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**Carbon sink strength of nodules but not other organs modulates photosynthesis of faba bean (*Vicia faba* L.) grown under elevated [CO<sub>2</sub>] and different water supply**

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## Summary

- Photosynthetic stimulation by elevated  $[\text{CO}_2]$  ( $e[\text{CO}_2]$ ) may be limited by the capacity of sink organs to use photosynthates. In many legumes,  $\text{N}_2$ -fixing symbionts in root nodules provide an additional sink, so that legumes may be better able to profit from  $e[\text{CO}_2]$ . However, drought not only constrains photosynthesis but also size and activity of sinks, and little is known about the interaction of  $e[\text{CO}_2]$  and drought on carbon sink strength of nodules and other organs.
- To compare carbon sink strength, faba bean was grown under ambient (400 ppm) or elevated (700 ppm) atmospheric  $[\text{CO}_2]$  and subjected to well-watered or drought treatments, and then exposed to  $^{13}\text{C}$  pulse-labelling using custom-built chambers to track the fate of new photosynthates.
- Drought decreased  $^{13}\text{C}$  uptake and nodule sink strength, and this effect was even greater under  $e[\text{CO}_2]$ , and associated with an accumulation of amino acids in nodules. This resulted in decreased  $\text{N}_2$  fixation, increased accumulation of new

photosynthates ( $^{13}\text{C}$ /sugars) in leaves, which in turn can feed back on photosynthesis.

- Our study suggests that nodule C sink activity is key to avoid sink limitation in legumes under  $e[\text{CO}_2]$ , and legumes may only be able to achieve greater C gain if nodule activity is maintained.

**Keywords:** Climate change,  $^{13}\text{C}$  allocation, drought, legume,  $\text{N}_2$  feedback mechanism, photosynthetic acclimation, sink limitation, root exudates

## Introduction

Atmospheric  $\text{CO}_2$  concentration ( $[\text{CO}_2]$ ) is the major driver of global climate change and has increased by 45% since pre-industrial times, from 280 ppm to currently 406 ppm (NOAA, 2018). According to the RCP 8.5 scenario, atmospheric  $[\text{CO}_2]$  may surpass 700 ppm by the end of this century (IPCC, 2014). Increased  $[\text{CO}_2]$  stimulates photosynthesis of  $\text{C}_3$  crops and increases growth and yield through the “ $\text{CO}_2$  fertilisation effect” (Leakey *et al.*, 2009). However, initial stimulation of photosynthesis may diminish during long-term exposure to elevated  $[\text{CO}_2]$  ( $e[\text{CO}_2]$ ), a phenomenon known as “photosynthetic (downward) acclimation” (Long *et al.*, 2004).

Photosynthetic acclimation is related to limited sink strength (Ainsworth *et al.*, 2004; Tausz-Posch *et al.*, 2020). Sinks are the organs/tissues that consume photosynthates and their ability to acquire and utilize photosynthates is termed sink strength (Farrer, 1993). High  $[\text{CO}_2]$  greatly increases the supply of carbohydrates, but if the sink strength is adequate, photosynthetic capacity may remain unaffected (Lewis *et al.*, 2002; Sanz-S  ez *et al.*, 2010). In contrast, restricting sink growth through environmental factors (Fatichi *et al.*, 2014) may exert a negative feedback on photosynthetic capacity, causing photosynthetic acclimation whereby maximum carboxylation rate of Rubisco ( $V_{\text{cmax}}$ ) and maximum rate of electron transport ( $J_{\text{max}}$ ) are downregulated under long-term exposure to  $e[\text{CO}_2]$  (Long *et al.*, 2004; Nowak *et al.*, 2004; Ainsworth & Rogers, 2007; Leakey *et al.*, 2009). Continuous production or growth of organs, such as sustained leaf growth (Ruiz-Vera *et al.*, 2017) or

indeterminate production of generative organs (Ainsworth *et al.*, 2004) has been shown to minimise acclimation. Faster export rate of C to the sink organ also modulates sink strength through greater sink activity (Ainsworth & Bush, 2011). In legumes, not only shoots and roots but also the symbionts in nodules are additional important sinks (Arp, 1991).

In conjunction or in addition to a source-sink mediated feedback, photosynthetic acclimation is associated with a decrease in tissue N concentration, commonly observed in e[CO<sub>2</sub>] grown plant (Rogers *et al.*, 1998; Tausz *et al.*, 2013). Among other mechanisms, lower tissue N concentrations may result from inhibition in nitrate assimilation or from excessive stimulation of biomass growth that is not matched by N uptake. Some experimental evidence shows that high or excessive N supply accelerates rather than mitigates photosynthetic downward acclimation of C<sub>3</sub>-crops (Farage *et al.*, 1998; Jifon & Wolfe, 2002) which would suggest that decreased N acquisition or availability does not fully explain photosynthetic acclimation under e[CO<sub>2</sub>] (Sims *et al.*, 1998). It seems that the balance between supply and demand for N during growth and equilibrium between production and utilization of carbohydrates at tissue and whole plant levels is crucial (Jifon & Wolfe, 2002; Leakey *et al.*, 2009).

Legumes as N<sub>2</sub>-fixers may be better able to constrain their C and N balance because they can allocate any additional fixed carbon to their nodule symbionts (*Rhizobia*) (Rogers *et al.*, 2009). This does not only constitute a potentially large and flexible carbohydrate sink, it also stimulates N<sub>2</sub> fixation in tune with carbohydrate supply (Rogers *et al.*, 2006; Aranjuelo *et al.*, 2008). In one experiment, photosynthetic acclimation to e[CO<sub>2</sub>] was observed as decreased V<sub>cmax</sub> and J<sub>max</sub> values in perennial rye grass, but not in clover grown in the same system (Rogers *et al.*, 1998; Ainsworth *et al.*, 2003). The legume clover maximized photosynthetic C gain by adjusting the balance between C and N metabolism at e[CO<sub>2</sub>] (Rogers *et al.*, 2009). That the additional C sink of nodules can help to avoid photosynthetic acclimation (Ainsworth *et al.*, 2004; Aranjuelo *et al.*, 2008) was demonstrated in an experiment where a non-nodulating soybean cultivar showed acclimation but a nodulated isogenic line did not (Ainsworth *et al.*, 2004; Rogers *et al.*, 2009).

Experimental evidence suggests that nodule number, mass and activity (C import per nodule mass) increase under e[CO<sub>2</sub>], which would increase their sink strength and enable greater C export from source leaves (Voisin *et al.*, 2003a). Moreover, C and N released by the roots as exudates also constitute a further sink, as this phenomenon contributes to resource drawdown (White *et al.*, 2016). Sink strength of different organs, whole plant C-movement and partitioning of recently fixed C can be determined directly with <sup>13</sup>CO<sub>2</sub> isotope labelling (Bromand *et al.*, 2001; Voisin *et al.*, 2003b; Oriens & Colin, 2006) but to date, there are only few such direct studies addressing how e[CO<sub>2</sub>] modifies C transport to nodules and how the relative sink strength of different organs of nodulated legumes is affected (Rogers *et al.*, 2009).

Direct effects of [CO<sub>2</sub>] on plants aside, e[CO<sub>2</sub>]-driven climate change predictions include changes in precipitation patterns, which are anticipated to cause more severe drought events in many regions (Knapp *et al.*, 2015). Such drought events may modify C-uptake capacity and will become more significant in Mediterranean dry-land environments (Parvin *et al.*, 2018; Parvin *et al.*, 2019b; Parvin *et al.*, 2020). Legumes are frequently cultivated in such drought prone agro-ecosystems, and their nodule C sink activity is likely to be more threatened by frequent and severe episode of droughts (Leport *et al.*, 1999; Peoples *et al.*, 2001; Maalouf *et al.*, 2015). A synthesis of manipulation experiments has revealed that sink and source regulate each other by feedback mechanisms (White *et al.*, 2016) but the number of studies investigating details of drought impacts on the sink strength of different organs is very limited. In legumes, drought limitation on photosynthesis may decrease carbohydrate supply to nodules, deplete carbohydrate concentrations in the nodules, and therefore constrain nodule activity and N<sub>2</sub> fixation (Parvin *et al.*, 2019b). Alternatively, soil drought can also directly decrease nodule function even if supply of photosynthates is still sufficient, a situation marked by an accumulation of sugars in nodules (Serraj *et al.*, 1999; McDowell, 2011). Drought limitation to shoot growth may limit N demand and export from nodules, leading to an accumulation of N-compounds in the nodules. Accumulating N might act as a feedback mechanism to downregulate N<sub>2</sub> fixation, which would then limit the C sink strength of nodules. Despite the critical role of the

nodule C-sink to regulate photosynthesis, its magnitude and limitation imposed by drought is unclear.

Elevated [CO<sub>2</sub>] can maintain nodule activity under drought by increasing photosynthetic C-export and decreasing drought-induced accumulation of N-compounds (Serraj *et al.*, 1998; Aranjuelo *et al.*, 2014). Elevated [CO<sub>2</sub>] may also reduce canopy water use through stomatal closure and thus counteract the drought impact on nodule functionality (Serraj *et al.*, 1998; De Luis *et al.*, 1999). An analysis by Gray *et al.* (2016) from a high rainfall agro-ecosystem with soybean demonstrated that e[CO<sub>2</sub>]-induced soil water savings under drought were variable and diminished in the longer-term. Regardless, a related study observed increased nodulation (+230 %) under the interactive effect of e[CO<sub>2</sub>] and drought (Gray *et al.*, 2013). Whether such a nodulation response can maintain activity, consume more photosynthate and therefore maintain a greater C-sink under drought has not been quantified yet.

