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Effects of elevated CO_2 and water stress on physiological responses of *Perilla frutescens* var. *japonica* HARA

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Abstract The aim of the present study was to investigate the interactive effects of expected environmental constraints, specifically elevated CO_2 and drought conditions, on the physiological responses of *Perilla frutescens* var. *japonica* Arum. *Perilla frutescens* var. *japonica* 'Arum' was exposed to 700 μmol mol^{-1} of CO_2 under both well-watered and water-stressed conditions. Photosynthetic rate was higher under elevated CO_2 conditions. Stomatal resistance increased while transpiration rates declined, which suggests that water-use efficiency rose under elevated CO_2 conditions. Under water-stressed conditions, elevated CO_2 concentrations induced much higher stomatal resistance than ambient CO_2 levels. This result implies that elevated CO_2 concentrations might increase plant sensitivity to water stress, thereby providing plants with increased protection against drought. Furthermore, elevated CO_2 concentrations alleviated drought-induced photosynthetic decline in the early stages of drought, although this effect was not sustainable. Water stress also elevated ascorbate peroxidase (APX) activity but had little effect on glutathione reductase activity. Water stress-induced APX activity was much lower under elevated CO_2 conditions compared to ambient CO_2 levels. Therefore, elevated CO_2 concentrations might increase plant resistance to water stress. Moreover, there is a possibility that increased ethylene evolution under elevated CO_2 conditions could improve the capacity of plants to scavenge reactive oxygen species by enhancing APX activity.

Keywords Elevated CO_2 · Water stress · Ethylene · Antioxidative enzymes · Stomatal resistance

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Introduction

Many concerns have arisen concerning the potential impact of climatic change induced by increasing greenhouse gas concentrations and rising annual temperatures on the ecological performance of both natural and artificial eco-systems (such as agriculture). Atmospheric CO_2 concentration has an indirect effect on plants through induction of climatic change as well as a direct effect on plant growth and physiology (Bettinger et al. 2013; Luong et al. 2013). It is commonly expected that the greenhouse effect will result in the modification of precipitation regimes. In fact, changing patterns of precipitation such as increased duration of drought periods in mid-summer as well as concentrated rainfall in autumn have been observed, especially in middle latitude regions (Ruiz-Sánchez et al. 2007). These shifts in precipitation patterns might increase the seasonal and local frequencies of drought stress in plants.

Water availability from nearby surroundings is one of the most important factors in terms of plant productivity and survival, especially in natural eco-systems. Innes (1992) reported that the most important factor affecting the growth of European beech is water availability from soil. Dry weather in 1976 and between 1983 and 1984 is regarded to be the major reason for the decline of the European beech (Ling et al. 1993). As present climatic conditions will assumedly be maintained in the future, it is important to investigate the interactive effects between high atmospheric CO_2

concentrations and drought stress on plant eco-systems, including agriculture. There have been many reports demonstrating the positive effects of elevated CO_2 concentrations on plant productivity mediated by improved energy efficiency caused by increased C assimilation rates (Boese et al. 1997; Reddy et al. 2010; Thomas et al. 1993) as well as reduced photorespiration (Havir and McHale 1989; Robredo et al. 2010; Roden and Ball 1996). It has also been reported that stomatal conductivity decreases under elevated CO_2 conditions (Ainsworth and Rogers 2007; Zhu et al. 1998), suggesting that water-use efficiency (WUE) of plants can be increased (Bunce 2004; Malmström and Field 1997; Oliveira et al. 2013). Therefore, among the factors influencing drought stress in plants, elevation of atmospheric CO_2 is considered to be one of the most important factors. Although elevation of atmospheric CO_2 concentrations may potentially increase drought stress in forest eco-systems by increasing total leaf area, it is generally assumed that photosynthetic limitations caused by drought are alleviated under high atmospheric CO_2 conditions, and leaf water potential under drought conditions can be maintained by increased water use efficiency under high atmospheric CO_2 levels (Ge et al. 2011; Oliveira et al. 2013; Roger et al. 1984).

