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Effects of elevated atmospheric CO_2 concentration on morphology of leaf blades in Chinese yam

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ABSTRACT

The effects of elevated carbon dioxide concentration on the morphology of leaf blades in two Chinese yam lines under different temperature conditions were determined. Plants were grown under two $[CO_2]$ levels, ambient (about 400 μmol mol^{-1}) and elevated (ambient + 200 μmol mol^{-1}) in the daytime, and two mean air temperature regimes, approximately ambient temperature (22.2°C) and high temperature (25.6°C). The palisade layer was thicker under elevated $[CO_2]$ than under ambient $[CO_2]$ in both temperature regimes, and the whole yam leaf blade was thicker under elevated $[CO_2]$ than under ambient $[CO_2]$ in the approximately ambient temperature regime. The numbers of chloroplasts per palisade cell and spongy cell as well as per unit profile area of palisade cell, number of starch grains per chloroplast, profile area of the starch grain, and starch-to-chloroplast area ratio in both palisade and spongy cells were higher under elevated $[CO_2]$ than under ambient $[CO_2]$ in both temperature regimes. Furthermore, the stomatal density on the abaxial side of the leaf blade in Chinese yam was greater under elevated $[CO_2]$ than under ambient $[CO_2]$ under both temperature regimes, and stomatal-pore length was higher under elevated $[CO_2]$ than under ambient $[CO_2]$ in the approximately ambient temperature regime. These results indicate that elevated $[CO_2]$ positively affects the photosynthetic apparatus. The results of this study provide information for understanding the response characteristics of the leaf blade under elevated $[CO_2]$ and a possible explanation for the positive photosynthetic responses of Chinese yam to elevated $[CO_2]$ in our previous study.

List of Abbreviations:[CO₂]: carbon dioxide concentration

1. Introduction

Yam (*Dioscorea* spp.) is a multi-species tuber crop cultivated widely in Africa, Asia, parts of South America, the Caribbean, and the South Pacific islands (Asiedu & Sartie, 2010) and is among the top 10 most consumed foods in the world, because its tubers contain high starch, protein, and micronutrient contents (Asiedu & Sartie, 2010). Among the various yam species, Chinese yam (*Dioscorea opposita* Thunb.) is largely cultivated in Japan, China, Korea, and Taiwan and is a very important tuber crop in the northern prefectures of Japan, such as Aomori and Hokkaido.

Since the industrial revolution period a couple of centuries ago, the atmospheric carbon dioxide concentration ($[CO_2]$) has increased from a stable 280 μmol mol^{-1} before the industrial revolution to 400 μmol mol^{-1} for the first time in March 2015, and reached 403.95 μmol mol^{-1} in July 2017 (National Oceanic & Atmospheric Administration [NOAA], 2017). It is predicted to continue increasing in the future (Intergovernmental Panel on Climate Change [IPCC], 2013). Because of the $[CO_2]$ increase, the global air temperature by the end of the twenty-first century is predicted to rise (IPCC, 2013) as a result of global warming. Thus, $[CO_2]$ is a key variable affecting plant growth, physiology, and morphology.

Many studies have shown that elevated $[CO_2]$ can accelerate the growth and development of some plant species such as rice (Cheng et al., 2009; Krishnan et al., 2007; Roy et al., 2012; Shimono et al., 2008), cassava (Cruz et al., 2014), soybean (Koti et al., 2007), sorghum (Ottman et al., 2001), and *Impatiens hawkeri* (Zhang et al., 2012); increase the rate of photosynthesis in rice (Shimono et al., 2008), potato (Aien et al., 2014), soybean (Koti et al., 2007), *Arabidopsis thaliana* (Li et al., 2008), and mulberry (Sekhar et al., 2015); and decrease the mineral nutrient concentration in wheat (Broberg et al., 2017), Scots pine (Luomala et al., 2005), silver birch (Riikonen et al., 2005), and *Arabidopsis thaliana* (Teng et al., 2006). However, few studies have considered the effects of elevated $[CO_2]$ on leaf ultrastructure, especially the photosynthetic apparatus (Hao et al., 2013; Radoglou & Jarvis, 1992; Wang et al., 2004).

In the case of Chinese yam, our previous studies on the effects of elevated [CO_2] on growth and photosynthesis showed that Chinese yam responds positively to elevated [CO_2] both at the early growth stage (Thinh et al., 2017b) and at the intermediary vegetative stage (Thinh et al., 2017a). Yam leaf number, leaf area, leaf dry weight (DW), and total plant DW were higher under elevated [CO_2] than under ambient [CO_2] (Thinh et al., 2017b). The photosynthetic rate was also enhanced by elevated [CO_2] (Thinh et al., 2017a). However, no possible mechanism was proposed to explain how yams positively respond to elevated [CO_2] and whether the yam response is related to leaf structure changes under elevated [CO_2]. These aspects are very important for an integrated understanding of the mechanism by which yams show a positive response to elevated [CO_2].

In this study, we hypothesized that elevated [CO_2] affects the leaf internal structure and the morphology of stomata and chloroplasts. To test this hypothesis, we observed the morphology of yam leaves collected from two dominant Chinese lines grown under two [CO_2] conditions (ambient and elevated). To our knowledge, this is the first study to investigate yam leaf structure changes at the tissue and subcellular levels in response to elevated [CO_2].

