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THE OPTIMAL TEMPERATURE FOR THE GROWTH OF BLUEBERRY (*VACCINIUM CORYMBOSUM* L.)

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Abstract

Blueberry (*Vaccinium corymbosum* L.), a perennial evergreen or deciduous shrub, has recently been introduced into Southern China, where the subtropical climate is hot and humid in summer. Identifying the optimal growth temperatures and understanding the mechanisms of thermal stress on blueberry are not only critical to determining suitably growing areas in Southern China, but also significantly important for selecting and breeding new heat tolerance blueberry cultivars for adapting to subtropical climates. In this study, we examined the optimal temperature for the growth of six blueberry cultivars ('Bluecrop', 'Duke', 'Brigitta', 'Gulfcoast', 'O'Neal', and 'Blue Ridge') with four growth chambers where the temperatures were controlled at 25, 30, 35, and 40℃, respectively. We found that initial increase in temperature dramatically enhanced the growth of four cultivars ('Bluecrop', 'Duke', 'Brigitta', and 'Blue ridge') through the warming effect, whereas this warming effect was substantially compromised with further increase in growth temperature, demonstrating an optimal temperature of 32.6, 30.4, 31.8, and 29.0℃ for the four cultivars respectively. By contrast, the aboveground, belowground, and total biomass of 'Gulfcoast' and 'O'Neal' were linearly declined with growth temperatures, indicating that elevating temperature above 25℃ had negative effects on blueberry growth. Meanwhile, we also found that the leaf photosynthesis, stomatal conductance, and transpiration of the six blueberry cultivars shared similar trends as plant growth in response to temperatures, suggested that leaf biochemical and photochemical processes affecting the optimal growth temperature of blueberry plants. Moreover, the temperature effects on blueberry growth was also attributed to the changes in the leaf number, leaf length and width, leaf biomass, as well as the leaf stomatal traits including density, openness, and spatial distribution pattern of stomata. In addition, high temperatures exceeding the optima also affected chloroplast structures through damaging grana lamella and stromal lamella as well as breaking chloroplast envelope. Our results suggested that the optimal growth temperature of blueberry was highly dependent on cultivars. Therefore, the optimal temperature found in this study can be used as an indicator in selecting and breeding new blueberry strains in adapting to high temperatures in subtropical China where the market demands for blueberry products have been skyrocketing.

Key words: Optimal temperature, Blueberry,*Vaccinium corymbosum*, Bluecrop', 'Duke, Brigitta', 'Gulfcoast'

Introduction

Plants exposed to environmental changes may modulate their growth and development mainly to their perennial lifestyle (Thomas *et al.,* 2004; Colwell *et al.,* 2008; van Mantgem *et al.,* 2009). Optimal plant growth usually takes place within more or less strict environmental conditions (Ruelland & Zachowski, 2010), and outside the optimal range the growth and productivity of plants may be limited by several abiotic stresses such as thermal stress (Rodríguez *et al.,* 2015). Previous studies have shown that different plants may have different optimal growth temperatures, and most plant species can only survive in a certain range of growth temperatures (van Mantgem *et al.,* 2009; Jin *et al.,* 2011; Zheng *et al.,* 2013a). Therefore, plants with higher optimal temperatures are likely to benefit most from higher temperatures, and meanwhile plants with lower optimal temperatures may suffer negative impacts, and thus cause severe damage to yield when exposed to long-term high temperatures (Malcolm *et al.,* 2006; Colwell *et al.,* 2008; Tacarindua *et al.,* 2013). For example, Tacarindua *et al.* (2013) reported that increasing temperature 3℃ above the ambient with growth chamber marginally reduced the aboveground biomass and seed yield of soybean by 27% and 40% respectively.

It is well demonstrated that temperature have profound effects on net photosynthetic rates through various processes including both up-regulations (Chapin & Shaver, 1996; Yin *et al.,* 2008; Prieto *et al.,* 2009; Zheng *et al.,* 2013a) and down-regulations (Callaway *et al.,* 1994; Roden & Ball, 1996; Djanaguiraman *et al.,* 2011) depending on the temperature below or above the optimal temperature for plants. Increasing temperature may dramatically enhance the photosynthetic rates by increasing both the Rubisco concentration and activity, and thus enhancing the carboxylation efficiency when the temperature is below the optimal temperature of plant growth (Apple *et al.,* 2000; Han *et al.,* 2009). Conversely, higher growth temperatures above the optimum may lead to photosynthesis reduction by disrupting the structure of chloroplasts, damaging the function of photosystem Π (PS Π), and suppressing the activation state of Rubisco (Roden & Ball, 1996; Javed *et*

al., 2014). Moreover, growth temperatures above the optimal temperature thresholds may also limit geographical distribution of plants/crops due to the growth reduction (Rodríguez *et al.,* 2015). It is noted that high temperatures also increase plant respiration which further reduces plant growth (Atkin & Tjoelker, 2003; Atkin & Macherel, 2009; Crous *et al.,* 2011).

Blueberry (*Vaccinium corymbosum* L.) is a perennial evergreen or deciduous flowering shrub originated from North America. Blueberry has been becoming a popular fruit in China and the world mainly due to its nutritional value of anthocyanins which may reduce the incidence of cardiovascular disease, cancer, hyperlipidemias and other chronic diseases (Pascual-Teresa & Sanchez-Ballesta, 2008). Human selection has lead to the development of many new cultivars in the past years (Kole, 2007). In general blueberry can tolerate a large range of temperature changes ranging from below zero to more than 40℃ (Chen *et al.,* 2012). Among various blueberry cultivars, the highbush blueberry is one of the best of its kind (Starast *et al.,* 2009) and widely grown in Northern China. However, it can not be grown in Southern China where the warm climate cannot meet its chilling requirements (Hancock *et al.,* 2008). Meanwhile several other cultivars have recently been introduced to subtropical China for commercial cultivation (Chen *et al.,* 2012). Given that the temperature in subtropical China often approaches 40℃ or even higher in summer, high temperature has become the most significant abiotic stress, limiting the growth and yield of blueberry in this area (Li *et al.,* 2013). In fact, the high temperature has already damaged the blueberry industry and caused severe economic losses in Zhejiang Province (Chen *et al.,* 2012). Therefore, investigating the optimal growth temperatures and understanding the mechanisms of thermal stress on blueberry cultivars are critical for blueberry zoning in China and for selecting and breeding new heat tolerance cultivars especially under future global warming with more frequent heat waves (Anon., 2013).

The objectives of this study are to: (1) examine the effects of high temperature on the growth of blueberry plants; (2) investigate the optimal growth temperatures of different blueberry cultivars; and (3) explore the physiological and biochemical processes affecting the growth of blueberry under high temperature stresses.

Materials and Methods

Plant material: Two-year-old seedlings of six highbush blueberry cultivars including 'Bluecrop', 'Duke', 'Brigitta', 'Gulfcoast', 'O'Neal', and 'Blue Ridge' were selected from field plots in the research farm at Dalian University in northeast China. Then the collected plants were transplanted into pots (10 cm diameter × 25 cm long) filled with fritted clay (one plant per pot) and grown in a greenhouse with an average temperature of 25/20℃ (day/night) and about 1000 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR) in natural sun light, and 65% relative humidity for 30 d (March-April 2014) to establish canopy. During the establishment period, plants were irrigated daily to water-holding capacity and fertilized twice per week with half-strength Hoagland's solution (Hoagland & Arnon, 1950).

High temperature treatments: We selected 20 pots with healthy and uniform growth plants for each cultivar (20 pots × 6 cultivars = 120 pots in total) and then randomly planted 30 pots (5 pots for each cultivar) into each of four walk-in growth chambers, where the temperature was set up to 25/20, 30/25, 35/30, or 40/35℃, respectively. Other environmental factors maintained throughout all four chambers including humidity (60-70%), light intensity (1000 μmol m^{-2} s^{-1} PAR), photoperiod (light on at 6 am, and off at 8 pm), soil type (fritted clay, same brand and package for all), water amount (200 mL per pot, watered every other day), and nutrition type (plain tap water). Plants were fertilized once weekly with half-strength Hoagland's solution throughout the growth period. In order to minimize confounding effects of environmental variation between different chambers, we randomly changed the temperature of each growth chamber every week, and then relocated the high temperature treated plants to the growth chambers with corresponding temperature. The large volume of the pot and frequent watering and fertilization ensured enough space for root growth and ample nutrient supply to avoid "bonsai effect". After a 90-day growth period, 3 pots of each cultivar were randomly chosen from each growth chamber as 3 replications for measurement of plant biomass, leaf structural characteristics, leaf stomatal traits, and leaf gas exchange.