On the other hand, an elevated atmospheric CO_2/O_2 ratio can reduce the rate of photorespiration, thereby decreasing generation of internal reactive oxygen species (ROS). This suggests that the capacity of plants to detoxify ROS might be improved under elevated atmospheric CO_2 conditions (Polle et al. 1993, 1997; Schwanz and Polle 2001). Both CO_2 and air pollutants such as SO_2 and NO_2 are byproducts of fossil fuel combustion, and ROS are regarded as a major substance inducing plant damage under air pollution or drought stress. On that point, the effect of CO_2 on plant oxidative stress is no less important than its effect on plant productivity under changing climatic conditions. Thus, the aim of the present study was to investigate the interactive effects of elevated CO_2 concentration and drought on the physiological responses of plant using *Perilla frutescens* var. *japonica* 'Arum' which has been known as an indicator species of environmental fluctuations having an important economic value in many of East Asian countries (Hur et al. 1995; Park et al. 2001; Kim et al. 2013).

Materials and methods

Plant culture

Perilla frutescens var. *japonica* 'Arum' was grown from seeds in plastic pots (top diameter 15 cm) containing a mixture of 1:1(v/v) of peat (Klasman, Potground-H, Germany): perlite for 60 days in a greenhouse maintained at 27 ± 0.5 °C during the day and 20 ± 0.5 °C at night. The pots were flushed once a day and fertilized twice a week with a nutrient solution developed for leafy vegetables by the University of Seoul (UOS) (Table 1).

Table 1 Composition of nutrient solution used during the experiment

Macroelement (meL^{-1})					
N	P	K	Ca	Mg	S
11.7	2.0	6.7	3.5	2.0	2.0
Microelement (ppm)					
Fe	Mn	B	Zn	Cu	Mo
2.0	0.3	0.5	0.3	0.1	0.05

CO_2 treatment and water regime

Plants were placed in a controlled environment chamber maintained at 27 ± 0.5 °C during the day, 20 ± 0.5 °C at night, 50 ± 5 % relative humidity, and 250 ± 30 μmol m^{-2} s^{-1} light intensity. After acclimation for a week, plants were treated with either ambient or elevated CO_2 concentrations under both well-watered and drought-stressed conditions. CO_2 concentrations of 350 ± 20 μmol mol^{-1} (ambient) and 680 ± 30 μmol mol^{-1} (elevated) were used. The elevated CO_2 concentration was maintained for 8 h per day for 1 week and was monitored by a computer-controlled NDIR (non-dispersive infrared) CO_2 analyzer (Mapo Technomax Ltd. Korea) and injection system (Genie, USA). Two watering regimes, drought-stressed and well-watered, were imposed under both CO_2 concentrations by withholding water from the beginning of CO_2 treatment for the drought-stressed regime and watering once a day for the well-watered regime. Plant water status was monitored by measuring the relative water content (RWC) of leaves.

Gas exchange measurement

All photosynthetic parameters of plants were measured without removing the plants from growth chambers to ensure that conditions during measurement did not differ from experimental conditions. Measurements were taken between 11:00 and 14:00 h. Leaf photosynthetic rate, CO_2 absorption rate, and stomatal resistance were measured using a Li-6200 (Li-Cor, USA), and transpiration rate was measured using a porometer (Li-1600, Li-Cor, USA) on fully expanded leaves.

Ethylene evolution measurement

The amount of ethylene evolution was determined according to the methods by Mathooko et al. (1998). Same-sized leaf discs were collected using a cork borer and enclosed in airtight vials containing wet filter paper to

prevent leaf desiccation. Following incubation at 25 °C for 2 h in the dark, 1 mL of headspace was withdrawn. Ethylene concentration in the gas sample was measured using a gas chromatograph (model GC-14B, Shimadzu, Japan) equipped with a flame-ionization detector and an activated alumina column (110 × 0.32 cm). A standard curve was used for quantification of ethylene, and total ethylene evolution was normalized to the fresh weight of leaves.