2. Materials and methods

2.1. Plant materials and growth conditions

We conducted our experiments in temperature-gradient chambers at the Tohoku Agricultural Research Center, NARO (39°74′N, 141°13′E) in Morioka, Japan. The details of the cultivation method are described in our previous paper (Thinh et al., 2017b). In short, we used two dominant lines of Chinese yam in north Japan: Enshikei 6 and Shojikei. For each line, seed bulbils were sown in plastic pots on 4 June 2016 and then placed immediately in the temperature-gradient chambers for treatment until 9 July 2016. Two temperature-gradient chambers were used under two [CO_2] conditions: ambient and elevated (ambient [CO_2] + 200 µmol mol^{-1} in the daytime (04:00 to 20:30)). In each temperature-gradient chamber, two treatment plots were set along an air temperature gradient. The first plot had a mean air temperature of 22.2 ± 1.7°C (1.4°C higher than the temperature outside the chamber) and the second plot had a mean air temperature of 25.6°C ± 1.7°C (5.2°C higher than the temperature outside the chamber). The average daytime (04:00 to 20:30) [CO_2] over the treatment period was 406 ± 9 µmol mol^{-1} in the ambient [CO_2] chamber and 603 ± 22 µmol mol^{-1} in the elevated [CO_2] chamber. The relative

humidity was about 58% and 39% for the approximately ambient temperature plots and the high temperature plots, respectively, in the chambers. In this study, we setup the treatments as described above because plants are generally more sensitive to air temperature changes than relative humidity changes. Air temperature, relative humidity, and [CO_2] were measured at 5°s intervals and averaged every 1 min, 30 min, and 24 h by a datalogger (CR 1000; Campbell Sci. Inc., Logan, UT, U.S.A.).

Both Enshikei 6 and Shojikei were treated in the same manner in the temperature-gradient chambers. The four treatments were abbreviated as follows:

(1) AA: ambient [CO_2] (406 µmol mol^{-1}) and approximately ambient air temperature (22.2°C)
(2) EA: elevated [CO_2] (603 µmol mol^{-1}) and approximately ambient air temperature (22.2°C)
(3) AH: ambient [CO_2] (406 µmol mol^{-1}) and high air temperature (25.6°C)
(4) EH: elevated [CO_2] (603 µmol mol^{-1}) and high air temperature (25.6°C)

For morphological observations, fully expanded leaves on the middle vines of the yam plants in each treatment were collected between 08:00 and 11:00 on 9 July 2016.

2.2. Observation of leaf blade tissues

Leaf blade tissues were observed by bright-field optical microscopy as follows. Segments of the center parts of the leaf blades were immediately immersed in 0.05 M sodium phosphate buffer (pH 7.2) containing 2% (v/v) paraformaldehyde and 1% (v/v) glutaraldehyde. Then, the samples were washed in 0.05 M sodium phosphate buffer. The materials were post-fixed in 1% (v/v) osmium tetroxide in 0.1 M sodium phosphate buffer at 4°C for 10 h and washed in 0.1 M sodium phosphate buffer and distilled water. After being dehydrated in a graded ethanol series and immersed in propylene oxide, the segments of the leaf blades were embedded in Spurr's resin at 70°C for 24 h. Then, the samples were cut with a glass knife into 0.7-µm-thick sections using an ultramicrotome (Leica EM UC7, Nussloch, Germany) and stained with 0.1% (w/v) toluidine blue-O. The sections were observed under a bright-field optical microscope (BX51; Olympus, Japan) and photographed.

2.3. Measurements of leaf blade thickness, profile area of mesophyll cells, and chloroplast numbers

From the photographs taken with the bright-field optical microscope, the thickness of the upper (adaxial)

epidermis, the thickness of the palisade parenchyma, the thickness of the spongy parenchyma, the thickness of the lower (abaxial) epidermis, the total leaf blade thickness, and the profile area of the mesophyll cells (palisade and spongy cells) were determined using the Image J software. The numbers of chloroplasts per palisade cell and per spongy cell in the yam leaves were also counted using the images. Three photographs per leaf blade; one leaf blade per plant, and three to five plants per treatment were used to investigate these parameters. Totally, 10 cells from each photograph were randomly selected to determine the number of chloroplasts.

2.4. Observation of chloroplasts

Chloroplasts were observed by transmission electron microscopy. First, samples were fixed as described above. After being embedded in Spurr's resin at 70℃ for 24 h, the samples were cut with a diamond knife into 80-nm-thick sections using the same ultramicrotome. These ultrathin sections were picked up on a 200-mesh copper grid. The grid-mounted sections were stained with aqueous uranyl acetate (4%) for 20 min and then washed in distilled water. The samples were put into lead-staining solution (Sigma-Aldrich Co. LLC, Tokyo, Japan) for 5 min and washed in distilled water. The stained sections were observed and photographed with a transmission electron microscope (JEOL-JEM 1230, Japan) at 80 kV.

2.5. Counting of starch grains and measurement of chloroplast profile area and starch grain profile area

Based on the images taken by transmission electron microscopy as mentioned in section 2.4 (Observation of chloroplasts), the number of starch grains per chloroplast in the mesophyll (palisade + spongy) was counted. Additionally, the chloroplast and starch grain profile areas in the palisade and spongy cells were determined using the Image J software. Six to ten chloroplasts per photograph, four to six photographs per leaf blade, one leaf blade per plant, and three plants per treatment were investigated.

