

## Minirhizotron imaging reveals that nodulation of field-grown soybean is enhanced by free-air CO<sub>2</sub> enrichment only when combined with drought stress

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**Abstract.** The rate of N<sub>2</sub> fixation by a leguminous plant is a product of the activity of individual nodules and the number of nodules. Initiation of new nodules and N<sub>2</sub> fixation per nodule are highly sensitive to environmental conditions. However, the effects of global environmental change on nodulation in the field are largely unknown. It is also unclear whether legumes regulate nodulation in response to environment solely by varying root production or also by varying nodule density per unit of root length. This study utilised minirhizotron imaging as a novel *in situ* method for assessing the number, size and distribution of nodules in field-grown soybean (*Glycine max* (L.) Merr.) exposed to elevated atmospheric CO<sub>2</sub> ([CO<sub>2</sub>]) and reduced precipitation. We found that nodule numbers were 134–229% greater in soybeans grown at elevated [CO<sub>2</sub>] in combination with reduced precipitation, and this response was driven by greater nodule density per unit of root length. The benefits of additional nodules were probably offset by an unfavourable distribution of nodules in shallow, dry soil in reduced precipitation treatment under elevated [CO<sub>2</sub>] but not ambient [CO<sub>2</sub>]. In fact, significant decreases in seed and leaf nitrogen concentration also occurred only in elevated [CO<sub>2</sub>] with reduced precipitation. This study demonstrates the potential of minirhizotron imaging to reveal previously uncharacterised changes in nodule production and distribution in response to global environmental change.

**Additional keywords:** elevated CO<sub>2</sub>, FACE, *Glycine max*, nodule, root.

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### Introduction

Most of the 20 000 species belonging to the family *Leguminosae* have the capacity to form symbiotic relationships with N<sub>2</sub>-fixing rhizobia bacteria. Enhanced availability of plant-usable N through this symbiosis results in high tissue N content and contributes to the importance of legumes ecologically, agriculturally and economically. The 40–60 million metric tons of N<sub>2</sub> that are fixed biologically by agricultural crops every year would cost an estimated \$7–10 billion annually to replace with chemical fertiliser (Peterson and Russelle 1991; Smil 1999). Consequently, understanding the responses of N<sub>2</sub> fixation and legume performance to global environmental change is important to predict future ecosystem goods and services as well as development of adaptation measures.

The total rate of N<sub>2</sub> fixation by a leguminous plant is a product of the capacity for N<sub>2</sub> fixation by individual nodules and the number of root nodules. Since nodule production and function are significant carbon sinks, and N availability often limits plant growth, optimising allocation of carbon to N<sub>2</sub> fixation is important to plant performance (Caetano-Anollés and Gresshoff

1991; Reid *et al.* 2011). Nodule initiation, development and function are all known to be sensitive to various factors of global environmental change and abiotic stress (Walsh 1995; Zahran 1999). However, this knowledge has only rarely been translated into quantitative understanding of the contributions that altered nodule production versus nodule activity make towards variation in total N<sub>2</sub> fixation (e.g. Serraj *et al.* 1998). The absence of experiments that integrate measures of physiology and structure to understand whole-plant function is particularly acute in the field. This reflects the difficulty in assessing nodulation status under field conditions. To date, nodulation status in the field has been assessed by destructively harvesting roots from soil by coring, trenching or uprooting of plants (Grubinger *et al.* 1982; Oyun 2007). In all cases, significant effort is required to excavate and separate roots from the soil. In addition, these processes are destructive, limiting the extent to which a single experimental plot can be sampled over time. This study overcomes these challenges by using minirhizotron imaging to assess nodulation responses of soybean (*Glycine max* (L.) Merr.) to elevated atmospheric CO<sub>2</sub> ([CO<sub>2</sub>]) and

reduced precipitation, which are two elements of global environmental change that are expected to have opposing effects on nodulation and legume performance.

Elevated CO<sub>2</sub> generally stimulates total N<sub>2</sub> fixation by legumes when other nutrients are not limiting (Zanetti *et al.* 1996; Schortemeyer *et al.* 2002; van Groenigen *et al.* 2006). In addition, legumes typically maintain N status in leaf and reproductive tissues at elevated [CO<sub>2</sub>] compared with ambient [CO<sub>2</sub>], whereas nonlegumes do not, implying that N<sub>2</sub> fixation is increased to maintain the balance between C and N metabolism (Rogers *et al.* 2006; Ainsworth *et al.* 2007; Taub *et al.* 2008; Rogers *et al.* 2009). This can lead to less photosynthetic acclimation, smaller reductions in yield quality and greater stimulation of productivity and yield in legumes versus nonlegumes at elevated [CO<sub>2</sub>] (Rogers *et al.* 2009). Numerous pot-based experiments suggest that the stimulation of N<sub>2</sub> fixation at elevated [CO<sub>2</sub>] is primarily driven by a greater number or size of nodules, and not by changes in the specific nodule N<sub>2</sub> fixation rate (Finn and Brun 1982; Murphy 1986; Norby 1987; Schortemeyer *et al.* 2002; Lee *et al.* 2003; Prévost *et al.* 2010; Cernusak *et al.* 2011). However, it is unclear the extent to which legumes achieve greater nodulation under elevated [CO<sub>2</sub>] by varying nodule density per unit of root length, the distribution of nodules on the root system or total root length. Furthermore, there are very few, if any, published reports of nodulation status for legumes in replicated free-air CO<sub>2</sub> enrichment (FACE) or open-top chamber experiments on plants growing in unrestricted soil. This is significant because it is increasingly recognised that plant responses to elevated [CO<sub>2</sub>] are different in controlled environments versus field conditions where interactions between the plant–microbe–soil complex are not disturbed (Körner 2006; Ainsworth *et al.* 2008).

Initiation, growth and activity of N<sub>2</sub>-fixing nodules are more sensitive to water stress than carbon assimilation or general metabolism of the host plant, but the magnitude of the reduction in N<sub>2</sub> fixation depends on the severity of the drought stress as well as the timing of the drought stress relative to plant growth and development (Durand *et al.* 1987; Zahran 1999). The effects of drought on total plant N<sub>2</sub> fixation may be attributed to reduced nodule numbers (Williams and De Mallorca 1984; Sangakkara *et al.* 1996; Antolin *et al.* 2010), reduced activity of individual nodules (Sprent 1971) or a combination of both.