Measuring plant biomass and leaf structural characteristics: We obtained the aboveground and belowground biomass by harvesting and de-potting the plants. The aboveground portion (leaves plus stem) of all plants were removed, placed in paper bags, and then oven-dried at 80℃ for 24h before measuring the dry biomass with an electronic scale. Leaf length was measured from the base of the leaf (excluding the stalk) to the tip of the leaf and leaf width was measured from the middle portion of each leaf with a ruler. At the same time, the leaf number of each plant was also counted and recorded.

Leaf gas exchange: Leaf gas exchange measurements were obtained using a portable photosynthesis system (Li-6400; LI-COR Inc. USA). We randomly selected three new fully expanded leaves from each pot under different high temperatures to measure the net CO_2 assimilation rate (P_n), stomatal conductance (g_s) transpiration rate (T_r), intercellular CO_2 concentration (C_i), and leaf dark respiration (R_d). Leaf gas exchange measurements were conducted with saturating light at 1000 μmol m^{-2} s^{-1} PAR, and CO_2 concentration of 400 μmol mol^{-1}. Leaf temperature in the cuvette was controlled at the same as growth temperature in each chamber. The cuvette was sealed with plasticine to prevent leakage, and then the gas exchange system was zeroed using H_2O and CO_2 free air every day. The vapor pressure deficit (VPD) in the foliar cuvette was controlled by the Li-cor 6400 system, and most of the measurements were conducted with VPD lower than 1.5 kPa, which means moisture was not a limiting factor. After the measurements of leaf photosynthesis, the red and blue light source was turned off at least 10 minutes, and then measured the leaf dark

respiration rates (R_d) with the portable photosynthesis system. All the other conditions were the same as leafphotosynthesis measurements.

Measuring leaf stomatal density, stomatal pore traits, and spatial distribution pattern of stomata: Given that all the stomata of blueberry plants distribute on the abaxial surface of leaves, we sampled imprints from the middle section of the abaxial surface with a colorless nail polish to characterize stomatal density pore traits. The epidermis of the leaves were cleaned first by a degreased cotton ball and then carefully smeared with nail varnish from the mid-area between the central vein and the leaf edge for about half an hour. The thin film (approximate 5 by 15 mm) was peeled off from the leaf surface and mounted on a glass slide. Then the thin film was immediately covered with a cover slip and pressured lightly with a fine-point tweezers. The sampling method is a widely adopted approach to measure stomatal traits as in previous studies on this topic (Zheng *et al.*, 2013b; Xu, 2015).

The imprints were observed in the laboratory with a microscope (DM2500, Leica Corp, Germany) and photographed them using a digital camera (DFC 300-FX, Leica Corp, Germany) with a scale of 100 μm burned onto each image. Then, stomata on the photographs were counted, recorded, and converted to stomatal density, which was expressed as the number of stomata per unit leaf area. To characterize the features of length, width, and area of stomatal pores, we imported the photographs into the Image J quantification software (NIH, USA) for measuring stomatal apertures length (SAL), stomatal apertures width (SAW), stomatal apertures area (SAA), stomatal apertures circumference (SAC). In addition, we also calculated stomatal aperture shape index (SASI), which is calculated by the function that shape index = $\sqrt{A}$ /P×100%, where A is the stomatal aperture area and P is the stomatal aperture circumference. In addition to stomatal density and pores traits, the spatial distribution pattern of stomata for each image was also obtained by digitizing the stomatal positions into a shape file in GIS with the ArcMap software (Xu, 2015). The center of each stoma was converted to a point in the shape file.

For visualizing and comparing the differences of single stoma among high temperature treatments, electronic microphotographs of stomata were also obtained using scanning electron microscopy (SEM). We snapped three pieces (2×2 mm) from the middle section of each leaf with a fixative solution consisting of 2.5% (v/v) glutaraldehyde (0.1 M phosphate buffer, pH 7.0). Samples were stored at 4 ℃ and transportedto the laboratory as soon as possible. Then the samples were washed six times with the same buffer and post-fixed in 1% (v/v) osmium tetroxide for 3 hours at room temperature. After being washed with the same buffer, leaf tissues were passed through an ethanol dehydration series. Then the samples were critical point-dried, mounted on stubs, and coated with gold in a high-vacuum evaporation unit. Samples were examined and photographed at 10 KV under a Quanta 200 scanning electron microscope (FEI Corp, USA).

Observation on ultra-structures of chloroplast: For examining the changes on ultra-structure of cellular organelles, we took the advantage of the transmission electron microscopes. New fully expanded leaves of each cultivar under high temperatures were selected and dissected, and then immediately fixed in 2.5% (v/v) glutaraldehyde (0.1 M phosphate buffer, pH 7.0) for 2 h at 4℃. The samples were washed four times with the same buffer and postfixed in 1% osmium tetroxide for 3 h. After being washed with the same buffer, leaf tissues were passed through an ethanol dehydration series, and then infiltrated and embedded in Spurr's resin. Sections were cut using an LKB-V ultramicrotome (LKB, Bromma, Sweden). Thin sections were stained with uranyl acetate and lead citrate, then observed and photographed under a transmission electron microscope (JEOL Ltd, Tokyo, Japan).

Statistical analysis: The differences of the physiological, anatomical, and biochemical variables between the warmed and control plots were tested with the Student's *t*-test (p<0.05) using software SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Plant biomass: The plant growth of 'Bluecrop', 'Duke', 'Brigitta', and 'Blue ridge' (belowground biomass, aboveground biomass, and total biomass) shared a bell-shaped curve in relation to growth temperature following a quadratic relationship with the maximal biomass accumulation around 30 ℃ (Fig. 1). As the growth temperature increased from 25℃ to 30℃, the total biomass production substantially stimulated by 51.9%, 20.3%, 14.2%, and 19.6% for 'Bluecrop', 'Duke', 'Brigitta', and 'Blue ridge', respectively (Fig. 1). However, with further increase of growth temperature, the stimulation of high temperature on plant growth declined and eventually vanished beyond a turning point, which is the optimal growth temperature where the total biomass reaches its maximum. The optimal growth temperature of 'Bluecrop', 'Duke', 'Brigitta', and 'Blue ridge' was 32.6℃, 30.4℃, 31.8℃, and 29.0℃, respectively. Beyond these optimal temperatures, we found obviously negative effects on the biomass accumulation with further elevating growth temperatures, indicating that the growth of these four blueberry cultivars was limited by higher temperatures over the optimum. In contrast to the four blueberry cultivars ('Bluecrop', 'Duke', 'Brigitta', and 'Blue ridge'), the growth of the other two cultivars ('Golfcoast' and 'O'Neal') in response to high temperature featured linear relationships with sharply decreases in plant biomass accumulation. The belowground biomass, aboveground biomass, and total biomass reduced 28.0%, 29.9%, 41.7% for 'Golfcoast' and 43.8%, 44.9%, 44.5% for 'O'Neal', respectively with the growth temperature increasing from 25℃ to 40℃ (Fig. 1).

Leaf characteristics: Our two-way ANOVA results showed that the leaf number, leaf length and width, as well as leaf biomass were statistically different among the six blueberry cultivars (all p<0.001) (Table 1). Meanwhile, high temperature substantially affected leaf number (p=0.012), leaf length and width (p<0.001), and leaf biomass (p=0.035). In addition, we also found significantly interactive effects of species and temperature on leaf length (p=0.024), leaf width (p=0.014), and leaf biomass (p=0.004), except for leaf number (p=0.066) (Fig. 2; Table 1).

