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ORIGINAL ARTICLE

Leaf morphology, wax composition, and residual (cuticular) transpiration of four poplar clones

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Abstract

Key message **We identified two poplar clones of the same species as highly comparable, yet clones of two further species of the same genus to be distinctly different regarding multiple morphological and ecophysiological traits.**

Abstract Leaf morphology, wax composition, and residual (cuticular) transpiration of four poplar clones (two clones of the hybrid species *P.* × *canescens*, *P. trichocarpa*, and *P. euphratica*) were monitored from the beginning to end of the growing season 2020. A pronounced epicuticular wax coverage was found only with *P. euphratica*. As the most prominent substance classes of cuticular wax primary alcohols, alkanes and esters were identified with *P.* × *canescens* and *P. trichocarpa*, whereas esters and alkanes were completely lacking in *P. euphratica*. Wax amounts were slightly decreasing during the season and significantly lower wax amounts were found for newly formed leaves in summer compared to leaves of the same age formed in spring. Residual (cuticular) transpiration was about five to tenfold lower for *P.* × *canescens* compared with the two other poplar species. Interestingly, with three of the four investigated species, newly formed leaves in summer had lower wax coverages and lower rates of residual (cuticular) transpiration compared to leaves of exactly the same age formed in spring. Our findings were especially surprising with *P. euphratica*, representing the only one of the four investigated poplar species naturally growing in very dry and hot climates in Central Asia. Instead of developing very low rates of residual (cuticular) transpiration, it seems to be of major advantage for *P. euphratica* to develop a pronounced epicuticular wax bloom efficiently reflecting light.

Keywords *P.* × *canescens* · *P. trichocarpa* · *P. euphratica* · Cuticular wax · Plant cuticle · Residual transpiration

Introduction

Due to its considerably fast growth and ease of vegetative propagation, the genus of *Populus* became an increasingly interesting taxon for short-rotation agroforestry (Sannigrahi and Ragauskas 2010), even despite its generally high needs for a constant water supply caused by low water-use efficiencies (Blake et al. 1984; Souch and Stephens 1997). Especially after the publication of the genome of *Arabidopsis thaliana* as the first plant genome in 2000 (The Arabidopsis Genome Initiative 2000), desires for a tree model organism arose with a species of the genus *Populus* being the favored candidate (Bradshaw et al. 2000; Taylor 2002). Ultimately, *Populus trichocarpa* became the first tree species with its genome sequence being published in 2006 (Tuskan et al. 2006), leading to a multitude of subsequent genetical studies (Jansson and Douglas 2007; Quesada et al. 2008) further strengthening the standing of *P. trichocarpa* as the new model organism of trees. In this study, we report on a subset of four commonly researched *Populus* clones covering two intra-sectional hybrids and two true species, belonging to three sections in total: two clones of the hybrid species *P.* × *canescens* (Aiton) Sm. (section Populus), *P. trichocarpa* Torr. & Gray ex Brayshaw (section Tacamahaca), and *P. euphratica* Oliv. (section Turanga). To date, genomes of these 4 genotypes are available, allowing for extensive genomic comparisons in the future.

The interface between leaves and the surrounding environment is formed by the plant cuticle (Mérida et al. 1981). It is composed of the cutin polymer and cuticular waxes

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(Jetter et al. 2006; Schreiber 2010; Stark and Tian 2006) deposited in the cutin polymer (intracuticular waxes) and on its outer surface (epicuticular waxes). Its main function is the protection of the living tissues inside the leaf from environmental stress factors. This includes (i) the restriction of uncontrolled water loss (cuticular transpiration) under drought conditions (Riederer and Schreiber 2001), (ii) protection from microbial pathogens (Andrews and Harris 2000) and herbivores (Alfaro-Tapia et al. 2007), and (iii) reflection of UV light (Shepherd and Griffiths 2006). The transpiration barrier of cuticles is essentially established by cuticular waxes, since upon wax extraction using organic solvents rates of cuticular transpiration increase by factors of 1–3 orders of magnitude (Schönherr and Lendzian 1981). When measuring cuticular transpiration using intact leaves instead of isolated astomatous cuticles, it has been suggested to use the term"residual transpiration" (Burghardt and Riederer 2003; Kerstiens 1996). This considers the fact that incomplete stomatal closure after leaf abscission can not always be excluded. Thus, cuticular transpiration (residual transpiration) measured with intact leaves must not but could potentially overestimate the true cuticular transpiration to some extent (Burghardt and Riederer 2003). Therefore, in the following, we will use the term "residual (cuticular) transpiration" to indicate that the transpiration was measured with intact leaves and not with isolated astomatous cuticles.

We investigated in detail the cuticular wax composition and residual transpiration of four poplar clones. We intended to find out to what extent the four investigated poplar clones are similar or different in wax composition and residual transpiration, and whether this is related in some way to the climate of their natural habitats. The information presented here could be helpful in future genetic approaches deciding which of the four clones will be best suited for molecular biological and ecophysiological investigations on wax biosynthesis and the effect of an altered wax composition on residual (cuticular) transpiration, or whether all 4 clones are equally suited.

Materials and methods

Poplar clones investigated

The four different clones were chosen because (i) they represent widely used models in poplar research, (ii) their genomes were sequenced and published and they can be genetically modified, and (iii) they reflect very different adaptations to their natural habitats. *P.* × *canescens* hybrids are frequently occurring in close geographical proximity to their parent species *P. alba* (van Loo et al. 2008) which prefers floodplain ecosystems of the Northern Temperate Zone (Eckenwalder 1996). One of the *P.* × *canescens* clones ("84 K", Qiu et al. 2019), a cross between *P. alba* and *P. tremula* var. *glandulosa*, is frequently researched in Asian geographical regions. The other clone ("INRA 717-1B4", Mader et al. 2016), a cross between *P. tremula* and *P. alba* is more prominently investigated in Europe. In the following, the two clones will be abbreviated A × T and T × A, respectively. *P. trichocarpa* naturally situates in Pacific coastal and adjacent inland areas of north-western North America (Isebrands and Richardson 2014). The genotype "Nisqually 1" of *P. trichocarpa* from North America was the first poplar species to be sequenced (Tuskan et al. 2006). In strong contrast, *P. euphratica* may be considered as the geographical outlier species. It is well adapted to semi-arid desert environments (Ottow et al. 2005) and may tolerate extreme light intensities and temperatures (Zhou et al. 2010) as long as its roots can reach a continuously available groundwater table (Aishan et al. 2015; Chen et al. 2006). Individuals of the species *P. euphratica* (clone "B2" from Israel was used in this study) have also been sequenced (Ma et al. 2013; Zhang et al. 2020).

Cultivation of plants

In early 2018, two individuals of each of the four clones from axenic tissue cultures were transferred to soil. They were slowly adapted to growth outside in an experimental field of the University of Bonn via stepwise growth in a climate chamber (6 weeks) and a greenhouse (6 weeks). Plants were left outside in pots for the first 2 years and were always strongly cut-back in autumn of 2018 and 2019.