Existing data suggest that elevated atmospheric [CO<sub>2</sub>] ameliorates drought-induced reductions in N<sub>2</sub> fixation by increasing the exchange of both carbon and N when the nodule is at a given soil moisture content (Serraj *et al.* 1998; De Luis *et al.* 1999). Additionally, the reduced transpiration of plants growing under elevated [CO<sub>2</sub>] often leads to conservation of soil moisture and it has been proposed that this may also delay the effects of drought on N<sub>2</sub> fixation by maintenance of a favourable water status in the soil surrounding the nodules (reviewed in Rogers *et al.* 2009). However, studies investigating the interactive effects of elevated [CO<sub>2</sub>] and drought have been conducted in controlled environments where plants were not exposed to natural gradients or fluctuations in soil moisture resources. Therefore, if nodule distribution were affected by elevated [CO<sub>2</sub>] in a way that altered their ability to fix nitrogen, these laboratory studies would have overlooked this complexity in plants' response to elevated [CO<sub>2</sub>]. Growth in soil

compared with growth in an artificial environment alters the number of root nodules, the distribution of root nodules between primary and lateral roots, and the depth distribution of nodules in seedlings of various legumes including soybean, cowpea (*Vigna unguiculata* (L.) Walp.) and siratro (*Macroptilium atropurpureum* (Moc. & Sessé ex DC.) Urb.) (Pueppke 1986), suggesting that field experiments are necessary to accurately quantify nodulation responses to environmental change. Over 76 million hectares of soybean were harvested in the USA in 2010, with a value of approximately \$39 billion (USDA; [www.nass.usda.gov](http://www.nass.usda.gov)). Production is primarily in the Midwest where soybean is grown in rotation with maize (*Zea mays* L.) to form the dominant land use. This study used the soybean free-air CO<sub>2</sub> enrichment (SoyFACE) facility, which is located at the centre of this region. The low environmental heterogeneity of the site and the inbred nature of soybean increased the statistical power of this experiment ( $n=4$ ). The objective of this study was to investigate the interactive effects of elevated [CO<sub>2</sub>] and reduced precipitation on nodulation status of field-grown soybean. Specifically, we aimed to test the hypotheses that: (1) elevated [CO<sub>2</sub>] will stimulate nodule production and nodule size; (2) reduced precipitation will inhibit nodule production; and (3) elevated [CO<sub>2</sub>] will ameliorate the negative effects of reduced precipitation on nodule production.

## Materials and methods

### Field site and treatments

This experiment was carried out at the SoyFACE facility in Champaign, Illinois (40°02'N, 88°14'W) during the summer of 2010. At SoyFACE, soybean (*Glycine max* (L.) Merr. cv. 93B15, Pioneer Hi-Bred International, Des Moines, IA, USA) were grown under fully open-air conditions at either current ambient atmospheric CO<sub>2</sub> (~385 parts per million (ppm)) or elevated atmospheric CO<sub>2</sub> (585 ppm). Elevated CO<sub>2</sub> treatment was achieved through the use of FACE technology (Miglietta *et al.* 2001). Detailed descriptions of the fumigation technology, field management practices and micrometeorological monitoring used at SoyFACE have been published previously (Leakey *et al.* 2004; Rogers *et al.* 2004). Plants were grown in experimental plots measuring 20 m in diameter. Whole plots were exposed to either ambient or elevated CO<sub>2</sub>, and within whole plots, 8 × 4 m subplots were exposed to either control precipitation or reduced precipitation. This resulted in four treatment combinations: ambient CO<sub>2</sub> with control precipitation (AC-CP), ambient CO<sub>2</sub> with reduced precipitation (AC-RP), elevated CO<sub>2</sub> with control precipitation (EC-CP) and elevated CO<sub>2</sub> with reduced precipitation (EC-RP). The replication level of our experiment was four. Palmer Crop Moisture Index (PCMI) data for control precipitation conditions were obtained from the National Oceanic and Atmospheric Administration ([http://www.cpc.ncep.noaa.gov/products/analysis\\_monitoring/cdus/pastdata/palmer/index.shtml](http://www.cpc.ncep.noaa.gov/products/analysis_monitoring/cdus/pastdata/palmer/index.shtml)).

The reduced precipitation treatment was imposed by intercepting rainfall with modified Solair motorised retractable fabric awnings (Glen Raven Inc., Glen Raven, NC, USA) mounted on lightweight metal scaffolding. Rainfall intercepted by awnings ran into gutters and was diverted 20 m away from the

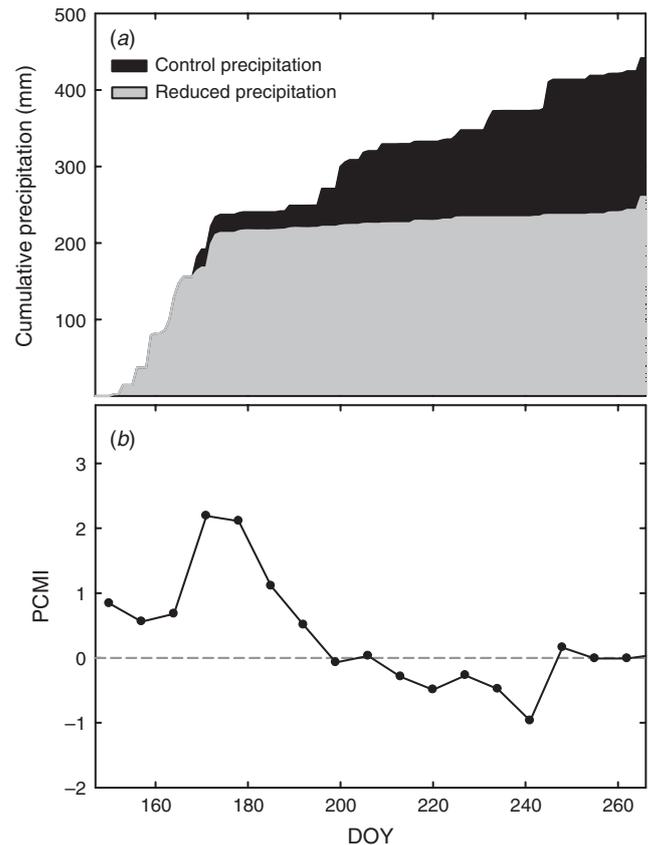
treatment plots using corrugated drain pipes. Awnings were automatically controlled to intercept rainfall events, primarily at night but also occasionally during daylight hours under very low light (<2.5% full sunlight). A central weather station and computer relayed a signal to the awnings to deploy when rainfall was detected, the wind speed was <10 m s<sup>-1</sup> and PAR was <50 μmol m<sup>-2</sup> s<sup>-1</sup>. This allowed interception of significant rainfall, but with minimal effects on solar radiation or CO<sub>2</sub> fumigation received by the crop. Overall, the awnings were deployed for 4% of the total growing season (109 h). During this time, 0.05% of the total growing season PAR was incident on the crop. However, this is likely to be an overestimate of the shading caused by the canvas awning due to diffuse radiation entering the plot from the gaps between the awning and the plant canopy. No sampling was performed within a 0.5-m perimeter of the edge of the plot, where the metal scaffolding and gutter cast shade. A rubber subsoil barrier was installed around the perimeter of the reduced precipitation subplot to 1 m depth, minimising horizontal inflow of subsurface water. Soil volumetric water content was measured throughout the growing season using a capacitance probe (Diviner-2000, Sentek Sensor Technologies, Stepney, SA, Australia) at 10-cm increments. Measurements were made between depths of 5–105 cm at each of four locations within a subplot. Two locations were within crop rows and two locations were between rows. Measurements were made every 2–6 days. Raw data from the probe were calibrated against gravimetric data using the method of Paltineanu and Starr (1997).

