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Contribution of leaf anatomical traits to leaf mass per area among canopy layers for five coexisting broadleaf species across shade tolerances at a regional scale



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ABSTRACT

Variability in leaf mass per area (LMA) is largely explained by leaf anatomical traits. However, few studies have been conducted to explore the anatomical characteristics and the contribution of leaf anatomical traits to LMA for coexisting broadleaf species with different shade tolerances in same forests at a regional scale. Here, we analyzed variations in LMA and three leaf anatomical traits and the bivariate relationships among four leaf traits within three canopy layers for five major coexisting broadleaf species with different shade tolerances in four mixed broadleaved-Korean pine (Pinus koraiensis) forests along a latitude gradient in Northeast China. We focused on assessing the relative weight of the leaf anatomical traits to predict LMA and on exploring the correlations among leaf anatomical traits, shade tolerance and LMA by using structural equation modeling (SEM). In comparison to shade-tolerant species, relative shade-intolerant species had higher LMA and length of minor veins per unit area (VLAmin). LMA, palisade tissue thickness (PT) and VLAmin increased with the height of the canopy. The bivariate relationships among the three anatomical traits and LMA were significantly different among the five species, but there were no significant differences among the three canopy layers. LMA was directly enhanced by PT, epidermis thickness (ET) and VLAmin and directly decreased by shade tolerance as well as indirectly decreased by shade tolerance via leaf anatomical traits. Our results clearly suggest that shade tolerance is important for driving variations in key leaf traits, which will provide a better understanding of the drivers of trait variation as well as the application of functional traits in outlining mechanisms of forest dynamics and management.

1. Introduction

For plant leaves, light and water are indispensable resources for photosynthesis, and light availability and hydraulic constraints can not only determine the expression of leaf traits (Scartazza et al., 2016; Poorter et al., 2019; Tinya et al., 2019), but also affect plant performance (Lilles et al., 2014; Coble et al., 2017). Plant leaves have the ability to adapt to environmental changes that may occur at large scales (e.g., the regional or global scale) or small scales (e.g., within canopies) (Coble and Cavaleri, 2017; He et al., 2018). The leaves have such an adaptive strategy is that plant performance is improved by regulating leaf traits, particularly the anatomical traits associated with resource acquisition and utilization (Oguchi et al., 2005). Compared with the worldwide variation in leaf traits, the leaf trait variation and the bivariate relationships across the canopy layer gradient among species with different shade tolerances in the same forest type are greatly compressed (Liu et al., 2019). A better understanding of leaf anatomical traits in species with different shade tolerances is necessary for modeling plant photosynthesis and has broad implications for the prediction of forest productivity (Niinemets et al., 2015; Coble et al., 2017). Substantial variability in leaf traits is largely explained by the shade tolerance of species and the local light conditions to which leaves are exposed (Niinemets, 1999; Lilles et al., 2014; Zhang et al., 2016). In recent years, the importance of shade tolerance and the canopy gradient has been increasingly emphasized (Lilles et al., 2014), and how the species with different shade tolerances adapt to light environments by adjusting anatomical traits has been a long-standing question (Portsmuth and Niinemets, 2007). However, few studies has been conducted to reveal the variation in leaf anatomical traits and the bivariate relationships between leaf anatomical traits and key leaf functional trait (e.g., leaf mass per area (LMA)) within canopy layers for coexisting broadleaf species with different shade tolerances in same forests at a regional scale.

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Interspecific differences in shade tolerance can not only explain a large proportion of variation in leaf traits but also determine species composition and dynamic changes in the succession process of temperate forests (Niinemets and Kull, 1994; Barbier et al., 2008). To compete for resources and occupy space to achieve rapid growth and longterm survival, species with different shade tolerances will have some typical characteristics, which enable them to better cope with the changes in the light environment in the forest (Portsmuth and Niinemets, 2007). In recent years, research on the typical characteristics of species with different shade tolerances is getting more attention, but most researches have mainly focused on morphological, chemical and physiological traits (Zhang et al., 2016). The results show that the leaves of shade-intolerant species are characterized by a higher leaf nitrogen concentration, leaf phosphorus concentration, leaf thickness (LT) and net photosynthetic rate and a lower LMA than those of leaves of shade-tolerant species, but the leaf anatomical traits of species with different shade tolerances are poorly understood.