In the present study, we grew faba bean under ambient [CO<sub>2</sub>] (~400 ppm) or elevated [CO<sub>2</sub>] (~700 ppm) environments and exposed them to one of two watering regimes, a high rainfall scenario or the simulation of a dry season in a typical Mediterranean agro-ecosystem. We chose faba bean (*Vicia faba* L.), because it has higher nodulation and N<sub>2</sub> fixation potential but also greater drought sensitivity than most other cultivated legumes (Dayoub *et al.*, 2017). Plants were grown in sand culture, which allows proper development of nodules and maximises their sink capacity by avoiding inhibition by soil N. Soil N arrests initiation, growth and activity of nodules, and also diverts photosynthate/energy to the NO<sub>3</sub><sup>-</sup> assimilation process instead of nodule symbionts for fixing atmospheric N<sub>2</sub> (Peoples *et al.*, 2012; Butterly *et al.*, 2016). A <sup>13</sup>CO<sub>2</sub> pulse labelling technique was deployed to test the following hypotheses:

1. Elevated [CO<sub>2</sub>] will increase the carbon sink-strength of nodules and other organs through greater sink size (i.e. sink biomass) and sink activity (carbon import rate per sink biomass).
2. Drought will decrease the carbon sink-strength of nodules and other organs, but the greater C supply under e[CO<sub>2</sub>] will mitigate the decrease.

3. Increased nodule carbon sink strength under e[CO<sub>2</sub>] will contribute to avoiding sugar-mediated acclimation of photosynthesis, even under drought conditions.

## Materials and Methods

### *Glasshouse conditions*

The experiment was conducted in a glasshouse at the Creswick Campus of the University of Melbourne, Victoria, Australia (37°25'24.2" S, 143°54'1.6" E, elevation 465 m) (Uddin *et al.*, 2018a). Temperature and humidity settings in the glasshouse chambers were chosen to approximate the average 2000-2015 climate data from Horsham, at the centre of the south-eastern Australian dryland cropping region. Climate data were sourced from the Australian Bureau of Meteorology (BOM) for the winter crop growing period from June to August (Station ID # 079100). The average temperature was 12±3°C, average humidity 75-80% and the photoperiod was 10±2h, whereby natural light was supplemented with fluorescent lamps (Sylvania DECOR 183, Professional-58W, Erlangen, Germany) (Uddin *et al.*, 2018b).

### *Plant growth*

Faba bean (*Vicia faba* cv. 'Fiesta') was grown in polyvinyl chloride (PVC) columns (15 cm diameter × 60 cm tall) containing white washed sand (10 kg per column) to ensure that the only N source was atmospheric N<sub>2</sub>. Seeds were inoculated with commercial Group F peat-based inoculum (WSM1455, *Rhizobium leguminosarum*, NoduleN™, New Edge Microbials Pty Ltd, Albury, NSW, Australia) before sowing. Inoculated seeds were hand sown at a depth of 2 cm (4 seeds per column). Seedlings were thinned to the two most vigorous plants per column one week after emergence. Thirty (30) ml of Hoagland N-free nutrient solution was added once per week (Hoagland & Arnon, 1950) containing the following macro and micro-nutrients: 3 mM KH<sub>2</sub>PO<sub>4</sub>, 3 mM CaCl<sub>2</sub>, 3 mM MgSO<sub>4</sub>, 0.015 mM MnSO<sub>4</sub>, 0.075 mM H<sub>3</sub>BO<sub>3</sub>, 60 gL<sup>-1</sup> FeSO<sub>4</sub>, 0.0015 mM CuSO<sub>4</sub>, 0.006 mM ZnSO<sub>4</sub>, 0.0003 mM H<sub>2</sub>MoO<sub>4</sub>. The pH of the Hoagland nutrient solution was 6.0 to 6.5, which was within the range of optimum nutrient availability for legumes (Islam *et al.*, 1980; Kane *et al.*, 2006). To ensure that well-watered and drought treated plants received the prescribed average of 40 ml and

15 ml of water day<sup>-1</sup>, respectively, well-watered plants received an additional 10 ml of water on the day when nutrients were applied, and drought treated plants did not receive any supplemental water on this and the following day.

#### *CO<sub>2</sub> treatments*

Two glasshouse chambers were used to apply two [CO<sub>2</sub>] treatments; ambient CO<sub>2</sub> (a[CO<sub>2</sub>]) (~400 ppm) and elevated CO<sub>2</sub> ([CO<sub>2</sub>]) (~700 ppm) from June to July in each of two consecutive years (2016 and 2017). To avoid unspecific chamber effects, columns and CO<sub>2</sub> treatments were shifted weekly among chambers. Moreover, columns were arranged randomly in the respective glasshouse chamber.

#### *Water treatments*

In each [CO<sub>2</sub>] chamber, columns were randomly assigned to two groups 10 days after sowing (DAS): eight columns to well-watered and eight to drought stress per CO<sub>2</sub> chamber each year. Water treatments approximated the variability of rainfall in the south-eastern Australian dryland cropping area, using June-July amounts of a high rainfall zone and a low rainfall zone as guidance. Well-watered plants received a mean of 40 ml water each day, corresponding to average precipitation of 2.4 mm day<sup>-1</sup>, whereas drought treated plants received 15 ml water equivalent to 0.9 mm rain day<sup>-1</sup>. Water status of the column was monitored using time domain reflectometer (TDR, Theta Probe, ML3, Delta-T Devices, Cambridge, UK) every three days from sowing to harvest at 20, 40 and 60 cm depth respectively. Averaged soil water within the column is reported in Fig. S1.

#### *<sup>13</sup>CO<sub>2</sub> pulse labelling*

At the stem elongation stage of faba bean (according to the BBCH-scale as described by Lancashire *et al.* (1991); about 60 DAS, days after sowing), on clear sunny days, plants were <sup>13</sup>C pulse-labelled. <sup>13</sup>CO<sub>2</sub> pulse-labelling was carried out in an air-tight chamber (100 cm long × 70 cm wide × 60 cm height; made of highly transparent plexiglass) fitted onto a stainless-steel frame of 50 cm height (Fuchslueger *et al.*, 2014). A detailed description and illustration of the chamber set up were given by



Butterly *et al.* (2015) and slight modifications were made to allow better utilisation of  $^{13}\text{CO}_2$  by the plant canopy. Briefly, eight columns with faba bean plants were sealed into fitting holes in the bottom of the plexiglass chambers so that the canopies were inside the chamber. The top of each column was sealed with plastic film around the plant stem to prevent direct  $\text{CO}_2$  absorption by the soil or below ground organs of the plants. Temperature and relative humidity (RH) were monitored and logged by a portable sensor (LU-MCH-383SD, ECEFast, Australia) mounted just above the canopy. Chamber conditions were  $20\pm 2^\circ\text{C}$  temperature,  $40\pm 3\%$  RH and  $\sim 1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetic photon flux density. After the canopies were sealed into the chamber, the soda-lime trap was started and once  $[\text{CO}_2]$  was approximating zero (trapped by soda lime) after 1 hour,  $^{13}\text{CO}_2$  was generated by the addition of  $\text{H}_2\text{SO}_4$  as described elsewhere (Bromand *et al.*, 2001). The  $^{13}\text{CO}_2$  concentrations were maintained at the chamber growth conditions of the plants, that is,  $400\pm 10$  or  $700\pm 10$  ppm, respectively (Palta *et al.*, 1994; Zong & Shangguan, 2016) by continuously adjusting the emission and ventilation of  $^{13}\text{CO}_2$  inside the chambers.  $[\text{CO}_2]$  inside the chamber was monitored and recorded during the labelling period with an infrared gas analyser (IRGA, Li-6400, Li-Cor Inc, Lincoln, NE, USA) (Fig. S2). At the end of the labelling period, air from the chamber was forced to pass through a soda-lime cartridge that trapped the unassimilated  $^{13}\text{CO}_2$ . Plants were exposed to a  $^{13}\text{CO}_2$  enriched atmosphere during active photosynthesis period for 6 hours (9.00-15.00) and then transferred to their respective growth conditions in the glasshouse.

#### *Photosynthetic parameters*

Light saturated photosynthetic  $\text{CO}_2$  uptake ( $A_{\text{sat}}$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), stomatal conductance ( $g_s$ ,  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) and internal  $[\text{CO}_2]$  ( $C_i$ , ppm) measurements were performed on the day after  $^{13}\text{CO}_2$  labelling. Fully expanded apical leaves were measured using an infrared gas analyser (IRGA) system (Li- 6400, Li-Cor, Lincoln, NE, USA). Briefly, leaves were placed in the measurement cuvette at their growth  $[\text{CO}_2]$  (i.e. 400 ppm or 700 ppm) at a saturating light level of  $1500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ . Block temperature was held constant at  $25^\circ\text{C}$ , and relative humidity was stabilized at 45–55% during measurements. Once steady state of photosynthesis ( $A$ ) was reached, measurements of  $A$ ,  $C_i$ , and  $g_s$  were recorded at the growth  $[\text{CO}_2]$ ;  $[\text{CO}_2]$

was then decreased step-wise to 50  $\mu\text{mol mol}^{-1}$ , increased again to the growth  $[\text{CO}_2]$ , and then increased stepwise to 1800 ppm  $[\text{CO}_2]$ . A minimum of 10 data points was collected for each plant and the  $\text{ACi}$  data were fitted to the biochemical model of photosynthesis (Farquhar *et al.*, 1980) to obtain maximum rate of carboxylation ( $V_{\text{cmax}}$ ), maximum electron transport rate ( $J_{\text{max}}$ ) and respiration ( $R_{\text{d}}$ ) following the methods outlined by Long and Bernacchi (2003). The 'plantecophys' package implemented in R was used to estimate  $V_{\text{cmax}}$ ,  $J_{\text{max}}$  and  $R_{\text{d}}$  with the function "fitaci" (Duursma, 2015).  $V_{\text{cmax}}$  and  $J_{\text{max}}$  were expressed per unit leaf area basis.