In 2010, soybeans were planted on 27 May and harvested on 23 September. CO<sub>2</sub> fumigation began on 9 June and rainfall interception in reduced precipitation plots began on 18 June. The growing season precipitation in control precipitation plots was 442 mm, but reduced precipitation plots received 260 mm, corresponding to a 41% reduction in growing season rainfall in reduced precipitation plots (Fig. 1).

#### Rhizotron image data collection and analysis

Cellulose acetate butyrate minirhizotron observation tubes with a 2-inch internal diameter (Bartz Technology Corp, Carpinteria, CA, USA) were installed within 4 days of planting, before the emergence of soybean seedlings. A tractor-mounted Giddings probe (Giddings Machine Co., Windsor, CO, USA) was used to bore holes and to set minirhizotron tubes. Four minirhizotron tubes were installed in each subplot at 30° from vertical to a soil depth of 90 cm, with two tubes within each subplot located within the soybean rows, and two tubes within each subplot located between the soybean rows. Minirhizotrons were placed in the portion of each subplot that was immediately adjacent to soil moisture access tubes, and with the same distribution and placement relative to crop rows. Tubes were wrapped heavily with tape above the soil surface to exclude light, and the ends were covered using aluminium cans to exclude light and water.

Images were collected along the upper face of the tubes every 2 weeks using a BTC100× minirhizotron video microscope and a BTC I-CAP Image Capture System (Bartz Technology Corp.). Images were collected every 1.3 cm for a total of ~80 images per tube per measurement date. Images were analysed using the WinRHIZO Tron MF manual root measurement program



**Fig. 1.** (a) Cumulative precipitation during the 2010 growing season plotted against day of year (DOY) for the control precipitation treatment (black area) and the reduced precipitation (grey area) treatments. (b) Palmer Crop Moisture Index (PCMI) for Illinois Climate Division 5 during the 2010 growing season plotted against DOY.

(Regent Instruments, Quebec, Canada). For each image, root length and nodule numbers were recorded, and nodule height and width were traced. These data were used to estimate the nodule number per unit of root length ( $\text{nod}\#_{\text{root\_length}}$ ) and combined with a standard estimate of depth of view (2 mm; Iversen *et al.* 2008) to estimate nodule numbers per unit of soil volume ( $\text{nod}\#_{\text{soil\_vol}}$ ). Nodule height and width were also used to estimate nodule cross-sectional area using the equation for the area of an ellipse ( $\pi \times (\text{width} \times 0.5) \times (\text{height} \times 0.5)$ ). Collectively, over 25 500 images were analysed for nodule numbers and root length (a representative image with a root nodule is shown in Fig. S1, available as Supplementary Material to this paper).

#### Leaf nitrogen analysis

Leaflets were collected at midday on six dates, ranging from early vegetative growth (pre-N<sub>2</sub> fixation) to reproductive growth. Immediately following harvest, leaf samples were dried to a constant mass in a 70°C oven. Leaves were powdered in a mechanical grinder (2000 Grinder, SPEX CertiPrep, Metuchen, NJ, USA), 1.5–2.5-mg aliquots of each sample were weighed and placed into tin capsules (AX26 DeltaRange Microbalance, Mettler Toledo, Columbus, OH, USA). Leaf N content was determined using an elemental analyser

following the manufacturer's instructions (2400 Series II CHNS/O Analyzer, Perkin Elmer, Waltham, MA, USA). Acetanilide was used as a standard to calibrate the analyser and to confirm instrument accuracy during analysis.

### Seed nitrogen analysis

Whole soybean plants were harvested from each treatment plot on 23 September. Seeds were hand-shelled, oven-dried at 70°C, and ground to a fine powder using a Geno/Grinder 2010 tissue homogeniser (SPEX SamplePrep, Metuchen, NJ, USA). For each treatment plot, three technical replicates were used, each consisting of a 3-mg aliquot of homogenised seed tissue. Aliquots were placed into tin capsules and analysed using an elemental analyser (ECS 4010, Costech Instruments, Valencia, CA, USA).

### Statistical analysis

Nodule density data and N data were analysed using mixed-model ANOVA (proc mixed; SAS ver. 9.2, SAS Institute, Cary, NC, USA). Analyses were performed on subplot means ( $n=4$ ). For nodule density data generated from rhizotron images, data from 5–55 cm depth were used in this analysis because 95% of the nodules that were found were at 45 cm depth or shallower. Significant depth  $\times$  CO<sub>2</sub>  $\times$  H<sub>2</sub>O  $\times$  date interactions were not detected, so all data in the 5–55 cm soil depth profile were averaged together for a given treatment plot and date combination, and this value was used in subsequent statistical analyses. A repeated-measures ANOVA was used, where date was treated as the repeated measure; CO<sub>2</sub> treatment, precipitation treatment and sampling date as well as their interactions were included as fixed effects; and block as well as block  $\times$  CO<sub>2</sub> were included as random effects. For soil volumetric water content data, each depth was analysed in a separate repeated-measures analysis of covariance, where saturated soil water content in each plot at the beginning of the growing season was used as the covariate; CO<sub>2</sub> treatment, precipitation treatment and date along with their interactions were included as fixed effects; and block as

well as block  $\times$  CO<sub>2</sub> were used as random effects. As in previous studies (Morgan *et al.* 2005; Leakey *et al.* 2006), differences from the control were considered significant at  $P < 0.1$  in order to avoid Type II errors.

