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Drought-induced changes in nitrogen partitioning between cyanide and nitrate in leaves and stems of *sorghum* grown at elevated CO_2 are age dependent

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ABSTRACT

Sorghum [*Sorghum bicolor* (L.) Moench] is the world's fifth most important crop, grown for forage, grain, and as a biofuel. Fast growing and drought tolerant, it is increasingly being planted as a climate change-ready alternative to maize. All parts of the *sorghum* plant except the grain contain the cyanogenic glucoside dhurrin, which breaks down to release hydrogen cyanide (prussic acid) when plant tissue is disrupted. Fresh forage, hay and silage may be toxic to stock when derived from plants that are young, droughted or heavily fertilized. *Sorghum* also stores nitrate, which can cause nitrite toxicity. The impact of elevated CO_2 on dhurrin and nitrate concentration is unknown. It is important to understand how global environmental change will affect composition in order to be able to predict the safety of the crop in coming decades. *Sorghum* was grown experimentally at elevated CO_2 in two free-air CO_2 enrichment (FACE) experiments at ambient and elevated CO_2 (ca. 550 ppm) and either irrigated regularly or only once after sowing in consecutive years and sampled at different stages of development. Since FACE-grown *sorghum* has been shown to have improved water status we hypothesized that they would contain less dhurrin. We found the most important factors governing cyanide concentration were (in decreasing order): plant age, irrigation treatment and tissue type. For nitrate, tissue type was by far the most important factor, followed by plant age, and then irrigation treatment. The concentration of CO_2 in the atmosphere had no significant effect on the total nitrogen concentration, or the concentrations of cyanide and nitrate. As *sorghum* is becomes more widely used for forage, it will be important to have simple methods to assess the cyanide levels in the field or to develop new, low cyanogenic varieties to ensure that it is safe for grazing.

1. Introduction

The concentration of CO_2 in the atmosphere recently reached 400 ppm, higher than at any time during the past 800,000 years and could reach 550 ppm by 2050 (A2 scenario, IPCC WG2) (IPCC, 2014). This will affect plants in two ways: directly through the process of photosynthesis and indirectly via changes in the climate. Predicted increases in the frequency and intensity of episodic drought in many regions of the world and higher global temperatures are likely to adversely affect food security (Funk et al., 2008), despite the increases in plant production as a result of the higher concentrations of CO_2 (Long and Ort, 2010). Increasingly attention is being paid to the impact of climate change on the nutritional value of crops, as well as total production (Cavagnaro et al., 2011; Tilman et al., 2011; Myers et al., 2014).

Sorghum bicolor (L.) Moench. is grown widely throughout the world as a grain or forage crop and increasingly for first and second-generation biofuel production (Xin et al., 2009; Zhao et al., 2009; Calvino and Messing, 2012). All parts of the *sorghum* plant except the grain contain the cyanogenic glucoside dhurrin, which breaks down to release hydrogen cyanide (HCN) when mixed with spe-

Abbreviations: FACE, free air CO_2 enrichment; FD, elevated CO_2/dry treatment; FW, elevated CO_2/wet treatment; CD, control CO_2/dry treatment; CW, control CO_2/wet treatment; DOY, day of year; DAS, days after sowing.

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cific endogenous β-glucosidases. Dhurrin can also be degraded by the microbes in the rumen, without the need for any plant-derived β-glucosidases. Cyanide concentrations of over 750 μg g^{-1} are considered toxic to cattle (Bennet et al., 1990). *Sorghum* also stores nitrogen as nitrate. Nitrate consumed in forage is converted to nitrite in the cattle rumen, which is toxic when tissue nitrate concentration is in excess of 6.6 mg g^{-1} (equivalent to Nitrate-*N* concentrations of 1500 μg g^{-1}) (Finnie et al., 2011).

