Enhancement in leaf photosynthesis and upregulation of Rubisco in the C₄ sorghum plant at elevated growth carbon dioxide and temperature occur at early stages of leaf ontogeny

P. V. Vara Prasad⁴, Joseph C. V. Vu⁵, Kenneth J. Boote⁶ and L. Hartwell Allen Jr⁵

⁴Agronomy Department, 2004 Throckmorton Hall, Kansas State University, Manhattan, KS 66506, USA.
⁵United States Department of Agriculture – Agricultural Research Service, Center for Medical, Agricultural and Veterinary Entomology, Chemistry Research Unit, Gainesville, FL 32608, USA.
⁶Agronomy Department, 304 Newell Hall, University of Florida, Gainesville, FL 32611, USA.
⁷Corresponding author. Email: vara@ksu.edu

Abstract. Rising atmospheric carbon dioxide (CO₂) concentration and temperature will influence photosynthesis, growth and yield of agronomic crops. To investigate effects of elevated CO₂ and high temperature on leaf gas exchanges, activities of Rubisco and phosphoenolpyruvate carboxylase (PEPC) and growth of grain sorghum (Sorghum bicolor L. Moench), plants were grown in controlled environments at day-time maximum/night-time minimum temperatures of 30/20°C or 36/26°C at ambient (350 μmol mol⁻¹) or elevated (700 μmol mol⁻¹) CO₂. Gas-exchange rates, activities of Rubisco and PEPC and growth parameters (leaf, stem and total dry weights) were determined at different stages of leaf development. Between 6 and 25 days after leaf tip emergence, leaf carbon exchange rate (CER) of elevated CO₂ plants was greater at 30/20°C and 36/26°C than that of ambient CO₂ plants at the same temperatures. The positive response of CER to elevated CO₂ was greater in young leaves than in old leaves. In young leaves, elevated CO₂ enhanced Rubisco activity at 30/20°C and 36/26°C, whereas PEPC activity was not affected by elevated CO₂ at 30/20°C but was marginally enhanced at 36/26°C. At 30/20°C, growth parameters were not affected by elevated CO₂ until 50 days after sowing (DAS); at 36/26°C, C, growth parameters were progressively enhanced by elevated CO₂ to as high as 49 to 62% by 50 DAS. Leaf CER and Rubisco activity were enhanced by elevated CO₂ at early stages of leaf ontogeny for the C₄ grain sorghum. Such enhancement should have a significant role in dry matter production under elevated CO₂.

Additional keywords: climate change, dry matter production, heat stress, leaf development, leaf growth, Sorghum bicolor.

Introduction
Concentrations of atmospheric carbon dioxide (CO₂) are projected to double the current levels of 380 μmol mol⁻¹ by the end of this century. This, together with increases in other trace gases, could cause an increase in global surface temperatures in the range of 1.6–4.5°C, relative to the mean from 1980 to 1999, averaged across various models (IPCC 2007). These changes will likely influence photosynthesis, dry matter accumulation and yield of plants. In plants with a C₃ photosynthetic pathway, the enzyme Rubisco-carboxylase (EC 4.1.1.39) catalyses the initial carboxylation and oxygenation (photorespiration) reactions (Bowes 1993). The balance of carboxylation and oxygenation depends on the CO₂ to O₂ ratio at the Rubisco site. Rubisco is not saturated at current atmospheric CO₂ levels in C₃ plants, and an increase in atmospheric CO₂ concentration will decrease photorespiration and increase photosynthesis (Ogren 1984; Bowes 1993; Drake et al. 1997; Osborne et al. 1998). In C₄ plants, CO₂ is fixed initially in the mesophyll cells by phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) to form C₄ acids (such as malic acid and aspartic acid) that are diffused into bundle sheath cells, where they are decarboxylated, and the released CO₂ is then used by Rubisco (Hatch 1987).

The C₄ photosynthetic cycle concentrates CO₂ concentration at the active site of Rubisco in bundle sheath cells, leading to an increase in carboxylation and suppression of photorespiration. Consequently, C₄ plants are able to reach a high photosynthetic rate at the current ambient CO₂ level (370 μmol mol⁻¹), and further increase in atmospheric CO₂ should have little or no effect on photosynthesis and growth (Furbank and Hatch 1987; He and Edwards 1996). However, many studies have shown increased growth and dry matter production of C₄ species at elevated CO₂ under well watered conditions (Poorter et al. 1996; Ghannoum et al. 1997; Wand et al. 1999; Ziska et al. 1999), and such C₄ growth stimulations occur with or without the concomitant enhancements in photosynthetic rates of fully expanded leaves (Roger et al. 1983; Ghannoum et al. 1997; Ziska et al. 1999).

