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The higher area-based photosynthesis in *Gossypium hirsutum* L. is mostly attributed to higher leaf thickness

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Abstract

There is a trade-off between leaf structures and photosynthesis physiology. While numerous reports have shown a difference in photosynthesis between *Gossypium hirsutum* L. (upland cotton) and *G. barbadense* L. (pima cotton), the potential contribution of leaf structures on this difference was not fully clarified. Here, we investigated the differences in area- and mass-based photosynthetic traits and leaf structures between upland and pima cottons. Our results showed that upland cotton had higher area-based net photosynthetic rate (P_{area}) than that of pima cotton, which was attributed to the coordination of stomatal conductance (g_s), area-based mesophyll conductance ($g_{m\text{-}area}$), and maximum carboxylation rate ($V_{cmax\text{-}area}$). P_{area}, $g_{m\text{-}area}$, and $V_{cmax\text{-}area}$ correlated positively with leaf mass per area. But upland and pima cotton had similar mass-based P_N (P_{mass}), g_m ($g_{m\text{-}mass}$), V_{cmax} ($V_{cmax\text{-}mass}$), suggesting that they have a similar photosynthetic capacity of single cells. Consequently, the higher area-based values in upland cotton were only due to a higher leaf thickness (T_{leaf}).

Additional key words: chlorophyll fluorescence; gas exchange; leaf density; leaf mass per area; nitrogen content.

Introduction

Leaf photosynthesis is determined by biochemical capacity (*i.e.*, the maximum carboxylation rate, V_{cmax}, and the maximum electron transport rate, J_{max}) and CO_2 diffusion conductance from the atmosphere to chloroplasts, including stomatal conductance (g_s) and mesophyll conductance (g_m) (Flexas *et al.* 2007, 2008, 2012, 2016; Evans *et al.* 2009, Terashima *et al.* 2011). These traits should be tightly coordinated, thus maximizing photosynthetic efficiency (Wright *et al.* 2004a). The g_s can be calculated relatively easily according to the concentration difference of water in and out of the stomata. But *in vivo* measurements of g_m are not as straightforward as those of net photosynthetic rate (P_N) and g_s (Gago *et al.* 2016). Therefore, in the last decades, g_m had been considered to be constant and infinite. Indeed, g_m is variable and sufficiently small as to significantly limit photosynthesis to an extent similar to that known for g_s (Flexas *et al.* 2006, 2008). Furthermore, it has been shown that stomatal, mesophyll conductance, and biochemical limitations to photosynthesis are of similar magnitude in leaves of angiosperm species (Flexas 2016), but especially in crops (Nadal and Flexas 2018). Currently, there are three common g_m estimation methods including the variable *J* method (Harley *et al.* 1992), the carbon isotope discrimination method (Evans *et al.* 1986), and the curve-fitting method (Ethier and Livingston 2004, Sharkey *et al.* 2007, Sharkey 2016, Gu and Sun 2014), but they employ different assumptions and have their own shortcomings because of the very complex mesophyll diffusion process affected by many structural diffusion paths (*e.g.*, leaf thickness, cell wall thickness, and chloroplast traits). Although a structures-based quantitative one-dimensional gas diffusion model of Niinemets and Reichstein (2003) as modified by Tosens *et al*. (2016) was also used to estimate g_m, Parkhurst (1994) has indicated intercellular diffusion as fundamentally a three-dimensional process. Generally, the area-based photosynthetic parameters were often used to analyze the effects of leaf structures on photosyn-thetic traits, but some studies have shown that mass-based photosynthetic parameters can better reflect the photosynthetic capacity of single cells (Westoby *et al.* 2013, Niinemets *et al.* 2015). Photosynthesis depends largely on CO_2 concentration at the sites of carboxylation within chloroplasts (C_c); CO_2 drawdown from the internal airspace to chloroplasts is a mesophyll-volume weighted average