Pot volumes were continuously adapted to plant size (about 2 m height in 2 years) leading to a final pot volume of 40 L filled with standardized soil (Einheitserde Classic Type Topf 1.5, Einheitserde Werksverband e.V., Germany). A constant drop irrigation system secured adequate water supply to the plants. Weather conditions were monitored by an MWS 9–5 system (Reinhardt System- und Messelectronic GmbH, Germany). The daily light integral (DLI) was calculated by integrating the photosynthetically active radiation (PAR) of a given day (Fig. 1). During the year 2020, the mean temperature ranged between 6 and 22 °C, the precipitation between 6 and 102 mm, and the DLI was between 122 and 1555 mol m^{-2} $month^{-1}$. Especially the study period (April to September) was characterized by arid climatic conditions with high light radiation, high temperature, and significantly reduced amounts of precipitation compared to other years.

Leaf sampling

Leaves were harvested in the third year of the plants growing outside (2020) from newly forming branches in spring. Both leaf growth and development were monitored starting from

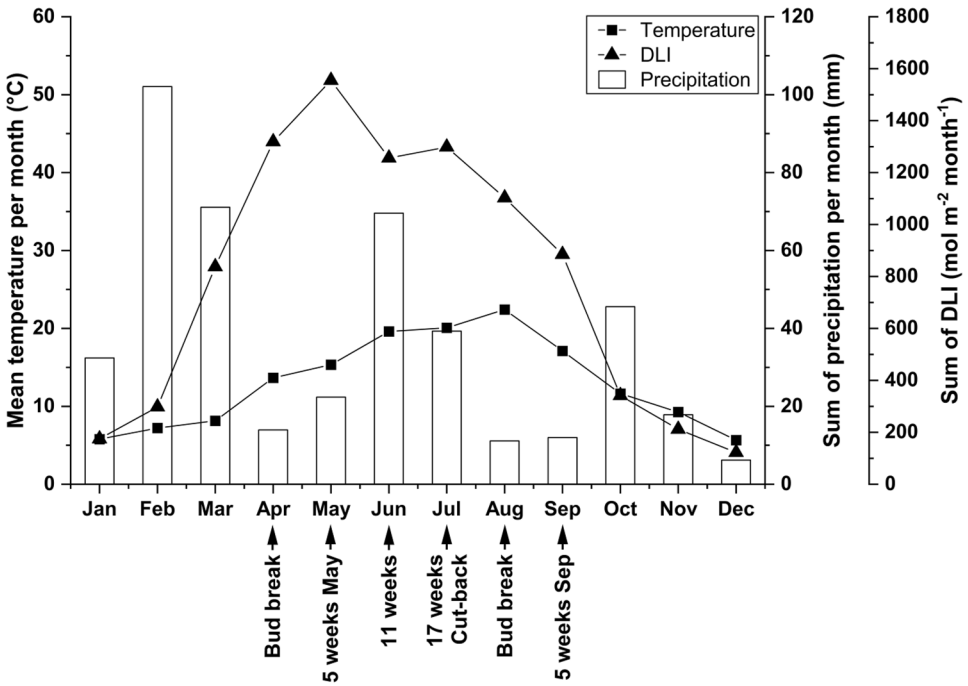


Fig. 1 Mean temperature per month (°C), sum of monthly precipitation (mm), and monthly sum of daily light integral (DLI, mol m^{-2} $month^{-1}$) during the year 2020. DLIs of each day of a given month are summarized to yield the monthly sum. Bud break and leaf harvesting times are indicated by arrows

bud break on a weekly basis. Thus, leaf age over the growing season could exactly be identified. Leaf areas were fully developed within the fifth week after bud break. Leaves were harvested at the age of 5, 11, and 17 weeks. Towards the end of the season (September 2020), another set of 5-week-old leaves, which started to grow mid of August 2020, was harvested (Fig. 1). To ensure that the harvested leaves did not show early macroscopically "invisible" signs of disease infection, the chlorophyll content of five leaves for each clone and harvesting time was estimated with a Force A device (Dualex Scientific, France).

Leaf anatomical and morphological analysis

The projected lamina surface areas of leaves from each harvest were measured using a scanner (Canon, Japan). Leaf fresh mass and leaf dry mass (also used to calculate the relative water content) of leaves from each harvest were measured using an analytical balance (Sartorius, Germany) with a resolution of ± 0.01 mg. Leaf mass per area (LMA, mg cm^{-2}) was calculated by dividing the leaf dry mass by its projected lamina surface area. The relative water content was calculated as a percentage: (fresh mass—dry mass)/fresh mass × 100.

Contrary to all other morphological parameters, the presence of stomata and contact angles of water were determined only with 11-week-old leaves. Stomatal presence was determined by light microscopy after taking imprints with nail polish from both leaf sides. Adaxial leaf surfaces of all four clones were analyzed by scanning electron microscopy (SEM; S200 Cambridge Instruments, Great Britain). 1 cm^2 leaf segments were mounted to aluminum sample stubs using double-sided adhesive tape and dried over silica gel for at least 24 h. Dried samples were sputter-coated (SCD 040, Balzers Union, Germany) with gold for 30 s using 30 mA current (ca. 15 nm thickness). Leaves were examined by SEM at 15 kV accelerating voltage and a working distance of 11 mm. Contact angles of water were measured on adaxial leaf sides using a Drop Shape Analyzer (Krüss, Germany). Leaf samples were fixed to glass slides using double-sided adhesive tape. Five droplets of 10 µl were placed on each leaf. Three independent leaves were investigated for each clone.

Cuticular wax composition analysis

The wax composition of 5-, 11- and 17-week-old leaves grown in spring and of 5-week-old leaves grown in summer was analyzed. Wax extraction was obtained by tightly placing chloroform-filled glass vials with broad rims on adaxial leaf sides. Thus, leaf areas for wax extraction were precisely defined (0.30 cm^2 for the narrow *P. euphratica* leaves and 1.28 cm^2 for the leaves of the other 3 clones). Glass vials closed with the leaves were turned upside down for 10 s and slightly shaken to ensure an efficient wax extraction. Previous tests with other species had shown that longer extraction would rather result in tissue damages by $CHCl_3$ leading to contaminations with internal lipids (Bringe et al. 2006; Richardson et al. 2005). For each biological replicate, four extracts from the adaxial leaf side were pooled. Four to six independent leaves of each clone were taken for analytical investigations. The internal standard for wax quantification

(10 µg C24 alkane, Tetracosane, Fluka, Germany) was added to chloroform before wax extraction. Chloroform was completely evaporated at 60 °C under a gentle stream of nitrogen. Samples were derivatized for 45 min at 70 °C after adding again 250 µl chloroform, 20 µl BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide, Macherey–Nagel, Germany) and 20 µl pyridine (Sigma Aldrich, Germany). Derivatization leads to the formation of trimethylsilyl (TMS) esters and ethers of alcohols and acids (Hauke and Schreiber 1998). 1 µl of each sample was analyzed by gas chromatography equipped with on-column injection. Flame ionization detection coupled to gas chromatography (GC-FID: 6890 N) was used for the quantitative determination of wax amounts. Mass spectrometry coupled to gas chromatography (GC–MS: 7890B) was used for the qualitative identification of wax compounds. Compounds were identified based on fragmentation patterns using an in-house created mass spectral library. Conditions after sample injection were 50 °C for 2 min, a temperature increase of 40 °C min^{-1} up to 200 °C, 2 min at 200 °C, 3 °C min^{-1} up to 310 °C and finally 30 min at 310 °C. The flow rate of the hydrogen (GC-FID) or helium (GC–MS) carrier gas was 2 ml min^{-1} and DB-1 columns (30 m length, 0.32 mm diameter, 0.2 µm coating thickness; Agilent J&W) were employed.