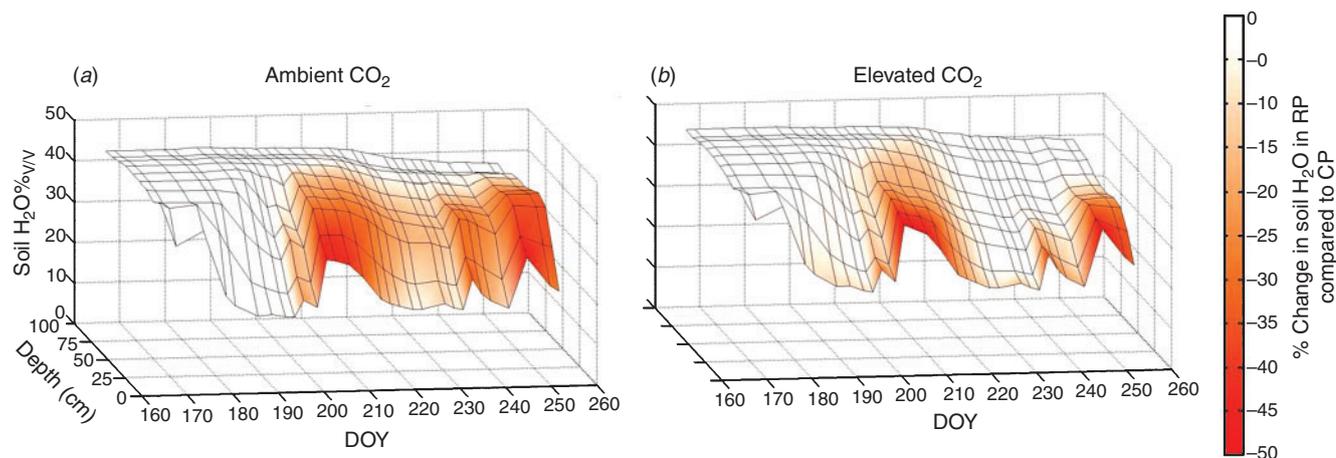
## Results

### PCMI data and microclimate

The PCMI is a dynamic measure of crop moisture demand relative to moisture availability in the soil (Palmer 1968). Negative values indicate when demand exceeds supply and the crop experiences drought stress. The 2010 growing season began and ended with favourable growing conditions, but the crop experienced a period of drought stress from day of year (DOY) 200 to 245 (Fig. 1). The average daily mean temperature in June to August was 24.6°C, which is greater than the long-term average daily mean temperature of 22.9°C from 1889 to the present day (Angel 2010). Precipitation in June to August was 348 mm, which is slightly above the long-term average of 337 mm (Angel 2010); however, this was not evenly distributed, with 212 mm falling in June and only 42 mm falling in August. Drought stress caused by the below-average rainfall in August was exacerbated by above-average temperatures, causing stressful conditions.

### Soil volumetric moisture content

Soil volumetric moisture content (H<sub>2</sub>O%<sub>v/v</sub>) was near field capacity at the beginning of the growing season in all treatments (Fig. 2). As the growing season progressed, plant water use depleted soil H<sub>2</sub>O%<sub>v/v</sub>. Within individual drying cycles, soil drying was greatest initially at shallow depths and became progressively deeper over time. In the control precipitation (CP) treatments, rain events led to significant rewetting of the soil. Interception of 41% of the growing season rainfall meant that rewetting of the soil was substantially reduced and drying cycles were extended in duration in the reduced precipitation (RP) treatments.



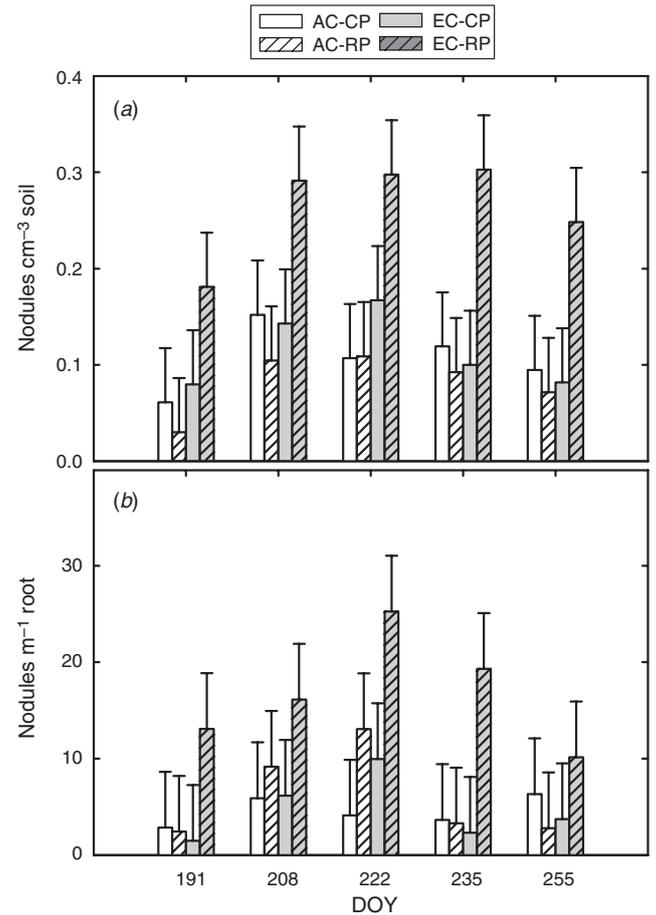
**Fig. 2.** Soil volumetric moisture content (H<sub>2</sub>O%<sub>v/v</sub>) for (a) ambient CO<sub>2</sub> with control precipitation and (b) elevated CO<sub>2</sub> with control precipitation during the 2010 growing season. In (a) and (b), the y-axis shows day of year (DOY), the x-axis shows soil depth (cm) and the z-axis shows soil volumetric moisture content (H<sub>2</sub>O%<sub>v/v</sub>). The colour scale represents the percent change in soil volumetric moisture content in the reduced precipitation treatment compared with the control precipitation treatment, with the darker shade representing a larger reduction in soil volumetric moisture content.

The RP treatment caused significant reductions in soil H<sub>2</sub>O%<sub>v/v</sub>, which were greatest in magnitude following two periods of substantial precipitation (DOY 200–220 and 225–250). In ambient [CO<sub>2</sub>], a reduction in soil H<sub>2</sub>O%<sub>v/v</sub> was also sustained at depths of 5–75 cm throughout the period separating these two wetting periods (Fig. 2, Table S1). In elevated [CO<sub>2</sub>], there were mild effects of the RP treatment on soil H<sub>2</sub>O%<sub>v/v</sub> at shallow depths, and the effects of RP treatment became apparent earlier in the growing season (DOY 185) in elevated [CO<sub>2</sub>] (EC) than in ambient [CO<sub>2</sub>] (AC; Fig. 2). In addition, the RP treatment effect on soil H<sub>2</sub>O%<sub>v/v</sub> almost completely disappeared from DOY 225 to DOY 235 in EC but not AC. These distinct patterns of soil H<sub>2</sub>O%<sub>v/v</sub> in ambient and elevated [CO<sub>2</sub>] resulted from equal rates of soil drying in AC–RP and EC–RP, but slower (DOY 175–195) and then faster (DOY 210–250) soil drying in EC–CP versus AC–CP (Fig. 2, Table S1). The demand for water from the crop growing in above-average temperatures meant that soil H<sub>2</sub>O%<sub>v/v</sub> approached the permanent wilt point in multiple soil layers of all treatments, but to a greater degree and for long periods of time in AC–RP and EC–RP compared with AC–CP and EC–CP.