Cyanogenic glucosides are effective in reducing herbivory by generalist herbivores, (Gleadow and Woodrow, 2002a), but they also play a role in nitrogen turnover and storage in many species and their concentration is, therefore, linked to the demand for nitrogen by other metabolic processes (Gleadow and Møller, 2014). Recently it has been proposed that they may also act to mitigate oxidative stress (Selmar and Kleinwächeter, 2013; Gleadow and Møller, 2014). The three most important factors driving high concentrations of dhurrin are ontogeny, tissue nitrogen concentration and drought stress (Wheeler et al., 1990; Miller et al., 2014). Less is known about the effect of the environment on nitrate production, although concentrations tend to be higher in water-limited plants and plants that have been supplied with nitrogenous fertilizers (Bennet et al., 1990). Very few studies have included measurements of both nitrate and dhurrin, and their results are somewhat contradictory. Neilson et al. (2015), for example, found higher concentrations of both nitrate and dhurrin in drought-stressed *sorghum*. O'Donnell et al. (2013), in a study of osmotically-stressed *sorghum* likewise found higher concentrations of dhurrin, but lower concentrations of nitrate.

Nothing is known about the effect of elevated CO_2 and drought on total nitrogen concentration in *sorghum*, or how nitrogen is partitioned between nitrate and dhurrin in the leaves and stems. We analysed samples collected during the landmark Maricopa FACE (Free Air CO_2 Enrichment). In that study, plants grown at elevated CO_2 and a reduced irrigation regime had a higher water use efficiency and decreased stomatal conductance and evapotranspiration, resulting in conserved soil moisture over the growing season and improved water status (Ottman et al., 2001; Rillig et al., 2001; Wall et al., 2001). Our hypothesis was that water-limited plants would have higher concentrations of dhurrin, but that this increase would be mitigated by FACE and also that there would be no significant direct CO_2 effects on tissue nitrogen, dhurrin or nitrate.

2. Materials and methods

2.1. Plant material and growing conditions

Sorghum [*S. bicolor* (L.) Moench cv. Dekalb 54] was grown in two experiments conducted at the University of Arizona Maricopa Agricultural Center (MAC), Maricopa, AZ, USA in 1998 and 1999, as described in Williams et al. (2001) and Rillig et al. (2001). Briefly, a free-air CO_2 enrichment (FACE) technique was used to enrich the air in circular plots within a 12-ha *Sorghum* field. FACE plots were enriched to a target 200 μmol mol^{-1} above ambient. The FACE treatment was applied continuously from the date when 50% of the plants emerged until grain maturity. The average daytime CO_2 concentrations in the FACE and control plots were 556 and 363 μmol mol^{-1}, respectively, while the nighttime values were 603 and 428 μmol mol^{-1}. Air blowers were used to dilute the CO_2 and distribute it around the FACE rings. Similar blowers were installed for the control rings, so that air movement was the same in both the control and FACE rings. Plants were sown on 16 July 1998 (Day 197) and 15 June 1999 (Day 166). Half of each circular plot was flood irrigated seven times (Wet treatment) during the season while the other half was water-limited and only received two irrigations (Dry treatment), 11 and 57 days after sowing (DAS) in 1998 and 13 and 51 DAS in 1999. All plots were fertilized prior to sowing and again midseason with Dry and Wet plots. The same amount of fertilizer nitrogen was applied to both Dry and Wet plots for the season (Ottman et al., 2001). Fertilizer [93 kg N ha^{-1} and 41 kg P ha^{-1} as urea (46–0–0) and monoammonium phosphate (11–52–0)] was applied by air on 10 June 1998 and 1 June 1999. A second application of fertilizer was applied to the Dry plots on 11 September 1998 and 6 August 1999 in the form of Uran-32 [urea ammonium nitrate (32–0–0)] in the irrigation water at a rate of 186 kg N ha^{-1} and 172 kg N ha^{-1}, respectively, to give a total of 265 kg N ha^{-1}. Based on historical measurements of the N concentrations in the irrigation water, the Dry plots received about an additional 26 and 18 kg N ha^{-1} in 1998 and 1999, respectively, from the irrigation water itself, while the Wet plots received about 68 and 42 kg N ha^{-1}.

Growth, yield, leaf area and stomatal conductance data have been published previously (Conley et al., 2001; Ottman et al., 2001; Rillig et al., 2001; Williams et al., 2001) (see summary in Supplementary Table S1). The overall effect of FACE was to ameliorate the adverse effects of drought on plant water status, and therefore yield. Yield, shoot biomass, relative water content, photosynthetic rate, evapotranspiration and stomatal conductance (g_s) were all significantly lower in Dry compared with Wet plots, but plants grown in FACE had improved water status (Supplementary Table S1).