Antioxidative enzyme assays

The activities of two antioxidative enzymes, ascorbate peroxidase (APX) and glutathione reductase (GR), were measured according to the methods by Asada (1992) and Kondo and Saji (1992) respectively. The overall procedure was carried out at 0–4 °C. Randomly sampled mature leaf tissues were ground in a chilled mortar using specific buffers and pH values for each enzyme. To measure APX activity, leaf tissue samples (0.2 g) were ground with 2 mL of ice-cold extraction buffer (1 M AsA, 100 mM K-P buffer pH 7.4). The homogenate was centrifuged at 4 °C for 3 min at 12,000 rpm. The supernatant (60 µL) was added to a reaction mixture containing 2.34 mL of H_2O, 300 µL of 1 M K-P buffer (pH 6.5), 180 µL of 10 mM AsA, and 120 µL of 5 mM H_2O_2. Enzyme activity was calculated based on the decrease in A_{290} of the mixture over a 30 s interval, as detected by a UV spectrophotometer (UV2100, Shimadzu, Japan). To measure GR activity, leaf tissue samples (0.15 g) were ground with 2 mL of cold extraction buffer containing 50 mM K-P buffer (pH 7.0), 5 % PVP, 5 mM AsA, 5 mM DTT, 5 mM EDTA, and 0.1 M NaCl. The homogenate was centrifuged at 4 °C for 5 min at 15,000 rpm, after which the supernatant (150 µL) was added to a reaction mixture containing 2.43 mL of H_2O, 300 µL of 1 M K-P buffer (pH 7.8), 60 µL of 10 mM GSSG, and 60 µL of 10 mM NADPH. Enzyme activity was calculated based on the decrease in A_{340} of the mixture over a 90 s interval. The protein content was quantified following the methods by Bradford (1976).

Statistical analysis

Statistical analysis was carried out on each measurement with SAS (9.13). Data was submitted to Duncan's multiple range test and the significance of differences between treatments was considered at the $P \leq 0.05$ level.

Results and discussion

Effect of high CO_2 on photosynthetic parameters

Single treatments of elevated CO_2 under well-watered conditions increased the photosynthetic rate of *P. frutescens* var. *japonica* 'Arum' by 39 and 30 % on the 2nd and 5th days of treatment, respectively (Fig. 1). As elevation of atmospheric CO_2 generally shifts the activity of RubisCO in favor of carboxylation in most C_3 species, net photosynthesis initially increases in response to increased CO_2 levels (Bowes 1991; Robredo et al. 2010; Stitt 1991). Along with an increase in photosynthetic rate, the stomatal response could be affected by elevated CO_2 concentrations (Ainsworth and Rogers 2007; Berryman et al. 1994; Kellomäki and Wang 1998; Robredo et al. 2010). Stomatal resistance of *P. frutescens* var. *japonica* 'Arum' increased by 29 and 19 % on the 2nd and 5th days of treatment, respectively (Fig. 2). WUE of a plant is the ratio of carbon gain to water loss. Thus, increased stomatal resistance under elevated CO_2 conditions might increase WUE (Gunderson et al. 1993; Idso and Idso 1994; Malmström

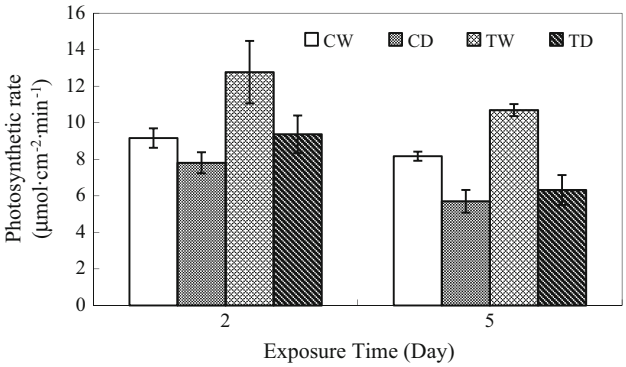

Fig. 1 Effect of elevated CO_2 on photosynthetic rate in *P. frutescens* var. *japonica* 'Arum' under both well-watered and drought-stressed conditions. *Each symbol* represents the mean of 10 observations ±SE (*CW* well-watered control at ambient CO_2 level, *CD* drought-stressed at ambient CO_2 level, *TW* well-watered at 700 µmol mol^{-1} CO_2 level, *TD* drought-stressed at 700 µmol mol^{-1} CO_2 level)