#### Nodule density on roots and in the soil, and nodule size

Nod#<sub>soil\_vol</sub> and nod#<sub>root\_length</sub> both increased in the 75 days after planting and peaked at the beginning of seed fill (DOY 222). Both nod#<sub>soil\_vol</sub> and nod#<sub>root\_length</sub> then declined only slightly by the time pods were beginning to mature. There was a strong interaction effect of EC and RP treatments, in which nod#<sub>soil\_vol</sub> and nod#<sub>root\_length</sub> were 134–229% greater in EC–RP than all the other treatments (Fig. 3, Table 1). Nodule area showed significant seasonal variation, increasing from DOY 191 to DOY 222, and then remaining stable through to the end of the growing season (Tables 1, 2). CO<sub>2</sub> and precipitation treatments, however, did not significantly affect nodule area (Tables 1, 2).

In addition to altering the density of nodules on roots and in the soil, EC and RP treatments altered the distribution of nodules relative to soil moisture resources. Under EC–RP, 48% of the total nodules counted were found in dry soil (~20–30% soil H<sub>2</sub>O%<sub>v/v</sub>; Fig. 4). This was a very large fraction of nodules exposed to dry soil compared with that observed under AC–RP (7%), AC–CP (14%) or EC–CP (11%). The effects of the EC–RP treatment on



**Fig. 3.** (a) Nodule number per cm<sup>3</sup> soil (nod#<sub>soil\_vol</sub>) and (b) nodule number per meter root (nod#<sub>root\_length</sub>) plotted against measurement date. For both panels, ambient CO<sub>2</sub> with control precipitation (AC–CP) is represented by white bars, ambient CO<sub>2</sub> with reduced precipitation (AC–RP) is represented by hatched white bars, elevated CO<sub>2</sub> with control precipitation (EC–CP) is represented by grey bars and elevated CO<sub>2</sub> with reduced precipitation (EC–RP) is represented by hatched grey bars. Bar height represents mean value for 5–55 cm soil depth, and error bars represent s.e.

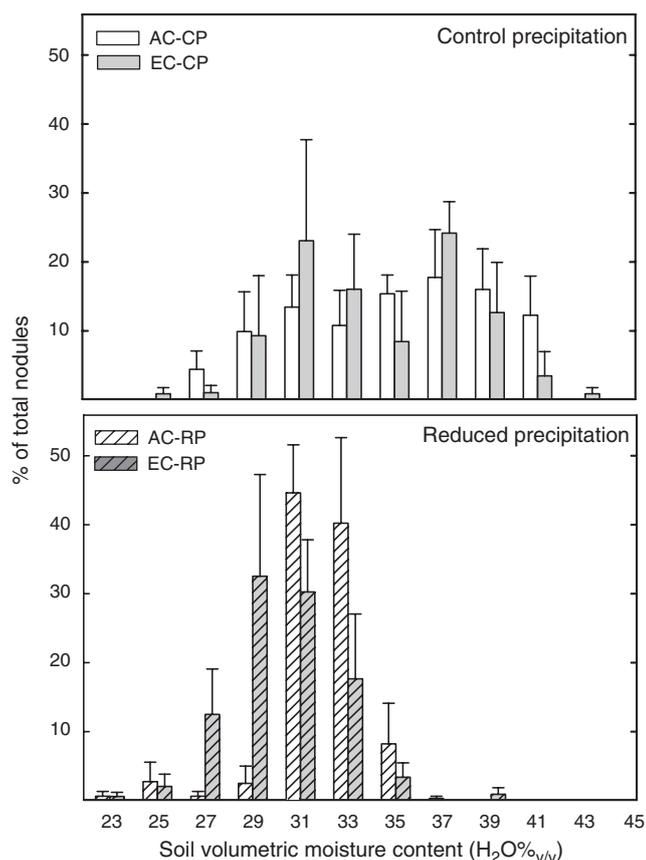
the distribution of nodules relative to soil moisture resources were associated with greater nod#<sub>soil\_vol</sub> in shallow soil depths in EC–RP (Fig. 5). Specifically, in the EC–RP treatment, 45% of

**Table 1.** ANOVA table showing the direct and interactive effects of CO<sub>2</sub>, precipitation (H<sub>2</sub>O) and date on nodules per cm<sup>3</sup> soil, nodules per metre of root and nodule cross-sectional area (mm<sup>2</sup>) in soybean  
Bold font indicates significant differences

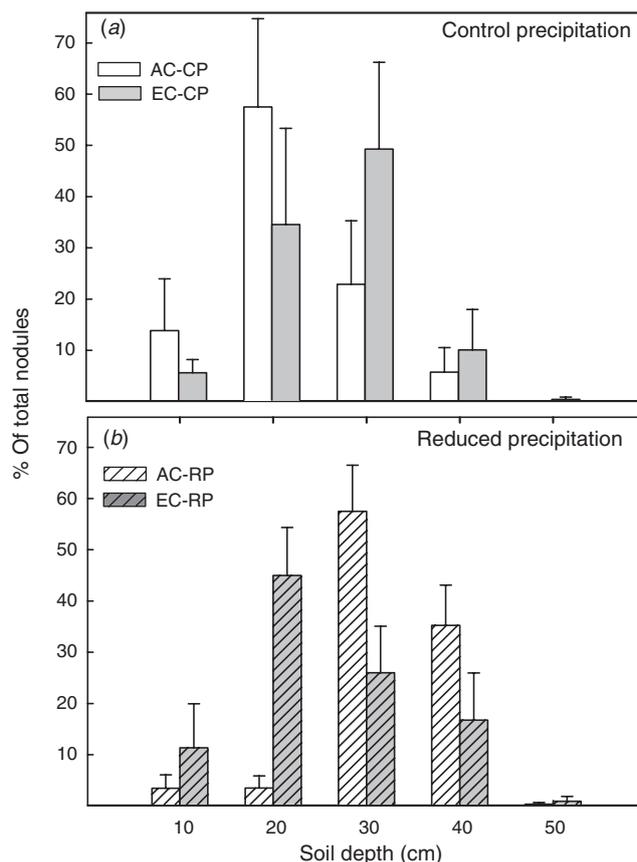
Effect	Nodules per cm <sup>-3</sup> soil		Nodules m <sup>-1</sup> root		Nodule cross-sectional area	
	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value
CO <sub>2</sub>	<b>3.80</b>	< <b>0.10</b>	1.53	0.26	0.98	0.35
H <sub>2</sub> O	2.24	0.17	<b>5.51</b>	< <b>0.05</b>	0.91	0.37
CO <sub>2</sub> × H <sub>2</sub> O	<b>4.35</b>	<b>0.07</b>	<b>9.06</b>	<b>0.02</b>	0.78	0.41
Date	<b>4.99</b>	< <b>0.01</b>	0.75	0.56	<b>15.11</b>	< <b>0.0001</b>
Date × CO <sub>2</sub>	0.51	0.73	0.30	0.87	1.05	0.40
Date × H <sub>2</sub> O	0.22	0.93	0.65	0.63	0.66	0.62
Date × CO <sub>2</sub> × H <sub>2</sub> O	0.53	0.71	0.79	0.54	0.51	0.73