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Abbreviations: C_c – CO_2 concentration at the sites of carboxylation within chloroplasts; C_i – intercellular CO_2 concentration; D_{leaf} – leaf density; g_m – mesophyll conductance; g_s – stomatal conductance; J_{flu} – electron transport rate; J_{max} – the maximum electron transport rate; LMA – leaf mass per area; l_b – biochemical limitation; l_m – mesophyll limitation; l_s – relative stomatal limitation; P_N – net photosynthetic rate; R_D – dark respiration; R_d – day respiration; T_{leaf} – leaf thickness; V_{cmax} – the maximum carboxylation rate; α – leaf absorptance; β – the partitioning of absorbed quanta between PSI and PSII.
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(Niinemets *et al.* 2005, Niinemets and Sack 2006). Therefore, Niinemets and Sack (2006) indicated that the mass-based g_m ($g_{m\text{-}mass}$) is the true determinant of the volume-weighted average chloroplastic CO_2 concentration. Ellsworth *et al.* (2018) also proposed that modeled V_{cmax} and J_{max} normalized on a per-gram basis were effective in separating biochemical from anatomical effects on P_N. Furthermore, the mass-based maximum P_N was more variable than the area-based maximum P_N in the global databases (Niinemets 1999, Wright *et al.* 2004b, Kattge *et al.* 2011), suggesting that the mass basis provides a more effective estimate of photosynthetic capacity for datasets with higher species coverage. An easily measurable leaf integrative structure trait, leaf mass per area (LMA), can be used to convert area-based photosynthetic parameters to mass-based ones. The relationships between area-based P_N (P_{area}) and LMA are often variable. This is partly because leaf thickness (T_{leaf}) and leaf density (D_{leaf}), which are the most important intrinsic drivers of LMA (Niinemets 2015), may influence leaf photosynthetic capacity in different ways (Niinemets 1999). But the relationship between mass-based P_N (P_{mass}) and LMA was found to be stronger than that between the P_{area} and LMA (Tosens *et al.* 2016). Numerous studies have indicated that low P_{mass} is associated with thick robust leaves and thus high LMA (Reich *et al.* 1997, Niinemets 2001, Wright *et al.* 2004b). Moreover, P_{mass} correlates positively with mass-based nitrogen content (N_{mass}), thus the leaves with high P_{mass} should have high nitrogen-use efficiency (Niinemets 2001, Wright *et al.* 2004a). The photosynthetic capacity per unit of nitrogen can be used to analyze the nitrogen investment proportion between anatomical structures and photosynthetic proteins. Consequently, the photosynthetic traits on the basis of mass should better reflect the trade-off between the physiological and structural characteristics of leaves (Westoby *et al.* 2013, Niinemets *et al.* 2015).

Gossypium hirsutum L. (upland) and *G. barbadense* L. (pima) are the most important fiber crops grown worldwide in more than 50 countries and play an important role in the global economy. They have obvious yield difference, fiber quality difference, and plant morphological difference. In previous works, it has been shown that upland cotton has higher photosynthesis than that of pima cotton due to larger g_s and much more photosynthetic tissues reflected by higher palisade thickness (Zhang *et al.* 2011). But these data are from area-based photosynthetic physiological analysis. At present, no studies analyzed the photosynthetic physiological and structural trading between these two species. The aims of the study were (*1*) to determine if g_m also plays an important role in the difference of photosynthesis between upland and pima cotton; (*2*) to reveal if there is trade-off between mass-based photosynthetic traits and leaf structures in upland and pima cotton.

Materials and methods

Plant materials: Four representative cotton cultivars (upland cotton – *Gossypium hirsutum* L. cv. Xinluzao 45 and Xinluzao 33 and pima cotton – *Gossypium barbadense* L. cv. Xinhai 28 and Xinhai 25) were used in this study. The experiment was conducted in an experimental field (a clay loam) of Shihezi Agricultural College, Shihezi University, Xinjiang, China (45°19'N, 86°03'E) in 2015. Seeds were sown on 21 April, 2015, in rows 12 cm apart at a plant density of 1.8×10^5 ha^{-1}. Cottons were drip irrigated according to local field irrigation level (495 mm). The plots were fertilized before sowing with 240 kg(N) ha^{-1} (urea), 170 kg(P_2O_5) ha^{-1} [$(NH_4)_3PO_4$], and 1,500 kg ha^{-1} of organic fertilizer [235 g(organic matter) kg^{-1}, 18 g(total N) kg^{-1}, 14 g(total P) kg^{-1}, and 22 g(total K) kg^{-1}]. An additional 120 kg(N) ha^{-1} (urea) was applied by drip irrigation during the growing season. Weeds and pests were controlled in the field using standard management practices. Measurements were conducted on the topmost fully expanded leaf on the main stem of the cotton selected at random at the boll formation stage (about 80 d after sowing). Meteorological data during the growing season are shown in Fig. 1.

Leaf gas exchange and Chl fluorescence parameters were measured simultaneously using an open gas-exchange system (*Li-6400*, *Li-Cor*, *Inc*., Lincoln, NE, USA) connected to leaf fluorometer chamber (2 cm^2, *Li-6400-40*, *Li-Cor*, *Inc*., Lincoln, NE, USA). At least three CO_2-response curves were measured per cultivars using light-adapted mature leaves. Leaf temperature was set to 30°C. The photosynthesis measurements started at a CO_2 concentration surrounding the leaf (C_a) of 400 μmol mol^{-1} and a saturating PPFD of 2,000 μmol m^{-2} s^{-1}. Once steady state was reached (usually 20 min after clamping the leaf), data were recorded. Immediately after that, the air inlet pipe was replaced with medical gas bag with 2% O_2 and 98% N_2, and a CO_2-response curve (P_N/C_i curve; C_i – the intercellular CO_2 concentration) was performed. After that, *Li-COR* inlet was disconnected from N_2 medical gas bag (*i.e*., air with 21% O_2 was supplied again to the plant). After reaching steady state, another P_N/C_i curve was performed. Gas exchange and Chl fluorescence were first measured at 400 μmol mol^{-1}, then C_a was decreased