For individual plants, the upper canopy layer supports leaves adapted to strong light, while the lower canopy layer produces leaves adapted to low light (Niinemets et al., 2015). The leaves located in the lower canopy layer become increasingly shaded by the leaves located in the upper canopy layer, leading to strong light gradients (Niinemets et al., 2015). The light gradients result in significant differences in key leaf functional traits at different canopy positions, and ultimately have important impacts on many aspects of tree biology and potentially contributes to whole-crown performance (Coble and Cavaleri, 2014; Niinemets et al., 2015). Previous studies have shown that leaf morphological, chemical and physiological traits vary from canopy top to bottom. For example, the LMA, LT, leaf nitrogen content per leaf area and net photosynthetic rate of the upper leaves are significantly higher than those of the lower leaves (Scartazza et al., 2016; Coble and Cavaleri, 2017). In contrast, the information on the canopy gradient of leaf anatomical traits needs more attention. The branching strategy and canopy structure are significantly different in species with different shade tolerances (Seiwa et al., 2006; Niinemets et al., 2015), leading to different light gradients range, thus, the strength of leaf traits canopy gradient may be different among species with different shade tolerances.

The correlations of leaf traits are still under discussion (Blackman et al., 2016; Muir et al., 2017; Liu et al., 2019). However, whether the intraspecific correlations among various leaf traits were consistent among different species was still controversial (Blonder et al., 2011; Blackman et al., 2016). Previous studies have found that shade tolerance of species plays a key role in the correlations among leaf traits (Ameztegui et al., 2017). As a collection of different traits determine species performance in varying light environments (Portsmuth and Niinemets, 2007), the tradeoffs of traits of leaves growing under different light environments were different (Poorter et al., 2009). Thus, we expect that relationships between leaf anatomical traits and LMA are different among species with different shade tolerances and among canopy layers.

LMA is the key integrated trait for characterizing the biomass cost of forming a unit of leaf area and can be explained by the variation in leaf density (LD) and LT (LMA = LT \times LD) (Poorter et al., 2009). They are

all affected by anatomical tissue composition (Oguchi et al., 2005; Sack et al., 2013; Onoda et al., 2017). For instance, leaf density decreases when the volumetric fraction of epidermal tissue or air spaces increases, and leaf density increases with the volumetric fraction of vascular or palisade tissue. LT increases with increasing in the epidermis thickness (ET) or palisade tissue thickness (PT) (Villar et al., 2013), thus a comprehensive understanding of how anatomical traits determine LMA is fundamental and necessary (Mason et al., 2016; John et al., 2017; Onoda et al., 2017). In recent years, an increasing amount of studies have emphasized that there is a critical need for a more detailed framework for the underlying anatomical basis of LMA and for the evaluation of the relative contribution of the different tissues to LMA (Villar et al., 2013; Kawai et al., 2016; Mason et al., 2016; John et al., 2017). Additionally, shade tolerance markedly affect the variation in leaf traits (Ameztegui et al., 2017), however, how leaf anatomical traits and shade tolerance affect LMA (directly or indirectly) for coexisting species at a regional scale have rarely been identified.

In this study, five coexisting broadleaf species with different shade tolerances in four mixed broadleaved-Korean pine (Pinus koraiensis) forests along a latitude gradient in northeast China were selected, including Populus davidiana, Betula platyphylla, Fraxinus mandshurica, Tilia amurensis and Acer mono (shade tolerance gradually increases). We measured LMA and three anatomical traits (i.e., PT, ET and the length of minor veins per unit area (VLA $_{min}$)) for the five species. The objective of this study was mainly to explore the variation pattern and the relationship of leaf traits with shade tolerance and canopy gradient and to evaluate the contribution of the three anatomical traits to LMA for five tree species that differ in their shade tolerances. Thus, we tested the following three hypotheses: (1) the four leaf traits and bivariate relationships among the three leaf anatomical traits and LMA have significant vertical variations in canopies and gradient variations in shade tolerance; (2) the relative contribution of PT and VLA_{min} to LMA are more than ET to LMA: and (3) shade tolerance also plays a key role in determining anatomical traits, and shade tolerance can indirectly affect LMA through anatomical traits.