2.6. Observation of stomata and measurement of stomatal pore and density

Stomata on the abaxial and adaxial sides of leaf blades were observed by scanning electron microscopy as follows. Samples were fixed as described above: segments of leaf blades were fixed

immediately in 0.05 M sodium phosphate buffer (pH 7.0) containing 2% (v/v) paraformaldehyde and 1% (v/v) glutaraldehyde, and then post-fixed in 1% (v/v) osmium tetroxide in 0.1 M sodium phosphate buffer at 4℃ for 12 h and washed in 0.1 M sodium phosphate buffer. The samples were dehydrated in a graded ethanol series and then immersed in 100% isoamyl acetate at 20℃ and dried using a critical point dryer (JCPD-5; JEOL). The dried leaf blade samples were mounted on stubs with conductive carbon tape and coated with platinum using an auto fine coater (JFC-1600; JEOL). The stomata were observed and photographed using a scanning electron microscope (JSM-7000F; JOEL) at an accelerating voltage of 5 kV. Stomata were not found on the adaxial side of the leaves in our observations. Therefore, the number of stomata per area unit (mm^2) only on the abaxial side of the leaves was determined, and the stomatal pore length and width were measured using the Image J software. All the stomata in each photograph (966 × 1276 μm in size) at a magnification of × 100 were counted for density and four to five stomata per photograph were measured for size; three photographs per leaf blade, one leaf blade per plant, and five plants per treatment were observed.

2.7. Statistical analysis

To test the significance of differences related to [CO_2] levels, air temperature conditions, Chinese yam lines, and their interactions, we applied three-way analysis of variance (ANOVA). When ANOVA produced a significant result, we performed a Tukey–Kramer's test for significant differences between means. All statistical analyses were performed with the SPSS statistical software (SPSS ver. 24.0; IBM Corp, New York, NY, U.S.A.).

3. Results

3.1. Effects of elevated CO_2 concentration on internal leaf tissues in Chinese yam

Differences in cell size and number of chloroplasts in the yam leaves between the ambient [CO_2] treatment (Figure 1(a,c,e,g)) and elevated [CO_2] treatment (Figure 1 (b,d,f,h)) were observed for both Enshikei 6 and Shojikei.

According to a Tukey–Kramer's test (Table 1), for both Enshikei 6 and Shojikei, the thickness of the palisade layer in the yam leaf blade was significantly higher in EA and EH than in AA and AH, respectively, and the thickness of the whole leaf blade was significantly higher in EA than in AA. Although no significant differences were observed, there was an increasing trend in
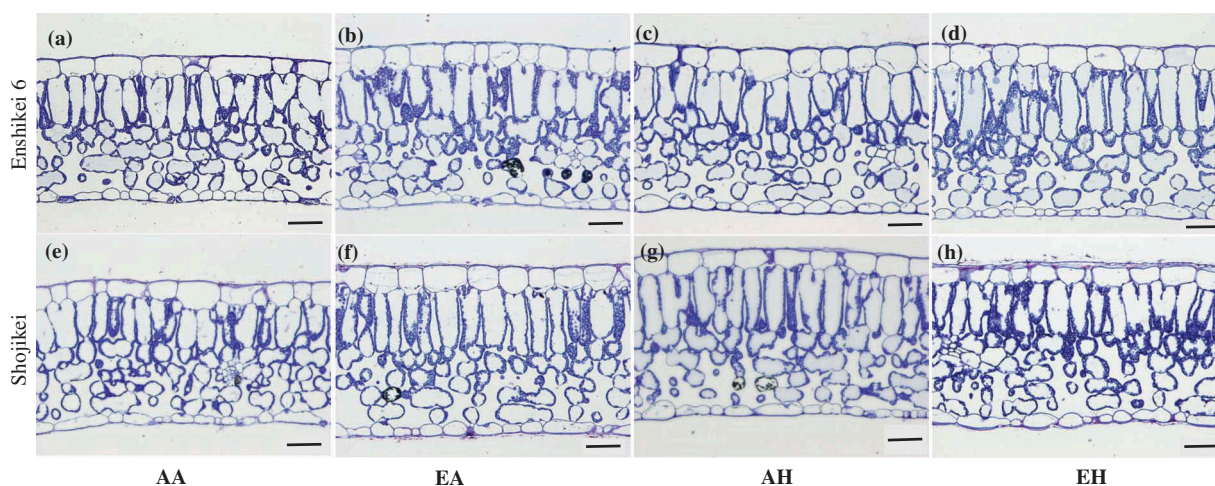
Figure 1. Optical micrographs of transverse sections of leaf blades showing the effects of elevated CO_2 concentration on the inner structures.

(a–d) Enshikei 6 and (e–h) Shojikei Chinese yams grown under AA, EA, AH, and EH treatments, respectively. AA: ambient [CO_2] and approximately ambient-temperature, EA: elevated [CO_2] and approximately ambient-temperature, AH: ambient [CO_2] and high temperature, EH: elevated [CO_2] and high temperature. Bars = 50 μm.

leaf thickness from AH to EH for both Enshikei 6 and Shojikei. There were no significant differences in the thickness of the adaxial epidermis layer, spongy layer or abaxial epidermis layer between ambient [CO_2] and elevated [CO_2] under either temperature regime.

According to the ANOVA results (Table 1), elevated [CO_2] had significant effects on the thickness of the palisade layer, abaxial epidermis layer, and whole leaf blade in Chinese yam. In addition, significant effects of air temperature on the thickness of the abaxial epidermis layer and of yam line on the adaxial epidermis thickness and whole leaf blade thickness were detected. There were interactions between air temperature and

yam line for adaxial epidermis layer thickness, palisade layer thickness, and spongy layer thickness, and among [CO_2], air temperature and yam line for palisade layer thickness.