Leaf stomatal and residual (cuticular) transpiration

All transpiration measurements have been performed with fully developed 5-week-old leaves formed in the beginning (April to May) and the end (August to September) of the growing season. Stomatal conductances were measured using an SC-1 Leaf Porometer (Decagon, USA) in the morning between 10 to 11 a.m. Residual (cuticular) transpiration was measured using intact leaves. Leaves were harvested at the experimental field in the morning, stored in sealed plastic bags, and transported within 60 min to the institute. With all stomata fully closing after cutting off leaves, subsequent measurements will mainly represent cuticular transpiration (Kerstiens 1996). Due to the unavoidable delays in measurements, stomatal transpiration of each leaf before abscission was evaluated right at the site of harvest using the leaf porometer. After measuring the fresh mass and determination of leaf areas in the institute, leaves were stored at 25 °C in plastic boxes over activated silica gel (2% relative humidity). Decreasing leaf masses were measured every 20 min up to 6 h. For each measurement, rates of water loss per area and time (g m^{-2} s^{-1}) were measured. Permeances (m s^{-1}) were calculated by dividing rates of water loss by the driving force (concentration gradient of water inside the leaf minus outside). Since leaves were kept at 2% humidity, the driving force for residual (cuticular) transpiration was essentially given by the water concentration inside the leaf, which was assumed to be close to 10^6 g m^{-3}. To allow direct comparison, stomatal transpirations (measured in the field in mmol m^{-2} s^{-1}) were converted into permeances (m s^{-1}) according to McDermitt (1990). The permeance of each measurement period was plotted against its corresponding estimated relative water deficit, which was calculated from water loss divided by the leaf fresh mass of the respective measurement period. Stomatal transpirations of intact leaves were assumed to be measured at a relative water deficit close to zero.

Statistical analysis

Numbers of investigated biological replicates were between 3 and 6 leaves for each clone, experiment, and harvest. Statistical tests (two-sample *t* test or one-way ANOVA with Fisher's LSD post hoc test) at a significance level of $p \leq 0.05$ were applied (OriginPro 20, OriginLab Corporation, USA). Statistical differences were tested on the 95% level. Boxplots or means with standard deviation are shown.

Results

Bud break and leaf morphology

Bud break (Fig. 1) and leaf development of *P. trichocarpa*, was always 1 week earlier (calendar week 14 in 2020) than the 2 clones of *P.* × *canescens* (calendar week 15). Bud break and leaf development of *P. euphratica* was 1 week delayed (calendar week 16). After a strong cut-back of the plants in calendar week 28, the very same pattern of bud break and development of the 4 clones was observed after a 3- to 5-week recovery period in calendar weeks 31, 32, and 33. In the fifth week leaves had fully expanded, resulting in mean projected leaf areas of about 32 cm^2 (both clones of *P.* × *canescens*), 16 cm^2 (*P. trichocarpa*), and 8 cm^2 (*P. euphratica*) at a leaf age of 5 weeks (Fig. 2). Dense trichomes were still visible on the abaxial sides of clones of *P.* × *canescens*, less for *P. trichocarpa*, and none for *P. euphratica*. Adaxial surfaces were devoid of any visible trichomes for all clones (Fig. 2). Stomata could only be identified in light microscopy and SEM on the lower leaf side of both *P.* × *canescens* clones (hypostomatous leaves), whereas *P. trichocarpa* and *P. euphratica* were amphistomatous.

Although fresh mass per leaf area varied significantly between 16 and 36 mg cm^{-2}, the LMA was very similar with 6–8 mg cm^{-2} for each clone (Fig. 3a). This was due to differences in the relative water content with *P. euphratica* having a water content of 80% compared to the 3 other clones with 61 to 65% (Fig. 3b). Chlorophyll contents for all clones and harvests were between 25 and 35 µg cm^{-2} with no obvious seasonal trends (no data shown). Also all other morphological parameters (projected lamina surface area,

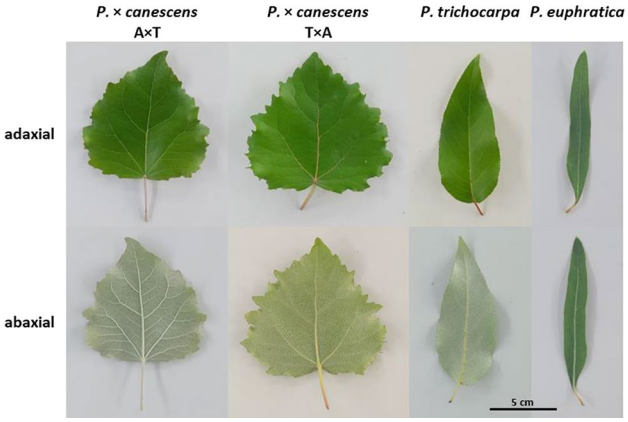


Fig. 2 Representative morphology of 5-week-old poplar leaves harvested in May. Leaves of all 4 clones were fully developed at this point. Abaxial leaf sides of all clones except isobilateral leaves of *P. euphratica* are covered by trichomes. Stomata were found on adaxial leaf sides of *P.*×*canescens* clones (hypostomatous) and on both leaf sides of *P. trichocarpa* and *P. euphratica* (amphistomatous)

chlorophyll content, mass per area, and relative water content) estimated for each clone and harvest were not changing after leaves were fully developed and also not for the newly grown leaves of 5 weeks age after the strong cut-back in July (no data shown).

Contact angles on adaxial leaf sides of both clones of *P.*×*canescens* varied between 85° to 90° whereas contact angles were slightly lower on leaves of *P. trichocarpa* (70.8°). Leaf surfaces of *P. euphratica* were hardly wettable since they showed contact angles of around 160° (Fig. 3c).

Scanning electron microscopic investigations

A low abundance of epicuticular wax crystals (granules and platelets) was visible on adaxial leaf surfaces of both clones of *P.*×*canescens* (Fig. 4a, b). *P. trichocarpa* had a smooth leaf surface with only very few visible wax crystals (Fig. 4c). *P. euphratica* showed a very dense coverage with epicuticular wax platelets (Fig. 4d). Folds and edges on the leaf surface of *P. trichocarpa* (Fig. 4c) are considered to be drying artifacts.