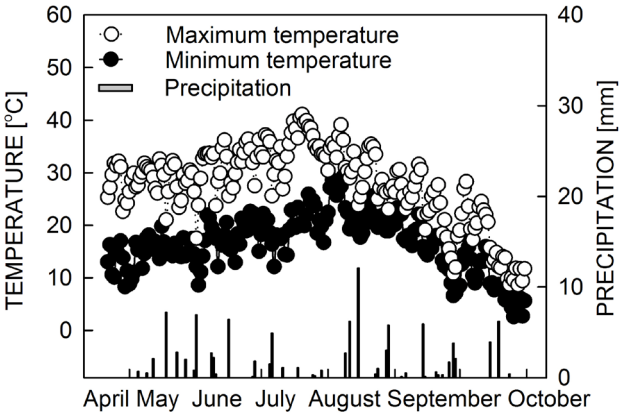

Fig. 1. Daily maximum (*open circles*) and minimum (*solid circles*) air temperature and precipitation (*bars*) during the growing season at the experimental field.

stepwise until 50 μmol mol^{-1}, upon completion of measurements at low C_a, this was returned to 400 μmol mol^{-1} to restore the original P_N. Then, C_a was increased stepwise to complete the curve. The number of different C_a values used for the curves was 12, and the time interval between two consecutive measurements at different C_a was restricted to 2–4 min, so that each curve was completed in 30–50 min. Leakage of CO_2 into and out the leaf cuvette was determined with photosynthetically inactive leaves of each species enclosed in the leaf chamber (obtained by heating the leaves until no variable Chl fluorescence was observed), and used to correct measured leaf fluxes (Flexas *et al*. 2007). The steady-state fluorescence (F_s) and maximum fluorescence during the multiphase saturation pulse flash [a light-saturating pulse at the end of phase 1 was *ca.* 8,000 μmol(photon) m^{-2} s^{-1}] (F_m') were estimated, and the actual photochemical efficiency of PSII (Φ_{PSII}) was calculated as (F_m'– F_s)/F_m' (Genty *et al.* 1989). The electron transport rate (J_{flu}) was then calculated as $\Phi_{PSII} \times \alpha \times \beta \times$ PPFD, where α is leaf absorptance and β reflects the partitioning of absorbed quanta between PSI and PSII. The term α was assumed to be 0.85 and β assumed value was 0.5. Numerous studies have shown that the estimation of J_{flu} is affected by PSI and the signal-to-noise ratio in the determination of F_m' at high light. To overcome the uncertainties, electron transport from gas exchange (J_A) under 2% O_2 conditions was used to calibrate J_{flu} (*see* Pons *et al*. 2009 for details).

Estimation of mesophyll conductance, g_m, by gas exchange and Chl fluorescence and by the P_N/C_i curve fitting: Mesophyll conductance (g_m) was estimated according to the method of Harley *et al*. (1992), as follows:

$$g_m = \frac{P_N}{C_i - \frac{\Gamma * \times (J_{flu} + 8 \times (P_N + R_d))}{J_{flu} - 4 \times (P_N + R_d)}} \quad (1)$$

where P_N and C_i were taken from gas-exchange measurements at saturating light. Γ* is the CO_2-compensation point in the absence of mitochondrial respiration, and R_d is day respiration. Γ* was taken according to Bernacchi *et al.* (2002) [Γ* = EXP(13.49 – 24,460/8.314/(273.15 + T_L)), where T_L is the leaf temperature in °C]. R_d was assumed to be 0.5 times of the measured dark respiration (R_d = R_D/2) (Piel *et al*. 2002, Niinemets *et al*. 2005). R_D was determined by gas-exchange measurement (*Li-6400*) after plants had been dark-adapted for more than half an hour in the evening. The maximum ribulose-1,5-bisphosphate carboxylation (V_{cmax}) and maximum electron transport rate (J_{max}) were calculated from the P_N/C_c curves, using the Rubisco kinetic constants and their temperature dependencies described by Bernacchi *et al*. (2002). The model of Farquhar *et al*. (1980) was fitted to the data by applying iterative curve-fitting (minimum least-square difference) using the Solver tool of *Microsoft Excel* (Sharkey 2016). Meanwhile, an alternative estimate of g_m was obtained by the curve-fitting method introduced by Sharkey (2016). This method is based on changes in the curvature of P_N *vs*. C_i response curves due to a finite g_m. By nonlinear curve fitting minimizing the sum of squared model deviations from the data, g_m can be estimated from observed data. The same leaves were used for estimation of g_m by the methods of Sharkey (2016) and Harley *et al*. (1992).