**Table 2.** Estimated nodule cross-sectional area ( $\text{mm}^2$ ) of root nodules of soybean grown in ambient  $\text{CO}_2$  with control precipitation (AC-CP), ambient  $\text{CO}_2$  with reduced precipitation (AC-RP), elevated  $\text{CO}_2$  with control precipitation (EC-CP) and elevated  $\text{CO}_2$  with reduced precipitation treatment (EC-RP) for each of five measurement dates (shown as day of year; DOY)  
Values shown are mean  $\pm$  s.e.

DOY	Treatment			
	AC-CP	AC-RP	EC-CP	EC-RP
191	2.4 $\pm$ 0.7	1.4 $\pm$ 0.7	1.9 $\pm$ 0.7	2.4 $\pm$ 0.6
208	2.8 $\pm$ 0.6	3.1 $\pm$ 0.9	3.0 $\pm$ 0.6	3.2 $\pm$ 0.6
222	5.2 $\pm$ 0.7	5.7 $\pm$ 0.7	4.2 $\pm$ 0.6	4.4 $\pm$ 0.6
235	4.7 $\pm$ 0.6	4.8 $\pm$ 0.7	3.8 $\pm$ 0.7	4.6 $\pm$ 0.6
255	4.6 $\pm$ 0.7	4.8 $\pm$ 0.6	2.9 $\pm$ 0.7	4.9 $\pm$ 0.6



**Fig. 4.** Percentage of nodules in a given treatment found in each category of soil volumetric moisture content. Nodules found at 22–24% soil volumetric moisture content are binned into the category labelled 23%, nodules found at 24–26% soil volumetric moisture content are binned into the category labelled 25%, and so on. (a) Control precipitation treatments; (b) reduced precipitation treatments. Ambient  $\text{CO}_2$  with control precipitation (AC-CP) is represented by white bars, ambient  $\text{CO}_2$  with reduced precipitation (AC-RP) is represented by hatched white bars, elevated  $\text{CO}_2$  with control precipitation (EC-CP) is represented by grey bars and elevated  $\text{CO}_2$  with reduced precipitation (EC-RP) is represented by hatched grey bars.



**Fig. 5.** Percentage of nodules in a given treatment found in each depth category. Nodules found at 5–15 cm depth are binned into the category labelled '10 cm', nodules found at 15–25 cm depth are binned into the category labelled '20 cm' and so on. (a) Control precipitation treatments; (b) reduced precipitation treatments. Ambient  $\text{CO}_2$  with control precipitation (AC-CP) is represented by white bars, ambient  $\text{CO}_2$  with reduced precipitation (AC-RP) is represented by hatched white bars, elevated  $\text{CO}_2$  with control precipitation (EC-CP) is represented by grey bars and elevated  $\text{CO}_2$  with reduced precipitation (EC-RP) is represented by hatched grey bars.

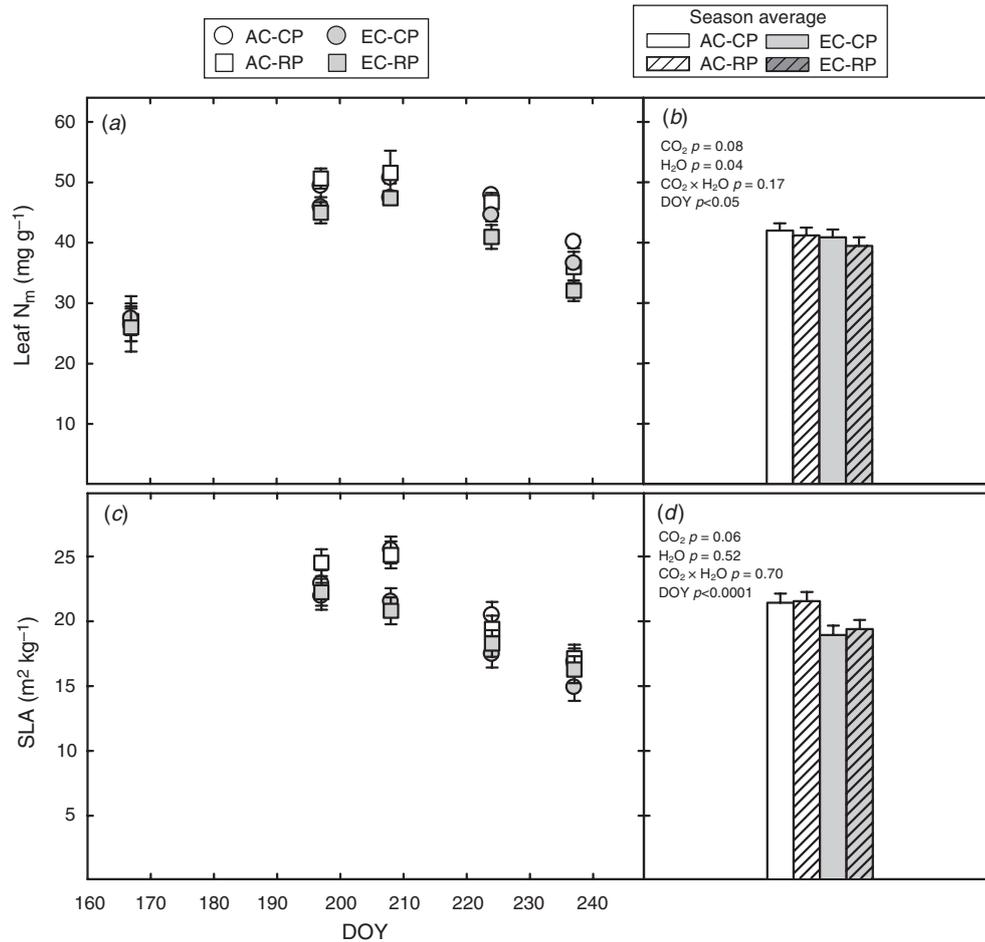
nodules were located at depths of 15–25 cm, but only 3% of nodules in the AC-RP treatment were located at this soil depth (Fig. 5).