C₄ plants have higher optimum temperature for photosynthesis than C₃ plants (Berry and Bjorkman 1980). C₄ plants also have greater photosynthetic capacity, particularly at
higher temperatures, than C₃ species (Matsuoka et al. 2001). In C₃ plants, high temperature decreases Rubisco activity (Sage et al. 1990; Vu et al. 1997) and limits Rubilose 1–5 bisphosphate (RuBP) generation (Sage et al. 1995) or Rubisco activation (Law and Crafts-Brandner 1999), which may result in lower photosynthetic rates. In C₄ plants, Rubisco does not appear to limit photosynthesis at elevated temperature because its in vitro capacity is well in excess of the net photosynthetic rate (Pittermann and Sage 2001). Recent studies on maize (Zea mays L.) have shown that inactivation of Rubisco was the primary constraint on the rate of photosynthesis as leaf temperature increased above 30°C (Crafts-Brandner and Salvucci 2002). Also in maize, the activation state of Rubisco decreased at temperatures exceeding 32.5°C and was completely inactivated at 45°C, whereas the activation state of PEPC decreased marginally at temperatures above 40°C (Crafts-Brandner and Salvucci 2002). However, these studies were conducted after a short-term heat stress, and such responses may vary when plants are continuously grown at high temperature throughout the growth period.

Plants’ responses to elevated CO₂ and temperature may also depend on leaf age, with differential responses with increased leaf age as the leaf expands to full size and matures. Comprehensive studies investigating time-series data on leaf photosynthesis, related enzyme activities and dry matter accumulation have not been fully investigated for C₄ crop species. In addition, season-long effects of high growth temperature and interactive effects of elevated CO₂ and growth temperature on physiology and growth of grain sorghum (Sorghum bicolor L. Moench) have received less attention and are not well understood. It is essential to characterise and document these effects to better understand responses of C₄ plants to elevated CO₂ and high temperature.

The objective of this research was to characterise effects of elevated CO₂ and high temperature on leaf gas exchange rates, Rubisco and PEPC activities and biomass production of grain sorghum at different stages of leaf ontogeny and crop development. We hypothesise that enhancements in leaf photosynthetic rates and total biomass production in C₄ grain sorghum are caused by developmental and/or enzymatic changes occurring during the early stages of leaf ontogeny, which include changes in activities of Rubisco and PEPC.

Materials and methods

Growth conditions and plant material

This research was conducted in sunlit controlled-environment chamber facilities at the University of Florida and USDA-ARS in Gainesville, FL, USA. Each growth chamber consists of an aerial chamber (2 m long, 1 m wide and 1.5 m high) with aluminum framework with clear, transparent polyethylene telephthalate (Six light) tops and sides on a soil lysimeter of the same dimensions but 0.60 m deep. The soil lysimeters contained field soil (Kendrick fine sand) from an adjacent site. Detailed descriptions of chambers and quality control of various measurements are published elsewhere (Allen et al. 2003; Prasad et al. 2003).

Air temperatures in each growth chamber were controlled on a sinusoidal wave mode during the day-time and decay function during the night-time, and dew point temperatures were set 10°C below the target air temperatures derived from Parton and Logan (1981). These environments provided nearly constant relative humidity (55–58%) at 1500 h in all treatments. Air temperatures were continuously measured in each chamber above the crop canopy by using shielded, aspirated thermocouples. Carbon dioxide concentrations in each chamber were measured and controlled at predetermined set points. Carbon dioxide was controlled by continuously measuring CO₂ with an infrared gas analyser and by injecting the required amount of CO₂ with mass flow controllers connected to pure CO₂ cylinders. The set-point and actual measured air temperatures, dew point temperatures and CO₂ concentrations were controlled and recorded by using CR10 data loggers (Campbell Scientific, Logan, UT, USA). Actual measured diurnal mean temperatures were within ±0.3°C of the target temperatures. Similarly, CO₂ concentrations were within ±5 μmol CO₂ mol⁻¹ of target levels in all chambers. There was no difference between vapour pressure deficit (VPD) between ambient and elevated CO₂ treatments. At 1500 hours when day-time maximum dry bulb temperatures were 30 and 36°C, and maximum dew point temperatures were 20 and 26°C occurred, the VPDs were 1.9 and 2.6 kPa, respectively. Similarly, at 0700 hours when day-time minimum dry bulb temperature were 20 and 26°C, and minimum dew point temperature of 10 and 20°C occurred, the VPDs were 1.1 and 1.6 kPa, respectively.

