Front. Biol. China 2007, 2(3): 333–339
DOI 10.1007/s11515-007-0049-8

RESEARCH ARTICLE

Interactive effects of elevated CO_2 and temperature on the anatomical characteristics of leaves in eleven species

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Abstract The anatomical features of leaves in 11 species of plants grown in a temperature gradient and a temperature + CO_2 gradient were studied. The palisade parenchyma thickness, the spongy parenchyma thickness and the total leaf thickness were measured and analyzed to investigate the effects of elevated temperature and CO_2 on the anatomical characteristics of the leaves. Our results show that with the increase of temperature, the leaf thickness of C_4 species increased while the leaf thickness of C_3 species showed no constant changes. With increased CO_2, seven out of nine C_3 species exhibited increased total leaf thickness. In C_4 species, leaf thickness decreased. As for the trend on the multi-grades, the plants exhibited linear or non-linear changes. With the increase of temperature or both temperature and CO_2 for the 11 species investigated, leaf thickness varied greatly in different plants (species) and even in different branches on the same plant. These results demonstrated that the effect of increasing CO_2 and temperature on the anatomical features of the leaves were species-specific. Since plant structures are correlated with plant functions, the changes in leaf anatomical characteristics in elevated temperature and CO_2 may lead to functional differences.

Keywords CO_2 concentration gradient, temperature gradient, leaf anatomical characteristics, C_3 species, C_4 species

1 Introduction

Due to the combustion of fossil fuels and deforestation, atmospheric CO_2 concentration ($[CO_2]$) has increased from the 280 μmol/mol before the onset of the Industrial Revolution to around 370 μmol/mol today (Mendelsohn and Rosenberg, 1994). It is projected to continue to rise at a rate of 1.8 μmol/mol every year and will have doubled by the year 2050 when compared to pre-industrial levels (Amther, 1991). As a result, global climatic changes have taken place with a significant increase in global air temperature. Under the current IPCC phase-out schedule, the annual mean temperature has increased by about 0.6° globally in the 20th century, and is predicted to increase 1.4–4.9°C by the year 2100 (Houghton et al., 2001). In view of the steadily rising global CO_2 concentrations and temperature, the interactive effects of the elevated $[CO_2]$ and temperature on plant growth and development are important.

A number of long-term and short-term experimental studies have examined the effects of elevated $[CO_2]$ and temperature on leaf characteristics, especially on the stomatal index due to its importance in determining historical changes of CO_2 concentration in the atmosphere (Beerling and William, 1993; Beerling et al., 1998; Poole et al., 2000; Chen et al., 2001; Royer, 2001; Woodward, 2002). However, studies concerning the effects of elevated $[CO_2]$ and temperature on the anatomical characteristics of leaves were rare and only changes of total leaf thickness and mesophyll thickness were generally demonstrated in those studies (Wilson and Cooper, 1969; Thomas and Harvey, 1983; Tepping and David, 1999). Comprehensive analyses concerning the effects of elevated $[CO_2]$ and temperature on the anatomical characteristics of leaves of different plant functional types are limited.

Previous researches analyzing the relationship between leaf anatomical characteristics and $[CO_2]$ generally applied two $[CO_2]$ concentration levels (current CO_2 concentration level and double current level) (Ferris et al., 1996). However, atmospheric $[CO_2]$ will not jump from the current levels of $[CO_2]$ to twice the current levels. The rise in $[CO_2]$ is going to be gradual though perhaps accelerating, as shown by long-term measurements. Therefore, predictions based on comparing plants grown under 350 and 700 μmol/mol may not necessarily occur in the future.

Translated from *Acta Ecologica Sinica*, 2006, 26(2): 326–333 [译自: 生态学报]

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It is currently unclear whether the response of the changes in the anatomical characteristics of leaves to atmospheric CO_2 concentrations differs with species and in functional groups, and whether or not the response is linear. Thus the aim of this study is: 1) to analyze the relationship between the anatomical characteristics of the leaves and gradual increasing CO_2 concentrations and temperature; 2) to test whether the responses of the anatomical characteristics of the leaves to elevated atmospheric CO_2 concentrations and temperature are species-specific and whether the responses are linear.