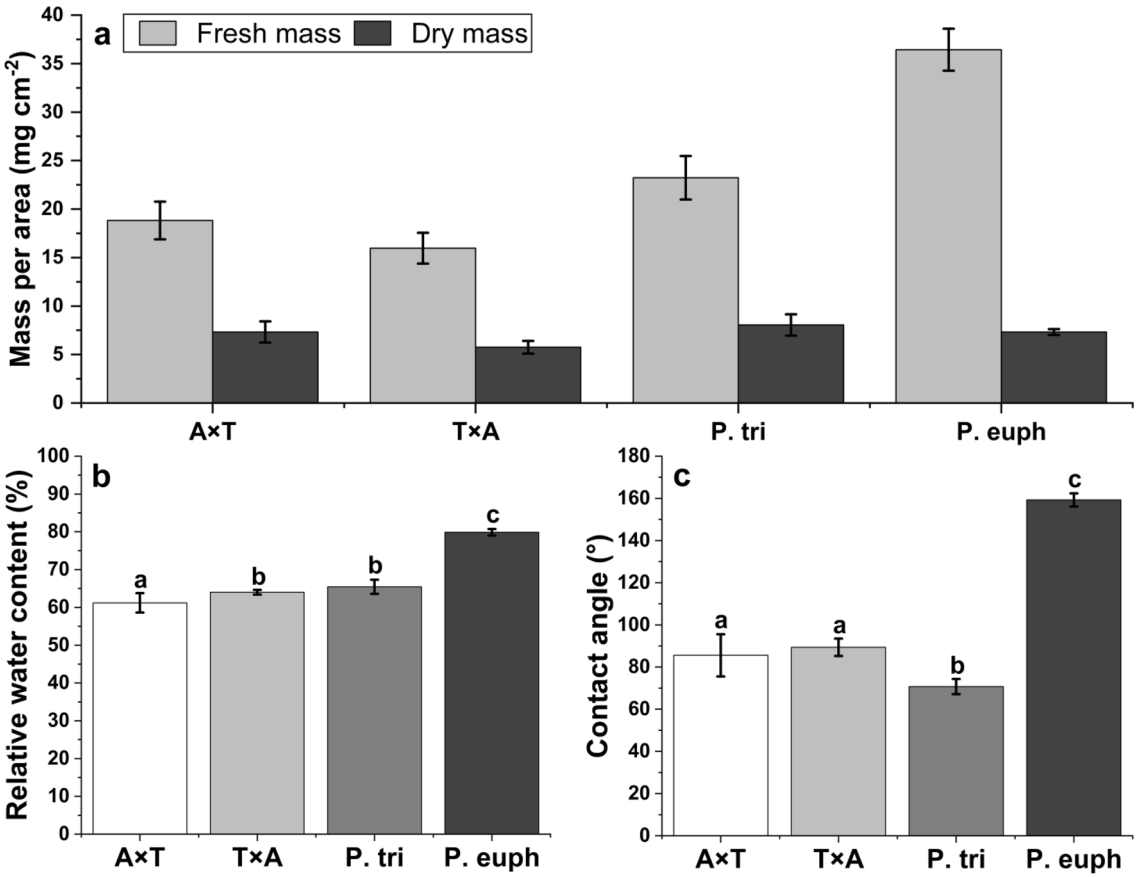


Fig. 3 Mass per surface area **a**, relative water content **b**, and contact angles of water **c** of 11-week-old poplar leaves (A×T and T×A: *P.*×*canescens*; P. tri.: *P. trichocarpa*; and P. euph.: *P. euphratica*). The data shown in **a** and **b** is representative of all evaluated leaf cohorts. Means with standard deviations are shown ($n=3$–6 for **a** and **b**; $n=15$ for **c**). Significant differences at $p \leq 0.05$ are indicated by differential letters (ANOVA)

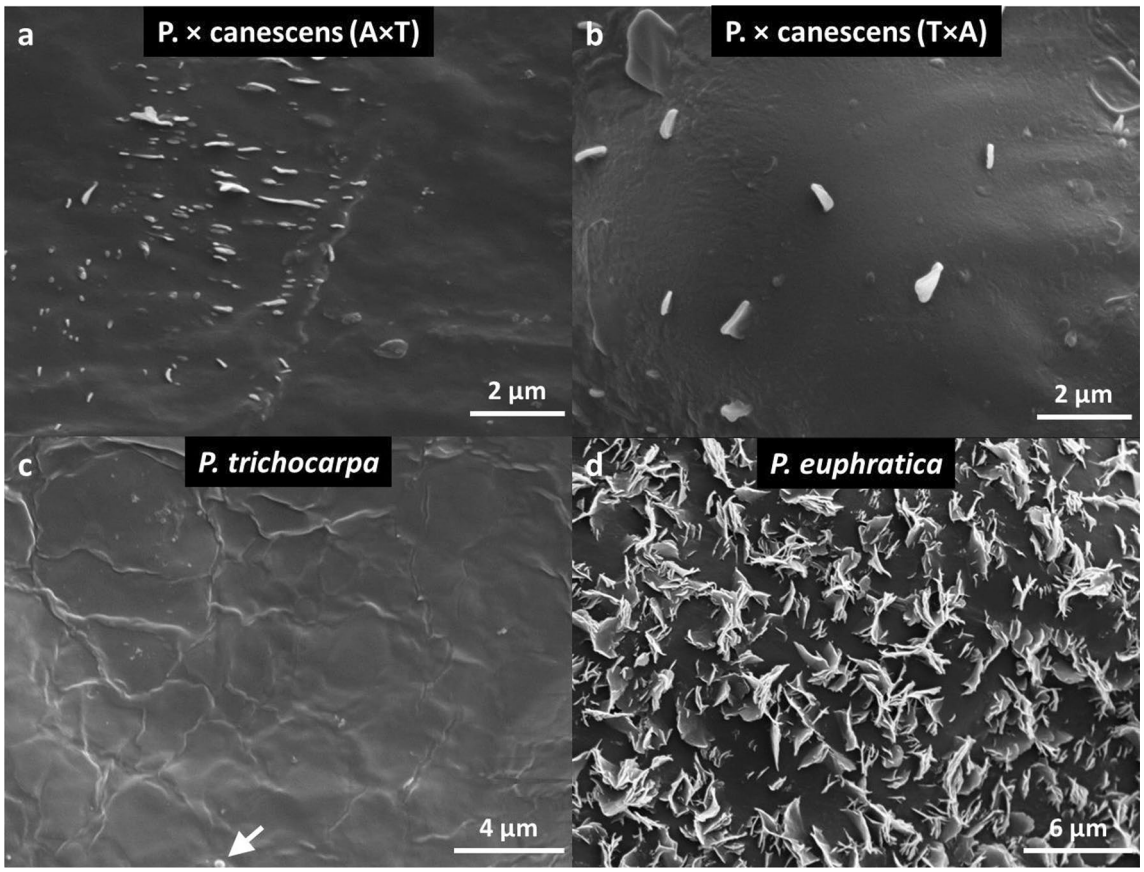


Fig. 4 Scanning electron pictures of adaxial leaf surfaces of 11-week-old poplar leaves. Clones of *P.* × *canescens* do show a low abundance of wax granules (**a** A × T; **b** T × A). The surface of *P. trichocarpa* (**c**) is smooth with very few wax crystals (arrow). *P. euphratica* is densely covered with wax platelets (**d**)

Cuticular wax composition

Five substance classes of linear long-chain aliphatic compounds (acids, aldehydes, alcohols, alkanes, and esters) of cuticular waxes were identified in 5-week-old leaves of both hybrids of *P.* × *canescens* and *P. trichocarpa* (Fig. 5a–c). Cuticular wax of *P. euphratica* was composed of only the three substance classes acids, aldehydes, and alcohols. Alkanes and esters were completely missing (Fig. 5d). Chain lengths of the esters ranged from C34 to C46, chain lengths of the 4 other substance classes ranged from C20 to C31 (Fig. 5). Alcohols, alkanes, and esters were the three prominent substance classes in both *P.* × *canescens* hybrids (Fig. 5e). In *P. trichocarpa*, only alcohols and alkanes were the two most prominent substance classes, whereas only alcohols were the most prominent substance class in *P. euphratica* (Fig. 5e). Total amounts of wax varied between 24 and 41 µg cm^{-2} with one of the *P.* × *canescens* clones (A × T) having the lowest wax amount and the other *P.* × *canescens* clone (T × A) having the highest wax amount (Fig. 5e).

