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Variations of cuticular wax in mulberry trees and their effects on gas exchange and post-harvest water loss

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Abstract Though mulberry (*Morus alba*) tree shows great adaptations to various climate conditions, their leaf water status and photosynthesis are sensitive to climate changes. In the current study, seven widely planted mulberry cultivars in Chongqing, Southwest China, were selected to analyze leaf cuticular wax characteristics, gas exchange index, post-harvest leaf water status and their relationships, aiming to provide new theory in screening high resistant mulberry cultivars. Mulberry trees formed rounded cap-type idioblasts on the adaxial leaf surface. Film-like waxes and granule-type wax crystals covered leaf surfaces, varying in crystal density among cultivars. The stomatal aperture on the abaxial surface of cultivars with high wax amount was smaller than that of cultivars with low wax amount. The amount of total wax was negatively correlated with the net photosynthetic rate (P_N), transpiration rate (E) and stomatal conductance (g_s) and positively correlated with the moisture retention capacity. It suggested that both cuticular wax and stomatal factor might be involved in regulating water loss in mulberry leaves under field conditions. The variability in moisture retention capacity and cuticular wax characteristics might be important in evaluating and screening mulberry cultivars for increasing silk quality and silkworm productivity.

Keywords Cuticular wax · Gas exchange index · Leaf water status · Mulberry (*Morus alba*) · Resistance

Introduction

Mulberry (*Morus alba*), as an important economic plant, is widely distributed in China from the North to the South, including 15 species and 4 subspecies. It is mainly used for silk production, food production, livestock production, and soil and water conservation with its developed root system. It shows great adaptations to various climate conditions from arid to humid and temperate to tropic. However, its leaf water status is sensitive to soil moisture conditions and drought stress might severely influence its production potential (Reddy et al. 2004). The growth of mulberry trees will be inhibited when subjected to drought stress (Huang et al. 2013). Apparent photoacclimatory changes were recorded in drought-exposed mulberry plants including decrease in electron transport and enhanced thermal dissipation from PS II (Guha and Reddy 2014).

Under certain climate conditions, mulberry leaf should be under strong selection to maximum physiological function in a plant's habitat (Agrawal et al. 2009). Mulberry plants might rapidly reduce photosynthesis via stomatal closure and thus minimize water loss and prevent cellular damage (Kottapalli et al. 2009). Stomatal closure is the earliest response to drought and the dominant limitation to photosynthesis at mild to moderate drought, protecting the plants from extensive water loss (Flexas and

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Medrano 2002). In drought-stressed trees, the rates of change in stomatal conductance tended to differ from the theoretical summed rates more than in well-watered trees (Aasamaa and Sober 2011). However, when the availability of soil water is strongly reduced, the only way for a higher plant to survive is to conserve sufficient amounts of water in the tissue, and the main pathway for transpiration is then through the cuticle (Bengtson et al. 1978). As one of the components of cuticle, cuticular wax has also been shown to be related to leaf water status.

Cuticular wax is the outermost of aerial plant tissues and consists of a very heterogenous mixture of lipophilic substances (Yeats and Rose 2013). It plays important roles in protecting plants against biotic and abiotic stresses (O'Toole et al. 1979). Induction of cuticle lipids was associated with reduced cuticle permeability and might be important for plant acclimation to subsequent water-limited conditions (Kosma et al. 2009). Compared to well-irrigated plants, drought treatment caused an increase in wax amount on most sesame cultivars (Kim et al. 2007). Under the water stress conditions, greater mean epicuticular wax load in barley plants favored their tolerance of drought and improved their yields (Gonzalez and Ayerbe 2010). The chemistry of the wax constituents was shown to be an important factor to determine the degree of resistance to evaporation (Oliveira et al. 2003). Cultivation at 20–30 % RH led to increased total amounts of wax and reduced surface wettability, and the wax crystal morphology and qualitative wax composition of *Brassica* were altered after cultivation at 98 % RH (Koch et al. 2006). Jordan et al. (1984) found that epicuticular wax greater than about 0.067 g m^{-2} provide an effective barrier to water loss through cuticles of sorghum leaves under most conditions. However, no consistent relationship was found between epicuticular wax production and cuticle transpiration as estimated by water loss rate of excised leaves of alfalfa and crested wheatgrass (Jefferson et al. 1989). Guo et al. (2011) found that wax crystalloid platelets in alfalfa melted and dispersed over leaf surface, decreased transpiration rate, and resulted in a reduction of water loss under low air humidity. In addition to total wax amount, the crystallization pattern of epicuticular wax deposition also affects surface hydrophobicity (Koch and Ensikat 2008). These studies implied that both wax composition and crystal structure might influence water loss from leaf surface.