2.2. Harvesting and sampling

Individual plants were harvested weekly, weighed, dried, and ground and stored in a dry atmosphere at room temperature at The University of Arizona. In order to make meaningful ontogenetic comparisons across years (Miller et al., 2014), samples were taken from plants at the 10 leaf-stage (Day 237 in 1998 and Day 215 in 1999), and at the 19-leaf stage (Day 265 in 1998 and day 236 in 1999). FACE had relatively minor effects on leaf, stem and plant development (Rillig et al., 2001). Plants harvested for chemical analysis were at a similar leaf stage across all treatments at the first harvest (ca. 10–11 leaves), but at the second harvest plants were ca. 18 and 21 leaf stage in the Dry and Wet plots, respectively (Supplementary Table S2). Dried, ground samples were stored in sealed bags in metal containers at room temperature, following standard practice and known to preserve elemental nitrogen and nitrate over the long term (Reuter et al., 1997a).

2.3. Chemical analyses

Foliar dhurrin (cyanogenic glucoside) concentrations were determined on leaves (i.e., leaf blade) and stems (comprising both the stem and the enclosing leaf sheaths) using the evolved cyanide method following Blomstedt et al. (2012). Exogenous β-glucosidase (1.12 units mL^{-1}) from almond (EC 3.2.1.21, Sigma) was used to ensure that all dhurrin was converted to cyanide during incubation. Evolved HCN was assayed colorimetrically and expressed as cyanide (μg HCN g^{-1} dry weight). One gram of cyanide is equivalent to 11.9 g dhurrin. LC–MS analysis of the samples from the Maricopa experiment confirmed that cyanide was present as dhurrin and showed a very high correlation between evolved cyanide and the concentration of dhurrin measured directly by LCMS ($y = 1.066x - 84.7$, $R^2 = 0.85$) (Gleadow et al., 2012). Previous experiments showed that dhurrin is preserved in oven-dried tissue over two years (Fox et al., 2012; Gleadow et al., 2012). Retesting of samples reported in (Gleadow et al., 2012) after storing under similar conditions for 8 years (2007–2015) was not significantly different, with a cyanide concentration of 1.20 ± 0.40, compared with the original value of 1.41 ± 0.10 ($P = 0.21$).

Foliar nitrate was measured on dried tissue (Cataldo et al., 1975). Samples (50 mg) were extracted in MilliQ H_2O, incubated at 45 °C for 1 h, centrifuged and the supernatant assayed colorimetrically following (Cataldo et al., 1975) as modified by O'Donnell et al. (2013) and the absorbance measured using a Lachat QuikChem 8000 autoanalyzer. This was converted to nitrate per dry mass using the molar mass of nitrate to give milligram nitrate per gram plant tissue.

Elemental nitrogen concentration (Total N) was determined by combustion analysis in a Carlo Erba 1500C/N analyzer. Foliar N was within the expected range reported for well- fertilized *sorghum* at similar stages of development (ca. 2%) (Reuter et al., 1997b).

2.4. Statistical analysis

The data were analysed as a strip-split-plot design using the 'Mixed' Procedure (Littell et al., 1996) with CO_2 as the main plot and irrigation as the strip-split.

3. Results

FACE alone had no significant effect on total nitrogen, nitrate or cyanide concentrations in leaves or stem on a per mass basis (Fig. 1a,b; Table 1; Supplementary Table S3). Overall, tissue nitrogen was higher in leaves than in stems, with an average across all treatments of 2.9% and 1.9% in young leaves and stems, respectively. Total nitrogen was significantly higher in Dry compared to Wet plants ($P = 0.0003$), although there was considerable variation with year and plant age (Fig. 1; Supplementary Table S3). While there was no main effect of CO_2, the $CO_2 \times$ water interaction was significant in stems at two time points (day 98/265 and 99/215) (Table 1).