Seeds of grain sorghum (Sorghum bicolor L. Moench cultivar DK 28E) were sown in two 2-m long rows running east–west on 4 May 2004 at a depth of 1.5–2 cm in the soil lysimeters with a plant-to-plant spacing of 9 cm, and 24 cm between the twin rows in four growth chambers. Plants in all four chambers were grown at a day-time maximum/night-time minimum air temperature regime of 30/20°C from sowing to appearance of the second leaf to remove effects of temperature on seedling emergence and establishment. Thereafter, plants in each chamber were exposed to an air temperature regime of 30/20°C or 36/26°C at either ambient (350 μmol mol⁻¹) or elevated (700 μmol mol⁻¹) CO₂ until final harvest at 63 days after sowing (DAS). Plants were irrigated with overhead sprinklers from sowing to 15 DAS and were dependent thereafter on subsurface irrigations provided by a constant watertable at 0.45 m below the soil surface controlled by an external bucket and float valve connected hydraulically to the bottom of each soil lysimeter.

Leaf tagging and sampling

After emergence, six plants were randomly chosen in each chamber, and leaf number 5 (numbered basipetally) was marked and followed throughout the experiment to obtain time-series measurements of leaf-element gas-exchange rates. In addition, leaf number 5 was tagged on all plants in the chambers for destructive samplings for enzyme studies and leaf area determinations.

At different days after leaf tip appearance (DALE), 3–4 tagged leaves were collected from different plants during midday on a clear, sunny day when PPFD was greater than 1500 μmol m⁻² s⁻¹. Leaf tips and bottom portions were removed to sample specific leaf-age material, and middle portions of leaves were immediately immersed in liquid nitrogen, ground to a fine power in liquid
nitrogen and then stored in liquid nitrogen until enzyme assays. Most of the midrib vein was removed while grinding the samples. Leaf samples were also collected at the same time for determination of area, fresh weight and dry weight. All data on enzyme assays are expressed on a unit leaf area basis.

Gas-exchange measurement of leaves

Leaf-element gas-exchange measurements (CO₂ exchange, stomatal conductance and transpiration rate) of individual attached leaf number 5 were measured at different DALE on three different tagged plants by using the LI-6400 Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA). The portion of the leaf element used for gas-exchange measurements was at a similar location as that used for enzyme assays. All measurements were made by using the standard LI-6400 leaf cuvette (6 cm²) and air temperature, carbon dioxide and relative humidity were set similar to growth conditions of respective growth chamber treatments. Growth chamber conditions were simulated in the leaf cuvette of the photosynthesis system through an integrated peltier temperature controller and CO₂ injection system. The internal light source (6400–02B red/blue) in the Li-Cor 6400 was set at a PPFD of 1800 μmol m⁻² s⁻¹ for constant, uniform light across all measurements. All measurements were made around midday (between 1100 and 1400 eastern daylight savings time) when chambers were at day-time maximum temperatures to avoid any diurnal changes. After each leaf-elemental area (6 cm²) was placed in the cuvette, a minimum of 3 min was allowed to obtain equilibrium, and measurements were made after a steady carbon exchange rate (CER) was obtained. Photosynthetic water use efficiency was estimated as the ratio of leaf CER to transpiration.

Extraction and assay of Rubisco and PEPC

About 90 mg of liquid nitrogen-frozen leaf powder was transferred to a pre-chilled (2°C) Pyrex Ten-Broeck tissue grinder (Fisher Scientific, Pittsburgh, PA, USA) and ground on ice for up to 2 min in 1.8 mL of cold extraction buffer containing 100 mM bicine (pH 8.0 at 25°C), 10 mM MgCl₂, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), 10 mM isooascorbate, 2% PVP-40 (w/v) and 0.1% TX-100 (w/v). The homogenate was micro-centrifuged at 12 000 g at 25°C for up to 2 min in 1.8 mL of cold extraction buffer containing 100 mM bicine (pH 8.0), 10 mM MgCl₂, 0.1 mM EDTA, 5 mM NaHCO₃, 5 mM DTT, 2.5 mM ATP, 5 mM phosphocreatine, 5 units creatine phosphokinase, 5 units each of the linking enzymes, 0.2 mM NADH and 0.6 mM RuBP. After a steady baseline absorbance was established, the reaction was initiated with 0.01 mL of activated enzyme. The linear decrease in absorbance resulting from oxidation of NADH was recorded over a period of 150 s.

Activity of PEPC was assayed by using a modification of the basic procedure by Ashton et al. (1990). The reaction mixture contained 100 mM bicine (pH 8.0), 10 mM MgCl₂, 0.1 mM EDTA, 10 mM NaHCO₃, 5 mM DTT, 2.5 units malate dehydrogenase and 0.2 mM NADH. After addition of 0.01 mL of enzyme extract, a steady baseline was established, and the reaction was initiated by addition of phosphoenolpyruvate to a final concentration of 5 mM. The linear decrease in absorbance was recorded over a period of 150 s.