2 Materials and methods

2.1 Experiment materials

In the present study, 11 plant species, including eight annual herbaceous species and three canopy tree species, were selected for analysis. The former were common species in Middle American old fields, including three functional types: three C_3 species, three legume C_3 species and two C_4 species; and the latter were the dominant canopy species in many hardwood forests in Northeastern American (Table 1).

Table 1 The species in the research and their functional group and growth habit

Species	Functional group	Growth habit
Abutilon theophrasti	C_3	forb
Ambrosia artemisiifolia	C_3	forb
Polygonum pensylvancium	C_3	forb
Chamaecrista fasciculate	C_3, Legume	forb
Medicago lupulina	C_3, Legume	forb
Trifolium pretense	C_3, Legume	forb
Setaria faberii	C_4	grass
Setaria italica	C_4	grass
Quercus rubra	C_3, Tree	deciduous
Acer rubrum	C_3, Tree	deciduous
Betula papyrifera	C_3, Tree	deciduous

2.2 Experiment methods

The seeds of the 11 selected species were sown during spring in two designed tunnels, a Temperature Gradient Tunnel (TGT) and a $[CO_2]$ + Temperature Gradient Tunnel (CTGT), at the campus of Harvard University. The tunnel designs and planting details followed the descriptions given in Zuo et al. (2005). Since at same district in CTGT and TGT, the grown condition were similar except for $[CO_2]$. We assumed that variations of leaf anatomical characteristics at the same district of two tunnels will mainly caused by $[CO_2]$ difference. Through the comparison of the anatomical characteristics of the leaves grown in these two tunnels, we can compare the effects of $[CO_2]$ to the anatomical characteristics of the leaves.

Leaves used for analysis were collected at the end of August. One mature healthy leaf at the similar position of plant was selected from each individual plant, and fixed in formalin-acetic acid-alcohol (FAA) fixative in the field. For each leaf, three 0.5 cm × 0.5 cm pieces were cut with a knife on-site at 1/3 of the distance from the leaf tip. The pieces were dehydrated in a graded series of ethanol, embedded in paraffin, and then sectioned with a Leitz microtome and stained with Safrania and Alcian green. All slides were mounted in Canada balsam after dehydration. Palisade parenchyma thickness, spongy parenchyma thickness and total leaf thickness were then measured with an Olympus BH_2 microscope equipped with a scaled grid. As for the isobilateral leaves, only the total leaf thickness was measured. All measurements were conducted near the middle of the leaf, avoiding midribs and margins. Five randomly chosen portion of leaf were measured for each slide. Data analyses were performed using SPSS software.

3 Results

3.1 Response of C_3 species to the elevated temperature and $[CO_2]$

Nine out of the eleven species are C_3 species and show the characteristics of bifacial leaves. The palisade parenchyma was tightly packed and contained numerous chloroplasts. Most species had 1–2 layers of palisade cells except for *Abutilon theophrasti* which had 2–3 layers of palisade cells. The spongy parenchyma was loosely packed and contained relatively fewer chloroplasts. The leaf thickness of the different species varied significantly. Among those species, *Ambrosia artemisiifolia* had the largest leaf thickness and *Acer rubrum* had the smallest leaf thickness.

The total leaf thicknesses of the three canopy C_3 species were positively correlated with air temperature. Among the three canopy species, *Quercus rubra* had the largest increase in leaf thickness when exposed to elevated air temperature. As for the palisade parenchyma thickness, *Betula papyrifera* and *Q. rubra* showed a strongly positive correlation with air temperature and *Acer rubrum* had no significant change (Fig. 1(a) and (b)). For the spongy parenchyma thickness, *B. papyrifera* and *A. rubrum* showed significant increases while *Q. rubra* had a decreasing trend when exposed to elevated air temperature. The total leaf thickness of the three canopy C_3 species also showed an increasing trend when exposed to elevated air temperature and $[CO_2]$. The increasing levels were larger than those only exposed to elevated air temperature. Among the three species, the total leaf thickness of *B. papyrifera* had a strong positive correlation with temperature and $[CO_2]$ (Fig. 2(c)). For palisade parenchyma and spongy parenchyma thickness, the three canopy species showed increasing trend and the increasing levels were larger than those exposed to elevated air temperature. The thickness of palisade parenchyma and spongy parenchyma of *B. papyrifera* had a strongly positive correlation with temperature and $[CO_2]$ (Fig. 2(a) and (b)).