Relative limitation analyses on P_N: According to Grassi and Magnani (2005), relative stomatal limitation (l_s), mesophyll limitation (l_m), and biochemical limitation (l_b) were investigated, respectively, in the cotton leaves. The quantitative changes in light-saturated assimilation can be expressed in terms of parallel changes in stomatal and mesophyll conductance and in biochemical capacity as follows:

$$l_s = \frac{\frac{g_{tot}}{g_s} * \frac{\partial P_N}{\partial C_c}}{g_{tot} + \frac{\partial P_N}{\partial C_c}} \quad (2)$$

$$l_m = \frac{\frac{g_{tot}}{g_m} * \frac{\partial P_N}{\partial C_c}}{g_{tot} + \frac{\partial P_N}{\partial C_c}} \quad (3)$$

$$l_b = \frac{g_{tot}}{g_{tot} + \frac{\partial P_N}{\partial C_c}} \quad (4)$$

where g_{tot} is total conductance to CO_2 between the leaf surface and the sites of carboxylation ($1/g_{tot} = 1/g_s + 1/g_m$); l_s, l_m, and l_b are the corresponding relative limitations ($0 < l_i < 1$; i = s, m, b; $l_s + l_m + l_b = 1$). $\partial P_N/\partial C_c$ was calculated as the slope of P_N/C_c response curves over a C_c range of 50–100 μmol mol^{-1} (Tomás *et al*. 2013).

Light microscopy: After the gas-exchange and Chl fluorescence measurements, sections of 2 × 2 mm were cut between the main veins and subjected to microscopic analysis. Leaf samples were fixed by infiltration of 4% glutaraldehyde and 3% paraformaldehyde in phosphate buffer (0.1 mol L^{-1}, pH 7.2) under vacuum. Leaf samples were fixed again in 1% osmium tetroxide overnight and dehydrated in a graded acetone series and embedded in Spurr's resin. Semi-thin (1 μm) cross-sections were prepared with an ultramicrotome (*Leica Ultracut R*). Semi-thin cross-sections for light microscopy were stained with 0.5% toluidine blue and observed under light microscope with a digital camera (*BH-2*, *Olympus*). Leaf thickness (T_{leaf}), the thickness of palisade and spongy tissue layers were obtained and six different positions were measured in each sample.

The mass per area and nitrogen content: Leaf mass per unit area (LMA) is the ratio of dry mass and leaf area. Dry mass was determined from oven-dried certain area of leaf discs after 48 h at *ca.* 80°C. Dividing LMA by leaf thickness is defined as leaf density.

For the measurement of the nitrogen content, leaves were harvested on the same day. Total nitrogen content of the dried tissues was determined according to the micro-Kjeldahl method (Schuman *et al.* 1972).

Statistical analysis was performed with *SPSS 17.0* for *Windows* (*SPSS Inc*., Chicago, IL, USA). All data were tested by analysis of variance (*ANOVA*). The significance of differences between treatment means were separated by using *Student-Newman-Keuls* (S-N-K) test at the 0.05 probability level. Data are presented as the means ± standard errors of three replicates. Interrelations between variables by means of linear regression analysis were investigated.

Results

The daily minimum and maximum temperature and precipitation during the growing season at the experimental field is shown in Fig. 1; we conducted this experiment in mid-July. The upland cotton had higher area-based net photosynthetic rate (P_{area}), mesophyll conductance ($g_{m\text{-}area}$), maximum carboxylation rate ($V_{cmax\text{-}area}$), and maximum electron transport rate ($J_{max\text{-}area}$) than that of pima cotton (Table 1). Pooling all the data, P_{area} positively correlated with g_s, $g_{m\text{-}area}$, and $V_{cmax\text{-}area}$. Moreover, there was close relationship between $g_{m\text{-}area}$ and $V_{cmax\text{-}area}$ and the drawdown in CO_2 between substomatal cavities and the sites of carboxylation within chloroplasts (C_i-C_c), respectively (Fig. 3). A quantitative limitation analysis following Grassi and Magnani (2005) revealed different weights for each potential limitation (Fig. 4). Only the relative mesophyll limitation (l_m) dominated in pima cotton, but l_m and biochemical limitation (l_b) appear to co-limit photosynthesis in upland cotton.

From the analysis of leaf structure (Table 2), upland cotton had higher leaf mass per area (LMA) contributed by higher leaf thickness (T_{leaf}) than that of pima cotton. Palisade thickness (T_p) and spongy thickness (T_s) in upland cotton were also larger than those in pima cotton. LMA was positively correlated with P_{area}, $g_{m\text{-}area}$, and $V_{cmax\text{-}area}$ (Fig. 5). Although upland cotton had higher area-based nitrogen content (N_{area}) than that of pima cotton, there was no significant difference in mass-based nitrogen content (N_{mass}) between upland and pima cotton. Furthermore, interestingly, we converted the area-based photosynthetic parameters into mass-based ones according to available leaf mass per area (LMA) and found there was no significant difference in P_{mass}, $g_{m\text{-}mass}$, and $V_{cmax\text{-}mass}$ between upland and pima cotton (Table 1). Also, pooling all of the data, a closer relationship between T_{leaf} and LMA was observed than that between D_{leaf} and LMA (Fig. 6), which was also shown clearly in Fig. 7.