#### *Plant harvest*

Plants were harvested 24 hours after pulse labelling. A 24 hours chasing period is well suited to characterise the allocation fate of new photosynthates (Kouchi *et al.*, 1986). Plants were separated into leaf, stem, root, and nodule. Sub-samples of leaf and nodule tissues were immediately frozen in liquid  $\text{N}_2$  and stored in  $-80^\circ\text{C}$  for amino acid analysis. Leaf area was measured using a leaf area meter (LI-3100C, LI-COR, Lincoln, NE, USA). All biomass was dried at  $65^\circ\text{C}$  for 72 hours and weighed. Leaf mass area (LMA) was calculated as the ratio of leaf mass (g) to leaf area ( $\text{m}^2$ ).

#### *$^{15}\text{N}$ and $^{13}\text{C}$ isotopic analysis*

Finely ground plant tissue samples (leaves, stems, roots, nodules) were analysed for total N (% of tissue dry weight), total C (% of tissue dry weight),  $^{15}\text{N}$  and  $^{13}\text{C}$  (atom %) by isotope ratio mass spectrometry (IRMS) (Hydra 20–20, SerCon, Crewe, UK) coupled to an elemental analyser (Thermo Scientific Flash 2000). As the plants were grown in pure sand without exogenous N sources, all N accumulated in the biomass was considered to have been fixed from atmospheric  $\text{N}_2$  (Aranjuelo *et al.*, 2008). Moreover,  $^{15}\text{N}$  atom % from all organs were equivalent to the atmospheric air value (0.3663 atom %) (Unkovich *et al.*, 1997), which confirmed that the only source of N in these faba bean plants was aerial  $\text{N}_2$ .

Nodule activity was calculated as the amount of  $\text{N}_2$  fixed (total plant N) divided by final nodule dry weight at harvest (Naudin *et al.*, 2011; Parvin *et al.*, 2019a).

The  $^{13}\text{CO}_2$  uptake by leaves and translocation to other organs was determined by multiplying the C content of an organ fraction with the  $^{13}\text{C}$  excess (atom %) of this fraction over the  $^{13}\text{C}$  (atom %) of an unlabelled reference group as described by Fischinger and Schulze (2010).

$$^{13}\text{C fixed (mg)} = \text{C (mg)} \times [^{13}\text{C labelled (mg}^{13}\text{C)} - ^{13}\text{C reference (mg}^{13}\text{C)}] / 100(\text{mg C})$$

The reference group consisted of plants grown under the same conditions and treatments in the glasshouse but not subjected to pulse labelling.

#### *Estimation of sink strength*

Sink strength of each organ was estimated by the following equation (Farrar, 1993):

$$\text{Sink strength (nmol C s}^{-1}\text{)} = \text{sink size (g)} \times \text{sink activity (nmol C g}^{-1}\text{ s}^{-1}\text{)}$$

where sink size was the total biomass of the sink tissues (g) and sink activity was the specific uptake rate of C per unit of time per gram tissue dry weight (nmol C g<sup>-1</sup> s<sup>-1</sup>). Sink activity was calculated from the amount of labelled  $^{13}\text{C}$  partitioned to each organ after the 24 hours chase period and expressed as nmol C g<sup>-1</sup> s<sup>-1</sup>.

#### *Measurement of root exudates*

After harvesting the plants, intact root systems including the rhizosphere sand adhering to the roots were removed from the columns and immersed in 100 mL deionized water for 30 minutes. Eluates were filtered through membrane filters (0.45  $\mu\text{m}$ , EMD Millipore TM, Thermo Fisher Scientific, NSW, Australia) to remove sand particles and root debris, and aliquots were analysed for total C and  $^{13}\text{C}$  excess (atom%) by isotope ratio mass spectrometry (IRMS) coupled to an elemental analyser. To estimate the amount of  $^{13}\text{C}$  released by root exudation, the below equation was followed (Fischinger & Schulze, 2010) and  $^{13}\text{C}$  in exudates was expressed per g root dry weight (Phillips *et al.*, 2009).

$$^{13}\text{C exudate (mg)} = \text{C (mg)} \times [^{13}\text{C labelled exudate (mg}^{13}\text{C)} - ^{13}\text{C reference exudate (mg}^{13}\text{C)}] / 100(\text{mg C})$$

As we did not detect  $^{13}\text{C}$  excess (atom %) in bulk sand, we assumed that all  $^{13}\text{C}$  exudated by the roots was present in the rhizosphere zone.

### *Biochemical analysis*

Leaves and nodule tissues were analysed for soluble sugars and starch as described by Parvin *et al.* (2019a). Briefly, total soluble sugars was determined from oven dried and finely ground leaves and nodule tissues with the anthrone method (Yemm & Willis, 1954) modified for use in a plate reader (Tecan Sunrise, Tecan, Austria; (Tausz-Posch *et al.*, 2015). Measurements were done at 600 nm using D-fructose as standard and total sugars concentration expressed as mg fructose-equivalent  $\text{g}^{-1}$  dry weight. The retained pellets (Sheng *et al.*, 1993) were used for analysis of starch at 505 nm as described by Edwards *et al.* (2011) using glucose as standard. Sugars and starch contents of leaves were expressed on a leaf surface area basis ( $\text{g m}^{-2}$ ) by multiplying concentration ( $\text{mg g}^{-1}$ ) with leaf mass area ( $\text{g m}^{-2}$ ).

Total free amino acid concentration (AA) of leaves and nodule tissues were determined by acid ninhydrin method (Yemm *et al.*, 1955). Absorbance (at 570 nm wavelength) was measured in a plate reader (Tecan Sunrise, Tecan, Austria). The concentration of AA was calculated as mixed amino acid standard (Amino Acid Standard AAS18, Sigma-Aldrich) equivalent and expressed as  $\mu\text{mol g}^{-1}$  dry weight.

Xylem sap was collected by the centrifugation method from the freshly harvested stem sections (Dannel *et al.*, 1995). For xylem sap collection, the stem was cut with a sharp blade and to avoid contamination, close the phloem and remove cell bleeding, the cut surface was rinsed with 1 M  $\text{CaCl}_2$  solution for 1 minute (Fischinger & Schulze, 2010). Immediately after collection, the concentration of AA in the sap was measured as above and was expressed as  $\mu\text{M}$ .

### *Statistical analysis*

The experiment consisted of 2 [ $\text{CO}_2$ ]  $\times$  2 levels of water supply (well-watered and drought)  $\times$  4 replications (columns with two plants each) in each of two years. A

similar set of reference plants was grown under similar [CO<sub>2</sub>] and watering supply level with four replications and therefore, the total experiment consisted of 32 columns (16 used for pulse labelling and 16 for reference) in each of the two years. As in a first analysis there were no statistical differences between years, data from both years were analysed jointly. Values presented in this experiment correspond to mean values of data collected during the two consecutive years (n=8).

Linear mixed-effect models were fit by REML using the R (R Core Team, 2019) package “nlme” (Pinheiro *et al.*, 2017) considering [CO<sub>2</sub>] and water regimes as fixed effects and replications as random effect. The results were accepted as significant at  $P < 0.05$ .  $P$  values between 0.05-0.1 are presented for discussion purposes.

## Results

### *Soil water*

Well-watered columns had soil water close to field capacity (Fig. S1). Drought decreased soil water as the season progressed and the soil reached permanent wilting point at harvest. Soil water content in well-watered columns was greater under e[CO<sub>2</sub>]. In contrast, drought decreased soil water to a greater extent under e[CO<sub>2</sub>] than a[CO<sub>2</sub>].

### *Leaf photosynthetic parameters*

Elevated [CO<sub>2</sub>] stimulated photosynthesis ( $A$ ) of faba bean and this stimulation was greater under well-watered (50%) than drought conditions (20%). Stomatal conductance ( $g_s$ ) was lower under e[CO<sub>2</sub>] which was further accentuated by drought. Elevated [CO<sub>2</sub>] increased intrinsic water use efficiency (iWUE; ratio of  $A$  and  $g_s$ ); this increase tended to be greater under drought than well-watered conditions (Table 1).

Under well-watered conditions, e[CO<sub>2</sub>] grown plants had no depression of maximum photosynthetic carboxylation capacity ( $V_{cmax}$ ) and appeared to have even greater maximum capacity for electron transport ( $J_{max}$ ) compared to a[CO<sub>2</sub>] grown plants (Fig. 1a, b). In contrast, in drought stressed plants there was a trend towards an acclimatory

response to  $e[\text{CO}_2]$  as demonstrated by lower (-29%)  $V_{\text{cmax}}$  and lower (-17%)  $J_{\text{max}}$  compared to  $a[\text{CO}_2]$  grown plants. The ratio of  $J_{\text{max}}$  to  $V_{\text{cmax}}$  was always greater under  $e[\text{CO}_2]$  and this pattern was maintained under drought (Fig. 1c). Respiration rate as derived from  $A/C_i$  curves ( $R_d$ ) was stimulated by  $e[\text{CO}_2]$  (+82%) when plants were well-watered but decreased by 55% under drought (Fig. 1d).

#### *Plant growth and N status*

Elevated  $[\text{CO}_2]$  significantly increased total biomass of faba bean plants and this increase was greater under well-watered (+49%) than drought conditions (+39%) (Table 1). Leaf and stem biomass increased under  $e[\text{CO}_2]$  and decreased under drought. Root biomass was affected by an interaction between  $[\text{CO}_2]$  and watering regimes such that  $e[\text{CO}_2]$  grown plant invested more biomass into roots, leading to an increased root:shoot ratio and this effect was greater under well-watered than drought conditions. Leaf area and leaf mass area (LMA) were decreased (by 6%) by drought.