3.2. Effects of elevated CO_2 concentration on chloroplast numbers in Chinese yam leaves

In the Tukey–Kramer's test (Table 2), the number of chloroplasts per palisade cell in the leaf blades was significantly higher in EA and EH than in AA and AH, respectively, in both Enshikei 6 and Shojikei, while the number per spongy cell in the leaf blades was higher in

Table 1. Effect of elevated CO_2 concentration on the thickness of the adaxial epidermis, palisade layer, spongy layer, abaxial epidermis, and whole leaf blade.

Chinese yam lines	Treatments	Adaxial epidermis (μm)	Palisade layer (μm)	Spongy layer (μm)	Abaxial epidermis (μm)	Whole leaf blade (μm)
Enshikei 6						
	AA 22.2°C[†]	33.1 a	73.4 a	115.7 a	14.9 a	240.4 a
	EA 22.2°C[†]	34.9 ab	83.9 b	120.3 a	15.7 ab	261.7 b
	AH 25.6°C[†]	38.1 b	77.5 ab	112.9 a	16.9 ab	248.1 ab
	EH 25.6°C[†]	38.1 b	96.3 c	113.3 a	17.0 b	255.7 ab
Shojikei						
	AA 22.2°C[†]	33.8 a	73.4 a	107.6 a	14.8 a	231.4 a
	EA 22.2°C[†]	35.9 a	90.7 c	113.6 a	16.0 a	248.4 b
	AH 25.6°C[†]	32.5 a	75.9 a	111.5 a	15.1 a	241.8 ab
	EH 25.6°C[†]	34.3 a	82.9 b	116.8 a	16.3 a	247.5 ab
ANOVA						
	CO_2 (C)	ns	***	ns	*	***
	Temperature (T)	ns	ns	ns	**	ns
	Lines (L)	*	ns	ns	ns	**
	C × T	ns	ns	ns	ns	ns
	C × L	ns	ns	ns	ns	ns
	T × L	**	**	*	ns	ns
	C × T × L	ns	**	ns	ns	ns

Different letters indicate significant difference at the 5% level (Tukey–Kramer's test). AA: ambient [CO_2] and approximately ambient air temperature, EA: elevated [CO_2] and approximately ambient air temperature, AH: ambient [CO_2] and high air temperature, EH: elevated [CO_2] and high air temperature. [†]: mean air temperature. ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; ns, not significant.

Table 2. Effect of elevated CO_2 concentration on the number of chloroplasts per mesophyll cell and per unit profile area of mesophyll cells.

Yam lines	Treatments	Number of chloroplasts per mesophyll cell (cell^{-1})		Number of chloroplasts per unit profile area of cell (mm^{-2})	
		Palisade	Spongy	Palisade	Spongy
Enshikei 6					
	AA 22.2°C[†]	20.6 a	11.2 a	9814.8 a	15607.7 a
	EA 22.2°C[†]	25.1 b	13.6 b	12204.1 bc	16141.8 a
	AH 25.6°C[†]	23.6 ab	12.7 ab	10468.1 ab	17009.6 a
	EH 25.6°C[†]	29.8 c	13.6 b	12990.2 c	17616.1 a
Shojikei					
	AA 22.2°C[†]	20.1 a	9.6 a	9862.7 a	15042.8 a
	EA 22.2°C[†]	23.9 bc	11.6 bc	10957.4 ab	15567.5 a
	AH 25.6°C[†]	23.1 b	10.3 ab	10763.3 a	15129.5 a
	EH 25.6°C[†]	26.1 c	13.1 c	12590.5 b	15830.5 a
ANOVA					
	CO_2 (C)	***	***	***	ns
	Temperature (T)	***	*	**	ns
	Lines (L)	***	***	ns	ns
	C × T	ns	ns	ns	ns
	C × L	*	ns	ns	ns
	T × L	*	ns	ns	ns
	C × T × L	ns	ns	ns	ns

Different letters indicate significant difference at the 5% level (Tukey–Kramer's test). AA: ambient $[CO_2]$ and approximately ambient air temperature, EA: elevated $[CO_2]$ and approximately ambient air temperature, AH: ambient $[CO_2]$ and high air temperature, EH: elevated $[CO_2]$ and high air temperature. [†]: mean air temperature. ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; ns, not significant.

EA than in AA for Enshikei 6, and higher in EA and EH than in AA and AH, respectively, for Shojikei. In addition, the number of chloroplasts per unit profile area of palisade cells was clearly higher in EA and EH than in AA and AH, respectively, for Enshikei 6, and higher in EH than in AH for Shojikei, but no significant difference in the number of chloroplasts per unit profile area of spongy cells was found between elevated $[CO_2]$ and ambient $[CO_2]$ in either Chinese yam line (Table 2).

The ANOVA (Table 2) revealed that elevated $[CO_2]$ and air temperature had significant effects on the numbers of chloroplasts per palisade cell and per spongy cell as well as per unit profile area of palisade cells in Chinese yam. No significant effects of $[CO_2]$, air temperature or yam line on the number of chloroplasts per unit profile area of spongy cells were detected. There were no interactions between $[CO_2]$ and air temperature, or among $[CO_2]$, air temperature, and yam line on the parameters for either yam line.

3.3. Effects of elevated CO_2 concentration on starch accumulation in chloroplasts in Chinese yam leaves

Differences in the size of starch grains accumulated in the chloroplasts in yam leaves between plants grown under ambient $[CO_2]$ treatment (Figure 2(a,c,e,g)) and

elevated $[CO_2]$ (Figure 2(b,d,f,h)) were observed in the palisade cells and the spongy cells (no images are shown) for both Enshikei 6 and Shojikei.