2. Materials and methods

2.1. Study site

The study was conducted in four old-growth mixed broadleaved-Korean pine forests along a south-north latitude gradient in Northeast China, i.e., Changbai Mountain ($41^{\circ}41' \sim 42^{\circ}51'$ N, $127^{\circ}42' \sim 128^{\circ}16'$ E), Muling ($44^{\circ}20' \sim 44^{\circ}30'$ N, $129^{\circ}40 \sim 129^{\circ}53'$ E), Fenglin ($48^{\circ}02' \sim 48^{\circ}12'$ N, $128^{\circ}59' \sim 129^{\circ}15'$ E) and Shengshan ($49^{\circ}25' \sim 49^{\circ}40'$ N, $126^{\circ}27' \sim 127^{\circ}02'$ E). The sites are characterized by a temperate continental monsoon climate. The mean annual temperature in these forests ranged from -2.0 to 3.6° C, and the mean annual precipitation varied from 514 mm to 700 mm.

2.2. Sample design

At the four sampling sites, five major coexisting broadleaf species were selected, including *P. davidiana*, *B. platyphylla*, *F. mandshurica*, *T. amurensis* and *A. mono*, and their shade tolerances gradually increased

Table 1

Shade tolerance index, means and standard errors of height, DBH and height of the canopy layer for five broadleaf species in northeast China.

Species	Shade tolerance index	DBH (cm)	Height (m)	Hei	Height of the canopy layer (m)		
				Lower	Middle	Upper	
Populus davidiana	0.65	34.1 ± 2.3	21.6 ± 1.1	12.5 ± 0.8	16.1 ± 0.8	19.8 ± 0.9	
Betula platyphylla	1.25	38.1 ± 1.8	20.0 ± 1.5	10.8 ± 0.7	15.1 ± 0.8	19.3 ± 1.0	
Fraxinus mandshurica	2.75	35.6 ± 3.3	24.3 ± 2.4	11.8 ± 1.3	16.8 ± 1.6	21.8 ± 2.2	
Tilia amurensis	3.68	47.2 ± 3.6	25.1 ± 1.7	11.6 ± 0.9	17.0 ± 1.1	22.4 ± 1.5	
Acer mono	4.30	$34.5~\pm~2.3$	15.7 ± 1.1	7.1 ± 0.5	$10.6~\pm~0.7$	$14.0~\pm~1.0$	

(Table 1). For each species, three adult individuals were randomly sampled at each site. The sample individuals were located in almost the same slope direction and similar slope conditions at the four sites in mid-July to mid-August in 2018. In total, twelve sample individuals were selected for each species at four sites. The canopy of each individual was divided into three layers (upper, middle and lower) based on the canopy height, and each layer was further divided into two horizontal positions based on the direction of the sampling branch (north and south). Each individual had six canopy positions, i.e., uppersouth, upper-north, middle-south, middle-north, lower-south and lower-north. The diameter at breast height (DBH), the tree height and the height of the canopy layer of each individual were measured (Table 1).

For each canopy position, mature leaves (40 < n < 60) were collected. Leaves were temporarily sealed in plastic bags and transported immediately to the laboratory. Fully expanded leaves (n = 5 per canopy position) were selected for measurements of leaf area and leaf dry mass. Fresh and fully expanded leaves (n = 20 per canopy position) were fixed into a buffered formalin-acetic acid-alcohol (FAA) fixation solution (70% ethanol: formalin: glacial acetic acid = 90: 5: 5) for the analyses of the anatomical traits.

2.3. Leaf trait measurements

2.3.1. Leaf mass per area

At each canopy position, five mature leaves were randomly selected for measuring the LMA. The leaf surface area was scanned using a portable scanner (Canon LiDE 120, Tokyo, Japan), and the leaf area was then calculated using a leaf area processing program (with a precision of 0.01 cm^2). The leaves were then oven-dried at 65 °C for 72 h to a constant mass and weighed to the nearest 0.1 mg. The LMA (g m⁻²) was calculated as the ratio of the leaf dry mass to the leaf area.