Regardless of watering regime,  $e[\text{CO}_2]$  increased nodule biomass (~40%), whereas drought decreased it by 25% irrespective of  $[\text{CO}_2]$ . Nodule number was stimulated by  $e[\text{CO}_2]$  with stronger stimulation under well-watered (50%) than drought (43%) conditions. Drought decreased nodule biomass by about a third in both  $a[\text{CO}_2]$  and  $e[\text{CO}_2]$  (Table 1). Elevated  $[\text{CO}_2]$  increased nodule activity (+28%) in well-watered plants and decreased it by 14% in drought treated plants (significant interaction, Table 1).

Drought depressed leaf [N] to a greater extent under  $e[\text{CO}_2]$  (29%) than  $a[\text{CO}_2]$  (5%). Drought also depressed root [N] and nodule [N] but had no effect on stem [N]. Elevated  $[\text{CO}_2]$  increased total  $\text{N}_2$  fixation to a greater extent under well-watered (+60%) than drought conditions (+21%) (Table 1).

#### *$^{13}\text{C}$ incorporation and C sink strength*

The total amount of  $^{13}\text{C}$  incorporated into biomass was greater in  $e[\text{CO}_2]$  grown plants, which was associated with greater photosynthesis and greater leaf biomass and area

(Fig. 2; Table 1). In leaves, e[CO<sub>2</sub>] grown and well-watered plants had more <sup>13</sup>C incorporated, and there was no interaction between [CO<sub>2</sub>] and drought treatment (Fig. 2a). Incorporation of <sup>13</sup>C in stems was not significantly affected by either water or CO<sub>2</sub> treatments (Fig. 2b), and incorporation in roots was greater under e[CO<sub>2</sub>] (Fig. 2c). Under well-watered conditions, nodules of e[CO<sub>2</sub>]-grown plants incorporated more <sup>13</sup>C than under a[CO<sub>2</sub>], but this was reversed under drought (Fig. 2d).

Elevated [CO<sub>2</sub>] increased nodule sink activity under well-watered conditions, but decreased it under drought, with a similar trend for leaves. Sink activity of roots and stems were unaffected by either drought or e[CO<sub>2</sub>] (Fig. 3). Elevated [CO<sub>2</sub>] increased <sup>13</sup>C released by roots, and this increase was greater under drought than well-watered conditions (significant interaction, [CO<sub>2</sub>] × W) (Fig. 4). Elevated [CO<sub>2</sub>] increased nodule specific <sup>13</sup>C fixation under well-watered conditions and decreased it under drought. A similar trend was also detected in nodule specific <sup>15</sup>N fixation (Fig. 5).

#### *Carbohydrate and amino acid concentrations*

Drought increased concentrations of sugars and starch in leaves to a greater extent under e[CO<sub>2</sub>] than a[CO<sub>2</sub>]. On a leaf surface area basis, similar trends were detected for both sugars and starch content (Table 2). In contrast, nodules contained higher concentrations of sugars under well-watered conditions than under drought (Table 2). Starch concentrations were on average slightly greater in both organs under e[CO<sub>2</sub>], but drought stimulated starch accumulation in leaves and depletion in nodules. Drought decreased total free amino acid (AA) concentrations in leaves but increased AA concentration in nodules (Table 2) and this increase was greater in e[CO<sub>2</sub>] (+46%) than a[CO<sub>2</sub>] (+19%). Concentrations of AA were also greater in the xylem sap of drought treated plants and whilst e[CO<sub>2</sub>] slightly depressed xylem sap concentrations of amino acids under well-watered conditions, it slightly increased them under drought (resulting in a significant [CO<sub>2</sub>] × W interactions, Table 2). In leaves, the ratio of amino acids to sugars was lower under drought and e[CO<sub>2</sub>]. Drought increased amino acids: sugars ratio in nodules, and this effect was greater in e[CO<sub>2</sub>] grown plants (significant interaction, [CO<sub>2</sub>] × W) (Fig. S3).

## Discussion

The drought treatment in our study decreased  $g_s$  by 50%, and the averaged value of  $g_s$  at or above  $0.1 \text{ mol m}^{-2} \text{ s}^{-1}$  (Table 1) suggested that plants were exposed to mild to moderate drought stress (Flexas *et al.*, 2006a). Stomatal closure limits photosynthesis under moderate stress, but direct metabolic impairments or physiological damage would only be expected when  $g_s$  falls below  $0.10 \text{ mol m}^{-2} \text{ s}^{-1}$  (Flexas *et al.*, 2006a; Flexas *et al.*, 2006b). Soil water content declined gradually with advancing plant growth and reached values close to the permanent wilting point at harvest (Fig. S1), indicating moderate drought conditions. Such drought could induce sink limitations by retarding sink growth and cause feedback on photosynthetic capacity (Grassi & Magnani, 2005; Pinheiro & Chaves, 2010).

Because  $e[\text{CO}_2]$  commonly decreases  $g_s$ , it has been assumed that the “ $\text{CO}_2$  fertilization effect” will be greatest under dry conditions (Kimball, 2016). Elevated  $[\text{CO}_2]$ -induced decreases in  $g_s$  may decrease overall plant water use, but stimulation by  $e[\text{CO}_2]$ -driven transpiring leaf area and elevated canopy temperature may overcompensate so that soil water use is greater under drought (Manea & Leishman, 2014). Our results are consistent with Manea and Leishman (2014), as the reduction in  $g_s$  (by 37%) under  $e[\text{CO}_2]$  was apparently overcompensated by the increase in leaf area (by 50%), so that we observed greater soil water depletion under drought conditions (Table 1). Greater stomatal sensitivity of  $e[\text{CO}_2]$ -grown plants to drying soils can ultimately eliminate the stimulation of C gain (Gray *et al.*, 2016).

*Hypothesis 1: Elevated  $[\text{CO}_2]$  increased sink strength of all organs due to increased sink size but greater sink activity was only detected in nodules*

This study investigated the sink strength (sink size  $\times$  activity) of different organs directly using the  $^{13}\text{CO}_2$  pulse labelling approach (Voisin *et al.*, 2003b). Sink size, i. e. the total C content of the sink organ reflects that larger organs are likely to accumulate more C than smaller ones, while sink activity (uptake rate per organ mass) indicates that metabolically more active tissues import more C per unit of time (White *et al.*, 2016). In the faba bean plants in our study, leaves had the greatest C sink strength, about double the strength of nodules, stems, or roots (Fig. 2). This ranking was



dominated by sink size, whereas nodules ranked first in terms of sink activity, i.e. they imported most C per g organ weight per unit of time. Previous experiments with  $^{13}\text{C}$  labelling also showed strong sink activity of nodules for recent photo-assimilates (Lawrie & Wheeler, 1973; Voisin *et al.*, 2003b), but these studies were conducted under  $a[\text{CO}_2]$ .

To test our first hypothesis, sink strengths of all organs were compared in well-watered plants, where  $e[\text{CO}_2]$  increased sink strength (i.e. total  $^{13}\text{C}$  incorporation) of leaves, roots and nodules by 40-50% over  $a[\text{CO}_2]$ . Increased sink strength might be correlated with greater sink size, sink activity or a combination of both. The present study showed that  $e[\text{CO}_2]$  increased the sink strength of leaves, roots and nodules, yet sink activity increased especially in nodules (Fig. 3). Development of new sinks such as new leaves, more roots and greater nodulation under  $e[\text{CO}_2]$  demonstrates increased sink size and confirmed findings on *Pisum sativum* L. (Voisin *et al.*, 2003a).

Increased sink activity of nodules under  $e[\text{CO}_2]$  suggested that nodules were the most competitive sink for recently fixed C, consistent with other reports (Voisin *et al.*, 2003b; Voisin *et al.*, 2003a; Aranjuelo *et al.*, 2008). This is probably associated with increased respiratory activity, the main C cost for  $\text{N}_2$  fixation. As we did not measure  $^{13}\text{CO}_2$  lost by respiration in our experiment, the actual  $^{13}\text{C}$  to nodules would have been even greater (by whatever fraction of the  $^{13}\text{C}$  was respired during the labelling and chase period) as reported elsewhere (Rainbird *et al.*, 1984; Voisin *et al.*, 2003a; Fischinger & Schulze, 2010). Despite this fact,  $^{13}\text{C}$  enrichment in nodules was linked with greater  $\text{N}_2$  fixation, which strengthens our findings.

Hypothesis 2: Drought decreased sink strength of all organs and the effect was greater in nodules grown under  $e[\text{CO}_2]$

In our study, the biomass growth of all organs decreased under drought compared to well-watered plants. In line with biomass, sink strength also decreased due to

decreasing sink size, leading to proportionally lower  $^{13}\text{C}$  assimilation in drought-stressed plants. Simultaneous decrease of photosynthetic C gain and sink capacity under drought has been observed previously (Muller *et al.*, 2011; Pallas *et al.*, 2013). One study suggested that drought decreased the distribution of assimilate through inhibition of vascular transport and reduction of sink activity rather than size (Galvez *et al.*, 2005). For nodules, our  $^{13}\text{C}$  partitioning data showed such a reduction in sink activity and a related decrease in the transport of recently fixed  $^{13}\text{C}$  transport to bacteroids. In contrast, sink activity of leaves was only slightly reduced under drought and that of stems and roots unchanged, which indicates that the sink strength of organs except nodules is determined by constraints on growth rather than by a more rapid change in C consumption.