The qualitative wax composition did not significantly change over the season with increasing leaf ages (5 weeks to 11 weeks and 17 weeks) of leaves formed in spring and with 5-week-old leaves formed in summer (Fig. 6b, d, f, h). There was a tendency that total amounts of wax in hybrid A × T (24, 22, and 19 µg cm^{-2}) and *P. trichocarpa* (27, 20, and 14 µg cm^{-2}) continuously decreased from 5-week- over 11-week- to 17-week-old leaves (Fig. 6a, c). Five-week-old leaves of all four clones newly formed in summer had about 1.5- (A × T) to twofold (T × A, P. tri, and P. euph) lower wax amounts compared to 5-week-old leaves formed in spring (Fig. 6a, c, e, g).

Stomatal and residual (cuticular) transpiration

Stomatal conductances of leaves harvested in May and September were between 250 and 350 mmol m^{-2} s^{-1}, which equals 75–115 × 10^{-9} m s^{-1} (Fig. 7). After leaf abscission, permeances (m s^{-1}) steeply decreased by 1–2 orders of magnitude (Fig. 7). The linear region between relative water deficits of 0.1 to 0.5 represents residual (cuticular) transpiration.

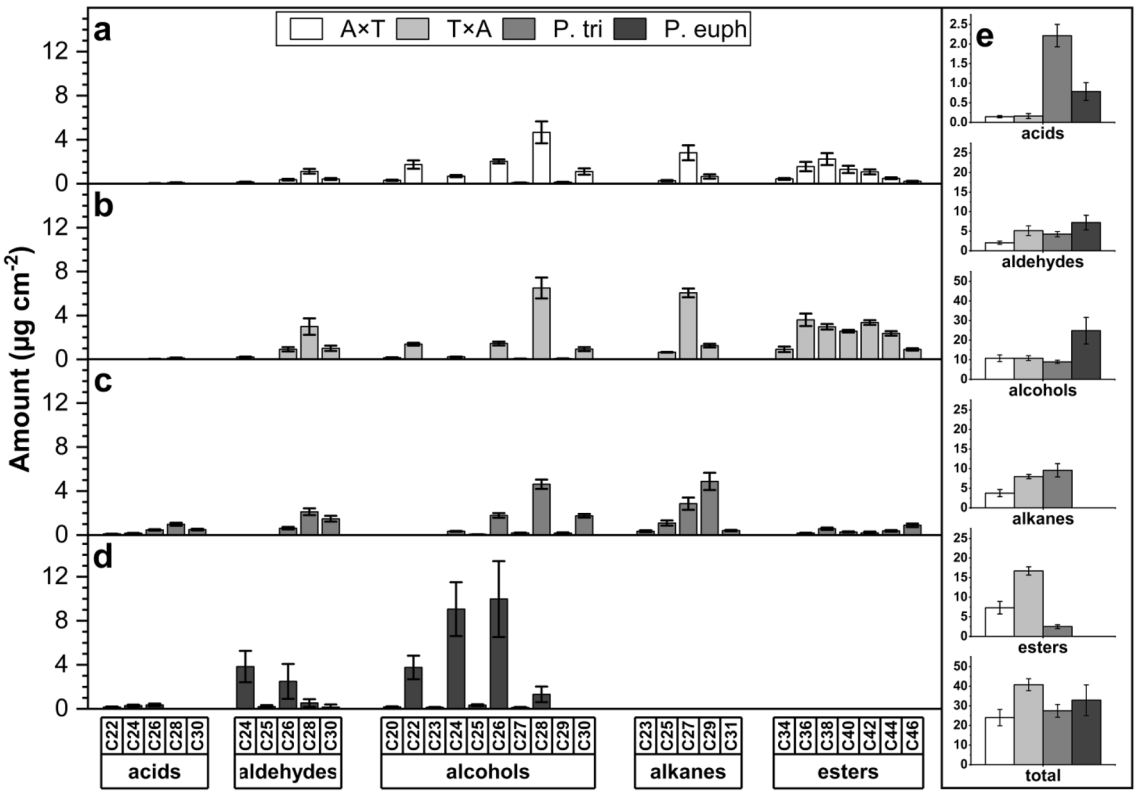


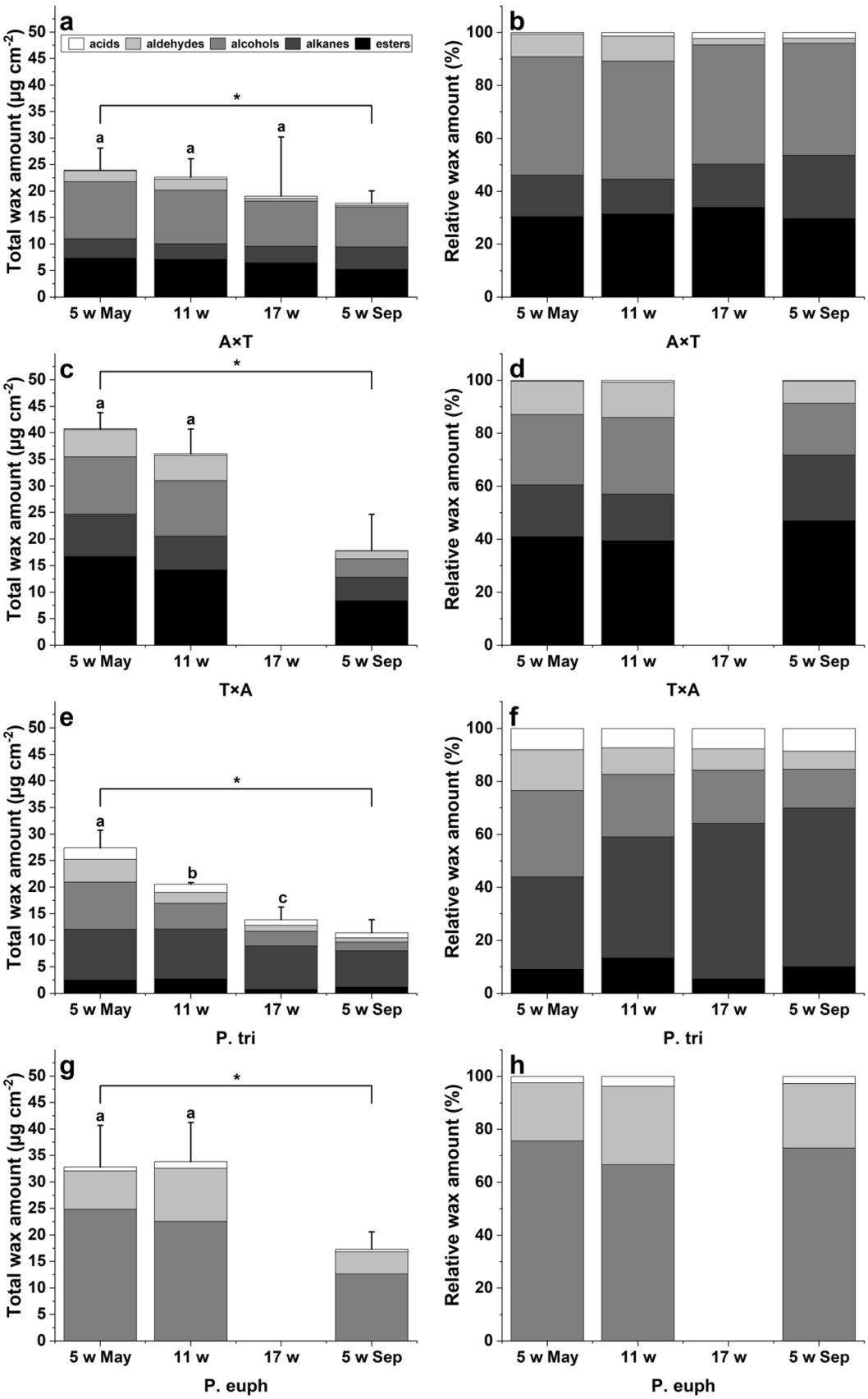
Fig. 5 Qualitative (**a**, **b**, **c**, and **d**) and quantitative (**e**) composition of adaxial cuticular wax of the 4 poplar clones (A×T and T×A: *P.*×*canescens*; P. tri.: *P. trichocarpa*; and P. euph.: *P. euphratica*). Cuticular wax was extracted from 5-week-old fully developed leaves and the qualitative wax composition is representative of all measured leaf cohorts. Means with standard deviations ($n=4$–6 leaves) are shown