A Tukey–Kramer's test (Table 3) showed that, for both Enshikei 6 and Shojikei, the number of starch grains per chloroplast, starch grain profile area, and ratio of starch to chloroplast area in palisade and spongy cells was significantly higher in EA and EH than in AA and AH, respectively. There were no differences in chloroplast profile area in palisade or spongy cells between the ambient $[CO_2]$ treatment and elevated $[CO_2]$ treatment under either air temperature regime.

According to the ANOVA results (Table 3), elevated $[CO_2]$ significantly affected the number of starch grains per chloroplast, starch grain profile area, and ratio of starch to chloroplast area in both palisade and spongy cells, while air temperature affected the number of starch grains per chloroplast and the ratio of starch to chloroplast area in spongy cells. There were also significant differences in the chloroplast profile area in spongy cells, the starch grain profile area in palisade cells, and the ratio of starch to chloroplast area in palisade cells according to yam line.

3.4. Effects of elevated CO_2 concentration on the density and pore size of stomata in Chinese yam leaves

Differences in stomatal density and pore size on the abaxial side of leaf blades in Chinese yam between plants grown under ambient $[CO_2]$ treatment (Figure 3(a,c,e,g)) and elevated $[CO_2]$ (Figure 3(b,d,f,h)) were observed for Enshikei 6 and Shojikei. The effect of elevated $[CO_2]$ on stomatal density and pore size was investigated by scanning electron microscopy (Figure 3).

According to a Tukey–Kramer's test (Table 4), the stomatal density in the leaf blade was clearly higher in EA and EH than in AA and AH, respectively, and stomatal pore length was higher in EA than in AA for both Enshikei 6 and Shojikei. There was no significant difference in stomatal pore length between ambient $[CO_2]$ and elevated $[CO_2]$ under the high temperature regime or in stomatal pore width under either temperature regime.

According to the ANOVA results (Table 4), elevated $[CO_2]$ significantly affected the stomatal density and stomatal pore length, while air temperature affected the stomatal pore width; yam line affected both the stomatal pore length and width. No interactions among $[CO_2]$, air temperature, and yam line for these factors were found except an interaction between $[CO_2]$ and air temperature for stomatal pore length.
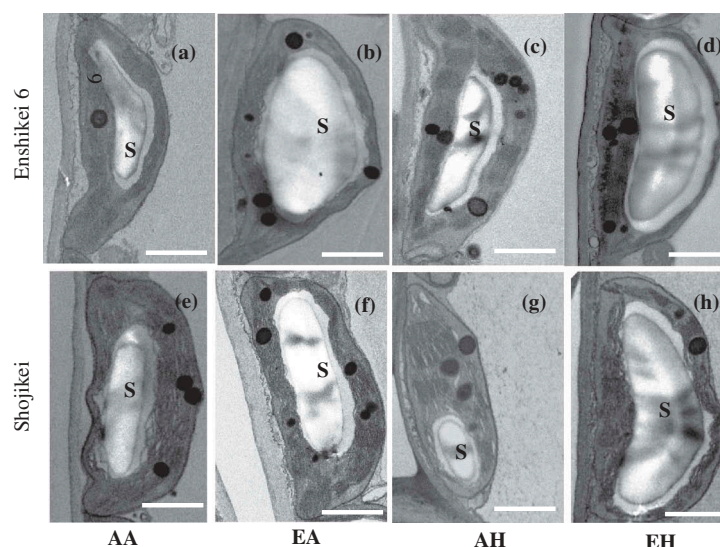
Figure 2. Transmission electron micrographs of transverse sections of chloroplast showing the effects of elevated CO_2 concentration on chloroplasts and starch grains in palisade parenchyma cells.

(a–d) Enshikei 6 and (e–h) Shojikei Chinese yams grown under AA, EA, AH, and EH treatments, respectively. AA: ambient $[CO_2]$ and approximately ambient-temperature, EA: elevated $[CO_2]$ and approximately ambient-temperature, AH: ambient $[CO_2]$ and high temperature, EH: elevated $[CO_2]$ and high temperature. S: starch grain. Bars = 2 μm.

Table 3. Effect of elevated CO_2 concentration on the inner structure of chloroplasts.

Yam lines	Treatments	Number of starch grains (Chloroplast^{-1})	Chloroplast profile area (μm^2 chloroplast^{-1})		Starch grain profile area (μm^2 chloroplast^{-1})		Starch grain to chloroplast area ratio (%)	
		palisade + spongy	Palisade	Spongy	Palisade	Spongy	Palisade	Spongy
Enshikei 6								
	AA 22.2°C[†]	0.9 a	14.2 a	13.9 a	3.9 a	3.2 a	27.4 a	22.4 a
	EA 22.2°C[†]	1.5 c	15.1 a	14.4 a	7.7 b	7.2 b	47.0 b	43.2 b
	AH 25.6°C[†]	1.0 a	14.2 a	13.8 a	3.7 a	3.6 a	26.6 a	29.5 a
	EH 25.6°C[†]	1.2 b	15.9 a	14.7 a	6.7 b	6.3 b	45.1 b	43.9 b
Shojikei								
	AA 22.2°C[†]	1.1 b	12.3 a	11.1 a	3.1 a	3.5 a	22.7 a	24.2 a
	EA 22.2°C[†]	1.4 c	12.9 a	12.9 a	5.1 bc	5.8 b	41.9 b	41.6 b
	AH 25.6°C[†]	0.8 a	13.8 a	12.1 a	3.7 ac	3.4 a	26.9 a	27.5 a
	EH 25.6°C[†]	1.2 cb	13.9 a	13.3 a	5.9 b	5.9 b	40.6 b	45.4 b
ANOVA								
	CO_2 (C)	***	ns	ns	***	***	***	***
	Temperature (T)	***	ns	ns	ns	ns	ns	*
	Lines (L)	ns	ns	*	**	ns	**	ns
	C × T	*	ns	ns	ns	ns	ns	ns
	C × L	ns	ns	ns	ns	ns	ns	ns
	T × L	ns	ns	ns	ns	ns	ns	ns
	C × T × L	***	ns	ns	ns	ns	ns	ns