Growth and dry matter measurements

Destructive harvests of three randomly selected plants were done at 20, 28, 34, 37, 42 and 50 DAS from each treatment to obtain data on growth and dry matter production. At each harvest, plant height, number of expanded leaves (nodes with ligules), leaf area and plant component (leaf, stem and roots) dry weights were measured after oven drying at 65°C for 7 days. Total plant dry weights were the sum of the dry weights of leaves and stems.

Data analyses

Data on measured and computed variables were analysed by using ANOVA procedures in SAS (SAS Institute Inc., Cary, NC, USA). The combinations of two CO₂ concentrations (ambient 350 μmol mol⁻¹ and elevated 700 μmol mol⁻¹) and two temperature treatments (30/20°C and 36/26°C) were regarded as treatments. There were three replications for all gas exchange rates, growth and dry matter production. There were two replications for enzyme assays. Growth temperature, growth CO₂ and leaf age (harvest time) were used as class variables. Mean and s.e. of means are shown in figures for comparisons.

Results

Main and interaction effects of different treatments on all measured and estimated variables are shown in Table 1.

Leaf gas exchange

The CER of the midportion elements of leaf 5 was highest at 11 DALE across all temperature and CO₂ treatments and gradually decreased thereafter with leaf age (Fig. 1). The response of leaf 5 to elevated CO₂ was positive with greater CER compared with ambient CO₂ until ~25 DALE; thereafter, response decreased with no effect at 35 DALE and negative effect at 45 DALE for both growth temperatures (Fig. 1a, b). At 6, 11, 18 and 25 DALE, leaf CER was increased 24, 19, 10 and 13%, respectively, by elevated CO₂ at 36/26°C. Corresponding increases at 30/20°C were 24, 19, 23 and 23%. The individual leaf area of leaf 5 in different treatments are shown in Fig. 1e, f. During first two measurements the leaf area of leaf 5 was ~30% of its total area.

There were significant effects of leaf age and CO₂ on stomatal conductance, but there were no effects of temperature or interactions between treatments (Table 1). Stomatal conductance decreased beyond 25 DALE either at ambient or elevated CO₂ at both temperatures (Fig. 1c, d). Elevated CO₂
Table 1. Probability (P) values and significance of main and interaction effects of different treatments on photosynthetic and growth traits of grain sorghum

<table>
<thead>
<tr>
<th>Trait</th>
<th>Leaf/crop age (A)</th>
<th>Carbon dioxide (CO2)</th>
<th>Temperature (T)</th>
<th>CO2 × T</th>
<th>CO2 × A</th>
<th>T × A</th>
<th>T × A × CO2</th>
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<tr>
<td>CER</td>
<td>0.001***</td>
<td>0.001***</td>
<td>0.137</td>
<td>0.013</td>
<td>0.014**</td>
<td>0.856</td>
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<tr>
<td>Stomatal conductance</td>
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<td>0.001***</td>
<td>0.077</td>
<td>0.243</td>
<td>0.806</td>
<td>0.407</td>
<td>0.899</td>
</tr>
<tr>
<td>Transpiration</td>
<td>0.001***</td>
<td>0.011**</td>
<td>0.001**</td>
<td>0.002**</td>
<td>0.093</td>
<td>0.001***</td>
<td>0.070</td>
</tr>
<tr>
<td>WUE</td>
<td>0.001***</td>
<td>0.001***</td>
<td>0.001**</td>
<td>0.001***</td>
<td>0.019*</td>
<td>0.211</td>
<td></td>
</tr>
<tr>
<td>PEPC</td>
<td>0.001***</td>
<td>0.075</td>
<td>0.001**</td>
<td>0.068</td>
<td>0.058</td>
<td>0.001***</td>
<td>0.016**</td>
</tr>
<tr>
<td>Rubisco/PEPC</td>
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<td>0.001***</td>
<td>0.001**</td>
<td>0.002**</td>
<td>0.103</td>
<td>0.035*</td>
<td>0.230</td>
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<td>Leaf area</td>
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<td>0.001***</td>
<td>0.001**</td>
<td>0.006**</td>
<td>0.078</td>
<td>0.045*</td>
<td>0.215</td>
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<tr>
<td>Leaf dry weight</td>
<td>0.001***</td>
<td>0.001***</td>
<td>0.001**</td>
<td>0.034*</td>
<td>0.115</td>
<td>0.771</td>
<td>0.181</td>
</tr>
<tr>
<td>Specific leaf area</td>
<td>0.001***</td>
<td>0.034</td>
<td>0.457</td>
<td>0.349</td>
<td>0.926</td>
<td>0.070</td>
<td>0.497</td>
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<tr>
<td>Stem dry weight</td>
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<td>0.002**</td>
<td>0.030*</td>
<td>0.034*</td>
<td>0.115</td>
<td>0.771</td>
<td>0.181</td>
</tr>
<tr>
<td>Total dry weight</td>
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<td>0.001***</td>
<td>0.001**</td>
<td>0.006**</td>
<td>0.008*</td>
<td>0.182</td>
<td>0.046*</td>
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Fig. 1. Influence of ambient ( ) or elevated ( ) CO2 at day-time maximum/night-time minimum growth temperatures of (a, c) 30/20°C, and (b, d) 36/26°C on (a, b) photosynthetic CO2-exchange rate, and (c, d) stomatal conductance of the midportion elements of leaf 5, and leaf area of leaf 5 (e, f) at different stages of leaf ontogeny (days after leaf tip emergence) of grain sorghum. Vertical bars denote ±s.e. of means of three replications.