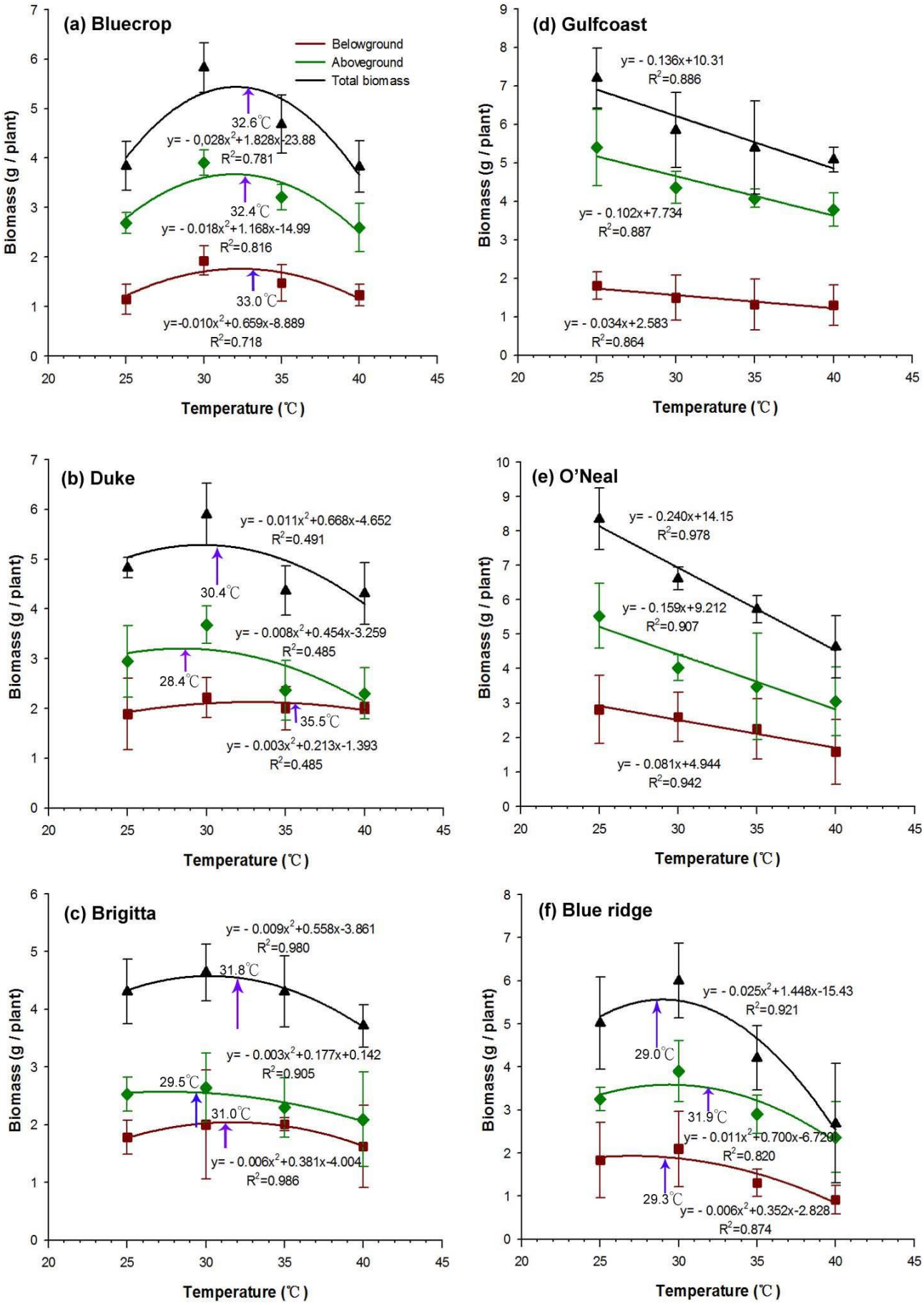

Fig. 1. Effects of growth temperature on the plant biomass of six blueberry cultivars.

Table 1. Effects of growth temperatures on leaf traits of six blueberry cultivars.

Cultivars	Leaf traits			
	Number (per plant)	Length (cm)	Width (cm)	Biomass (g)
		25℃		
Bluecrop	11.7	5.2	2.8	0.2
Duke	18.8	4.9	3.0	0.4
Brigitta	17.3	5.4	3.2	0.5
Gulfcoast	65.3	3.5	2.1	1.1
O'Neal	23.0	4.2	2.6	1.0
Blue Ridge	25.5	4.6	2.6	0.3
		30℃		
Bluecrop	17.7	5.5	3.2	0.6
Duke	14.7	5.0	2.9	0.9
Brigitta	15.0	5.5	3.2	0.4
Gulfcoast	40.5	3.4	1.8	0.8
O'Neal	18.0	3.6	2.2	0.8
Blue Ridge	26	4.7	2.7	0.9
		35℃		
Bluecrop	17.5	4.6	2.5	0.5
Duke	22.5	5.0	3.0	0.5
Brigitta	21.0	4.2	2.5	0.4
Gulfcoast	79.0	2.8	1.6	0.4
O'Neal	25.3	3.0	2.0	0.4
Blue Ridge	27.8	4.0	2.4	0.4
		40℃		
Bluecrop	17.3	4.2	2.4	0.3
Duke	17.0	4.6	2.7	0.2
Brigitta	24.0	4.5	2.5	0.6
Gulfcoast	56.0	4.1	2.3	0.2
O'Neal	8.5	4.1	2.4	0.2
Blue Ridge	27	4.3	2.4	0.2
Species (*S*)	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001
Temperature (*T*)	*P*=0.012	*P*<0.001	*P*<0.001	*P*=0.035
S×*T*	*P*=0.066	*P*=0.024	*P*=0.014	*P*=0.004

Stomatal density, anatomy and spatial distribution pattern: The stomata of the six highbush blueberry cultivars was significantly different in stomatal density (p<0.001), stomatal pore length (p<0.001), stomatal pore width (p<0.001), stomatal pore area (p<0.001), stomatal pore perimeter (p=0.001), and stomatal pore shape index (p=0.003) (Table 2). Moreover, high temperature substantially changed the stomatal density (p=0.025), stomatal pore width (p=0.035), and stomatal pore shape index (p=0.003), while had little effect on stomatal pore length (p=0.866), stomatal pore area (p=0.214), and stomatal pore perimeter (p=0.275) of the six blueberry cultivars (Table 2), indicating that stomata in response to high temperature mainly by adjusting both the stomatal density and stomatal pore shape attributed to the openness of stomtal pores (stomatal pore width). However, we found that no obviously interactive effect of species and temperature on the stomatal pore width (p=0.065) and stomatal pore shape index (p=0.215) (Table 3).

High temperature not only affected stomatal anatomy, but also changed the spatial distribution pattern of stomata (Fig. 3). The spatial pattern analysis with the Ripley's K function showed that stomatal distribution on leaf surface was highly scale dependent. The stomata on leaves followed a regular distribution at small scales (*c.* <150μm) and a random distribution at larger scales for the six blueberry cultivars grown at ambient temperature (25℃) or high temperatures (30℃, 35℃, and 40℃). Meanwhile, the spatial distribution pattern of stomata on leaves also species dependent, evidenced by the different L (d) values (an expectation of zero for any value of d) among the six blueberry cultivars (Fig. 3). The stomatal distribution on the leaves of 'Bluecrop' tended to be more regular than that of the other five cultivars, because the stomata of 'Bluecrop' shared a regular pattern at the lower L (d) value and the smaller threshold scale than those of the other cultivars under ambient temperature. Moreover, we also found that high temperature, in general, would make the stomata more regularly distributed on blueberry leaves, although the stomatal distribution in response to high temperature slightly varied with cultivars. Specifically, elevating growth temperature from 25℃ to 40℃ made the stomatal distribution more regular at small scales (*c.* <180μm) demonstrating by the obviously decreased minimum L (d) values of the blueberry cultivars except for 'Bluecrop'. However, high temperatures had adverse impacts on the stomatal distribution pattern of 'Bluecrop' because the enhanced temperatures from 25℃ to 35℃ substantially increased both the minimum L (d) value and threshold scale, but further elevating growth temperature from 35℃ to 40℃ resulted in dramatically decreases in the minimum L (d) value and scale range of the regular pattern (Fig. 3).