Discussion

Area-based net photosynthetic rate (P_{area}) was significantly higher in upland cotton than that in pima cotton (Table 1), which is consistent with other studies (Zhang *et al.* 2011). Zhang *et al*. (2011) have shown that stomatal conductance (g_s) mainly contributed to the difference in P_{area} between upland and pima cotton (*see* also Fig. 2*A*). But in this study, we found that the low internal CO_2 diffusion, *i.e*., area-based mesophyll conductance ($g_{m\text{-}area}$) was also an important factor causing a low P_{area} in pima cotton (Table 1,

Table 1. Area- and mass-based net photosynthetic rate (P_{area} and P_{mass}), stomatal conductance (g_s), area- and mass-based mesophyll conductance ($g_{m\text{-}area}$ and $g_{m\text{-}mass}$) estimated by two independent methods: using gas-exchange plus fluorescence measurements following Harley *et al.* (1992) and the curve-fitting method of Sharkey (2016); area- and mass-based maximum carboxylation rate ($V_{cmax\text{-}area}$ and $V_{cmax\text{-}mass}$), area-based maximum electron transport rate ($J_{max\text{-}area}$), and V_{cmax} expressed on the basis of nitrogen content (V_{cmax}/N) in upland and pima cotton. Values are means ± SE of three replicates. *Different letters* indicate significant differences at the 0.05 probability level. ** and *** indicate significant differences between species at the 0.01 and 0.001 probability level, respectively. ns indicates no significant differences among species.

	P_{area} [μmol(CO_2) m^{-2} s^{-1}]	P_{mass} [μmol(CO_2) g^{-1} s^{-1}]	g_s [mol(H_2O) m^{-2} s^{-1}]	$g_{m\text{-}area}$ (Harley) [mol(CO_2) m^{-2} s^{-1}]	$g_{m\text{-}mass}$ (Harley) [μmol(CO_2) g^{-1} s^{-1}]	$g_{m\text{-}area}$ (fitting) [mol(CO_2) m^{-2} s^{-1}]	$V_{cmax\text{-}area}$ [μmol(CO_2) m^{-2} s^{-1}]	$V_{cmax\text{-}mass}$ [μmol(CO_2) g^{-1} s^{-1}]	$J_{max\text{-}area}$ [μmol(CO_2) m^{-2} s^{-1}]	V_{cmax}/N [μmol(CO_2) g^{-1}(N) s^{-1}]
Upland-45	37.23 ± 0.85^{a}	0.37 ± 0.01^{a}	0.56 ± 0.06^{ab}	0.43 ± 0.04^{a}	4.27 ± 0.34^{a}	0.38 ± 0.03^{a}	315.8 ± 5.91^{a}	3.11 ± 0.10^{a}	309.6 ± 8.95^{a}	60.93 ± 1.93^{ab}
Upland-33	35.88 ± 1.49^{a}	0.36 ± 0.01^{a}	0.63 ± 0.03^{a}	0.40 ± 0.04^{a}	3.99 ± 0.40^{a}	0.33 ± 0.02^{ab}	331.0 ± 6.00^{a}	3.31 ± 0.06^{a}	297.0 ± 28.92^{a}	59.39 ± 1.00^{ab}
Pima-28	29.46 ± 1.83^{b}	0.38 ± 0.03^{a}	0.40 ± 0.08^{b}	0.26 ± 0.03^{b}	3.33 ± 0.47^{a}	0.29 ± 0.01^{b}	252.7 ± 15.70^{b}	3.20 ± 0.28^{a}	283.3 ± 21.84^{ab}	67.49 ± 5.83^{a}
Pima-25	27.43 ± 1.04^{b}	0.37 ± 0.01^{a}	0.38 ± 0.05^{b}	0.29 ± 0.01^{b}	3.89 ± 0.19^{a}	0.25 ± 0.02^{b}	213.0 ± 5.51^{b}	2.87 ± 0.11^{a}	225.0 ± 4.36^{b}	51.59 ± 2.02^{b}
Species (*P* value)	***	ns	**	**	ns	**	***	ns	*	ns

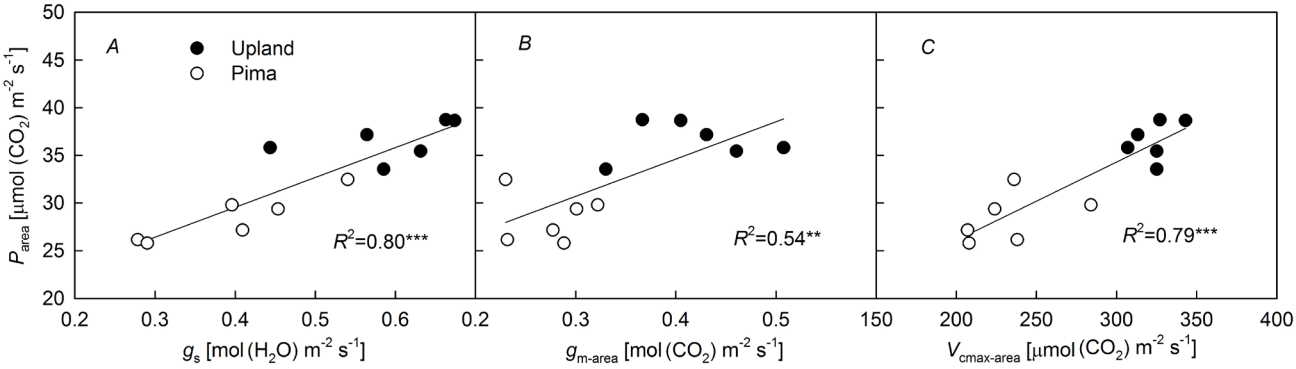

Fig. 2. Area-based net photosynthetic rate (P_{area}) as a function of stomatal conductance (g_s) (*A*), area-based mesophyll conductance ($g_{m\text{-}area}$) (*B*), and the maximum carboxylation rate ($V_{cmax\text{-}area}$) (*C*), respectively, in upland cotton (*solid circles*) and pima cotton (*open circles*). ** and *** indicate significance at the 0.01 and 0.001 probability level, respectively.