Fig. 2. Influence of ambient ( ) or elevated ( ) CO2 at day-time maximum/night-time minimum growth temperatures of (a, c) 30/20°C, and (b, d) 36/26°C on (a, b) transpiration, and (c, d) and photosynthetic water use efficiency (WUE) of the midportion elements of leaf 5 at different stages of leaf ontogeny (days after leaf tip emergence) of grain sorghum. Vertical bars denote ±s.e. of means of three replications.

decreased stomatal conductance by ~17% across all temperatures and leaf ages.

Leaf transpiration rates of the midportion elements of leaves were significantly affected by leaf age, CO2, temperature and interactions between temperature and CO2 and between temperature and leaf age (Table 1). Elevated CO2 decreased transpiration rates at 30/20°C by 36 and 38% at 6 and 11 DALE, respectively (Fig. 2a). There was no effect of elevated CO2 on transpiration rates after 25 DALE at 30/20°C, and no effect of elevated CO2 on transpiration at any stage of leaf growth at 36/26°C (Fig. 2b).

Photosynthetic water use efficiency (WUE), as measured by the ratio of leaf CER to transpiration, was significantly increased by elevated CO2 at 30/20°C but not at 36/26°C (Fig. 2c, d). At 30/20°C, increases in WUE due to elevated CO2 were 81, 101, 53, 50 and 19% at 6, 11, 18, 25 and 35 DALE, respectively.

Leaf position and crop age

When measured at either 35 or 50 DAS plant age, CER decreased gradually with decreasing leaf (node) position (or increasing leaf age) across all treatments (Fig. 3). The CER of top leaves (younger) were more responsive to CO2 than those of lower (older) leaves (Fig. 3). In addition, the positive responses of CER to elevated CO2 were greater early in the crop life cycle (35 DAS, Fig. 3a, b) compared with the later period (50 DAS, Fig. 3c, d).
Furthermore, positive effects of elevated CO\textsubscript{2} on CER of top (younger) leaves were greater at 36/26°C (37% on leaf 10 at 35 DAS and 23% on leaf 13 at 50 DAS) than at 30/20°C (19% on leaf 10 at 35 DAS and 13% on leaf 13 at 50 DAS).

**Rubisco and PEPC activities**

Elevated CO\textsubscript{2} significantly increased Rubisco activity of the midportion elements of leaves in early stages of leaf ontogeny at both growth temperatures. At 30/20°C, elevated CO\textsubscript{2} increased Rubisco activity by 22 and 23% at 6 and 12 DALE, respectively (Fig. 4a). At higher growth temperatures of 36/26°C, elevated CO\textsubscript{2} increased Rubisco activity by 17, 74, 21 and 37% at 6, 12, 20 and 23 DALE, respectively (Fig. 4b). There was no effect of elevated CO\textsubscript{2} on Rubisco at 28 DALE at 36/26°C.

Elevated CO\textsubscript{2} did not affect PEPC activity across all stages of leaf development at 30/20°C (Fig. 4c). At 36/26°C, elevated CO\textsubscript{2} increased PEPC activity at only 6 DALE by 13% (Fig. 4d). Compared with the growth temperature of 30/20°C, the elevated temperature of 36/26°C increased PEPC activity at 6 DALE by 42% when averaged across both CO\textsubscript{2} treatments.

The ratio of Rubisco/PEPC was significantly influenced by leaf age, CO\textsubscript{2} and temperature (Table 1). Rubisco/PEPC activity increased with increasing age, and overall elevated CO\textsubscript{2} increased Rubisco/PEPC activity, the response was generally greater at elevated temperature. Elevated temperature decreased Rubisco/PEPC ratio.