Cuticular wax on leaf surface might also influence stomatal status and gas exchange parameters. Holroyd et al. (2002) reported that the composition of the epidermal wax influenced the degree of stomatal development in the leaf epidermis. Increasing the air exchange rate for in vitro-grown carnation plants caused increases in epidermal waxes and reductions in stomatal density (Majada et al. 2001). Induction of cuticular wax on *Arabidopsis*

conferred tolerance to drought and recovery from drought, and was correlated with reduced numbers of stomata (Yang et al. 2011). These studies suggested that cuticular wax might also be involved in controlling water loss from leaf surface by influencing stomatal status under drought stress.

Morphology and chemical composition of cuticular wax on mulberry leaf surface might be an adaptation to local climate. Mamrutha et al. (2010) investigated the post-harvest leaf moisture retention capacity and wax characteristics of 290 mulberry accessions in India and found that accessions having elevated leaf surface wax amount and crystal size and density exhibited reduced leaf post-harvest water loss. Cameron et al. (2002) reported that *Salix* species and *Populus* species grown under the same environmental conditions produced measurably different cuticular waxes and that regulation of wax production appeared to be different in each species, suggesting that the characteristics of the cuticular wax might prove to be useful selectable traits in a breeding program. Therefore, evaluating mulberry cultivars according to characteristics of cuticular wax on leaf surface might be an alternative approach for screening cultivars tolerant to abiotic stresses. Mulberry trees grown in Chongqing, Southwest China, are often confronting seasonal drought in spring and summer and high temperature stresses in summer. In the current study, we selected seven mulberry cultivars widely planted in this area to evaluate their difference in wax characteristics, gas exchange index, and leaf water status under field conditions. The following three aspects were addressed: (1) Variations of cuticular wax among different mulberry cultivars; (2) Are the amount of total wax and wax constituents and crystal structure related to gas exchange index? (3) What is the relationship between cuticular wax and the post-harvest water loss of mulberry leaves?

Materials and methods

Plant material

The experiment was conducted in Chongqing Sericulture Science and Technology Research Institute of China, where a collection of about 200 mulberry germplasms around the world was cultivated. In the current study, seven cultivars widely used for silkworm were used to measure leaf water index, gas exchange index and cuticular waxes. The seven cultivars included Kanva-2 (KV) from Sri Lanka, Xinyizhilai (XY) from Japan, Yusang101 (YS) from Chongqing, China, Shanxitiansang (SX) from Shanxi, China, Junyou5 (JY) from Sichuan, China, Husang 32 (HS) from Zhejiang, China, and Tongxiangqing (TX) from Zhejiang, China. The plants were 15-year-old and cut three times every year. At the time of sampling, the height of the

twig ranged from 2–3 m. The 20-year daily average lowest and highest temperature were 6 and 10 °C in January, 19 and 27 °C in May, 25 and 33 °C in July, and 16 and 22 °C in October, respectively. The average annual rainfall is 1208 mm. The measurements were done in May, 2012. The plants were cultivated in rows and we separated the rows into three plots for each cultivar according to plant positions. Each plot represented one replicate. All measurements were done in three plants for each replicate.

Gas exchange measurements

Gas exchange measurements were taken with a portable photosynthesis measurement system *LI-6400* (*LI-COR*, Lincoln, NE, USA). The value of net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were the means of 6 individual measurements in each replicate. Only the eighth to tenth leaves from the top of branches (recently matured leaf) without disease lesion were selected. Measurements were conducted before midday between 09:30–11:30 with light intensity of PAR 1000 μmol m^{-2} s^{-1}. Throughout the whole measurements, the atmospheric CO_2 concentration was 420 μmol mol^{-1} and the leaf temperature was 30–35 °C. The relative air humidity was 60–70 %.

Estimation of leaf moisture retention capacity (MRC)

For MRC measurement, same plants were selected as in gas exchange measurements in each replicate. In total, six leaves between 8th to 10th positions were randomly collected in each replicate early in the morning (08:30–9:30 a.m.) and the fresh weight (FW1) was recorded immediately. The leaves were air-dried for 5 h, weighed second time (FW2), and then dried to a constant weight (DW) at 75 °C for 48 h. The leaf moisture retention capacity (MRC) was estimated using the following formula, MRC (%) = $\{(FW_2 - DW)/(FW_1 - DW)\} \times 100$.