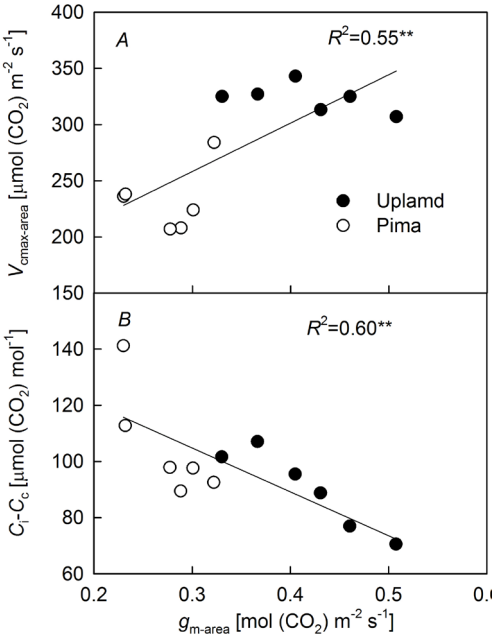

Fig. 3. The relationships between area-based mesophyll conductance ($g_{m\text{-}area}$) and the area-based maximum carboxylation rate ($V_{cmax\text{-}area}$) (*A*), and the drawdown in CO_2 between substomatal cavities and the sites of carboxylation within chloroplasts (C_i-C_c) (*B*) in upland (*solid circles*) and pima cotton (*open circles*). *The solid lines* are the linear regressions; *** indicate significance at the 0.001 probability level.

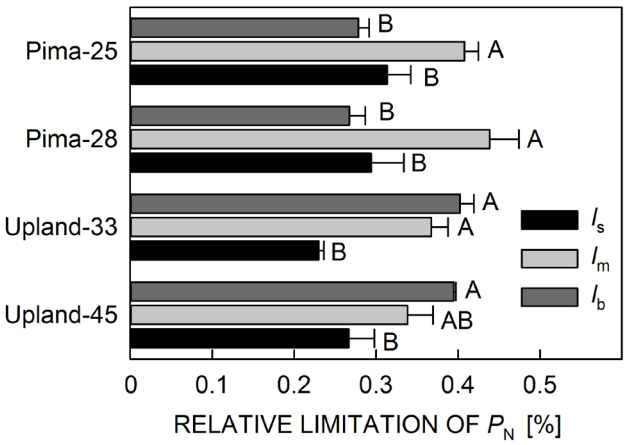

Fig. 4. The relative limitations analysis to net photosynthetic rate (P_N) imposed by stomatal conductance (l_s), mesophyll conductance (l_m), and biochemistry (l_b) for upland and pima cotton species. Values are means ± SE of three replicates. *Different letters* indicate significant differences between three limitations within cultivar at the 0.05 probability level.

Fig. 2*B*,*C*). Ellsworth *et al.* (2018) indicated that the drawdown in CO_2 between substomatal cavities and the sites of carboxylation within chloroplasts (C_i-C_c) should always be estimated to assess the influence of $g_{m\text{-}area}$ on P_{area}. There was a negative correlation between C_i-C_c and $g_{m\text{-}area}$ (Fig. 3), suggesting that upland cotton leaves with greater $g_{m\text{-}area}$ do have lower mesophyll diffusion limitations of photosynthesis (Niinemets and Sack 2006, Warren and Adams 2006, Warren 2008) and then higher CO_2 concentration at the sites of carboxylation within chloroplasts (C_c) and P_N. Besides the CO_2 diffusional limitation, P_{area} correlated strongly with the area-based maximum carboxylation rate ($V_{cmax\text{-}area}$) (Fig. 2*C*) that can reflect biochemical capacity just like Rubisco activity (Flexas *et al.* 2014). Study has shown that Rubisco activity was regulated by C_c (Galmés *et al.* 2011), and $V_{cmax\text{-}area}$ was closely related with $g_{m\text{-}area}$ in this study, suggesting that the CO_2 diffusion limitation and biochemical limitation tightly co-regulated photosynthesis. A quantitative limitation analysis following Grassi and Magnani (2005) revealed different weights for each potential limitation (Fig. 4). Only the relative mesophyll limitation (l_m) dominated in pima cotton, but l_m and biochemical limitation (l_b) appeared to co-limit photosynthesis in upland cotton. Indeed, from this limitation weight analysis, none of these three limitations can be ignored in setting the differences between upland and pima cotton.