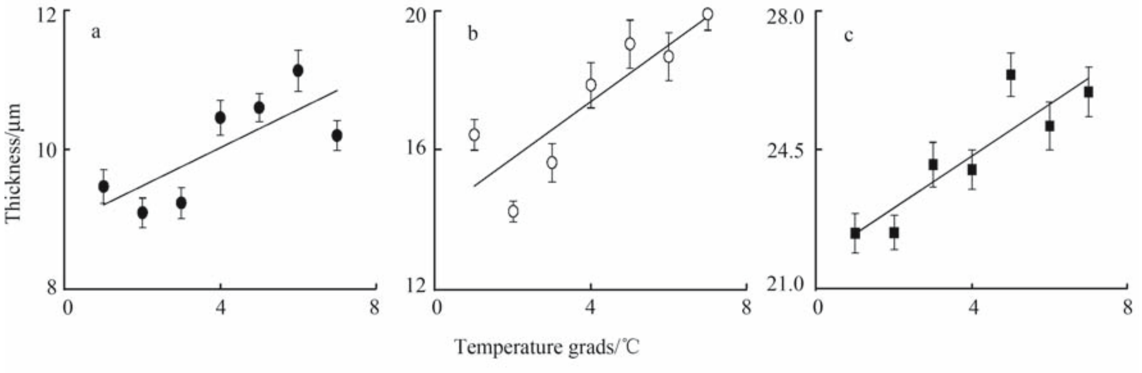

Fig. 1 The trend for the leaf thickness with the increase of temperature
(a) Palisade parenchyma thickness of *Betula papyrifera* ($R = 0.76$, $P = 0.046$); (b) Palisade parenchyma thickness *Quercus rubra* ($R = 0.86$, $P = 0.013$); (c) Total leaf thickness of *Setaria italica* ($R = 0.89$, $P = 0.007$) 1–7 is the seven levels of the temperature, increasing gradually.