Previous studies suggested that  $e[\text{CO}_2]$  can ameliorate the effects of drought on nodule activity either by maintaining greater water content in the soil surrounding root nodules (through improved plant water use efficiency translating into reduced transpiration) or by maintaining greater supply of C to the nodules and greater N demand in growing plant organs (Rogers *et al.*, 2009; Gray *et al.*, 2013). The drought treatment applied in our study,  $e[\text{CO}_2]$  did not lead to any soil water savings (Fig. S1). Soil water may have been depleted even more under  $e[\text{CO}_2]$  and this coincided with decreased nodule  $\text{N}_2$  fixation activity (Table 1). As we used sand with low water holding capacity in our experiment, cumulative effects of improved water use efficiency on stored soil moisture may have been precluded, nevertheless, our results are in line with a multiyear FACE study in soybean (Gray *et al.* 2016). In that study,  $e[\text{CO}_2]$ -stimulation of leaf area offset the potential water savings via lower  $g_s$ , leading to greater soil water depletion under drought with little to no stimulation of  $\text{N}_2$  fixation (Gray *et al.*, 2016).

The second hypothesis we tested was that  $e[\text{CO}_2]$  will maintain C sink strength of nodules under drought conditions. In contrast to our original hypothesis,  $^{13}\text{C}$  labelling in our study showed that nodule C sink strength decreased markedly under drought, and even more so at  $e[\text{CO}_2]$ , and while sink activity was nearly unchanged by drought alone, it was depressed by the combination of drought and  $e[\text{CO}_2]$  (Fig. 3). Several

mechanisms might affect nodule sink activity under  $e[\text{CO}_2]$  and drought such as decreased sugars export to the nodules, or feedback by amino acid accumulation in nodules (Galvez *et al.*, 2005; Aranjuelo *et al.*, 2014), which could also result from limitations to amino acid export (Garneau *et al.*, 2018) through decreased xylem flow (Robredo *et al.*, 2011). Although leaves had higher concentrations of  $^{13}\text{C}$ /sugars under  $e[\text{CO}_2]$  in our study, we observed a significant decline of  $^{13}\text{C}$  incorporation and sugars in nodules under drought, especially in  $e[\text{CO}_2]$  grown plants (Fig. 3 and Table 2). Despite overall decreases of  $^{13}\text{C}$  in nodules,  $^{13}\text{C}$  export to root exudates was greater under  $e[\text{CO}_2]$ . Apparently, C transport to the root exceeded C demand of roots and nodules, and some of the excess C was exuded into the rhizosphere under drought (Palta & Gregory, 1997). Some of the  $^{13}\text{C}$  signal in our exudate fraction may have been respiratory  $\text{CO}_2$  trapped in the soil solution as  $\text{HCO}_3^-$  according to the pH dependent  $\text{CO}_2 - \text{HCO}_3^-$  equilibrium in aqueous systems (Manahan, 2017). Although we did not specifically measure pH in the soil solution, it was likely in the range between 6 and 7 (as the pH of the added nutrient solution was just above 6), where about 50% or less of the dissolved  $\text{CO}_2$  pool would be in the form of  $\text{HCO}_3^-$ . As increased  $\text{N}_2$ -fixation in nodules comes at increased respiratory cost, some of the measured increased  $^{13}\text{C}$  signal in the root exudate fraction may have resulted from greater root (and nodule) respiration under  $e[\text{CO}_2]$ . This would however not explain the even greater increase under  $e[\text{CO}_2]$  of  $^{13}\text{C}$  in the exudate fraction in drought treated plants, where  $\text{N}_2$ -fixation was less stimulated by  $e[\text{CO}_2]$ . Furthermore, we did not detect excess  $^{13}\text{C}$  in soil outside the root zones, suggesting that the observed signal was mainly due to exudates remaining closely associated with roots rather than highly mobile compounds (Walker *et al.*, 2003).

A decrease in nodule C demand seemed associated with decreased N export from the nodules towards the aboveground organs, marked by the enhanced accumulation of free amino acids in nodules and xylem sap. As xylem flow would be lower under  $e[\text{CO}_2]$  (due to decreased  $g_s$ ), the export of fixation products may have been impaired even more under  $e[\text{CO}_2]$ . Increased accumulation of amino acids in nodules and xylem sap could exert feedback on nodule activity (Serraj *et al.*, 1998). Long-distance transport of amino N and signal transduction involves amino acid transporters, as described in depth elsewhere (Koch *et al.*, 2003; Tegeder & Masclaux-Daubresse,

2018). There is evidence from experiments on common bean and soybean (where the fixed N is transported in form of ureides rather than amino acids) that overexpressing relevant ureid transporters can increase N<sub>2</sub> fixation, apparently overcoming a key limitation and alleviating any feedback inhibition (Carter & Tegeder, 2016). How amino acid transporters influence N fixation, fluxes and feedback under drought and e[CO<sub>2</sub>] would be interesting to examine, and may help to optimise legume cultivars for a future climate (Tegeder & Masclaux-Daubresse, 2018).

*Hypothesis 3: Decreased nodule sink activity under e[CO<sub>2</sub>] and drought was associated with negative feedback mechanisms, thus led to photosynthetic acclimation*

In well-watered plants, the concentration of soluble sugars in leaves was not increased by e[CO<sub>2</sub>]. Sugars concentrations increased in nodules, indicating rapid export of additional assimilate to the nodules, which showed increased C sink strength under e[CO<sub>2</sub>]. Under e[CO<sub>2</sub>], increases in C transport to nodules may enhance nodular activity and in turn supply more N for plant growth (Guo *et al.*, 2013; Irigoyen *et al.*, 2014). However, decreased sink capacity/activity may lead to increased leaf carbohydrate concentration under e[CO<sub>2</sub>] growth conditions (Ainsworth *et al.*, 2004; Rogers *et al.*, 2004), which then can act as a signal to down-regulate the capacity of the photosynthetic apparatus.

To test our third hypothesis, we investigated how nodule sink strength was related to photosynthetic acclimation in e[CO<sub>2</sub>] grown plants in response to drought. In contrast to our hypothesis, growth under e[CO<sub>2</sub>] did not maintain sink activity of nodules under drought, and photosynthetic acclimation was observed (Fig. 1). Drought depressed nodule sink activity even more under elevated e[CO<sub>2</sub>] (Fig. 3). The question arises whether this was due to a direct drought effect on nodules specifically of e[CO<sub>2</sub>] grown plants, a limitation of C supply to the nodules, or whether a decrease in aboveground N demand led to feedback inhibition on N<sub>2</sub> fixation in nodules. Given that soil water contents were affected by drought, but not strongly different between e[CO<sub>2</sub>] and a[CO<sub>2</sub>] grown and drought treated plants, it seems unlikely that nodules of e[CO<sub>2</sub>] grown plants had experienced (much) stronger direct soil drought effect. And although carbohydrate concentrations were moderately lower in nodules of drought

stressed e[CO<sub>2</sub>]-grown plants compared to those in the a[CO<sub>2</sub>] and drought treatment, high concentrations of leaf carbohydrates in these plants suggest that there would have been sufficient carbohydrates available for translocation to nodules. Increases in root exudation also corroborate that it was not a limitation to carbohydrate availability alone that triggered the strong effect of drought × e[CO<sub>2</sub>] on nodule sink activity. This leaves a feedback mechanism on nodule activity as illustrated in Fig. 6 and also suggested by previous work on drought-treated soybean (Serraj *et al.*, 1998) and faba bean in a FACE experiment (Parvin *et al.*, 2019b). Drought inhibits growth and organ expansion, and therefore might decrease the N demand of roots and aboveground organs. Import of N into these organs decreases, hampering export of N from nodules, where accumulation of amino acids marks such a feedback on N<sub>2</sub>-fixation. This effect may be stronger for e[CO<sub>2</sub>] grown plants with their greater organ biomass. As nodule activity is downregulated, an important C sink becomes inactive, leading to sink limitation and photosynthetic acclimation to e[CO<sub>2</sub>].

In conclusion, our results confirm that the ability to enhance nodule sink strength is an important mechanism that can enable legumes to maximise C gain and N<sub>2</sub> fixation in a future e[CO<sub>2</sub>] atmosphere. Even though the experiments for this study were done in a glasshouse under moderate drought conditions, the results are consistent with the concepts developed in field studies under e[CO<sub>2</sub>] on this (Parvin *et al.*, 2019b) and other legume species (Parvin *et al.*, 2018; Parvin *et al.*, 2019a). Consistent with those field experiments, our results show that interactions with climate change related stresses such as drought may obliterate a positive effect of e[CO<sub>2</sub>] on legume N<sub>2</sub>-fixation. It will be important to fully understand environmental effects on trade-offs between C assimilation and N<sub>2</sub> fixation in legumes under e[CO<sub>2</sub>], as this could allow to select for traits related to legume source and sink strengths with a view to optimise crop selection for future conditions. The drought treatment in our experiment was modelled on realistic scenarios in Australian dryland cropping regions representative for similar agroecosystems worldwide. Even when droughts become more severe, optimising source-sink balances in legume varieties based on such experimental results seems promising, because it would still confer benefits during the transition period through mild and moderate drought, before severe drought may obliterate any positive effect of e[CO<sub>2</sub>] (Gray *et al.*, 2016). Although there is no reason to believe

that the principles shown in this study on faba bean would not apply to other legumes and hold over a range of environmental conditions, it is important to note that trade-offs between C assimilation and N<sub>2</sub> fixation depend on a number of finely balanced quantitative traits, which may be different between species and will require investigations across a greater variety of environmental conditions to evaluate the practical potential for targeted selection.