Cuticular wax extraction and analysis

Six fresh leaves were sampled in each replicate, dipped in 50 ml of chloroform containing 0.25 μg of hexadecane (Sigma) as internal standard for 30 s at room temperature. The wax extractions were dried under nitrogen stream, derivated with 100 μl of BSTFA (*N,O*-bis (trimethylsilyl) trifluoroacetamide) for 1 h at 80 °C, and the surplus BSTFA was evaporated under nitrogen. The extract was redissolved in 1 ml of hexane for wax analysis using GC-2010 (Shimadzu Technologies Co., Japan) equipped with a flame ionization detector (FID) as described by Kim et al. (2007). The GC column was a 30 m × 0.25 mm × 0.2 μm

DM-5 capillary column and the carrier gas was nitrogen. The injector and FID detector temperatures were set at 300 and 320 °C, respectively. The oven temperature for the GC was programmed with an initial temperature of 80 °C and increased at 15 °C min^{-1} to 260 °C, where the temperature remained unchanged for 10 min. The temperature was then increased at 5 °C min^{-1} to 320 °C, where the temperature was held for 15 min. Quantification was based on FID peak areas and internal standard. Compound identification was based on co-injection with commercial standards and analysis of a subsample in a GC/MS-GP2010. The total amount of unknown constituents was calculated from the cumulative peak areas for all unidentified peaks. Total wax amount was expressed as micrograms per total leaf area (μg cm^{-2}). After wax extraction, the leaf was scanned with an Epson Expression/STD 4800 Scanner and the leaf area was measured using WinFOLIA leaf analysis software (Regent, Quebec, Canada).

Scanning electron microscopy (SEM)

Leaves from each replicate were sampled, air-dried, and then affixed to an aluminum stub with double-sided adhesive tape. Stub was coated with gold and placed in the low-vacuum, variable pressure chamber of the Hitachi S3500 Scanning electron microscopy and photographed with a digital camera at approximately 5000× magnification.

Data analysis

The data obtained were subjected to analysis of variance (ANOVA) using GenStat Release 13.0. The contents of total wax and wax constituents, gas exchange index and leaf moisture retention capacity were tested by a one-way ANOVA analysis. Mean values were separated by protected Fisher's least significant difference at $P < 0.05$. The Pearson correlations between wax constituents, gas exchange index and leaf moisture retention capacity were determined using SPSS 14.0.

Results

Crystal structure of cuticular wax on mulberry leaves

Film-like waxes and irregular granule-type wax crystals covered the leaf surfaces of all mulberry cultivars (Fig. 1). Granule-type wax crystal existed mainly on adaxial leaf surfaces of the tested cultivars except HS and TX where Granule-type wax crystal could be found on both abaxial and adaxial surfaces. Cultivar XY and SX had relatively higher density of wax crystals compared to the other