**Growth and dry matter production**

There were significant effects of harvest time (DAS), temperature, CO\textsubscript{2} and interaction between temperature and CO\textsubscript{2} on leaf area and dry weights of leaves, stems and total plant (Table 1). There was a gradual increase in leaf area and dry weights of leaf, stem and total plant across all temperature and CO\textsubscript{2} treatments during the season (Figs 5, 6). There was a greater positive response to elevated CO\textsubscript{2} at higher temperature (36/26°C) than at cooler temperature (30/20°C). At 30/20°C, there was no response to elevated CO\textsubscript{2} until 50 DAS, when elevated CO\textsubscript{2} increased leaf area and dry weights of leaf, stem and total plant by 32, 37, 32 and

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**Fig. 3.** Influence of ambient (○) or elevated (□) CO\textsubscript{2} at day-time maximum/night-time minimum growth temperatures of (a, c) 30/20°C, and (b, d) 36/26°C on leaf photosynthetic rates of the midportion leaf elements of different leaves with respect to leaf position at (a, b) 35, and (c, d) and 50 days after sowing (DAS) of grain sorghum. Vertical bars denote ±s.e. of means of three replications.

**Fig. 4.** Influence of ambient (○) or elevated (□) CO\textsubscript{2} at day-time maximum/night-time minimum growth temperatures of (a, c) 30/20°C, and (b, d) 36/26°C on activities of (a, b) Rubisco, and (c, d) PEPC, and (e, f) Rubisco/PEPC of the midportion elements of leaf 5 at different stages of leaf ontogeny (days after leaf tip emergence) of grain sorghum. Vertical bars denote ±s.e. of means of two replications.

**Fig. 5.** Influence of ambient (○) or elevated (□) CO\textsubscript{2} at day-time maximum/night-time minimum growth temperatures of (a, c) 30/20°C, and (b, d) 36/26°C on activities of (a, b) Rubisco, and (c, d) PEPC, and (e, f) Rubisco/PEPC of the midportion elements of leaf 5 at different stages of leaf ontogeny (days after leaf tip emergence) of grain sorghum. Vertical bars denote ±s.e. of means of three replications.
49\% \text{ respectively, compared with ambient CO}_2. In contrast, at 36/26°C, elevated CO\textsubscript{2} progressively enhanced leaf area and dry weights of leaf, stem and total plant until 42 DAS. The response at 50 DAS was similar to that at 42 DAS. The percentage increases in leaf area and dry weights of leaf, stem and total plant due to elevated CO\textsubscript{2} at 36/26°C were 55, 50, 49 and 62\%, respectively, at 50 DAS. Specific leaf area (leaf area/leaf dry weight) decreased with increasing leaf age at all treatments (Fig. 5e, f). There was no influence of temperature, CO\textsubscript{2} or their interaction on specific leaf area.

The significant effect of the interaction between temperature and harvest time on leaf area and leaf dry weight was due to relatively larger increases at high temperature at a later harvest date (stage of crop development). The interaction between harvest, CO\textsubscript{2} and temperature was significant for total plant dry weight because of a greater response to elevated CO\textsubscript{2} at high temperature at 42 DAS compared with all other treatments.

Discussion

There were strong interactions of leaf age and growth environment (CO\textsubscript{2} and temperature) on CER and photosynthetic carboxylating enzyme activities of the midportion of leaf elements and on dry matter production of grain sorghum. Leaf CER of sorghum showed an optimum at 11 DALE and gradually declined thereafter with increasing leaf age. In addition, CER of top (younger) leaves of sorghum was more responsive to elevated CO\textsubscript{2} than lower (older) leaves. An increase in photosynthesis during leaf expansion followed by a gradual decrease in mature leaves is the usual ontogenetic course (Catsky and Sestak 1997). Our study also demonstrates that the enhancement in leaf CER and Rubisco activity in response to elevated growth CO\textsubscript{2} were greater in early stages of sorghum leaf ontogeny.

Expression of the C\textsubscript{4} photosynthetic characteristics has been shown to be controlled by leaf age. In C\textsubscript{4} species, expression of C\textsubscript{4} photosynthetic genes does not occur until Kranz anatomy has been established, and use of the C\textsubscript{3} photosynthetic pathway may exclusively occur before full differentiation of Kranz anatomy (Nelson and Langdale 1992). It has been suggested that, in some C\textsubscript{4} species, the ‘immature’ C\textsubscript{4} pathway in young leaves has C\textsubscript{3}-like photosynthetic characteristics, and this may cause these species to be more responsive to elevated CO\textsubscript{2} (Poorer et al. 1996; Ghannoum et al. 1997). However, Ghannoum et al. (1998) argued against this hypothesis by showing that gas exchange parameters of young leaves of Panicum antidotale (C\textsubscript{4}, NADP-ME) and Panicum coloratum (C\textsubscript{4}, NAD-ME) do not have C\textsubscript{3}-like characteristics. It was also suggested that young C\textsubscript{4} Panicum leaves have lower bundle sheath conductance relative activity of the C\textsubscript{4} and C\textsubscript{3} cycles and/or lower ratios of activities of the C\textsubscript{4} to C\textsubscript{3} enzymes, which may be responsible for the positive response to elevated CO\textsubscript{2} (Ghannoum et al. 1998).