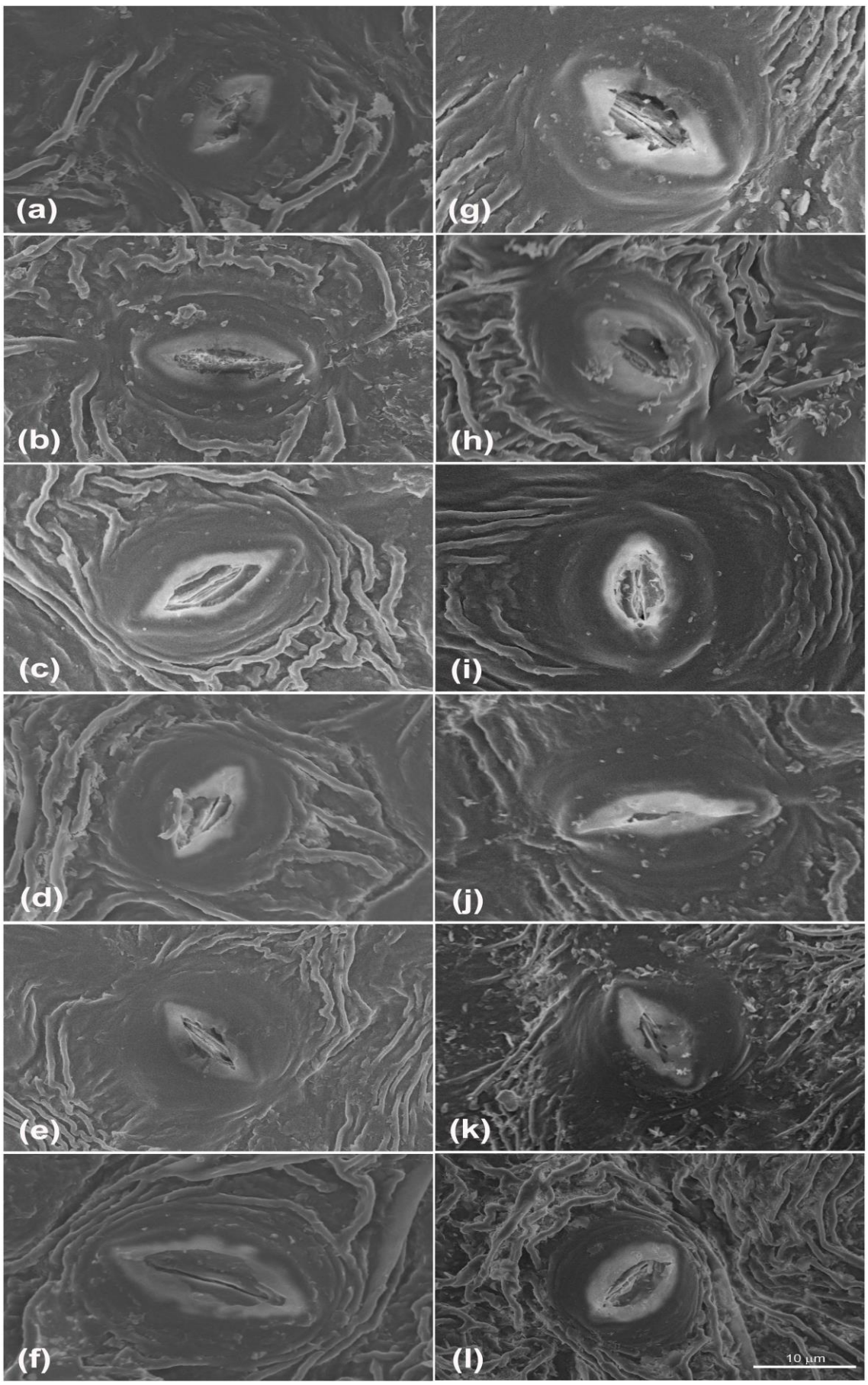

Fig. 2. Scanning electron micrographs (SEM) showed the leaf stomatal traits of six blueberry cultivars grown at 25℃ (a-f) and 40℃ (g-l) . Bars, 10 μm.

Table 2. Effects of growth temperatures on stomatal characteristics of six blueberry cultivars.

Cultivars	Stomatal characteristics	Growth temperatures			
		25℃	30℃	35℃	40℃
'Bluecrop'	Stomatal density (number mm^{-2})	124a	113a	96a	91a
	Stomatal pore length (μm)	15.2b	17.8 ab	21.5a	20.7a
	Stomatal pore width (μm)	10.5b	11.6b	14.9a	13.3ab
	Stomatal pore area (μm^2)	133c	159 b	246 a	211ab
	Stomatal pore perimeter (μm)	41.9c	47.3b	58.3 a	54.4 ab
	Stomatal pore shape index	1.08a	1.08a	1.06a	1.08a
'Duke'	Stomatal density (number mm^{-2})	97a	84a	96a	85a
	Stomatal pore length (μm)	19.4a	18.3a	18.7a	17.1a
	Stomatal pore width(μm)	10.7a	10.6a	11.5a	11.0a
	Stomatal pore area (μm^2)	163a	162 a	161a	148a
	Stomatal pore perimeter (μm)	44.9a	47.3a	48.2a	45.3a
	Stomatal pore shape index	1.12a	1.12a	1.09a	1.08a
'Brigitta'	Stomatal density (number mm^{-2})	72b	108ab	124a	84b
	Stomatal pore length (μm)	18.3ab	16.1ab	20.4a	13.4b
	Stomatal pore width (μm)	11.5a	9.9ab	12.7a	7.3b
	Stomatal pore area (μm^2)	174a	119ab	202a	72b
	Stomatal pore perimeter (μm)	48.2a	41.8ab	53.4a	33.3b
	Stomatal pore shape index	1.09b	1.09b	1.08b	1.12a
'O'Neal'	Stomatal density (number mm^{-2})	62.9a	82.5a	67.3 a	72.0 a
	Stomatal pore length (μm)	23.3a	18.7 a	20.1a	22.9a
	Stomatal pore width (μm)	14.6a	11.9a	14.3a	13.6a
	Stomatal pore area (μm^2)	258a	167a	238a	245a
	Stomatal pore perimeter (μm)	60.7a	48.9a	55.8a	58.8a
	Stomatal pore shape index	1.09a	1.09a	1.07a	1.09a
'Gulfcoast'	Stomatal density (number mm^{-2})	76ab	81ab	98a	61b
	Stomatal pore length (μm)	25.8a	23.8a	18.2b	24.2a
	Stomatal pore width (μm)	15.0a	13.5a	12.1a	13.3a
	Stomatal pore area (μm^2)	301a	248ab	171b	251ab
	Stomatal pore perimeter (μm)	66.3a	60.6a	48.4b	61.3a
	Stomatal pore shape index	1.09ab	1.11a	1.07b	1.10a
'Blue Ridge'	Stomatal density (number mm^{-2})	78a	99a	100a	85a
	Stomatal pore length (μm)	19.3b	19.5b	19.7b	22.9a
	Stomatal pore width (μm)	12.4ab	11.9 b	14.2a	13.9a
	Stomatal pore area (μm^2)	184b	171b	220a	242a
	Stomatal pore perimeter (μm)	51.2b	50.2b	54.7ab	58.8a
	Stomatal pore shape index	1.07a	1.09a	1.06a	1.08a

Table 3. Integrative effects of species and temperature on stomatal traits of blueberry plants.

Stomatal traits	Species (*S*)	Temperature (*T*)	*S*×*T*
Stomatal density (number mm^{-2})	$p<0.001$	$p=0.025$	$p=0.044$
Stomatal pore length (μm)	$p<0.001$	$p=0.866$	$p=0.020$
Stomatal pore width (μm)	$p<0.001$	$p=0.035$	$p=0.065$
Stomatal pore area (μm^2)	$p<0.001$	$p=0.214$	$p=0.030$
Stomatal pore perimeter (μm)	$p=0.001$	$p=0.275$	$p=0.022$
Stomatal pore shape index	$p=0.003$	$p=0.003$	$p=0.215$