Fig. 1 Cuticular wax crystallization patterns on adaxial and abaxial leaf surface of mulberry trees viewed by scanning electron microscope at 50 and 10 μm. **a** Tongxiangqing (TX), **b** Xinyizhilai (XY), **c** Yusang 101 (YS), **d** Husang 32 (HS), **e** Shanxitiansang (SX), **f** Kanwa-2 (KV), **g** Junyou 5 (JY), *1* adaxial surface at 50 μm, *2* adaxial surface at 10 μm, *3* abaxial surface at 10 μm
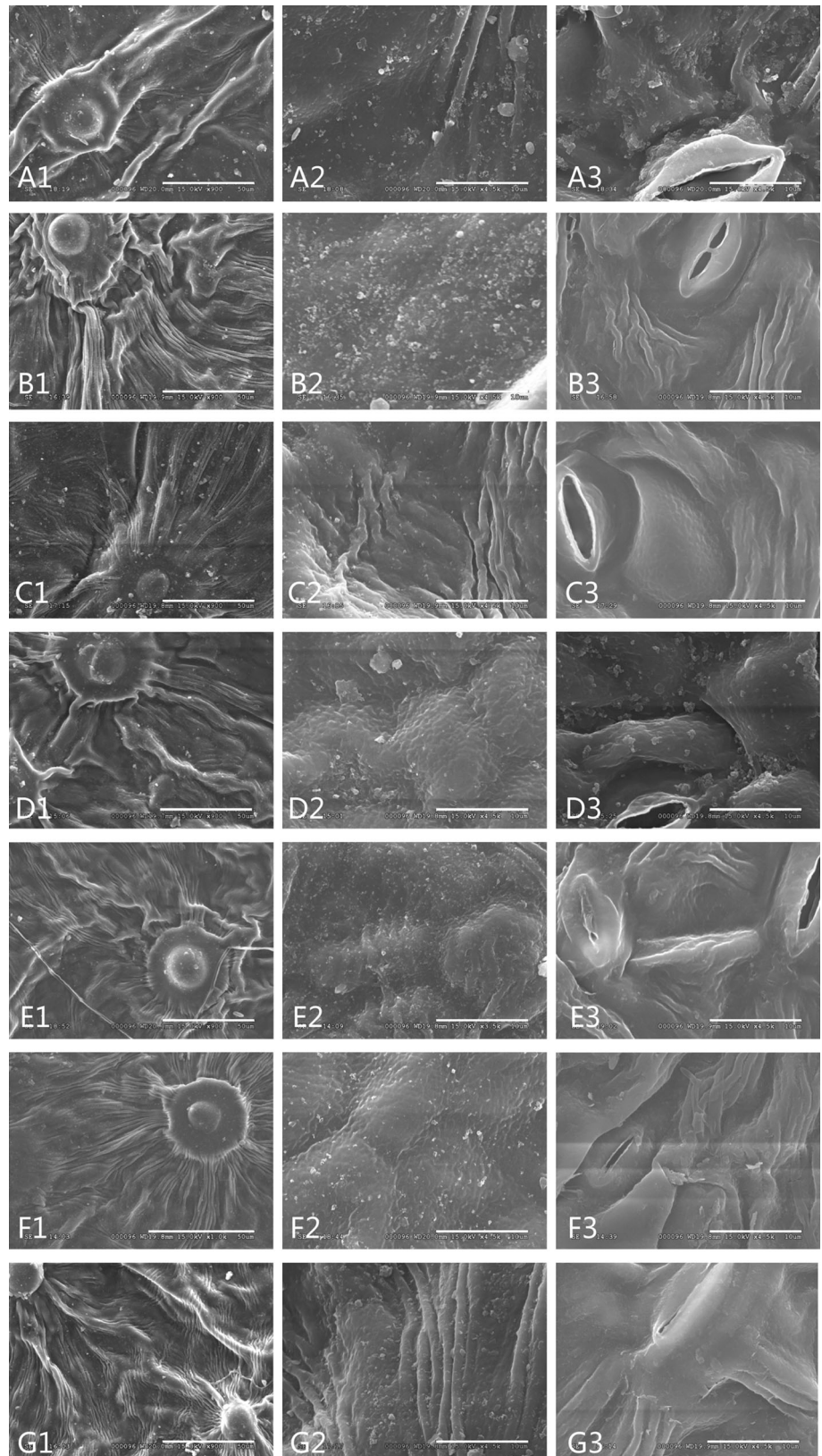
cultivars. No apparent difference in wax morphology was observed among cultivars. Some rounded cap-type idioblasts were observed only on the adaxial surface of all tested mulberry leaves. Stomata mainly distributed on the abaxial leaf surface and no stomata could be found on adaxial surface. The stomatal aperture was apparently different among the tested cultivars and some stomata on SX were almost covered by film-like wax.

Cuticular wax contents and constituents

Across all 7 mulberry cultivars, the average total amount of leaf cuticular wax was 248 µg dm^{-2} (Fig. 2). The identified wax components on mulberry leaves were mainly *n*-alkanes (6–30 % of total wax), alcohols (2–24 % of total wax), and esters (5–17 % of total wax). Aldehydes were only detectable in TX, KW and JY. Trace amounts of ketones were detectable in most cultivars except TX. A group of unknown constituents were quantified, and the amounts of unknown constituents of XY were significantly higher than that of the other cultivars. However, the amounts of *n*-alkanes and alcohols in XY were the lowest among all cultivars. Cultivar variations of the total amounts of cuticular wax were relatively high, with the highest for XY (325 µg dm^{-2}) and the lowest for TX (215 µg dm^{-2}) and HS (170 µg dm^{-2}).

Leaf gas exchange index

Among all 7 mulberry cultivars, the net photosynthesis rates (P_N), stomata conductance (g_s), intercellular CO_2 (C_i), and transpiration rates (E) in YS, HS and KW were significantly higher than those in other cultivars (Fig. 3). Correlation analysis indicated that the amount of total wax was negatively correlated with the P_N, g_s and E (Fig. 4). No significant correlation was observed between the amounts of wax constituents and gas exchange parameters.

Leaf moisture retention capacity (MRC)

There was significant variation among the MRC of mulberry cultivars, ranging from 46 to 72 % (Fig. 5). The MRC of XY, YS and KW was significantly higher than that of TX, HS and JY. Correlation analysis indicated that the amount of total wax was positively correlated with the MRC (Fig. 6).

Discussion

Many plants produce crystalline calcium deposits in specialized cells called idioblasts (Bauer et al. 2011). In the leaves of mulberry, calcium carbonate was deposited in large, rounded idioblast cells located in the upper epidermal layer of mulberry leaves (Nitta et al. 2006). In the current study, some rounded cap-type idioblasts were also observed on the adaxial surface of all tested mulberry leaves, which might play important role in Ca metabolism and the pathogen-defense mechanism (Sugimura et al. 1999). Though Sugimura et al. (1999) reported that the cystolith-containing idioblast morphology might be useful as a criterion for mulberry varietal classification, no difference in idioblast morphology was observed among the tested cultivars. In the study of Mamrutha et al. (2010), no cap-type idioblasts could be observed from the SEM pictures. This implied that, for the mulberry trees, the formation of cap-type idioblasts might be related to the environmental conditions and the cap-type idioblasts might be a common response of the tested cultivars under local field conditions.