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### **Author contribution**

SP, SU, MT: designed and conducted experiments, data collected, analysed, interpreted and wrote the manuscript.

STP, RA: supported experimental settings, reviewed and edited the draft manuscript

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**Table 1.** Gas exchange parameters, biomass growth and nitrogen concentration ([N]) of faba bean grown under two CO<sub>2</sub> concentrations (ambient [CO<sub>2</sub>]: a[CO<sub>2</sub>], ~400 μmol mol<sup>-1</sup> and elevated [CO<sub>2</sub>]: e[CO<sub>2</sub>], ~700 μmol mol<sup>-1</sup>) and two water (W) regimes (well-watered and drought ) and harvested at 60 DAS.

Growth parameters	Well-watered		Drought		P-value		
	a[CO <sub>2</sub> ]	e[CO <sub>2</sub> ]	a[CO <sub>2</sub> ]	e[CO <sub>2</sub> ]	[CO <sub>2</sub> ]	W	[CO <sub>2</sub> ] × W
<b>Gas exchange parameters</b>							
Photosynthesis (A)	20.33±0.48	30.64±0.62	15.04±0.52	18.29±1.50	<0.001	<0.001	<0.001
Stomatal conductance (g <sub>s</sub> )	0.29±0.01	0.21±0.03	0.16±0.03	0.10±0.01	<0.001	0.001	0.073
iWUE	70.14±2.89	159.08±18.89	140.58±38.69	210.57±15.7	<0.001	0.002	0.079
<b>Growth attributes</b>							
Total biomass (g plant <sup>-1</sup> )	4.77±0.22	7.12±0.21	3.55±0.13	4.95±0.19	<0.001	<0.001	0.013
Leaf biomass (g plant <sup>-1</sup> )	1.23±0.13	1.81±0.11	1.08±0.08	1.63±0.09	<0.001	0.091	ns
Stem biomass (g plant <sup>-1</sup> )	2.27±0.14	2.58±0.08	1.74±0.04	2.12±0.15	0.004	<0.001	ns
Root biomass (g plant <sup>-1</sup> )	1.04±0.13	2.44±0.13	0.58±0.11	0.97±0.09	<0.001	<0.001	<0.001
Root: shoot (R/S) ratio	0.374±0.045	0.628±0.039	0.265±0.040	0.334±0.043	0.001	<0.001	0.034
Leaf area (cm <sup>2</sup> plant <sup>-1</sup> )	377.10±10.33	550.32±20.63	273.66±20.06	411.01±19.7	<0.001	<0.001	ns
Leaf mass area (LMA)	32.98 ±3.52	32.99±2.22	40.59±3.55	40.68±3.51	ns	0.017	ns
Nodule biomass (g plant <sup>-1</sup> )	0.231±0.02	0.290±0.02	0.159±0.01	0.246±0.01	<0.001	<0.001	ns
Nodule number (plant <sup>-1</sup> )	150.76±8.89	227.13±8.24	101.85±5.13	145.67±6.04	<0.001	<0.001	0.033



Nodule activity	765.15±56.36	942.25±91.89	663.89±51.94	627.28±34.9	ns	<0.023	0.025
N <sub>2</sub> fixation (mg plant <sup>-1</sup> )	164.88±7.15	250.28±8.66	123.08±7.17	139.29±4.79	<0.001	<0.001	<0.001
<b>N concentration</b>							
Leaf [N] (% dwt)	5.19±0.28	5.31±0.30	4.92±0.19	3.75±0.16	ns	<0.001	0.008
Stem [N] (% dwt)	2.12±0.18	2.43±0.17	2.19±0.12	1.98±0.11	ns	ns	0.090
Root [N] (% dwt)	2.94±0.05	3.14±0.15	2.66±0.15	2.52±0.11	ns	0.001	ns
Nodule [N] (% dwt)	6.85±0.27	6.83±0.17	6.33±0.27	5.78±0.15	0.071	<0.001	0.086
N <sub>leaf</sub> (g N m <sup>-2</sup> )	1.70±0.13	1.65±0.11	1.96±0.14	1.64±0.16	ns	ns	ns

Gas exchange parameters were measured at growth [CO<sub>2</sub>]. Means ±SE (n=8) and statistical results (mixed effect model with [CO<sub>2</sub>] and W as fixed factors) are shown. DAS, days after sowing; dwt, dry weight; N<sub>leaf</sub>, leaf-area based N; LMA, leaf mass area; ns, not significant (P≥0.1). Units for A, μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>; g<sub>s</sub>, mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>; iWUE, μmol CO<sub>2</sub> mol<sup>-1</sup> H<sub>2</sub>O; LMA, g leaf m<sup>-2</sup> leaf area; nodule activity, mg N<sub>2</sub> fixed g<sup>-1</sup> final nodule dry mass.

**Table 2.** Carbohydrates and amino acid concentrations of faba bean grown under two CO<sub>2</sub> concentrations (ambient [CO<sub>2</sub>]: a[CO<sub>2</sub>], ~400 μmol mol<sup>-1</sup> and elevated [CO<sub>2</sub>]: e[CO<sub>2</sub>], ~700 μmol mol<sup>-1</sup>) and two water (W) regimes (well-watered and drought) and harvested at 60 DAS.

Growth parameters	Well-watered		Drought		P-value		
	a[CO <sub>2</sub> ]	e[CO <sub>2</sub> ]	a[CO <sub>2</sub> ]	e[CO <sub>2</sub> ]	[CO <sub>2</sub> ]	W	[CO <sub>2</sub> ] ×
<b>Leaf</b>							
Sugars conc. (mg g <sup>-1</sup> dwt)	120.69±7.47	129.39±6.22	158.22±10.04	250.68±11.96	0.001	<0.001	<0.001
Sugars content (g m <sup>-2</sup> )	3.99±0.50	4.28±0.42	6.28±0.44	10.36±1.26	0.001	<0.001	0.005
Starch (mg g <sup>-1</sup> dwt)	159.23±6.93	160.90±5.83	191.56±9.97	283.80±6.19	0.001	<0.001	<0.001
Starch content (g m <sup>-2</sup> )	5.23±0.54	5.32±0.44	7.63±0.53	11.63±1.16	0.002	<0.001	0.004
Free AA conc. (μmol g <sup>-1</sup> )	129.20±5.881	133.59±7.06	102.31±6.49	83.21±8.60	ns	0.005	ns
<b>Nodule</b>							
Sugars conc. (mg g <sup>-1</sup> dwt)	304.03±18.87	358.95±22.9	275.26±17.84	231.42±15.85	ns	<0.001	0.015
Starch conc. (mg g <sup>-1</sup> dwt)	185.74±20.59	250.92±20.5	159.95±18.73	182.62±14.50	0.036	0.025	ns
Free AA conc. (μmol g <sup>-1</sup> )	239.44±19.27	229.37±5.17	285.78±15.20	335.97±18.16	ns	<0.001	0.042
<b>Xylem sap</b>							
Free AA conc. (μM)	10.97±0.349	9.08±0.202	13.70±0.651	15.36±0.553	ns	<0.001	<0.001

Means ±SE (n=8) and statistical results (mixed effect model with [CO<sub>2</sub>] and W as fixed factors) are shown. DAS, days after sowing; conc., concentration; dwt, dry weight; ns, not significant (P≥0.05). Sugars and starch contents of leaves were expressed on a leaf surface area basis (g m<sup>-2</sup> leaf area).

**Figure legends:**

**Figure 1** (a) Maximum velocity of RuBP carboxylation by Rubisco ( $V_{\text{cmax}}$ ), (b) maximum rate of photosynthetic electron transport ( $J_{\text{max}}$ ), (c) ratio of  $J_{\text{max}}$  to  $V_{\text{cmax}}$  and (d) respiration ( $R_d$ ) in leaves of faba bean grown under ambient [ $\text{CO}_2$ ] ( $a[\text{CO}_2]$ ,  $\sim 400 \mu\text{mol mol}^{-1}$ , open bars) or elevated [ $\text{CO}_2$ ] ( $e[\text{CO}_2]$ ,  $\sim 700 \mu\text{mol mol}^{-1}$ , closed bars) and two water (W) regimes (well-watered and drought) and measured next day after pulse labelling. Each bar represents means  $\pm$ SE ( $n=8$ ). Only significant effects ( $P<0.05$ ) are shown.

**Figure 2** Total  $^{13}\text{C}$  incorporation in different sinks organs (a) leaf, (b) stem, (c) root and (d) nodule of faba bean grown under ambient [ $\text{CO}_2$ ] ( $a[\text{CO}_2]$ ,  $\sim 400 \mu\text{mol mol}^{-1}$ , open bars) or elevated [ $\text{CO}_2$ ] ( $e[\text{CO}_2]$ ,  $\sim 700 \mu\text{mol mol}^{-1}$ , closed bars) and two water (W) regimes (well-watered and drought) and exposed to  $^{13}\text{CO}_2$  pulse labelling (for 6 h and harvested after a 24 h chase period). Each bar represents means  $\pm$ SE ( $n=8$ ). Only significant effects ( $P<0.05$ ) are shown.

**Figure 3** Activity of different sink organs (leaf, stem, root and nodule) of faba bean grown under ambient [ $\text{CO}_2$ ] ( $a[\text{CO}_2]$ ,  $\sim 400 \mu\text{mol mol}^{-1}$ , open bars) or elevated [ $\text{CO}_2$ ] ( $e[\text{CO}_2]$ ,  $\sim 700 \mu\text{mol mol}^{-1}$ , closed bars) and two water (W) regimes (WW: well-watered and DT: drought) and harvested after pulse labelling with  $^{13}\text{CO}_2$  for 6 h and a 24 h chase period. Each bar represents means  $\pm$ SE ( $n=8$ ). Effects are considered significant at  $P<0.05$ . WW, well-watered; DT, drought; ns, non-significant.