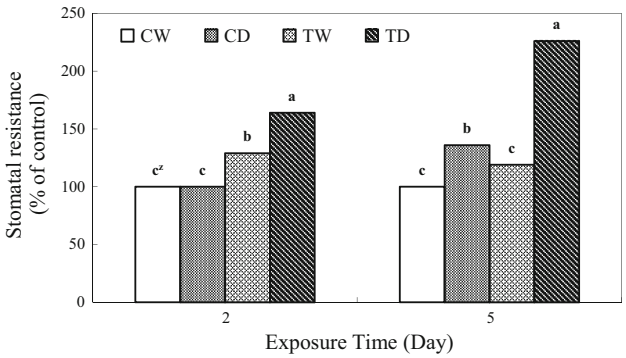

Fig. 2 Effect of elevated CO_2 on stomatal resistance in *P. frutescens* var. *japonica* 'Arum' under both well-watered and drought-stressed conditions. [z]Mean separation (n = 10) within treatments by Duncan's multiple range test at $P \leq 0.05$ (*CW* well-watered control at ambient CO_2 level, *CD* drought-stressed at ambient CO_2 level, *TW* well-watered at 700 µmol mol^{-1} CO_2 level, *TD* drought-stressed at 700 µmol mol^{-1} CO_2 level)

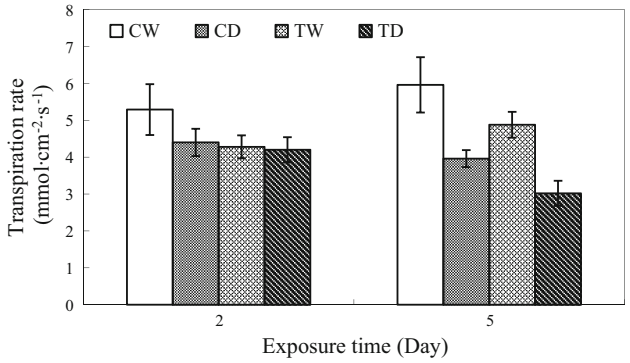

Fig. 3 Effect of elevated CO_2 on transpiration rate in *P. frutescens* var. *japonica* 'Arum' under both well-watered and drought-stressed conditions. *Each symbol* represents the mean of 10 observations ±SE (*CW* well-watered control at ambient CO_2 level, *CD* drought-stressed at ambient CO_2 level, *TW* well-watered at 700 μmol mol^{-1} CO_2 level, *TD* drought-stressed at 700 μmol mol^{-1} CO_2 level)

and Field 1997) as the result of a reduced transpiration rate. Transpiration rates in well-watered plants under elevated CO_2 conditions decreased significantly during treatment (Fig. 3). This result shows that the WUE of *P. frutescens* var. *japonica* 'Arum' might be improved by increased net photosynthesis and a decreased transpiration rate, suggesting resistance to drought might be increased. Robredo et al. (2007) and Rogers et al. (1984) also reported reduction of the transpiration rate due to high atmospheric CO_2 levels even under drought conditions as well as delayed onset of irreversible water stress.