#### Leaf and seed nitrogen content

The EC treatment significantly decreased leaf nitrogen on a mass basis (mg N per g leaf tissue; leaf  $N_m$ ; Fig. 6a, b). This was associated with significantly lower specific leaf area (SLA) under EC (Fig. 6c, d). RP also decreased leaf  $N_m$ , but the effect was limited to late in the growing season and was not accompanied by significant changes in SLA (Fig. 6a). There was a significant interaction effect of EC and RP in which seed nitrogen on a mass basis (seed  $N_m$ ) under EC-RP was significantly lower than in all other treatments ( $\text{CO}_2 \times \text{H}_2\text{O}$ ;  $P=0.06$ ; Table 3).

#### Discussion

This study demonstrated the use of minirhizotron imaging to provide repeated nondestructive assessment of the nodulation



**Fig. 6.** (a, b) Leaf N on a mass basis and (c, d) specific leaf area (SLA) for (a, c) each measurement date and (b, d) the average for the growing season. Ambient CO<sub>2</sub> with control precipitation (AC-CP) is represented by white bars (white circles in (c) and (d)), ambient CO<sub>2</sub> with reduced precipitation (AC-RP) is represented by hatched white bars (white squares in (c) and (d)), elevated CO<sub>2</sub> with control precipitation (EC-CP) is represented by grey bars (grey circles in (c) and (d)) and elevated CO<sub>2</sub> with reduced precipitation (EC-RP) is represented by hatched grey bars (grey squares in (c) and (d)). Symbols and bars represent mean values and error bars represent s.e.

**Table 3.** Seed N on a mass basis (N<sub>m</sub>; mg N per g dry seed mass) of soybean grown in ambient CO<sub>2</sub> with control precipitation (AC-CP), ambient CO<sub>2</sub> with reduced precipitation (AC-RP), elevated CO<sub>2</sub> with control precipitation (EC-CP) and elevated CO<sub>2</sub> with reduced precipitation (EC-RP) at final harvest during the 2010 growing season. Values shown are mean ± s.e. Means sharing a common superscript letter do not differ significantly ( $P < 0.1$ )

Treatment	AC-CP	AC-RP	EC-CP	EC-RP
Seed N <sub>m</sub>	60.1 ± 0.6 <sup>a</sup>	59.1 ± 0.6 <sup>a</sup>	60.1 ± 0.6 <sup>a</sup>	56.0 ± 0.6 <sup>b</sup>

status of field-grown soybean by characterising a strong interactive effect of elevated [CO<sub>2</sub>] and reduced precipitation on nod#<sub>soil\_vol</sub>. Nodules were found at soil depths of up to 55 cm. Nodule density (nodules per cm<sup>3</sup> soil) estimates generated from minirhizotron images in this experiment were consistent with previously published values for field-grown soybean generated

from soil cores (Chen *et al.* 1992). We also found that nodule density and size estimates generated from minirhizotron images varied significantly throughout the season in a manner that was consistent with previously published data. For example, nodule size and nodule density on the roots and in the soil increased from DOY 191 to DOY 222 and then remained stable or decreased slightly thereafter (Fig. 3, Table 2). In our experiment, DOY 222 corresponded with the beginning seed stage (stage R5; Fehr and Caviness 1977), corroborating the finding of Weil and Ohlrogge (1975) that the number and size of nodules per soybean plant peaked in early pod-filling stage and declined afterwards. Effective sampling of soils up to 55 cm depth by traditional excavation methods would have been highly destructive and consequently difficult or impossible to repeat on multiple dates through the growing season in relatively small (8 × 4 m) treatment plots with an infrastructure that restricts access for mechanised excavation equipment. The repeated sampling using a minirhizotron camera increased the statistical

power to detect treatment effects, which is always challenging in the heterogeneous soil environment. The potential for the minirhizotron imaging approach to be combined with existing techniques that estimate rates of N<sub>2</sub> fixation in order to advance a mechanistic understanding of the environmental influences on legume performance in the field was emphasised by treatment effects on: (a) the distribution of nodules within the soil profile, (b) the local soil conditions experienced by the nodules, and (c) the density of nodules per unit of root length (nod#<sub>root\_length</sub>). Collectively, these findings contradict conclusions drawn from controlled environment experiments and indicate the need for further field experimentation to determine how nodulation status influences rates of plant N acquisition and C use.

The finding that nod#<sub>soil\_volume</sub> was 134–229% greater in EC–RP compared with all of the other three treatments was contrary to the predicted individual and combined effects of elevated [CO<sub>2</sub>] and reduced precipitation (e.g. Finn and Brun 1982; Williams and De Mallorca 1984; Serraj *et al.* 1998). Controlled environment experiments have repeatedly found direct stimulation of nodulation, rather than higher nodule N<sub>2</sub> fixation rates, to be the primary driver of greater total N<sub>2</sub> fixation by elevated [CO<sub>2</sub>] (Finn and Brun 1982; Murphy 1986; Norby 1987; Cabrerizo *et al.* 2001; Schortemeyer *et al.* 2002; Lee *et al.* 2003; Prévost *et al.* 2010; Cernusak *et al.* 2011). The absence of stimulated nod#<sub>soil\_vol</sub> or nodule size under EC–CP in the field may be a function of genetic variability in the response, since there is variation among species as to whether N<sub>2</sub> fixation is stimulated by elevated [CO<sub>2</sub>] (West *et al.* 2005). However, several lines of evidence suggest that N<sub>2</sub> fixation was probably stimulated under EC–CP compared with AC–CP. In this study, there was no difference between EC–CP and AC–CP in leaf N<sub>m</sub> or seed N<sub>m</sub>, indicating that greater biomass production was matched by greater N assimilation. In addition, previous studies of the same soybean cultivar under CP at SoyFACE discovered that N metabolism was stimulated to match enhanced photosynthetic C gain under elevated [CO<sub>2</sub>] (Rogers *et al.* 2006) and changes in the δ<sup>15</sup>N of aboveground tissue were consistent with a stimulated N<sub>2</sub> fixation pathway under elevated [CO<sub>2</sub>] (Decock *et al.* 2012). If this is the case, it suggests that soil water status plays a hitherto unrecognised role in determining the relationship between nodulation status and N<sub>2</sub> fixation under elevated [CO<sub>2</sub>]. This will need to be confirmed by combining further assessment of nodulation status with isotopic methods (Unkovich and Pate 2000).

The lack of any reduction in nod#<sub>soil\_vol</sub> in AC–RP compared with AC–CP may indicate that the drought stress resulting from RP was relatively mild since previous studies have observed that inhibition of nodule number requires severe drought stress (Sinclair *et al.* 1988). Translating an understanding of plant responses to treatments in controlled environment conditions into knowledge of key thresholds that constrain plant function under field conditions is a major challenge for predicting plant responses to global environmental change. Therefore, it is valuable to discover that inhibition of nodulation must require a >41% reduction in growing season precipitation, even in a year where some drought stress has already occurred. However, the response of nodulation to reduced precipitation will probably depend on the timing of the reduction in precipitation, as well as

the magnitude. It is also important to note that this site is likely to be more resilient to RP than others because of the high water-holding capacity of its deep soils and the substantial precipitation before the growing season that is characteristic of this region in most years (Hollinger and Isard 1994).