**Figure 4** Amount of  $^{13}\text{C}$  released by root exudation after 24-hr chasing period of faba bean grown under ambient  $[\text{CO}_2]$  (a $[\text{CO}_2]$ ,  $\sim 400 \mu\text{mol mol}^{-1}$ , open bars) or elevated  $[\text{CO}_2]$  (e $[\text{CO}_2]$ ,  $\sim 700 \mu\text{mol mol}^{-1}$ , closed bars) and two water (W) regimes (well-watered and drought) and harvested after pulse labelling with  $^{13}\text{CO}_2$  for 6 h and a 24 h chase period. Each bar represents means  $\pm$ SE (n=8). Only significant effects ( $P < 0.05$ ) are shown. DW: dry weight.

**Figure 5** Nodule (a) specific  $^{13}\text{C}$  fixation and (b) specific  $\text{N}_2$  fixation of faba bean grown under ambient  $[\text{CO}_2]$  (a $[\text{CO}_2]$ ,  $\sim 400 \mu\text{mol mol}^{-1}$ , open bars) or elevated  $[\text{CO}_2]$  (e $[\text{CO}_2]$ ,  $\sim 700 \mu\text{mol mol}^{-1}$ , closed bars) and two water (W) regimes (well-watered and drought) and harvested at 60 DAS. Means and  $\pm$ SE (n=8) in each bar. Only significant effects ( $P < 0.05$ ) are shown. NWD: nodule dry weight.

**Figure 6** Summary of the interactive effect of elevated  $[\text{CO}_2]$  and drought on nodule C sink strength. In well-watered plants, faba bean avoided photosynthetic acclimation to elevated  $[\text{CO}_2]$  mainly due to a greater C sink strength of nodules rather than other organs, as confirmed by the  $^{13}\text{C}$  enrichment (greater sink activity) of nodules tissues. In contrast, as a result of drought stress, leaves imported less N and the reduction in N demand fed back on the nodules. Decreased nodule sink activity was marked by an accumulation of respective fixation products in the respective organs, carbohydrates in leaves and amino acids in nodules. Accumulation of leaf carbohydrates appeared to have eventually triggered photosynthetic acclimation to elevated  $[\text{CO}_2]$  under drought. Lower nodule sink strength also tended to increase  $^{13}\text{C}$  allocation towards roots and exudates but this would not overcome photosynthetic acclimation.

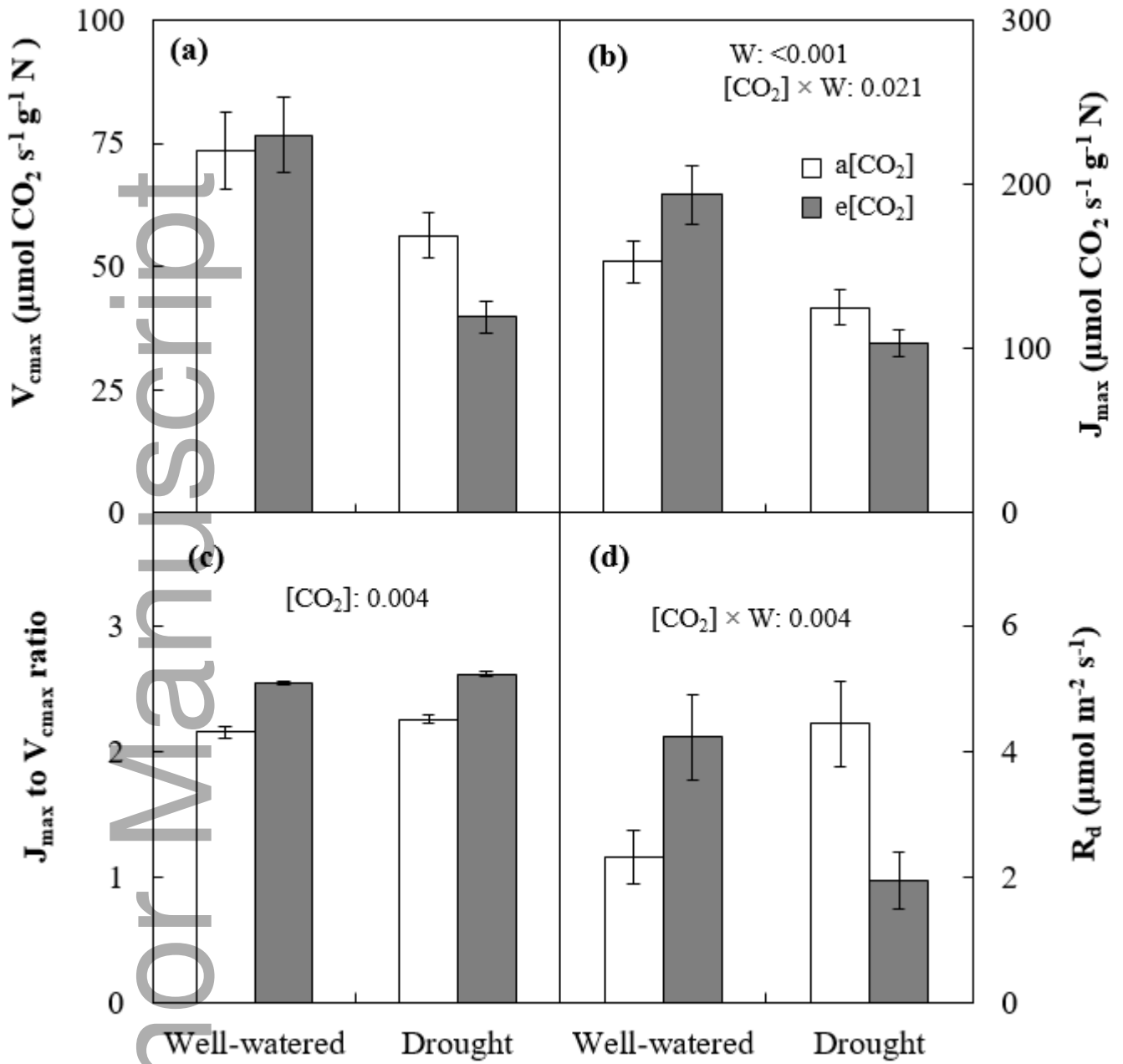
The following Supporting Information is available for this article:

**Fig. S1** Volumetric soil water content (v/v %) during the growth period of faba bean under two  $\text{CO}_2$  concentrations and two water regimes.

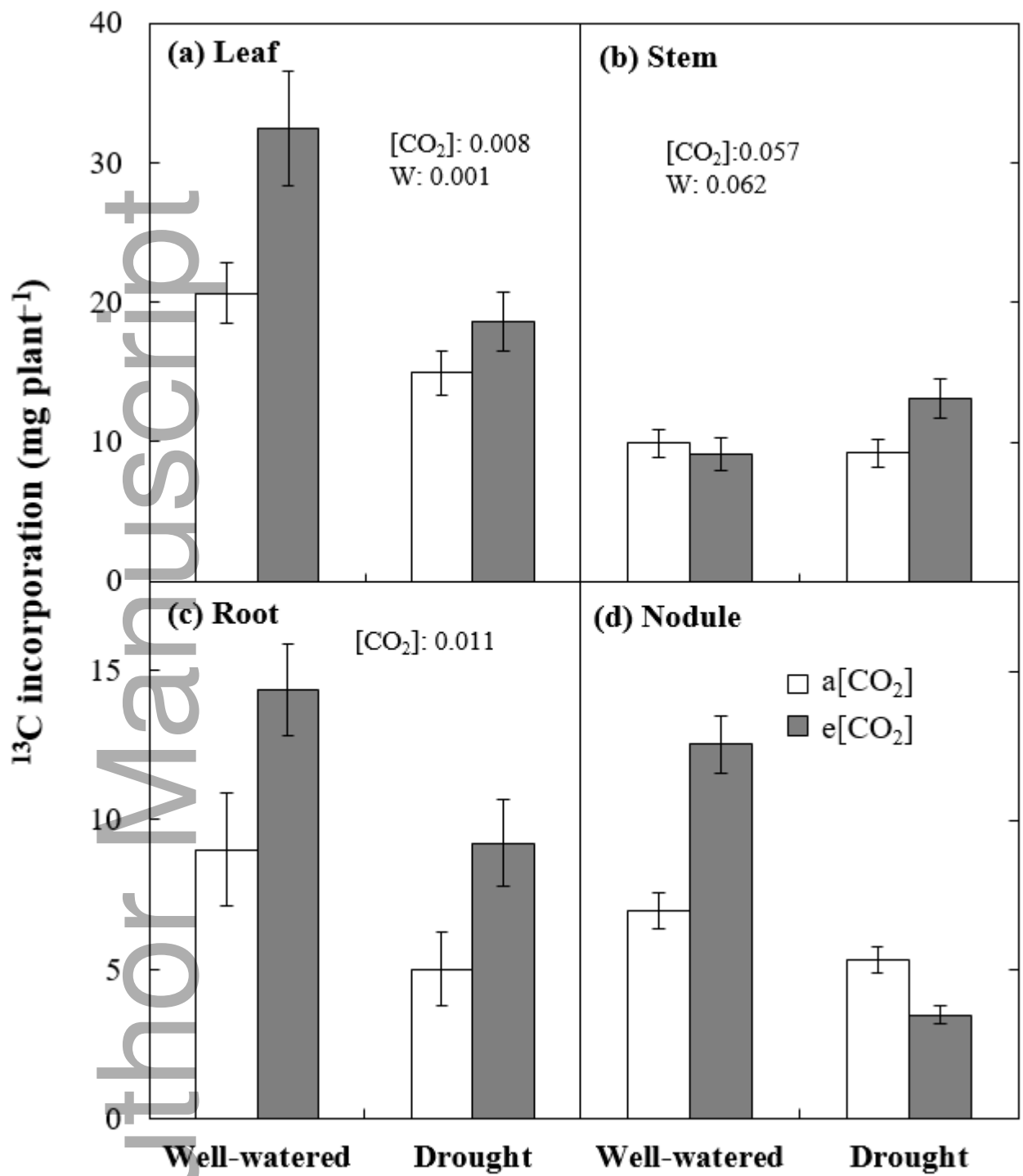
**Fig. S2**  $^{13}\text{CO}_2$  concentration inside the ambient  $[\text{CO}_2]$  and elevated  $[\text{CO}_2]$  chambers during  $^{13}\text{CO}_2$  pulse labelling period.