2.3.2. Leaf anatomical traits

The leaf anatomical traits included PT (µm), ET (µm) and VLAmin (mm mm⁻²). Three sample leaves were selected from the buffered FAA fixation solution. For the measurement of PT and ET, based on the paraffin section technique (He et al., 2018), leaf section samples were obtained. The rectangular sections $(1.0 \times 0.8 \text{ cm}^2)$ were cut from the sample leaves. The leaf sections were progressively dehydrated in an ethanol series (70%, 85%, 95%, and 100%, 2 h at each concentration) and infiltrated with warm paraffin. The leaf transverse section (7 µm thickness) was then cut using a rotary microtome (KD-2258, Zhejiang, China). The lignified tissues were red-stained using safranin, and the nonlignified cell walls were green-stained with fast green stain. The leaf sections were then mounted on slides and sealed with neutral glue. After obtaining the sections of the leaves, the slides were observed under a light microscope (Olympus Electronics, Inc., Tsukuba, Japan), photographed and measured using electronic image analysis equipment (cellSens Standard 1.11 software, Olympus Electronics Inc., Tsukuba, Japan). The clearest and most complete image of each slide was selected for measuring the adaxial epidermis thickness (µm), abaxial epidermis thickness (µm) and PT. Each trait was measured by 3 times on each slide. By averaging the values of each trait, the adaxial epidermis thickness, abaxial epidermis thickness, and PT of the leaves were obtained. The summed value of the adaxial epidermis thickness and abaxial epidermis thickness was taken as ET.

For the measurement of VLA_{min}, the remaining portions of leaves that were used to measure the PT and ET were selected and were placed in 5% sodium hydroxide for malacia, and then, the adaxial epidermis and palisade mesophyll were carefully brushed away using a banister brush to expose the minor veins. The sections were then placed in 5% sodium hypochlorite for bleaching for several hours to several days, depending on the species, until clear. After clearing, the sections were carefully rinsed to remove bleach and then stained in 1% safranin (Sack et al., 2012; Caringella et al., 2015). Finally, each section was observed using a light microscope, and 3 fields of view were photographed for each section using electronic image analysis equipment. By manually counting the total length of the minor veins in the images by using ImageJ software (NIH, Bethesda, MD, USA), the VLA_{min} was measured as the total length of the minor veins per unit area.

2.4. Statistical analysis

All statistical analyses were performed using R-3.2.5 (R Core Team 2016). The differences in the mean values of leaf traits among five species were tested using one-way ANOVA with least significant difference (LSD), which was also used to assess the differences in the mean values of leaf traits among the three canopy layers. We assessed the difference in the relationship between anatomical traits and LMA by comparing the slopes of the LMA-PT, LMA-ET, and LMA-VLAmin linear regression models for the five species and three canopy layers by using standardized major axis (SMA) regression analysis (Warton et al., 2006). We used a multiple regression method on log-transformed trait values to establish linear mixed models for the three leaf anatomical traits against LMA (West et al., 1984). To quantify the contribution of anatomical traits to LMA, we calculated the relative weight of the predictors (Lebreton and Tonidandel, 2008). They reflected the explained degree of PT, ET and VLA_{min} to the variance (R²) of the full model (LMA = $\beta_1 PT + \beta_2 ET + \beta_3 VLA_{min} + \beta_0$) for predicting the LMA of all species and each species. Additionally, we ignored effects of repeated measurements for each individual that may lead to intercorrelation of residuals within any individual on our results (West et al., 1984, 1986).

We finally used structural equation modeling (SEM) to examine the effects of shade tolerance and anatomical traits on LMA. The overall model fit was evaluated by comparing the expected variance-covariance structure with the observed variance-covariance structure using the χ^2 test (a small χ^2 value with P > 0.05 means that there is no sufficient evidence to reject the model) (Grace, 2006). The framework was classified into three levels: shade tolerance, leaf anatomical traits and LMA (Fig. S1). The shade tolerance can affect leaf traits (Zhang et al., 2016; Ameztegui et al., 2017), we constructed the paths from shade tolerance to four leaf traits. The epidermis, mesophyll and veins together form a complete leaf and can affect the LMA (John et al., 2017), thus we constructed the paths from leaf anatomical traits to LMA. The vascular tissue supports the mesophyll tissue, whereas the epidermal tissue protects mesophyll tissue (John et al., 2017), and there may interplay between three leaf anatomical traits, thus we constructed the paths from VLA_{min} to PT, from ET to PT (Fig. S1). According to the framework, we constructed four models with a total model including all species and all canopy layers. One model was fit separately for each canopy layer for the five species (upper, middle and lower). Furthermore, we determined the indirect effect and total effects (direct and indirect effects) of each factor on LMA. The SEM model was performed using the R package 'lavaan' (Rosseel, 2012).