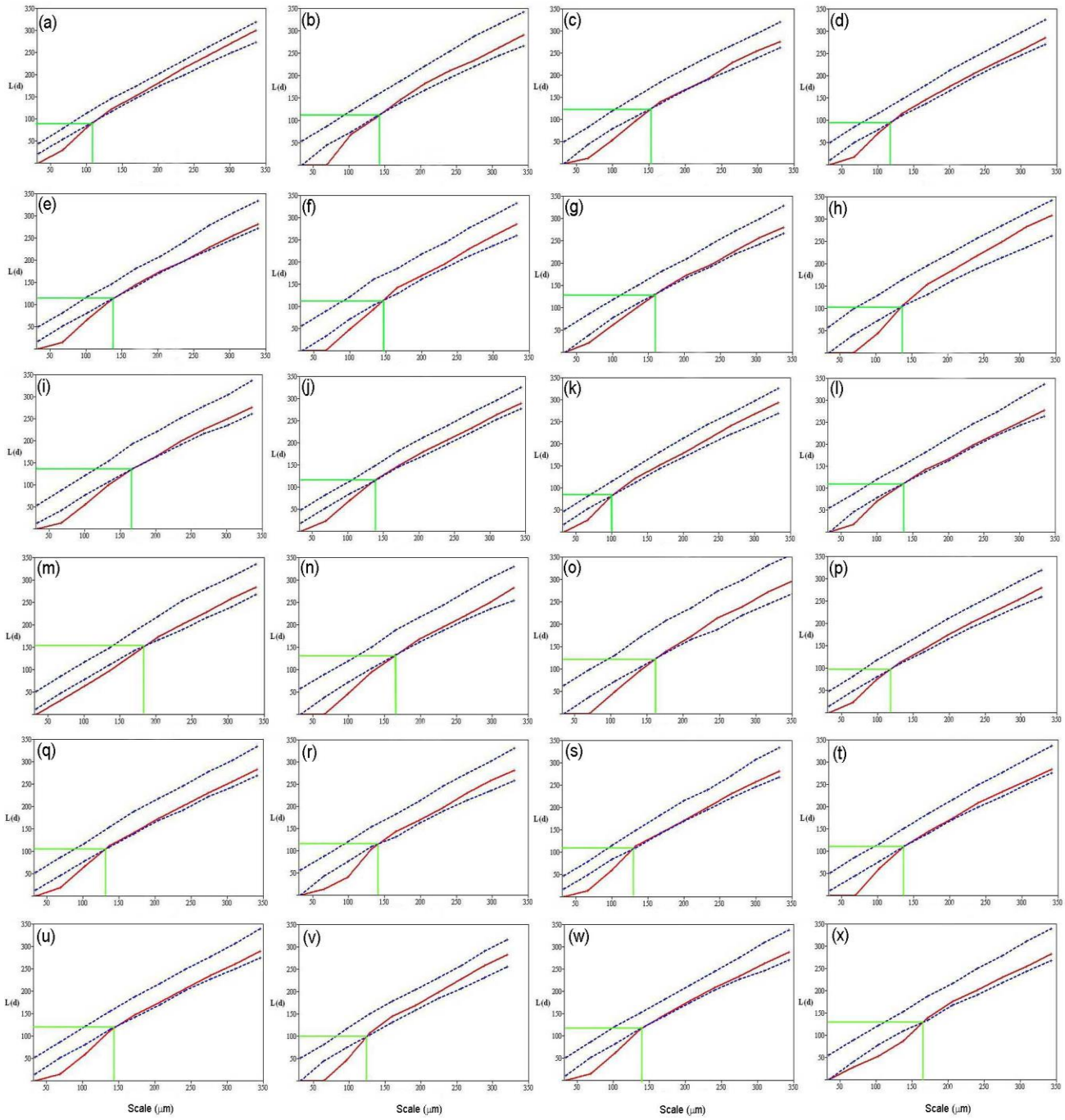

Fig. 3. Spatial pattern of stomatal distribution on leaf adaxial surface of Bluecrop (a-d), Duke (e-h), Brigitta(i-l), Golfcoast (m-p), O'Neal (q-t), and Blue ridge (u-x) grown at 25℃, 30℃, 35℃, and 40℃.

Leaf gas exchange: The leaf photosynthesis (P_n) and stomatal conductance (g_s) both demonstrated a bell-shaped curve in relation to growth temperatures. The maximum P_n of the six blueberry cultivars occurred at about 34℃ (Fig. 4), while the optimal temperature for g_s ranged from 30℃ to 33℃ (Fig. 5), which is apparently lower than the optimal temperature for P_n. Meanwhile, both relationships between temperature and P_n or g_s can be quantified through quadratic models. Similar with P_n and g_s, the relationship between growth temperature and transpiration rates (T_r) also featured distinct bell-shaped curves for the six blueberry cultivars. The T_r increased rapidly with the initial enhancement in growth temperature, and gradually leveled off with continuous increase in growth temperature (Fig. 6). Further increase in growth temperature would dramatically decrease the T_r of the six blueberry cultivars. However, it is noted that the optimal temperatures for the T_r of the three north highbush blueberry cultivars was 37.8, 35.5, and 38.1℃ for 'Bluecrop', 'Duke', and 'Briggita', which is much higher than those of the three south highbush blueberry cultivars where 'Glucoast' and 'O'Neal' had an optimal temperature around 34 ℃ and Blue ridge had even lower optimal temperature, merely 31.2℃ (Fig. 6). In addition, the leaf dark respiration (R_d) of the three north highbush blueberry cultivars exponentially increased with the enhancement of growth temperature from 25℃ to 40℃ (Fig. 7). However, we did not find significantly different temperature sensitivity (Q_{10}) of leaf dark respiration among the six cultivars (Fig. 7).

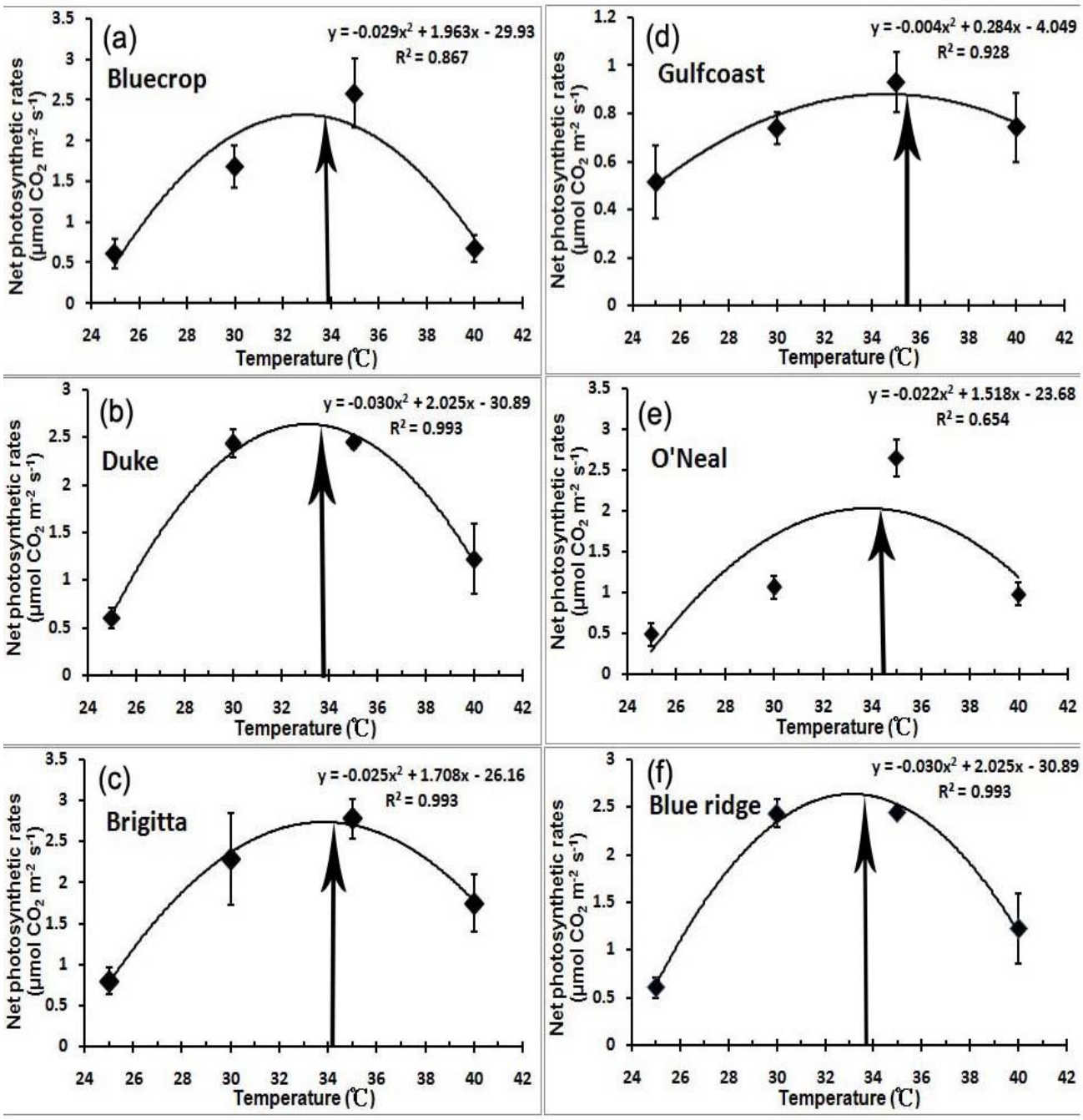

Fig. 4. The optimal temperature for net photosynthetic rates of six blueberry cultivars.