The most common wax morphologies on plant leaf surface are thin films and several three-dimensional structures such as massive crusts, granules, plates, platelets, filaments, rods, and tubules with a hollow center (Koch and Ensikat 2008). In the current study, film-like waxes and granule-type wax crystals covered the mulberry leaf surfaces. Granule-type wax crystals are distributed on both abaxial and adaxial surfaces of HS and TX but only on abaxial surface for other cultivars. Though Gulz (1994) reported that the surface wax ultrastructures of deciduous trees differed from species to species according to wax composition, no obvious difference of wax composition was observed within the mulberry cultivars, except for relatively lower amount of wax in HS and TX.

The stomatal aperture on the abaxial leaf surface differed among the tested mulberry cultivars. The stomatal aperture was relatively smaller in high wax amount cultivars such as XY, SX and JY than in low wax amount cultivars such as HS and TX. Some stomata on SX were even covered by film-like wax (Fig. 1). This implied that wax on leaf surface might be involved in regulation of the stomatal aperture. The wax coverage at the entrance of stomata in *Leucadendron lanigerum* increased resistance to gas diffusion, resulting in decreased stomatal conductance, transpiration, and photosynthesis (Mohammadian et al. 2007). Epicuticular wax load helped in maintaining stomata regulation and leaf water relations, thus affording adaptation to wild *Arachis* species to thrive under water-limited environments (Nautiyal et al. 2008). Yang et al. (2011) reported that drought tolerance caused by the induction of WIN1/SHIN gene might be due to reduced numbers of stomata as well as to cuticular wax accumulation in *Arabidopsis*. In the current study, wax film covered the whole adaxial surface, resulting in invisible stomata on adaxial surface. This might protect leaf from direct strong radiation, particularly in hot summer
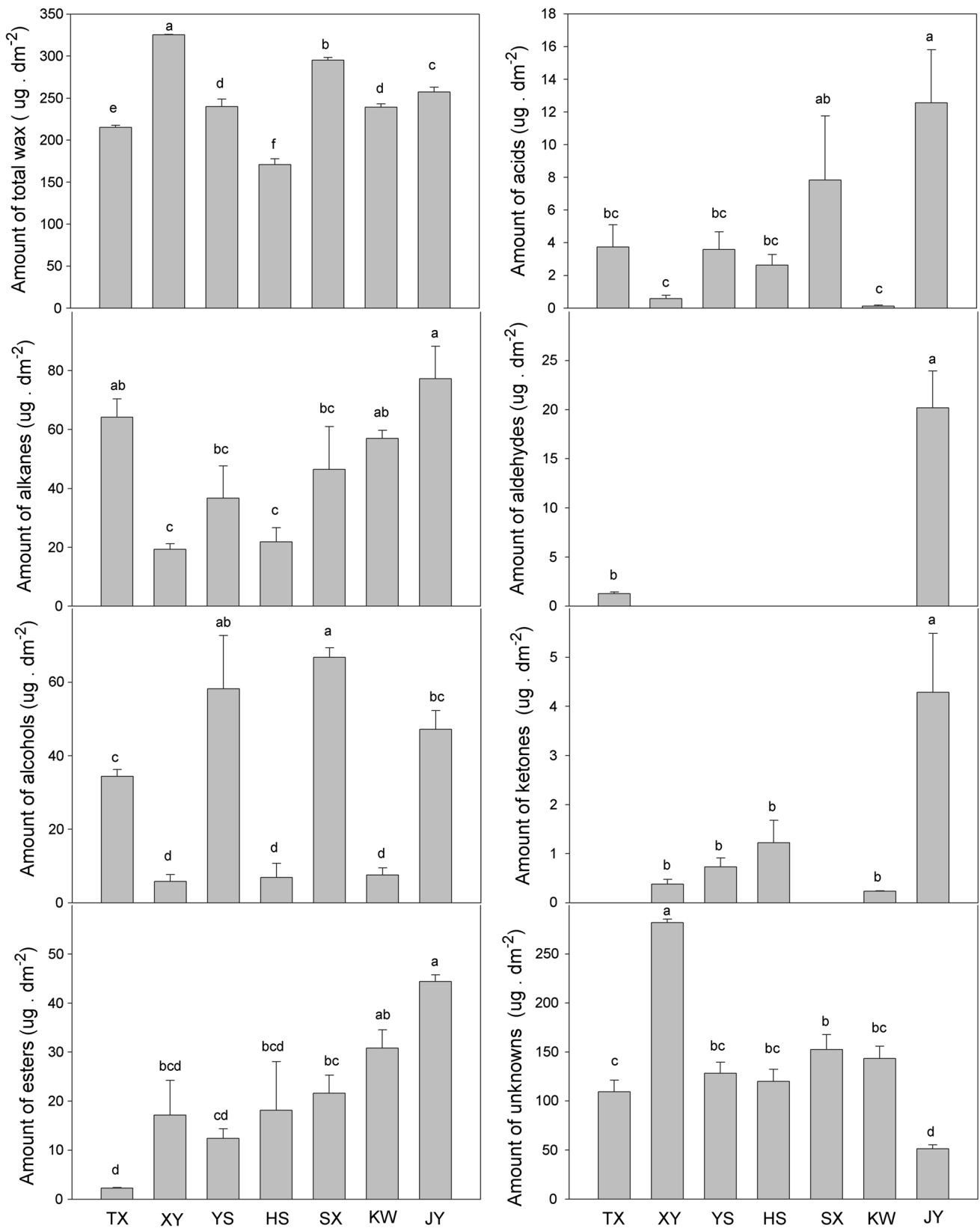
Fig. 2 Amounts of total cuticular wax and wax constituents on leaves of mulberry cultivars. *TX* Tongxiangqing, *XY* xinyihzilai, *YS* Yusang 101, *HS* Husang 32, *SX* Shanxitiansang, *KW* Kanwa-2, *JY* Junyou 5
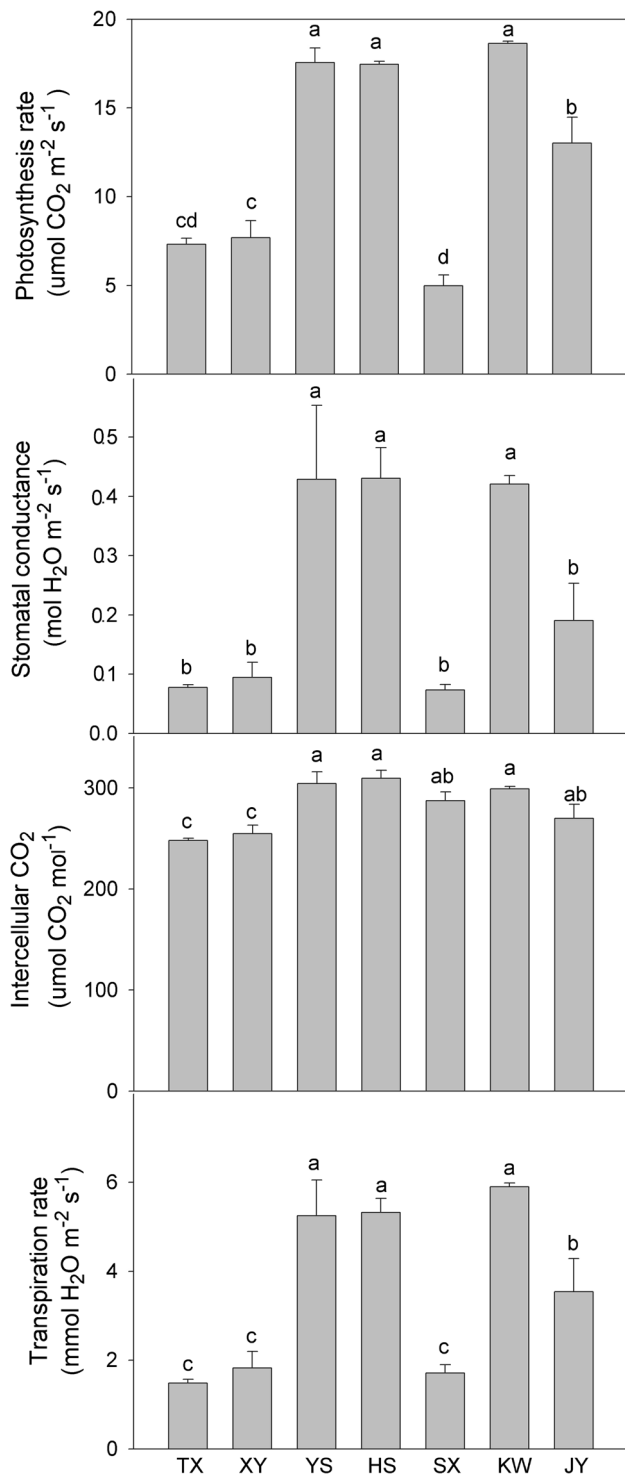
Fig. 3 Gas exchange index in leaves of mulberry cultivars. *TX* Tongxiangqing, *XY* xinyihzilai, *YS* Yusang 101, *HS* Husang 32, *SX* Shanxitiansang, *KW* Kanwa-2, *JY* Junyou 5