3. Results

3.1. Variations in leaf traits in terms of shade tolerance and canopy layer

There were significant differences in four leaf traits among five species, relative shade-intolerant species had higher LMA and VLA_{min} in comparison to shade-tolerant species (Fig. 1). For five species with different shade tolerances, the species with thicker palisade tissue had thinner epidermal tissue (Fig. 1). LMA, PT and VLA_{min} increased with increasing height of canopy (Fig. 1). The relative shade-tolerant species had stronger canopy gradient of leaf traits than shade-intolerant species



Fig. 1. Boxes of the leaf traits of the three canopy layers for five species with different shade tolerances. The boxes indicate the differences in the mean values of leaf traits among the three canopy layers for each species. Different capital letters below the boxes indicate that the leaf traits were significantly different among the five broadleaf species with different shade tolerances at the 0.05 level. Different lowercase letters on the boxes indicate that the leaf traits were significantly different among the canopy layers for each species at the 0.05 level. Different lowercase letters on the boxes indicate that the leaf traits were significantly different among the canopy layers for each species at the 0.05 level. LMA, leaf mass per area; PT, palisade tissue thickness; ET, epidermis thickness; VLA_{min}, length of minor veins per unit area; PD: *Populus davidiana*; BP: *Betula platyphylla*; FM: *Fraxinus mandshurica*; TA: *Tilia amurensis*; and AM: *Acer mono*.

(Fig. 1), reflecting the adaptation strategies of LMA, PT and VLA_{min} in response to the canopy light gradient varying with shade tolerance of species.

3.2. The bivariate relationships among three leaf anatomical traits and LMA

For each species and each canopy layer, there was a significant linear relationship between anatomical traits and LMA (Table 2). There were no significant differences in the LMA-PT and LMA-VLA_{min} slopes among the three canopy layers, and the slope of the lower canopy layer was higher than that of the upper and middle canopies, whereas the intercept of the lower canopy was smaller than that of the upper and middle canopies (Table 2). The LMA-ET slopes were significantly different among the three canopy layers (P < 0.05). There were significant differences in the LMA-PT, LMA-ET, and LMA-VLA_{min} slopes among the five species (P < 0.01). The LMA-PT slope and the LMA-ET slope first increased and then decreased with increasing shade

tolerance, and the LMA-VLAmin slope had an opposite trend (Table 2).

3.3. Relative contributions of leaf anatomical traits to LMA

According to the relative weight method, PT explained 50.51% of R^2 ($R^2 = 0.70$), VLA_{min} explained 46.81% of R^2 , and ET explained 2.68% of the total variation in LMA for the five species (Table S1, Fig. 2), indicating that the changes in LMA are primarily determined by PT and VLA_{min}. When we divided the total variation in LMA into five species, we found that PT explained more variation in LMA than VLA_{min} did, and the contribution of ET to LMA was relative small (Table S1, Fig. 2), suggesting that PT is more important than VLA_{min} and ET in explaining intraspecific variation in LMA.

3.4. Correlation networks of shade tolerance and leaf traits

In the SEM, we found that each canopy model had similar results to those of the total model (including three canopy layers) including five species (Fig. 3). In the four models, the three anatomical traits (i.e., VLA_{min}, PT and ET) had significant positive direct effects on LMA (Fig. 3). Shade tolerance had a significant negative direct effect on the LMA and significant negative indirect effects through the three anatomical traits on LMA (Table S2, Figs. 3 and 4). ET had a significant negative indirect effect on the LMA through PT, but VLA_{min} had a non-significant positive indirect effect through PT (Table S2, Fig. 4). Shade tolerance also had a negative direct effect on PT and VLA_{min} but had a positive direct effect on ET (Fig. 3). ET had a negative direct effect on PT, whereas VLA_{min} did not significantly affect PT (Fig. 3).

4. Discussion

4.1. Variations in leaf traits in term of shade tolerance and canopy layer

Consistent with our first hypothesis, we found that leaf traits significantly varied with species along a shade tolerance gradient (Fig. 1), probably related to the differences in stand conditions and resource availability (light and nutrition) for shade-intolerant species and shadetolerant species (Portsmuth and Niinemets, 2007). The stand conditions of shade-intolerant species are relative open, and the environmental resources are generally abundant, while the stand conditions of shadetolerant species are relative closed and the environmental resources are generally limited (Niinemets et al., 2015). For five species with different shade tolerances, the species with thicker palisade tissue had thinner epidermal tissue (Fig. 1), probably because of the optimization strategy in resource allocation (Prentice et al., 2014), when leaves are constructed, more investment in palisade tissue may necessarily involve tradeoffs that decrease investments in epidermal tissue (Evans, 1999; Coble et al., 2017).