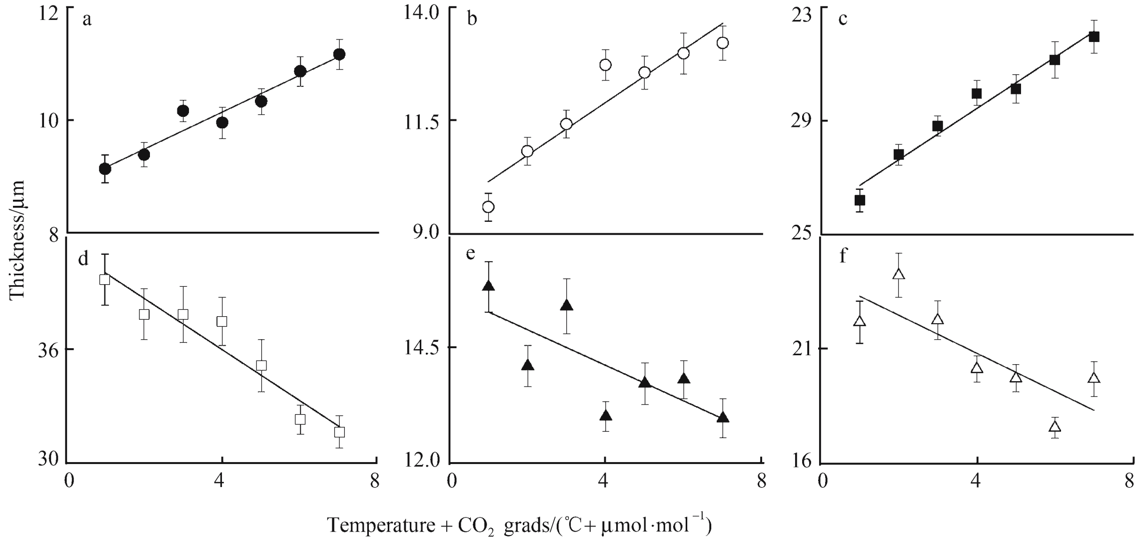

Fig. 2 The trend for the leaf thickness with the increase of temperature + CO_2
(a) Palisade parenchyma thickness of *Betula papyrifera* ($R = 0.97$, $P = 0.00$); (b) Spongy parenchyma thickness of *Betula papyrifera* ($R = 0.94$, $P = 0.002$); (c) Total leaf thickness of *Betula papyrifera* ($R = 0.98$, $P = 0.00$); (d) Total leaf thickness of *Medicago lupulina* ($R = -0.96$, $P = 0.001$); (e) Spongy parenchyma thickness of *Abutilon theophrasti* ($R = -0.75$, $P = 0.05$); (f) Total leaf thickness of *Setaria faberii* ($R = -0.82$, $P = 0.025$) 1–7 is the seven levels of the temperature, increasing gradually.

The response of the three legume C_3 species to elevated air temperature varied specifically. As the air temperature increased, the total leaf thickness of *Chamaecrista fasciculate* and *Trifolium pretense* as well as the palisade parenchyma and spongy parenchyma thickness of *C. fasciculate* showed an increasing but non-linear change (first increased then decreased) (Fig. 3(a)–(c)), while the total leaf thickness of *Medicago lupulina* showed a decreasing trend. The response of the three legumes to elevated air temperature and [CO_2] were also varied. The palisade parenchyma and spongy parenchyma thickness and the total leaf thickness of *C. fasciculate* all showed an increasing trend when exposed to elevated [CO_2] and air temperature. The increased levels were larger than those exposed to elevated air temperature. The total leaf thickness of *T. pretense* showed a non-linear change (first increased then decreased, and the leaf thickness of districts 1 and 7 are similar) (Fig. 3(d)). The total leaf thickness of *M. lupulina* remained the same, but the rate of decrease weakened (Fig. 2(d)).

The responses of the three non-legume C3 species to the elevated temperature were also varied. As the air temperature increased, the total leaf thickness of *Ambrosia artemisiifolia* tended to decrease, but the total leaf thickness of *Abution theophrasti* and *Polygonum pensylvancium* remained the same. The mesophyll thickness of the three non-legumes all had non-linear correlations with temperature, but the changing trend varied, especially for the thickness of spongy parenchyma (Fig. 3(e)). As both the air temperature and [CO_2] increased, the total leaf thickness of *P. pensylvancium* increased significantly. The total leaf thickness of *A.*

