicuticular waxes on abaxial leaf surfaces cause surface roughness resulting in low wettability (Neinhuis and Barthlott 1997).

4.4 *Implications*

The wettability of leaves plays an important role in several processes, such as interactions of vegetation with precipitation and gaseous and particulate pollutants. On the one hand, leaves of plant species with low wettability may increase removal of particulates resulting in pristine leaf surfaces (Neinhuis and Barthlott 1997), and quantities of throughfall, stemflow, and precipitation by reduced water storage capacity and evaporation, resulting in greater hydrological inputs beneath the canopy (Haines et al. 1985; Holder 2007). On the other hand, an increase in leaf wettability enhances dry deposition of gaseous and particle pollutants (Cape 1983; Cape et al. 1989; Neinhuis and Barthlott 1997, 1998; Beckett et al. 1998; Nowak et al. 2006, 2013; Muhammad et al. 2019) and canopy exchange of dissolved nutrients (Adriaenssens et al. 2011, Wuyts et al. 2015). Considering the particle pollution, wettable leaf surfaces exhibit an increased residence time for particles resulting in low particle re-suspension rates (Litschke and Kuttler 2008) taking into account

that re-suspension of particles rapidly reduces with time (Litschke and Kuttler 2008). Moreover, leaves with high wettability which are unable to shed excess water, creating thin water films on their leaf surfaces may likely enhance the germination and development of phyllosphere microbial communities (Martin and Juniper 1970; Knoll and Schreiber 1998; Marcell and Beattie 2002) but may impede gas exchange (Holder 2007). In terms of canopy storage capacity, plants with high leaf wettability can result in a higher canopy water storage capacity compared to plants with low leaf wettability (Klamerus-Iwan and Witek 2018). The water storage capacity defines the amount of water available for evaporation, and thus may influence the mitigation of the urban heat island effect through evaporative cooling. Hiemstra et al. (2017) emphasized that mitigation of urban heat island effect can be achieved through cooling by evapotranspiration which is highly dependent on the soil water availability. Our study provided a thorough analysis of EWS types and their association to leaf wettability and found significant similarities in EWS types between functional plant types and families. However, no conclusive relationship between a change in EWS type with a change in leaf wettability with time was achieved. Nonetheless, an increase in leaf wettability from June to September for most investigated plant species was observed which highlights the possibility that plants may become more effective in particle capture, canopy exchange and evaporative cooling later in the growing season. Moreover, an increase in leaf wettability later in the season may serve as a good provision for phyllosphere microbial communities.

5. Conclusion

This research has proven to be exceptional because it includes a large number of plant species (n = 96) commonly found in urban environments for comprehensively investigating an association between leaf wettability and epicuticular wax structures. The common-garden approach enabled us to expose all plants to similar atmospheric and meteorological conditions.

The investigated plant species showed four distinct EWS types namely thin film, platelets, crusts and tubules in both June and September. The EWS identified on leaves of investigated plant species in June were fairly similar to the EWS types identified in September thus providing a basis that repeated measurements for identification of EWS types may not be required. Functional plant types and families were significantly associated with distinct EWS types possibly due to the phylogeny of the respective plant members. A significant association between EWS types and leaf wettability was indicated. The EWS types varied in DCA in the following order, thin film < platelets = crusts < tubules. In view of the fact that a significant association between EWS and DCA was indicated, the DCA does not solely depend on EWS. Other leaf traits such as trichome density also influence the wettability of a leaf surface. We conclude that DCA cannot be a good indicator to identify the different EWS types because of the overlapping DCA intervals between the identified EWS types. The effect of time on leaf wettability was significant with an average decrease in DCA of 15° from June to September between the investigated plant species. The change in DCA with time differed between EWS types, tubules were found to have the largest decrease in DCA of 22° while crusts showed the smallest decrease in DCA of 13°. A change in EWS (type or clustering) within a given species does not influence the time-dependent wettability increase. We did not find a significant association between a change in EWS from June to September and functional plant type but observed with plant families. Plant species for example, within Cupressaceae and Pinaceae having tubules as EWS type showed a decrease in clustering of wax crystals, possibly because tubules are found to be thermodynamically unstable, while plant species within Betulaceae, Cornaceae, and Malvaceae were more prone to a change in EWS type. An increase in leaf wettability for all investigated plant species can be considered as of ecological significance, e.g., with respect to reduction in air pollution and productivity of microbial ecosystems.

Acknowledgments

The research was funded by the Ontario Student Assistance Program (OSAP # 15103399). The SEM equipment was funded by FWO/Hercules. The authors acknowledge the help of the ENdEMIC group involved in the upkeep of the experimental site. The authors would like to thank the anonymous reviewers for their critical comments and constructive suggestions in improving the quality of the article.

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