It is difficult to capture gas exchange of immature C\textsubscript{4} leaves, as the photosynthetic rates are high as soon as leaves emerge from the whorl (Ghannoum et al. 1998). Our data shows that maximum photosynthetic rates were observed at 5–10 DALE for all treatments (Fig. 1). This suggests that by 10 DALE, the leaves have established their photosynthetic pathway. At 10 DALE, the leaf was still expanding and had only ~30\% of its final leaf size (Fig. 1e, f). Nevertheless, in sorghum, young leaves exhibit more than twice the rate of photorespiration of old leaves, and young leaves of plants at elevated growth CO\textsubscript{2} have a higher rate of photosynthesis than counterpart plants at the ambient CO\textsubscript{2} level (Cousins et al. 2001). Although activities of Rubisco and PEPC increase rapidly as the leaf differentiates and emerges from the surrounding whorl, Rubisco accumulates well before significant amounts of PEPC are detectable in early developmental stages of sorghum leaves, making the youngest leaf tissues more C\textsubscript{3}-like and more responsive to elevated CO\textsubscript{2} (Cousins et al. 2003). However, in our study the ratio of Rubisco/PEPC increased as the leaf age progressed across all treatments (Fig. 4e, f). This clearly suggests that the sorghum leaves had higher photosynthetic capacity (more Rubisco), which is one of the reasons for greater responsiveness to elevated CO\textsubscript{2} and increased biomass production. Overall, elevated CO\textsubscript{2} increased Rubisco/PEPC ratio across both temperatures. The responses were particularly obvious at higher temperature (36/26°C).

Despite increases in sorghum leaf CER at early stages of leaf development and younger leaves at 30/20°C (Fig. 1a), there was no significant influence of elevated CO\textsubscript{2} on dry matter production of sorghum, particularly at early stages of development until 50 DAS (Figs 5, 6). Some studies have shown positive photosynthetic responses but no growth benefits (Ziska et al. 1999); others have shown positive growth responses with no increases in photosynthesis (Ghannoum et al. 2000). In our study, elevated CO\textsubscript{2} increased dry matter production of sorghum at all stages of development at 36/26°C but only at the later stage of development at 30/20°C (Figs 5, 6). Water conservation and improved shoot water relations, due to partial closure of stomata, are among the mechanisms commonly attributed to positive growth responses at elevated CO\textsubscript{2} in C\textsubscript{4} plant species (Drake et al. 1997). Despite greater increased photosynthetic WUE under elevated CO\textsubscript{2}, particularly at early stages of development (Fig. 2c), there was no positive response in biomass accumulation at the 30/20°C growth temperature (Figs 5, 6). In contrast, at growth temperatures of 36/26°C, there were positive growth responses even with no significant
increases in WUE (Fig. 2d). This is likely because there was no decrease in transpiration despite significant reduction in stomatal conductance at high temperatures (Figs 1, 2). Previous studies have shown that high growth temperatures and increases in tissue temperatures due to partial closure of stomata increase transpiration rates because of higher vapor pressure deficits (Allen et al. 2003). Our experiments were conducted under fully irrigated conditions and with no nutrient stress; thus, there were no confounding effects of these two environmental conditions. However, the higher growth and leaf area development at elevated CO$_2$ and higher temperatures will more likely increase the total water use by the plants. These responses will have significant negative implications under predicted climate change scenarios, particularly if the future increases in CO$_2$ and higher temperatures are associated with drought or limited soil moisture conditions. Elevated CO$_2$ can also influence vegetative growth via other mechanisms, including raising intercellular CO$_2$ and consequently enhancing CER (Ghannoum et al. 2000), increasing tillering and leaf area thus increasing light capture and CER (Bowes 1993) and increasing growth rates in young, but not old, leaves (Geiger et al. 1999). Nevertheless, any firm conclusion on the mechanisms responsible for greater photosynthesis and growth responses at elevated CO$_2$ and high temperatures needs further investigation.