Different letters indicate significant difference at the 5% level (Tukey–Kramer's test). AA: ambient $[CO_2]$ and approximately ambient air temperature, EA: elevated $[CO_2]$ and approximately ambient air temperature, AH: ambient $[CO_2]$ and high air temperature, EH: elevated $[CO_2]$ and high air temperature. [†]: mean air temperature. ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; ns, not significant.

4. Discussion

Leaf thickness is a very important morphological parameter because it has a direct bearing on photosynthesis and water use efficiency (Ashton & Berlyn, 1994; Murthy & Dougherty, 1997). Chinese yam plants exhibited a significant increase in total leaf blade thickness under elevated $[CO_2]$ in this study (Figure 1, Table 1). This is consistent with previous studies on various crops such as soybean (Vu et al., 1989), loblolly pine, and sweet gum (Thomas & Harvey, 1983), *Arabidopsis thaliana* (Teng et al., 2006), and *Phaseolus vulgaris* (Bray & Reid, 2002). However, the causes of increased leaf thickness are not totally consistent. Radoglou and Jarvis (1992) found that the increase of *P. vulgaris* leaf thickness was mainly due to an increase in the spongy parenchyma thickness. Vu et al. (1989) reported that elevated $[CO_2]$ caused an increase in soybean leaf thickness due to an increased number of palisade cells. In the case of Chinese yam, we found that the palisade layer thickness
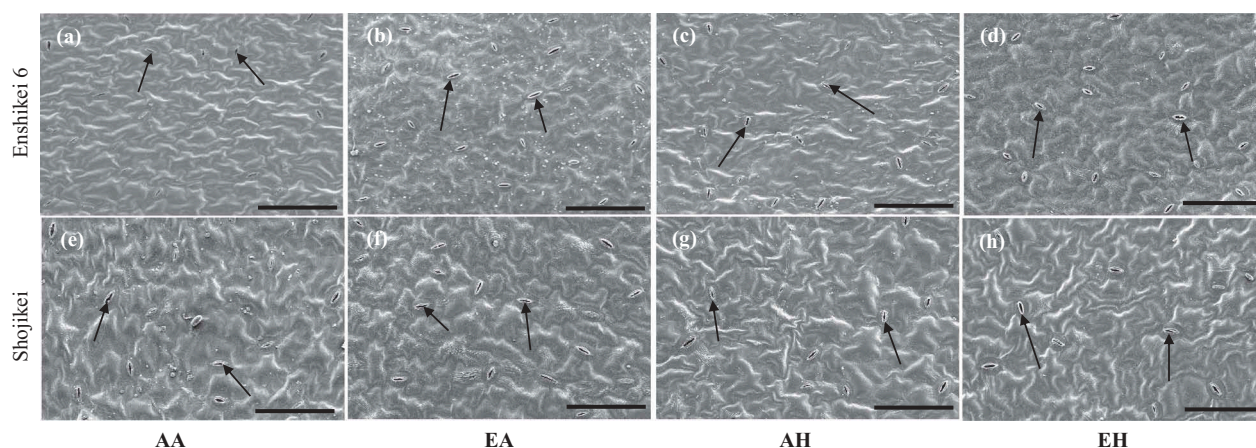
Figure 3. Scanning electron micrographs showing the effect of elevated CO_2 concentration on stomatal density on the abaxial side of leaf blades.

(a–d) Enshikei 6 and (e–h) Shojikei Chinese yams grown under AA, EA, AH, and EH treatments, respectively. AA: ambient [CO_2] and approximately ambient-temperature, EA: elevated [CO_2] and approximately ambient-temperature, AH: ambient [CO_2] and high temperature, EH: elevated [CO_2] and high temperature. Bars = 100 μm. Arrow: stomata

Table 4. Effects of elevated CO_2 concentration on stomatal density and pore length and width on the abaxial side of leaf blades.