Fig. 4 Regression analysis between gas exchange index and total cuticular wax amount

(29–39 °C for 1–2 months) in Chongqing and reduce leaf water loss. Wax erosion induced by enhanced UV-B radiation resulted in different stomatal status on abaxial and adaxial leaf surface of *Brassica napus*, causing decrease of P_N, and increase of g_s, C_i and E in leaves (Ni et al. 2014).
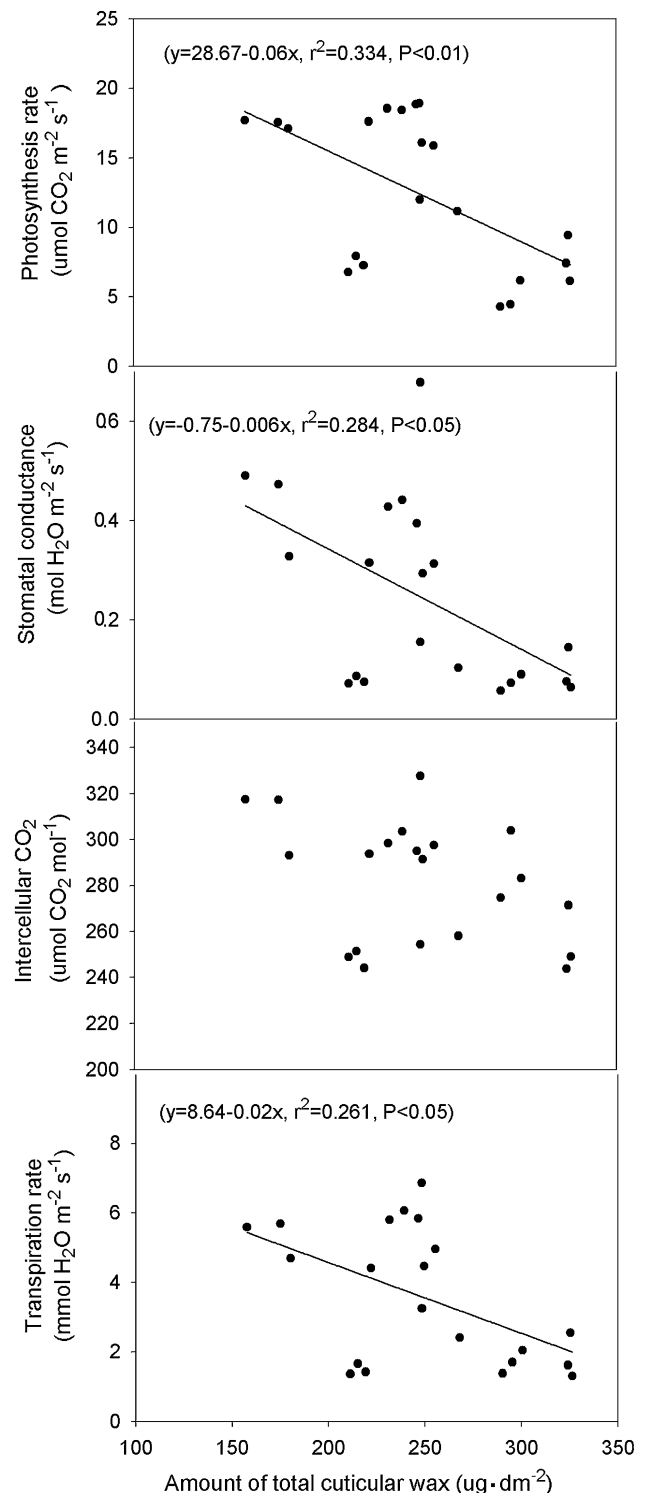
In the current study, the amount of total wax was negatively correlated with the P_N, g_s and E. High amount of wax reduced the stomatal aperture, which might subsequently affect the gas exchange. The stomatal aperture
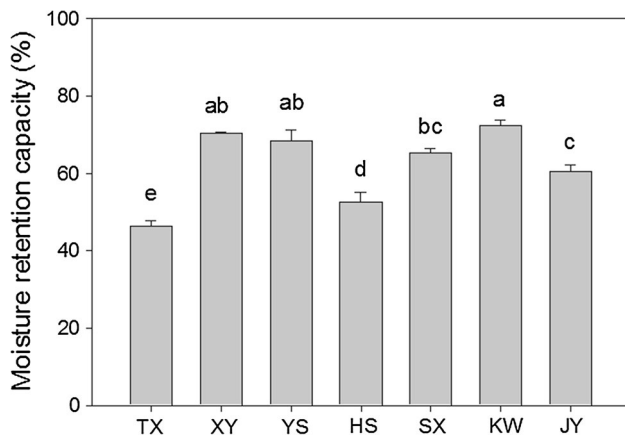
Fig. 5 Moisture retention capacity in leaves of mulberry cultivars. *TX* Tongxiangqing, *XY* Xinyihzilai, *YS* Yusang 101, *HS* Husang 32, *SX* Shanxitiansang, *KW* Kanwa-2, *JY* Junyou 5
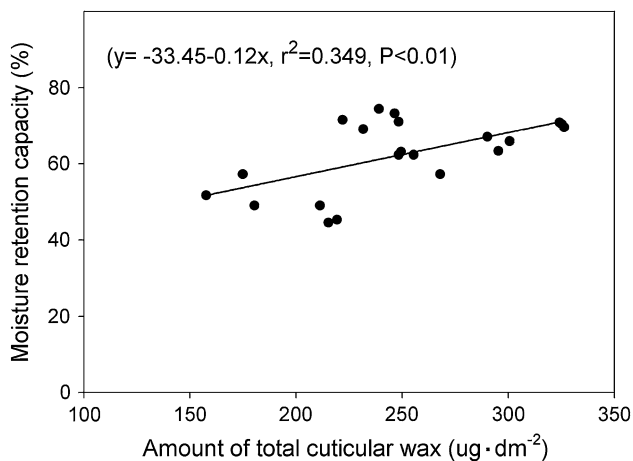