Although very few studies have tested the response of nodulation of legumes to factorial treatments of elevated [CO<sub>2</sub>] and drought, the observation that nod#<sub>soil\_vol</sub> was stimulated in EC–RP under field conditions contradicts previous controlled environment studies (Serraj *et al.* 1998; De Luis *et al.* 1999). In soybean, drought stress imposed by variable watering over a 17-day period resulted in significantly decreased nod#<sub>soil\_vol</sub>, with a smaller effect observed in elevated [CO<sub>2</sub>] than in ambient [CO<sub>2</sub>] (Serraj *et al.* 1998). These treatment effects on nod#<sub>soil\_vol</sub> were, in turn, the primary drivers of changes in total nodule mass per plant and whole-plant N<sub>2</sub> fixation. In alfalfa (*Medicago sativa* (L.)), elevated [CO<sub>2</sub>] had no effect on the inhibition of nodulation by drought stress imposed by variable watering over a 30-day period (Aranjuelo *et al.* 2009). The different treatment effects observed in the three studies may be a consequence of the manner in which treatments were applied. Although the current study imposed both elevated [CO<sub>2</sub>] and RP treatments from early in plant development and throughout the growing season, in the controlled environment studies, soybean or alfalfa were grown with a plentiful water supply at ambient [CO<sub>2</sub>] for 28–30 days before elevated [CO<sub>2</sub>] and drought treatments were imposed (Serraj *et al.* 1998; Aranjuelo *et al.* 2009). In this experiment, the interactive effects of EC and RP on nodulation began early in the growing season (DOY 191; Fig. 3). This is notable because at this time, RP had caused only marginally significant effects on soil moisture in shallow soils in the EC treatment, but no effect on soil moisture in the AC treatment (Fig. 2). The initial nodulation response to these conditions appears to have propagated across the entire growing season despite complex spatial and temporal variation in soil H<sub>2</sub>O%<sub>v/v</sub> among treatments.

Although soybean grown under EC–RP had many more nodules than in the other treatments, it also had the lowest leaf N<sub>m</sub> and seed N<sub>m</sub>. This could only occur if there was a decrease in N<sub>2</sub> fixation per nodule under EC–RP. Reduced N<sub>2</sub> fixation per nodule under EC–RP needs to be tested with direct measurements of the N<sub>2</sub> fixation rate, but may be explained by an altered distribution of nodules relative to soil water resources. Only under EC–RP was a substantial fraction of nodules located in shallow, dry soils. Exposure of nodules to dry soil is likely to have significant functional consequences because drought stress directly inhibits nodule metabolism (Durand *et al.* 1987) and nodule N<sub>2</sub> fixation activity is more sensitive to local soil conditions than overall plant water status (Abdelhamid *et al.* 2011). Further work will be needed to determine whether more nodules were made in response to tissue N shortages, and whether this compensation mechanism was only partially successful in maintaining N supply, or if the change in nodulation was a response to some other element of the EC–RP treatment that led to production of extra nodules that subsequently were partly or wholly dysfunctional. An associated knowledge gap is whether functionality could be restored to nodules in the EC–RP treatment with rewatering, or whether their location in dry, shallow soils led to irreversible impairment of N<sub>2</sub> fixation. In

either case, plants under EC–RP must have allocated more photosynthate into nodule production but still received a reduced return of N for the investment of C.

In addition to stimulation of total nodule numbers, EC–RP increased the number of nodules per unit of root length. The number of nodules on the roots of soybean and other legumes is systemically regulated by autoregulation of nodulation, which ensures a balance between the level of nodulation and other aspects of plant development (Caetano-Anollés and Gresshoff 1991). Previously, it was found that the ratio of nodule dry weight to total plant dry weight was the same under ambient [CO<sub>2</sub>] and elevated [CO<sub>2</sub>] (Serraj *et al.* 1998), but our finding that EC–RP alters the number of nodules per metre of root demonstrates that soybean can alter investment in nodules not only by increasing the overall size of the root system in response to elevated [CO<sub>2</sub>] but also by altering the ratio of nodules to the root system size. Changes in  $\text{nod}\#_{\text{root\_length}}$  are known to occur in response to variation in N availability. The stimulation of plant biomass by elevated [CO<sub>2</sub>] intensifies through the season, requiring ever greater amounts of N in addition to carbon. The reduction in leaf N<sub>m</sub> in soybean under EC–RP was not evident until late in the growing season. This argues against a mechanism sensing tissue N status as the driver for greater nodulation early in the growing season. One alternative possibility would be that the inhibition of N mineralisation that occurs when soils dry (Larsen *et al.* 2011; Decock *et al.* 2012) provides a direct signal to the root. Previous experiments at soyFACE have demonstrated that elevated CO<sub>2</sub> treatment did not affect bulk soil N content (1.83 ± 0.07 g N per kg soil and 1.83 ± 0.05 g N per kg soil in ambient and elevated CO<sub>2</sub>, respectively; Pereira *et al.* 2011), but we cannot rule out that interactions between altered soil water availability and elevated [CO<sub>2</sub>] may have affected local soil N dynamics in the rhizosphere and therefore nodulation status in the current experiment. In either case, the ability to increase nodule numbers independently of increases in root system size may allow plants to respond to elevated [CO<sub>2</sub>] and drought with greater plasticity than previously recognised.

## Conclusions

Most previous studies assessing legume nodule numbers and their distribution in response to environmental factors have been limited to laboratory experiments in which plants were grown in pots. In these restricted soil environments, soil water distribution does not reflect the dynamic changes in soil water availability and distribution through the soil depth column that occur in nature. Few experiments have assessed nodulation responses to environmental treatments in the field because whole-root system excavation is destructive and time-consuming (Grubinger *et al.* 1982; Oyun 2007). This study demonstrates a novel minirhizotron imaging technique to quantify the nodulation status of field-grown plants. This method provided an *in situ* analysis of the effects of elevated [CO<sub>2</sub>] and reduced precipitation on the number and distribution of root nodules in soybean. Contrary to previous controlled environment studies, elevated [CO<sub>2</sub>] did not affect nodule number or size in plants exposed to control precipitation, but

elevated [CO<sub>2</sub>] increased nodule numbers and nodule density per unit of root length only in plants exposed to reduced precipitation. Surprisingly, EC–RP plants also had the lowest leaf N content and the lowest seed N content, despite having the greatest number of nodules. This may have been due to a shift in the depth distribution of nodules, which may have caused inhibition of N<sub>2</sub> fixation activity, because 48% of the nodules in EC–RP occurred in shallow, drier soil layers.

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