LMA, PT and VLA_{min} increased with increasing canopy height (Fig. 1), which also supports our first hypothesis. Palisade tissue can be described as 'light pipes' that help direct light to the interior of cells, ensuring chlorophyll absorption (Ollinger, 2011). The vascular tissue can be described as 'water pipes' that can affect water supply and substance transportation (Nardini et al., 2012; Kawai et al., 2016). Thus, leaves growing in high light conditions often contain a thicker palisade layer and a higher VLA_{min} could supply the indispensable resources for leaf photosynthesis. Upper canopy layer leaves generally experience heat, desiccation and excessive irradiance stresses in high sunlight and physical damage from wind and rain, in order to cope with these stresses requires a high level of investment in protection (Demmig-Adams and Adams, 2006). Thus, the leaves with higher LMA, PT and VLA_{min} would enhance leaf toughness in the upper canopy (Blonder et al., 2011; Onoda et al., 2011; Poorter et al., 2018). Additionally, canopy height affects LMA, as the increasing canopy height, the limited osmotic adjustments and leaf turgor may restrict the further expansion and development of the leaf (Lilles et al., 2014; Coble et al.,

Table 2

The slopes, intercepts and R^2 values of the LMA-PT, LMA-ET and LMA-VLA_{min} relationships for each species and each canopy layer and the significance (*P*) of the difference in the slopes across five broadleaf species with different shade tolerances and three canopy layers.

Bivariate relationships	Species/Canopy	Slope	Intercept	R^2	Significance (P) of the differences in slopes
LMA-PT	Populus davidiana	0.58***	0.76***	0.40***	0.001
	Betula platyphylla	0.61***	0.71***	0.38***	
	Fraxinus mandshurica	0.97***	-0.126	0.40***	
	Tilia amurensis	0.99***	0.038	0.63***	
	Acer mono	0.70***	0.44***	0.58***	
	Upper	0.52***	0.84***	0.42***	0.670
	Middle	0.51***	0.82***	0.41***	
	Lower	0.61***	0.62***	0.50***	
LMA-ET	Populus davidiana	0.06	1.78***	0.00	0.008
	Betula platyphylla	0.63***	0.83***	0.21***	
	Fraxinus mandshurica	1.00**	0.359	0.16**	
	Tilia amurensis	0.90***	0.311	0.26***	
	Acer mono	0.83***	0.439	0.16***	
	Upper	-0.29*	2.19***	0.05*	0.047
	Middle	-0.29*	2.14***	0.04*	
	Lower	-0.41***	2.27***	0.05**	
LMA-VLA _{min}	Populus davidiana	1.63***	0.24***	0.10**	0.001
	Betula platyphylla	0.32***	1.48***	0.16***	
	Fraxinus mandshurica	0.072	1.70***	0.001	
	Tilia amurensis	0.74***	1.10***	0.22***	
	Acer mono	0.72***	1.18***	0.29***	
	Upper	0.46***	1.41***	0.33***	0.396
	Middle	0.53***	1.31***	0.45***	
	Lower	0.66***	1.18***	0.54***	





Fig. 2. Histograms indicate the relative weight of PT, ET and VLA_{min} to explain the variance (R^2) in the full model for predicting LMA of all species, and each species. Numbers on the histograms are the proportions of each anatomical trait interpretation. The R^2 values in the ordinate title are the variance in the full model for predicting LMA. *P < 0.05; **P < 0.01; and ***P < 0.001. The full model: LMA = $\beta_1 PT + \beta_2 ET + \beta_3 VLA_{min} + \beta_0$ (Supplementary information in Table S1). The abbreviations of the traits and species are shown in Fig. 1.

2017; Azuma et al., 2019). These morphological or anatomical effects are consistent with those seen by Coble and Cavaleri (2014) and maybe gravitational potential gradient is involved.