Fig. 6 Regression analysis between moisture retention capacity and total cuticular wax amount

regulated by cuticular wax affected water loss through the reduction of stomatal transpiration for mulberry trees. The barrier to water vapor under reduced stomatal conductance was highest in the native species (*Jatropha mollissima*) which showed a higher content of long-chain *n*-alkanes compared to exotic species (*Jatropha curcas*) subjected to a water deficit (Figueiredo et al. 2012). However, no significant relationship was observed between the amounts of wax constituents and gas exchange index in this study.

As the main feed for silkworm, the post-harvest leaf moisture content of mulberry influenced their feed quality and intake (Khurana and Checker 2011). In the current study, the MRC value varied greatly among mulberry cultivars, with XY, YS and KW having relatively higher MRC. A significant positive relationship between the total amount of cuticular wax and MRC was observed, showing the relevance of cuticular waxes and leaf water

conservation. TX and HS, both cultivated from Zhejiang, China, had significant lower amount of total wax and MRC than the other cultivars. It suggested that the leaf cuticular resistance with the involvement of wax amount might be important in regulating water loss in harvested leaves as reported by Mamrutha et al. (2010). The research on the relationship between leaf cuticular wax of tree tobacco and dehydration stress indicated that there was a negative relationship between total wax load and epidermal conductance (Cameron et al. 2006). The epicuticular wax load on leaves of *Arachis hypogaea* reduced surface transpiration and thus improved crop water use efficiency (Samdur et al. 2003). Mamrutha et al. (2010) reported that cuticular waxes on mulberry might be an important component affecting water use efficiency through the reduction of epidermal transpiration and indirectly assist in maintenance of conditions optimum for carbon fixation under field situations. In the current study, both cuticular wax and stomatal factor are involved in regulating water loss in mulberry leaves under field conditions.

Mulberry cultivars originated from different places exhibited variance in wax deposition, gas exchange and moisture retention. Such variance might be used in estimating the adaptation of mulberry cultivars in certain environmental conditions. Rice cultivars originated in dry environments showed greater amount of cuticular wax than those originated in wet environment (O'Toole et al. 1979). The positive correlation between amount of total wax and MRC suggested that selecting mulberry cultivars with high wax deposition might be necessary in improving leaf MRC.

In conclusion, high amount of wax and wax film covering the adaxial surface reduced the stomatal aperture, which subsequently affected the gas exchange. The stomatal aperture regulated by cuticular wax affected water loss through the reduction of stomatal transpiration for mulberry trees. The higher leaf moisture retention capacity was related with the higher total wax amount. It suggested that both the variability in moisture retention capacity and cuticular wax characteristics might be important in evaluating and screening mulberry cultivars for increasing silk quality and silkworm productivity.

Author contribution statement Dr. Yu Ni contributed to conception and design, interpretation of data, and drafting the article and final approval of the version to be published. Mr. Zhengyuan Sun contributed to field sampling, acquisition of data, analysis of data, and final approval of the version to be published. Dr. Xianzhi Huang contributed to acquisition of data, experiment design, and final approval of the version to be published. Mr. Chuanshu Huang contributed to acquisition of data, experiment design, and final approval of the version to be published.

Professor Yanjun Guo contributed to conception and design, data analysis, revising the article, and final approval of the version to be published.

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