In our study, there was a positive interaction between elevated CO$_2$ and temperature with respect to CER of the midportion of leaf elements (Fig. 3) and vegetative dry matter production (Table 1; Figs 5, 6). In other words, benefits of elevated CO$_2$ in terms of vegetative growth were greater at high temperatures (36/26°C) than at cooler temperatures (30/20°C). Sufficient caution should be taken to extrapolate increased vegetative growth to reproductive growth (economic yield) because most reproductive processes are more sensitive to higher temperatures and elevated CO$_2$ (Prasad et al. 2002, 2006). Moreover, economic yield losses were greater at elevated CO$_2$ than at ambient CO$_2$ (Prasad et al. 2002). Activities of Rubisco and PEPC were also greater under elevated CO$_2$ at high temperature than at cooler temperature, particularly at early stages of development when the greatest CO$_2$ enhancements of photosynthesis were noticed (Fig. 4). Studies on the C$_4$ sugarcane plant (Saccharum officinarum) showed that growth at double-ambient CO$_2$ and 5°C above ambient temperature increased leaf area by 56%, total aboveground dry weight by 74% and juice volume by 164% compared with respective enhancements of 31, 21 and 83%, respectively, in plants grown at both ambient CO$_2$ and temperature (Vu et al. 2002). These results are in agreement with suggestions that photosynthesis and growth responses of C$_4$ plants under a combination of elevated CO$_2$ and high temperature may be greater than responses under elevated CO$_2$ alone (Long 1999; Morison and Lawlor 1999).

In contrast to results of our controlled-environment study, field studies from free-air-carbon dioxide enrichment (FACE) experiments on maize (Leakey et al. 2006) showed that in the absence of water stress growth at elevated CO$_2$ did not stimulate photosynthesis, biomass or yield. Nor were there any CO$_2$ effects on activities of key photosynthetic enzymes (Rubisco and PEPC). The field responses are more realistic due to naturally occurring water stress or non-water stress conditions. However, the differences in responses could be related to growth temperatures or species differences. The study by Leakey et al. (2006) had maximum air temperatures of <30°C. VPD at 1500 hours ranged between 0.6 and 1.8 kPa and elevated CO$_2$ levels were 550 μmol m$^{-2}$ s$^{-1}$; in our study the day-time maximum growth temperatures were 30 and 36°C, the VPD at 1500 hours were between 1.9 and 2.6 kPa and elevated CO$_2$ levels were 700 μmol m$^{-2}$ s$^{-1}$. The wind speed under natural field conditions would be generally greater than those in the enclosed growth chambers which may cause differential boundary layer conditions at leaf interface.

Some studies have shown that elevated CO$_2$ increased the leaf temperature in green house growth C$_4$ plants (Siebke et al. 2002). Our previous study with same sorghum genotype under same growth chamber conditions showed that elevated CO$_2$ increased the seasonal canopy temperatures by 1.2 and 2.6°C at air temperature regimes of 32/22 and 36/26°C, respectively (Prasad et al. 2006). The enhanced photosynthesis under elevated CO$_2$ and high growth temperatures in our present study was not related to faster sink production (developing leaves or seed numbers or seed size). Investigating the interaction of elevated CO$_2$ at different growth temperatures on same sorghum genotype showed that there were no significant differences in seed numbers or seed size at ambient or elevated CO$_2$ at growth temperatures of 36/26°C (Prasad et al. 2006). However, the percent seed-set was decreased under elevated CO$_2$ (700 μmol m$^{-2}$ s$^{-1}$) compared with ambient CO$_2$ (350 μmol m$^{-2}$ s$^{-1}$) at either 32/22 or 36/26°C (Prasad et al. 2006). Photosynthetic rates were not significantly affected by season-long growth temperatures of 30/20 and 36/26°C (Table 1; Fig. 1). This suggests that there was thermal acclimation at these temperature ranges. This is in agreement with other studies on C$_4$ plant species that suggest photosynthetic rates undergo thermal acclimation when temperature are within the optimal range, and C$_4$ plants will not simply increase their photosynthetic rates as has been predicted, but will acclimate by adjusting capacity and reallocating nitrogen resources between photosynthetic components (Dwyer et al. 2007). The interaction effects of elevated CO$_2$ and high temperature in C$_4$ plants are still poorly understood, and study of additional C$_4$ species is necessary before generalising these effects.

In summary, our study showed that the enhancement in CER and upregulation of Rubisco activity of the midportion of leaf elements in the C$_4$ sorghum plant due to elevated CO$_2$ occurred to a greater extent at early stages of leaf ontogeny and at high growth temperatures. Such enhancements in CER and Rubisco activity may contribute to the greater vegetative growth and dry matter production observed at elevated CO$_2$ and high temperatures.

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