Interactive effect of high CO_2 and drought on photosynthetic parameters

Leaf RWC was reduced by only 3 % during the 7 days of drought period (data not shown), which suggests that *P. frutescens* var. *japonica* 'Arum' is relatively resistant to drought. However, there were significant differences in the physiological responses, including photosynthetic parameters. Photosynthetic rate decreased by 15 and 31 % on the 2nd and 5th days of drought treatment, respectively, under ambient CO_2 levels (CD) (Fig. 1). The photosynthetic rate on the 2nd day of treatment in plants exposed to both drought and elevated CO_2 levels (TD) was higher than that in drought-stressed plants under ambient CO_2 conditions (Fig. 1). As drought stress progressed, the photosynthetic rate decreased by 23 % by day 5 even under elevated CO_2 conditions. This rate was similar to that in plants subjected to drought treatment under ambient CO_2 levels. That is, elevated CO_2 concentrations alleviated drought-induced reduction of photosynthesis in the early stages of drought, although this effect was not sustainable. Drought treatment under ambient CO_2 conditions increased stomatal resistance by about 36 % by the 5th day of treatment (Fig. 2). It is known that both stomatal closing induced by the ABA-mediated signal transduction pathway (Wilkinson et al. 1998) as well as decreased ATP-synthase activity (Tezara et al. 1999, 2008) are involved in reduction of photosynthesis under drought conditions. In plants subjected to combined elevated CO_2 and drought treatment, stomatal resistance was 64 % higher by day 2 and twofold higher by day 5 compared to plants subjected to ambient CO_2 and drought treatment (Fig. 2). This result suggests that elevated atmospheric CO_2 concentrations increase the sensitivity of stomata to drought stress, which helps plants respond to drought stress more effectively. Rogers et al. (1984) also reported that elevation of stomatal resistance with decreasing leaf water potential in soybeans is exacerbated under elevated CO_2 conditions. Similar results have been observed in tropical tree species (Berryman et al. 1994; Heath 1998). The CO_2 absorption rate was higher in plants exposed to both drought and elevated CO_2 than in drought-stressed plants under ambient CO_2 conditions, although stomatal resistance was much higher in plants subjected to combined drought and elevated CO_2 treatment (Table 2). Therefore, the high photosynthetic rates under drought and elevated CO_2 conditions in the early stages of drought can be attributed to increased carbon fixation capacity resulting from higher WUE and CO_2 absorption rates in response to elevated CO_2 concentrations. Malmström and Field (1997) reported that increased WUE resulting from reduced transpiration rates in response to elevated CO_2 concentrations results in higher whole-plant carbon gain. However, after 5 days of drought treatment, the CO_2 absorption rate under high CO_2 conditions had

Table 2 Effect of elevated CO_2 concentrations on CO_2 absorption rate in *P. frutescens* var. *japonica* 'Arum' under well-watered and drought-stressed conditions

CO_2 level (μmol mol^{-1})	CO_2 absorption rate (μg cm^{-2} min^{-1})			
	3rd day		7th day	
	Well-watered	Drought	Well-watered	Drought
Ambient	1.05 ± 0.14[z]	0.87 ± 0.15	0.84 ± 0.09	0.52 ± 0.12
700	1.38 ± 0.23	1.10 ± 0.23	1.06 ± 0.13	0.49 ± 0.12

[z] Means ± SE of result from 10 observations are shown for each treatment

decreased to the same rate as that seen under ambient CO_2 levels (Table 2) along with reduction of the photosynthetic rate. Tezara et al. (1999, 2008) reported that drought stress decreases leaf ATP and RuBP contents as a result of reduced ATP-synthase activity. This photosynthetic limitation is not compensated for by elevation of the atmospheric CO_2 concentration. This suggests that expected improvement of plant productivity via increased atmospheric CO_2 concentrations might be limited by shifts in precipitation patterns induced by the greenhouse effect.