Chinese yam lines	Treatments	Stomatal density (mm^{-2})	Stomatal pore length (μm)	Stomatal pore width (μm)
Enshikei 6				
	AA 22.2°C[†]	73.5 a	11.1 a	1.9 a
	EA 22.2°C[†]	85.7 bc	13.2 b	2.1 a
	AH 25.6°C[†]	76.6 ab	11.5 ab	2.2 a
	EH 25.6°C[†]	89.3 c	12.5 ab	2.3 a
Shojikei				
	AA 22.2°C[†]	74.9 a	11.6 a	2.2 a
	EA 22.2°C[†]	92.6 b	13.7 b	2.3 a
	AH 25.6°C[†]	75.1 a	13.1 ab	2.4 ab
	EH 25.6°C[†]	88.8 b	13.2 ab	2.8 b
ANOVA				
	CO_2 (C)	***	***	ns
	Temperature (T)	ns	ns	*
	Lines (L)	ns	*	**
	C × T	ns	*	ns
	C × L	ns	ns	ns
	T × L	ns	ns	ns
	C × T × L	ns	ns	ns

Different letters indicate significant difference at the 5% level (Tukey–Kramer's test). AA: ambient [CO_2] and approximately ambient air temperature, EA: elevated [CO_2] and approximately ambient air temperature, AH: ambient [CO_2] and high air temperature, EH: elevated [CO_2] and high air temperature. [†]: mean air temperature. ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; ns, not significant.

of Chinese yam leaf blades was significantly greater under elevated [CO_2] than under ambient [CO_2], and thus the total leaf blade thickness was also noticeably higher in yams grown at elevated [CO_2] than at ambient [CO_2] under the approximately ambient temperature regime. Our results indicate that elevated [CO_2] increased the yam leaf blade thickness by increasing the thickness of palisade tissue layer.

The chloroplast is the primary photosynthetic organelle in plants (Sharma et al., 2014) and its development influences plant development (Pogson et al., 2015). Thus, the effects of elevated [CO_2] on chloroplasts have been more extensively studied than any other organelle. Teng et al. (2006) reported that the number of chloroplasts per mesophyll cell was significantly higher (17.9%) in the leaves of *A. thaliana* plants grown under elevated [CO_2] than under ambient [CO_2]. Wang et al. (2004) reported a 71% increase in chloroplast number per unit cell area in *Nicotiana sylvestris* leaves under elevated [CO_2] of 730 ppm in a growth chamber. Bockers et al. (1997) also reported that elevated [CO_2] increased the number of chloroplasts in *Marchantia polymorpha*. Similarly, Thomas and Harvey (1983) showed an increase in chloroplast density at high [CO_2] in the leaves of *Zea mays, Glycine max,* and *Liquidambar styraciflua*. In Chinese yam, we found that the numbers of chloroplasts per cell were significantly higher in both the palisade and spongy layers but the numbers per unit cell profile area were higher only in the palisade layer, not the spongy layer, in yams grown under elevated [CO_2] compared with ambient [CO_2] (Table 2). The ANOVA results also showed that [CO_2] and air temperature had significant effects on chloroplast numbers per unit cell profile area for palisade but not spongy cells (Table 2). Thus, the results of this study suggest that palisade cells might be more sensitive to elevated [CO_2] than spongy cells. The mechanisms that regulate chloroplast numbers in

Chinese yam cells cannot be determined from this study. However, by researching *Nicotiana sylvestris* plants grown at elevated [CO_2], Wang et al. (2004) reported that one reason for the higher number of chloroplasts is that elevated [CO_2] may stimulate chloroplast biogenesis. They also found a concomitant increase in chloroplast number and photosynthesis in *N. sylvestris* and a close correlation between them – they concluded that the higher rate of photosynthesis was the result of an adjustment of the photosynthetic apparatus, including chloroplast numbers, under higher [CO_2] conditions (Wang et al., 2004). Conversely, chloroplasts are one of the organelles in which photorespiration take places (Brautigam & Gowik, 2016), which causes a decrease in net photosynthesis (Chollet & Ogren, 1975). Net photosynthesis can be increased up to 50% by stopping photorespiration and the associated oxygen inhibition of photosynthesis (Chollet & Ogren, 1975). In the case of sunflower plants, Bravdo and Canvin (1979) showed that photorespiration could continue under 21% O_2 at high [CO_2] and decreased little as the [CO_2] increased from 20 to 1150 ppm. However, it is widely assumed that photorespiration is suppressed at high [CO_2]. In our previous study (Thinh et al., 2017a), we found that the net photosynthetic rate was 36–73% higher in Chinese yams grown under elevated [CO_2] than under ambient [CO_2]. These results suggest that an increase in the number of chloroplasts in Chinese yam leaves is one of the factors underlying the increase in photosynthesis under elevated [CO_2].

Some studies on starch grain accumulation in chloroplasts have shown an increase in the number of starch grains per chloroplast and the area per starch grain under elevated [CO_2] (Hao et al., 2013; Kumar et al., 2013; Oksanen et al., 2001; Teng et al., 2006; Vu et al., 1989; Zhang et al., 2012). Changes in starch grains in chloroplasts under elevated [CO_2] depend on many factors such as species, cultivar, developmental age, leaf position, and duration of CO_2 exposure (Sharma et al., 2014). For example, Teng et al. (2006) found that the average size and number of starch grains in *A. thaliana* leaf chloroplasts were increased under elevated [CO_2] and that the starch grains in leaves occupied 34.4% of the chloroplast profile under elevated [CO_2], which was significantly higher than under ambient [CO_2]. Additionally, Wang et al. (2004) reported that starch grains occupied as much as 67% of the chloroplast profile under elevated [CO_2] in *N. sylvestris* leaves. Similarly, Hao et al. (2013) observed increases in the number and size of starch grains in chloroplasts in *Isatis indigotica* leaves at elevated [CO_2] compared with ambient [CO_2]. This is consistent with our results, which showed an increase in the number of starch

grains per chloroplast in both palisade and spongy cells, and a greater starch grain profile area in yam leaf chloroplasts at elevated [CO_2] than at ambient [CO_2] (Table 3). Some studies have reported that the accumulation of starch at elevated [CO_2] can vary with the photosynthetic mode of carbon assimilation (Sharma et al., 2014) and serve as a transient sink in leaves to accommodate excess photosynthate production (Wolfe et al., 1998). Tipping and Murray (1999), in researching *Panicum trichanthum, P. antidotale, and P. decipiens*, attributed increased starch accumulation at elevated [CO_2] to enhanced photosynthesis. In this study, our results suggested that the increased starch grain accumulation in the chloroplast under elevated [CO_2] may be caused by an increase in the leaf photosynthetic rate under elevated [CO_2] (Thinh et al., 2017a) and act as a mechanism for storing carbon.