The concentration of dhurrin was measured as evolved cyanide per dry mass in leaves (Fig. 1c) and stems (Fig. 1d). There are three key noteworthy points to be made. First, there was a large and highly significant effect of plant age on cyanide concentration. This was most strongly seen in the stems where the concentration decreased almost an order of magnitude from 1202 to 193 μg CN g^{-1} in young and old plants, respectively, from the Dry treatment (Fig. 1d; Table 1; Supplementary Table S3). Second, cyanide concentrations were significantly higher in both leaves and stems of Dry than Wet plants ($P < 0.0001$; Table 1). In Wet plants the overall foliar cyanide concentration was 709 μg CN g^{-1}, compared with 1155 μg CN g^{-1} in Dry plants. Third, there was no main effect of CO_2 on tissue cyanide concentration, and no significant $CO_2 \times$ water interaction was detected (Table 1).

Significant age and treatment effects were also observed for tissue nitrate concentration (Fig. 1e,f; Table 1). Stem nitrate concentration was six-fold higher than foliar nitrate concentration, with overall averages of 19.6 and 3.2 mg NO_3 g^{-1}, respectively (Fig. 1). Nitrate concentrations were also significantly higher in Dry than Wet plants in both leaves ($P = 0.0083$) and stems ($P < 0.0001$), although there was quite a lot of variation between years and harvest dates (Supplementary Table S3). There was no main effect of FACE on tissue nitrate and there was no significant interaction between CO_2 and water.

The partitioning of nitrogen was assessed by dividing the molecular mass of nitrogen occurring as cyanide and nitrate by the total mass of N to give proportion of tissue nitrogen occurring as cyanide (HCN-N/N%) or nitrate (NO_3-N/N%) in leaves and stems (Fig. 2; Table 1; Supplementary Table S4). In leaves, the proportion of nitrogen allocated to nitrate and cyanide was similar (approx. 2%), whereas in stems the proportion of nitrogen allocated to nitrate was almost ten-fold higher (approx. 20%). Dry plants allocated relatively more nitrogen to cyanide, particularly in the stems where HCN-N/N% was four- to five-fold higher in Dry plants, but there was no obvious trend for nitrate-nitrogen (NO_3-N/N%). Importantly, no trade-off between the partitioning of nitrogen to cyanide versus nitrate was detected between treatments (Figs. 1 and 2).

4. Discussion

Sorghum is an important forage crop, particularly in hot, dry regions. In order to reduce the risk of cyanide poisoning, farmers are advised against feeding grazing stock with *sorghum* that is young, heavily fertilized or suffering from drought (Stuart, 2002). In addition, there is also the risk of nitrate poisoning when there is rain after a period of drought when it may be mistaken for cyanide intoxication (Stuart, 2012). Understanding the environmental drivers affecting the partitioning of nitrogen to cyanide and nitrate under present and future environmental challenges will help to reduce the risk of inadvertent poisoning. Here, we measured the concentrations of nitrogen, nitrate and dhurrin in leaves and stems of *sorghum* grown under drought stress and elevated CO_2 for the first time. By measuring plants at two different ages, we were able to compare the relative importance of ontogenetic and environmental factors. The most important factors governing cyanide concentration were, in decreasing order: age, irrigation treatment and tissue type. For nitrate the most important factor was tissue type, followed by plant age, and then irrigation treatment. The concentration of CO_2 in the atmosphere had no significant effect on tissue nitrogen, cyanide or nitrate concentration or on the proportion of nitrogen allocated to cyanide and nitrate.

4.1. Cyanide and nitrate toxicity thresholds for forage

The strong effect of irrigation on dhurrin is consistent with studies in other cyanogenic species (Gleadow and Woodrow, 2002b; Vandegeer et al., 2013). Given the relatively limited capacity for C4 photosynthesis to act as a significant alternative electron sink in water stressed plants (Ghannoum, 2009), dhurrin synthesis and turnover may provide an alternative mechanism for mitigating stress, as proposed by Gleadow and Møller (2014).