Chloroplast ultra-structure: The chloroplasts of the six highbush blueberry cultivars showed regular ellipsoidal shape with well-organized chloroplast thylakoid and parallel lamellae under 25℃ (Fig. 8). After heat stress (40℃), however, these chloroplasts became swollen (Fig. 8, g, h, i), and even seriously damaged with disordered grana lamella and stromal lamella (Fig. 8 h) as well as broken chloroplast envelope, especially for the three south highbush cultivars (Fig. 8, j, h, j, k, l). Meanwhile, the number of plastoglobulus (Pl) for 'Bluecrop', 'Duke', and 'Blue ridge' was increased by heat stress (Fig. 8, g, h, l). Moreover, we also found large starch grains in the blueberry leaves grown under 40℃ conditions, whereas few starch grain was observed for the plants under 25℃ (Fig. 8 i). In addition, it was interesting that more and larger mitochondria were observed near the chloroplasts from the plants grown at 40℃ than those of plants at 25℃ (Fig. 8 j).

Discussion

It is well known that most plants can only grow in a certain range, and thus some species may adapt to warmer environment by shifting their ranges or changing the growth and development to ensure that the optimal growth temperature are not exceed (Jin *et al.,* 2011; Rodríguez *et al.,* 2015). By contrast, other species may fail to adapt to the warmer temperatures and may even be extinct under too high air temperature (Thomas *et al.,* 2004; Malcolm *et al.,* 2006; Colwell *et al.,* 2008). Previous studies have reported that the growth temperature range for highbush blueberries is cultivar dependent (Chen *et al.,* 2012) whereas little information was known about the optimal growth temperature for highbush blueberry, which is ecologically classified into two groups including north highbush and south highbush cultivars according to the chilling

requirements and winter hardiness (Kole, 2007; Hancock *et al.*, 2008). In the current study, we employed linear and non-linear (quadratic equations) regressions to examine the relationships between growth temperature and plant biomass, and then estimated the optimal growth temperature of blueberry cultivars. Our results showed that the biomass accumulation of 'Bluecrop', 'Duke', 'Brigitta', and 'Blue ridge' shared a bell-shaped curve in relation to growth temperature with maximal values around 30℃ (Fig. 1), suggesting that the optimal growth temperature of these four blueberry cultivars was 30℃. However, it is noted that the biomass accumulation of the other two cultivars ('Golfcoast' and 'O'Neal') was linearly decreased with the enhanced growth temperatures, indicating that plant growth was limited by the higher temperatures over 25℃. Meanwhile, earlier studies have claimed that the thresholds of growth temperature for north highbush blueberries are much lower than those of south highbush blueberries (Kalt *et al.*, 2001*;* Starast *et al.*, 2009), because the south highbush blueberries contain much more hot resistance genes (Starast *et al.*, 2009; Šterne *et al.*, 2011). However, our results showed that the optimal temperature of biomass accumulation for the three north highbush cultivars were much higher than those of the two south highbush cultivars ('Golfcoast' and 'O'Neal'), except for 'Blue ridge'. Meanwhile, it should be noted that the six highbush cultivars have grown more than ten years at Dalian City featured with a very cold climate in Northeast China. In this study, the south highbush blueberry cultivars may fully adapt to the cold climates during the long-term survive at cold environments and thus may lost the resistance ability on heat tolerance, even more heat susceptible than the north highbush cultivars. Our results suggested that some south highbush cultivars may be more susceptible to high temperatures than north highbush cultivars, and thus heat-tolerance blueberry cultivars should be selected according to their optimal growth temperatures before introducing to Southern China (featured with a hot climate in summer).

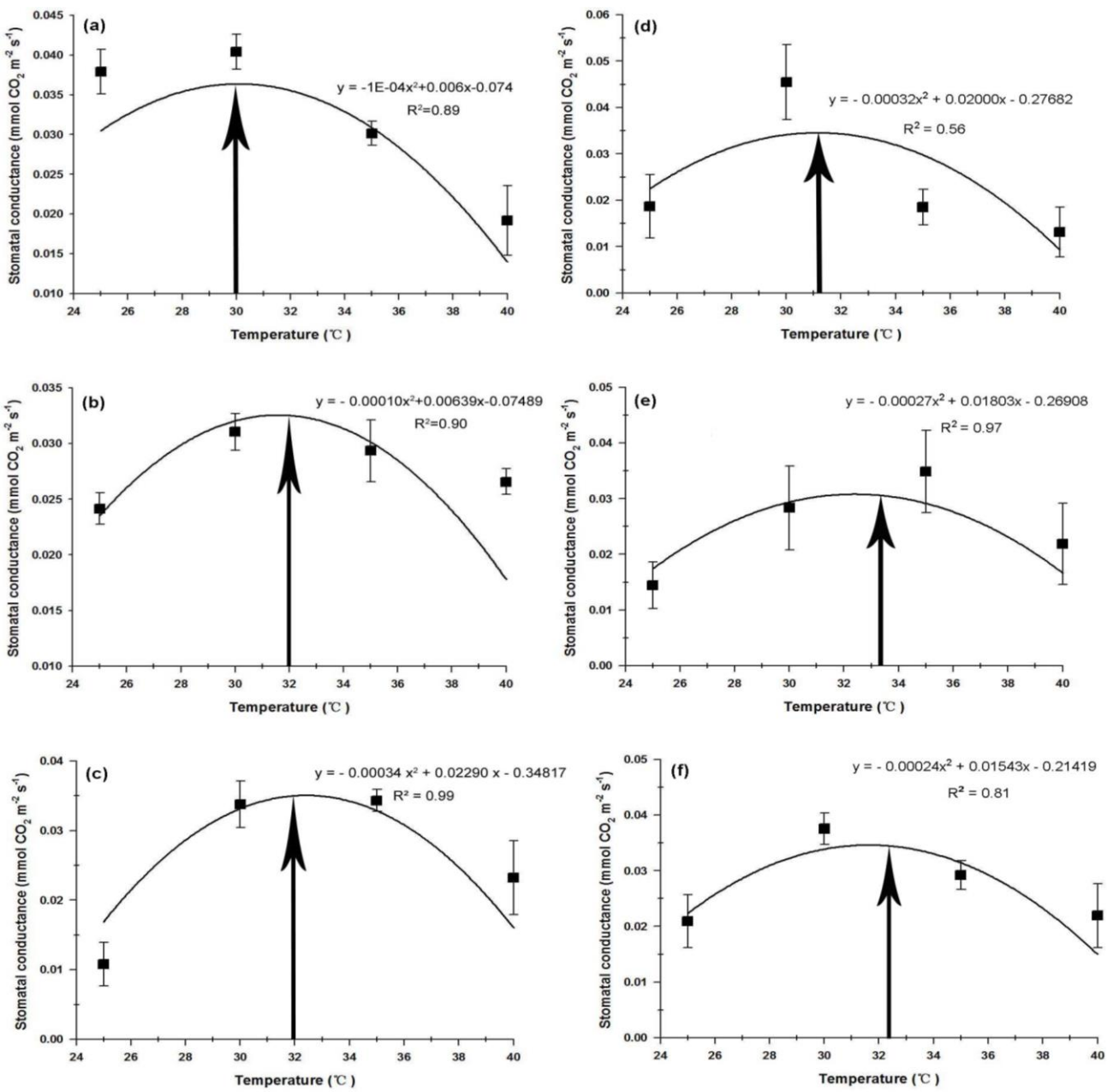

Fig. 5. The optimal temperature for stomatal conductance of six blueberry cultivars.