Shade-intolerant species are often characterized by strong vertical light gradients (Poorter, 2009), indicating that the canopy gradient of leaf traits of shade-intolerant species is stronger than shade-tolerant species. However, our results showed that the canopy gradients of LMA, PT and VLA_{min} of relative shade-tolerant species were stronger than

that of shade-intolerant species (Fig. 1). Shade-intolerant species have higher leaf turnover rates, shorter leaf lifespans (Kitajima et al., 2012), more uniform production and fall of leaves. The branches of shadeintolerant species usually distributed more sparsely to avoid selfshading. The rapid leaf turnover and sparse branch designs keep leaves in resource-rich light environments (Seiwa et al., 2006). Shade-tolerant species often have a slower leaf turnover rate and a peak of leaf production (Kitajima et al., 2012), resulting in a more closed canopy



Fig. 3. Structural equation model (SEM) relating shade tolerance, leaf anatomical traits and LMA. (a) Model exploring the effects of shade tolerance and leaf anatomical traits on LMA for the five broadleaf species; (b) model exploring the effects of shade tolerance and anatomical traits on LMA for the upper canopy layer of the five broadleaf species; (c) model exploring the effects of shade tolerance and anatomical traits on LMA for the middle canopy layer of the five broadleaf species; and (d) model exploring the effects of shade tolerance and anatomical traits on LMA for the middle canopy layer of the five broadleaf species; and (d) model exploring the effects of shade tolerance and anatomical traits on LMA for the middle canopy layer of the five broadleaf species. The coefficients are standardized prediction coefficients for each causal path. Solid arrows represent significant paths (p < 0.05) (gray: positive; blue: negative), and dashed arrows indicate nonsignificant paths (p > 0.05). Numbers on the arrows are standardized prediction coefficients. R² values around each variable represent the proportions of variation explained (Supplementary information in Table S2). Trait abbreviations are the same as those in previous figures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

structure, which may increase the shade from the upper leaves to the lower leaves (Niinemets et al., 2015). These factors may be the reason why the vertical gradient of leaf traits becomes more significant with increasing of shade tolerance.

4.2. Importance of anatomical traits and shade tolerance for LMA

The LMA-PT, LMA-ET, and LMA-VLA_{min} slopes significantly differed among the five broadleaf species, but there were no significant differences among the three canopy layers (Table 2). These results highlight the importance of shade tolerance when analyzing the relationship among leaf traits (Ameztegui et al., 2017). We found that the LMA-PT slope of F. mandshurica was higher, probably because of the highest PT (Fig. 1), and the increasing PT of F. mandshurica leads to increase in LMA more rapid than that in the other four species. The LMA-PT and LMA-VLA_{min} slopes of the lower canopy were higher than those of the upper and middle canopies, whereas the intercepts were smaller than the upper and middle canopies. These results mean that for the same individual, the LMA of the upper and middle canopy leaves would be smaller than that of the lower canopy layer for a leaf with a certain value of PT or VLAmin from the three canopy layers. This maybe because the other anatomical tissue maybe also vary with increasing canopy layers, such as the spongy tissue and the proportion of air space, which result in the variation strength of LMA with the increasing PT and VLA_{min} was different among three canopy layers (Mullin et al., 2009).

There appeared to be more evidence to support the second hypothesis. The variations in LMA were primarily determined by changes

in PT and VLA_{min}, whereas the contribution of ET was minimal for each species except *F. mandshurica* (Fig. 2). This case is consistent with many previous studies (Coble and Cavaleri, 2017; John et al., 2017). The epidermis has a very low density, that of the mesophyll is intermediate, and the vascular tissue has a high density (Poorter, 2009), thus, palisade cells expanding into the available air space and greater investments in vascular tissue could increase leaf density and further promote the LMA (Villar et al., 2013; Li et al., 2018). It is worth noting that the contribution of leaf anatomical traits had interspecific differences (Fig. 2), leaves with sparse vein distributions and smaller vein diameters, the contribution of VLA_{min} may be weakened for some species. For example, *F. mandshurica* had the highest PT and the lowest VLA_{min} (Fig. 1), and the relative weight of VLA_{min} was only 1.04%, whereas the relative weight of PT reached as high as 78.10%.

The SEM results showed that shade tolerance had a direct negative effect on LMA and VLA_{min} and had a direct promotion effect on ET (Figs. 3 and 4), which suggests that shade-tolerant species usually have lower LMA, VLA_{min} and higher ET. The results provide information that species with different shade tolerances have not only typical morphological and physiological characteristics but also typical anatomical characteristics. Relative shade-intolerant species may develop higher VLA_{min} for a greater hydraulic supply to get higher gas exchange rates (Sack et al., 2013), to compensate for water loss (Nardini et al., 2012; Kawai et al., 2016; Li et al., 2018) and maintain a suitable leaf temperature (Demmig-Adams and Adams, 2006; Onoda et al., 2011; Muir et al., 2017). For shade-tolerant species, their survival strategy is to minimize respiratory loss and maximize investment in storage organs