There is always a trade-off between photosynthetic physiological and structural characteristics as reported by most studies. Leaves with higher leaf mass per area (LMA) tend to have higher investment in nonphotosynthetic tissue and this leads to lower photosynthetic efficiency (Niinemets *et al.* 2009a, Hassiotou *et al.* 2010). Meanwhile,

Table 2. Leaf mass per unit area (LMA), leaf thickness (T_{leaf}), leaf density (D_{leaf}), palisade thickness (T_p), spongy thickness (T_s), and mass- and area-based nitrogen (N_{mass} and N_{area}) in upland and pima cotton leaves. Values are means ± SE of three replicates. *Different letters* indicate significant differences at the 0.05 probability level. ** and *** indicate significant differences between species at the 0.01 and 0.001 probability level, respectively. ns indicates no significant differences among species.

	LMA [g m^{-2}]	T_{leaf} [μm]	D_{leaf} [g cm^{-3}]	T_p [μm]	T_s [μm]	N_{mass} [%]	N_{area} [g m^{-2}]
Upland-45	101.58 ± 1.37^a	432.63 ± 10.98^a	0.236 ± 0.01^c	199.44 ± 6.39^a	191.19 ± 13.85^a	5.11 ± 0.17^a	5.20 ± 0.06^b
Upland-33	100.02 ± 0.59^a	404.95 ± 7.70^b	0.245 ± 0.00^c	175.95 ± 4.02^b	184.61 ± 7.97^a	5.57 ± 0.36^a	5.53 ± 0.05^a
Pima-28	79.02 ± 2.70^b	266.92 ± 1.58^c	0.293 ± 0.00^a	115.54 ± 2.24^c	103.83 ± 7.59^b	4.76 ± 1.66^a	3.72 ± 0.09^d
Pima-25	74.43 ± 3.08^b	269.60 ± 1.66^c	0.275 ± 0.00^b	107.27 ± 5.81^c	122.98 ± 9.07^b	5.56 ± 0.11^a	4.12 ± 0.10^c
Species (*P* value)	***	***	***	***	***	ns	***

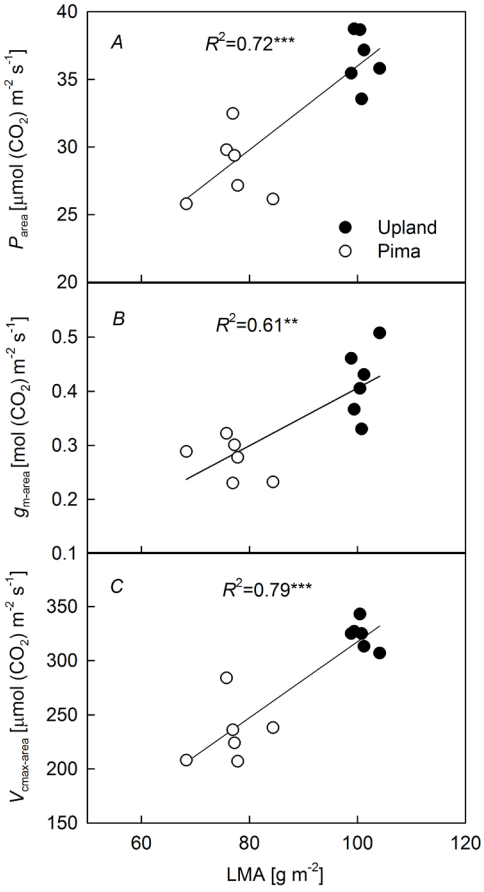

Fig. 5. The relationships between leaf mass per area (LMA) and area-based net photosynthetic rate (P_{area}) (*A*), mesophyll conductance ($g_{m\text{-}area}$) (*B*), and the maximum carboxylation rate ($V_{cmax\text{-}area}$) (*C*), respectively, in upland (*solid circles*) and pima cotton (*open circles*). *The solid lines* are the linear regressions; ** and *** indicate significance at the 0.01 and 0.001 probability level, respectively.

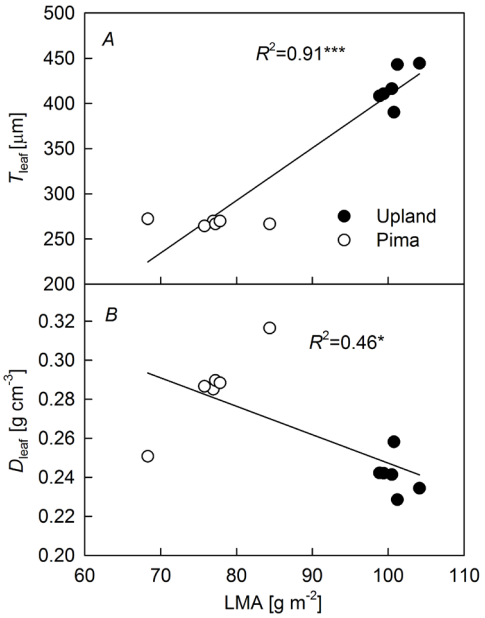

Fig. 6. The relationships between leaf mass per area (LMA) and leaf thickness (T_{leaf}) (*A*) and leaf density (D_{leaf}) (*B*), respectively, in upland (*solid circles*) and pima cotton (*open circles*). *The solid lines* are the linear regressions; *** and * indicate significance at the 0.001 and 0.05 probability level, respectively.