The foliar cyanide concentrations reported here for grain *sorghum* are marginally higher than for published results for forage *sorghum* varieties of similar age (Gleadow et al., 2012; Miller et al., 2014). Concentrations were well above the toxicity threshold of 750 ppm cyanide in leaves of young plants, and at the marginally toxic level (600–750 ppm) in older plants (Duncan, 1996). In stems, by contrast, the cyanide concentration was below the threshold in all plants except for young plants grown in the Dry plots (Fig. 1). This is consistent with other studies of water-limited (Wheeler et al., 1990; Neilson et al., 2015) or osmotically-stressed *sorghum* (O'Donnell et al., 2013). Burke et al. (2013, 2015), by contrast, did not observe any increase in dhurrin concentration with drought. We are not sure why their results are so different, but their use of a different analytical method and the very low concentrations, e.g., <10 μg g^{-1} dwt for BTx632 (Burke et al., 2015), may be contributing factors.

Stem nitrate concentrations of all plants at the 10-leaf stage were well above the toxicity threshold of 750 μg g^{-1} (Finnie et al., 2011). In older plants, the concentrations of nitrate in the stem were lower, only exceeding the toxicity threshold in Dry plants (Fig. 1; Finnie et al., 2011). Leaf nitrate concentrations, by contrast, were consistently below the toxicity threshold in all plants, even in plants that received less water. Previous published reports on the effect of water stress on nitrate levels are inconsistent (Bennet et al., 1990; O'Donnell et al., 2013; Neilson et al., 2015).

Approximately the same proportion of nitrogen was allocated to both nitrate and cyanide in the leaves (approx. 2%), whereas in the