Fig. 4. Beta coefficients of explanatory variables on LMA. Different colors represent different explanatory variables. The solid-color bars show the direct effects, and the dashed-color bars show the total indirect effects of each predictor on the response variable. (a) Beta coefficients of shade tolerance and anatomical traits on LMA for the five broadleaf species (b) beta coefficients of shade tolerance and anatomical traits on LMA for the upper canopy of the five broadleaf species (c) beta coefficients of shade tolerance and anatomical traits on LMA for the upper canopy of the five broadleaf species (c) beta coefficients of shade tolerance and anatomical traits on LMA for the middle canopy of the five broadleaf species and (d) beta coefficients of shade tolerance and anatomical traits on LMA for the lower canopy of the five broadleaf species. Level of significance *P < 0.05, **P < 0.01, and ***P < 0.001, while no sign indicates nonsignificance (P > 0.05). Trait abbreviations are the same as those as in previous figures.

such as stems and roots rather than in leaves obtaining light resources (Poorter, 2009). The lower LMA and VLA_{min} are the embodiment of their decreased investment in leaves. A thicker epidermis is helpful for resisting against physical hazards for shade-tolerant species (Wright and Westoby, 2002; Poorter, 2009), which may contribute to enhance leaf lifespan (Wright and Westoby, 2002). Whereas the thinner epidermis of shade-intolerant species can better promote the penetration of light into the deep leaf (Coble et al., 2017; He et al., 2018).

We also found that PT, ET and VLA_{min} had a direct promotion effect on LMA (Figs. 3 and 4). Previous studies found that a higher LMA correlated with a lower ET for deciduous species, maybe because epidermal tissue has a low density (Villar et al., 2013; John et al., 2017). However, we found that ET had a positive effect on LMA. For some species, the density of epidermal cells is high, as their cell walls are thicker, which can enhance the influence of epidermal tissue on LMA

(Poorter, 2009). Many previous studies hypothesized that LMA was strongly driven by minor vein traits or by total vein length per area (VLA), but the results were unexpected (Sack et al., 2014; Li et al., 2015; John et al., 2017). Our results were different from those of Sack et al. (2014), Li et al. (2015) and John et al. (2017), but were consistent with Blonder et al. (2011). The former three groups of authors found that LMA and VLA were not correlated, particularly with $\ensuremath{\mathsf{VLA}_{\min}}\xspace$, the last author proposed the 'vein origin' hypothesis, where the vein traits would predict LMA according to specific equations. Although the minor veins account for a relatively small volume inside the lamina (Sack et al., 2013), VLA_{min} largely determined (> 80%) the VLA (Sack et al., 2012, 2013; Kawai et al., 2016). A high VLA was positively related to the concentration of total structural carbohydrates (Blonder et al., 2011), contributed a high carbon concentration in a leaf (Villar et al., 2013). Another observation was the negative effect of ET on PT (Figs. 3 and 4). This result is consistent with previous research results that found a negative correlation between these two tissue types (Niinemets, 1999), which represents a biological trade-off. The results from the SEM indicated that leaf anatomical trait combinations and shade tolerance can drive interspecific differences in LMA.

5. Conclusions

In our study, relative shade-intolerant species had higher LMA and VLAmin, and LMA, PT and VLAmin increased with increasing canopy height for five study species, which providing evidence that leaf anatomical traits have a vertical gradient within canopies and that there are typical anatomical characteristics for species with different shade tolerances. Our results also confirm that leaf anatomical traits can serve as predictors of LMA, which provide supplementary evidence for the anatomical basis of LMA variation. We also confirm that the relationships among leaf traits have an universality across canopy layers but they have a specificity among species with different shade tolerances, which highlight the importance of shade tolerance on tradeoffs among leaf traits in trait-based ecology. We also provide the evidences that shade tolerance is a good predictor for functional traits, which is helpful for better understanding ecological response mechanism of key leaf functional traits in faced of environmental conditions change and further revealing the mechanisms of forest dynamics via functional traits.

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Author contributions

Zhili Liu and Guangze Jin planned and designed the research. Xueshuang Zhang and Zhili Liu performed experiments. Xueshuang Zhang analysed data and wrote the manuscript. Zhili Liu and Guangze Jin provided helpful comments in the draft.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foreco.2019.117569.

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