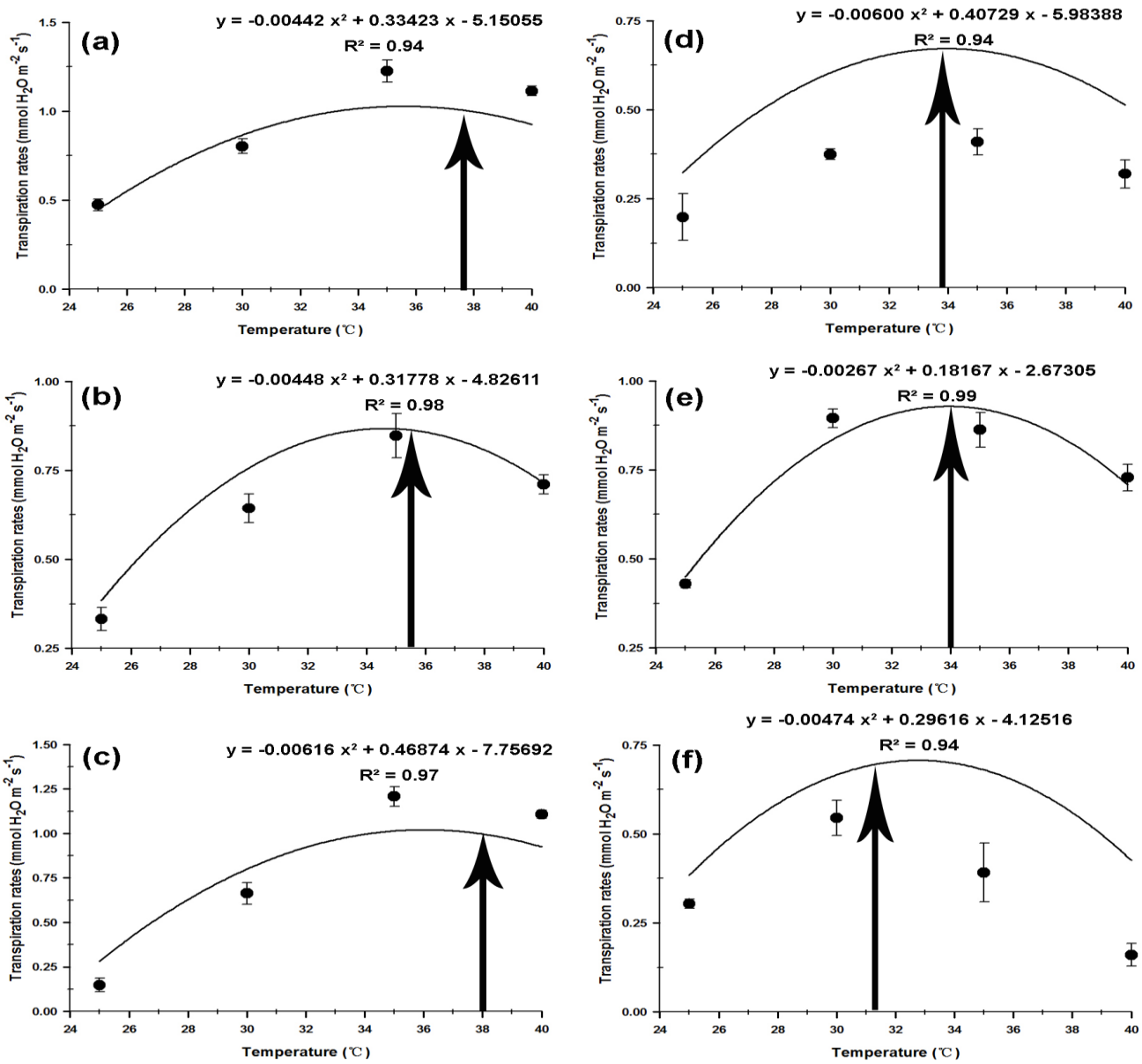

Fig. 6. The optimal temperature for transpiration rates of six blueberry cultivars.

Similar with plant biomass of blueberry cultivars, stomatal conductance also featured with optimal temperatures around 32℃ (Fig. 5). The stomatal conductance-temperature relationship followed a similar bell-shaped curve like the biomass-temperature relationship (Fig. 5), suggesting that the decline of biomass at high temperature might be attributed to the decrease of stomatal conductance at high temperature conditions. Further analysis showed that the anatomy structure and spatial distribution pattern of stomata played a critical role in determining the optimal temperature of stomatal conductance. We found that temperature had significant effects on stomatal density (p=0.035) and stomatal pore shape index (p=0.003) (Table 2), indicating that the thermal stress induced reduction in stomatal density and changes in stomatal pore shape may have contributed to the decline of biomass by reducing stomatal conductance at high temperature. Meanwhile, our results from spatial pattern analysis showed that increasing growth temperature from 25℃ to 30℃ made the stomatal distribution more regular, but the regular distribution pattern became weaken with further increasing growth temperature from 30℃ to 40℃ (Fig. 3), suggested that the changes in spatial distribution pattern of stomata under temperatures may have also contributed to the optimal temperature for stomatal conductance, and thus affecting the plant growth. In addition, our results also showed that transpiration has higher optimal temperature than stomatal conductance (Figs. 5 and 6), suggesting that CO_2 intake may be more sensitive to high temperature than water release. Meanwhile, the higher optimal temperature for transpiration than stomatal conductance may be a strategy of plants to take much more heat through water loss for cooling and protecting the reaction site of carbon assimilation under high temperatures (Wahid *et al.*, 2007; Ben-Asher *et al.*, 2008). Interestingly, our results also showed that the north highbush cultivars have higher optimal temperatures of transpiration (about 38℃) than the south highbush cultivars (around 34 ℃) (Fig. 6), suggesting that north highbush cultivars may less suffer from high temperature due to more efficiency of "cooling effect" from water loss than south highbush cultivars, which may also partly contributed to the higher biomass accumulation of north highbush cultivars than those of the south highbush cultivars.

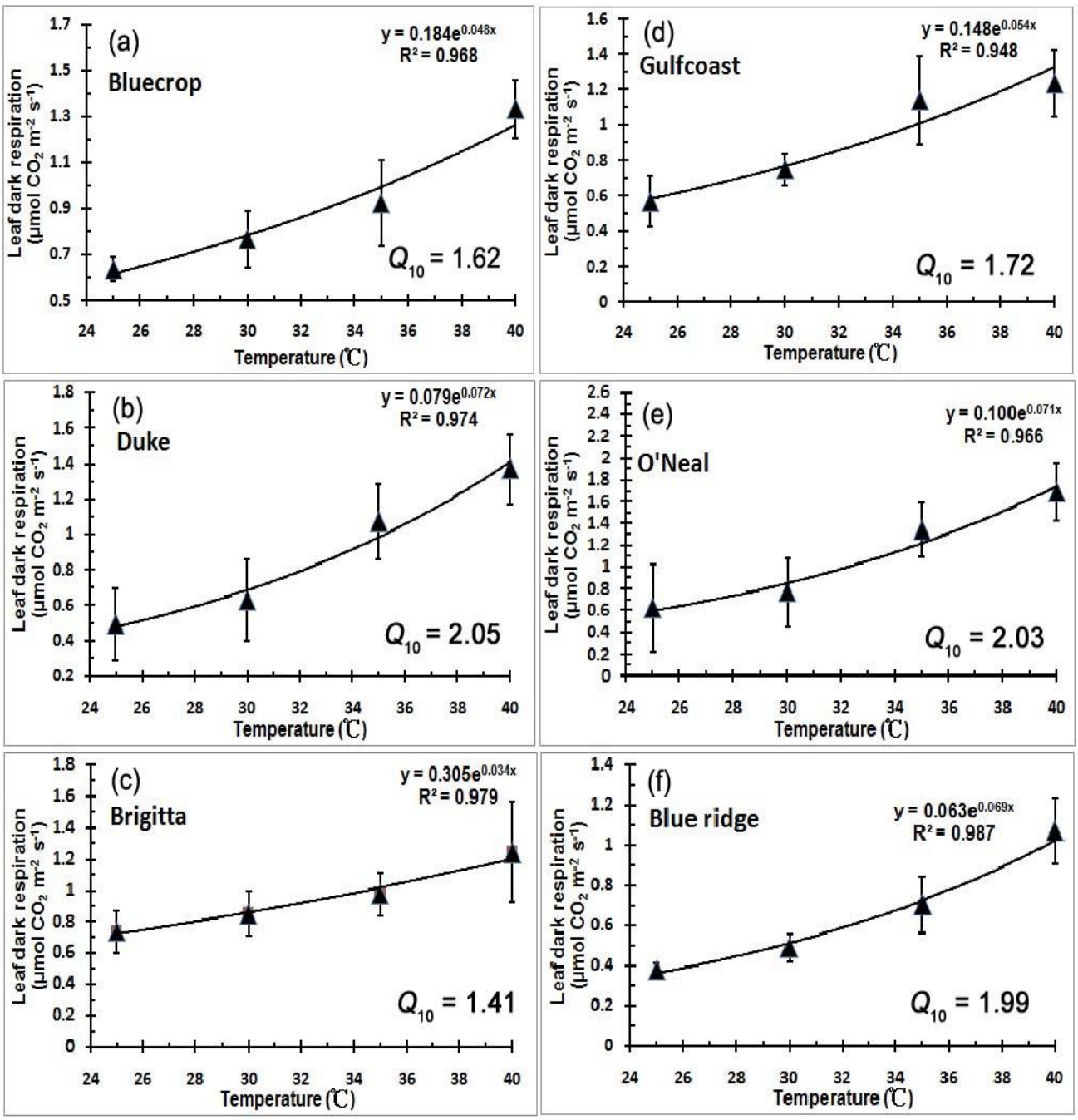

Fig. 7. Effects of growth temperature on leaf dark respiration of six blueberry cultivars.