LMA has been suggested to negatively affect the $g_{m\text{-}area}$ and limit area-based photosynthesis (Flexas *et al.* 2008). However, in this study, LMA was positively correlated with physiological traits (*i.e.*, P_{area}, $g_{m\text{-}area}$, $V_{cmax\text{-}area}$) (Fig. 5). It is well established that LMA is an integrative trait of leaf structural characteristics and the product of leaf density (D_{leaf}) and thickness (T_{leaf}) (Poorter *et al.* 2009, Niinemets 2015). Our results showed that the variation in LMA is primarily driven by variations in T_{leaf} and to a lesser degree in D_{leaf} (Fig. 6). It is likely that upland cotton with higher T_{leaf} had more Rubisco carboxylation sites (Flexas *et al.* 2014) and/or higher surface of chloroplasts exposed to intercellular airspace (S_c/S) (Hanba *et al.* 1999, 2002; Terashima *et al.* 2006, Peguero-Pina *et al.* 2016), thereby increasing photosynthetic physiological traits.

Generally, photosynthetic characteristics and CO_2 diffusion properties were measured on the basis of leaf area, but it is reported that photosynthetic process inside the leaves is based on a three-dimensional structure (Parkhurst 1994, Niinemets *et al.* 2009b). The mass-based photosynthetic traits can be better used to measure the trade-off between the physiological and structural characteristics of leaves (Niinemets *et al.* 2015). The trait values on the mass basis can be calculated according to available LMA. Interestingly, it was documented that

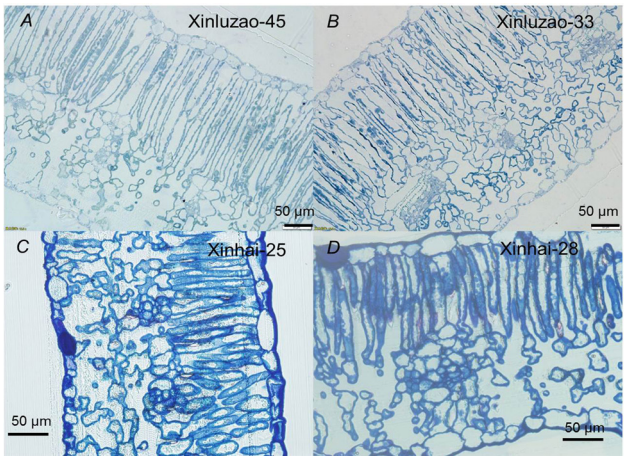

Fig. 7. Light microscopy images of upland Xinluzao 45 (*A*) and Xinluzao 33 (*B*), and pima Xinhai 25 (*C*) and Xinhai 28 (*D*) cotton leaves. *The black lines* represent measuring scales of 50 μm.

mass-based V_{cmax} ($V_{cmax\text{-}mass}$) is more effective in separating biochemical from anatomical effects on P_N than $V_{cmax\text{-}area}$ (Ellsworth *et al.* 2018); $g_{m\text{-}mass}$ is more strongly related to photosynthetic tissue volume than to the area; the P_{mass} is the key player in the trade-off between the physiological and structural characteristics of leaves (Westoby *et al.* 2013, Niinemets *et al.* 2015). In our study, no difference in P_{mass}, $g_{m\text{-}mass}$, and $V_{cmax\text{-}mass}$ between upland and pima cotton was observed, indicating that both of upland and pima cotton had the similar photosynthetic capacity of single cells. Moreover, there was no significant difference in nitrogen investment. A similar carboxylation capacity per unit of nitrogen (*i.e.*, V_{cmax}/N) suggests that upland and pima cotton divert a similar proportion of their N to nonphotosynthetic compounds. Therefore, the higher trait values on the basis of area in upland cotton were only due to higher T_{leaf} or LMA. This was inconsistent with the change rule of data in the global databases, but keeping in mind that only four species were studied and these belonged to the trade-off within species that has not been proved to be consistent with the laws along species.

Conclusion: Upland cotton had higher area-based net photosynthetic rate (P_{area}) than that of pima cotton, which was attributed to the coordination of area-based stomatal conductance (g_s), mesophyll conductance ($g_{m\text{-}area}$), and biochemical capacity (*i.e.*, $V_{cmax\text{-}area}$ and $J_{max\text{-}area}$). P_{area}, $g_{m\text{-}area}$, and $V_{cmax\text{-}area}$ correlated positively with leaf mass per area (LMA). But there was no difference in mass-based P_N (P_{mass}), g_m ($g_{m\text{-}mass}$), and V_{cmax} ($V_{cmax\text{-}mass}$) between upland and pima cotton, suggesting that upland and pima cotton had a similar photosynthetic capacity of single cells. Therefore, we can conclude that the higher trait values on the basis of area in upland cotton were only due to higher T_{leaf} or LMA.

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