All values on the plateaus, between relative water deficits ranging from about 0.1 to 0.5, were used to calculate the permeance means of 5-week-old leaves of each clone, harvested in spring (May) and summer (September) (Fig. 8). Mean residual permeances varied between 1.7 to 1.9×10^{-9} m s^{-1} (T×A and A×T) and 10 to 11.3×10^{-9} m s^{-1} (P. tri and P. euph) for leaves harvested in spring. In most instances, lower permeances were calculated for leaves harvested in summer. They were 0.9 to 2.4×10^{-9} m s^{-1} for T×A and A×T and 4.8 to 6.5×10^{-9} m s^{-1} for *P. trichocarpa* and *P. euphratica*, respectively (Fig. 8).

Discussion

As the spring bud break of a full-grown poplar tree is under tight genetic control and needs a specific temperature sum for initiation (Jansson and Douglas 2007), parallel leaf development leads to all primary leaves being almost the same age at any given date. Leaf morphology of both *P.*×*canescens* clones appears to be intermediate between those of parent species *P. alba* and *P. tremula* (Eckenwalder 1996; Isebrands and Richardson 2014; Lexer et al. 2005) and also morphological parameters of *P. trichocarpa* and *P. euphratica* leaves are in accordance to those previously reported (Al Afas et al. 2007; Calagari et al. 2006; Ceulemans et al. 1987; Liu et al. 2015; Xu et al. 2016). The succulent, lanceolate leaves of *P. euphratica* observed here are known to fulfill a water storage function especially in young trees that do not yet develop into the reproductive phase (Ottow et al. 2005; Xu et al. 2016). This higher water content obviously represents an adaptation to the hot and dry climate where *P. euphratica* is naturally growing. Amphistomaty of *P. euphratica* leaves is broadly reported as a key morphological trait (Liu et al. 2015; Mirzaie-Nodoushan et al. 2015; Zheng et al. 2007; Zhou et al. 2010) potentially to compensate for thicker leaf laminas and reduce distances of diffusion into the tissue. In *P. trichocarpa*, which normally is hypostomatic, amphistomaty is hypothesized to be introduced by hybridizations (Al Afas et al. 2006, 2007; Ceulemans et al. 1984; Dillen et al. 2008; Dunlap and Stettler 2001; Gornall and Guy 2007; McKown et al. 2014; Schulte and Hinckley 1987).

Each cuticular surface structure observed in this study is in concordance with previous reports in the literature (Alfaro-Tapia et al. 2007; Guzmán et al. 2014; Huang et al.


a
acids
aldehydes
alcohols
alkanes
esters
Total wax amount (μg cm-2)
5 w May
11 w
17 w
5 w Sep
A×T
b
Relative wax amount (%)
A×T
c
T×A
d
T×A
e
P. tri
f
P. tri
g
P. euph
h
P. euph

◂**Fig. 6** Absolute (**a**, **c**, **e**, **g**) and relative (**b**, **d**, **f**, **h**) amounts of adaxial leaf wax over the growing season of 2020 (A × T and T × A: *P.* × *canescens*; P. tri.: *P. trichocarpa*; and P. euph.: *P. euphratica*). Means with standard deviations ($n = 4$–6) of the total wax amounts are shown. Differential letters (ANOVA) and asterisks (*t* test) indicate significant differences at $p \leq 0.05$

2018; Meng et al. 2019; Schreiber et al. 2006; Szuba et al. 2019; Xu et al. 2016; Zheng et al. 2007) but to our knowledge, their influence on wettability has not been reported for poplar before. The observed low abundance of wax granules of both *P.* × *canescens* clones and *P. trichocarpa* resulted in moderate contact angles varying between 70° and 90°. In contrast to this, the very rough leaf surface with dense wax platelets of *P. euphratica* resulted in contact angles of almost 160°. On such a superhydrophobic surface (Wang and Jiang 2007) water immediately forms spherical droplets that will roll off even at the lowest angles of tilt, commonly referred to as the "Lotus effect" (Barthlott and Neinhuis 1997). This is typically associated with self-cleaning abilities of plants when subjected to rain (Barthlott and Neinhuis 1997). However, this property appears to be useless if the plant is growing in environments with yearly precipitations close to zero. Instead, we suggest that these wax platelets on *P. euphratica* leaves must accomplish a different function. In a hot and dry habitat pronounced three-dimensional epicuticular wax crystalloids should help in efficiently reflecting light and thereby protect leaves from heat and harmful UV radiation (Shepherd and Griffiths 2006). The cuticular wax morphology of *P. euphratica* is dominated by wax platelets, which have been described to be formed by primary alcohols (Barthlott et al. 1998; Ensikat et al. 2006; Koch and Barthlott 2006) that are also representing the major substance class in *P. euphratica* wax as it was found here. For example barley, a member of the *Graminaceae* family also originating from arid regions, is known to exhibit a comparable epicuticular wax structure and alcohol-dominated wax chemistry (Richardson et al. 2005). For the other three clones, the formation of epicuticular wax crystalloids by self-assembly (Koch et al. 2004) may be less favored, as they are composed of a broader mixture of substance classes. Nonetheless, wax crystals on adaxial sides of *P. trichocarpa* leaves have also been reported to consist of mainly alcohols, as analyzed by secondary ion mass spectrometry (SIMS) (Kulkarni et al. 2018).