Stomata, the pivotal doors, control the flow of gases between vegetation and the atmosphere (Xu et al., 2016). To adjust CO_2 intake for photosynthesis and water release for transpiration, plants need to mediate stomatal development and behavior to balance CO_2 and water exchange via the leaf epidermis. Thus, stomatal density is an important physiological parameter that affects gas exchange and stomatal conductance (Ceulemans et al., 1995) and strongly influences water use efficiency in plant species (Woodward, 1987). According to Woodward and Kelly (1995), changes in [CO_2] might induce changes in leaf stomatal density that varies greatly in different species. A number of previous studies (Beerling & Chaloner, 1993; Lin et al., 2001; Madsen, 1973; Teng et al., 2006) have shown a decrease in stomatal density in plant species grown under elevated [CO_2]. Woodward (1987) also reported a dramatic (67%) decrease in stomatal density in the leaves of herbarium specimens as [CO_2] increased from the pre-industrial level of 280 ppm to the ambient level of 340 ppm in 1987. Similarly, Woodward and Kelly (1995) investigated stomatal density in as many as 100 plant species and found a reduction of 14.3% due to elevated [CO_2]. Ainsworth and Rogers (2007) reported a 5% decrease in stomatal density due to elevated [CO_2] from a meta-analysis on stomatal responses. Relatively few studies – on poplar clones (Ceulemans et al., 1995; Tricker et al., 2005) and *Alnus glutinosa* (Poole et al., 2000) – have reported no changes in stomatal density with elevated [CO_2]. However, in this study, we found that the stomatal density on the abaxial side of leaf blades in Chinese yam was significantly higher (16.5–16.6% in Enshikei 6 and 18.2–23.5% in Shojikei) at elevated [CO_2] than at ambient [CO_2] (Table 4, Figure 3). This result is consistent with the results of Reid et al. (2003), who observed a higher stomatal

density in 15 species (*Bothriochloa ischaemum, Bromus japonicas, Convolvulus equitans, Eriogonum trichopes, Larrea tridentata, Lepidium lasiocarpum, Liquidambar styraciflua, Lonicera japonica, Parthenocissus quinquefolia, Paspalum pubiflorum, Pinus taeda, Polygonatum biflorum, Solanum dimidiatum, Solidago canadensis*, and *Sorghum halepense*) exposed to elevated $[CO_2]$ for 4 years in free air CO_2 enrichment experiments. However, the mechanism of this response of Chinese yam needs to be further studied in future research.

In our previous studies, we showed that all growth parameters related to size (number of leaves, vine length, and leaf blade area) and weight (dry weight of leaves, vines, roots, tubers, and whole plants), and the net photosynthetic rate in Chinese yam were greater under elevated $[CO_2]$ than under ambient $[CO_2]$ (Thinh et al., 2017a, 2017b). In this study, we found that the thickness of the leaf blade and palisade tissue layer, the number of chloroplasts, and the stomatal density, and pore length in Chinese yam were greater under elevated $[CO_2]$ than under ambient $[CO_2]$. A possible mechanism to explain why Chinese yam positively responds to elevated $[CO_2]$ is that elevated $[CO_2]$ increases the thickness of the leaf blade, number of leaves and leaf area; and positively supports photosynthesis apparatus such as chloroplasts and stomata in Chinese yam. Thus, the photosynthetic rate in Chinese yam was enhanced under elevated $[CO_2]$ and higher than that in rice plants (Thinh et al., 2017a). Because of the increase in the photosynthetic rate, the dry weight of post-treatment seed bulbils, seedling growth, and seedling dry weight in Chinese yam increased under elevated $[CO_2]$ (Thinh et al., 2017b). Our results indicated that elevated $[CO_2]$ is a positive resource for growth in Chinese yam from an early growth stage.

The experiments in this study were conducted in temperature-gradient chambers. As shown by Okada et al. (2000), both air temperature and vapor pressure deficit increase from the inlet to the outlet in temperature-gradient chambers and these two factors are closely associated. In our experiments, Chinese yams were grown in approximately ambient- and high-temperature plots in each temperature-gradient chamber. The increase in air temperature resulted in a decrease in relative humidity in the high-temperature plots in the chambers. However, the difference in relative humidity between the two plots in this study was about 19%, and plant growth is generally much more sensitive to changes in air temperature than in relative humidity. Shimizu et al. (1996), who researched the effects of $[CO_2]$ (500 and 1000 ppm) and relative humidity (79% and 37%) on the growth of several plants, reported that there were no obvious interactive effects between CO_2 and relative humidity and that these environmental factors may affect the growth of plants independently. Therefore, relative humidity would have no relevance to the conclusions of this study.

The data gathered in this study provide important information and a possible explanation of the mechanism by which Chinese yams responded positively to elevated $[CO_2]$ and yam photosynthesis was enhanced at elevated $[CO_2]$ in our previous studies.

Disclosure statement

No potential conflict of interest was reported by the authors.

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