**Fig. S3** Ratio of free amino acids to sugars in leaf and nodule of faba bean grown under two  $\text{CO}_2$  concentrations and two water regimes.

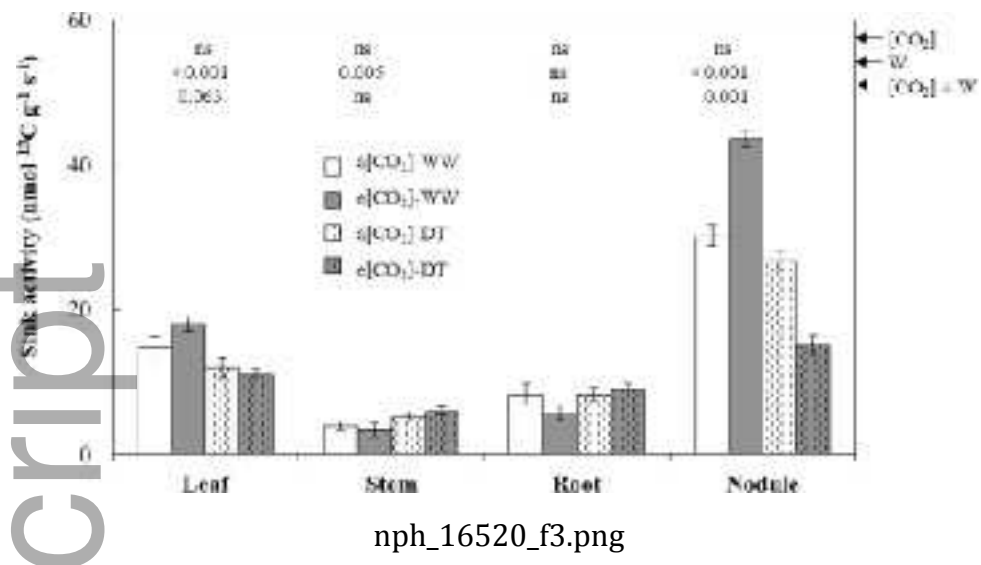
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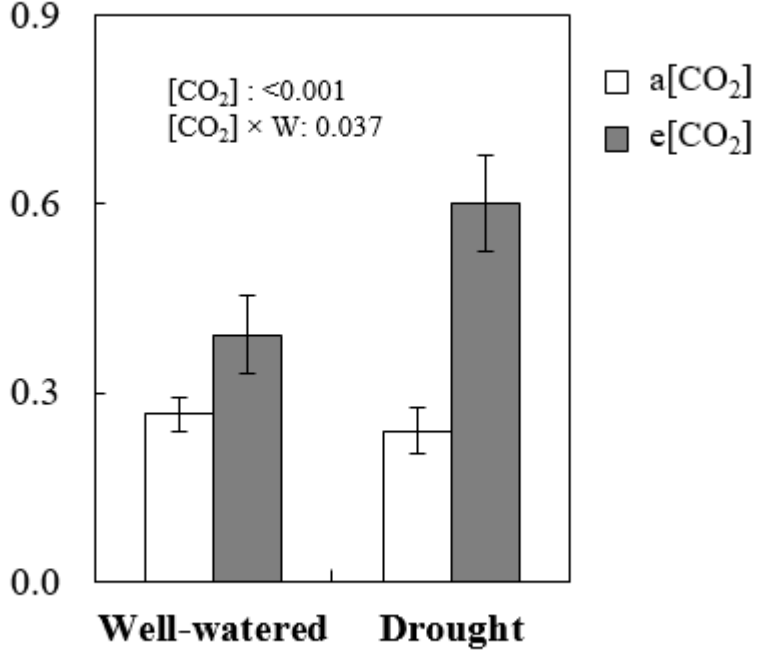


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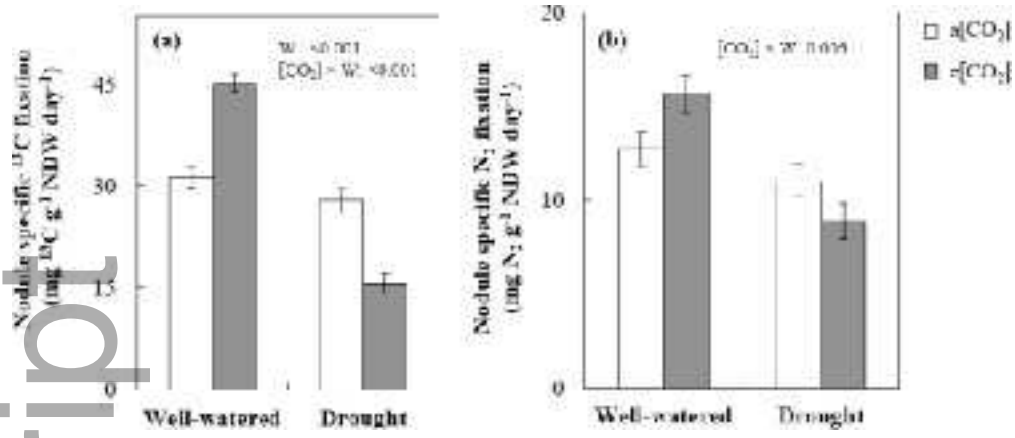
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Root exudate  
( $\text{mg } ^{13}\text{C g}^{-1}\text{root-DW}$ )

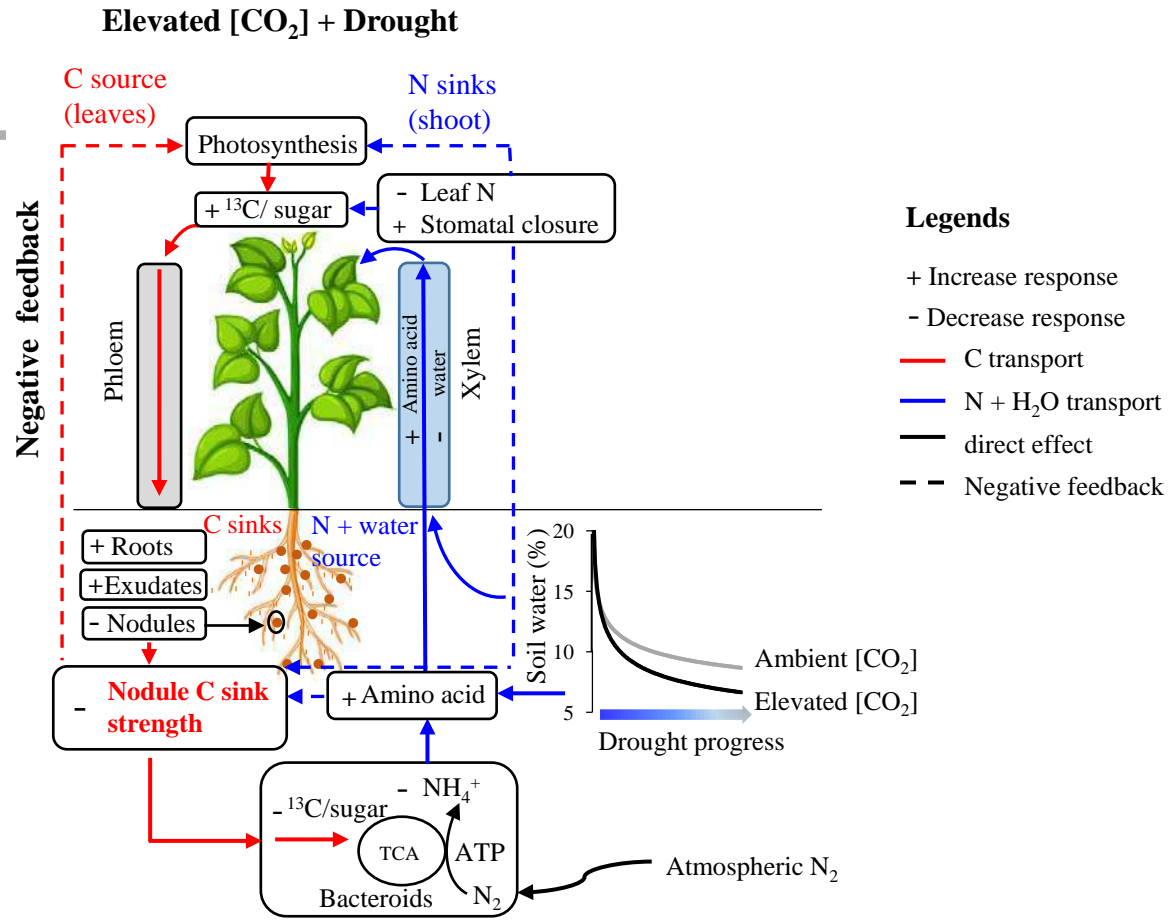


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