In our study, both clones of *P.* × *canescens* had a very similar wax composition which was dominated by alcohols, alkanes, and esters. This is different from a previous analysis of the wax composition of clone A × T where wax esters were completely missing, but considerably longer chain lengths (C31 to C35) of all other functional groups were described (Meng et al. 2019). These differences in composition and quantity of each substance class might be attributed to different environmental growth conditions and leaf ages investigated. But differences might also arise from the methodological approaches used. Unlike our approach using on-column injection for cuticular wax analysis, a split/splitless injection for GC analysis will not recover long-chain wax esters since they will be trapped in the liner of the injector. Similar methodological limitations were encountered when analyzing cuticular waxes of *P. trichocarpa* since in a qualitative isotope ratio mass spectrometry (IRMS) analysis only alkanes were described as the wax fraction (Kahmen et al. 2011). However, the alkanes were very similar to the alkanes found in our study. In addition, a MALDI-TOF-MS analysis after cryo-adhesive-isolation of epicuticular waxes of *P. trichocarpa* identified 19 individual compounds belonging to the functional groups of alcohols, alkanes, and esters (Kulkarni et al. 2018), again sharing a high similarity to our own results. Further qualitative and quantitative differences are encountered when comparing the leaf wax composition of *P. euphratica* trees cultivated in Germany to plants grown outside in their natural habitat in Inner Mongolia (Xu et al. 2016). Acids, aldehydes, and alcohols but not aldehydes and esters were identified as main substance classes when investigating juvenile (lanceolate) leaves grown in Germany in our study, whereas considerable amounts of alkanes were detected after GC-FID/MS analysis of leaves sampled from their natural habitat in Mongolia (Xu et al. 2016). Reductions in wax amounts of older leaves (11 and 17 weeks) observed in our study are attributed to potential wax degradation. Abrasion or erosion of surface lipids has been reported (Cameron et al. 2006; Hauke and Schreiber 1998; Neinhuis and Barthlott 1998), especially if plants are grown outside where mechanical stimuli as wind and rain are significantly higher (Shepherd and Griffiths 2006). When leaves of the exact same age (5-week-old) that developed in spring (May) or summer (September) were compared, significantly lower total wax amounts were found for the summer leaves of all 4 clones. We hypothesize that a much lower irradiance in the second growth period of the year might be one of the key factors leading to a reduced wax accumulation (Baker 1974).

Values of permeances measured here for intact poplar leaves are in a similar range when compared to permeances of intact leaves, leaf disks, or isolated astomatous cuticular membranes of different species (Kirsch et al. 1997; Schreiber 2001; Schreiber and Riederer 1996; Schreiber and Schönherr 2009). The hypostomaty of *P.* × *canescens* offers the advantage of isolating astomatous cuticles of the adaxial leaf side. This allows measuring true rates of cuticular transpiration for *P.* × *canescens* (Schreiber et al. 2006). Permeances of *P.* × *canescens* clones measured here vary between 0.9 and 2.4×10^{-9} m s^{-1} and they are very similar to permeances of 2.7×10^{-9} m s^{-1} measured with isolated astomatous *P.* × *canescens* cuticles from a mature

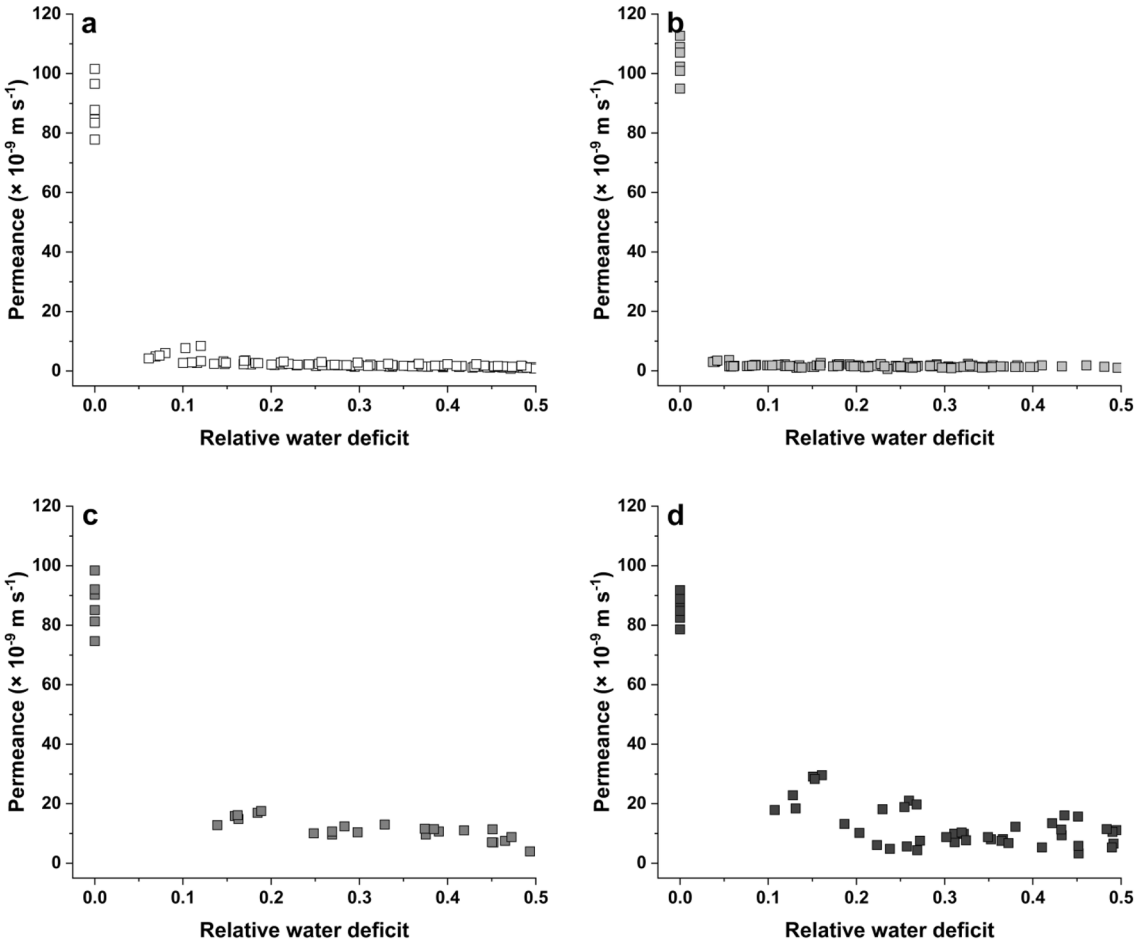


Fig. 7 Representative stomatal and residual (cuticular) transpiration measured with 5-week-old leaves harvested in May (A × T and T × A: *P.* × *canescens*; P. tri.: *P. trichocarpa*; and P. euph.: *P. euphratica*). Stomatal transpirations of leaves (shown at relative water deficits of 0) were measured in the field. Residual (cuticular) transpirations were measured with detached leaves at relative leaf water deficits between 0.1 and 0.5. Single values of 6 leaves per clone are shown