In addition to stomatal controls, plant growth is also highly dependent on the biochemical and photochemical processes (such as photosynthesis and respiration), which may also play a critical role in determining the optimal temperature of plants. A large number of works has shown that elevated temperature can stimulate plant photosynthesis and increase plant productivity (Baker *et al.*, 1992; Shaw *et al.*, 2002; Zheng *et al.*, 2013a). However, other studies reported that plant photosynthesis may be deceased by higher temperatures (Callaway *et al.*, 1994; Roden & Ball, 1996; Ben-Asher*et al.*, 2008; Djanaguiraman *et al.*, 2011). This apparent discrepancy may be partly attributed to the growth temperature below or above the optimal temperature of photosynthesis (Shaw *et al.*, 2002). Specifically, elevated temperatures can stimulate plant photosynthesis when the growth temperature below the optimal temperature, whereas photosynthesis may also be decreased by higher temperatures above the optimal temperature. For example, the net photosynthetic rate of maize increases with temperature up to a maximum around 35 ℃, then decreases at higher temperatures (Lizaso *et al.*, 2005; Ben-Asher*et al.*, 2008). In the current study, we also found bell-shaped relationships between leaf net photosynthesis and growth temperature for six blueberry cultivars with a very similar optimal temperature around 34 ℃ (Fig. 4), suggesting that the temperatures above 34℃ may also have adverse impacts on the growth and yield of blueberry plants affecting biochemical and photochemical processes such as photosynthesis.

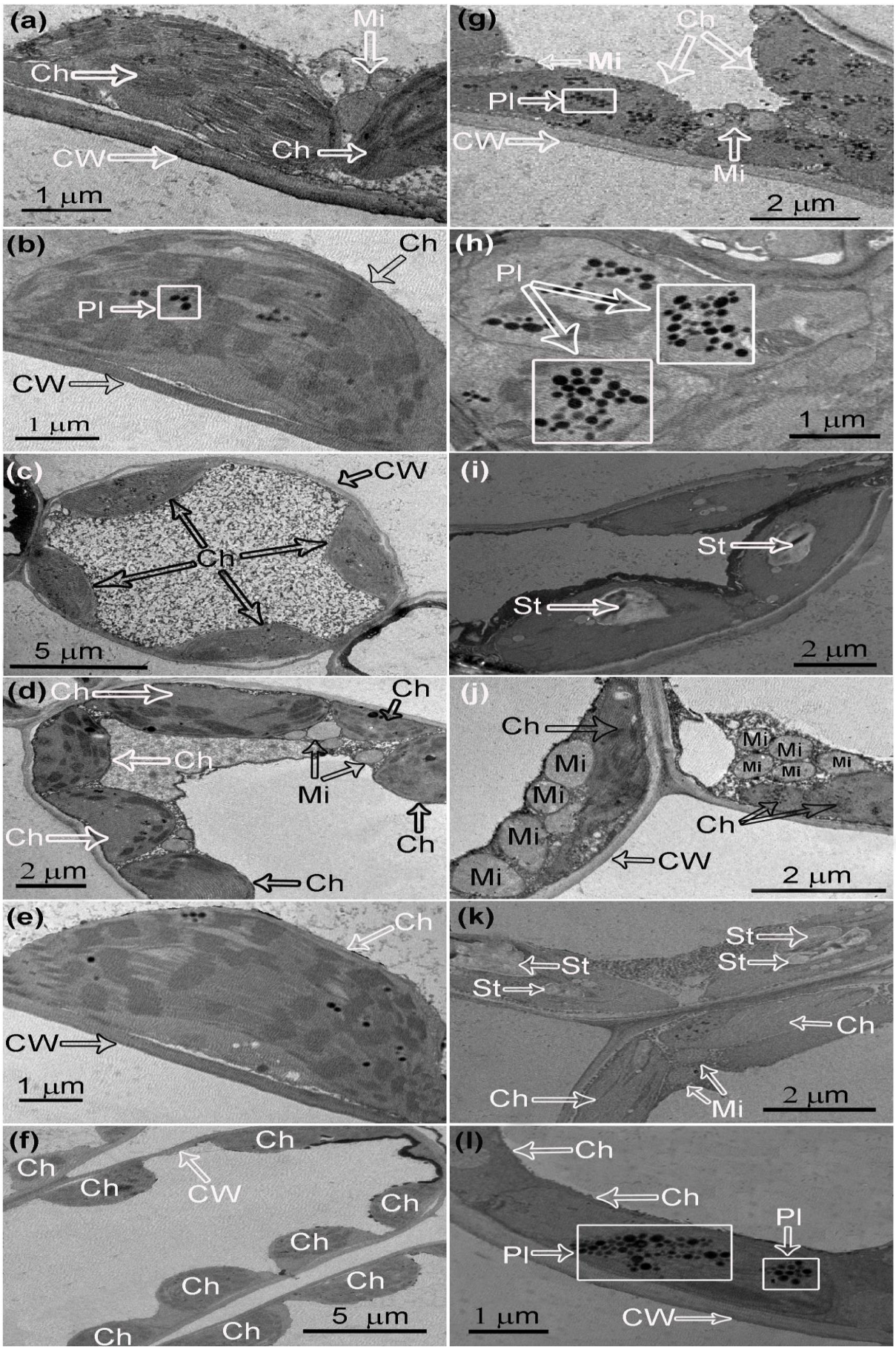

Fig. 8. Transmission electron micrographs (TEM) showed ultra-structure of blueberry leaves grown under 25℃ (a-f) or 40℃ (g-l). Ch, chloroplast; Mi, mitochondria; CW, cell wall; Pl, plastoglobuli. Bars, 1 μm (a-b, e, h, and i), or 2 μm (d, g, i, j and k), or 5 μm (c and f).

The high temperature effect on plant growth may confound with other factors such as elevated CO_2 concentration, nitrogen deposition, ozone concentration, and water availability (Fiscus *et al.,* 2002; Shaw *et al.*, 2002; Prieto *et al.,* 2009; Zhao & Liu, 2009; Mishra *et al.*, 2013; Aljazairi & Nogues, 2015). For example, Yu *et al.* (2012) reported that thermal stress or drought could be compensated by elevated CO_2 concentration through enhancing plant water status, cellular membrane stability, and photosynthesis capacity. Unfortunately, this confounding effect is already happening and is most likely to become worse in the future because climate change drivers like elevated atmospheric CO_2 concentration, high temperature, possibly precipitation, and N deposition in most temperate regions where blueberry grows (IPCC, 2013). In addition, the response of plants to high temperature is further complicated by considering the acclimation and adaption of plants (Gunderson *et al.,* 2000; Atkin & Tjoelker, 2003; Atkin *et al.,* 2006; Yamori *et al.*, 2014; Zhou *et al.,* 2015). Thus, the optimal temperature for blueberry plants in the real world under climate change might be different from the findings of the current study. Nevertheless, the current study focused on high temperature effect on the vegetative growth, a foundation stage for yield production. Therefore, further studies with long-term multi-factor experiments are needed to fully understand the mechanisms and processes governing the interactions between blueberry growth and high temperatures for improving the predictions of heat stress impacts on blueberry production.

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