Effect of high CO_2 on evolution of secondary toxic substances

ROS are some of the most important secondary toxic substances inducing oxidative damage to plants (such as lipid peroxidation) under environmental stress. Metabolic changes induced by increased atmospheric CO_2 concentrations might affect the activities of antioxidative enzymes and thus plant resistance to environmental stresses (Polle et al. 1997; Schwanz and Polle 2001). Therefore, the effects of high atmospheric CO_2 concentrations on activities of GR and APX, which play a central role in protecting chloroplasts and other cellular components from oxidative damage, were examined. The results show that GR activity was not affected significantly by CO_2 concentration under well-watered conditions during treatment (Fig. 4). APX activity increased in response to high CO_2 concentrations under well-watered conditions, although the difference was not very significant (Fig. 4). After they observed that activities of superoxide dismutase (SOD) and catalase were decreased in the response to high atmospheric CO_2 concentrations, Polle et al. (1997) suggested that the ROS detoxification capacity of plants might be improved under high atmospheric CO_2 concentrations by providing increased amounts of substrate for detoxification and repair processes. However, peroxidase is known to be related to various metabolic processes in plants such as the biosynthesis of hormones and lignification, which suggests its activity might increase in response to metabolic changes related to increasing atmospheric CO_2 concentrations (Polle et al. 1993). Ethylene can also affect the activities of various antioxidative enzymes such as APX (Ievinsh et al. 1995; Lafuente et al. 2004; Mehlhorn 1990), and ethylene biosynthesis can increase under high atmospheric CO_2 concentrations (Mathooko et al. 1998). It was therefore, suspected that APX activity might be increased under high atmospheric CO_2 concentrations. Ethylene evolution in well-watered plants also increased in response to elevated CO_2 concentrations, especially in the early stage of treatment (Table 3). CO_2 can stimulate ACC oxidase activity so that an increase in intercellular CO_2 concentration under high atmospheric CO_2 enhances ethylene biosynthesis in plants (Mathooko et al. 1998). Mehlhorn (1990) reported that plants pre-exposed to ethylene showed increased resistance to ozone and that this response was related to the ethylene-induced stimulation of APX activity. Therefore, it is suspected that an increase in APX activity under high CO_2 concentration in the present study does not result from an increase in ROS but rather from metabolic changes induced by elevated CO_2 concentration or increased amounts of ethylene evolution. In the latter case, increased

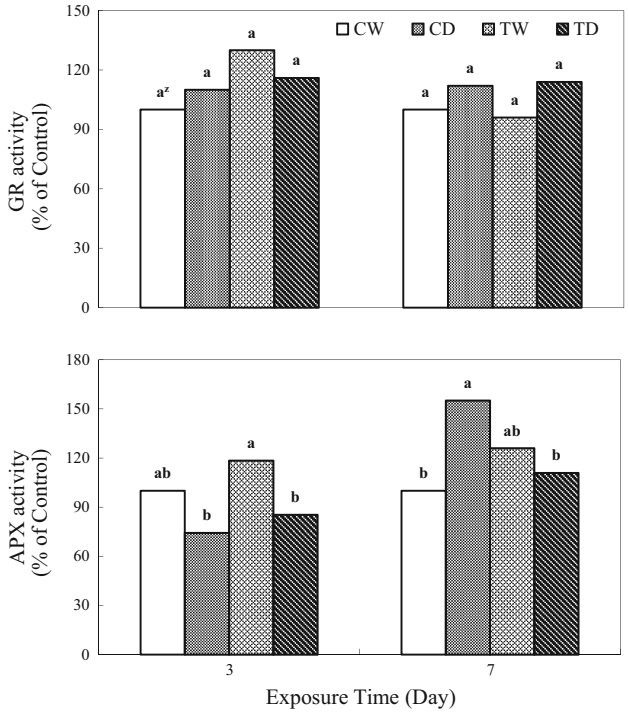

Fig. 4 Effects of elevated CO_2 on activities of glutathione reductase (GR) and ascorbate peroxidase (APX) in *P. frutescens* var. *japonica* 'Arum' under both well-watered and drought-stressed conditions. [z]Mean separation (n = 5) within treatments by Duncan's multiple range test at $P \leq 0.05$ (*CW* well-watered control at ambient CO_2 level, *CD* drought-stressed at ambient CO_2 level, *TW* well-watered at 700 μmol mol^{-1} CO_2 level, *TD* drought-stressed at 700 μmol mol^{-1} CO_2 level)

Table 3 Effect of elevated CO_2 on ethylene evolution in *P. frutescens* var. *japonica* 'Arum' under well-watered and drought-stressed conditions

CO_2 level (μmol mol^{-1})	Ethylene evolution (nL g^{-1} h^{-1})			
	3rd day		7th day	
	Well-watered	Drought	Well-watered	Drought
Ambient	3.07 b[z]	4.51 ab	2.35 b	3.87 a
700	5.10 a	4.93 ab	3.82 a	3.79 a

[z] Different letters indicate significant differences in mean separation among treatments at $P \leq 0.05$ by Duncan's multiple range test

APX activity could allow plants to become more resistant to oxidative damage induced by environmental stresses such as air pollution. On the other hand, ethylene is known to be a major secondary toxic substance to induce lipid peroxidation under stress conditions. However, it has also been reported that ACC treatment, which increases ethylene evolution, induces no significant changes in ion leakage as an index of membrane damage by lipid peroxidation. This suggests that the actions of ethylene may vary depending on the cause of induction. Thus, the effect of increased ethylene evolution due to elevated atmospheric CO_2 levels on plant responses to environmental stress remains unclear.