grey poplar tree growing outside in Bonn (Schreiber et al. 2006). This is a very good indication that residual rates of transpiration measured with intact *P.* × *canescens* leaves are in fact reflecting cuticular rates of transpiration. Different from both *P.* × *canescens* clones, it was described that some clones of *P. trichocarpa* are unresponsive in stomatal closure towards stress (Schulte and Hinckley 1987), and also for *P. euphratica* stomata have been reported to show a non-uniform behavior (Zheng et al. 2007). This described incomplete closure of stomata of *P. trichocarpa* and *P. euphratica* might explain why residual permeances were about 10 times higher compared to both *P.* × *canescens* clones. Thus, residual transpiration measured with *P. trichocarpa* and *P. euphratica* might to some extent represent overestimations of the true cuticular transpiration (Burghardt and Riederer 2003; Kerstiens 1996), whereas those measured for both clones of *P.* × *canescens* fit very well to the previously reported cuticular transpiration (Schreiber et al. 2006).

When comparing both sets of 5-week-old leaves grown in spring and summer, it is evident that the leaves of three of the four poplar clones harvested in September show twofold lower permeances, even though total wax amounts of all clones are between 1.5- and twofold lower. This is a further example that higher wax amounts do not necessarily correlate with lower rates of cuticular transpiration and vice versa (Jetter and Riederer 2016; Sánchez et al. 2001; Schreiber and Riederer 1996; Zeisler-Diehl et al. 2018). Instead of cuticular wax amounts, it has been suggested that the mean carbon chain length of wax molecules might represent a potential feature negatively correlating with the transpiration properties of cuticles (Hauke and Schreiber 1998; Leide et al. 2007; Macková et al. 2013; Riederer and Schneider 1990). Except for one *P.* × *canescens* clone (A × T), lower rates of residual (cuticular) transpiration of leaves harvested in summer were in fact observed alongside higher weighted mean carbon chain lengths of the cuticular wax (Fig. 9). Since

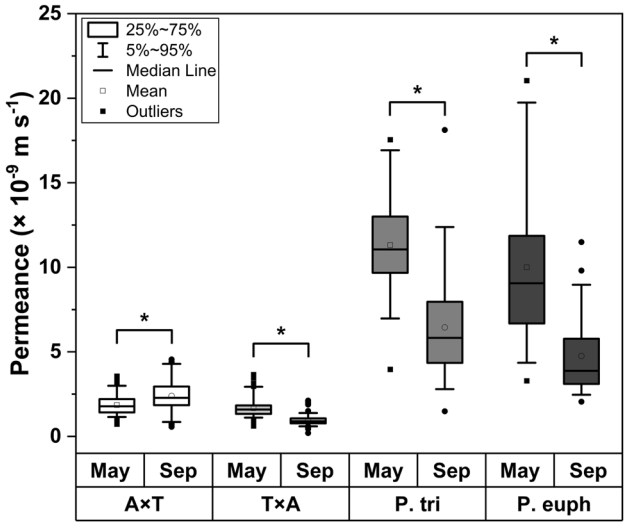


Fig. 8 Residual (cuticular) transpiration of detached 5-week-old leaves harvested in May and September (A×T and T×A: *P.*×*canescens*; P. tri.: *P. trichocarpa*; and P. euph.: *P. euphratica*). Boxplots are based on single permeances ($n = 23–106$) measured at relative leaf water deficits between 0.1 and 0.5 (Fig. 7). Significant differences at $p \leq 0.05$ are indicated by asterisks (*t* test)

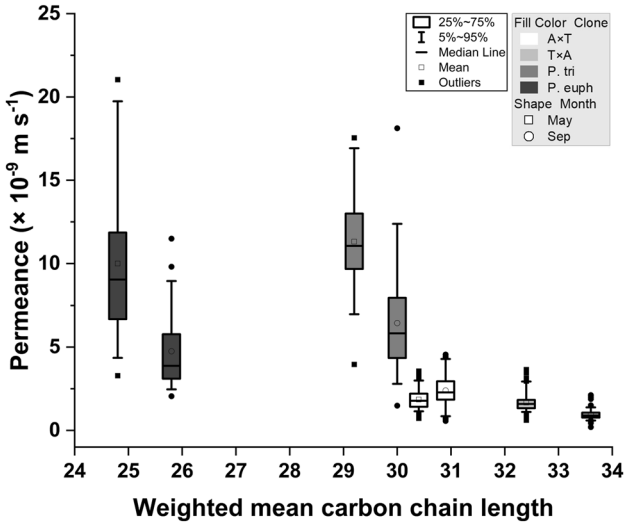


Fig. 9 Residual (cuticular) transpiration of detached 5-week-old leaves harvested in May and September as a function of the weighted mean carbon chain length of the cuticular wax (A×T and T×A: *P.*×*canescens*; P. tri.: *P. trichocarpa*; and P. euph.: *P. euphratica*). Boxplots are based on single permeances ($n = 23–106$) measured at relative leaf water deficits between 0.1 and 0.5 (Fig. 7)

P. euphratica is growing in an arid and hot climate, it was initially hypothesized by us that *P. euphratica* should be characterized by a considerably lower residual (cuticular) transpiration compared to *P.*×*canescens* and *P. trichocarpa*. But the typical xeromorphic adaptations of *P. euphratica* such as smaller and succulent leaves, which can help to survive after long periods of stomatal closure in response to stress (Bueno et al. 2019), are obviously more important than low rates of residual (cuticular) transpiration. In addition, roots of *P. euphratica* in their natural environment must have constant contact with the groundwater table (Aishan et al. 2015; Chen et al. 2006; Zhou et al. 2010), making true water limitations even in an arid climate a less abundant scenario. As a consequence, this will allow keeping both, stomatal and residual rates of transpiration high, which is essential in cooling the leaves in a hot and dry habitat (Lange 1959).

Conclusion

In terms of leaf morphology, wax composition, and residual (cuticular) transpiration both clones of *P.*×*canescens* are not very different, which ensures good comparability of ecophysiological data acquired with either clone. The genetically more distant *P. trichocarpa* and *P. euphratica* (Cervera et al. 2005) had different leaf morphologies, cuticular wax compositions, and significantly higher residual (cuticular) transpiration rates. Physiological transport experiments that might require isolated astomatous cuticles can be performed with clones of *P.*×*canescens*. In addition, highly efficient transformations of both parent species of *P.*×*canescens* (Fladung et al. 1997; Ma et al. 2019) make this hybrid a valuable candidate for the creation and investigation of different mutant lines in the future, whereas *P. trichocarpa* is the best genetically characterized *Populus* species to date and may serve as a reference for extensive genome studies. In contrast, the environmentally driven specialization of cuticular wax structure and composition together with a fairly high residual (cuticular) transpiration of *P. euphratica* make it an interesting species for future stress-physiological studies.

Author contribution statement LS and PG designed the experiments. LH and PG performed the experiments. LS supervised the experiments. LS, PG, and LH analyzed the data. PG wrote the manuscript. LS and LH revised the manuscript. All authors read and approved the final manuscript.

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Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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