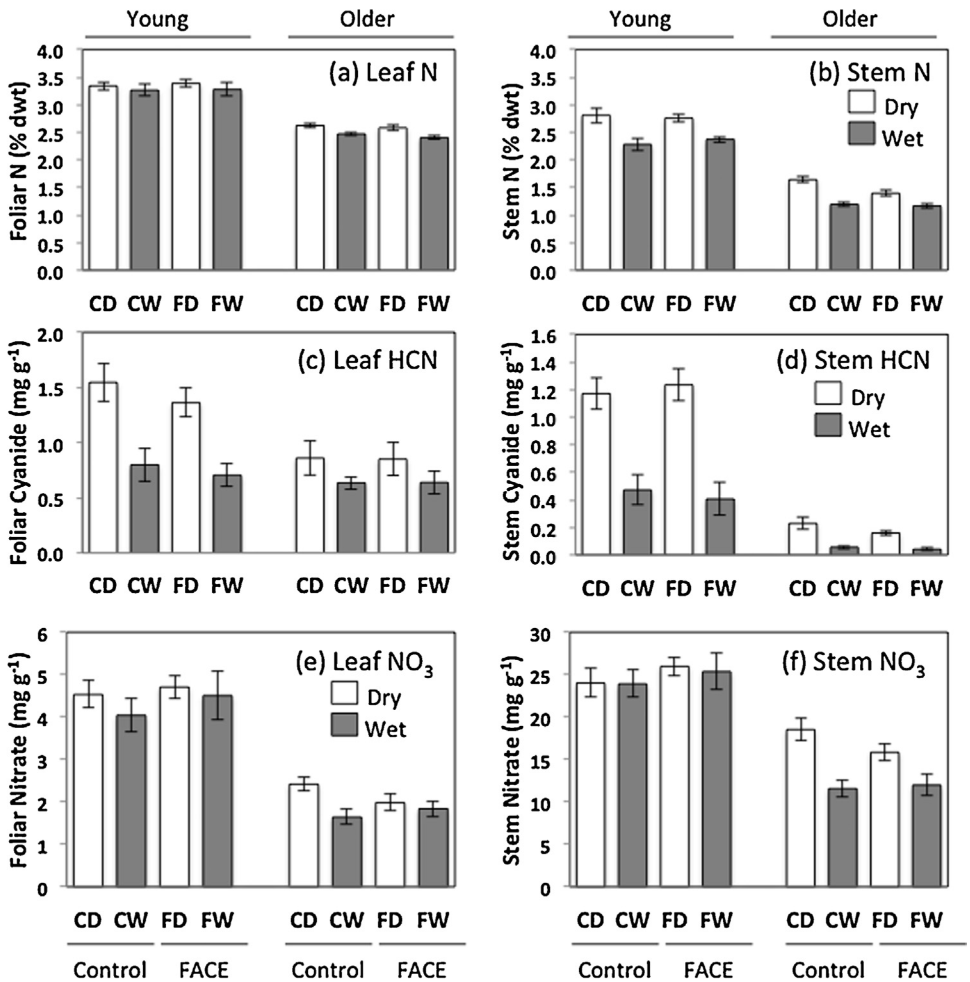

Fig. 1. Chemical analysis from FACE experiment of leaves (a, c, e) and stems (b, d, f) of *Sorghum bicolor* grown at ambient (control) and elevated CO_2 (FACE) and two different irrigation regimes (Wet and Dry): Total nitrogen (a, b); Cyanide; (c, d); Nitrate (e, f). Values are the mean ± 1SE ($n = 4$). Plants were harvested at the 10- and 19-leaf stages in two successive years. All values are on a dry mass basis. Statistical analysis is presented in Table 1. CW: Control Wet; CD: Control Dry; FW: FACE Wet; FD: FACE Dry.

Table 1
Statistical analysis (*P* values) of chemical analysis of *sorghum* leaves and stems presented in Fig. 1. Plants grown under ambient (control) and elevated CO_2 (FACE) and two different irrigation regimes (Wet and Dry). Statistically significant *P* values at the 5% probability level are indicated with bold text. Overall average (Avg) *P* values for each treatment are listed in the bottom section of the table.

Year	DOY	Effect	N Leaf	N Stem	NO_3 Leaf	NO_3 Stem	HCNp Leaf	HCNp Stem
1998	237	CO_2	0.9807	0.6695	0.4337	0.1922	0.6390	0.2608
		Water	0.5978	0.0783	**0.0024**	0.3988	0.0626	**0.0005**
		CO_2xWater	0.6682	0.1218	**0.0487**	0.0632	0.9189	0.1090
	265	CO_2	0.3584	**0.0120**	0.5230	0.1695	0.2410	0.0642
		Water	**0.0017**	**<0.0001**	0.0194	**0.0004**	0.6829	**0.0280**
		CO_2xWater	0.7439	**0.0003**	0.0838	0.1961	0.5153	0.2574
1999	215	CO_2	0.5223	0.8130	0.2020	**0.0391**	0.1798	0.3026
		Water	**0.0005**	**<0.0001**	0.8626	0.6109	**<0.0001**	**0.0002**
		CO_2xWater	0.4937	**0.0014**	0.2424	0.1935	0.5977	0.5974
	236	CO_2	0.3389	0.4234	0.2246	0.9836	0.2973	0.2728
		Water	0.0857	0.1054	**0.0228**	0.0768	**0.0415**	**0.0298**
		CO_2xWater	0.8489	0.2987	**0.0151**	0.2258	0.7958	0.3717
Avg	Avg	CO_2	0.6327	0.2666	0.3435	0.4700	0.4688	0.6393
		Water	**0.0003**	**0.0055**	**0.0083**	**<0.0001**	**0.0216**	**<0.0001**
		CO_2xWater	0.7423	0.0762	0.1436	0.4390	0.5902	0.6991

stem there was relatively more nitrate. Approximately 20% of stem nitrogen was allocated to nitrate but less than 2% was allocated to cyanide. These values for field-grown plants are of the same order as found in *sorghum* grown hydroponically at different levels of osmotic stress (O'Donnell et al., 2013).

4.2. Allocation of nitrogen to cyanide and nitrate unchanged in FACE-grown plants

FACE had no overall effect on tissue N, consistent with extensive studies on maize (Leakey et al., 2006, 2009). Contrary to our