Interactive effect of high CO_2 and drought on evolution of secondary toxic substances

Drought stress induces damage to the electron transport system in thylakoid membranes, resulting in increased superoxide production (Kitao et al. 2003; Price and Hendry 1991). The rates of ROS production induced by drought stress under both ambient and elevated CO_2 levels were measured in this experiment based on changes in GR and APX activities. The activity of GR in plants exposed to drought under ambient CO_2 levels showed no significant increase during treatment, whereas APX activity increased by 50 % by day 7 of drought treatment (Fig. 4). The antioxidant defense system of plants consists of a variety of antioxidant enzymes but their activities are not all increased under stress conditions that lead to the formation of ROS in plants. For example, GR activity in response to salt or drought stress might not increase until the stress level becomes severe (Hernandez et al. 1999; Zhang and Kirkham 1996). In our study, drought stress, elevated CO_2 levels, as well as their combined treatment all had no significant effect on GR activity, whereas APX activity in plants exposed to combined drought and elevated CO_2 treatment was much lower compared to plants under drought at ambient CO_2 conditions (Fig. 4). Therefore, elevated CO_2 conditions might allow plants to become more resistant to drought stress with respect to oxidative damage. The activities of antioxidative enzymes in response to drought stress generally increase with decreases in leaf water potential (Hernandez et al. 1999; Zhang and Kirkham 1996). Price and Hendry (1991) reported that reduction of chlorophyll content is strongly correlated to reduction of RWC. Production of ROS in response to drought stress is the major cause of chlorophyll destruction. In the present study, reduction of RWC and chlorophyll content as the result of drought treatment was not significant (data not shown), suggesting *P. frutescens* var. *japonica* 'Arum' could respond to drought stress properly by increasing stomatal resistance and ROS scavenging metabolisms. Drought stress also increases ethylene evolution, thereby inducing leaf senescence and retarding the growth rate. Drought treatment increased ethylene evolution under both ambient and elevated CO_2 conditions (Table 3). Under elevated CO_2 conditions, ethylene evolution was higher in well-watered plants than in drought-stressed plants. The effect of elevated CO_2 levels on ethylene evolution might be limited under drought conditions as the result of elevated stomatal resistance. Leaf abscission induced by drought stress imposed by withholding water generally appears after re-watering since the transpiration stream is restricted under drought conditions, which prevents the transfer of ACC, the precursor of ethylene, to the leaf (Nilsen and Orcutt 1996). Therefore, ethylene evolution could immediately increase after plants are released from drought stress. This may be possible under elevated CO_2 conditions, considering that CO_2 may activate ACC oxidase. These phenomena might affect plant responses to environmental stresses stress in relation to the action of ethylene mentioned above. In summary, elevation of atmospheric CO_2 levels increased the photosynthetic rate as well as decreased the transpiration rate, resulting in elevated plant WUE. Under elevated CO_2 conditions, stomata of *P. frutescens* var. *japonica* 'Arum' showed much higher sensitivity to drought. This result suggests that plants are afforded increased protection against drought stress under elevated CO_2 conditions. The extent of these responses will be important to determine plant productivity and survival in relation to climatic changes induced by increasing atmospheric CO_2 concentrations. Furthermore, elevated CO_2 concentrations reduced drought-induced oxidative damage and seemed to have an indirect effect on APX activity by enhancing ethylene evolution. Therefore, this effect of elevated CO_2 levels on antioxidative defense systems should be considered as an important factor in the present environment in which plant stressors are gradually increasing.

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