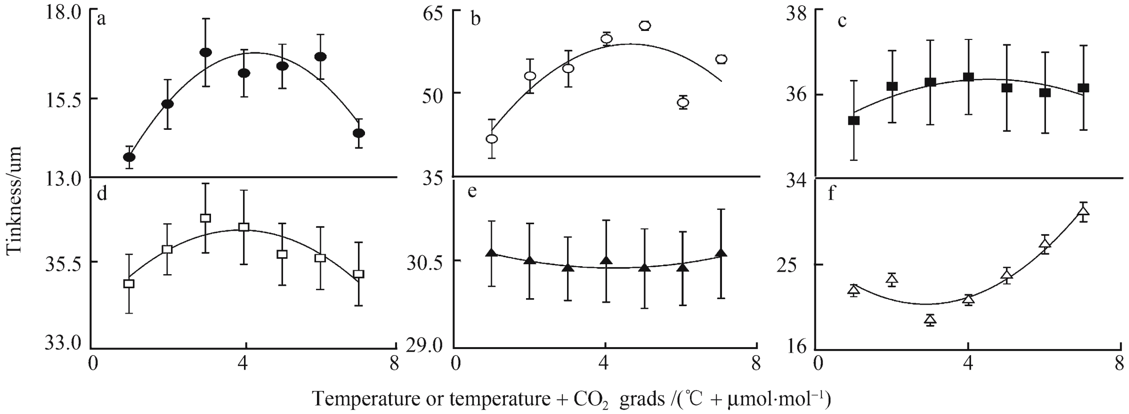

Fig. 3 The trend of leaf thickness with the increase of temperature and temperature + CO_2 in the North and South tunnel
(a) Spongy parenchyma thickness of *Chamaecrista fasciculate* with the increase of temperature ($R = 0.93$, $P = 0.019$); (b) Total leaf thickness of *Chamaecrista fasciculate* with the increase of temperature ($R = 0.84$, $P = 0.083$); (c) Total leaf thickness of *Trifolium pretense* with the increase of temperature ($R = 0.83$, $P = 0.092$); (d) Total leaf thickness of *Trifolium pretense* with the increase of temperature + CO_2 ($R = 0.88$, $P = 0.049$); (e) Total leaf thickness of *Polygonum pensylvancium* with the increase of temperature ($R = 0.81$, $P = 0.118$); (f) Total leaf thickness of *Setaria faberii* with the increase of temperature ($R = 0.95$, $P = 0.002$); 1–7 is the seven level of the temperature or temperature + CO_2, increasing gradually.

theophrasti and *A. artemisiifolia* decreased. In respect of mesophyll thickness, the palisade parenchyma and spongy parenchyma thickness of *A. theophrasti* and *A. artemisiifolia* tended to decrease while the spongy parenchyma thickness of *A. theophrasti* had a prominently negative correlation with air temperature and [CO_2] (Fig. 2(e)).

3.2 Response of C_4 species to the elevated temperature and [CO_2]

The leaves of two C_4 species are characterized with isobilateral leaves, small intercellular space, well developed sclerenchyma tissues in the area between vascular bundles and epidermal cells and a large and tightly arranged vascular bundle sheath cell around the vascular bundle.

As air temperature increased, the total leaf thickness of *Setaria faberii* indicated a non-linear increasing trend, decreasing at first and then increasing from district 4 (Fig. 3(f)). The total leaf thickness of *Setaria italica* indicated a prominently linear increasing trend (Fig. 1(c)). As both the air temperature and [CO_2] increased, the total leaf thickness of *S. faberii* indicated a strongly negative correlation and *S. italica* showed a weakly negative correlation (Fig. 2(f)). Therefore, elevated CO_2 concentration can lessen leaf thickness.

In summary, the response of leaf thickness to elevated temperature and [CO_2] varied with different functional groups (Table 2). The leaf thickness of the same species grown at the same plot also varied with individual. For example, in *C. fasciculate*, the palisade parenchyma thickness of different individual plants grown at the same plot varied from the thinnest 25.64 to the thickest 64.1um, and the spongy parenchyma thickness from 12.82 to 64.1um, and the total leaf thickness from 64.1 to 115.38um.

4 Discussion

4.1 Effects of elevated temperature and [CO_2] on leaf anatomical characteristics

Previous studies showed that most plant species would increase their leaf thickness when exposed to elevated [CO_2]. Pritchard et al. (1999) analyzed the relationship between leaf thickness of 16 species and elevated [CO_2] and found that about 81% of them enhanced their leaf thickness and about 19% had no significant change. Roger et al. (1983) and Gaudillére and Mousseau (1989) showed that the palisade parenchyma layers of *Glycine max* and *Castanea sativa* would increase when exposed to elevated [CO_2]. In our experiment, seven C_3 species (three canopy species, three legume species and *P. pensylvancium*) enhanced their total leaf thickness and mesophyll thickness when exposed to elevated [CO_2]. Two C_4 species and two non-legume C_3 species (*A. theophrasti* and *A. artemisiifolia*) decreased their leaf thickness when exposed to elevated CO_2 concentration. This suggested that most C_3 species would increase and most C_4 species would decrease their leaf thickness when exposed to elevated CO_2 concentration. Since previous works were mainly concentrated on C_3 species, our results are in agreement with the previous conclusion.

Generally, in our experiment, the responses of leaf thickness to elevated [CO_2] are different among functional groups, and are similar within functional groups. As the [CO_2] increases, the leaf thickness of C_4 species tends to decrease; the leaf thicknesses of canopy C_3 species and legume C_3 species tend to increase; the leaf thickness of most non-legume C_3 species tends to decrease. The different responses of C_3 and C_4 species can be interpreted by their difference in physiological and structural characteristics. In C_3 species,

Table 2 Leaf anatomical characteristics of 11 species under different temperature and CO_2