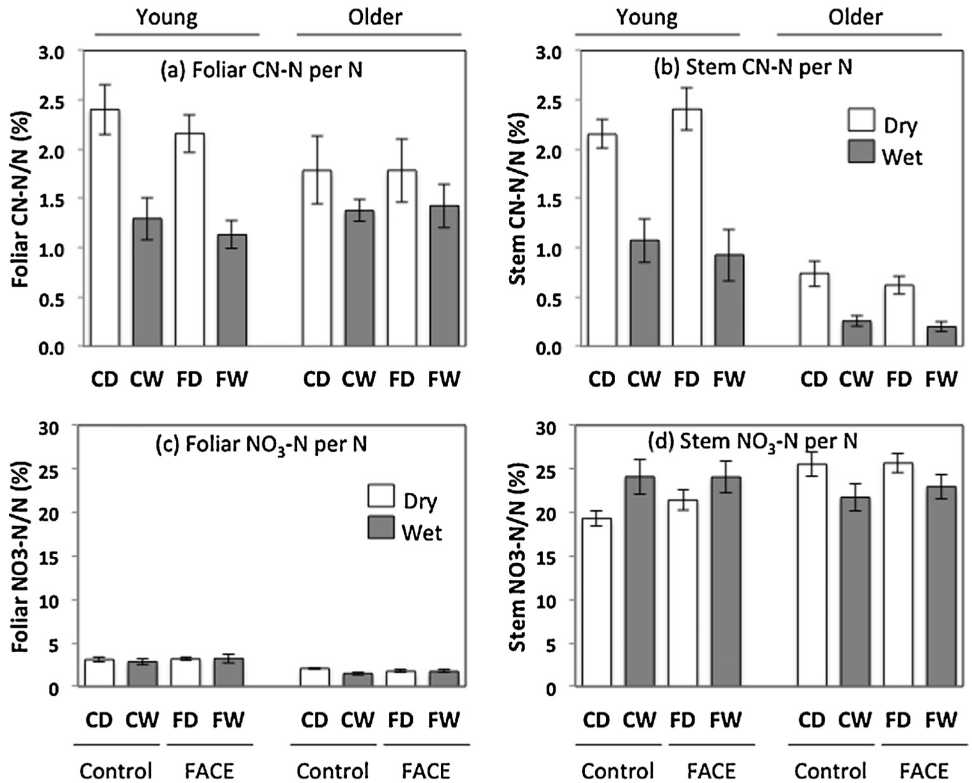

Fig. 2. Proportion of total nitrogen found as cyanide (a, b) or nitrate (c, d) or in leaves and stems of *Sorghum bicolor* grown under ambient (control) and elevated CO_2 (FACE) and two different irrigation regimes (Wet and Dry). Values are the mean ± 1SE ($n = 4$). Plants were harvested at the 10- and 19-leaf stages in two successive years. All values are on a dry mass basis. Statistical analysis is presented in Table 1. CW: Control Wet; CD: Control Dry; FW: FACE Wet; FD: FACE Dry.

hypothesis, FACE did not affect the proportion of tissue N allocated to cyanide or nitrate either (Fig. 2). In C_3 plants the higher allocation of nitrogen to cyanide (HCN-N/N) in plants grown at elevated CO_2 (Gleadow et al., 1998, 2009; Rosenthal et al., 2012) is primarily driven by the concentration of elemental nitrogen. The main impact of CO_2 on C_4 plants is via the direct effect of CO_2 on the guard cells, and the resultant decrease in stomatal aperture and evapotranspiration (Conley et al., 2001; Triggs et al., 2004; Leakey et al., 2006; Ghannoum, 2009) preserving soil moisture further into the growing season (Wall et al., 2001; Leakey et al., 2009). However, this did not significantly mitigate the drought-driven increase in dhurrin seen in water-limited plants.

4.3. Implication for grazing and crop management

To our knowledge this is the first time that both nitrate and cyanide concentrations have been measured in an elevated CO_2 experiment and the first time total nitrogen data has been reported in a FACE study on *sorghum*. Drought, plant age and nitrogen supply are the most important determinants of cyanide and nitrate toxicity (Fig. 1; Gleadow and Møller, 2014). While no significant direct effect of elevated CO_2 on nutritional value of *sorghum* was detected here, it is possible that effects may become apparent when N is limited.

Sorghum is being promoted as a more drought tolerant alternative to maize for forage in regions that face environmental challenges but our data show that care should be taken to reduce the possibility of cyanide and nitrate intoxication. The present study examined grain *sorghum*. While the grain itself is not cyanogenic, the stover is used for forage, hay and silage and the results, therefore, of direct relevance to graziers. The development of low cyanide forage varieties (e.g., Blomstedt et al., 2012), would remove the element of risk during periods of prolonged drought, although it will be important to monitor where there are any changes in foliar nitrate. Low cyanide varieties may have the added benefit of improving the nitrogen use efficiency of the crop.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fcr.2015.10.010.

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