		North							South						
T		27.37	27.78	28.19	28.24	28.29	28.72	29.14	27.41	28.01	28.60	28.76	28.92	29.32	29.73
CO_2		370	370	370	370	370	370	370	370	440	510	580	650	720	800
RM	PPT	11.74	12.28	12.18	11.54	12.5	12.64	11.14	12.12	11.32	14.13	11.65	12.01	14.71	12.26
	SPT	8.89	8.42	9.33	9.29	8.22	9.53	9.24	9.71	9.49	11.67	9.96	9.26	12.27	10.18
	TLT	24.47	24.29	25.12	24.62	25.73	26.28	24.84	25.86	24.01	29.54	25.47	25.15	30.98	26.15
GB	PPT	9.47	9.09	9.24	10.46	10.61	11.14	10.21	9.15	9.40	10.17	9.96	10.34	10.87	11.17
	SPT	11.79	9.68	10.75	12.12	11.58	12.13	12.14	9.60	10.83	11.42	12.72	12.55	12.98	13.21
	TLT	29.06	26.39	27.08	30.28	29.47	30.58	29.23	26.20	27.80	28.81	29.96	30.12	31.14	31.95
RO	PPT	16.43	14.23	15.63	17.86	19.05	18.68	19.91	18.10	14.78	16.72	20.16	19.15	16.2	21.13
	SPT	14.61	13.95	12.29	14.74	14.25	13.53	14.29	11.66	14.55	14.09	18.00	16.77	14.8	15.71
	TLT	36.97	34.16	34.04	38.53	39.76	38.38	40.19	34.94	34.52	36.61	45.52	41.93	36.65	44.16
Cham	PPT	25.94	28.94	28.67	28.05	30.19	29.47	26.59	29.09	24.2	26.31	29.73	33.00	35.64	31.30
	SPT	13.64	15.21	16.73	16.12	16.33	16.61	14.35	15.50	13.69	13.73	15.24	16.43	17.56	16.19
	TLT	42.09	53.21	54.53	59.80	62.12	48.52	56.19	60.53	53.53	56.06	61.20	65.45	69.87	63.82
Trif	TLT	35.38	36.18	36.28	36.41	36.15	36.04	36.15	34.90	35.90	36.79	36.54	35.75	35.64	35.18
Med	TLT	41.41	34.47	32.31	32.69	32.56	33.08	31.41	39.61	37.82	37.82	37.43	35.13	32.31	31.67
Ambr	PPT	41.37	32.14	35.81	35.99	38.97	36.15	39.19	41.79	40.13	39.66	41.02	37.36	37.43	39.06
	SPT	33.59	25.55	28.63	27.61	30.77	28.67	28.66	32.39	28.71	30.60	29.23	28.57	26.27	29.14
	TLT	100.7	83.33	90.42	77.43	95.72	91.14	88.12	101.8	95.59	96.41	96.49	88.97	89.35	94.36
Abut	PPT	21.72	18.89	20.88	19.73	19.59	21.59	22.27	22.8	21.17	23.05	20.39	21.84	20.63	19.12
	SPT	14.64	12.31	14.54	13.44	13.10	13.88	16.51	15.81	14.10	15.39	13.01	13.73	13.81	12.98
	TLT	54.04	46.80	50.69	39.25	47.96	47.72	47.61	55.12	51.96	54.48	46.55	51.88	51.00	48.30
Poly	TLT	30.64	30.51	30.38	30.51	30.38	30.38	30.64	29.36	29.49	29.87	30.13	30.51	30.26	30.00
S.fab	TLT	22.21	23.35	19.09	21.22	23.81	27.11	30.51	22.15	24.19	22.25	20.12	19.72	17.57	19.69
Si	TLT	22.40	22.42	24.13	24.00	26.40	25.11	25.96	25.98	25.85	24.94	22.59	24.43	24.86	23.17

Note: All data in table are the average of 10 samples.

T: original temperature (°C); CO_2 concentration unit: umol·mol^{-1}; RM: *Acer rubrum*; GB: *Betula papyrifera*; RO: *Quercus rubra*; Cham: *Chamaecrista fasciculate*; Trif: *Trifolium pretense*; Med: *Medicago lupulina*; Ambr: *Ambrosia artemisiifolia*; Abut: *Abutilon theophrasti*; Poly: *Polygonum pensylvancium*; S.fab; *Setaria faberii*; Si;*Setaria italica*

PPT: Palisade parenchyma thickness; SPT: Spongy parenchyma thickness; TLT: Total leaf thickness.

carbon fixation occurs in the mesophyll and the ratio of the mesophyll [CO_2] to air [CO_2] is approximately 0.7:1. As the atmosphere [CO_2] changes, mesophyll [CO_2] would also change to maintain this ratio. In C_4 species, carbon fixation occurs in vascular bundle sheath that is surrounded by tightly arranged parenchyma and the CO_2 exchange between bundle sheath cells and outer space is difficult, and the [CO_2] in vascular bundle sheath remains when the atmosphere [CO_2] changes (Polley et al., 1993; Ehleringer and Cerling, 1995; Beerling and Woodward, 1996; Bettarini et al., 1997). Therefore, the C_3 species are sensitive to the changes of air [CO_2]. But for C_4 species, the effect of changed air [CO_2] is limited.

Generally, C_4 species have a higher photosynthetic efficiency and productivity rate and have high potential values in agriculture. Recent studies concerning the relationship between air [CO_2] and the leaf anatomical characteristics of C_4 species have attracted more and more attention (Furbank and Foyer, 1988; Yang et al., 1997). Some studies state that the leaf thickness of C_4 species have no significant change when exposed to elevated [CO_2] (Downton et al., 1980; Thomas and Harvey, 1983). However, in our experiment, the leaf thickness of two C_4 species tends to decrease when exposed to higher [CO_2]. It is likely that the response of C_4 species to elevated CO_2 concentration varies with species. Considering that only two species were investigated in our study, more C_4 species should be investigated to illustrate the relationship between leaf thickness and air [CO_2].

The response of C_3 species varies within functional groups which may relate to leaf age and growth rate. In *Trifolium perenne*, the volume of mesophyll cells is positively correlated with [CO_2] when the plant grows quickly and negatively correlated with [CO_2] when the plant grows slowly (Ferris et al., 1996). In the present study, the canopy species are at juvenile stage and grow quickly. Therefore, the total leaf thickness and mesophyll thickness increased prominently when exposed to elevated [CO_2]. The different responses of legume and non-legume C_3 species can also be interpreted by their difference in the growth rate. Generally, legume plants live in symbiosis with nodule bacteria and can capture nitrogen from the air. As the air CO_2 concentration increases, they can provide more nitrogen to meet the need of their fast growth (Rogers et al., 1983). Therefore, they have fast developmental rates and increasing their leaf thickness sounds reasonable.

Most reports indicated that the changes in the anatomical characteristics of the leaves were species-specific when exposed to elevated air temperature (Bazzaz, 1990). In our experiment, the changes in leaf thickness of plants exposed to elevated temperatures were also varied and species-specific. For the leaf thickness of C_3 species we explored, there was no consistent trend when exposed to elevated air temperature. Although two C_4 species all showed the increasing trend, they had some differences. The leaf thickness of *S. italica* has a prominently positive correlation with air temperature while the leaf thickness of *S. faberii* shows a non-linear changing trend (decreasing initially followed by an increase). As for the non-linear changing trend, we hypothesize that the growth rate of *Setaria faberii* may alter when exposed to elevated air temperature for a period of time and its leaf thickness changes correspondingly.

Huang et al. (2001) stated that C_4 species could get more profit from elevated air temperature than elevated [CO_2]. Our experiment indicates that the leaf thickness increases when exposed to elevated air temperature and decreases when exposed to elevated CO_2 concentration. Since the leaf size of C_4 species changes slightly in our experiment, larger leaf thickness means a large production. Therefore, our results are in agreement with Huang's statement.

4.2 Other factors affecting leaf anatomical characteristics

In the present study, the response variability of the anatomical characteristics of the leaves to elevated air temperature and [CO_2] appears to be high. This may relate to the individual difference. It has been proven that the anatomical characteristics of the leaves of the same species show a difference within individual and development stages and leaf position (Blue and Jensen, 1988; Ashton et al., 1998; Bruschi et al., 2003). For the purpose of comparison, we selected leaves using leaf parts from the same position of the leaf taken from the same position of plants and collected at the same time. However, variations among different individuals may more or less override their response. Except for the canopy species whose seeds come from one single plant, the seeds of other species come from several individual seeds. The genetic difference may weaken or even overwhelm the intrinsic relationship between leaf anatomical characteristics and air temperature and [CO_2]. For instance, a large variation occurred in the palisade parenchyma thickness and spongy parenchyma thickness and the total leaf thickness of *Chamaecrista fasciculate*. Those three characteristics are poorly correlated with air temperature.

4.3 Response of leaf anatomical characteristics to gradual increasing air temperature and [CO_2]

In the present research, the anatomical characteristics of the leaves of some species show a changing curve when exposed to gradually increasing air temperature and [CO_2]. This may be associated with their feedback regulation ability. When plants are exposed to elevated air temperature and [CO_2] for a certain period of time, they may produce singles changing their anatomical characteristics to adapt to new living conditions. The anatomical characteristics of some species show an increasing or decreasing trend with a non-linear correlation (Table 3). Except for simply changing the thickness when exposed to gradually increasing air temperature and [CO_2], plants can use some other means to adapt to new living conditions.

Table 3 Slope, intercept and R value in unitary linear regression equation of different anatomical parameters with the increase of temperature and temperature + CO_2 for each species

		Temperature			Temperature + CO_2		
		a	b	R	a	b	R
RM	PPT	–0.03	12.11	–0.11	0.18	11.88	0.30
	SPT	0.08	8.68	0.33	0.16	9.71	0.30
	TLT	0.20	24.23	0.61	0.29	25.66	0.25
GB	PPT	0.27	8.93	0.76*	0.33	8.85	0.97 ***
	SPT	0.24	10.49	0.56	0.58	9.58	0.94 **
	TLT	0.40	27.26	0.55	0.90	25.82	0.98 ***
RO	PPT	0.81	14.15	0.86*	0.51	15.98	0.49
	SPT	0.01	13.93	0.01	0.55	12.89	0.58
	TLT	0.85	34.04	0.73†	1.33	33.87	0.63
Cham	PPT	/	/	/	1.29	24.72	0.72†
	SPT	/	/	/	0.45	13.69	0.68†
	TLT	/	/	/	1.86	52.13	0.65
Med	TLT	–1.16	38.64	–0.74†	–1.34	41.34	–0.96**
Ambr	PPT	0.17	36.42	0.20	–0.57	41.77	–0.73†
	SPT	–0.23	29.99	–0.12	–0.59	31.65	–0.68†
	TLT	–0.60	91.95	–0.17	–1.51	100.74	–0.76†
Abut	PPT	/	/	/	–0.48	23.19	–0.86*
	SPT	/	/	/	–0.38	15.65	–0.75*
	TLT	/	/	/	–0.89	54.89	–0.62
Poly	TLT	/	/	/	0.15	29.36	0.77*
S.fab	TLT	/	/	/	–0.83	24.12	–0.82*
Si	TLT	0.65	21.73	0.89 **	–0.39	26.11	–0.66

† $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

RM: *Acer rubrum*; GB: *Betula papyrifera*; RO: *Quercus rubra*; Cham: *Chamaecrista fasciculate*; Ambr: *Ambrosia artemisiifolia*; Abut: *Abutilon theophrasti*; Poly: *Polygonum pensylvancium*; S.fab: *Setaria faberii*; Si: *Setaria italica*

PPT: Palisade parenchyma thickness; SPT: Spongy parenchyma thickness; TLT: Total leaf thickness; " / " represents that anatomical parameters exhibit non-linear change.

Previous conclusions concerning the relationship between the anatomical characteristics of the leaves and elevated [CO_2] are generally based on two [CO_2] levels (current level and twice the current level) (Ferris et al., 1996). However, the rise in [CO_2], as shown by measurements, is going to be gradual. When permanently exposed to gradually increasing [CO_2], the response of the plant may be non-linear (Bazzaz and Garbutt, 1988). Our experiment shows that plants may have linear, non-linear or curved-shape response when exposed to gradually increasing [CO_2]. Therefore, investigation only based on two CO_2 concentrations is necessary but not sufficient.

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